Programa de Doctorado en Recursos y Tecnologías Agrarias, Agroambientales y Alimentarias

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INNOVATIVE PRE- AND POST-HARVEST TREATMENTS WITH MELATONIN AND γ-AMINOBUTYRIC ACID TO INCREASE YIELD AND QUALITY OF LEMONS AT HARVEST AND DURING STORAGE

Fátima Badiche El Hilali

Director: **Dr. D. Daniel Valero Garrido**

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La presente Tesis Doctoral, titulada "INNOVATIVE PRE- AND POST-HARVEST TREATMENTS WITH MELATONIN AND γ-AMINOBUTYRIC ACID TO INCREASE YIELD AND QUALITY OF LEMONS AT HARVEST AND DURING STORAGE" se presenta bajo la modalidad de **tesis por compendio** de las siguientes **publicaciones**:

- Badiche, F., Valverde, J.M., Martínez-Romero, D., Castillo, S., Serrano, M. & Valero, D. Preharvest use of γ-aminobutyric acid (GABA) as an innovative treatment to enhance yield and quality in lemon fruit. Horticulturae, 2023, 9, 93. https://doi.org/10.3390/horticulturae9010093

- Badiche-El Hilali, F., Valverde, J.M., Díaz-Mula, H., Serrano, M., Valero, D. & Castillo, S. Potential Preharvest Application of γ-Aminobutyric Acid (GABA) on Improving Quality of 'Verna' Lemon at Harvest and during Storage. Agriculture, 2023, 13, 1397. https://doi.org/10.3390/agriculture13071397

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- Badiche-El Hilali, F., Medeiros-Fonseca, B., Silva, J., Silvestre-Ferreira, A.C., Pires, M.J., Gil da Costa, R.M., Peixoto, F., Oliveira, P.A. & Valero, D. The Effect of Lemon Juice (Citrus limon L.) Treated with Melatonin on the Health Status and Treatment of K14HPV16 Mice. Antioxidants, 2024, 13, 588. https://doi.org/10.3390/antiox13050588

- Badiche-El Hilali, F., García-Pastor, M.E., Castillo, S., Valverde, J.M., Serrano, M. & Valero, D. Melatonin as an Efficient and Eco-Friendly Tool to increase yield and to maintain quality attributes during Lemon Storage. International Journal of Molecular Sciences, 2024, 25, 10025. https://doi.org/10.3390/ijms251810025

El Dr. D. Daniel Valero Garrido, director, y el Dr. D. Salvador Castillo García, codirector/a de la tesis doctoral titulada **"INNOVATIVE PRE- AND POST-HARVEST TREATMENTS WITH MELATONIN AND γ-AMINOBUTYRIC ACID TO INCREASE YIELD AND QUALITY OF LEMONS AT HARVEST AND DURING STORAGE"**

INFORMA/N:

Que Dña. Fátima Badiche El Hilali ha realizado bajo nuestra supervisión el trabajo titulado **"INNOVATIVE PRE- AND POST-HARVEST TREATMENTS WITH MELATONIN AND γ-AMINOBUTYRIC ACID TO INCREASE YIELD AND QUALITY OF LEMONS AT HARVEST AND DURING STORAGE"** conforme a los términos y condiciones definidos en su Plan de Investigación y de acuerdo al Código de Buenas Prácticas de la Universidad Miguel Hernández de Elche, cumpliendo los objetivos previstos de forma satisfactoria para su defensa pública como tesis doctoral.

Lo que firmamos para los efectos oportunos, en Orihuela a 24 de septiembre de 2024

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Dr. D./Dra. Dña. *"nombre y apellidos"*

Codirector/a de la tesis

Dr. D./la Dra. Dña. *"nombre y apellidos"*

Dr. Dña. Juana Fernández López, Catedrática de Universidad y Coordinadora del Programa de Doctorado en Recursos y Tecnologías Agrarias, Agroambientales y Alimentarias (ReTos-AAA) de la Universidad Miguel Hernández de Elche (UMH),

CERTIFICA:

Que la Tesis Doctoral titulada "**Innovative pre- and post-harvest treatments with melatonin and γ-aminobutyric acid to increase yield and quality of lemons at harvest and during storage"** de la que es autor/a el/la graduado/a en Ciencia y Tecnología de los Alimentos **Dña. Fátima Badiche El Hilali**, ha sido realizada bajo la dirección del/de la **Dr. Daniel Valero Garrido** y la codirección del/de la **Dr. Salvador Castillo García**, actuando como tutor/a de la misma el/la Dra. **María Serrano Mula**. Considero que la Tesis es conforme, en cuanto a forma y contenido, a los requerimientos del Programa de Doctorado ReTos-AAA, siendo por tanto apta para su exposición y defensa pública.

Y para que conste a los efectos oportunos firmo el presente certificado en Orihuela a 24 de septiembre de 2024.

Dra. Dña. Juana Fernández López

Coordinadora del Programa Doctorado ReTos-AAA

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"Me parece haber sido solo un niño jugando en la orilla del mar, divirtiéndose y buscando una piedra más lisa o una concha más bonita de lo normal, mientras el gran océano de la verdad yacía ante mis ojos con todo por descubrir"

Isaac Newton (1643-1727)

PUBLICATIONS CATEGORY

Publication 1

Badiche, F.; Valverde, J.M.; Martínez-Romero, D.; Castillo, S.; Serrano, M.; Valero, D. Preharvest use of γ-aminobutyric acid (GABA) as an innovative treatment to enhance yield and quality in lemon fruit. Horticulturae, 2023, 9, 93. Doi: 10.3390/horticulturae9010093

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- Publications image, page 54. Own-source related lemons images.
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DOCTORAL THESIS STRUCTURE

This Doctoral Thesis has been structured following the final format of the PhD thesis by compendium of publications of Miguel Hernández university:

- **Abstract/Resumen:** A brief description of the most relevant results and conclusions obtained in this PhD Thesis has been presented.
- **Introduction:** The scientific background and object of this PhD Thesis has been briefly tackled, relating it to the state of the art of colour problems and market requirements on table grape and pomegranate fruit. Additionally, production facts and crop importance has been studied to justify the use of these crops. Finally, postharvest group research experience and elicitation strategies have been deeply reviewed.
- **Aim and Objectives:** The main aim and specific objectives have been established in this section.
- **Materials and Methods:** The plant material, experimental design about the studied treatments and the analytical methods used to carry out the experiments included in this PhD Thesis have been briefly explained and referenced.
- **Publications:** The 4 publications and 1 under-review used for this PhD Thesis are presented in the following order:

Publication 1. Badiche, F., Valverde, J.M., Martínez-Romero, D., Castillo, S., Serrano, M., Valero, D. Preharvest use of γ-aminobutyric acid (GABA) as an innovative treatment to enhance yield and quality in lemon fruit. Horticulturae, 2023, 9, 93. Doi: 10.3390/horticulturae9010093

Publication 2. Badiche-El Hilali, F., Valverde, J.M., Díaz-Mula, H., Serrano, M., Valero, D., Castillo, S. Potential Preharvest Application of γ-Aminobutyric Acid (GABA) on Improving Quality of 'Verna' Lemon at Harvest and during Storage. Agriculture, 2023, 13, 1397. Doi:10.3390/agriculture13071397

Publication 3. Badiche-El Hilali, F., Valverde, J.M., García-Pastor, M.E., Serrano, M.; Castillo, S., Valero, D. Melatonin Postharvest Treatment in Leafy 'Fino' Lemon Maintains Quality and Bioactive Compounds. Foods, 2023, 12, 2979. Doi: 10.3390/foods12152979

Publication 4. Badiche-El Hilali, F., Medeiros-Fonseca, B., Silva, J.; Silvestre-Ferreira, A.C., Pires, M.J., Gil da Costa, R.M., Peixoto, F., Oliveira, P.A., Valero, D. The Effect of Lemon Juice (*Citrus limon* L.) Treated with Melatonin on the Health Status and Treatment of K14HPV16 Mice. Antioxidants, 2024, 13, 588. Doi: 10.3390/antiox13050588

🍊**Publication 5.** Badiche-El Hilali, F., García-Pastor, M.E., Valverde, J.M., Castillo, S., Valero, D. & Serrano, M. Melatonin as an Efficient and Eco-Friendly Tool to increase yield and to maintain quality attributes during Lemon Storage. International Journal of Molecular Sciences, 2024, 25(18), 10025. Doi:10.3390/ijms251810025.

- **Results and Discussion:** In this section, the main results obtained in this PhD Thesis are explained, discussed and summarized. In addition, a comparative fold analysis among the effect of the treatments tested in this PhD Thesis on increasing total anthocyanin content of table grape and pomegranate fruit at harvest has been carried out.
- **Conclusions/Conclusiones:** The main conclusions obtained in this PhD Thesis have been listed.
- **References:** Literature used for writing and justifying this PhD Thesis in the complementary sections to 'Publications' has been referenced.

Abstract / Resumen

ABSTRACT

Citrus fruits, and particularly lemons, are a group of fruits that are highly appreciated throughout the world both for their organoleptic and nutritional quality, as well as for their various technological uses, which make them a highly valuable product for the food industry. However, if lemons have gained relevance in recent years, it is undoubtedly due to their high content of bioactive compounds of diverse nature, such as L-ascorbic acid (commonly known as vitamin C), flavanones, flavones or hydroxycinnamic acids, which have proven to have multiple health benefits due to their strong antioxidant activity.

Citrus fruits have conquered the whole world and their cultivation is centralised around 40º north latitude and 40º south latitude, which allows us to understand why the Mediterranean Levant is undoubtedly the star location for citrus fruits in Spain. Of all Spanish fruit and vegetable production in 2022, the citrus sub-sector was second in terms of quantity, with 23% of national production. With regard to the fruit of the lemon tree (*Citrus limon* (L.) Burm. F), Spain, with a production of 900,000 tonnes in the 2022/2023 season, is the third largest producer in the world and the first in the European Union. The commercial value of the lemon is beyond any doubt if we consider that, of this national production, almost 70% is exported outside our borders, with Germany as the main importer. Low temperature preservation, one of the most widely used technologies to preserve the post-harvest quality of fruits, is necessary for marketing. However, fruits of certain citrus species and varieties are sensitive to develop chilling damage at temperatures below 5 °C, as well as to fungal infections, factors that depreciate their quality and marketability. All this, together with the growing concern of consumers and the limitation by European legislation on the use of traditional phytochemicals, opens up new lines of research.

Recently, research has been carried out to find pre- and post-harvest treatments with natural compounds to replace those traditionally used, in order to increase the quality of the fruit at the time of harvesting and maintain it during storage, due to consumer concerns and legal restrictions on the use of chemical treatments both before and after harvesting. In this regard, the application of naturally occurring and plant-derived compounds as pre- or post-harvest treatments to delay ripening and senescence, preserving the quality of fruits and vegetables, has received considerable attention. Among these compounds, γ-aminobutyric acid (GABA) and melatonin (MEL) are compounds that are ubiquitous in plants and are found in the fruit and vegetable sector and are involved in numerous physiological processes of their development, such as defence against biotic and abiotic stresses. The scientific literature on the effect of pre- and post-harvest application of these compounds on lemon quality is limited. Therefore, the aim of this PhD Thesis is to solve lemon quality problems by pre-harvest treatments with γ-aminobutyric acid and melatonin, and post-harvest treatments with MEL to solve mainly the common physiological

problems of two different landraces ('Fino' grafted on *Citrus macrophylla* and 'Verna' grafted on *Citrus aurantium*) at the time of harvesting, as well as their quality losses during cold storage due to the incidence of chilling injury and/or fungal infections, as well as dehydration and over-ripening, with the ultimate aim of increasing their shelf-life. In addition, it was assessed whether the treatments had an effect on the total crop yield.

Pre-harvest treatments were carried out by foliar spraying at 10, 50 and 100 mM concentrations for the GABA compound on both lemon varieties, containing 0.5 % Tween 20 as surfactant. For MEL, the concentrations used in both lemon cultivars were 0.1, 0.3 and 0.5 mM, also with 0.5 % Tween 20.

The treatments applied after fruit harvesting were divided into two different experiments: on the one hand, freshly harvested fruits of the Fino variety were immersed in melatonin solutions at concentrations of 0.01, 0.1 and 1 mM. On the other hand, Verna lemon fruits were immersed in 10 mM melatonin to make juice that would later be used in an *in vivo* trial with mice to test its effect on health. In all the pre-harvest experiments, the crop yield in the field and the quality of the fruit at the time of harvesting were evaluated and storage experiments were carried out at 2 and 10 °C for 28 days, evaluating each week, from the first day, the organoleptic (colour and firmness), nutritional (total soluble solids content (TSS) and total acidity (TA)) and functional quality (total phenol content (TPC) in the skin and in the juice and total antioxidant activity (TAA)). Cold storage was also carried out in the first post-harvest experiment.

The results of this PhD thesis have shown that pre-harvest GABA and MEL treatments increased the yield of lemon trees in both varieties by increasing the number of fruits per tree. In addition, lemon quality parameters such as weight, firmness, total soluble solids (TSS), total acidity (TA) or bioactive compound content were also influenced by the treatments, leading to a delay in the post-harvest ripening of lemons and to a longer period of storage with optimum fruit quality properties for consumption, being the doses of 50 and 100 mM GABA and 0.5 mM MEL the most effective.

Regarding post-harvest treatment with MEL at 0.01, 0.1 and 1 mM, the results obtained showed that the most effective dose for prolonging the shelf life of lemon fruit and preserving its quality parameters was 1 mM, mainly maintaining membrane integrity, phenol content and total antioxidant activity after storage at 10 °C for 21 days. It was also observed that the post-harvest application of MEL on leafy lemons was better than on leafless lemons, increasing their shelf life for a longer period of time. The *in vivo* test with mice treated with lemon juice extracted from fruits previously treated with 10 mM MEL showed that the compound did not present any toxicity or health risk to the animals after consumption, while a lower incidence of oxidative stress and weight gain was observed.

Finally, this research has shown that pre- and post-harvest treatments with GABA and MEL, applied at appropriate concentrations, could be considered as a safe strategy, based on natural compounds, to improve crop yield and quality attributes of lemon at the time of harvest and during cold storage. Overall, a solution to a global problem is provided by helping horticultural companies to increase their economic performance by obtaining higher yields and higher quality fruit at all stages from harvest to consumer.

RESUMEN

Los cítricos, y particularmente los limones, son un grupo de frutos muy apreciados en el mundo entero tanto por su calidad organoléptica y nutricional, como por sus diversos usos tecnológicos, que los convierten en un producto de gran valor para la industria alimentaria. No obstante, si el limón ha ganado relevancia en los últimos años es sin duda debido a su alto contenido en compuestos bioactivos de diversa naturaleza, como el ácido L-ascórbico (comúnmente conocido como vitamina C), las flavanonas, flavonas y ácidos hidroxicinámicos, que han demostrado tener múltiples beneficios para la salud por su fuerte actividad antioxidante.

Los cítricos han conquistado todo el mundo y su cultivo está centralizado en torno a los 40º de latitud norte y 40º de latitud sur, lo que nos permite entender por qué el levante mediterráneo es sin duda el lugar estrella para los cítricos en España. De toda la producción hortofrutícola española en 2022, el subsector de los cítricos ocupó el segundo puesto por detrás de las hortalizas en cuanto a cantidad, con un 23% de la producción nacional. En lo que se refiere al fruto del limonero (*Citrus limon* (L.) Burm. F), España, con una producción de 900.000 toneladas en la campaña 2022/2023, ocupa la tercera posición como productor mundial, y la primera dentro de la Unión Europea. El valor comercial del limón queda fuera de toda duda si tenemos en cuenta que, de esa producción nacional, casi el 70% se exporta fuera de nuestras fronteras, con Alemania como principal importador. Para la comercialización es necesaria la conservación a bajas temperaturas, una de las tecnologías más ampliamente utilizadas para preservar la calidad post-recolección de los frutos. Sin embargo, los frutos de ciertas especies y variedades de cítricos son sensibles a desarrollar daños por frío a temperaturas inferiores a 5 °C, además de ser susceptibles a infecciones fúngicas, factores que deprecian su calidad y comercialización. Todo esto, junto a la creciente preocupación de los consumidores y la limitación por parte de la legislación europea en relación al empleo de fitoquímicos tradicionales, abre nuevas líneas de investigación.

Recientemente, se han realizado investigaciones encaminadas a encontrar tratamientos pre y post-recolección con compuestos naturales que sustituyan los tradicionalmente utilizados, para incrementar la calidad de la fruta en el momento de la recolección y mantenerla durante el almacenamiento, debido a las preocupaciones de los consumidores y las restricciones legales sobre el uso de tratamientos químicos tanto antes como después de la recolección. En este sentido, la aplicación de compuestos de origen natural y derivados de las plantas, como tratamientos pre o post-recolección para retrasar la maduración y la senescencia, preservando la calidad de las frutas y hortalizas, ha recibido una atención considerable. Entre estos compuestos, el ácido γ-aminobutírico (GABA) y la melatonina (MEL) son compuestos que se encuentran ubicuos en las plantas y participan en numerosos procesos fisiológicos de su desarrollo, como en la defensa contra estreses bióticos y

abióticos. Es limitada la literatura científica sobre el efecto de la aplicación pre y post- recolección de estos compuestos sobre la calidad del limón. Por tanto, el objetivo de esta Tesis Doctoral es dar solución a los problemas de calidad del limón mediante tratamientos pre- recolección con GABA y MEL, y tratamientos post-recolección con MEL para resolver principalmente los problemas fisiológicos comunes de dos variedades autóctonas distintas ('Fino' injertado sobre *Citrus macrophylla* y 'Verna' injertado sobre *Citrus aurantium*) en el momento de la recolección, así como sus pérdidas de calidad durante el almacenamiento en frío debidos a la incidencia de daños por frío y/o de infecciones fúngicas, así como a la deshidratación y sobremaduración, con el objetivo final de aumentar su vida útil. Además, se evaluó si los tratamientos tenían efecto sobre la producción total del cultivo.

Los tratamientos pre-recolección se realizaron mediante pulverización foliar a las concentraciones de 10, 50 y 100 mM, para el compuesto GABA, en ambas variedades de limón, conteniendo un 0.5 % de Tween 20 como surfactante. En cuanto al compuesto MEL, las concentraciones empleadas en ambos cultivos fueron 0.1, 0.3 y 0.5 mM, también con Tween 20 al 0.5 %. Los tratamientos aplicados después de la recolección de los frutos se dividieron en dos experimentos diferentes. Por un lado, se sumergieron frutos de la variedad Fino, recién recolectados, en disoluciones de melatonina a concentración 0.01, 0.1 y 1 mM. Por otro lado, frutos de limón Verna se sumergieron en melatonina 10 mM para realizar zumo que sería empleado posteriormente en un ensayo *in vivo* con ratones con el fin de comprobar el efecto del mismo sobre la salud. En todos los experimentos pre-recolección, se evaluó el rendimiento del cultivo en campo y la calidad de los frutos en el momento de la recolección y se realizaron experimentos de conservación a 2 y 10 °C durante 28 días evaluando cada semana, desde el primer día, la calidad organoléptica (color y firmeza), nutritiva [contenido en sólidos solubles totales (SST) y acidez total, (AT)] y funcional [contenido en fenoles totales (CFT) en la piel y en el zumo y actividad antioxidante total (AAT)]. La conservación en frio, también se realizó en el primer experimento post-recolección.

Los resultados de esta Tesis Doctoral han demostrado que los tratamientos pre-recolección con GABA y MEL incrementaron el rendimiento de los limoneros en ambas variedades, mediante el aumento del número de frutos por árbol. Además, los parámetros de calidad de los limones, como el peso, la firmeza, los sólidos solubles totales (SST), la acidez total (AT) o el contenido de compuestos bioactivos, también se vieron influenciados por los tratamientos, lo que condujo a un retraso en la maduración post-recolección de los limones y un mayor periodo de conservación de sus propiedades tras el almacenamiento en frio, siendo la dosis de 50 y 100 mM de GABA y 0.5 mM de MEL las más efectivas. Con respecto al tratamiento post-recolección con MEL a 0.01, 0.1 y 1 mM los resultados obtenidos mostraron que la dosis más efectiva para prolongar la vida útil del fruto de limón y preservar sus parámetros de calidad fue 1 mM, manteniendo principalmente la integridad de la membrana, y su contenido en fenoles y actividad antioxidante total tras el

almacenamiento a 10 °C durante 21 días. También se observó que la aplicación postcosecha de MEL en limones con hoja fue mejor con respecto a los sin hoja, aumentando su vida útil por más tiempo. El ensayo *in vivo* con ratones tratados con zumo de limón extraído de frutos previamente tratados con MEL 10 mM mostró como el compuesto no presentaba toxicidad ni riesgo tras su consumo sobre la salud de los animales, mientras redujo la incidencia de estrés oxidativo y ganancia de peso.

Finalmente, esta investigación ha demostrado que los tratamientos pre y post-recolección con GABA y MEL, aplicados en las concentraciones adecuadas, podrían considerarse una estrategia segura, basada en compuestos naturales, para mejorar la producción del cultivo, así como los atributos de calidad del limón, en el momento de la recolección y durante el almacenamiento en frio. En general, se aporta una solución a un problema de índole mundial ayudando a las empresas hortícolas a aumentar su rendimiento económico, al obtener mayor producción y frutos con mayores niveles de calidad en todas las etapas, desde la recolección hasta su llegada al consumidor.

1 Introduction

1. INTRODUCTION

1.1. Scientific background and object

Lemons are fruits with a high qualitative value due to their excellent organoleptic, refreshing and nutritional characteristics, both in their fresh state and in their derivatives, such as juices, jams, jellies, concentrated juices, essential oils, essences, etc. In addition to its versatility when consumed, lemons are highly appreciated by consumers due to their high content of bioactive compounds that can provide health benefits. However, these fruits are highly sensitive to develop a series of physiopathologies such as fungal rotting, especially species of the genus *Penicillium* or cold damage, also known as chilling injuries, when stored at low temperatures. On the other hand, these fruits are mostly exported, which makes them more perishable and with a reduced shelf life after transport and storage at low temperatures. This causes undesired changes in organoleptic and sensory attributes, such as acidity, firmness, colour changes and loss of bioactive compounds.

This PhD Thesis is part of the research line that the Postharvest Group of Fruits and Vegetables (Miguel Hernández University of Elche) has been developing in recent years on the application of elicitors in preharvest. In addition, it has been funded by an I+D+i Spanish project titled 'INNOVATIVE PRE- AND POST-HARVEST TREATMENTS WITH MELATONIN AND MELATONIN AND YAMINOBUTYRIC ACID TO INCREASE THE QUALITY OF LEMON AND POMEGRANATE AT HARVEST AND DURING STORAGE POMEGRANATE AT HARVEST AND DURING STORAGE', reference RTI2018-099664-B-100, co-founded with FEDER funds inside the I+D+i projects announcement of the 'State Program of Research, Development and Innovation' oriented to the society challenges. In this sense, the PhD Thesis is framed within the activities of the aforementioned project. Besides, Fátima Badiche El Hilali was funded by a research scholarship (reference number: PRE2019-090805) from Spanish Minister of Science and Innovation.

The objective of this research is to provide solutions to the problem of the preservation and limited shelf life of lemons through the application of preharvest treatments with GABA and melatonin (MEL) to maintain the quality of the fruit for a longer period of time, mainly preventing the development of the common damage of this fruit. The quality problems of the fruit cause agricultural companies to encounter many problems in marketing them on national and international markets. This line of research also aims to use pre- and postharvest treatments to improve the nutritional quality and functional composition of lemons, as there is currently a growing awareness of the relationship between fruit consumption and health, which requires great efforts in research activities related to the production of high quality products, from the field to the table. On the other hand, the effects of climate change pose a real challenge for plant-based food production, as they negatively affect both

plant growth and crop physiology and productivity. Citrus cultivation is expected to be affected in the near future.

All this, together with the growing consumer concern about the use of unnatural compounds that may be harmful to health, forces researchers to find sustainable strategies capable of increasing fruit quality and/or maintaining it during post-harvest storage. In recent years, research has been carried out with innovative compounds or inducers that have effects on the process of fruit development in the plant and on the evolution of ripening and changes related to quality loss during postharvest storage. Our research group has a wide scientific knowledge within the subject of this Doctoral Thesis and has obtained beneficial effects with the application, either as preharvest or postharvest treatments, of natural elicitors such as Methyl Jasmonate (MeJa), Salicilic Acid (SA) and its derivates, or Gibberellin (GA3) in the increase of quality at harvest and in the maintenance of organoleptic, nutritional and functional quality during storage in fruits such as lemon, pomegranate, sweet cherry or plum, and in vegetables such as artichoke. As well as pre- and postharvest treatment with melatonin and GABA in other fruits such as pomegranate or sweet cherry. Also, recently, with the use of polyamines such as brassinosteroids or putrescine in pre-harvest treatments on fruits such as blood orange, sweet cherry or pomegranate. On the other hand, the essential oils carvacrol and thymol have been studied for the control of fungal diseases in lemons. In all these previous projects, satisfactory results have been obtained in terms of maintaining quality, and so we have continued with this line of research based on the study of natural elicitors to preserve the quality of the fruit.

The main hypothesis of this Doctoral Thesis is that elicitors applied before and/or after harvesting of the lemon fruits could increase their shelf life while preserving their nutritional and organoleptic properties, as well as reducing quality losses during post-harvest storage. During fruit growth and development, both cell division and cell expansion occur rapidly, and the fruit is more sensitive to external elicitors. Therefore, the pre-harvest strategy based on the application of elicitors induces a more active metabolism compared to untreated freshly harvested fruit, as well as prolonged storage at low temperatures. This would provide a solution to the problem of chilling injury that is often associated with fruit preservation, whether for export to international markets or not, and would have a net benefit in terms of increased consumer acceptance. The results will help fruit and vegetable companies by providing fruit with higher quality standards at harvest and after post-harvest handling, storage and marketing.

1.2. Lemon crop and production facts

Around the 4th-5th century BC, the lemon tree arrived in the Mediterranean area from Asia, being introduced through Greece. However, its arrival in Spain was from North Africa, during the time when the Arabs inhabited the Iberian Peninsula. It is clear that Muslims played a crucial role in the spread of cultivated citrus to North Africa and southern Europe, which was possible because they controlled an extensive

territory and trade routes stretching from India to the Mediterranean (Langgut, 2017). This introduction marked the beginning of lemon cultivation in Spain, with the Ricote Valley in the region of Murcia probably being one of the first places where it was cultivated (Westerveld, 2014). It is considered a crop with an outstanding tradition, whose fruit is highly appreciated by consumers all over the world due to its sensory, nutritional and traditional medicine-related properties (Saini et al., 2022).

Lemon is obtained from the lemon tree (*Citrus limon* (L.) Burm. f), a species of fruit tree belonging to the kingdom Plantae, division *Magnoliophyta* (also known as Angiosperms), class *Magnoliopsida*, order Sapindales and family *Rutaceae* (Stevens, 2020). The family *Rutaceae* includes a wide variety of flowering plants, many of which are important in horticulture and medicine. The subfamily to which the lemon belongs is the *Aurantioideae*, within the family *Rutaceae* (Ollitrault et al., 2020). This subfamily includes six genera of citrus plants, such as *Citrus, Fortunella* or *Poncirus*. *Citreae* is the tribe which lemon genus belongs. This tribe groups different genera of citrus plants that share similar characteristics (García-Lor., 2013). It is an evergreen tree with marked veins on both the upper and lower sides, with a long gestation period that can reach a height of between 3 and 6 metres depending on the species (Ortiz, 2002). Its branches have hard thorns and the crown is rounded. The fruit it produces is the lemon, which is oval-shaped with a length of 7 to 12 cm, with colours ranging from green during the early stages of fruit development due to the presence of chlorophylls, to the final yellow, the result of the degradation of chlorophylls, which leaves the lower layer formed by carotenoids visible (Conesa et al., 2019). *Citrus* is a tropical crop and therefore tolerates water deficit poorly and requires temperate climates (Colmenero-Flores et al., 2020). The ideal climate for the proper development of lemon trees is the Mediterranean. Lemon trees are planted with two objectives in mind; to absorb as much incident light as possible and to facilitate the operation of machinery inside the tree. Therefore, several planting frames are usually used depending on the variety, plantation or crop conditions: 6.5 x 5; 6.5 x 6; 7 x 5. The peculiar root system of lemon trees, sensitive to root asphyxia, means that the best soils for their cultivation are permeable, deep, low salinity, low limestone and well drained. In addition, lemon trees prefer slightly acid soils rich in organic matter.

1.2.1. Varieties of lemon tree

The number of varieties of lemon trees cultivated in the world is rather small compared to orange or mandarin trees; moreover, many local varieties from different countries are very similar. Some of the most important varieties worldwide are: ‘Eureka’, ‘Lisbon’, ‘Verna’, ‘Fino’, ‘Femminello’, ‘Villafranca’, ‘Interdonato’ or ‘Génova’ (Soler-Aznar & Soler-Fayos, 2006). Among the most relevant varieties of Spanish origin of lemon trees, the native varieties of the region of Murcia ‘Fino’ and ‘Verna’ stand out. These two varieties concentrate 98.4% of the cultivated area of lemons in Spain (Aguilar-Hernández et al., 2020). The importance of adequate rootstocks in the cultivation of citrus fruits is vital for the development of the plantation. Citrus rootstocks affect many external and internal characteristics of the

fruit, such as size, shape, skin thickness, juice content, total soluble solids, and phytonutrient composition.

- "Fino" lemon crop

It probably derives from 'Comunes' type lemons from the Vega Alta del Segura. It is the most important variety in Spain. The 'Fino' variety is mainly divided into two clones; 'Fino' 95 and 'Fino' 49 (Soler-Aznar, 1999). They flower intensively only once a year, normally between the first ten days of April and the beginning of May. Flowering starts later than that of the 'Verna' lemon tree and for a shorter period. There is also a second summer flowering ('redrojos'), which is very scarce. The harvesting period for clone 95 usually extends from the end of September to mid-January while clone 49 extends from the end of December to April (Pérez-Tornero & Porras, 2009). The fruits of this flowering are much thicker than those of normal harvest. The first fruits achieve high prices in international markets due to the lack of production at this time in competing countries, hence the interest in obtaining early production of this variety (Pérez-Pérez et al., 2005). In general, plantations do not present production problems.

Its leaves are longer and wider and the fruits are better formed with a smoother and thinner rind. Its shape varies from spherical to oval (elongated) and its mamelon is pointed and small. At the insertion of the fruit to the peduncle, the base has no neck. The fruit is medium-sized and the pale yellow flesh is very juicy (García-Lidón et al., 2003). Due to its high juice content and high acidity, this variety is highly appreciated for the citrus derivatives industry. The fruit keeps less well on the tree than 'Verna' and is less resistant to transport. However, the modernisation of refrigeration, handling and transport systems is making this variety more and more demanded in the market, so its cultivated area is continuously increasing.

- "Verna" lemon crop

It is the second most important variety in Spain and the fifth in the world. It is also cultivated in North African areas such as Algeria and Morocco. Depending on the climatology of the place where it is found and the cultural techniques, this variety can flower again with greater or lesser intensity. The main flowering period, which gives rise to the fruit to be harvested, is very long, as it extends from March to May, depending on the climatology, location and physiological state of the trees, this evolution is faster, with the period of maximum flowering occurring with the increase in temperature in April-May (García-Lidón et al., 2003). In any case, from the appearance of the first flowers to the last ones may take one to two months, so the development of the fruit to which they give rise is very uneven. Harvesting is staggered, starting in March-April and ending in July (Pérez-Pérez et al., 2005). Another flowering takes place in August-September, the fruits of which are called 'rodrejos' and are harvested in the summer of the following year. These fruits have a thinner and smoother skin, are more rounded and have a pale green colouring, unlike the first flowering. On the other hand, between spring and summer there is

occasionally a flowering whose fruits are known as 'seconds' or 'sanjuaneros'. They are rougher and do not keep well on the tree and are not highly valued on the market.

The leaves are acute at the apex and smaller than in 'Fino'. The fruits are oval-shaped, elongated, with a neck at the base, large, elongated and pointed apical mamelon (Agustí-Fonfría, 2003). The size of the fruit varies depending on the area, climate and crop practices. The colour of the fruit is intense yellow when ripe. The rind is very rough and adherent, the central axis is medium and solid, the pulp is juicy and the acidity is not very high. The number of seeds is low. The fruit is highly resistant to transport and can be kept on the tree for a long time.

1.2.2. Socio-economic context

Citrus is one of the most important fruit crops in the world. Lemon (*Citrus limon* (L.) Burm. f) is a fruit with a great acceptance by consumers which leads to an increase in the production of their crops. The third most important species of citrus after orange and mandarin are lemons, with a total production more than 21.5 million (m) tonnes during 2022 year around the world and a planted area of 1.3 m ha for lemons and limes, according to the Food and Agriculture Organisation of the United Nations (FAO). Nowadays, the main lemon producing countries are located in tropical and subtropical areas, being India and Mexico the 1th and 2nd world lemon producers. Europe, with 1.4 m tonnes in 2022, is the 6th main producer in the world ranking Spain the 1th producer country with 863.2 k tonnes (which accounts for about 60% of all EU production) and 52.6 k ha cultivated, ahead of Italy and Greece (FAO, 2022). Spain is both the main lemon producing country in the Mediterranean basin and is the greatest exporter of fresh fruit in the world. The areas under citrus in Spain are in the coastal zones of the east and the south, mainly Andalucía, Valencia and Murcia, zone which is defined by the absence of frosts.

Argentina	Spain	USA	Turkey	Italy	South Africa	Other countries
Fresh	Fresh	Fresh	Fresh	Fresh	Fresh	Fresh
311471	788188	623000	758100	423700	258342	650000
Processed	Processed	Processed	Processed	Processed	Processed	Processed
1159958	247522	211000	36600	79800	95873	240000
Production	Production	Production	Production	Production	Production	Production
1471429	1035711	834200	794700	503500	354216	890000

Total: Fresh: **3813002** Processed: **2070753** Production: **5883755**

Figure 1. World lemon production and processing average 2010-2020 in metric tonnes. Source: Ailimpo. Accesed April 2024.

According to MAPA data, the forecasts for the 2022/23 season will produce a volume of 5.8 m tonnes of citrus fruit, which 900.5 k would correspond to lemons. This represents a drop of more than 1m tonnes compared to the previous season, making it the lowest since the 2012/2013 season. This is due to the adverse and extreme weather conditions in the Spanish production areas from flowering and fruit set (excess rainfall), as well as during fattening (very high temperatures, no rainfall

and limited irrigation). In Andalucia, lemon production was reduced by 30% compared to the previous season due to the impact of the drought and the limitations imposed on irrigation. This situation is also observed in the other lemon producing countries with lower yields due to unfavourable weather in Argentina, Mexico and Turkey (Source: USDA).

For other hand, Spain is the major exporter of citrus fruits, with a very marked difference in volume between exports and imports (Spain is the world's leading exporter of fresh citrus fruits, accounting for around 25% of world exports). The evolution of exports in terms of volume shows a downward trend, more pronounced in the 2022/23 season, while in terms of value it is positive. The evolution of the value of Spanish citrus exports in recent seasons has shown an upward trend, tending towards stability in the last four seasons and even a moderate decrease in the last season as a result of a short production. It presents an average trade balance in the 2017/18 to 2021/22 seasons of almost 3.2 m euros. The total volume of citrus exported during the 2022/23 season amounted around 3 m tonnes, with a value of more than 3.5 billion euros. Imports amounted to 352.8 k tonnes, resulting in a trade balance of 3.2 million euros. Lemons represent 22.5% of this value with 786.6 k euros.

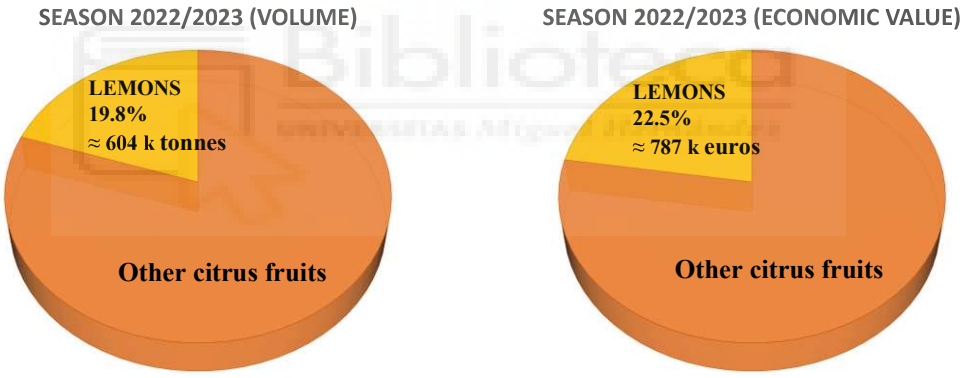


Figure 2. Spanish lemon exports in volume and economic value for season 2022/2023. Source: MAPA. Accesed April 2024.

In 2020, an EU-funded campaign called 'Welcome to the Lemon Age' was created in view of the importance of lemon consumption in Europe. Of the citrus exported, almost 86% is within Europe, with Germany being the main destination, and 14% outside Europe. If we consider the United Kingdom, this rises to 93.7%. And if we add the Norway-Switzerland-Liechtenstein aggregate, it rises to 96.8%. In economic value, the percentage reported by the EU is similar. The monthly distribution of lemon exports remains homogeneous throughout the season. The quantities imported in the 2022/23 season have increased significantly, both in relation to the previous season and to the average, as a result of the reduction in national production. The same occurs in relation to the value, being in both cases the highest of the last ten seasons. However, in the case of lemons, this increase is not representative, as it was rather reduced compared to the last seasons.

According to the 2023/2024 crop estimate on the map, the data provided by the main producing regions show that the citrus crop for the 2023/24 season, with 5.8 million tonnes, will be slightly higher than last year, but 14.4% below the average of the last five years. However, while oranges and small citrus fruits fall back significantly in relation to the average (more marked in the case of the former), lemons and grapefruit increase their production, notably lemons with 28% more production compared to the previous season, rising from 913 k in 2022/2023 to 1.2 m tonnes in 2023/2024. Although the trend in volume of exports is moderately downward, the value and balance show a slightly upward trend. According to the USDA, 10 million tonnes of lemons could be reached in the 2023/24 season with increased production from the EU, Turkey and South Africa. This increase in the European Union would be mainly provided by Spain, as Italy decreases and Greece remains stable.

1.3. Lemon fruit characteristics

1.3.1. Fruit anatomy and nutritional properties

The fruit of the lemon tree, commonly known as lemon, is a hesperidium berry, which arises as a consequence of the growth of the ovary and is formed by approximately ten carpel units or segments joined around the floral axis by which they contact each other, thus forming locules (or segments in mature fruits) containing the seeds and the juice sacs or vesicles (Agustí-Fonfría et al., 2000). The exocarp or flavedo is the external part of the fruit, consisting of an external waxy layer with a mottled appearance due to the presence of oil glands. It contains the pigments, chlorophylls or carotenoids, and is formed by an epidermis, covered with cuticle and several layers of cells joined to them forming a compact parenchyma (Ancillo & Medina et al., 2015). This is the most external part of the fruit and the one that acquires the characteristic colouring, depending on the ripeness of the fruit. The endocarp is the innermost part of the pericarp and consist of the membrane of the locules with the juice sacs. Between exocarp and endocarp is located the mesocarp or albedo, of spongy appearance as it is formed by a parenchymatous tissue with large intercellular spaces. At the ends of the fruit is the pedicle (calyx), the absence of which is a serious problem during marketing, and the opposite part, known as mamelon, which is the source of many quality problems due to its exposure to mechanical damage.

Lemons have long been recognized not only for their rich nutritional profile but for their potential health benefits. Extensive research has delved into the functional composition of *C. limon* fruit, covering not just the entire fruit but also its individual components such as the pericarp, juice, pomace, and essential oil. Analysis has also been extended to the leaves and the fatty oil extracted from *C. limon* seeds. The primary constituents of the fruit, namely the essential oil and juice, are renowned not only for their delightful flavour but also for their abundance of bioactive compounds, including carotenoids, flavonoids, and ascorbic acid (Ma et al., 2020). The content of phenolic compounds increases during the early stages of fruit development to decrease in the cell enlargement phase and during ripening (Multari et al., 2020). In seeds, total limonoids always remain in high concentration inside the seeds. These bioactive compounds found in lemons have been extensively studied, showing a myriad of health-promoting properties. The most important group of bioactive compounds in both *C. limon* fruit peel and juice, determining their biological activity, are flavonoids, such as eriodictyol, hesperidin, hesperetin, and naringin; flavones, apigenin and diosmin; and flavonols such as quercetin, and their derivatives (Saini et al., 2022). The whole fruit contains additional flavonoids, such as limocitrin, spinacetin, orientin, and vitexin. The flavonoids neohesperidin, naringin, and hesperidin are characteristic of *C. limon* fruit. Eriocitrin content is notably elevated in *C. limon* compared to other *Citrus* species (Klimek-Szczykutowicz, et al., 2020). Phenolic acids are another important group of compounds found both in the juice and peel. There are mainly two cinammic acids in the concentrated juice, ferulic acid and synapic acid, and their derivatives (Russo et al., 2014). In contrast, the presence of p-hydroxybenzoic acid has been confirmed in the peel. In the peel, there are also coumarin compounds, carboxylic acids, carbohydrates, as well as amino acids, a complex of B vitamins, and, importantly, ascorbic acid (vitamin C).

Regarding its nutritional composition, lemon is a fruit with a very low caloric value (29kcal/100g) and with a highwater content, in the pulp it reaches 90 % (USDA, 2020). The most abundant macronutrient is carbohydrate, of which the sugar content, when ripe, is composed of 44 % sugars, 33 % celluloses (lignins) and 20 % pectins and glycosides in the dry matter. Potassium, followed by calcium and phosphorus, are the most abundant minerals. Small amounts of zinc, iron, copper and manganese have also been found. As it is well known, the main vitamin is ascorbic acid or vitamin C, which usually ranges between 20-50 mg/100 ml juice. For the marketing of lemons, the most important parameter is the percentage of juice, according to Regulation (EU) 543/2011, which must be higher than 25 % in the case of 'Fino' lemons and 30 % in the case of 'Verna' lemons (Pardo et al., 2015).

1.3.2. Lemon maturity process and quality parameters

The process of maturity is defined as the set of biochemical changes in taste and texture that a fruit undergoes during the last phase of development (Agustí-Fonfría et al., 2000). Lemon maturity is a genetically programmed physiological process involving a series of biochemical changes, which modify physical, chemical,

nutritional and functional properties. Many biochemical pathways during growth and ripening are critical factors in determining ripening rates and harvest timing, which are also affected by climatic conditions and agronomic practices (Agustí-Fonfría et al., 2000). The determination of maturation criteria for citrus fruit is complex, as it involves two quite different tissues and systems: the internal changes that occur in the fruit flesh and the external colour modifications that take place in the skin of the fruit (Lado et al., 2014). Therefore, they are considered ripe when their external colouring, juice content and soluble solids: acidity ratio and other internal constituents have reached a minimum level of organoleptic acceptability and palatability. Moreover, citrus fruits show a non-climacteric ripening behaviour and should be harvested when internal maturity has been reached, as no further relevant changes in fruit composition occur after harvesting (Agustí et al., 2003).

Color is an important attribute in citrus fruit quality which influences consumer perception and acceptation. Immature fruits are green in colour and during ripening, chlorophylls degrade and yellow pigments in the skin start to increase their presence (Lado et al., 2014). The same trend is observed in the colour of the juice. Therefore, the total carotenoid content of both rind and juice increases as the fruit ripens. When, for commercial reasons, the fruits are harvested before reaching the yellow colour, the post-harvest treatment with ethylene is the most applied method, since it is able to activate enzymes responsible for the degradation of chlorophylls producing a uniform external colouring. Firmness is another of the most important attributes for the consumer. During the initial development of the fruit there is a rapid increase in pectin content in both rind and flesh (Tadeo et al., 2008). Their total content remains almost constant until ripening, after which the water-soluble pectin content increases. The acidity of the juice is largely due to citric acid. Free acids increase in the fruit during the first stages of development and remain practically constant until ripening, unlike what happens with other citrus fruits such as oranges and mandarins, where they decrease when they reach ripening. Citric acid remains constant during this process and can reach levels of 60-70% of the total soluble solids. The rest of the soluble solids content are mostly sugars, the most abundant being sucrose, glucose and fructose (Di Matteo et al., 2021). In early varieties, the sugar content increases rapidly as the fruit ripens. But in late varieties, ripening occurs when the temperature tends to rise and the sugar content hardly increases at all. The ratio of total soluble solids to acidity is called the maturity index and is a parameter that is the basis for determining the commercial maturity of the fruit, as well as its organoleptic index (Lado et al., 2018).

The fruit juice content is also related to the ripening process, as it increases as the fruit ripens until it reaches a maximum value at full maturity. The criteria for using this quality parameter as a commercial standard index depends on the citrus species and the market destination. In the EU, juice percentage is the only fruit maturity standard applied to lemons, which must be at least 20-25% (Lado et al., 2014).

1.3.3. Potential health benefits in lemon fruit

Lemon has a high biological activity mainly due to its phenolic compounds, although some chemical groups such as lignins or pectins also play a role. Among the many potentially beneficial activities for health, we highlight the following:

- Antioxidant activity

The antioxidant activity of lemon is primarily attributed to its rich content of bioactive compounds, including vitamin C, flavonoids such as hesperidin and hesperetin, and limonoids. These compounds exhibit potent antioxidant properties by scavenging harmful free radicals, reducing oxidative stress, and protecting cells from damage (Rafique et al., 2020). Vitamin C, also known as ascorbic acid, is a powerful antioxidant present in high concentrations in lemon juice. It neutralizes free radicals by donating electrons, thereby preventing oxidative damage to cells and biomolecules like DNA and lipids (Xavier et al., 2020). Flavonoids, particularly hesperidin and hesperetin, possess strong radical scavenging abilities and can enhance the body's antioxidant defenses by activating cellular signaling pathways like the ERK/Nrf2 pathway (Parhiz et al., 2015). For other hand, coumarins have been shown to possess strongly antioxidant activities because of their phenolic hydroxyl groups in molecule structure and direct decrease the cellular free radical production by inhibiting xanthine oxidase (Zou et al., 2016).

- Anti-obesity and Cardiovascular activity

Certainly, lemon exhibits several properties that may contribute to its potential anti-obesity effects. Lemon juice was used in a low-calorie diet study (‘lemon detox diet’) and the results showed that *C. limon* juice caused a reduction in serum high-sensitive C-reactive protein (hs-CRP) in comparison with the placebo and normal diet group. Hemoglobin and hematocrit levels remained stable in the group on the lemon detox diet, while they decreased in the placebo and normal diet groups (Kim, M.J. et al., 2015). Other studies have shown that *D*-limonene is beneficial to people with dyslipidaemia and hyperglycaemia preventing the accumulation of lipids, and affects the blood sugar level (Millet, 2014). Its antioxidant action enhances these effects. Dietary supplementation with *D*-limonene would restore pathological alteration of the liver and pancreas. Furthermore, the use of lemon fermented products can reduce the body weight of rats and lipid accumulation by regulating the mRNA expression of some genes that play critical roles in adipogenesis, lipogenesis, lipolysis, and energy metabolism. This ability to regulate lipid metabolism, is presumed to be caused by the increase in the content of limonene and total polyphenol (Wu et al., 2021). These findings suggest that lemon products could help in the prevention of obesity. For other hand, the plasma triglyceride levels of the high-cholesterol-fed rats administered with the citrus fruit juices reveal a decrease in triglyceride level with increased administration of the citrus fruit juices when compared to untreated ones (Oboh et al., 2015). There was a decrease in total cholesterol which is in line with earlier reports where citrus juices were shown to possess cholesterol lowering ability (Actis-Goretta et al., 2003;

Gorinstein et al., 2005). This hypocholesteromic effect could be part of the mechanism by which the *Citrus* could prevent cardiovascular diseases

- Anticancer activity

The anticancer properties of Citrus limonoids have been demonstrated in aqueous extracts of the fruit, containing compounds that protect cells from the damage implicated in cancer development. Research has indicated that isolating *C. limon* nanovesicles from fruit juice can inhibit the proliferation of cancer cells across various tumor cell lines by triggering TRAIL-mediated apoptotic cell death (Raimondo et al., 2015). Furthermore, studies have revealed that extracts from lemon seeds induce apoptosis in human breast adenocarcinoma (MCF-7) cells, thereby impeding proliferation. These findings suggest that both aglycones and glycosides of limonoids and flavonoids present in the extract hold potential as chemopreventive agents for breast cancer. Additionally, polymethoxyflavones (PMFs) found in citrus peels, as reported by Wang et al. (2008), have demonstrated activity against cancer incidence in both in vivo and in vitro systems. These studies shed light on the phytochemical content, antioxidant properties, anticancer effects, immunomodulatory potential, and antigenotoxic activities of lemon, grapefruit, and mandarin citrus peels (Diab, 2016).

1.3.4. Ripening and Senescence

The metabolic process continues during post-harvest leading to a quality losses stage, in a more or less accelerated way depending on the variety and the stage at which the fruit has been harvested. It involves a series of changes in the chemical composition of the fruit, such as hydrolysis of polysaccharides, hydrolysis of cell wall components, increased permeability of cell membranes, changes in organic acids, increased aroma or changes in pigmentation (Martínez-Hernández et al., 2017). More specifically, some of the quality parameters that are affected during postharvest storage in lemons are weight loss or texture (Sen et al., 2020). These losses vary greatly depending on storage conditions and are mainly due to losses in juice content, which gradually decreases during this stage (Lado et al., 2014). Both total soluble solids and acidity suffer a decrease in their content, however, this loss requires many days to be observed. Ascorbic acid content of stored fruits also decreased during storage, as well as other bioactive compounds such as phenols (Lu et al., 2023). All these changes are not accompanied by increases in respiration or ethylene production. Some enzymes such as peroxidase (POD), ascorbate peroxidase (APX) and catalase (CAT) decrease during this stage (Zapata et al., 2014), relating to this process in which overripening occurs, decreasing quality and shelf life and increasing the incidence of decay.

1.4. Changes in fruit quality during post-harvest storage

The application of cold contributes to slowing down metabolism, reducing transpiration, and consequently delaying senescence (Lado et al., 2014). Low temperatures also allow another of the causes of quality loss during post-harvesting,

namely the emergence and proliferation of microorganisms, to be reduced (Schreuder et al., 2018). However, applying low temperatures can also negatively affect the fruit, favouring the appearance of skin blemishes or dehydration (Lafuente et al., 2005). According to the optimal storage conditions recommended by Kader et al., (2002), the most suitable temperature for refrigerated storage of lemons is between 10 and 13 °C. Although low temperatures are essential to maintain the quality of the fruit, additional treatments are necessary to improve the results, as cooling strategies alone do not sufficiently stop the metabolic processes that determine ripening and senescence, or the attack of pathogenic micro-organisms (Schreuder et al., 2018).

1.4.1. Cold Damage or Chilling Injury

Spain is one of the main exporters of fresh lemon fruits worldwide (FAOSTAT, 2022). This situation imposes high internal and external quality standards on lemon production in Spain. It becomes critical every step of the post-harvest chain, from harvesting to transport and storage, in order to prevent losses, guarantee quality, meet market requirements and supply fruits that not only have an adequate commercial presentation, but also high organoleptic and nutritional quality standards.

Throughout the production chain, there are several causes that can affect fruit quality, resulting in significant losses in their commercialisation (Porat et al., 2018). These alterations, caused by abiotic factors, are known as physiological disorders, and can occur both during pre-harvest due to climatic factors, growing conditions, or nutritional and water deficiencies, and during post-harvest handling of the fruit (Lado et al., 2019a). The most common physiological disorders in citrus fruit postharvest can be divided into two groups: those related to chilling injury (DF), caused by exposure to very low storage temperatures (< 5 °C), and those that develop at room temperature or above 10 °C (Lado et al., 2019b; Zacarias et al., 2020).

Storage at low temperatures (1-5 °C) is one of the most widely used post-harvest technologies to preserve the quality and extend the post-harvest life of citrus fruits during transport and marketing, as it slows down cell metabolism, delaying fruit ripening and senescence (Lado et al., 2019b; Zacarías-García et al., 2020). Certain countries, such as the United States or China, require quarantine treatment at very low temperatures (1-2 °C) for fruit export as a preventive degree to eliminate pests (Lado et al., 2019b). These low temperature conditions can last for periods of more than two weeks during transport, and pose a challenge for fruits of tropical or subtropical origin, such as lemons, which are sensitive to low temperatures and can develop lesions of different symptomatology during prolonged exposure to cold (Zacarías et al., 2020), which becomes more visible when fruits are transported at warmer temperatures (Lafuente et al., 2005). The damage may vary according to the species, agronomic techniques applied in the crop and climatic conditions (Lo'ay & Dawood., 2019). The set of these lesions is known as "chilling injury" (DF), and is a disorder of great importance in postharvest, as it reduces the external quality of the

fruit and, consequently, its marketability, as well as predisposing the fruit to pathogen infection.

1.4.2. Peteca and Oleocellosis

The symptomatology of chilling injury on citrus fruits is diverse, but one of the most common macroscopic symptoms is "peel pitting". In lemons they appear as individual indentations distributed over the surface, which is known as "peteca". Peel pitting is a physiological disorder in which the cells of the fruit epidermis collapse, showing reduced size, irregular shape, smaller and ill-defined organelles, and vacuoles filled with a dense, dark material, while the cells of undamaged fruit or areas of the fruit remain intact, with their structures and organelles well-defined. The most characteristic symptoms are stinging, sagging or darkening of the skin, which may worsen if the glands containing the essential oils are ruptured (Cronjé, 2015). Both weather and cold conditions prior to harvest influence the occurrence of butterbur. In addition, nutritional imbalances have been shown to contribute to an increase in the disorder, e.g. with high calcium and low potassium levels (Undurraga-Martínez et al., 2006). Using edible coatings with low moisture permeability increases the occurrence of butterbur. However, the causal mechanisms and factors influencing fruit susceptibility remain unclear (Cronjé, 2015).

On the other hand, oleocellosis is one of the most common pathophysiologies of lemons. It is a disorder that commonly occurs in all citrus fruits, but is more sensitive when the fruit is harvested when green (Knight et al., 2002). Due to the hydration of the skin, the glands with the oil are more exposed so harvesting is avoided early in the day, when dew is very abundant or after rainfall (Scherrer-Montero et al., 2011). The release of the essential oil has a toxic effect on the surrounding cells producing characteristic stains, resulting in a brown necrotised area with an irregular appearance as can be seen in Figure 4. At the biochemical and physiological level, it seems that the development of oleocellosis is slightly influenced by the antioxidant activity of certain compounds present in the flavedo, such as carotenoids (Yongqiang et al., 2010).

1.4.3. Fungal Diseases

Fungal diseases are the most important source of fruit damage and post-harvest losses (Palou, 2014). The main microorganisms that develop rots in citrus are fungi, since the low pH prevents the proliferation of other microorganisms (Eckert & Eaks, 1989). They are usually filamentous, heterotrophic fungi, which absorb nutrients obtained from the release of extracellular enzymes. The hyphae form the vegetative structure of the fungi, giving them a cottony appearance due to their apical and branched growth (Valencia-Chamorro, 2011). The amount of inoculum is decisive for effective disease development, but other factors such as the susceptibility of the fruit itself or environmental conditions also play a role (Martínez & Hernández, 2007). Most of the fungi that cause diseases in lemon come from an infection produced in the field, however, there are some species whose development is associated with post-harvest, the main ones being:

- *Penicillium digitatum* (Green Mold)

P. digitatum is the main pathogenic fungus of citrus fruits. The main characteristic of this fungus lies in the green colouring of the spores it produces when it infects a fruit, which results in a relatively simple visual identification, as can be seen in Figure 4A. Infection can be initiated at different points during the processing of the lemon, e.g. during its stay in the fruit and vegetable plant or during the marketing chain (Palou, 2014). Infection usually occurs through open wounds where nutrients are available to stimulate fungal growth and spore production (Davies & Albrigo, 1994), being unable to infect other adjacent fruit unless they are previously damaged (Barmore & Brown, 1982). The initial stages of the disease appear as a white, watery and soft spot due to the degradation of pectins, but as it progresses the mycelium (white) starts to be observed and the sporogenous structures appear, changing the colour of the mycelium to greenish tones, when sporulation occurs (Barkai-Golan, 2011). The conidia or spores (main source of contamination) are dry and can be easily separated from the rest of the structure, causing powdery clouds to form, which facilitate their spread (Smilanick et al., 2019). The fungus develops rapidly at temperatures around 20-25°C, however, below 10°C and above 32°C and growth stops almost completely (Smilanick & Mansour, 2007).

- *Penicillium italicum* (Blue Mold)

P. italicum (moho azul) is another of the main causal agents of citrus rot, and is the second most prevalent after *P. digitatum*. This pathogenic fungus infects fruit through skin lesions, similar to *P. digitatum*, but its development is notably slower (Parra & Martinez, 2017). It is common to observe several pathogens developing simultaneously on the same surface of the fruit due to their similarities, but one of the most evident distinctions is the light blue colouring as can be seen in Figure 4B, which is produced due to the spores that form a dry layer, easily separable and that, due to their abundance, constitute small powdery masses (Tuset, 1987). This peculiarity allows it to be visually differentiated from green mould. At the same time as the asexual reproductive part of the fungus forms on the outside, the fruit tissues invaded by the mycelium of the pathogen become disorganised as a result of the dissolution of the pectins that make up the cell walls. Liquids are lost and the fruit becomes soft and completely wrinkled. Another difference between the two fungi is that *P. italicum* prefers developing under refrigerated conditions, especially at a temperature of about 10°C (Palou et al., 2008).

- *Geotrichum citri-aurantii*

Sour rot is an important disease that occurs in citrus fruit after harvest and is caused by the fungus *Geotrichum citri-aurantii* (Ferraris) (Butler et al., 1988). This fungus, like those mentioned above, infects through lesions in the skin of the lemon. However, its development is concentrated in the innermost layers of this tissue, specifically under the albedo. The first signs of infection appear as watery and transparent lesions (Figure 4C). These lesions progress to rupture of the skin due to

the accumulation of gases generated during infection, eventually resulting in the "peeling" of the lemon. During infection, affected fruit often emit a distinctive bitter odour, which attracts flies and contributes to the spread of the disease. (Pompeo-Ferraz et al., 2016). This pathogen tends to develop rapidly in warm conditions, reaching its optimum growth at 27 ºC. However, its development is significantly suppressed at temperatures below 10 ºC (Rodri et al., 2004a). It is the third most prevalent pathogenic fungus on lemon, although in periods of high humidity in the field its incidence may exceed that of green and blue moulds (Nazerian & Alian, 2013). It is a fungus that is present in the soil of plantations and after rainfall, due to splashing, its load on the surface of the fruit increases drastically (Suprapta et al., 1995).

Figure 4. A) Lemon infected by *Penicillium digitatum.* B) Set of lemons infected by *Penicillium italicum.* C) Lemon infected by *Geotrichum citri-aurantii.*

1.5. Postharvest technologies for lemon quality maintenance

Traditionally, there has been a search for safe and effective alternatives to control quality losses both before and after harvest. Plant pathogens and pests can have a major impact on agricultural productivity. Plant diseases reduce yields by 21-30% in several important crops worldwide (Savary et al., 2019). Therefore, finding a functional option for a wide range of horticultural products that is accepted by both industry and consumers is an urgent problem to solve. The control of pathologies occurring during the post-harvest of fruit and vegetables requires multiple interventions at different points in the process. It is therefore necessary to make a symbiosis between the use of biological agents and alternative technologies, which can be physical, chemical and biological, and integrate them into the production chain to achieve better results (Wieniewski et al., 2016).

1.5.1. Pesticides

In Spain there was a law (Real Decreto 3349/1983) in which pesticides is defined as any substance or mixture of substances intended to protect crops or plant products against all harmful organisms or to prevent their action, or to exercise control over them, provided that they do not include plant protection products of an exclusively physical or mechanical nature. Although they are effective, European

legislation is currently restricting their use as they can be harmful to health. In addition, there is a high level of public sensitivity towards chemically synthesised substances. (Wieniewski et al., 2014). There is a clear trend towards banning many of the compounds used in conventional production, such as the recent case of propiconazole. In order to regulate the sustainable use of pesticides in Spain and to establish degrees to reduce the risks and negative effects connected to their use, Royal Decree 1311/2012 was created. The most widely used pesticides for the control of fungi in fruit and vegetables are fungicides. They can be applied in different ways, through showers or waxes, immersing the fruit in the solution, spraying it with aerosols or with gas, which is the least common way. (Ons et al., 2020). Imazalil is one of the most widely used fungicides in citrus as it is very effective in inhibiting the growth of *P. digitatum* (Ismail & Zhang, 2004). Other compounds authorised by the European Union are also used to reduce the incidence of pathogenic fungi during production and post-harvest of conventional lemons, such as Pyrethamethanil, Orthophenylphenol or Thiabendazole.

1.5.2. Physical, Chemical and Biological Methods

Heat treatments: Postharvest heat treatments of fruit are used for insect disinfestation, disease control, to modify fruit responses to other stresses and to maintain fruit quality during storage (McDonald et al., 1999). It is a strategy that achieves very significant inhibition of pathogenic fungal growth by applying physical heat treatments in baths or through the air. Above 30°C, Penicillium fungi slow down their growth and can therefore be very effective in controlling this infection (Gocheva et al., 2006). However, in order to apply heat treatment, high humidity conditions must be used so that dehydration does not negatively affect the quality of the fruit. (Paull & Chen, 2000)

Ultraviolet-C light (UV-C): This treatment is considered an alternative to chemical approaches with great potential to control post-harvest diseases (Cisneros-Zevallos, 2003) as at low doses it can induce disease resistance, delay ripening and improve quality attributes (Charles & Arul, 2007). By applying light at a certain wavelength, a damaging effect is produced on the cells of pathogenic fungi by modifying the membrane, causing mutations in the genetic material that affect their growth and propagation (Charles & Arul, 2007).

Modified Atmospheres (MAP): Modified Atmosphere Packaging (MAP) involves packaging food in a container whose atmosphere has been altered to increase shelf life and maintain quality. This process involves sealing through the use of plastic films with a particular permeability to gas diffusion. The composition of the gases O_2 and CO_2, which are essential for respiration, can be modified. The only gas with an effect on microbial growth is CO_2, which at certain concentrations can inhibit it. In addition, these can contain antimicrobial substances that diffuse into the atmosphere of the packaging and contribute to the optimal preservation of the product (Thompson et al., 2018).

Ozone (O_3) treatments: Ozone is a powerful oxidising agent with a pleasant odour in very dilute concentrations. O_3 treatments can be an interesting option, as it is a powerful oxidising agent, and has a harmless decomposition product, oxygen (Skog & Chu, 2001). It does not produce halogenated (toxic) compounds and its action is fast and effective against a wide range of micro-organisms. However, its management is sometimes difficult, since its effectiveness depends on a multitude of variables such as the type of microorganism, quantity or organic matter present, among others (Brodowska et al., 2018). In addition, ozone is irritating and harmful at higher concentrations. Therefore, this technology requires a particular optimisation for each fruit and/or vegetable, so that the effect on microorganisms and the control of rots is adequate.

Inorganic salts: Combinations of metal cations with single anions such as halides, sulphates, nitrates, carbonates or phosphates generate an overwhelming amount of salts, double salts and salt hydrates. These are chemicals with very low toxicity, most of them being recognised as safe compounds (GRAS). Good results have been obtained using carbon and bicarbonate salts by immersion at 2-3 % to reduce the incidence of rot in citrus (Youssef et al., 2017). The mode of action of salts on pathogenic fungi is complex, as they have a direct effect by inhibiting spore formation, and an indirect effect by stimulating defence mechanisms in the fruit (Deliopoulos et al., 2010).

Antagonistic microorganisms are those that compete with pathogenic organisms for resources or space, or produce antimicrobial compounds that inhibit the growth of pathogens. These micro-organisms can be useful in preventing or controlling post-harvest diseases in fruits and vegetables, thus helping to maintain their quality and prolong their shelf life. For this purpose, fruit-friendly micro-organisms are used. Pantoea agglomerans and Pseudomonas syringae are microorganisms that have proven to be effective in the control of *P. digitatum* and *P. italicum* in citrus (Plaza et al., 2004b).

Edible coatings: it is a common practice during the processing of citrus fruits to use edible coatings, because during the washing and handling process the fruits may lose the external waxy layer that protects them. They are usually anionic microemulsions containing resins and/or waxes. However, coatings can be adversely affected if gas exchange is excessively restricted, which can lead to an overproduction of volatiles affecting flavour. The use of essential oils or gels with different Aloe vera species have also been shown to be effective in reducing the incidence of rot and controlling senescence in citrus and pomegranate (Martínez-Romero et al., 2013; Castillo et al., 2014). The use of chitosan as a protective barrier for fruits and vegetables is increasing, it has a number of amino groups that interact with microbial cell walls causing cell lysis. In addition, it also exerts control over the rate of respiration, which is reflected in reduced weight loss (El Guilli et al., 2016).

Essential oils: a group of compounds with high antimicrobial activity. The use of essential oils such as thymol, eugenol or carvacrol to reduce and/or control the

incidence of fungal infections has been studied. *In vitro* and *in vivo* application of thymol, carvacrol and a mixture of both on lemon fruits reduced the incidence of *P. digitatum* and *P. italicum* (Pérez-Alfonso et al., 2012). Moreover, when applied in packing lines, they prevent lemon spoilage and maintain quality during storage (Castillo et al., 2014). However, essential oils are very volatile, so application by encapsulation in cyclodextrins was tested. The encapsulation of thymol and carvacrol applied to lemon showed to be effective in the reduction of *Geotrichum citri-aurantii* (Serna-Escolano et al., 2019a; 2020).

Elicitors: are classified as biotic, abiotic and plant hormones (Baenas et al., 2014). The plant hormones include among others: Gibberellin (GA3), ethylene (ET), methyl jasmonate (MeJa), methyl salicylate (MeSa), salicylic acid (SA), acetyl salicylic acid (ASA) and oxalic acid (OA). Post-harvest application of GA3 to lemons resulted in increased firmness and endogenous polyamine levels, also delaying colour loss during storage (Valero et al., 1998). In lemon, pre-harvest application of MeJa increased antioxidant systems without affecting yield or fruit quality (Serna-Escolano et al.,2019b). The diamine putrescine and the polyamines spermidine and spermine are ubiquitous in plant cells and have regulatory effects on various plant growth and development processes. Putrescine influences the postharvest physiology of fruits according to research carried out by our group. Post-harvest vacuum infiltration of putrescine in lemon showed an effect of increased firmness and decreased weight loss (Valero et al., 1998; Martínez-Romero et al., 1999).

1.6. New elicitation strategies

In the last few years, γ-aminobutyric acid (GABA) and melatonin was recognised as an elicitor for its application in crops affected by different abiotic stresses related with the climate change such as low temperatures (chilling or freezing), high and fluctuated temperatures or flooding, among others. However, as far as we know, its application on lemon fruits has not been well studied.

1.6.1. γ-aminobutiric acid (GABA)

Research has revealed the effect of certain phytohormones such as gibberellins, abscisic acid, cytokines, auxins and ethylene on plant metabolism, growth, development and fruiting, among others. This has made it possible to identify the presence of important metabolites, including γ-aminobutyric acid (GABA). It is a 4-carbon molecule considered to be a non-protein amino acid. Initially, GABA was discovered in potato tubers (Dent et al., 1947) and later in animals, where it was first identified in the brain (Roberts & Frankel, 1950). The ubiquity of GABA can be established by the fact that it has been found in higher plants, lower plants, animals and bacteria. Research gained further momentum in plants, when it was found that GABA concentration increases under abiotic stress (Fait et al., 2008). Some studies indicate that its function is necessary for proper growth in response to abiotic stresses, such as lack of light and salinity (Michaeli et al.,2011; Renault et al., 2013).

γ-aminobutyric acid is produced via the GABA shunt pathway, a metabolic pathway first described in plants (Dent et al., 1947). The importance of GABA shunts has been reported, both for the production of GABA as a metabolite and for maintaining optimal endogenous levels (Hasan et al., 2021). The synthesis consists of three key reactions catalysed by the cytosolic enzyme glutamate decarboxylase (GAD) and the mitochondrial enzymes GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) (Shelp et al., 1999). Initially, GAD catalyses the α-decarboxylation of glutamate to GABA in an irreversible reaction that is the rate-limiting step in GABA synthesis (Figure 5). This GABA is converted to succinic semialdehyde (SSA) by GABA-T and subsequently SSADH converts SSA to succinate (Fig. 5). GABA-T depends on pyruvate and glyoxylate as amino acceptors, thus producing alanine and glycine, respectively. Succinate produced by SSADH can enter the Krebs cycle and/or participate in the electron transport chain in the mitochondrion as an electron donor (Shelp et al., 2021). It has also been observed that SSA can be converted to γ-hydroxybutyrate (GHB) via the enzyme GHB dehydrogenase (GHBDH) in plants, animals and E. coli (Breitkreuz et al., 2003; Saito et al., 2009). Alternative pathways of GABA synthesis from putrescine and other polyamines have been described (Seiler et al., 1980; Caron et al., 1987). In these, γ-aminobutyraldehyde, an intermediate of the polyamine degradation reaction, through the combined activities of diamine oxidase (DAO) and 4-aminobutyraldehyde dehydrogenase (ABALDH), leads to GABA synthesis (Wakte et al., 2011; Kim et al., 2015). On the other hand, other routes of GABA synthesis in response to abiotic stress have also been described, such as from proline via the formation of the δ1-pyrroline intermediate and via a non-enzymatic reaction (Signorelli et al., 2015).

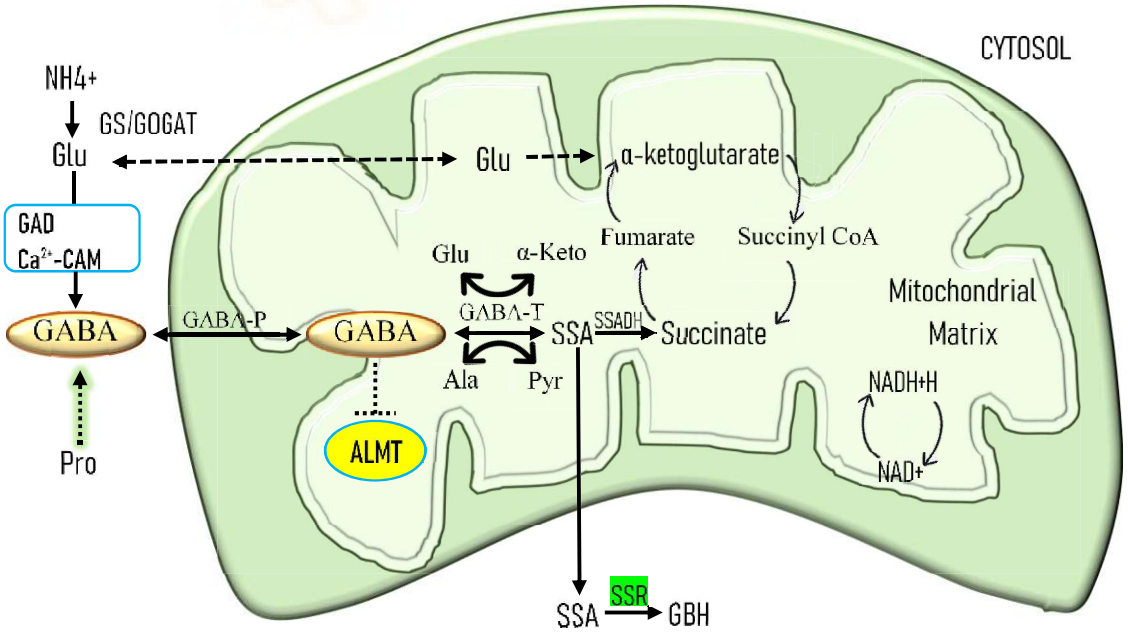


Figure 5. GABA biosynthesis in plants mediated by GABA shunts as adapted from Ramos-Ruiz et al. (2019). Abbreviations: GAD, glutamate decarboxylase; GABA-P, GABA permease; GABA-T, GABA transaminase; ALMT, aluminum-activated malate transporter; Glu, glutamate; Ala, alanine; Pyr, pyruvate; SSADH, succinic semialdehyde dehydrogenase; SSR, succinic semialdehyde reductase; SSA, succinic semialdehyde.

GABA plays a much broader role in organisms than previously thought, whether as a common signalling molecule, as a compatible solute in stress situations or as a defensive molecule in the host. Some of the main functions of GABA in plants include osmoprotective activity, defence or communication, as well as pH regulation, participation in the Krebs cycle or its role in nitrogen accumulation and C:N balance.

Glutamate decarboxylase (GAD) activity is stimulated under acidic conditions as it consumes H+ protons, suggesting a possible role for GABA in pH regulation under stress conditions (Kinnersley & Turano, 2000; Ramesh et al., 2017). GABA accumulation in response to acidic conditions has also been successfully demonstrated in the cytosol (Roleira et al., 2018). The tricarboxylic acid (TCA) cycle or Krebs cycle is another function in which the GABA molecule is involved. Under stress conditions such as hypoxia, the activity of the NADH-dependent enzyme succinic semialdehyde dehydrogenase decreases and as a consequence, succinate is not formed, so plants tend to accumulate more GABA. When stress is removed, GABA acts as a source of substrates for the Krebs cycle (Roleira et al., 2018). Another function attributed to this molecule is its involvement in nitrogen accumulation and C:N balance. GABA is considered as a temporary source of nitrogen storage, as it is produced from glutamate under stressful conditions. It has been shown that plants can grow on GABA-containing medium as the sole source of nitrogen (Li et al., 2021) thus exhibiting its role as a source of N/C needed for growth. Furthermore, the contribution to the C:N balance of GABA can be deduced from the fact that GABA can constitute almost 50% of the free amino acids in cherry tomato fruits (Rolin et al., 2000).

Several studies have shown a positive effect of pre- and postharvest application of GABA on fruits. Postharvest treatments with GABA can increase fruit firmness as in the case of blueberry or kiwifruit (Ge et al., 2018; Aghdam et al., 2019; Yan et al.,2024), and maintain or improve other quality attributes to increase fruit shelf life (Sheng et al., 2017; Asgarian et al., 2022; Aghdam et al., 2016). In addition, it can also suppress the activity and/or expression of some genes involved in fruit senescence as occurs in apples (Li et al., 2021). Another positive effect of postharvest GABA treatment is the increase of cold tolerance in fruits such as banana, cucumber or persimmon (Wang et al., 2014; Malekzadeh et al., 2017; Niazi et al., 2021; Ali et al., 2022). Antioxidant systems and functional compounds have also been affected in some GABA-treated fruits (Jin et al., 2019; Rastegar et al., 2020), showing an increase in phenolic compounds relative to untreated fruits in the case of wheat or germinated hulless barley (Li et al., 2016; Ma et al., 2019). Pre-harvest treatments have been less studied but the results showed similar effects to post-harvest treatments in tomato, apple, pistachio or sweet sherries (Zarei et al., 2020; Cheng et al., 2023; Jalali et al., 2023; Carrión-Antolí et al., 2023). In addition, they have also been shown to improve yields in crops such as pomegranate or cherry (Lorente-Mento et al., 2023; Carrión-Antolí et al., 2023).

1.6.2. Melatonin (MEL)

Melatonin was discovered in 1958 from the bovine pineal gland and identified as N-acetyl-5-methoxytryptamine (N-acetyl-5-methoxytryptamine). It is an indolamine structurally related to other important substances, such as tryptophan, serotonin, indole-3-acetic acid (IAA), etc. It is widely recognised as an animal hormone, but its recent discovery in plant biology has generated considerable interest. This is due to its widespread presence in the biological kingdom and recent data suggesting a possible physiological function in plants (Arnao & Hernández-Ruiz., 2006).

The melatonin biosynthesis pathway in plants is thought to start with tryptophan and involves four enzymatic steps catalysed by different enzymes (Back et al., 2016). The first two enzymes that contribute to the hydroxylation and decarboxylation of tryptophan, which are necessary for melatonin biosynthesis, are tryptophan decarboxylase (TDC) and tryptamine 5-hydroxylase (T5H) (Kang et al., 2010; Park et al., 2012). The penultimate step in melatonin biosynthesis is serotonin acetylation, catalysed by serotonin N-acetyltransferase (SNAT) in plants (known as AANAT in animals) (Back et al., 2016). It is now believed that the resulting serotonin N-acetylation is catalysed in plants by paralogues of AANAT rather than by homologues inherited from chlorophyceae or obtained by horizontal gene transfer (Tan et al., 2012), but the isoenzymes and gene structures involved in this process are still unclear. The final step in melatonin biosynthesis involves the O-methylation of N-acetylserotonin. N-acetylserotonin methyltransferase (ASMT) and caffeic acid O-methyltransferase (COMT) also have substrate affinity towards serotonin and N-acetylserotonin, as they first methylate serotonin to form 5-methoxytryptamine, followed by a reaction with SNAT to form melatonin (Arnao & Hernández-Ruiz, 2015; Back et al., 2016). Taken together, these observations indicate that the melatonin biosynthetic pathways in plants are more complex than those in bacteria, yeast and animals. Moreover, there may be alternative melatonin biosynthetic pathways in which as yet unidentified enzymes may be involved. These alternative pathways deserve further investigation because they are serotonin-independent, which may be beneficial for accelerating melatonin production by avoiding the inhibitory role of serotonin in melatonin biosynthesis (Tan et al., 2016).

Melatonin appears to play a more significant role in plants than in other organisms, influencing seed germination, root development, and environmental stress tolerance. Some of its most studied functions include circadian rhythm, growth regulation, and antioxidant capacity. The biosynthesis of melatonin in plants is not directly regulated by light but follows a circadian rhythm. Melatonin (MEL) seems to be involved in regulating circadian changes in the physiological processes of plants (Arnao & Hernández-Ruiz ,2006; Posmyk & Janas,2009). It also plays a regulatory role in the growth and development of plants, as demonstrated in different plant species. MEL promotes root and coleoptile growth, although its effectiveness may vary compared to auxin, depending on the concentration and type of tissue (Hernández-Ruiz et al., 2005). Regarding its antioxidant capacity, it is considered a

molecule with a powerful antioxidant power present in a wide range of organisms, from bacteria to plants and animals. Its ability to easily migrate between cells makes it effective in protecting against oxidative stress (Arnao & Hernández-Ruiz, 2015). MEL can protect against oxidative damage caused by environmental factors such as UV radiation and pollution by acting as a direct antioxidant, stimulating antioxidant enzymes, and promoting glutathione synthesis.

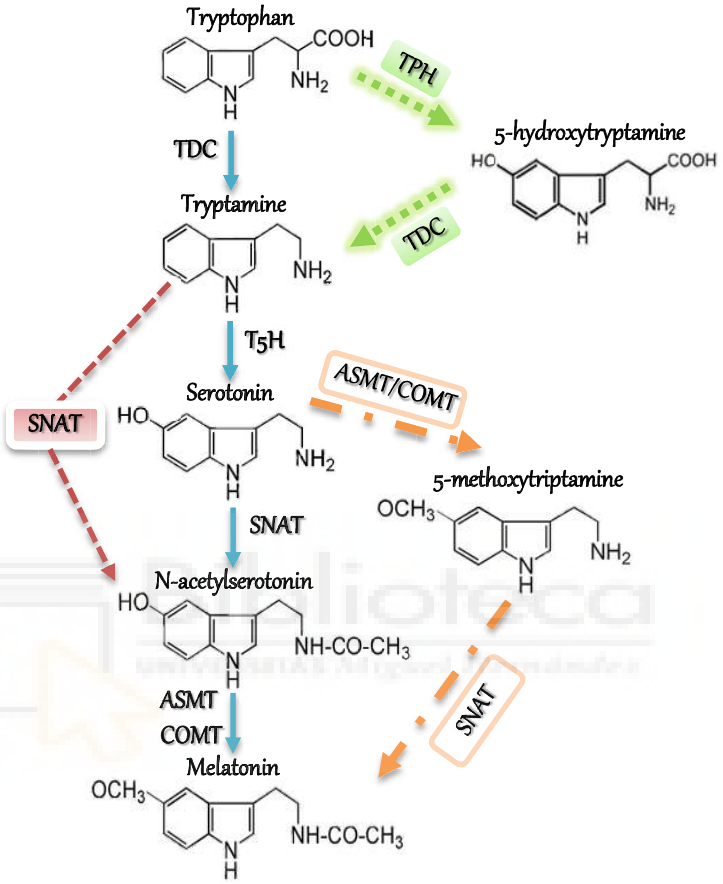
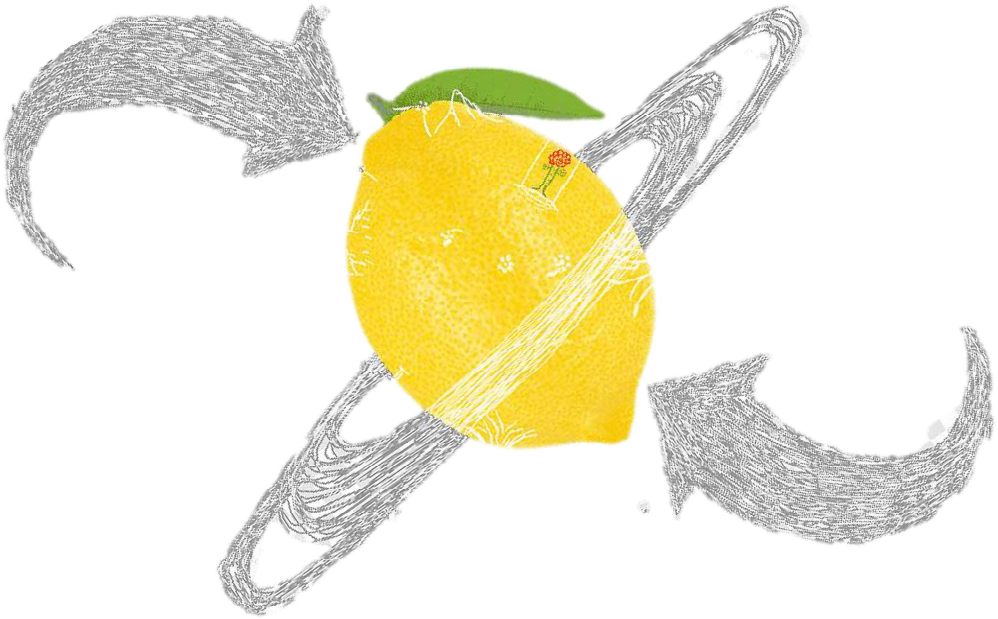


Figure 6. Biosynthetic pathway of melatonin in plants adapted from Sun et al. (2020). Abbreviations: TPH, tryptophan hydroxylase; TDC, tryptophan decarboxylase; SNAT, serotonin N-acetyltransferase; ASMT, N-acetylserotonin methyltransferase; COMT, caffeic acid O-methyltransferase. Dotted lines represent alternative reactions that occur in particular cases.

The exogenous application of MEL has been widely studied in various fruits, both pre-harvest and post-harvest. Melatonin not only can eliminate reactive oxygen species (ROS) in plants but also induces the genetic expression of antioxidant enzymes in fruits such as cherries (Xia et al., 2020; Sharafi et al., 2021). Post-harvest treatments with MEL have increased the quality of apples (Onik et al., 2021), banana (Li et al., 2019), citrus fruits such as orange or mandarin (Wang et al., 2019; Lin et al., 2019b), grapes (Gao et al.,2020), kiwi (Hu et al., 2018) and mango (Liu et al.,2020) Among other fruits, MEL treatments have been shown to decrease senescence, ripening, or decay. It also induced greater cold tolerance in various fruits (Cao et al., 2016; Jannatizadeh, et al., 2019; Kong et al., 2020; Guillen et al., 2022).

Regarding the bioactive compounds in fruits, melatonin induced an increase in anthocyanin levels, which correlated with changes in skin color in cherries (Miranda et al., 2020). In pre-harvest studies, foliar applications of melatonin have been shown to affect the levels of reactive oxygen species (ROS), counteracting oxidative damage caused by drought in tomatoes (Ibrahim et al., 2020), as well as reducing malondialdehyde (MDA) levels and relative membrane permeability in cherries (Carrión-Antolí et al., 2022a). This exogenous application is also capable of improving certain quality parameters during storage, as well as at the time of harvest, such as fruit yield or weight (Abd-El Naby et al., 2019; García-Pastor et al., 2019; Asif et al., 2020; Lorente-Mento et al., 2021; Medina-Santamarina et al., 2021). Melatonin has also demonstrated potential as a post-harvest treatment by delaying senescence in fruits and vegetables. It acts as an intermediary in the γ-aminobutyric acid metabolism pathway in strawberries (Aghdam & Fard, 2017), regulates anthocyanin accumulation in tomatoes (Sun et al., 2016), and influences ethylene metabolism in bananas (Wang et al., 2021). Additionally, a recent study suggests that melatonin maintains the normal functioning of mitochondrial structures in lotus seeds by regulating nitric oxide production through increased activity of nitric oxide synthase enzyme (Sun et al., 2021). This mitochondrial activity is crucial for maintaining cellular metabolism and providing energy to cells.

2 Aim and Objectives



2. AIM AND OBJECTIVE

Competitive markets and consumer demands oblige producers to achieve the highest levels of fruit quality at the time of harvesting and to maintain this quality during storage and distribution until it reaches the consumer. At the same time, new food consumption trends must be considered, which include a healthier and more sustainable eating style, free of chemical products that are also being limited by the health authorities.

Citrus fruits face a number of difficulties during production, processing and marketing that affect fruit quality. Crop yields without phytochemical treatments, as well as the sensitivity of lemons to chilling injury during storage, are key problems that require in-depth studies and for which solutions are still needed. There is very little information on the pre- and post-harvest effects of γ-aminobutyric acid (GABA) and melatonin (MEL) on the fruits proposed in this study. Therefore, to maintain the quality and prolong the shelf-life of citrus fruits, it is necessary to implement systems that monitor the condition of the fruit during post-harvest. For all of these reasons, we are considering the need to find new technologies capable of providing both producers and consumers with solutions of natural origin in the production and preservation of these fruits with a very low impact on the environment and on human and animal health, while complying with the requirements of the new regulations.

Therefore, the general objective of this Doctoral Thesis is to use alternative methods to traditional ones to increase crop yield, as well as to increase the organoleptic, nutritional and functional quality of lemon fruit (organic and conventional) at the time of harvesting and to maintain it during storage, applying pre-harvesting treatments with MEL and GABA and post-harvesting with MEL. To achieve this aim, the following partial objectives were set:

. To evaluate the effect of different concentrations of MEL and GABA, applied by foliar spraying throughout lemon development on yield and organoleptic, nutritional and functional characteristics in "Verna y Fino" lemons, at the time of commercial harvesting.

2. To determine the dose of each compound that produces the greatest increase in crop yield as well as the highest quality fruit.

3. To check whether these treatments affect the ripening process of the fruit on the tree.

4. To check the efficacy of the treatments on the incidence of rotting during post-harvest storage, as well as the incidence of chilling injury.

5. To evaluate the effect of the treatments on the shelf life of the fruit during storage under refrigerated conditions, analysing the quality parameters, total antioxidant activity and total phenolic compound content.

6. To check if post-harvest treatments with MEL are more effective than treatments during fruit development on the tree in maintaining fruit quality during storage.

7. To analyse the effects of post-harvest treatments with MEL on the health of animals fed with the juices of treated lemons.

3 Material and Methods

3. Material and methods

In this section the main characteristics of the plant material, experimental conditions, preharvest and postharvest treatments, in vivo assays, analytical determinations and statistical design used in this PhD Thesis are included. For more detailed aspects, the publications that constitute the result section could be consulted.

3.1. Pre-harvest plant material and experimental design

The pre-harvest studies proposed in this PhD thesis were carried out during two consecutive growing seasons, 2019-2020 and 2020-2021, with two different lemon cultivars, "Fino-95" and "Verna". The experiments were carried out in a commercial orchard located in Orihuela (Alicante, Spain, 38°7′49.09″ N, 0°59′54.38″ W) where the lemon trees cultivars were grafted onto *Citrus macrophylla* rootstock. This area is identified by a Mediterranean climate that gradually shifts to a desert climate, characterized by hot, dry conditions and minimal rainfall. Monthly historical data of average, maximum and minimum temperature and average rainfall from 1999 to 2021, including the whole experimental years, are shown in Table 1. It is worth noting that in September 2019 a COL event occurred that marked the fruit and vegetable production. The average temperature and rainfall per year is 18-19 °C and 230 mm, respectively. The soils were characterized by a clear predominance of a semi-arid morphogenetic system, highlighting accumulation processes with glacis-type forms and terrain with a slight slope. The lemon trees were grown in accordance with current organic farming regulations, using a planting frame of 7 x 5 m and 15 years of age. The rows were selected, and 6 trees were marked in them, grouped in blocks of 3 units for each treatment, aiming for a homogeneous design. The treatments applied were control and γ-aminobutyric acid (GABA) at three concentrations 10, 50 and 100 mM for GABA treatments and control and melatonin (MEL) at three concentrations 0.1, 0.3 and 0.5 mM for MEL treatments.

	January	February	March	April	May	June	July	August	September	October	November	December
Tª average (ºC)	10.9	11.3	13.5	15.7	18.8	22.9	25.3	25.6	22.9	19.5	14.4	11.6
Tª Min. (ºC)	7.4	7.6	9.6	11.7	14.7	18.9	21.5	22.1	19.6	16.2	11.2	8.3
Tª Max. (ºC)	14.7	15.2	17.4	19.5	22.4	26.6	29	29.2	26.3	22.9	17.9	15.2
Rainfall (mm)	28	22	26	29	20	6	2	7	35	40	36	28

Data: 1991 – 2021 [Min. Temperature. (°C), Max. Temperature (°C), Rainfall (mm)].

Table 1: Average, minimum and maximum temperatures estimated for Alicante from 1991 to 2021. Source: Climate-Data.org (https://es.climate-data.org). Accesed June 2024

Elicitor treatments used in the experiments were prepared under similar procedure and conditions. GABA and MEL were obtained from Sigma (Sigma-Aldrich, Madrid, Spain). Treatments were performed early in the morning and during favourable weather conditions, where rainfall or winds were not forecasted for the following 24 h, by foliar spray application dosing 5 L per tree of freshly prepared solutions (enough to moisten the aerial part of the tree) with a 15-L backpack sprayer. All solutions contained 0.5 % Tween 20 as surfactant, although MEL was previously diluted with absolute ethanol. Control treatment was prepared by mixing water with 0.5 % Tween 20. Treatments were applied three times, at monthly intervals, starting at the end of the physiological fruit drop stage, and the last treatment being applied 3 days before harvesting.

Specifically, the experiments assayed in both lemon cultivars were the following (Figure 7):

3.1.1. Fino-95

In November 2019 and February 2020, the first and second harvest of Fino lemons was carried out after being treated with GABA (control, 10, 50 and 100 mM), and after reaching commercial maturity (> 55 mm). The effect of the pre-harvest treatments on yield was evaluated by counting the number of fruits per tree and the production in kilograms (kg) per tree, as well as the number of fruits with some inconvenience or non-commercial fruits. Then the average weight of the fruits (grams) was calculated. On the other hand, 600 lemon fruits were taken randomly from the different locations around the tree canopy from each of the treatments and immediately transported to the laboratory in order to carry out a series of analytical determinations on day 0 and after a preservation experiment. For this purpose, 5 batches of 15 homogeneous fruits, from each treatment, were prepared, weighed and stored at 10 °C with a relative humidity of 85 to 90 % for 0, 7, 14, 21 and 28 days. On each sampling date, a batch of each treatment was taken at random to carry out the following analytical determinations: weight loss, colour, firmness, total soluble solids (TSS) and total acidity (TA). In addition, from each fruit of the different treatments, six 1 cm wide longitudinal strips of flavedo were taken from each fruit and immediately frozen, at the same time juice samples were taken, both were stored at -20 °C. In the following days, the content of total phenolic compounds, in both peel and juice of lemon and the total antioxidant activity of the lemon juice were quantified in duplicate.

In the second growing season (2020-2021) the same concentrations of GABA treatments were repeated again to confirm the effect observed in the previous season. Harvesting dates were November 2020 and February 2021 in which the effects of the treatments on production (via the same parameters of the first season) were evaluated. After that, fruits previously randomly selected were transported to the laboratory and, as before, homogeneous fruits were selected from each treatment to make 5 batches, of 15 fruits in the first harvest and 30 in the second. Subsequently, they were stored under the same relative humidity conditions as the previous season, while the storage

temperatures varied. In the first harvest they were stored at 10 °C and in the second harvest at 2 and 10 °C. The same analytical parameters as in the first season were evaluated.

3.1.2. Verna

For *Citrus limon* (L) Burm. var. Verna grafted on *Citrus macrophylla,* harvest date was in May and June of 2020 in the first growing season and May and June of 2021 in the second growing season. After being monthly treated with GABA at 10, 50 and 100 mM and MEL at 0.1, 0.3 and 0.5 mM from the physiological fruit drop until 3 days before harvesting, the fruits were harvested when they reached the commercial maturity stage (> 55 mm). The effect of the pre-harvest treatments on production was evaluated in both harvest dates and both growing seasons by determining the number of fruits per tree and the production in kilograms (kg) per tree, as well as the number of non-commercial fruits. Then the average weight of the fruits (grams) was calculated. On the other hand, for the laboratory determinations the conditions were different between the first and the second growing season.

In the first harvest, more than 600 lemons randomly selected from the different locations around the tree canopy for each treatment were transported to the laboratory. There, batches of 15 fruits were homogeneously selected from each of the treatments for each day of the storage experiment; 0,7,14,21 and 28 days. Once the batches to be cold preserved were prepared, the fruits were weighed and stored in a cold room at 10 °C and stored in a cool conditions and relative humidity of 85 to 90 %. On each sampling date, a batch of each treatment was taken at random to carry out the following analytical determinations: weight loss, colour, respiration rate, firmness, total soluble solids (TSS) and total acidity (TA). In addition, from each fruit of the different treatments, six 1 cm wide longitudinal strips of flavedo were taken from each fruit and immediately frozen, at the same time juice samples were taken, both were stored at -20 °C. In the following days, the content of total phenolic compounds, in both peel and juice of lemon and the total antioxidant activity of the lemon juice were quantified in duplicate.

In the second harvest, a larger number of lemons than in the first growing season of each of the treatments was transferred to the laboratory. There, homogeneously batches of 30 fruits from each of the treatments were made for each day of the two storage experiments; 0,7,14,21 and 28 days. Once the batches to be used for the cold storage test were prepared, the fruits were weighed and stored in a cold room at both 2 and 10 °C and a relative humidity of 85 to 90 %. On each sampling date, a batch of each treatment was taken at random to carry out the following analytical determinations: weight loss, colour, respiration rate, firmness, total soluble solids (TSS) and total acidity (TA). In addition, from each fruit of the different treatments, six 1 cm wide longitudinal strips of flavedo were taken from each fruit and immediately frozen, at the same time juice samples were taken, both were stored at -20 °C. In the following days, other functional determinations such as

the total content of phenolic compounds and the total antioxidant activity in lemon juice were evaluated in duplicated.

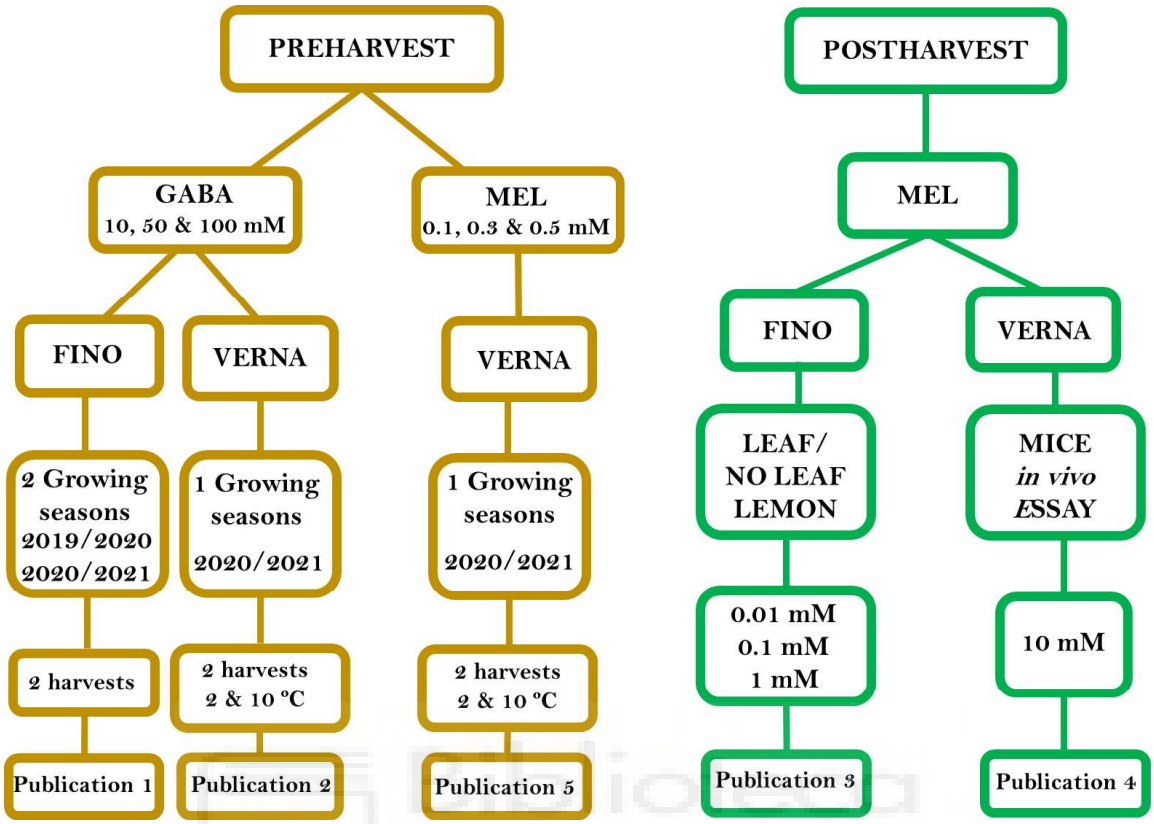


Figure 7. Experimental design scheme of the different experiments included in this PhD Thesis.

3.2. Post-harvest plant material and experimental design

The post-harvest studies realized in this PhD thesis were carried out during the growth cycle of 2021–2022 (November-December 2021 and July 2022), with two different lemon fruits varieties, "Fino-95" and "Verna". In Figure 7 it can be observed a summarize of these experiments. The essays were carried out in a commercial orchard located in Orihuela (Alicante, Spain, 38°07'54.5" N 0°59'33.0" W). The Fino lemon cultivar was grafted onto *Citrus macrophylla* rootstock while the Verna lemon cultivar was grafted onto *Citrus aurantium*. This region is characterized by being Mediterranean type and the same other factors as the commercial orchard of preharvest experiments.

3.2.1. Lemon with leaf or without leaf

In November and December of 2021 two experiments were carried out with organic lemon trees that were 20 years old and grafted on *Citrus macrophylla* rootstock (*Citrus limon* (L.) Burm. f) 'Fino-95', growing in a commercial orchard. The experiments were divided into Harvest-1 or Experiment 1 and Harvest-2 or Experiment 2. Within the orchard, the first experiment was designed using 30 trees in good vegetative state. From each tree, 10 fruits were picked at random, 5 of them with part of the stalk and a couple of leaves and 5 cut by the stalk in a standard way

for common marketing, as shown in Figure 7. Therefore, for the first experiment, a total of 300 lemons (10 per tree), 150 lemons with leaves and 150 without leaves, were harvested. Were fruits with commercial quality, without physical damage and homogeneous in size and colour, with a calibre between 70 and 90 mm. At laboratory, 8 lots of 30 fruit were selected (240 fruits), 4 lots with leaves and 4 without leaves. 30 fruits with leaf and 30 without leaf, without treatments, were used for day 0 analytical determinations. First, melatonin obtained from Sigma (Sigma-Aldrich, Madrid, Spain) was prepared by dissolving it in absolute ethanol and adding Tween 20 as a surfactant. Lemons were treated with melatonin at 0.01 mM, 0.1 mM and 1 mM concentrations and control (immersed in distilled water) in leaf and leafless lemons by dipping for 15 minutes. After the immersion, the fruits were air-dried before being stored in the cold room for post-harvest storage at 2 °C and a relative humidity of 90%. Cold storage was prolonged for 3 weeks (21 days) plus 2 days at 20 °C. Analytical determinations such as weight loss, colour or firmness among others were performed after the storage. In addition, from each fruit of the different treatments, six 1 cm wide longitudinal strips of flavedo were taken from each fruit and immediately frozen, at the same time juice samples were taken, both were stored at -20 °C. In the following days, the content of total phenolic compounds and the total antioxidant activity as well as total carotenoids were measured in duplicated.

In the Harvest-2, the experiment was repeated using the most effective dose. The conditions were similar with some differences. The number of lemons used was reduced from 4 to 2 batches of 30 lemons with leaves and from 4 to 2 batches of 30 lemons without leaves, since only 1 concentration of melatonin (Control and 1 mM) was used. Another difference is the dipping time; in this second experiment, in addition to dipping the fruits for 15 minutes, the fruits were also immersed for 30 minutes. Analytical determinations were performed after the 21 days storage + 2 days of shelf life. Some of the determinations were electrolyte leakage, TSS or TA and also from each fruit juice and longitudinal strips of flavedo were taken and immediately frozen at -20 °C. After that, we measured the content of bioactive compounds.

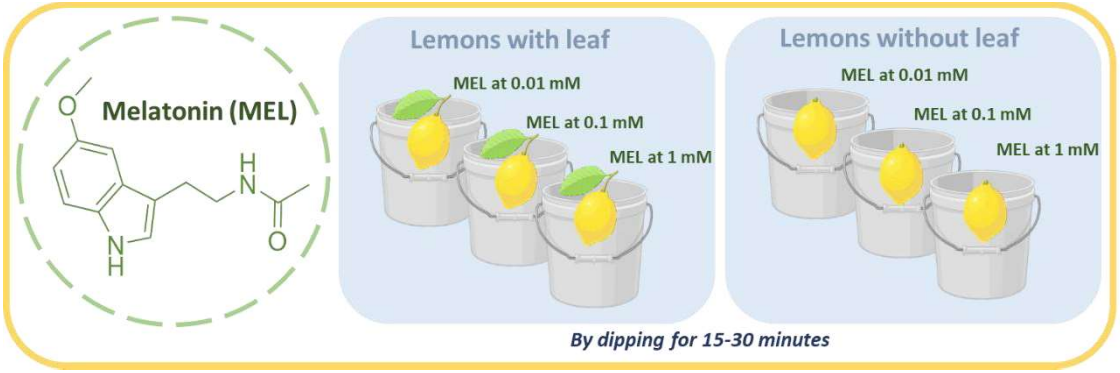


Figure 8. Graphical experimental design of the leaf and non-leaf lemon experiment

3.2.2. Mice in vivo essay

During the summer of 2022, Verna lemons were harvested from a commercial organic *citrus* orchard in Orihuela (Alicante), Spain. Fifteen trees were chosen, and ten lemons were randomly selected from each. The harvested fruit was promptly transported to the laboratory, where it was washed with distilled water. After drying at room temperature, the lemons were immersed in a 10 mM aqueous solution of melatonin (purchased from Sigma, Sigma-Aldrich, Madrid, Spain) for 30 minutes. The melatonin solution was prepared by dissolving it in absolute ethanol and adding Tween 20 as a surfactant. Following the immersion, the lemons were placed on a table to dry at room temperature. Once completely dried, the lemons were freshly squeezed for juice. The juice was then lyophilized to preserve its chemical composition until further use. Subsequently, the lyophilized sample was reduced to a dense, doughy mass and stored in a freezer at −80°C until further analysis. In the following days, two main analyses were conducted on the rehydrated freeze-dried lemon juice. The first was a shelf-life study to determine the duration for which the functional compounds in the juice retained their bioactive properties. This involved monitoring the evolution of total phenolic content, total antioxidant activity, and vitamin C content over 96 hours. Additionally, the melatonin content was measured using HPLC.

In September 2022, we transported the freeze-dried lemon in dry ice to the University of Trás-os-Montes and Alto Douro (UTAD, Vila-Real, Portugal), where we performed the *in vivo* tests with animal models as part of the international pre-doctoral research stay of this thesis. In this study we realized 2 differents experiments. In the first one, called “Drink assay”, we administered freeze-dried lemon through drinking water. Forty female mice were used for the study: twenty transgenic (HPV16+/−) and twenty wild-type (WT) (HPV16−/−), aged 18–20 weeks old. In order to minimize aggressive behavior between animals, frequently observed in male animals, female mice were selected. The mouse strain was donated by University of California through the National Cancer Institute Mouse Repository (Frederick, Maryland, USA). In the oral intervention study with mice using drinking water, we employed a concentration of freeze-dried lemon juice of 38.6, 57.8 and 77.1 mg/100 mL to obtain a dose equivalent to 1, 1.5 and 2 mL, respectively, of lemon juice reconstituted in the final volume of water administered to the mice. From lower to higher juice concentrations, the melatonin dose was 154, 231 and 308 ng, respectively, based on the concentration of melatonin in this proportion of freeze-dried lemon juice. Related with the concentration, animals were divided into eight groups (each with n = 5). Groups 1 to 4 were wild-type (WT), and groups 5 to 8 were transgenic (HPV16): group 1 (G1, WT, control), group 2 (G2, WT, 1 mL lemon), group 3 (G3, WT, 1.5 mL lemon), group 4 (G4, WT, 2 mL lemon), group 5 (G5, HPV16, control), group 6 (G6, HPV16, 1 mL lemon) group 7 (G7, HPV16, 1.5 mL lemon) and group 8 (G8, HPV16, 2 mL lemon). Mice were fed a standard diet (4RF21 GLP, Mucedola, Italy) ad libitum.

For the second experiment, the "Food assay", only twenty female mice were used: 10 WT and 10 HPV16+/−, aged 30 weeks old. Female mice were selected in order to minimize aggressive behavior between animals, frequently observed in male animals. The mouse strain was donated also by Drs Jeffrey Arbeit and Douglas Hanahan from the University of California. The animals were genotyped in the same way as in the Drink assay. For the diet assay, a commercial rodent feed (certified Mucedola 4RF21, Milan, Italy) was used as the basis for the preparation of modified diets containing melatonin-treated lemon extracts. Diets were prepared using an industrial mixer (CPM Europe, model C-300, Zaandam, The Netherlands) and adding 5% (w/w) water to the mixture to form new pellets (4.2 mm diameter). The control diet was prepared following the same method but without the addition of lemon extract. Subsequently, all batches of feed were dried in an oven at 40 °C for 48 h and stored at 4 °C until the feed was ready for use. We dissolved 8 g of freeze-dried lemon juice in 100 mL of water and added it to 2 kg of normal food. In this way, we ensure that the concentration of freeze-dried juice in the food was 4 mg/g, which is equivalent to 4 mL of rehydrated lemon juice, in which we can find 9.96 ng of melatonin. For the diet assay, mice were divided into four groups of 5 animals each: group 1 (G1, WT, control), group 2 (G2, WT, 4 mL lemon), group 3 (G3, HPV16, control) and group 4 (G4, HPV16, 4 mL lemon).

In both experiments the animals were kept under controlled experimental conditions. All mice were acclimated for four weeks in a controlled environment (20 ± 2 °C, 12 h light/dark cycle and relative humidity 50 ± 10%) and had free access to food and water. Animals' body weight, water and food intake were recorded and monitored every 5 days. At the same time, animals were carefully observed to confirm their well-being through their humane endpoint evaluation. The lemon juice melatonin-enriched extract was administered in drinking water for 30 days at different concentrations and was renewed every 48 h. The diet test also lasted 30 days, and the animal's diet food was added every 72 h. At the end of the 30 days, all animals (both experiments) were sacrificed by intraperitoneal administration of a mixture of xylazine and ketamine, followed by cardiac puncture exsanguination, according to FELASA guidelines, and biological samples of blood and organs (heart, lung, liver, spleen, kidneys, as well as chest and ear skin samples) were collected for analysis in the following days.

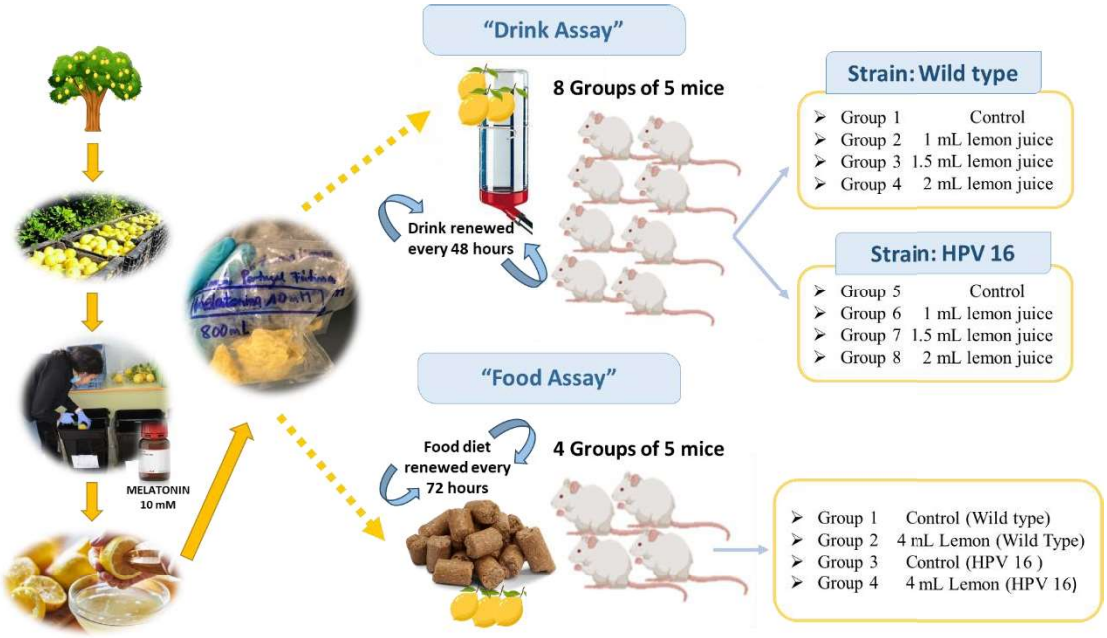


Figure 9. Summary graphical experimental design of the mice *in vivo* experiment.

3.3. Analytical determinations

3.3.1. Respiration rate

To quantify respiration rate, groups of 5 fruits were placed in a 5 L flask for 60 min and hermetically sealed. The cap of the flask incorporated a septum, which allowed sampling of the head air with 1 mL syringes. Afterwards, 4 samples of 1 mL of the atmosphere were extracted, 2 samples were used to quantify, CO_2 in duplicate, using a Shimadzu 14B gas chromatograph (Shimadzu Europe Gmbh, Duisburg, Germany), equipped with a thermal conductivity detector. The remaining 2 samples is taken for safety reasons in case any of the measurements are wrong. The chromatographic conditions have been described previously (Martínez-Romero et al, 2002). The chromatograph was connected to a computer that recorded and integrated the peak area, allowing quantification. To calculate the CO_2 produced, the weight of the fruit, the free volume of the container and the time in the container were used. Results for respiration rate were mean ± SE and expressed as mg CO_2 kg $^{-1}$ h $^{-1}$.

3.3.2. Weight loss

The weight was determined using a Radwag WLC 2/A2 (Radwag wagi Elektroniczne) balance with 2 decimal's accuracy expressed in grams. On the day of harvesting (day 0), all the fruit that formed part of the batches were weighed and reweighed on the different sampling dates during the storage period. Weight loss was expressed as percentage (%) with respect to initial weight.

3.3.3. External and internal colour

The external colour of the fruit of each batch was determined at equidistant 3 points of the equatorial perimeter of the fruit using a Minolta tristimulus colorimeter (CRC200, Minolta Camera Co., Tokyo, Japan), using the CIELab system (L*, a* and b*). Internal colour, was measured on the surface of the internal part of the fruit after being cut along the transverse axis using the same colorimeter and at 3 equidistant points. The results were expressed such as citrus colour index (CCI) using CCI the formula (1000 a*/(L*b*)) or with the a* parameter, which represents the axis from green to red colours. The data are the result of the mean ± ES of the total fruit of each batch. In addition, in some of the experiments, external and internal colour was measured by taking photos of the external and internal fruit to visually compare the colour. For this, we used a digital camera (Nikon D3400) in a light box with a black background. For external and internal colour measure, two images of the front and the back side, and another of the cut surface of lemon fruit were captured.

3.3.4. Firmness

Firmness was determined individually on each fruit from each lot using a TX-XT2i Texture Analyzer (Stable Microsystems, Godalming, UK) coupled to a probe with a flat steel disc plate. The steel disc of the probe applied a constant force to the surface of the fruit until a deformation of 5 % of the equatorial diameter was produced. The results were expressed as the ratio between the force applied and the distance covered (N mm^{-1}), being the mean ± SE of the measurements made on each fruit in each of the batches.

3.3.5. Total Soluble Solids (TSS) and Total Acidity (TA)

Total soluble solids (TSS) of freshly squeezed juice filtered through gauze were measured in duplicate by refractometry. Juice samples were obtained by mixing the juice from one lemon half made from all fruits of the replicates. A digital refractometer (model Atago PR-101, Atago Co. Ltd., Tokyo, Japan or Hanna Instruments, Rhode Island, EEUU, depending on the experiment) was used, which was calibrated with distilled water and readings were taken at room temperature (20 °C). This technique is based on the different refractive indixes of two media with different dissolved substances, juice and distilled water. The results were expressed as g 100 g^{-1} of fresh weight and represented the mean ± SE of the determinations carried out in duplicate in each sample.

Total acidity (TA) was determined in duplicate in the same juice by automatic potentiometric titration with a 785 DMP Tritino (Metrohm), with a sensitivity of ± 0.01. The quantity of juice employed was 0.5 mL that was diluted in 25 mL of distilled water. The potentiometer added 0,1 N NaOH until pH = 8,1 was reached (AOAC, 1990). The results were expressed as g citric acid content equivalent per 100 g^{-1} fresh weight, and were the mean ± SE of the determinations made on duplicate in three replicates.

3.3.6. Electrolyte Leakage (EL)

Electrolyte leakage (EL) was evaluated in the peel tissue, following the method described by McCollum & McDonald (1991), with some modifications. Fifteen peel discs were extracted for each replicate using a 0.5 cm corkscrew diameter from the equatorial zone of lemon fruit. After 3 rinses of 3 min for each replicate with deionized water, were made by constant shaking with 50 mL of deionized water at room temperature. After 30 min, the initial electrical conductivity (C1) was measured using a Crison conductivity meter. The samples were frozen and then brought to 121 °C for 15 min. Total conductivity (C2) was evaluated with samples at room temperature (20 °C). EL was calculated as (C1/C2) ×100.

3.3.7. Total Phenolic Content (TPC)

Total phenols were extracted and quantified according to Martínez-Espla et al., 2014, with minor modifications. In a centrifuge tube, 2 g of flavedo, cut into smaller sections of different lengthwise strips, were weighed and 15 mL of a water:methanol (2:8) solution containing 2 mM sodium fluoride (NaF) (to inactivate polyphenol oxidase activity and prevent phenolic degradation) were added. Homogenised with a Polytron (Ultraturrax, T18 basic, IKA, Berlin, Germany) for 60 seconds and centrifuged at 10,000 x g in a C30P centrifuge (B. Braun Biotech international) for 15 min at 4 °C. The final volume of the supernatant was then measured and sufficient sample was taken for subsequent quantification. Extractions were performed in duplicate for each of the processed samples.

After centrifugation and volume measurement, determination of total phenol content was performed using the Folin-Ciocalteau reagent, as previously reported Sayyari et al., 2011. The samples were read using a UV-1700 Pharma Spec spectrophotometer (Shimadzu), measuring absorbance at 760 nm. It was quantified based on a standard line with gallic acid, expressing the results as mg gallic acid equivalents (GAE) in 100 g-1 fresh weight, and the data were mean ± ES.

3.3.8. Individual phenols quantification

To quantify the individual phenols, 5 ml of lemon juice of each replicate was centrifuged at 10,000 g for 10 min. After that the supernatant was filtered throughout a 0.45 µm PVDF filter (Millex HV13, Millipore, Bedford, MA, EE.UU.) Identification of phenols was performed by liquid chromatography coupled to mass spectrometry (HPLC-DAD-ESI/MSn) by using an Agilent HPLC1100 series machine equipped with a photodiode array detector (Agilent Technologies, Waldbronn, Germany) and a mass detector in series (Bruker Daltonics Ultra HCT-ESI Ion Trap, Bremen, Germany) and a Luna C18 column (250 × 40 mm, 5 µm particle size). To quantify individual phenols 20 µL of two samples from each extract were injected into a HPLC system working with the chromatographic conditions previously reported (Martínez-Esplá et al., 2014). Chromatograms were recorded at 320 and 360 nm and for quantitative analysis, a calibration curve of two standards, 5-

O-caffeoylquinic acid and 3-luteolin-O-rutinoside (Sigma Aldrich, Germany), was used for the quantification of hydroxycinnamic acids and luteolin derivatives.

3.3.9. Total Antioxidant Activity (TAA)

Total antioxidant activity (TAA) was quantified in pomegranate arils and table grape skin according to the procedure described in Sayyari et al., 2011, which enables to determine TAA due to both hydrophilic (H-TAA) and lipophilic (L-TAA) compounds in the same extraction. Briefly, 5 g of arils and 1 g of berry skin tissue were homogenized in 5 and 10 mL of 50 mM phosphate buffer pH 7.8, respectively, and 5 mL of ethyl acetate. The skin tissue was manually homogenized in a mortar. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C and the upper and lower fractions were used to quantify L-TAA and H-TAA, respectively. In both cases, TAA was determined using the enzymatic system composed of the chromophore 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), the horseradish peroxidase enzyme and its oxidant substrate (hydrogen peroxide), in which ABTS+ radicals are generated and monitored at 730 nm. The decrease in absorbance after adding the pomegranate or grapes extract was proportional to TAA of the sample. A calibration curve was performed with Trolox ((R)-(+)-6-hydroxy-2, 5, 7, 8-tetramethyl-croman-2-carboxylic acid) (0-20 nmol) from Sigma-Aldrich (Madrid, Spain), and results were expressed as g of Trolox Equivalent (TE) kg^{-1}.

3.3.10. Determination of Microhematocrit and Total Plasma Proteins (TPP)

The microhaematocrit method was used to measure the haematocrit. The samples were first centrifuged at 9000 rpm for 5 minutes and then the height of the packed red blood cells was measured with a scale. Results were expressed as a percentage of red cell volume. In addition, blood samples were allowed to clot and centrifuged at 3000 rpm for 15 min (4°C) for serum biochemistry and total plasma proteins. Serum TPP concentrations were determined on an autoanalyser (Prestige 24i, Cormay PZ, Marynin, Poland).

3.3.11. Histological Analysis

Samples of heart, liver, kidney, lung and spleen were collected and immediately fixed in 10% neutral buffered formalin for at least 24 hours. Subsequently, using a graded ethanol series, the fixed tissues were dehydrated. They were then rinsed with xylene and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin (H&E) to assess organ histology. A qualified histopathologist examined the stained sections using a light microscope. Evaluation criteria included the presence of inflammation, fibrosis, necrosis, cellular infiltrates and any other histopathological changes that might indicate tissue damage or disease. The histopathological analysis was performed blindly, without the histopathologist knowing the treatment groups or experimental conditions.

3.3.12. Hepatic and Kidney Oxidative Stress

Oxidative stress markers levels were measured in a homogenate of liver tissue and kidney tissue. The organs tissues were homogenized in cold buffer solution (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of MgCl2 and 0.5 mM of phenylmethyl sulfonyl fluoride PMSF, prepared in ethanol to prevent protein degradation, pH 7.4) using a motor-driven Teflon and glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C (Sigma model 3K30, Osterode, Germany), and supernatants were collected for analysis. Superoxide dismutase activity (Cu/Zn-SOD) was determined by the nitroblue tetrazolium (NBT) reduction generated by superoxide radicals generated by xanthine oxidase system at 560 nm. For quantitative analysis, a calibration standard curve constructed by SOD from bovine erythrocytes was used (0–3.75 U mL^{-1}). The activity of catalase (CAT) was determined at 240 nm in accordance with a previously published method (Bendou et al., 2022) and was calculated using bovine catalase as a standard (0–5 U mL^{-1}).

3.3.13. Statistical analysis

In this PhD Thesis the results were expressed as mean ± SE of three replicates and as mean ± SD in the 4th publication. Data were subjected to analysis of variance (ANOVA), HSD Tukey's test or HSD Duncan's test to examine mean comparisons and Student's t-test. And also, the Bonferroni test was performed in the 4th publication. These are specified in each of the publications in chapter 4. Sources of variation varied according to the experimental design of each study. Differences were considered statistically significant at $p<0.05$ and were indicated using different lowercase or capital letter designations in each experiment or, in some cases, were expressed as * symbol placed in the corresponding bars for each parameter. All analyses were performed by using SPSS software package v. 17.0 and 22.0 for Windows.

4 Publications

4. PUBLICATIONS

4.1. PUBLICATION 1

PUBLICATION 1 (Literal transcription)

Preharvest use of γ-aminobutyric acid (GABA) as an innovative treatment to enhance yield and quality in lemon fruit.

Badiche, F., Valverde, J.M., Martínez-Romero, D., Castillo, S., Serrano, M., Valero, D.

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MDPI

Article

Preharvest Use of γ-Aminobutyric Acid (GABA) as an Innovative Treatment to Enhance Yield and Quality in Lemon Fruit

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Abstract: γ-Aminobutyric acid (GABA) occurs naturally at a low concentration in fruits, but can be increased following several stress events, playing a physiological effect. Lemon trees were preharvest treated with GABA at three concentrations (10, 50, and 100 mM) during two consecutive seasons (2019–2020 and 2020–2021). Fruit growth (diameter) and crop yield (kg tree^{-1} and number of fruits tree^{-1}) and quality traits were evaluated at harvest. Results showed that treatments were effective at increasing lemon size (a 5% higher) and yield, especially for GABA at 100 mM, for the two assayed seasons. Thus, yield was increased between 13 and 18% with respect to the control trees for the two harvest dates. With respect to the quality traits, GABA treatments did not impact any negative effects on the quality attributes, since the total soluble solids (7–8° Brix), total acidity (5–6 g 100 g^{-1}), and fruit firmness (13–14 N mm^{-1}) were similar to the control fruits. Therefore, GABA applied as preharvest treatment could be considered as a potent tool to enhance the yield of lemon fruits.

Keywords: elicitor; fruit growth; quality at harvest; climate change; citrus fruit

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1. Introduction

The five biggest exporters of lemons are Spain, Mexico, South Africa, the Netherlands and Turkey, with Spain being the first exporter worldwide and a value of more than EUR 4 billion. According to the latest statistics (2020/2021), Spain will produce 1,035,000 t, broken down into 820,000 t of 'Fino' lemons and 215,000 t of 'Verna' lemons [1].

The lemon market is subjected to EU regulations, with safety issues derived from using different contaminants, and thus not exceeding the MRL (maximum residue levels) and avoiding to reach the consumers [2]. In this sense, although consumers demand organic foods, organic lemon production is very small compared to conventional lemons. However, the total area for producing organic lemons has grown in Spain, reaching 8300 hectares in 2020, which represents a 14% of the total lemon production in Spain, with Murcia, Andalucía, and Valencia the main producing regions.

In lemon, as in other citrus fruits, the growth and development pattern follow a single sigmoid curve (either measured by weight, length, volume, or diameter), which can be divided into three stages [3]. In Stage 1, anthesis (increased cell number) and fruit drop occur, with little or moderate growth. Stage 2 corresponds to from fruit drop to the initial color changes, with a rapid fruitlet growth (cell expansion) and reaching the final size. Finally, at Stage 3, fruit maturation takes place, at which acidity decreases and sweetness increases and the color changes (from green to yellow) [4]. During lemon growth and development, the maximum accumulation of sugars takes place when the fruit size is around 50% of the final volume and then declines as maturation advances. In most

cultivars, the highest accumulation of sugars followed the acidity losses. Quality traits in lemon include both external (size, shape, skin thickness, visual appearance, and color), and internal quality (percentage of juice, number of seeds, sugars, acidity, flavor, taste, and bioactive compounds with antioxidant activity), which contribute to the consumer acceptability [5].

γ-Aminobutyric acid (GABA) is a non-proteinaceous amino acid of four carbons that occurs naturally in both plants and animals. Other isomers such as α-aminobutyric acid (AABA), also known as homoalanine, and β-aminobutyric acid (BABA) have also been reported to play a physiological role in animals (AABA), while BABA is a naturally-occurring moiety in plants [6]. GABA is found naturally in small concentrations in many plant sources including fruits, vegetables, and cereals. In plant cells, GABA accumulates under several conditions of abiotic stress, and plays a physiological role in redox balance, osmoprotection, osmotic adjustment, and antioxidant functions, among others [7].

In humans, it is well-known that GABA has multiple health-promoting properties including that GABA plays a role as an inhibitory neurotransmitter of the neuronal cortex acting on the central nervous system, but is also considered as a bioactive compound in foods with different roles such as anti-inflammatory, anti-diabetic, anti-hypertensive, and anti-cancer [8].

The impact of climate change and other environmental factors such as biotic and abiotic stress affect crop production and quality [9]. Citrus production is affected by several environmental conditions such as low and high temperatures, drought, and flooding, among others, that have negatively impacts and are considered as a big challenge for humans [10]. Accordingly, GABA exogenous application to the crop has demonstrated a significant enhancement of the endogenous content of GABA, which in turn alleviates the consequences of the stress [11]. On the other hand, there is some evidence that the accumulation of GABA occurred during the developmental stages of plants including fruit growth and ripening.

Tomato is used as a model since it accumulates GABA at higher rates during growth and ripening. GABA levels increase from flowering to the mature green stage, and then rapidly diminishes during the ripening [11]. Accordingly, GABA was found at elevated concentration near the breaker stage and rapidly catabolized, reaching low levels [12]. In higher plants, GABA is synthesized from glutamate by the enzyme glutamate decarboxylase, and metabolized through the GABA shunt pathway in two consecutive steps, first the oxidation to α-ketoglutarate, and then to succinate and enter the tricarboxylic acid cycle (TCA). The GABA shunt is involved in a wide range of physiological responses through the mitigation of reactive oxygen species (ROS) and plays a key role either as metabolites or endogenous signaling molecules in several regulatory mechanisms under stress conditions [13].

As a preharvest treatment, the role of GABA on growth and development has been reported in several fruits and vegetables, which has recently been reviewed [14], the most studied fruit being tomato. In addition, the preharvest application of GABA to apple trees led to the inhibition of fruit soft scald development depending on spray timing, but with benefits during postharvest storage [15].

On the other hand, the application of elicitors such as methyl jasmonate (MeJA) or salicylic acid (SA) induced the accumulation of endogenous GABA, and in turn, an increase in the total yield per plant was found in tomato with higher fruit quality attributes such as firmness, total soluble solids, and titratable acidity [16]. In an early report, the first quantitative content of GABA in lemon juice was lower (7 mg 100 mL^{-1}) than those reported for orange juices of several cultivars (18–32 mg 100 mL^{-1}) such as 'Valencia' and 'Washington Navel' [17].

As far as we know, there is no literature on the role of GABA preharvest application on citrus fruits. In this sense, the present study aimed to explore the impact of the foliar application of GABA in two consecutive years (2019 and 2020) in lemon trees on fruit

growth and yield (kg tree^{-1} and number of fruits) and to evaluate the role of GABA on the lemon quality attributes at harvest time.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The experiments were carried on a commercial orchard located in Orihuela (Alicante, Spain, 38°7′49.09″ N, 0°59′54.38″ W) during two consecutive years (2019–2020 and 2020–2021) on lemon trees (*Citrus limon* (L.) Burm. f) of the 'Fino-95' cultivar grafted on *C. macrophylla* rootstock and 15 years old. For both years, three blocks of three trees were selected at random for each treatment: GABA, purchased from Sigma (Sigma-Aldrich, Madrid, Spain) at 10, 50-, and 100-mM concentrations and the control (distilled water).

Geomorphologically, it is characterized by a clear predominance of a semi-arid morphogenetic system, highlighting accumulation processes with glacis-type forms and terrain with a slight slope. This region is characterized by being of the Mediterranean type transitioning to the desert climate, hot and dry, with little rainfall. The monthly temperature and rainfall for the whole experimental years is shown in Figure 1, at which the COL event is marked with an arrow, and the average temperature of 19 °C and rainfall of 230 mm.

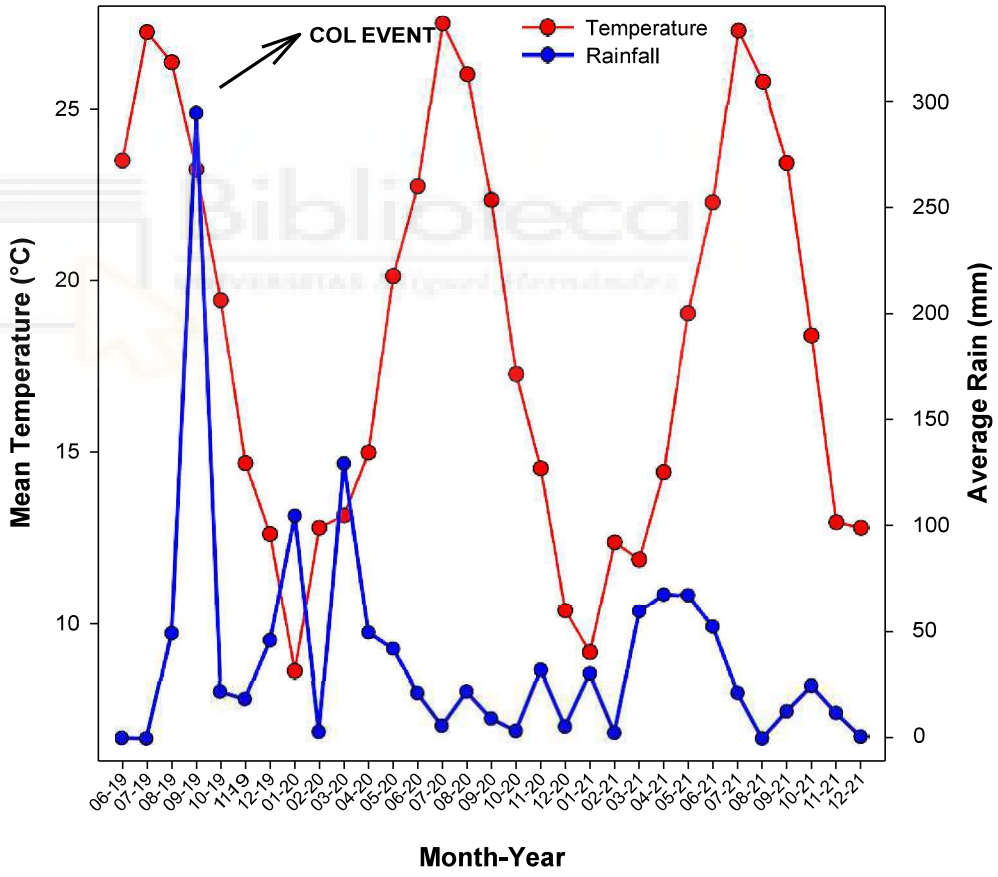

Figure 1. Mean temperature (°C) and rainfall (mm) along the experimental period (2019 and 2020).

Treatments were performed by foliar spray application with freshly prepared solution (5-L per tree) containing 0.5% Tween 20 as the surfactant and the phenological stage (BBCH-scale) was stage 71. The sprays were carried out with a 15-L backpack sprayer until runoff. Each treatment was repeated three times, starting after the typical fruitlet drop, at the onset of color change, and four days before harvest.

2.2. Fruit Growth and Crop Yield

For both years (2019 and 2020), before the first application, five fruits from each tree were marked at random. Fruit growth was followed by measuring the diameter with a digital caliper every 2 weeks, and the results were expressed in mm ± SE. Production was evaluated based on the total yield (kg tree^{-1}) and recording the number of fruits per tree. Two harvest dates were performed (November and February), which are the normal practices for the 'Fino-95' cultivar at which three categories were chosen: green, yellow, and wasted (non-commercial). From each tree and treatment, three lots of 15 fruits were picked and transported to the laboratory for the following analysis.

2.3. Fruit Quality Traits

All the parameters were measured individually in the 15 fruits of each replicate and the results expressed as the mean ± SE. Total soluble solids (TSS), total acidity (TA), firmness, and color were analyzed according to a previous report [18]. In brief, TSS was determined in the juice using with a digital refractometer at 20 °C (model Atago PR-101, Atago Co. Ltd., Tokyo, Japan) with the results being expressed as g 100 g^{-1}. After recording the pH of the juice, the TA was measured by potentiometric titration with 0.1 N NaOH up to reaching pH 8.1, using 1 mL of diluted juice in 25 mL distilled H_2O, and the results were expressed as g citric acid content equivalent per 100 g^{-1}. Fruit firmness was determined as the force–deformation ratio (N mm^{-1}) by using a TA-XT2i Texture Analyzer (Stable Microsystems, Godalming, UK). The external color was determined by the use of a Minolta colorimeter (CRC-400, Konica Minolta Co., Tokyo, Japan), recording L, a, and b coordinates, and color was expressed as the citrus color index (CCI, 1000 × a/L × b).

2.4. Statistical Analysis

A factorial design with GABA treatments (0, 10, 50, and 100 mM) with three triplicates of three trees per replicate was performed for both years (2019 and 2020). An analysis of variance (ANOVA) was performed by using the SPSS software statistic version 21.0 (IBM® SPSS®, USA). and means were compared by Tukey's test to find significant differences among treatments at $p < 0.05$.

3. Results

3.1. Fruit Growth and Crop Yield

Fruit growth was evaluated during the two consecutive years of the experiments by measuring the fruit diameter, and the results showed the typical single-sigmoid growth pattern of the citrus fruits (Figure 2). No significant differences were found between the control and GABA-treated trees for the 10- and 50-mM (average 46 and 57 mm for 2019 and 2020, respectively), although the preharvest application of GABA at 100 mM showed fruits with larger size (52 and 59 mm for 2019 and 2020, respectively). In September 2019, aa cold drop occurred, also known as a cut-off low (COL), which created unsettled weather and produced many thunderstorms with torrential rain and flash flooding [19].

With respect to the crop yield, two parameters were evaluated: total yield per tree (Figure 3) and the total fruit number (Figure 4). Two harvest dates is the normal procedure for this lemon cultivar, the first in November and the second in February, and three lemon categories were established: green, yellow, and waste (non-commercial), according to marketing procedures. In 2019 and harvest-1, the yield was similar for the control and 10- or 50-mM GABA (≅30 kg tree^{-1}), although the 100 mM GABA significantly increased ($p < 0.05$) the yield of green lemons (37.5 ± 4 kg tree^{-1}).

With respect to yellow lemons, all GABA treatments showed significantly ($p < 0.05$) higher yield (≅5 kg tree^{-1}) than the controls (3.31 ± 0.4 kg tree^{-1}), while the waste category was very variable (2.5–4.5 kg tree^{-1}), although the GABA at 10 mM doses significantly showed ($p < 0.05$) a lower rate of wasting lemons (Figure 3). During harvest-2, lemons acquired the full color and two categories were established (yellow and waste). In harvest-2, all GABA-treated trees produced a higher ($p < 0.05$) yield (≅63–74 kg tree^{-1}) compared

to the controls (47.4±2 kg tree^{-1}), and again, the wasting lemons remained variable (14–19 kg tree^{-1}).

In 2020, the experiment was repeated and the results were very similar, Thus, the 100 mM GABA significantly ($p < 0.05$) exhibited the highest yield (89.1 ± 1 kg tree^{-1}) of green lemons at harvest-1, and 193±21 kg tree^{-1} of yellow lemons at harvest-2. With respect to waste (non-commercial), all GABA treated trees significantly ($p < 0.05$) showed lower wasting lemons (≅21–28 kg tree^{-1}) than the control trees (37.1 ± 2 kg tree^{-1}). It is worthy to highlight that net production was much higher during 2020 than 2019 due to the fatal incidence of the COL event (Figure 3).

In relation to the number of fruits, results showed that GABA at 100 mM was also effective on increasing ($p < 0.05$) the number of green lemons in 2019 for both harvest dates (Figure 4). In 2020, there were not significant differences ($p < 0.05$) in the number of green fruits at harvest-1 (≅500 fruits), with the exception of 50 mM GABA, where the number of fruits was significantly ($p < 0.05$) lower (445 ± 12 fruits tree^{-1}). At havest-2, GABA at 10 or 100 mM showed the highest number of fruits (≅1350 fruits) and was significantly lower ($p < 0.05$) in the control trees (990± 22 fruits tree^{-1}). The number of wasting fruits was also very variable, and only for harvest-2 in 2020, all GABA treated trees showed significantly ($p < 0.05$) lower number of wasting fruits (≅155) than the controls (217 ± 11 fruits tree^{-1}).

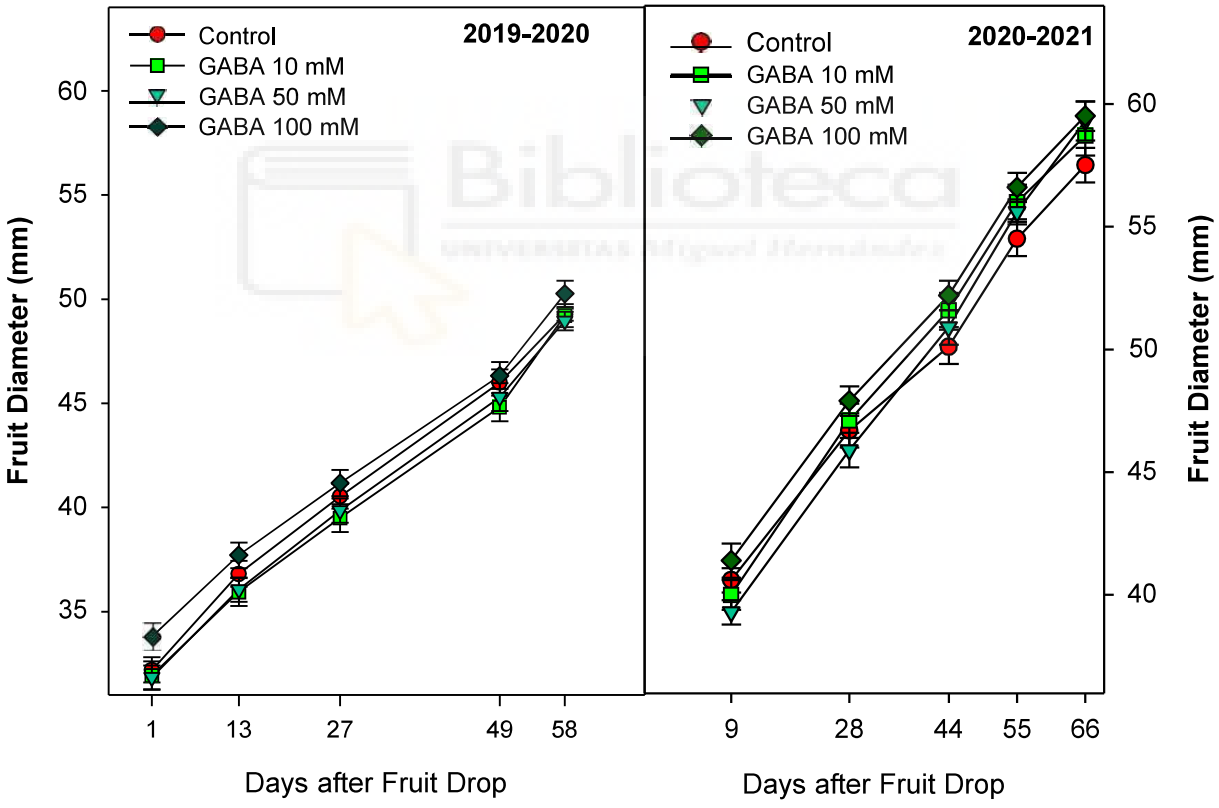

Figure 2. Fruit diameter (mm) in the 'Fino-95' lemon fruit during on-tree fruit development in the control and GABA-treated trees at 10, 50, and 100 mM. Data are the mean ± SE.

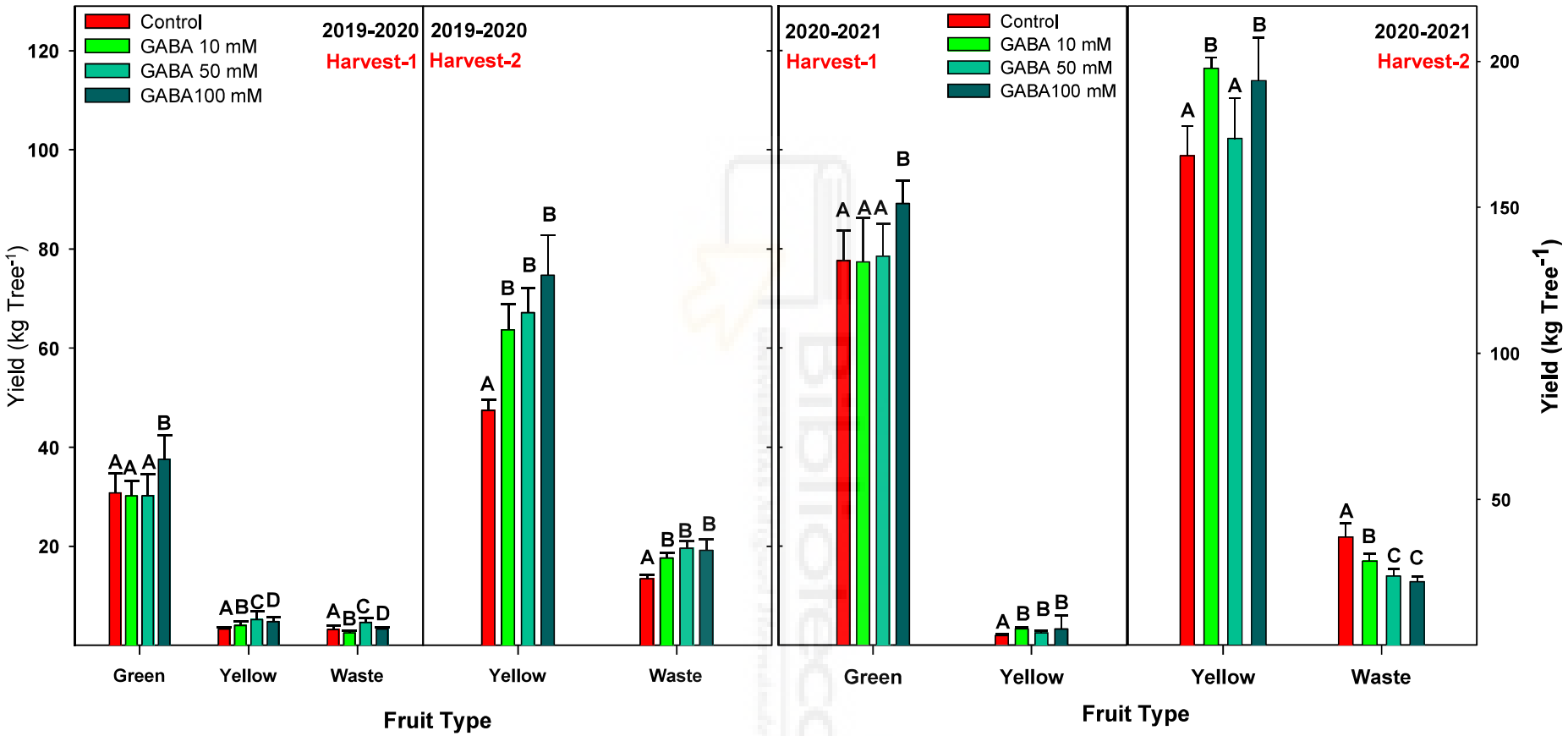

Figure 3. Crop yield (kg tree^{-1}) for the two harvest dates and two seasons as affected by GABA treatment and according to different categories. Data are the mean ± SE. Different letters (A, B, C, and D) on the bars denote significant differences at $p < 0.05$ among treatments for each fruit type.

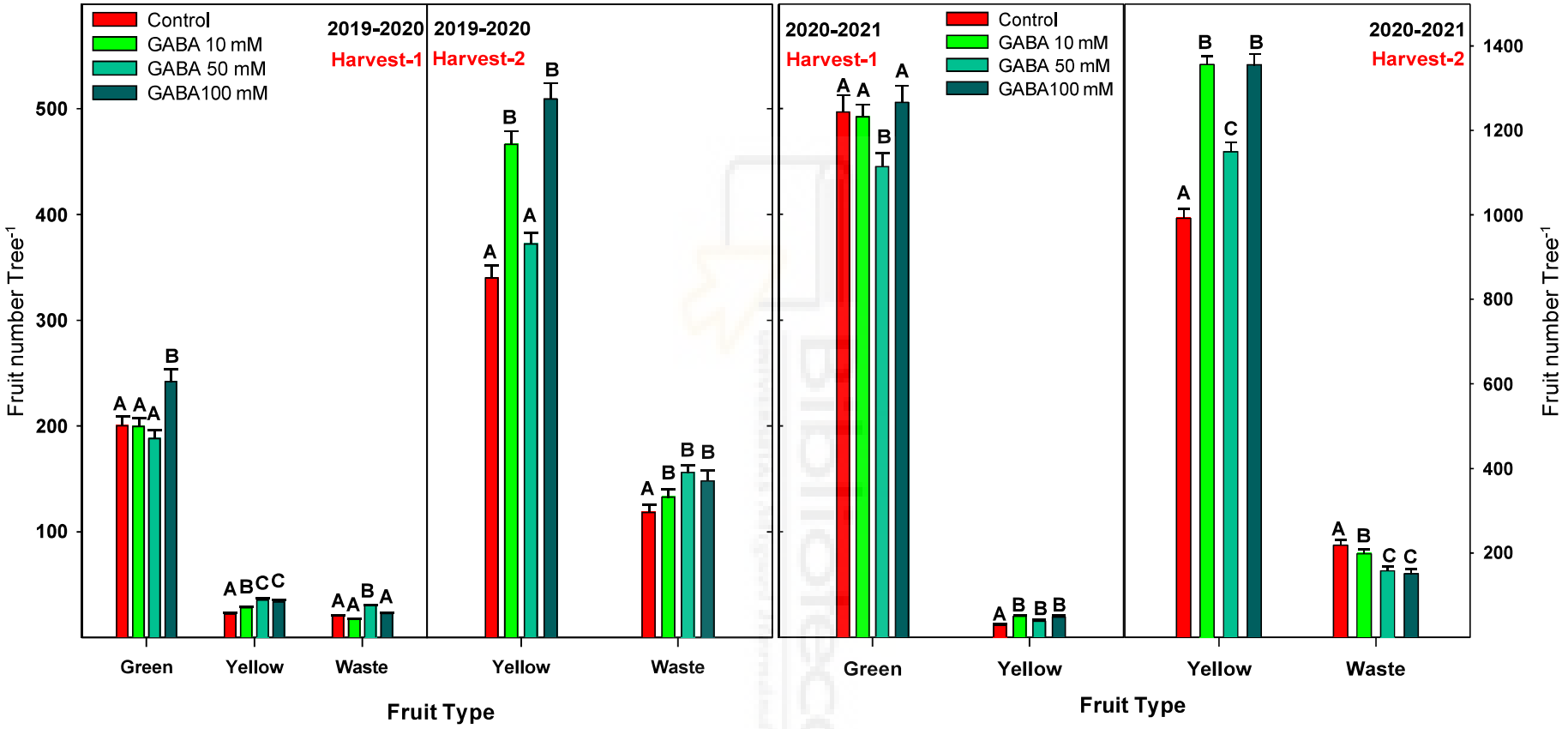

Figure 4. Crop yield (number of fruits tree^{-1}) for the two harvest dates and 2 seasons as affected by GABA treatment and according to different categories. Data are the mean ± SE. Different letters (A, B, and C) on the bars denote significant differences at $p < 0.05$ among treatments for each fruit type.

3.2. Fruit Quality Traits

Lemon fruit quality parameters were evaluated for the two seasons (2019 and 2020) from the picked fruits at both harvest dates. As reported above, at the first harvest date (November), the 'Fino-95' cultivar was picked at the green stage, which had negative values of CCI (Figure 5); the values being slightly lower in the GABA-treated (≅−7.5 and ≃−5.5) than in the control lemons (≃−6 and =−5 for the 2019–2020 and 2020–2021 seasons, respectively. At harvest-2 (February), CCI was ≅0.4 in 2019 for all of the treated and control fruits, while in 2020, GABA at 100 mM significantly ($p < 0.05$) had the highest CCI.

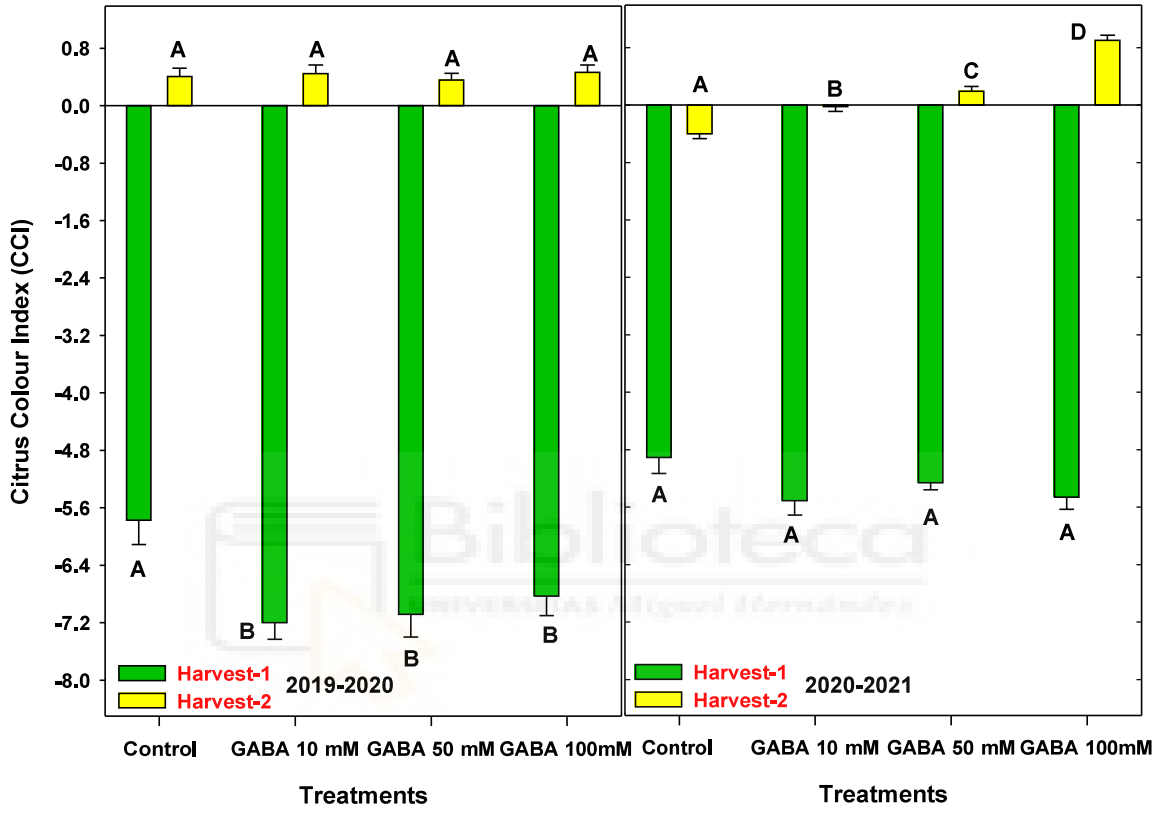

Figure 5. External color (expressed as the citrus color index) of lemons for the two harvest dates and two seasons as affected by GABA treatment. Data are the mean ± SE. Different letters (A, B, C, and D) on the bars denote significant differences at $p < 0.05$ among the treatments.

Other quality parameters were the concentration of total soluble solids (TSS) and total acidity (TA) as well as fruit firmness (Figure 6). No significant ($p < 0.05$) effect on TSS was observed for both seasons and the two harvest dates with the concentration being ≅7.7–8 g 100 g^{-1} for all fruits. Regarding the TA, in general, there were no significant differences ($p < 0.05$) between the control and treated lemons, with the exception of GABA at 50 mM. In addition, the TA levels were significantly ($p < 0.05$) higher in those fruit picked at harvest-1 (6.9–7.2 and 5.9–6.4 g 100 g^{-1}) for 2019 and 2020, respectively) compared with those at harvest-2 (5.9–6.4 and 5–5.6 g 100 g^{-1}, for 2019 and 2020, respectively). Similarly, fruit firmness did not show significant differences ($p < 0.05$) for all fruits, the only exception being found in lemons treated with GABA at 50 mM, in which firmness was significant lower ($p < 0.05$). Furthermore, as occurred for TA, the firmness values were significantly ($p < 0.05$) higher in harvest-1 (13.5–16.3 N mm^{-1}) with respect to harvest-2 (13.5–9.6 N mm^{-1}).

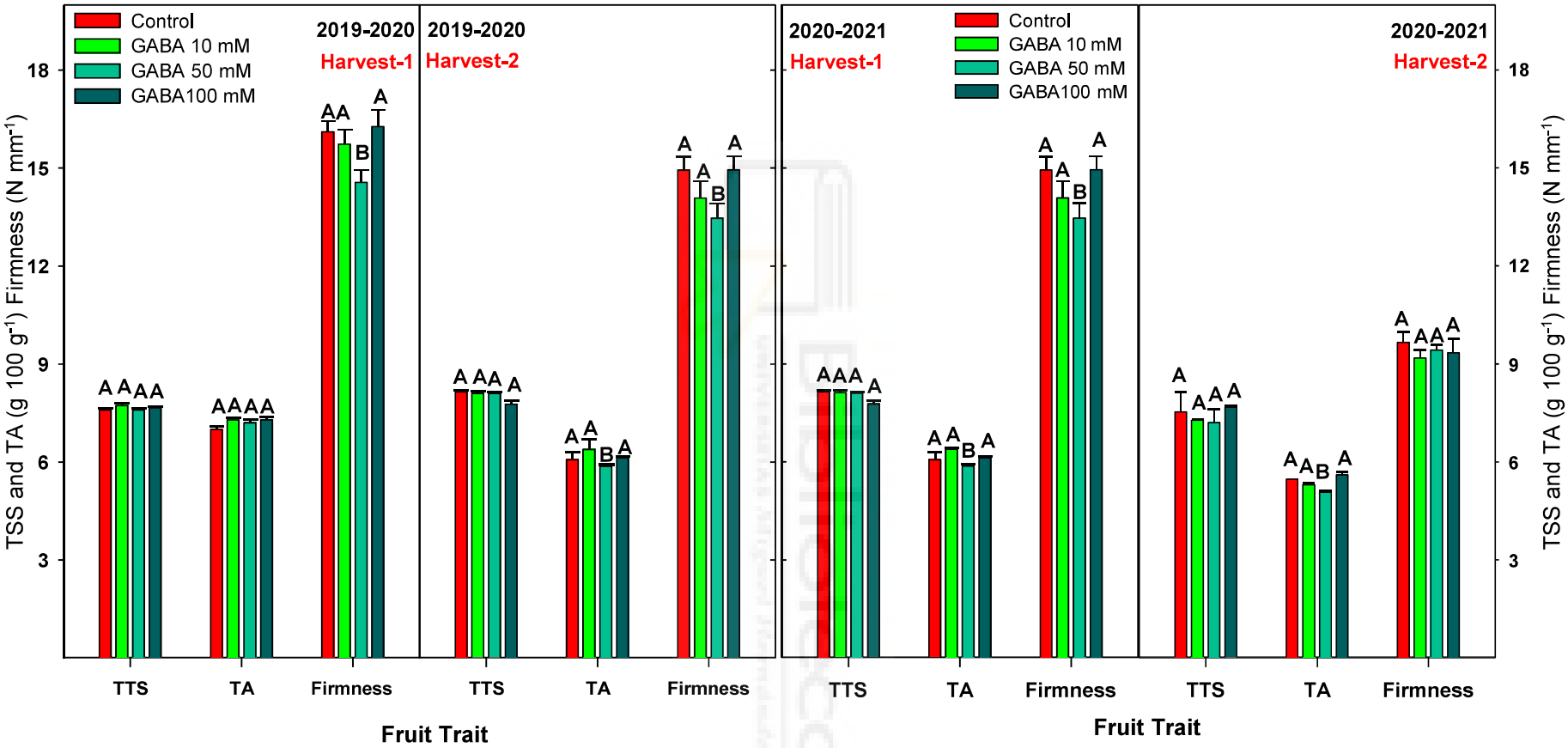

Figure 6. Quality traits of lemons for the two harvest dates and two seasons as affected by GABA treatment. Data are the mean ± SE. Different letters (A and B) on the bars denote significant differences at $p < 0.05$ among treatments for each quality parameter.

4. Discussion

With the aim to increase the productivity of the citrus industry, lemon trees were preharvest treated with GABA at three concentrations: 10, 50, and 100 mM. Overall, the results showed that GABA increased the fruit size and yield, the best concentration being 100 mM. The growth curve of thee 'Fino-95' lemon, which is characterized as having abundant seeds, is represented by a sigmoidal curve [20]. The differences in fruit size between the 2019 and 2020 growing cycles can be attributed to the unexpected cold drop, which provoked a reduction in fruit size. However, the application of GABA could increase the sink capacity of the fruits with a net increase in lemon size, as has been reported for other plant hormones such as auxins and gibberellins [4].

GABA treatments on lemon trees were very effective at enhancing the crop yield, determined by both production (kg tree^{-1}) and the number of fruits tree^{-1} for the two seasons and the two harvest dates, the effect being higher after the application of GABA at 100 mM. It was very noticeable that 2019 rendered a lower production than 2020 due to the incidence of the COL event, which did not occur in 2020. It has been reported that flooding can severely affect the crop yield, resulting in a 20–25% reduction in yield on average [21]. The decline in crop yield has been attributed to a reduction in the photosynthetic rate accompanied by the damage of protective enzymes [22], and then the exogenous application of GABA, especially at 100 mM, could partially increase the lemon yield, since the accumulation of GABA has been described as a response to several abiotic stresses including waterlogging. Moreover, under this stress situation, the effect of GABA at 100 mM on improving fruit production acquired special importance, since during harvest-2, the yield was almost 2-fold compared with the control fruits. A similar behavior was observed for the number of fruits per tree, for which the GABA treatments also showed a positive effect on increasing the number of fruits compared with those obtained in the control lemons, especially for 100 mM GABA. It is worthy to point out that the effects for both crop yield parameters were not dose-dependent, since GABA at 10 mM showed higher proficiency than at 50 mM. There is no literature on the role of GABA in fruit productivity, although some evidence exists in cereals and vegetables. In onion, the application of GABA at 0.5, 1.0, and 2.0 mg L^{-1} increased the bulb yield and other morphological characters, the highest effect being reported for GABA at 1.0 mg L^{-1} [23] in agreement with the results reported herein. In line with this report, foliar application of GABA at 0.5, 1, and 2 mM ameliorated drought stress and improved the yield of snap bean [24].

Citrus fruit is botanically considered as a hesperidium with a specific type of modified berry that is divided internally into segments containing the juice. The rind or peel of lemon is formed by the exocarp or colored flavedo and the colorless or white albedo. The ripening process is defined as the set of external flavor and texture changes that a fruit experiences when it completes its growth. This phase of the development includes several processes such as coloration of the pericarp, an increase in the concentration of sugars, a reduction in acid concentration, loss of firmness, and other physical and chemical changes [5].

Lemon fruits are generally harvested at different maturity stages to fulfil the market requirements. In the case of the 'Fino-95' cultivar, two harvest dates are the normal procedure, one in November and the other in February. The size and the color of the fruit are two of the main characteristics that determine when the fruit should be harvested. During autumn, as the temperature starts to decrease (below 13 °C), the fruit starts to change in color due to chlorophyll breakdown and the occurrence of the peel yellow color, from which the main pigments are carotenoids, while in the second harvest, the fruit is fully colored [25].

Other important parameters in lemon quality are related to the content of total soluble solids (TSS), total acidity (TA), and fruit firmness. From the point of view of the market, TSS and TA are undoubtedly the most important parameters. In mature lemons, sugars account for 80–90% of TSS depending on cultivars, the rest corresponded to TA, the major being citric acid, which is the main organic acid present in citrus fruits [26]. The content of TSS and TA were not affected by the GABA treatments, and the content agrees with previous reports

for 'Fino-95' compared with other cultivars such as 'Verna' and 'Fino-49' [27]. Fruit firmness is also considered as very important, which determines the evaluation of the consumers related to the quality of fresh lemons, but is also related to the potential storability during postharvest operations. In this report, GABA treatments (at 10 or 100 mM) did not impact any negative effect of firmness, although the 50 mM showed a significant reduction in fruit firmness at thee time of harvest. Information about preharvest GABA treatments on the quality of horticultural products is limited to cut flowers, although some evidence exists for when GABA is applied as postharvest treatments.

5. Conclusions

This is the first report that studied the effect of GABA preharvest application on citrus fruits. The application of GABA as preharvest treatments demonstrated a significant effect in enhancing the yield of 'Fino-95' lemons during two consecutive seasons (2019–2020 and 2020–2021), the most significant results being obtained for GABA at 100 mM. In addition, this GABA concentration did not impart any negative effect on the lemon quality attributes such as TSS, TA, and firmness. In future, the effect of GABA in other lemon cultivars such as 'Verna' and its role in the bioactive compounds and antioxidant enzymes requires further investigation. Thereafter, the possible role of preharvest GABA treatments on improving lemon quality during postharvest storage will provide a wide scenario of this natural elicitor compound.

Author Contributions: M.S. and D.V. conceived and designed the work; S.C., D.M.-R. and J.M.V. performed the field treatments; F.B. and J.M.V. performed most of the analytical determination, in collaboration with the other authors; M.S. and D.V. analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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4.2. PUBLICATION 2

PUBLICATION 2 (Literal transcription)

Potential Preharvest Application of γ-Aminobutyric Acid (GABA) on Improving Quality of 'Verna' Lemon at Harvest and during Storage

Badiche-El Hilali, F., Valverde, J.M., Díaz-Mula, H., Serrano, M., Valero, D.; Castillo, S.

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Article

Potential Preharvest Application of γ-Aminobutyric Acid (GABA) on Improving Quality of 'Verna' Lemon at Harvest and during Storage

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Abstract: γ-aminobutyric acid (GABA) is a naturally occurring plant compound that acts as a signalling agent during stress conditions, mainly due to unstable events, although sometimes the endogenous content does not reach sufficient levels. Thus, the main aim of this study was to apply GABA preharvest treatments in lemon and to study its effects on quality attributes at harvest and during postharvest storage. GABA was applied as foliar spray at 10, 50, and 100 mM, and quality traits during 28 days of storage at two temperatures (at 2 and 10 °C) were determined. Results show that all GABA treatments had a positive effect on reducing the weight losses and fruit softening. In addition, crop yield in terms of kg tree^{-1} and fruit number tree^{-1} was improved for the first and second harvest as well as the total phenolics content and total antioxidant activity (TAA). In conclusion, GABA at 50 mM concentration was the most effective preharvest treatment, enhancing shelf life being enhanced for 14 and 7 days at 2 and 10 °C, respectively, with respect to control lemons.

Keywords: crop yield; elicitor; firmness; postharvest; total phenolics

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1. Introduction

Citrus limon (L) is a yellow fruit originated from an important evergreen fruit crop belonging to the *Rutaceae* family, mainly cultivated in tropical and subtropical regions. World lemon production significantly increased in the last decade, with countries such as Mexico, Turkey, and Spain leading crop production [1]. Spain is one of the main exporters and producers worldwide and Europe accounts for about 60% of its cultivation area [2]. The Valencian Community, Andalucía, and Murcia lead the production, with the 'Fino' and 'Verna' autochthonous cultivars being the most widespread.

Lemon is a fruit highly appreciated for its composition and is rich in bioactive compounds, such as ascorbic acid and phenolic compounds (flavonoids and hydroxycinnamic acids), which contribute to its total antioxidant activity and has beneficial health effects [3]. However, this fruit is susceptible to physiological, biochemical, and pathological disorders.

The effects of the climate change are a real challenge to produce plant-based food commodities, negatively affecting both plant growth and crop physiology and productivity. It was predicted that citrus cultivation will be affected in the near future [4]. Although climate change will be differentially affected depending on the region, the main problem of the citrus growing area of the Mediterranean basin will be related to drought problems attributed to the increase in average temperatures [5].

During fruit growth and development, both cell division and expansion occur rapidly, and the fruit is more sensitive to external elicitors. Therefore, the preharvest strategy based on the application of elicitors induces a more active metabolism compared to the

recently non-treated harvested fruit, as well as prolonged storage at low temperatures [6]. Accordingly, the application of preharvest elicitors could improve the fruit resistance better than any postharvest tool, which is usually subjected to regulations.

In the last few years, γ-aminobutyric acid (GABA) was recognised as an elicitor for its application in crops affected by different abiotic stresses: low temperatures (chilling or freezing), high and fluctuated temperatures, solar radiation, flooding, and drought, among others [7,8]. GABA is a non-protein aminoaceus of four carbon atoms with a wide range of roles and modulates several physiological processes, including the cytosolic pH, redox status, osmotic moiety, plant growth, and senescence processes, among others [9], the latter being attributed to GABA shunt, which is the conversion of glutamate into GABA by glutamate decarboxylase enzyme [10]. At molecular level, GABA shunt acts as a signalling moiety, which is accumulated in the cytosol, playing a key role as a scavenger of reactive oxygen species (ROS), and thus, protecting the plant against the oxidative damage [11].

The association between fruit ripening and GABA was also proven in tomato. During fruit growth, GABA shows the higher concentration at the green mature stage and then sharply diminishes when the ripening processes advances [8]. Furthermore, the crosstalk between GABA and other plant hormones shows that the preharvest application of sal- icylates or jasmonates enhanced the endogenous concentration of GABA, which could be responsible for the observed increase in the yield and total antioxidant capacity [12]. Recently, GABA was postulated as a novel crop tool to increase quality and yield of lemon fruit (cv. Fino), even under undesired conditions of stress due to flash flooding as a conse- quence of torrential rain [13]. In 'Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck] trees, the application of GABA increased the endogenous GABA level, some amino acids, and several phytohormones, including auxins, salicylates, jasmonic acid, and abscisic acid. This fact suggests that these plant hormones are upregulated in GABA-treated plants and could improve the plant response to abiotic stress conditions [14].

The exogenous application of GABA leads to increasing its endogenous level and promotes the GABA shunt, which increases the carbon flux to the respiration pathway with the enhancement of NADH, NADPH, and ATP, which is being postulated as a good strategy to prolong storability in fruits [15]. Accordingly, GABA treatment was effective in maintaining quality traits during postharvest storage of oranges by reducing both the consumption of the respiratory substrates, mainly citrate and amino acids, and reducing the decay incidence rots and by increasing the antioxidant enzyme activities [16].

As far as we know, the literature about the use of GABA preharvest treatment and its effect during postharvest storage of fruit is limited; thus, to our better knowledge, this is the first evidence in lemon fruit. Therefore, the main aim of this study was to apply different concentrations of GABA (10, 50, and 100 mM) throughout lemon growth on yield, and to observe its influence on quality and functional traits (weight loss, fruit firmness, total phenolics content, and total antioxidant activity) as well as the evolution of the total phenolics in 'Verna' lemon stored at 2 and 10 °C for 28 days of postharvest storage.

2. Materials and Methods

2.1. Experimental Design and Plant Material

Lemon trees of 15 years old and grafted on *Citrus macrophylla* rootstock (*Citrus limon* (L.) Burm. F. cv. Verna) were used for the experiment in the growing cycle of 2020–2021 from a commercial plot located in La Matanza (Alicante, Spain). The 'Verna' lemon tree usually blooms twice a year in the spring and summer season. The crop was under organic certification, and the climatic conditions were typical of Southern Spain: semi-arid climate with mean temperature and rainfall of 19 °C and 230 mm, respectively. Within the orchard, the experiment was randomly designed by using 3 blocks of 3 trees and planted at 4 × 5 m for GABA treatments (GABA was acquired from Sigma™ company, Madrid, Spain) and was applied at concentrations of 10, 50, and 100 mM, while control trees were only sprayed with distilled water. GABA (containing 0.5% Tween-20 as surfactant) solutions were freshly prepared and applied by foliar spray, using 5 L solution per tree. Along the growing cycle,

treatments were repeated three times (T = 3): the first, being applied once, occurred at the normal physiological fruit drop (T1), the second application coinciding with the onset of colour changes (T2), while the third one was applied 4 days before harvest (T3). 'Verna' lemon was harvested at 3 dates (from May to June), and fruits from the 1st and 2nd harvests were used for the storage experiments. For each harvest date, 360 (10 lemons for each block of trees and treatment) lemons, that were uniformly mature and with colouration (full yellow), were manually harvested. At laboratory, lots of 30 fruits (grouped into 3 replicates of 10 fruits for each treatment; n = 3) were randomly selected at random for storage at 2 temperatures conditions (2 and 10 °C), and samples were transferred to a chamber at 20 °C for shelf life, in which the analytical measurements were carried out.

2.2. Lemon Fruit Yield and Quality Characteristics

Two measurements were carried out to evaluate the crop yield in terms of total yield (in kg tree^{-1}) and the number of lemons per tree (data are the mean ± SE). Quality parameters (weight loss, fruit firmness, total soluble solids, and titratable acidity content) were individually measured in each lemon fruit (data are the mean ± SE, n = 3). The weight loss was determined by weighing the recently harvested fruit and the weight obtained at each sampling date and results are expressed in percentage (data are the mean ± SE, n = 3). Regarding fruit firmness, a texturometer (TA-XT2i Texture Analyzer, Stable Microsystems^{TM}, Godalming, UK) was used with a flat probe of 10 cm. A force deformation was recorded by applying a 3% of the fruit deformation and results are expressed as N mm^{-1} (data are the mean ± SE, n = 3).

2.3. Extraction and Quantification of Total Phenolics and Total Antioxidant Activity (TAA)

The method of Folin-Ciocalteu was used following the protocol previously described by García-Pastor et al. (2020) [17]. A stainless steel lemon peeler was used to obtain the flavedo tissue by homogeneously splitting several 0.5 mm-thick slices of lemon peel. To obtain the lemon juice, a domestic squeezer ('Citromatic', Braun Española S.A., Barcelona, Spain) was used for the preparation of the juice by carefully squeezing the fruits manually in order to avoid contamination by components in the albedo tissue. Thus, 2 g of flavedo or 2 mL of juice were added to water:methanol (2:8) solution with 2 mM NaF (which inhibits the degradation of phenolics by the enzyme polyphenoloxidase). Then, the homogenized was obtained by using an Ultraturrax homogenizer (T18 basic, IKA, Berlin, Germany) at maximum speed for 1 min. The extract was centrifuged at 4 °C and 10,000× *g* for 10 min. An aliquot of 200 µL was mixed with the Folin reagent and the absorbance was quantified at 760 nm in a UV-Vis spectrophotometer (UV-1900i, Shimadzu, Duisburg, Germany). Total antioxidant activity (TAA) was measured in duplicate in each extract sample by using the ABTS peroxidase system, previously described in Martínez-Esplá et al. (2018) [18]. Results (mean ± SE) are expressed as mg equivalents of Trolox 100 g^{-1} of fresh weight.

2.4. Statistical Analysis

The software IBM SPSS Statistics 22.0 (IBM Corp., Armong, NY, USA) was used for the statistical analyses. Data for crop, quality, and functional parameters were subjected to analysis of variance (ANOVA) using the treatment studied and storage time as the factors. Thus, one-way ANOVA was used to determine the significance of mean differences among the four treatments: control and GABA at 10, 50, and 100 mM in 'Verna' lemon. Mean comparisons of analytical determinations were performed using a multiple range test (Tukey's test) to examine if differences among the treatments were significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$.

3. Results

3.1. Crop Yield

Crop yield was expressed in terms of kg tree^{-1} (Figure 1) and the total number of lemon trees^{-1} (Figure 2). Three harvest dates is the normal harvest schedule for this

lemon cultivar (between May and June). For each harvest date, two lemon categories were established: commercial (yellow colour) and waste (non-commercial) fruits, according to the marketing procedure's date (harvest 3). Results show that the lower yield was obtained in the June harvest compared to the yield for harvest 1 and 2. However, the preharvest application of GABA significantly increased the yield for harvest 1 and 2 ($p < 0.01$ and $p < 0.001$, respectively; Supplementary Table S1), while remaining unaffected for harvest 3. The treatment of GABA at 50 mM significantly showed the highest yield of commercial lemons (105.2 ± 5.05 and 108.63 ± 6.1 kg tree^{-1} for harvest 1 and 2, respectively) compared to control (78.6 ± 6.2 and 63.56 ± 5.1 kg tree^{-1} for harvest 1 and 2, respectively) ($p < 0.001$; Supplementary Table S1). Generally, the yield of waste (non-commercial lemons) did not show significant differences among all treatments ($p \geq 0.05$; Supplementary Table S1).

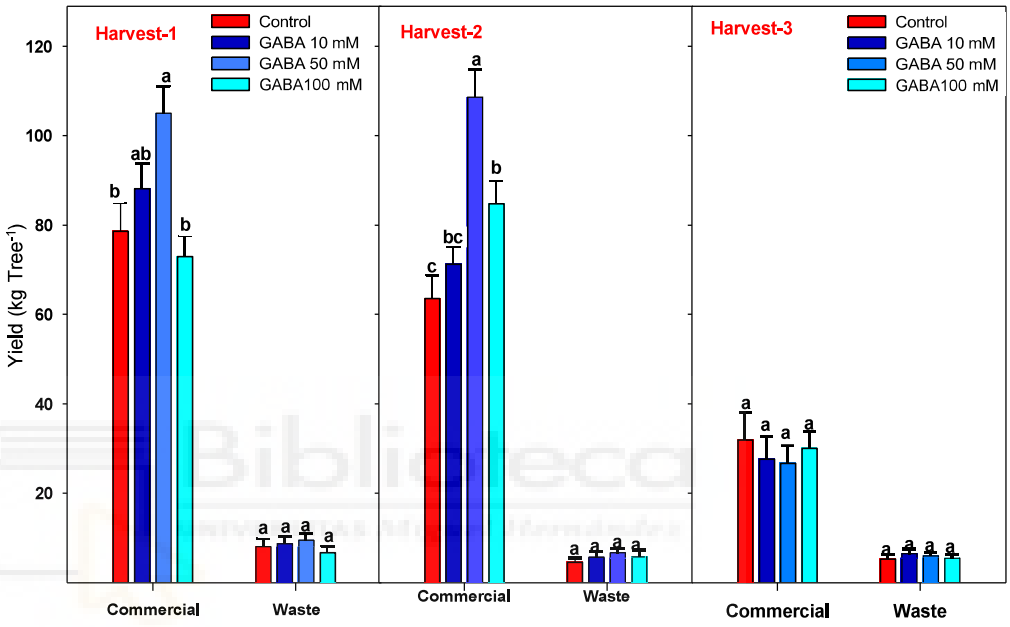

Figure 1. Influence of GABA preharvest treatment on yield (kg tree^{-1}) in three harvest dates as according to two categories: commercial and waste. Data are the mean ± SE. Different letters are significantly different at $p < 0.05$ according to Tukey's test.

The same behaviour was shown in relation to the number of fruit trees−1, where the preharvest treatment of 50 mM GABA significantly produced the highest number of commercial lemons (550.67 ± 25.4 and 642 ± 30.8 number of fruits for harvests 1 and 2, respectively) compared to control fruits (367.7 ± 12.9 and 407.2 ± 34.2 kg tree^{-1}, respectively) ($p < 0.001$; Supplementary Table S1).

1.1. Postharvest Storage

Lemon fruits from each preharvest treatment (control and GABA at 10, 50, and 100 mM), which were harvested in May and June (harvest 1 and 2), were stored dur- ing 28 days at two temperatures (2 and 10 °C) and samples were weekly transferred to shelf life conditions for 2 days at 20 °C. During postharvest storage, the percentage of weight loss was higher at 10 than 2 °C for both harvest dates. Control fruits showed the highest weight loss percentage (≈6 and 7%) at both temperatures compared to GABA-treated fruits, which showed (Figure 3) the lowest weight losses ($p < 0.001$; Supplementary Table S2). Among GABA treatments, the concentration of 50 mM significantly showed the lowest percentage of weight loss (3–4 and 5% for 2 and 10 °C, respectively) compared to that obtained for 10 mM GABA-treated ($p < 0.001$; Supplementary Table S2) and control lemons.

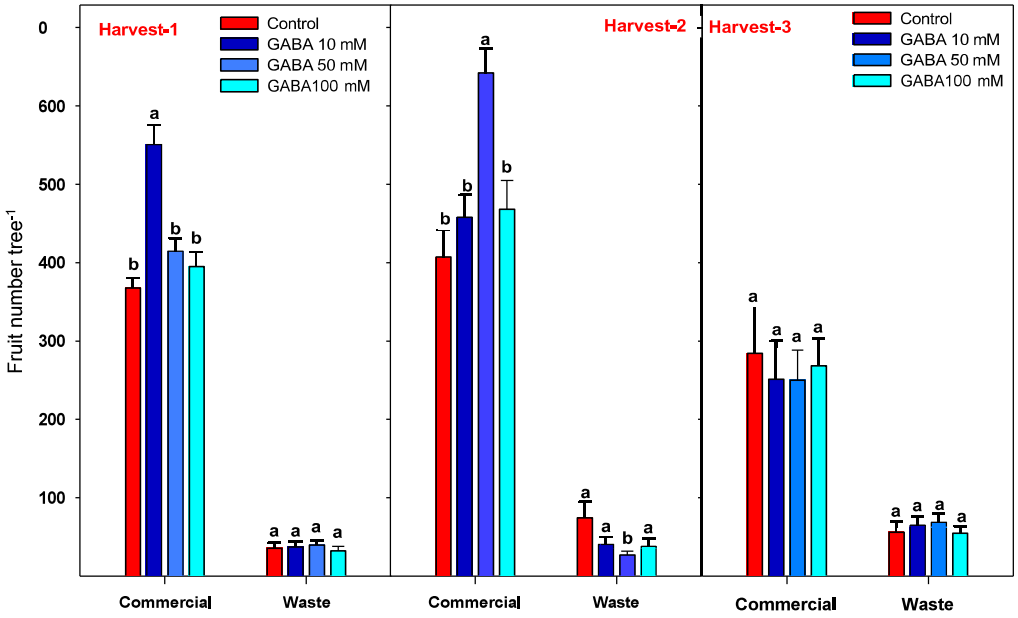

Figure 2. Influence of GABA preharvest treatment on fruits number tree^{-1} in three harvest dates as according to two categories: commercial and waste. Data are the mean ± SE. Different letters are significantly different at $p < 0.05$ according to Tukey's test.

Firmness values were higher in lemons harvested in May (harvest 1) than those picked in June (harvest 2). Firmness was expressed as fruit deformation (N mm^{-1}), and as expected, this parameter diminished during postharvest storage, for both harvest dates and storage temperatures (Figure 4). Control lemons showed the highest softening process compared to GABA-treated fruits. The most effective GABA concentration on maintaining lemon firmness was 50 mM.

Regarding total soluble solids (TSS). Control fruits showed a content of 6.33 ± 0.03 and 6.12 ± 0.05 g 100 g^{-1} at harvest for 10 and 2 °C, respectively, in the first harvest date, and no significant increment occurred during postharvest. During the storage, TSS slightly decreased for both temperatures and harvest dates. GABA preharvest treatments showed similar results to control lemons without significant differences among them for both harvest dates and temperatures. Concerning the TA content, in general, there were no significant differences during the storage between the control and treated lemons at the two temperatures and in both harvest dates.

Total phenolic content in the flavedo of lemon fruit ranged from 180 to 196 mg and 100 g^{-1} at the first sampling date and increased during storage, reaching final values of 182 and 197 mg 100 g^{-1} in the control and GABA 50 mM-treated fruit, respectively, in harvest 1 at 10 °C (Figure 5). During the second harvest (at 10 °C) phenol content showed an overall increase compared to the first harvest, while in GABA treatments this bioactive compound increased significantly compared to the control ($p < 0.001$; Supplementary Table S2), being that this effect was also observed at 2 °C.

On the other hand, the total phenolic content analyzed in the juice of lemon fruits showed a higher concentration in the harvest 1 compared to harvest 2 (Figure 6). During storage, total phenolics showed a decrease at 10 °C and an increase at 2 °C. All GABA-treated lemons showed a significantly higher content of total phenolics compared to control fruits ($p < 0.001$; Supplementary Table S2), and especially for the 50 mM concentration, in both harvest dates and storage temperatures (2 and 10 °C).

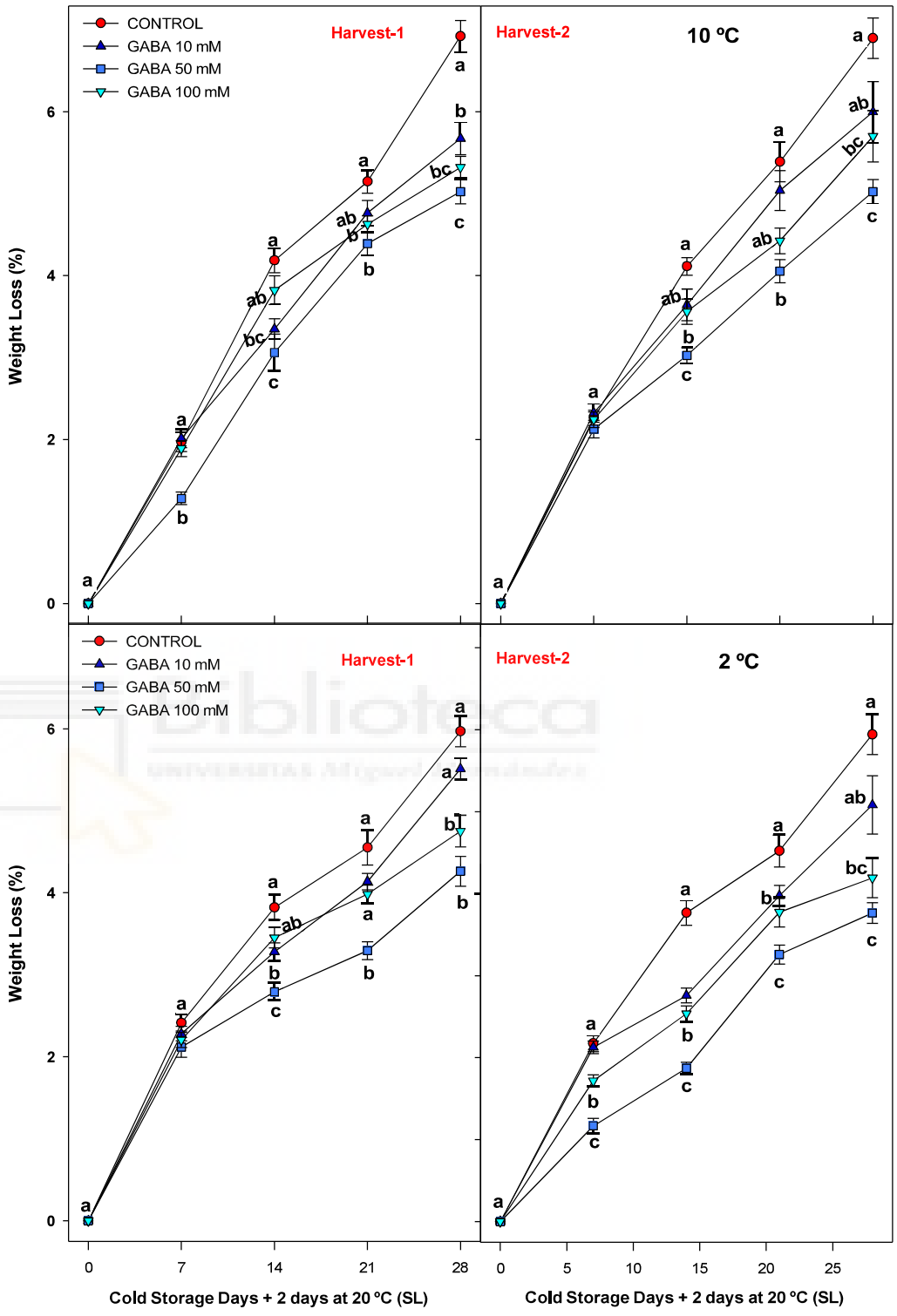

Figure 3. Weight loss (%) during 28 days of storage + shelf life (SL) at two temperatures (2 and 10 °C) from two harvest dates in lemon fruit. Data are the mean ± SE (n = 3). Different letters are significantly different at $p < 0.05$ according to Tukey's test.

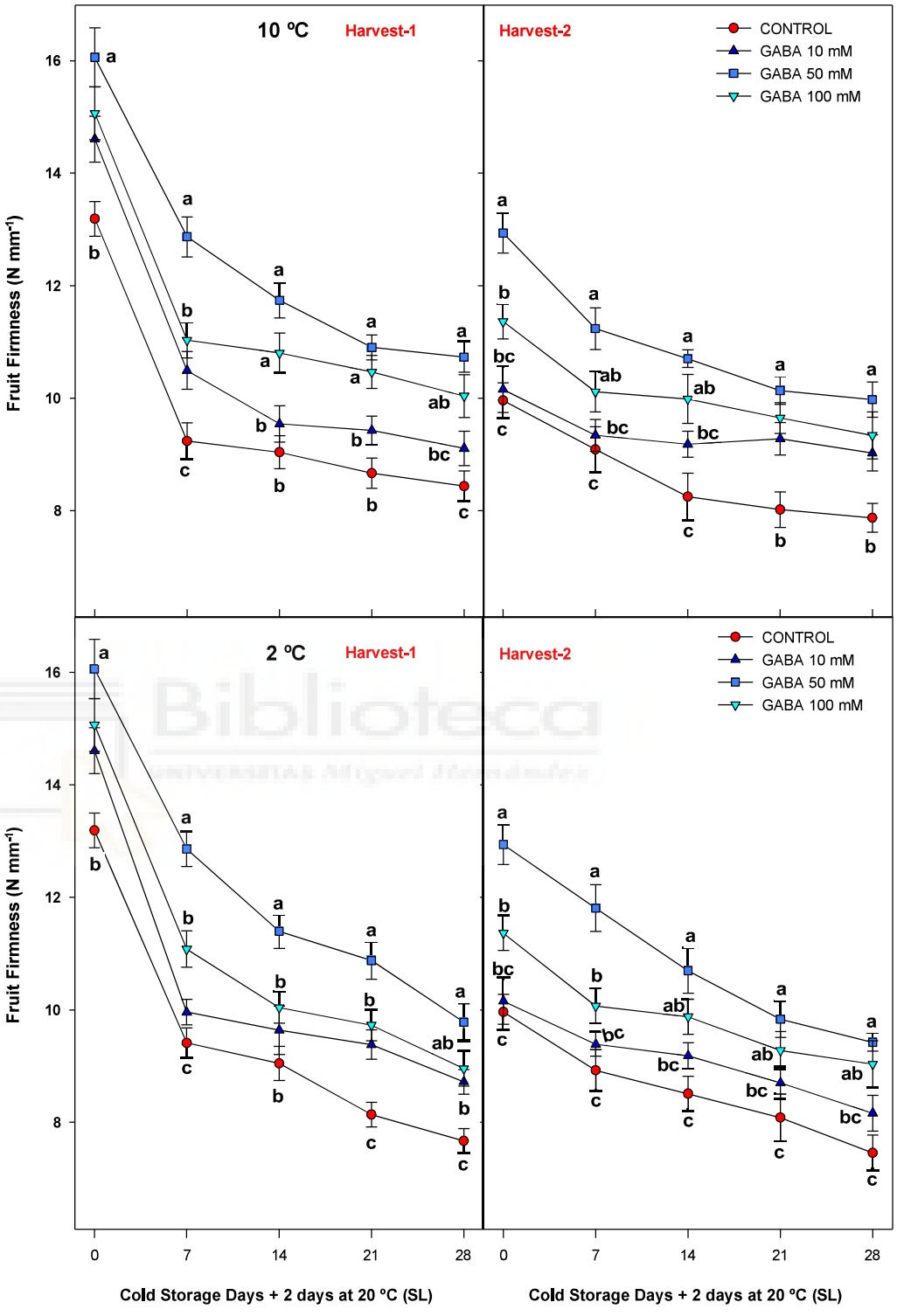

Figure 4. Firmness (N mm^{-1}) during 28 days of storage + SL at two temperatures (2 and 10 ˚C) from two harvest dates. Data are the mean ± SE (n = 3). Different letters are significantly different at $p < 0.05$ according to Tukey's test.

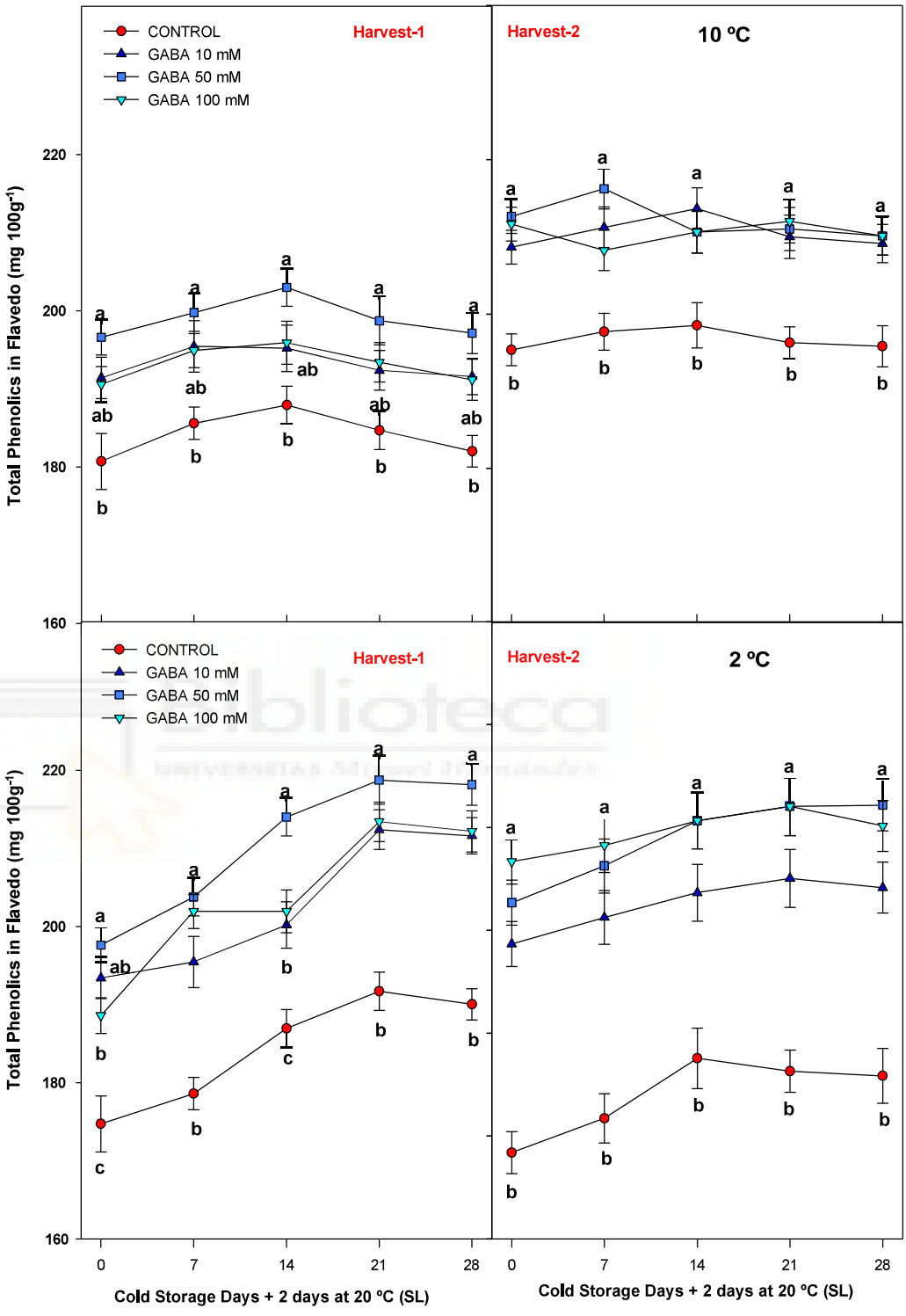

Figure 5. Total phenolics in the flavedo of lemon (mg 100 g^{-1}) during 28 days of storage + SL at two temperatures (2 and 10 °C) from two harvest dates. Data are the mean ± SE (n = 3). Different letters are significantly different at $p < 0.05$ according to Tukey's test.

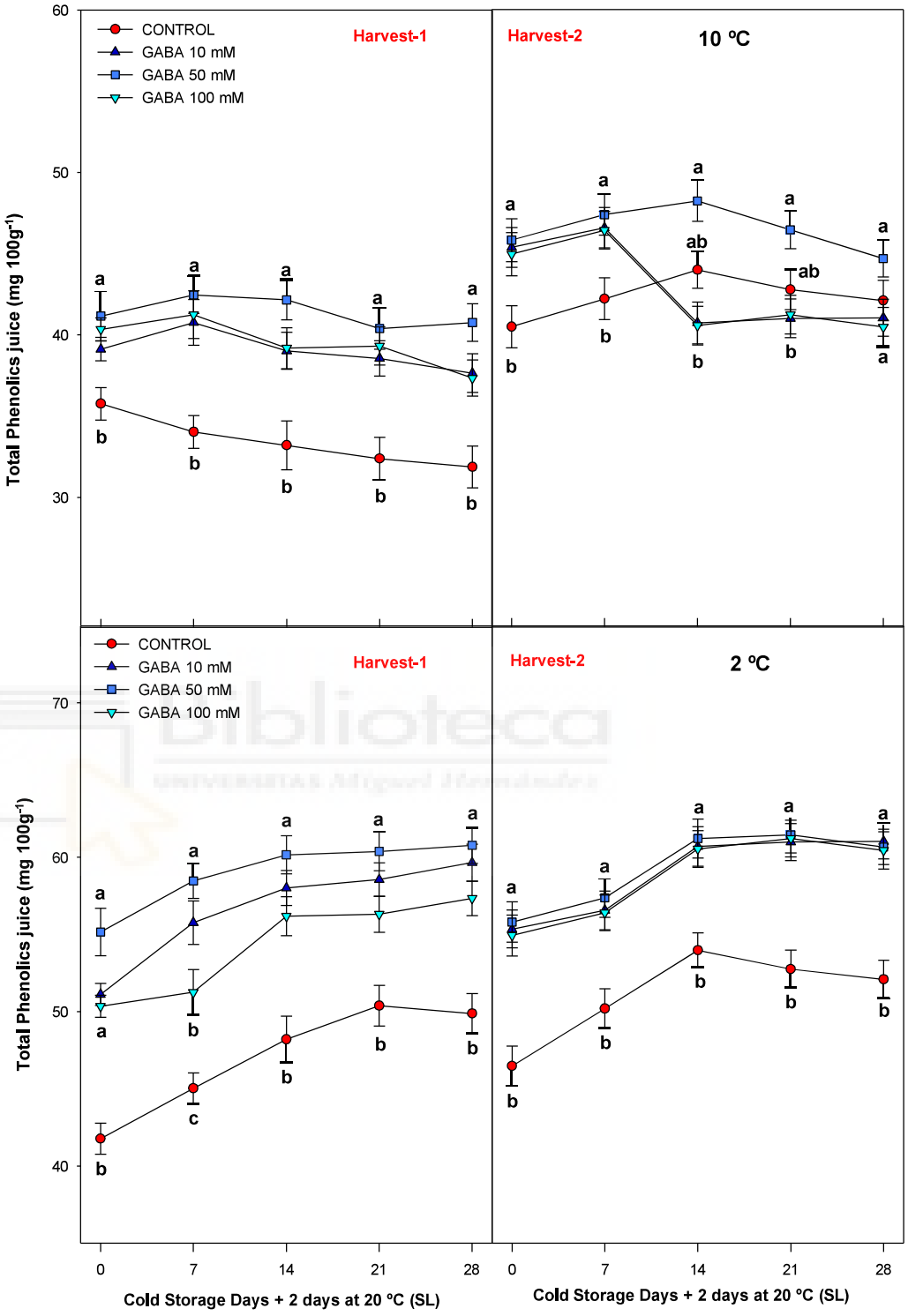

Figure 6. Total phenolics in the lemon juice (mg 100 g^{-1}) during 28 days of storage + SL at two temperatures (2 and 10 °C) from two harvest dates. Data are the mean ± SE (n = 3). Different letters are significantly different at $p < 0.05$ according to Tukey's test.

Total antioxidant activity (TAA) in the lemon juices was also significantly increased ($p < 0.001$; Supplementary Table S2) during storage at 2 °C from the first to fourth week at both harvest dates as a consequence of GABA treatments ($p < 0.001$; Supplementary Table S2). This increment was from 26.2 ± 1.7 to 35.7 ± 1.6 mg 100 g^{-1} in control and 50 mM in GABA-treated fruits, respectively, in the first harvest. For harvest 2, the TAA was from 31.7 ± 0.96

to 40.8 ± 1.7 mg 100 g^{-1} in control and 50 Mm GABA, respectively, also in the first harvest date. The values were significantly higher in the treated fruits than in the control, 50 mM being the most effective concentration of GABA treatment (Figure 7) ($p < 0.001$; Supplementary Table S2). Total antioxidant activity showed more variance in data during the storage at 10 °C in both harvest dates, although GABA at 50 and 100 mM exhibited significantly higher levels of TAA compared to control ($p < 0.001$; Supplementary Table S2) in the first harvest date.

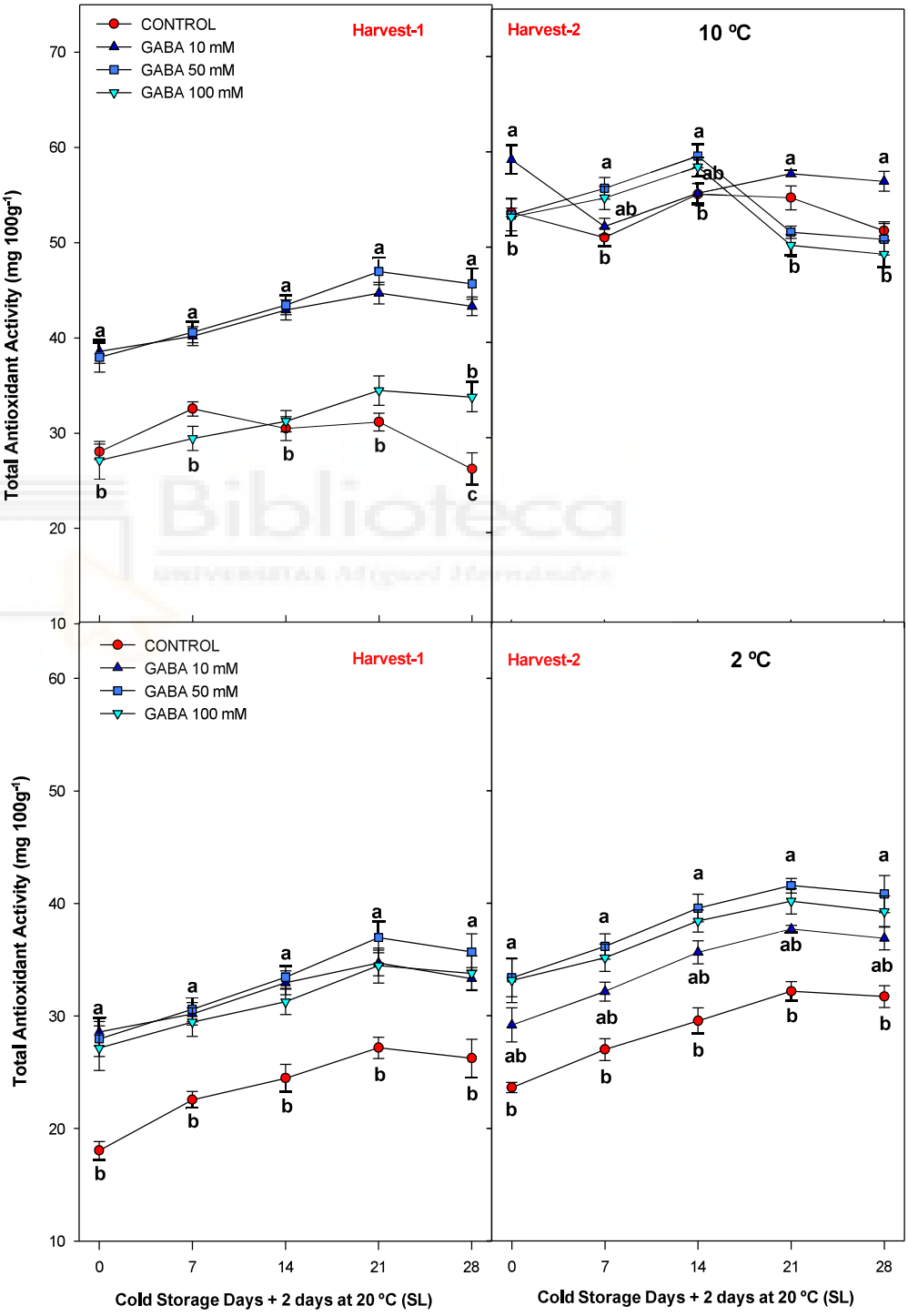

Figure 7. Total antioxidant activity in lemon juice (mg 100 g^{-1}) during 28 days of storage + SL at two temperatures (2 and 10 °C) from two harvest dates. Data are the mean ± SE (n = 3). Different letters are significantly different at $p < 0.05$ according to Tukey's test.

4. Discussion

It is clear that the consequence of climate changes associated with global warming is negatively affecting the fruit trees in general, and particularly in the citrus sector. In Southern parts of Spain, such as Alicante and Murcia, the scarcity of water is ruining crops and forcing trees to be uprooted. Apart from the episodes of extreme heat, the main problem for citrus is the mild winter temperatures, since the plant needs cold so that the skin of the fruit gains consistency, which directly affects the storability time once fruit is harvested. Therefore, it is necessary to deal with these stress situations in order to mitigate them, and among the different tools, we propose the use of preharvest elicitors, such as γ-aminobutyric acid (GABA), as this molecule is related to different growth environments [8].

Preharvest GABA application was effective in increasing crop yield as determined by the kg tree^{-1} and the number of lemons per tree, with the most effective GABA treatment being 50 mM. It is interesting to highlight that the improvement of crop yield was not dose dependent, since preharvest GABA at 10 mM showed better proficiency than 100 mM. The literature about the possible role of preharvest GABA applied in preharvest fruit productivity is scarce. The present results agree with the previous report on 'Fino' lemon, in which GABA at 100 mM GABA was the most effective dose in enhancing the yield during two consecutive seasons [13]. On the contrary, in 'Mollar de Elche' pomegranate trees, GABA preharvest treatments increased crop yield in a dose-dependent way [19]. The enhancement of lemon yield could be related to a higher content of photoassimilates through the developing fruits. Accordingly, the effects of GABA on enhancing the net photosynthesis rate were demonstrated in plants under stress conditions, the photosynthesis improvement was attributed to the exogenous application of GABA [8]. Furthermore, the relationship between kg tree^{-1} and number of fruit trees^{-1} in which similar results were observed, clearly shows that GABA treatments increased binding strength of the fruit branch, leading to reduction in normal fruit drop, which occurs throughout the fruit developmental cycle and is associated with environmental factors [19]. Current global warming predictions indicate that field temperature is expected to increase by 1.5–2.4 °C by 2050, and fruit crops will be subjected to more extreme weather conditions and increased abiotic stress, which in turn will reduce crop yield [20]. Therefore, the exogenous application of GABA could be considered an effective and sustainable strategy against different types of stress. An example of this effect was observed in creeping bentgrass, in which there was an alleviation of detrimental effects of heat stress, by reducing chlorophyll content, net photosynthesis, and water use efficiency among others, which was related to a GABA-induced increase in the activity of antioxidant enzymes (SOD, CAT, APX, POD, and DHAR) and with a reduction in ROS damage in the photosynthetic tissues [21].

Nowadays, the food market trends are based on fruits and vegetables with high-quality attributes that must be maintained for a long time during storage and commercialization. Fruits are often exposed to several stresses during postharvest storage, such as low temperature, affecting the ripening process, the onset of senescence, the loss of nutritive and bioactive compounds, and the reduction in shelf life. In this experiment, we stored the lemon fruits at two temperatures (2 and 10 °C), the 10 °C being considered as optimal and the 2 °C as the non-suitable temperature for lemon storage because it could lead to produce slightly retaining chilling injury damages. However, for this experiment, we observed that both temperatures maintained good results for lemon fruits after being treated with GABA. Two of the main parameters determining fruit deterioration during postharvest are weight loss and fruit firmness [22]. Even so, the weight loss was significantly reduced in GABA-treated than control lemons for both storage temperatures. Weight loss was even greater at 10 °C than at the 2 °C temperature. Thus, the fruit treated with GABA at 2 °C increased the quality of Verna lemon, preserving the weight for almost 14 days more than the control, which could reach a total of 42 days of shelf life. The visual quality of lemons is compromised (dehydration, shriveling, and decay) when the percentage of weight loss is higher than 6–7%. Similarly, fruit firmness was maintained by the GABA preharvest application when lemons were stored at 2 or 10 °C. One of the most important factors that

determine the commercial acceptance of lemons is fruit firmness, which is related to cell turgidity and thickness of skin [23]. For instance, GABA treatment can induce chilling tolerance in postharvest kiwifruit by regulating the ascorbic acid metabolism, keeping the firmness longer [24]. For both parameters, the best concentration of GABA was 50 mM with a non-dose dependence effect. In a highly sensitive zucchini fruit cultivar (Sinatra), the postharvest application of GABA improved zucchini quality during storage at 4 °C determined by a lower chilling injury index and weight loss [25]. In a similar way, GABA alleviated the chilling injury symptoms in peach fruit during cold storage due to the higher accumulation of ascorbic acid and glutathione [26]. In GABA-treated grapes, the percentage of weight loss was reduced, as well as stem browning and decay [27], while in tomato, GABA was able to obtain fruits with higher firmness [28]. TSS and TA are other important quality parameters in lemon fruit. In mature lemons, sugars account for 80–90% of TSS depending on cultivars, the rest being corresponded to TA and the major one, which was citric acid, which is the main organic acid present in citrus fruits [29]. TSS and TA content were not affected by GABA treatments and the slight decrease shown in both parameters during storage is to be expected, as this may occur due to increased fruit ripening [30].

The peel and the juice of lemon fruit have a wide range of natural phytochemical compounds, the most important being phenolic compounds (mainly flavonoids) that contribute to the total antioxidant activity. Carotenoids, vitamins, and essential oils are some of them and the content of these bioactive compounds is related to health properties attributed to their antioxidant nature [31]. In the present study, preharvest GABA treatments induced a higher concentration of total phenolics both in peel and juice. However, in both harvest dates and temperatures, GABA treatment significantly increased total phenolic concentration in flavedo and juice, with 50 mM being the most effective concentration. Furthermore, results of the present study show that the phenolic concentration was higher in the flavedo than in the juice, which is typical in lemon fruits [32]. The main factor responsible for the antioxidant properties attributed to lemon fruit is the concentration of phenolic compounds together with ascorbic acid. Thus, the effect of the treatment on the increase in the phenolic concentration in the juice and flavedo tissue of the lemon fruit would result in an increase in its antioxidant activity [33]. Therefore, GABA preharvest treatment of lemon trees increased total antioxidant activity at both harvest dates and was maintained at higher levels during storage at both temperatures, delaying the postharvest ripening process. This higher content of total phenolics could be attributed to a stimulation of activity of phenylalanine ammonia-lyase (PAL), which is the key enzyme in the phenolic biosynthesis pathway and a lower activity of polyphenol oxidase (PPO) as was reported for strawberries [34] and pomegranates [19]. In blood oranges stored at low temperatures (2 °C), GABA treatment increased PAL activity and enhanced or maintained total phenolic compound content [35]. In some GABA-treated fruits, some of the mechanisms that could explain the increased antioxidant capacity could also be that exogenous GABA alleviates cold damage through the maintenance of energy status by supplying nicotinamide adenine dinucleotide (NAD)+H+(NADH), adenosine triphosphate (ATP), and inhibition of cytoplasmic acidification, as well as by the endogenous accumulation of GABA during cold stress in the fruit. On the other hand, the reduction in cold damage is accompanied by the stimulation of the activity of antioxidant enzymes under cold storage conditions to scavange ROS, which could be another mechanism of action [35].

5. Conclusions

This study investigated the role of GABA in preharvest and postharvest, and overall, results indicate an improvement in the marketability of lemon fruits as a consequence of GABA preharvest treatments, the most effective concentration being 50 mM. Thus, GABA increases crop yield and quality and functional parameters in both temperatures and harvest dates. On average, shelf life could be prolonged 2 weeks more than that obtained for control lemons, leading to an increase in its economic value as well as its health beneficial effects for consumers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13071397/s1, Table S1: Analyses of variance (ANOVA) of crop parameters (kg tree -1 and fruit number tree^{-1}) for different harvest dates (1st, 2nd and 3rd harvest) as according to 2 categories: commercial and waste in 'Verna' lemon using the treatment studied as the factor. Table S2: Analyses of variance (ANOVA) of quality and functional parameters [weight loss, firmness, total phenolics in flavedo and juice and total antioxidant activity (TAA)] for different harvest dates (1st and 2nd harvest) in'Verna' lemon during 28 days of storage + shelf-life at 2 temperatures (2 and 10 °C) using the treatment studied and storage time as the factors.

Author Contributions: M.S. and D.V. conceived and designed the work; S.C. and J.M.V. performed the field treatments; F.B.-E.H., H.D.-M. and J.M.V. performed most of the analytical determination, in collaboration with the other authors; M.S. and D.V. analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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MDPI

Supplementary material

Potential Preharvest Application of γ-aminobutyric Acid (GABA) on improving Quality of 'Verna' Lemon at Harvest and during Storage

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Supplementary Table S1. Analyses of variance (ANOVA) of crop parameters (kg tree^{-1} and fruit number tree^{-1}) for different harvest dates (1st, 2nd and 3rd harvest) as according to 2 categories: commercial and waste in 'Verna' lemon using the treatment studied as the factor.[1]

Crop Parameters	¥ANOVA (F Value for treatment source)
Crop yield (kg tree^{-1}) for the 1st harvest	6.45**
Waste (kg tree^{-1}) for the 1st harvest	0.59 NS
Crop yield (kg tree^{-1}) for the 2nd harvest	14.94***
Waste (kg tree^{-1}) for the 2nd harvest	0.67 NS
Crop yield (kg tree^{-1}) for the 3rd harvest	0.25 NS
Waste (kg tree^{-1}) for the 3rd harvest	0.48 NS
Crop yield (fruit number tree^{-1}) for the 1st harvest	18.61***
Waste (fruit number tree^{-1}) for the 1st harvest	0.29 NS
Crop yield (fruit number tree^{-1}) for the 2nd harvest	9.82***
Waste (fruit number tree^{-1}) for the 2nd harvest	7.28**
Crop yield (fruit number tree^{-1}) for the 3rd harvest	0.12 NS
Waste (fruit number tree^{-1}) for the 3rd harvest	0.39 NS

[1] NS = not significant; *, ** and *** significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; data were previously tested for normality test.

Supplementary Table S2. Analyses of variance (ANOVA) of quality and functional parameters [weight loss, firmness, total phenolics in flavedo and juice and total antioxidant activity (TAA)] for different harvest dates (1st and 2nd harvest) in 'Verna' lemon during 28 days of storage + shelf-life at 2 temperatures (2 and 10 °C) using the treatment studied and storage time as the factors.[1]

Quality and Functional Parameters	ANOVA (F Value for treatment source)	ANOVA (F Value for storage time source)	ANOVA (F Value for interaction)
Weight loss for the 1st harvest at 2 °C	42.60***	1018.44***	7.04***
Weight loss for the 2nd harvest at 2 °C	69.82***	657.80***	7.32***
Weight loss for the 1st harvest at 10 °C	40.26***	1264.35***	7.37***
Weight loss for the 2nd harvest at 10 °C	22.95***	689.95***	3.90***
Firmness for the 1st harvest at 2 °C	60.77***	208.98***	1.02 NS
Firmness for the 2nd harvest at 2 °C	46.92***	38.49***	1.046 NS
Firmness for the 1st harvest at 10 °C	62.68***	161.27***	0.79 NS
Firmness for the 2nd harvest at 10 °C	47.53***	24.90***	1.13 NS
Total Phenolics flavedo for the 1st harvest at 2 °C	97.60***	47.52***	1.025 NS
Total Phenolics flavedo for the 2nd harvest at 2 °C	110.06***	6.32***	0.23 NS
Total Phenolics flavedo for the 1st harvest at 10 °C	28.12***	3.33*	0.08 NS
Total Phenolics flavedo for the 2nd harvest at 10°C	42.30***	0.50 NS	0.59 NS
Total Phenolics juice for the 1st harvest at 2 °C	96.02***	26.32***	0.65 NS
Total Phenolics juice for the 2nd harvest at 2 °C	54.71***	20.06***	0.20 NS
Total Phenolics juice for the 1st harvest at 10 °C	41.05***	3.35*	0.43 NS
Total Phenolics juice for the 2nd harvest at 10°C	12.98***	5.08***	2.65**
TAA for the 1st harvest at 2°C	63.81***	27.87***	0.41 NS
TAA for the 2nd harvest at 2°C	69.27***	35.12***	0.11 NS
TAA for the 1st harvest at 10°C	180.74***	16.78***	3.35***
TAA for the 2nd harvest at 10°C	7.36***	10.71***	5.67***

[1] NS = not significant; *, ** and *** significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; data were previously tested for normality test.

4.3. PUBLICATION 3

PUBLICATION 3 (Literal transcription)

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Melatonin Postharvest Treatment in Leafy 'Fino' Lemon Maintains Quality and Bioactive Compounds

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Abstract: Spain is a great producer of organic lemon; however, it is necessary to reduce the losses caused by post-harvest diseases. Melatonin (MEL) is a naturally occurring compound with phys- iological functions in fruit growth and ripening and is able to modulate postharvest ripening and senescence, most of it being concentrated in climacteric fruit. Thus, the aim of this study was to apply MEL to organic lemon fruit with stems and leaves (LEAF) and to organic lemon without those components (LEAFLESS) after harvesting and storage during 21 days at 2 °C to understand the effects of this treatment on the fruit quality. For this purpose, two experiments were carried out. First, MEL was applied at 0.01 mM, 0.1 mM and 1.0 mM by immersion for 15 min on lemon fruits, and the quality parameters and bioactive compounds of the fruit were analysed. Subsequently, a second experiment was carried out where the best concentration (1 mM) was selected and another time (15 and 30 min) was added, with the same quality parameters being analysed. As a result, we observed that all MEL treatments showed positive effects on weight loss reduction, softening (higher fruit firmness), total acidity and lower colour changes. Total phenols increased in MEL-treated lemons, both in peel and juice. For the three concentrations tested, the best efficiency was obtained with MEL at 1.0 mM, while LEAF lemons were the most effective. In conclusion, lemons containing stems and leaves (LEAF) improved preservability by using MEL at 1.0 mM with better organoleptic quality and enhanced phenolic compounds.

Keywords: bioactive compounds; firmness; melatonin; organic; postharvest; quality

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1. Introduction

Nowadays, in the marketing of some citrus fruits, the option of selling the fruit together with stem and leaves is possible, mainly for clementines and oranges. The presence of the leaves with the fruit means that the product is more natural and fresher [1]. There is not enough knowledge about the role of the leaf in maintaining quality during post-harvest. However, some studies have been carried out on pineapple and have concluded that the crown plays an important role in post-harvest quality maintenance. The fruit's detachment from the pineapple crown after harvest increases internal browning, reactive oxygen species (ROS), cell membrane damage, biosynthesis and oxidation of compounds [2]. These events directly influence the quality of the fruit.

Size, shape, skin thickness, visual appearance or colour as well as the percentage of juice, sugars, acidity or bioactive compounds are quality parameters that determine consumer acceptability [3]. One of the major postharvest disorders that affects lemon fruit quality is decay incidence, which is mainly caused by *Penicillium digitatum* and causes huge economic losses for the lemon industry worldwide [4]. Currently, postharvest diseases have been controlled mainly by the application of conventional fungicides such as imazalil (IZ),

sodium ortho-phenyl phenate (SOPP), thiabendazole (TBZ) or different mixtures of these compounds [5]. The use of fungicides is prohibited in organic crops. Several non-thermal technologies have also emerged in recent decades for the treatment of food crops, such as UV-C irradiation or electrical pulses, although their potential to negatively influence sensory attributes has been highlighted [6]. Therefore, compared to conventional lemons, organic lemons present additional problems until they reach the final consumer in optimum quality conditions [7]. They are more susceptible to postharvest diseases, so in order to consolidate a more sustainable agriculture without adverse effects on consumer health, new alternatives have emerged in food production, such as the use of elicitors.

Melatonin (MEL) or N-acetyl-5-methoxytryptamine is an indoleamine that was first discovered from the pineal gland of vertebrates [8] and, five decades later (1995), in the plant kingdom. MEL, as a phytohormone, is involved in a wide range of physiological processes in plants, from germination to ripening and senescence as well as amelioration of several abiotic stresses [9–11]. The biosynthetic pathway of MEL starts with the amino acid tryptophan, which is converted into serotonin and via N-acetylserotonin produces MEL and is regulated by six enzymes that are essential for maintaining the endogenous levels [12]. In fruits, the endogenous concentration of MEL changes during development and maturation, reaching a peak after fruit setting and progressively with the onset of ripening, as has been observed in sweet cherries [13]. Based on this statement, preharvest applications of MEL were effective on increasing yield, the content of bioactive compounds (polyphenols and anthocyanins), as has been reported for pomegranate [14,15], and enhancing the quality attributes of sweet cherries such as colour, firmness, total soluble solids (TSS) and titratable acidity (TA) [16]. As a postharvest treatment, MEL could be useful to maintain and improve the postharvest quality traits in fruit and to reduce the spoilage and decay, although different effects have been obtained depending on the fruit species [17]. In addition, MEL was also effective on increasing the content of bioactive compounds and the antioxidant activity of several fruit commodities [18] but also alleviating several abiotic stresses, the most studied being chilling injury [19].

However, the use of MEL during postharvest storability of citrus fruits has been poorly studied, and few data have been reported. In 'Newhall' navel oranges, MEL induced a reduction in weight loss and respiration rate and increased the organoleptic quality traits [20]. The immersion with MEL (0, 10, 100 and 1000 nmol) of 'Washington' navel oranges and storage at chilling temperature was effective in reducing the CCI index (the standard parameter used in the citrus industry to determine the ripening stage of citrus fruit by colour [21]) and water loss and preserved the skin colour, the best results being obtained for MEL at 1000 nmol [22]. The citrus industry is providing new formats of several citrus fruit with the presence of stalks and leaves; for that reason, the demand for this new citrus presentation by consumers is growing. However, lemons are normally harvested without stems and leaves and are susceptible to quality losses during post-harvest storage. Therefore, the aim of this work was to evaluate the role of melatonin as an elicitor agent in the behaviour of lemon fruits, harvested both with stems and leaves (LEAF) and without them (NO LEAF), in order to know if it can preserve or improve the quality of the fruit during postharvest storage.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The experiments were carried out with organic lemon trees that were 20 years old and grafted on *Citrus macrophylla* rootstock (*Citrus limon* (L.) Burm. f) 'Fino-95', growing in a commercial orchard located in La Matanza (Alicante, Spain). The crop was located in an area with a Mediterranean climate, characterised by an average annual temperature of 18.2 °C and a rainfall of 283 mm. The lemon trees were cultivated in accordance with the current organic farming regulations, using a planting pattern of 6 × 5 m and during the growth cycle of 2021–2022. Two experiments were carried out, Harvest 1 and Harvest 2. For experiment 1, or Harvest 1, 30 lemon trees in good vegetative condition were used,

and 10 fruits were picked at random from each tree: 150 of them were cut with a part of the stalk and a couple of leaves (LEAF) and the other 150 were cut by the stalk (NO LEAF) according to the standard procedure for commercialisation, as shown in Figure 1.

Figure 1. Photography of 'Fino' lemons with stalks and leaves (**right**, LEAF) and without (**left**, NO LEAF) used in the experiments.

At the laboratory, 8 lots of 30 fruits were selected (240 fruits) in the same day, from the same sub-region and the same producer, of which 4 lots were with leaves and 4 were without leaves. Both types of lemons were treated with melatonin at 0.01 mM, 0.1 mM and 1.0 mM concentrations by dipping for 15 min (based on previous experiments [16]), while control fruits were immersed in distilled water. After treatments, the fruits stood on the bench for air drying before being stored under cold conditions at 2 °C and an Relative Humidity (RH) of 90%. Analytical determinations were performed after 21 days plus 2 days at 20 °C (shelf-life), and, additionally, 30 fruits with leaves and 30 without leaves were used for day 0 determinations. For the second experiment, or Harvest 2, the conditions were the same; we used new lemons with the same features. The only difference between the harvests was the number of lemons used, which was reduced from 8 lots to 4 lots, 2 lots of 30 lemons with leaves and 2 lots of 30 lemons without leaves, since only one concentration of melatonin (1 mM) was used because it was the one with the best results. In addition, two different times for dipping (15 and 30 min) were performed.

2.2. Lemon Fruit Quality Characteristics

Quality parameters (physiological weight loss, fruit firmness, respiration rate, colour, total soluble solids content, titratable acidity and electrolyte leakage) were individually measured for each lemon fruit (data are the mean ± SE, n = 30). Physiological weight loss was determined by weight of the recently harvested fruit and that obtained at each sampling date and expressed in percentage (data are the mean ± SE, n = 30). For fruit firmness, a texturometer (TA-XT2i Texture Analyzer, Stable Microsystems, Godalming, Surrey,, UK) was used with a flat probe of 10 cm. The percentage of deformation was 3% of the fruit diameter, and results were expressed as N mm^{-1} (Data are the mean ± SE, n = 30). The respiration rate was analysed at room temperature; each lemon fruit was placed in a 0.5 L glass jar for 60 min. Then, 1 mL of the atmosphere generated in the headspace was sampled, and the CO_2 content was quantified using a gas chromatograph (Shimadzu: Kyoto, Japan) coupled with a thermal conductivity detector. The results wereexpressed in mg CO_2 kg^{-1} h^{-1}. Colour was measured at three points along the equatorial fruit perimeter by using a Minolta colorimeter (CRC400, Minolta, Osaka, Japan), and the results were expressed using the Citrus Colour Index (CCI), which was determined by measurements of the basic parameters of L*, a* and b* using a colorimeter. C*, Ho and CCI were calculated using the formulas as follows: C* = (a*2 + b*2)1/2, Ho = arctan (b*/a*) and CCI = 1000 a*/(L*·b*), respectively. The content of TSS was quantified in each juice sample using a digital refractometer (Hanna Instruments, Woonsocket, RI, USA). Moreover, TA was measured in the same juice sample by the automatic titration (785 DMP Titrino, Metrohm, Herisau, Switzerland) of 0.5 mL juice neutralised with different volumes of 0.1 N NaOH until a pH of around 8.1 was achieved. Electrolyte leakage (EL) was evaluated in the peel tissue, following the method described by McCollum and McDonald [23] with some modifications. First, slices of the three replicates per treatment were cut to 4 mm thickness in the equatorial zone of the lemon fruit. Fifteen discs were extracted for each

replicate using a 0.5 cm diameter cork borer. After 3 rinses of 3 min for each replicate with deionized water, they were subjected to constant shaking with 50 mL of deionized water at room temperature. After 30 min, the initial electrical conductivity (C1) was measured using a Crison conductivity meter. The samples were frozen and then brought to 121 °C for 15 min. Total conductivity (C2) was evaluated with samples at room temperature (20 °C).EL was calculated as (C1/C2) × 100.

2.3. Extraction and Quantification of Total Phenolic Content

The Folin-Ciocalteu method was used as previously described by García-Pastor et al. in 2020 [24]. A stainless-steel lemon peeler was used to obtain the flavedo tissue by homogeneously splitting several 0.5 mm thick slices of lemon peel. For lemon juice extraction, a domestic squeezer ('Citromatic', Braun Española S.A., Barcelona, Spain) was used for the preparation of the juices by carefully squeezing the fruits by hand to avoid contamination by the components in albedo. Thus, 2 g of flavedo or 2 mL of juice were added to a solution of water:methanol (2:8) and 2 mM NaF (to inhibited the degradation of the phenolics by the enzyme polyphenoloxidase) and a homogeniser with an Ultraturrax homogeniser (T18 basic, IKA, Berlin, Germany) at maximum speed for 1 min. The extract was centrifuged (at 4 °C and 10,000×*g* for 10 min). An aliquot (200 µL) was mixed with the Folin reagent and the absorbance was quantified at 760 nm in a UV-Vis spectrophotometer (UV-1900i, Shimadzu, Duisburg, Germany).

2.4. Statistical Analysis

The software IBM SPSS Statistics 22.0 (IBM Corp., Armong, NY, USA) was used for statistical analyses. Data are the mean SE for each replicate (n = 3). The data of the analytical determinations were submitted to two-way analysis of variance with treatment, storage time and type of leaf (Leaf vs. No Leaf) as factors. Means were compared using a multiple range test (Tukey's test) to examine if differences among treatments, storage time and type of leaf were significant, and the level of significance was established at p-value < 0.05.

3. Results and Discussion

3.1. Effect of Melatonin on Respiration Rate and Weight Loss

Respiration consists of a series of enzyme-catalysed reactions, the rate of which is related to temperature [25]. Furthermore, it is a complex of several oxidative reactions due to the breakdown of carbohydrates, mainly glucose, to produce the necessary energy during the storage of fruits in which H_2O and CO_2 are produced [26]. Citrus fruits are non-climacteric with slow respiration rates, although respiration of citrus fruits is affected by several factors, including temperature, humidity, gas composition of the atmosphere and handling practices, among others [27]. The respiration rate of 'Fino' lemon fruit gradually increased during the 21 days of storage significantly (p-value < 0.05), for both Harvest 1 and Harvest 2 and with LEAF and NO LEAF (Figure 2). At 21 days + 2 days of shelf life, control leafless lemons showed a significant lower respiration rate of 13.46± 0.34 mg kg^{-1} h^{-1} compared to 10.17±0.15 mg kg^{-1} h^{-1} on day 0 (p-value < 0.05). Melatonin-treated NO LEAF lemons tended to have a lower respiration rate than LEAF lemons, with the exception of those treated with 0.01 mM. This result suggests that the lowest dose of 0.01 mM has no effect on this physiological parameter. The respiration rate of fruits with leaves also increased compared to the day of harvest (Day 0). After storage, control LEAF lemons showed a respiration rate of 14.65±0.30 mg kg^{-1} h^{-1}, which was about 1% higher than NO LEAF lemons. Leafy lemons treated with melatonin increased their respiration rate, with the exception of those treated with 1 mM that had significantly (p-value < 0.05) lower results than the control with 13.55± 0.15 mg kg^{-1} h^{-1}. In addition, lemons from Harvest 2 showed greater respiration rates than those from Harvest 1.

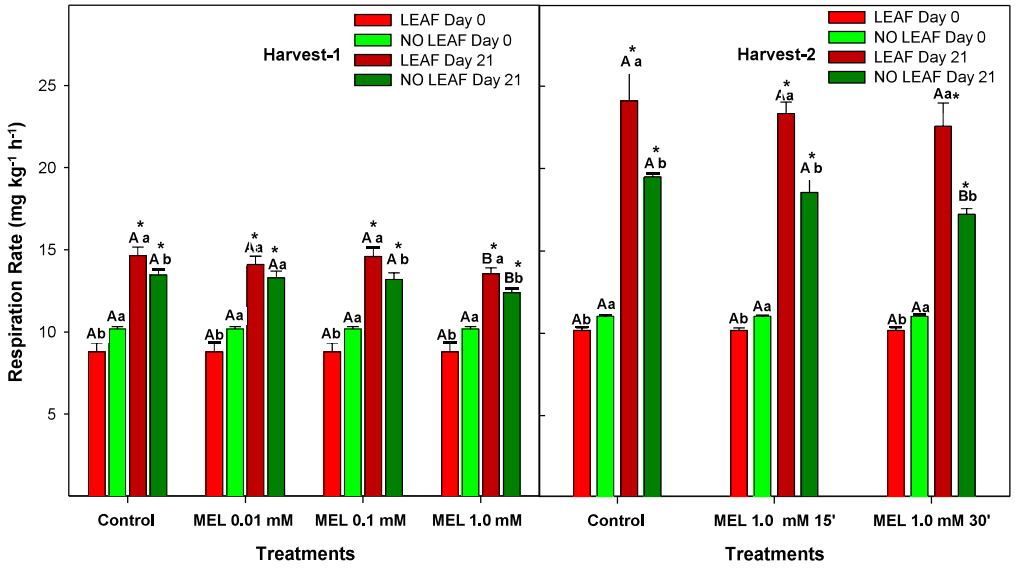

Figure 2. Respiration rates of 'Fino' lemons with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest 1) and MEL at 1.0 mM during 15 and 30 min (Harvest 2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letters denote significant differences between control and MEL treatments, while bars with different small letters denote significant differences between lemons with LEAF and NO LEAF. The asterisk symbol denotes significant differences between both storage days (0 and 21 days) for each type of leaf and treatment.

The respiration rate was significantly higher (p-value < 0.05) in lemons with LEAF than it was in those with NO LEAF for both experiments apart from the case of MEL at 0.01 mM. This result was expected since the respiration rate is the sum of both leaves and fruits. It has been reported that the leaves exhibited a significantly higher respiratory intensity (20 fold times) than the fruit [28]. However, the application of MEL at 0.01, 0.1 and 1.0 mM during 15 min (Harvest 1) showed that 1.0 mM was most effective in reducing the respiration rate in both LEAF and NO LEAF lemons. Considering this result, in the second experiment (Harvest 2), 2 dipping times (15 and 30 min) and the MEL concentration of 1 mM were assayed. The results were similar, but the dipping time of 30 min led to a significantly lower respiration rate than that obtained for 15 min. A high respiration rate during storage leads to excessive consumption of nutrients such as sugars, organic acids and amino acids, thus affecting the lemon quality, as occurred in control lemons. The application of MEL at 1.0 mM could retard lemon quality deterioration and delay the senescence process by reducing the respiration intensity, as has been observed for navel oranges [20], pears [29] and sweet cherries [30]. In addition, the oranges study concluded that melatonin significantly delays physiological senescence through a reduction of post-harvest respiration. Similar results were also found in [31]. However, the mechanism of action on inhibiting the respiration rate during postharvest storage of fruits, as well as the molecular and biochemical regulation, deserves further investigation.

The weight loss of fruits and vegetables during storage is mainly due to the decrease of water during transpiration, which is one of the main causes of the damages of lemons that results in quality losses. Once the lemon has been harvested, transpiration processes continue, where water in a vapour state goes through the stoma and the epidermis, causing the loss of fruit weight [25]. Then, physiological water loss is considered one of the most important factors related to fruit quality and various postharvest disorders. There are many factors affecting water loss, which are classified into 3 categories: pre-harvest, harvest and postharvest factors. At postharvest, the most important are cold temperature and relative humidity of the chamber [32]. During 21 days of storage, and regardless of treatment, the weight loss of 'Fino' lemons exhibited a progressive enhancement, the increase being significantly higher (p-value < 0.05) in LEAF than NO LEAF fruits in both harvests (Figure 3).

In LEAF lemons from Harvest 1, both control and MEL at 0.01 mM reached the highest weight loss (≈6%), while the lowest was obtained for those fruits treated with MEL at 1.0 mM. In lemons from Harvest 2, MEL-treated fruits at 1.0 mM and 30 min showed the lowest weight losses (≈4.5%), with a similar behaviour for NO LEAF lemons. However, at Harvest 1, NO LEAF lemons show higher weight losses than the control (p-value < 0.05), with the exception of 0.1 mM melatonin.

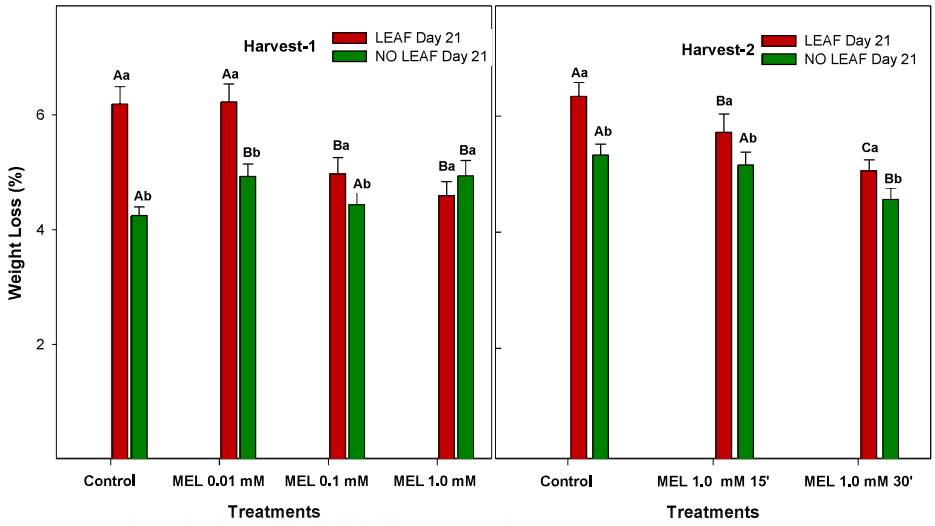

Figure 3. Percentage of weight loss of 'Fino' lemons with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest 1) and MEL at 1.0 mM during 15 and 30 min (Harvest 2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letters denote significant differences between control and MEL treatments, while bars with different small letter denote significant differences between lemons with LEAF and NO LEAF.

It has been reported that water loss over 5% of the initial fruit weight will affect the visual appearance, with major symptoms being wilting, peel desiccation, membrane disruption and onset of the senescence, which will limit fruit marketability and cause economic losses [32,33]. Comparing the two preservation methods (LEAF, NO LEAF), it has been observed that lemons without leaves showed lower weight losses than when the fruit kept the leaves. This could be due to the larger surface of the leaf promoting greater transpiration [34]. The results show that melatonin treatment does not affect lemons without leaves; however, it does have a beneficial effect on lemons with leaves. In the orange study [20], the researchers concluded that MEL treatment caused a significant decrease in weight loss compared to the control. Similar results have also been found in mandarins and strawberries as well as in sweet cherries and other 'Kinnow' fruits and vegetables [30,31,35,36]. Physiological postharvest weight loss is usually attributed to both water loss (by transpiration) and respiration. In this report, since all the fruits were in the same cold rooms with identical temperature and relative humidity, the MEL treatment on reducing the weight loss could be attributed to the reduced respiration rate, as has been observed in navel oranges [20].

3.2. Effect of Melatonin on Lemon Quality Characteristics

The quality of fruits refers to a series of characteristics that determine their degree of acceptance by the consumer, and in the case of lemon colour, firmness, electrolyte leakage, total soluble solids and titratable acidity are the most important and are related to organoleptic quality.

Softening is one of the main causes affecting quality deterioration and reduced shelf life of fruits, both climacteric and non-climacteric. Results about firmness are shown in Figure 4. After 21 days of storage, firmness decreased in both control and MEL-treated lemons and also in LEAF and NO LEAF lemons (p-value < 0.05) in Harvest 1 and Harvest 2.

However, those fruits treated with MEL at 1 mM showed higher firmness compared with the control, with MEL at 0.01 and 0.1 mM (p-value < 0.05) being the most effective dose related to this parameter. In Harvest 2, the MEL treatment in LEAF fruits presented higher firmness with respect to the control (p-value < 0.05) for both 15 and 30 min. In NO LEAF fruits, firmness was greater in treatment with MEL 1 mM 30' than in the control and MEL1 mM 15' (p-value < 0.05).

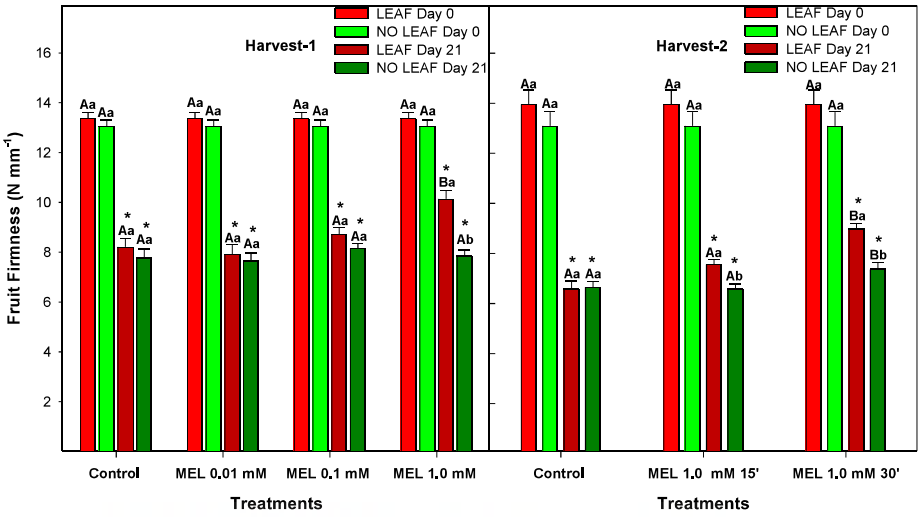

Figure 4. Firmness of 'Fino' lemons with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest 1) and MEL at 1.0 mM during 15 and 30 min (Harvest 2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letters denote significant differences between control and MEL treatments, while bars with different small letters denote significant differences between lemons with LEAF and NO LEAF. The asterisk symbol denotes significant differences between both storage days (0 and 21 days) for each type of leaf and treatment.

Comparing LEAF and NO LEAF lemons, there were not significant differences (p-value > 0.05), with the exception of MEL at 1 mM, since LEAF lemons showed significantly higher firmness (≈ 11 N mm^{-1}) than NO LEAF (≈7 N mm^{-1}) in Harvest 1, with the same behaviour being obtained in Harvest 2 when 15 and 30 min were used.

Firmness of fruit and vegetables is modified throughout post-harvest storage. These changes in texture are related to the turgidity of tissues and, therefore, to the hydrolytic activity of enzymes that are responsible for degrading pectins, celluloses and hemicelluloses of the cell wall [37]. The maintenance of firmness as a consequence of postharvest MEL treatments is a major factor that contributes to preserving fruit quality during storage. Accordingly, MEL at 0.5 mM by dipping 1 h delayed the postharvest ripening of man- goes [33], as well as for bananas, in a concentration-dependent manner in the range of 0.05–0.5 mM [38]. In pomegranates, preharvest application of MEL at 0.1, 0.3 and 0.5 mM was effective in maintaining firmness during 60 days of storage [15]. Thus, results demon- strate that MEL applications, especially at 1.0 mM, retarded the decrease of firmness, which could be related to a delay in cell wall degradation [29].

The organoleptic properties that are essential related to consumer appreciation of lemons are aroma, taste and colour. However, the senescence process during postharvest is manifested by the deterioration of sensory quality attributes, with changes in the levels of sugars, organic acids, and colour deterioration. The total soluble solids (TSS) and titratable acidity (TA) are key parameters for evaluating the quality of lemon juice. They are used as a maturity index in quality measurements [39]. With respect to total soluble solids in our samples, no significant differences (p-value > 0.05) were shown between the control and any MEL concentration, neither LEAF and NO LEAF lemons nor between day 0 and day 21 at Harvest 1. At Harvest 2, apart from LEAF and NO LEAF lemons showing significant

differences at MEL 1 mM 15 and 30 min (p-value < 0.05), we observed the same behaviour (Figure S1). In general, there was not an increase in sugar content or a decrease in acidity of non-climacteric fruits throughout the storage, which is consistent with the progress of the ripening process. The application of MEL on titratable acidity (Figure S2) showed that a reduction (p-value > 0.05) was produced in control and MEL-treated lemons after 21 days of storage, being higher in LEAF than in NO LEAF fruits at Harvest 2.

With regard to our results, the application of postharvest melatonin in nectarine fruit by 30 min of immersion showed no significant difference in TSS content between untreated and melatonin-treated fruit after 30 d of storage [35]. However, after 40 days of storage, nectarines showed a decrease in TSS at the highest applied melatonin concentration(1000 μmol L 1), which is consistent with other studies in plums and mangoes [33,40]. Regarding the TA content, several studies show similar results; the application of MEL in nectarines, mangoes and plums showed that there were no differences between the control and treatments. The role of MEL in maintenance acidity has been already reported in other fruits such as navel oranges [20], pears [29] and sweet cherries [30].

We measured colour (expressed as the Citrus Colour Index, CCI) in the peel of lemons. With respect to peel colour (Figure 5), CCI at Harvest 1 was 4, showing that the skin had a light yellow colour. During storage, CCI decreased in both control and MEL- treated lemons, although LEAF fruits treated with MEL at 1.0 mM did not significantly (p-value > 0.05) change with respect to values at harvest (Harvest 1), contrary to NO LEAF lemons since they had the lowest CCI (3). However, in Harvest 2, the lowest CCI (p-value < 0.05) was obtained in NO LEAF lemons, independently of duration of treatment (15 or 30 min).

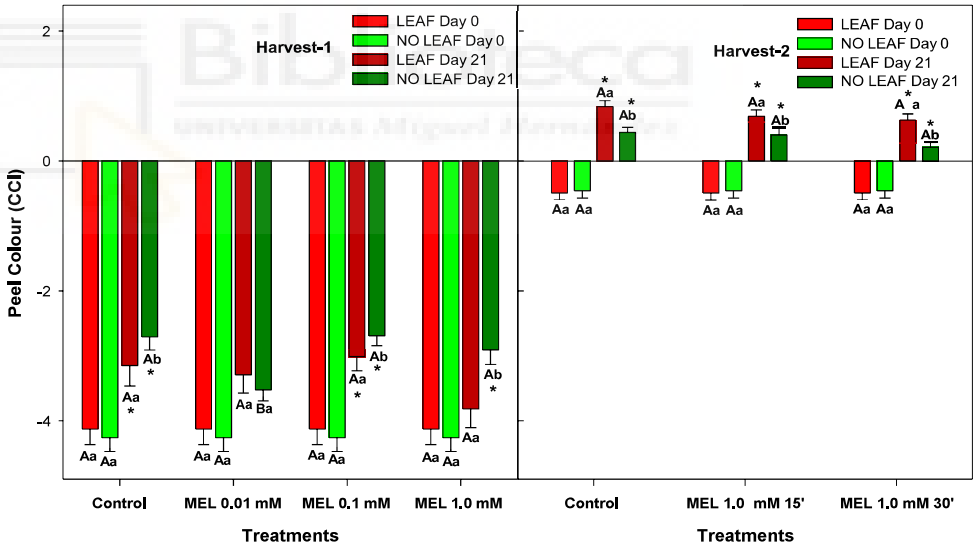

Figure 5. Peel colour of 'Fino' lemons with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest 1) and MEL at 1.0 mM during 15 and 30 min (Harvest 2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letters denote significant differences between control and MEL treatments, while bars with different small letters denote significant differences between lemons with LEAF and NO LEAF. The asterisk symbol denotes significant differences between both storage days (0 and 21 days) for each type of leaf and treatment.

The peel colour is usually associated with the internal quality (flavour and texture), which can affect the purchase decision by the consumers [41]. It is well known that the change in colour from green to yellow in lemon fruits is due to some modifications in the composition and concentration of chlorophylls and carotenoids. According to several studies, melatonin significantly decreases chlorophyll degradation and increases the biosynthesis of carotenoids (including α, β-carotene and lycopene) at transcriptomic and metabolic levels in broccoli, tomatoes and cabbage [42–44]. In our work, MEL treatment

enhanced colour changes of lemon fruit, as indicated by the higher values of CCI, consistent with navel oranges [20] treated with MEL at 200 µM. In lemons (yellow-coloured citrus), contrarily to oranges, the changes in carotenoids during fruit growth and ripening have been less investigated [45], which might be due to greater activity of phytoene synthase than of phytoene desaturase [46]. In another study of melatonin post-harvest treatment in oranges, something similar occurred, and the color of the control fruit exhibited a strong increase in storage. However, the oranges treated with melatonin at a concentration similar to ours accelerated and significantly improved this change of color [20]. It also occurs in strawberries and sweet cherries [30,31].

The incidence of CI symptoms results from primary and secondary responses to cold temperatures. Changes in the membrane structures and permeability are primary responses. On the other hand, secondary responses consist of ion leakage, a decrease in cellular energy and oxidative damage [47]. Electrolyte leakage (EL) is among the first defence activation events in plants, and membrane permeability can be evaluated by measuring this parameter. Figure 6 shows that EL significantly increased (p-value < 0.05) from the beginning to the end of storage in both harvests for controls and Mel 0.01 mM. However, those fruits treated with MEL at 0.1 and 1 mM did not show significant differences during storage. LEAF lemons EL showed significant differences (p-value < 0.05) between all MEL-treated fruits and the control, with values of 18.99±0.57% for the control and 10.24±0.26% in lemons treated with 1 mM MEL. Comparing LEAF and NO LEAF lemons, there were significant differences (p-value < 0.05), with an exception for the control after storage at Harvest 1. Nevertheless, there were no significant differences at Harvest 2.

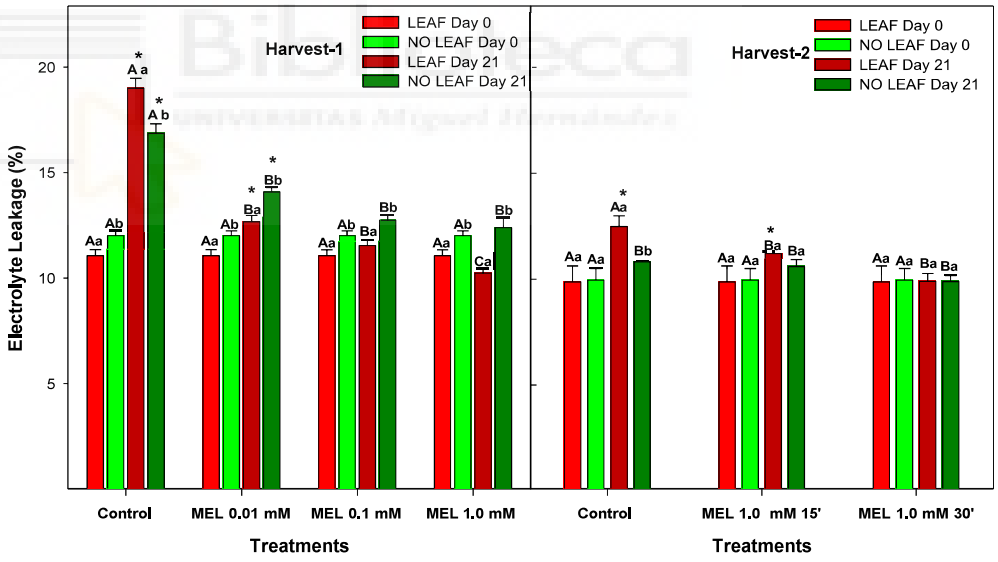

Figure 6. Electrolyte leakage (EL) of 'Fino' lemons with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest 1) and MEL at 1.0 mM during 15 and 30 min (Harvest 2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letters denote significant differences between control and MEL treatments, while bars with different small letters denote significant differences between lemons with LEAF and NO LEAF. The asterisk symbol denotes significant differences between both storage days (0 and 21 days) for each type of leaf and treatment.

Chilling injury is a physiological disorder that manifests itself in fruit after it is exposed to temperatures close to the freezing point. Cold tolerance depends on several factors such as the species, cultivar, harvest time, temperature and time of exposure to cold storage [48,49]. In a study in which the crown plays an important role in maintaining the quality of the harvested pineapple, it was appreciated that the crown offered greater protection from cold damage [50]. This may be due to the higher contribution of metabolic compounds from the pineapple leaf. MEL treatments produced significant reductions

in electrolyte leakage, showing a dose-dependent effect. Other studies have shown that melatonin is able to reduce lipid peroxidation of membranes by improving cell integrity and the ability in peaches and cabbage to maintain a balance in the cells of oxidative metabolism [51,52].

3.3. *Effect of Melatonin on Total Phenolic*

Citrus fruits are known to contain a wide variety of phytochemical compounds with benefits for human health demonstrated by clinical and epidemiological studies, such as reduction of cardiovascular diseases, cancers and diabetes, among others [53]. In lemons, total phenolic content was analysed in both the peel (Figure 7) and the juice (Figure 8), and concentration was significantly higher (10 fold) in the peel than in the juice (p-value < 0.05), although they share the same behaviour, which is an increase during 21 days of storage; levels were significantly higher (p-value < 0.05) in LEAF that in NO LEAF fruits, and MEL treatment induced greater accumulation of total phenolics.

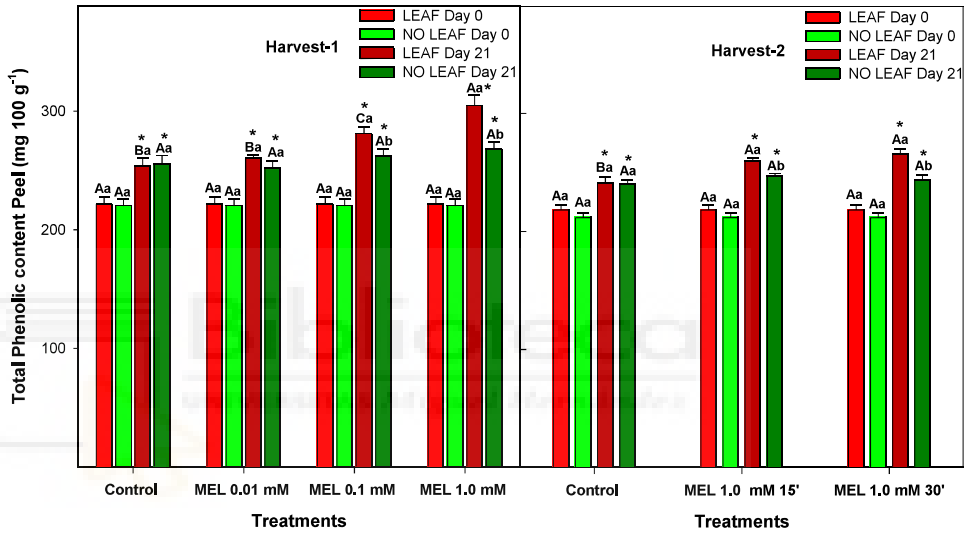

Figure 7. Total phenolic content in peels of 'Fino' lemons with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest 1) and MEL at 1.0 mM during 15 and 30 min (Harvest 2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letters denote significant differences between control and MEL treatments, while bars with different small letters denote significant differences between lemons with LEAF and NO LEAF. The asterisk symbol denotes significant differences between both storage days (0 and 21 days) for each type of leaf and treatment.

In lemons, the main phytochemicals are flavonoids and are found at high concentration in both peel and pulp [54]. The biological activities of phenolic compounds are well documented as acting as antioxidant moieties. The role of MEL modulating the contentof total phenolics has been documented in citrus and other fruits. Thus, in oranges, MEL also showed an accumulation of total phenolics during storage, leading to maintenance of postharvest quality and in turn alleviating physiological senescence [20]. In mandarins,MEL at 250, 500 and 1000 µM showed that the 1000 µM treatment was more effective than other doses during storage on maintaining higher total antioxidant activity as well as total phenolics [36]. In the same way, mangoes treated with MEL at 10, 100 or 1000 µM showed significantly higher total phenol content for MEL at 1000 µM than the control, while 10 or 100 µM did not significantly influence total phenol content [55], which agrees with ourresults in 'Fino' lemons, where MEL at 1.0 mM was the best concentration for increasing total phenolics.

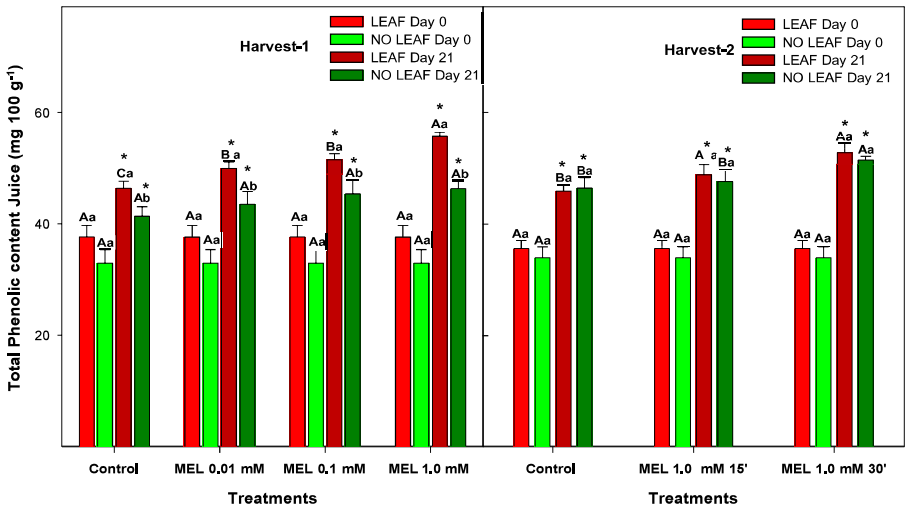

Figure 8. Total phenolic content in juice of 'Fino' lemons with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest 1) and MEL at 1.0 mM during 15 and 30 min (Harvest 2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letters denote significant differences between control and MEL treatments, while bars with different small letters denote significant differences between lemons with LEAF and NO LEAF. The asterisk symbol denotes significant differences between both storage days (0 and 21 days) for each type of leaf and treatment.

4. Conclusions

The postharvest treatments with melatonin by immersion in 'Fino' lemons had a positive effect on the improvement of the quality and in the late ripening of the lemon, reducing weight loss or the rate of breathing of the fruit as well as increasing firmness or colour levels and decreasing electrolyte leakage. The most effective dose was MEL1 mM, which showed better results. In addition, LEAF lemons showed in general that they could maintain better quality attributes with respect to NO LEAF lemons, possibly due to a greater absorption of melatonin through the leaf. For the content of phenolic compounds in both skin and juice, we observed the same positive effect on both parameters (treatment and type of leaf). However, further research including the effect of the combination of these treatments with different times of immersion and the effect of MEL application by immersion in LEAF and NO LEAF fruits in order to elucidate the way of introduction inside them is needed.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/foods12152979/s1: Figure S1: Total soluble solids of 'Fino' lemons with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest 1) and MEL at 1.0 mM during 15 and 30 min (Harvest 2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letters denote significant differences between control and MEL treatments, while bars with different small letters denote significant differences between lemons with LEAF and NO LEAF; Figure S2: Titratable acidity of 'Fino' lemon with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest-1) and MEL at 1.0 mM during 15 and 30 minutes (Harvest-2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letter denote significant differences between control and MEL treatments, while bars with different small letter denote significant differences between lemons with LEAF and NO LEAF. Asterisk symbol denotes significant differences between both storage days (0 and 21 days) for each type of leaf and treatment.

Author Contributions: M.S. and D.V. conceived and designed the work; S.C., J.M.V. and F.B.-E.H. performed the field treatments; F.B.-E.H. and M.E.G.-P. performed most of the analytical determination in collaboration with the other authors; M.S., D.V. and F.B.-E.H. analysed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author (daniel.valero@umh.es/fbadiche@umh.es). The data are not publicly available because we are still pursuing this line of study, and their publication must be authorised by the Ministry of Science and Innovation.

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Conflicts of Interest: The authors declare no conflict of interest.

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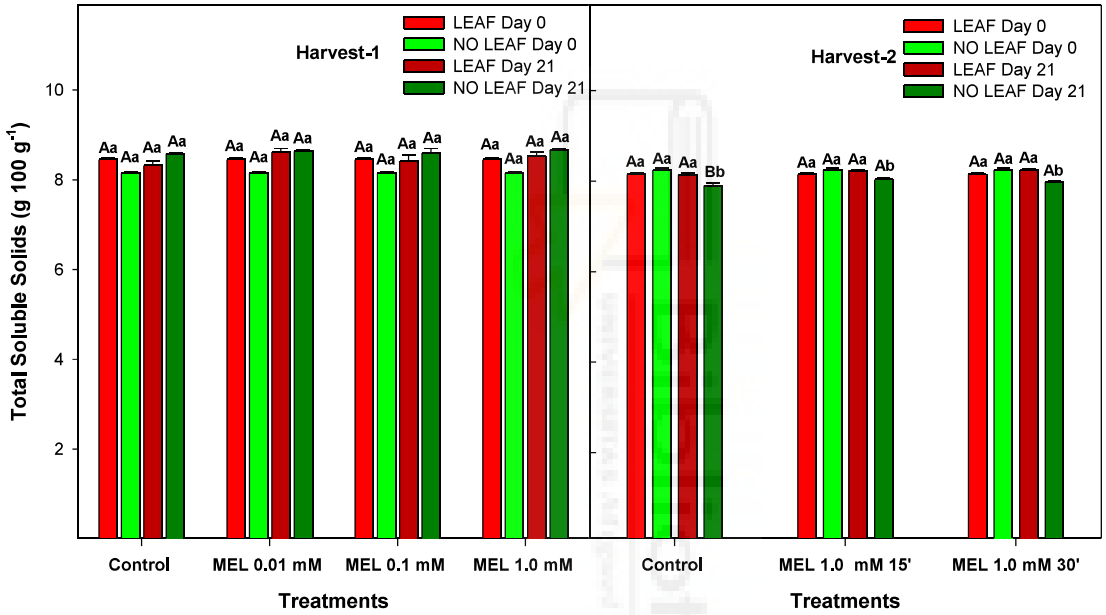

Figure S1. Total soluble solids of 'Fino' lemon with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest-1) and MEL at 1.0 mM during 15 and 30 minutes (Harvest-2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letter denote significant differences between control and MEL treatments, while bars with different small letter denote significant differences between lemons with LEAF and NO LEAF. Asterisk symbol denotes significant differences between both storage days (0 and 21 days) for each type of leaf and treatment.

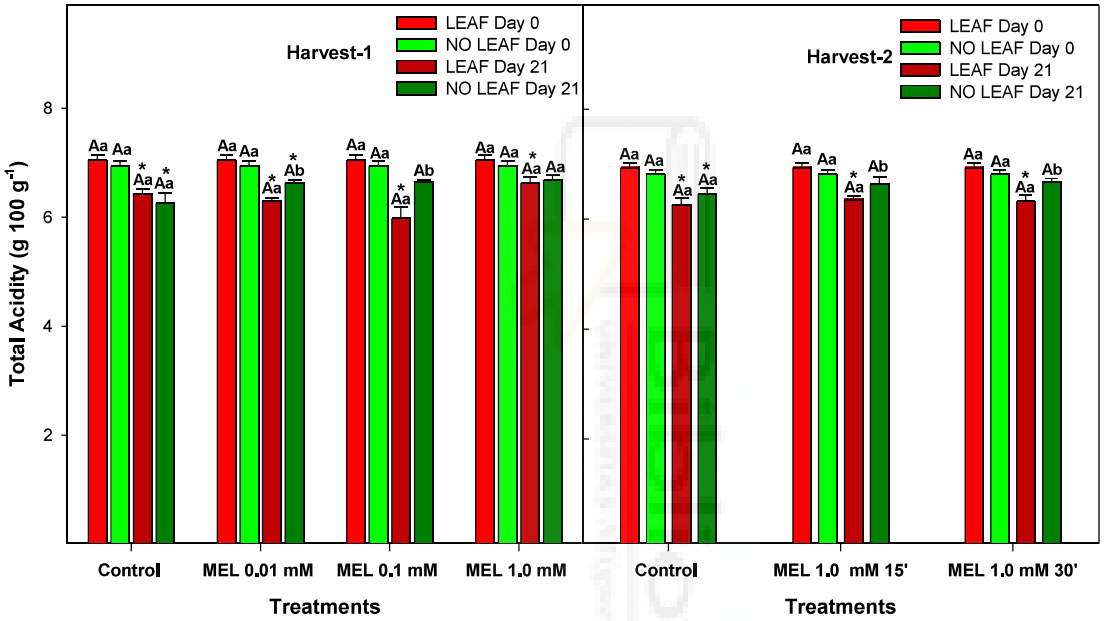

Figure S2. Titratable acidity of 'Fino' lemon with LEAF and NO LEAF as affected by MEL treatments at 0.01, 0.1 and 1.0 mM (Harvest-1) and MEL at 1.0 mM during 15 and 30 minutes (Harvest-2) after 21 days of storage. Data are the mean ± SE. Bars with different capital letter denote significant differences between control and MEL treatments, while bars with different small letter denote significant differences between lemons with LEAF and NO LEAF. Asterisk symbol denotes significant differences between both storage days (0 and 21 days) for each type of leaf and treatment.

4.4. PUBLICATION 4

PUBLICATION 4 (Literal transcription)

The Effect of Lemon Juice (Citrus limon L.) Treated with Melatonin on the Health Status and Treatment of K14HPV16 Mice

Badiche-El Hilali, F., Medeiros-Fonseca, B., Silva, J., Silvestre-Ferreira, A.C., Pires, M.J., Gil da Costa, R.M., Peixoto, F., Oliveira, P.A., Valero, D.

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antioxidants

Article

The Effect of Lemon Juice (*Citrus liMon* L.) Treated with Melatonin on the Health Status and Treatment of K14HPV16 Mice

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Abstract: Lemon is a fruit rich in antioxidant properties and has several health benefits, namely the reduction of skin edema and anticarcinogenic properties, which are due to its high content of bioactive compounds. Melatonin can improve and preserve the properties of lemon for longer and also has health benefits. The aim of this study was to evaluate the effects of oral administration of lemon juice after melatonin treatment on murinometric parameters of wild-type (WT) mice and transgenic mice carrying human papillomavirus (HPV). Two trials were performed for oral administration of the lemon extract compound: in drinking water and in diet. First of all, lemons were treated by immersion with melatonin at 10 mM. Then, lemons were squeezed, and the juice obtained was freeze-dried and stored to be subsequently added to drinking water or diet, according to the assay. Thus, mice were divided into eight groups in the drink assay (each with n = 5): group 1 (G1, WT, control), group 2 (G2, WT, 1 mL lemon), group 3 (G3, WT, 1.5 mL lemon), group 4 (G4, WT, 2 mL lemon), group 5 (G5, HPV16, control), group 6 (G6, HPV16, 1 mL lemon) group 7 (G6, HPV16, 1.5 mL lemon) and group 8 (G6, HPV16, 2 mL lemon). The diet assay was divided into four groups: group 1 (G1, WT, control), group 2 (G2, WT, 4 mL lemon), group 3 (G3, HPV16, control) and group 4 (G4, HPV16, 4 mL lemon). In the drink assay, the highest concentration of melatonin (308 ng/100 mL) was for groups 4 and 8, while in the food assay, there was only one concentration of melatonin (9.96 ng/g) for groups 2 and 4. Both trials lasted 30 days. During this time, body weight, food and water were recorded. Afterward, they were sacrificed, and samples were collected for different analyses. At the concentrations used, the lemon juice with melatonin had no adverse effects on the animals' health and showed a positive outcome in modifying weight gain and enhancing antioxidant activity in mice. Moreover, a reduction in the incidence of histological lesions was observed in treated animals. Further research is needed to better understand the effects of lemon extract on health and treatment outcomes in this animal model.

Keywords: lemon; melatonin; valorization; antioxidant; HPV16; human papillomavirus

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1. Introduction

Lemon is a citrus fruit widely used throughout the world due to its versatility and unique flavor. In addition to being a rich source of vitamin C, lemons also contain other

beneficial compounds, such as citric acid and flavonoids, which may have positive effects on health [1]. Regular consumption of lemon and other vitamin C-rich foods has been associated with a lower incidence of chronic diseases, such as cardiovascular disease, cancer and neurodegenerative diseases [2]. Furthermore, the citric acid present in lemons may help prevent the formation of kidney stones by increasing the excretion of calcium in the urine [3]. However, lemons can suffer from various pathologies that can reduce their shelf life and nutritional quality. For example, rot caused by fungi and bacteria can damage lemons and render them unusable. To address this issue, various preservation techniques have been studied, including the use of post-harvest treatments with melatonin, which has been shown to improve fruit quality and prolong shelf life [4]. Melatonin (MEL), or N-acetyl-5-methoxytryptamine, is an indoleamine that was first discovered in the pineal gland of vertebrates [5] and, five decades later (1995), in the plant kingdom. MEL, as a phytohormone produced naturally in plants, including citrus fruits, has been found to have a role in a wide range of physiological processes in plants [6]. Furthermore, the application of MEL to lemons has been found to reduce the incidence of fungal and bacterial diseases in the fruit, maintaining its nutritional quality and prolonging shelf life [7–9]. Also, melatonin has been shown to have antioxidant and anti-inflammatory effects on human health that may help prevent cancer and other HPV-induced diseases [10]. In addition, exposure of SARS-CoV-infected cells to melatonin has been shown to inhibit the growth and proliferation of the virus [11]. The use of melatonin as a potential treatment in animal models has also shown promising results in other studies [12].

On the other hand, in vivo animal studies, particularly those conducted on mice and rats, are an important tool in biomedical research due to the similarities between these animals and humans in terms of genetics, physiology and anatomy [13]. These studies can provide valuable information on the safety and efficacy of treatments, as well as insight into the underlying mechanisms of diseases. The K14HPV16 mouse is a transgenic model of cancer induced by human papillomavirus (HPV). HPV is the most common sexually transmitted infectious agent worldwide [14]. The HPV16 is responsible for the majority of cases of cervical cancer, as well as other anogenital and head and neck cancers [15]. The potential link between HPV infection and lemon consumption has not been extensively studied, but some research has suggested that citrus fruits such as lemon may have anti-cancer properties due to their high levels of flavonoids and other bioactive compounds [16]. However, more research is needed to fully understand the relationship between citrus fruit consumption and HPV-related diseases.

Often, post-harvest studies are carried out on fruits to enhance and extend their useful life. However, it is usually not studied if there is any effect on human health after eating these fruits or foods. Therefore, the aim of this study was to study the effects of lemon with aqueous melatonin extract in an in vivo mouse model to see if there is any effect on the health and well-being of HPV16 pathology mice and wild-type mice. This model was previously used by our team to test other natural compounds, making it a suitable model for testing both the efficacy and possible hepatotoxicity of lemon extracts.

2. Materials and Methods

2.1. Lemon, Lemon Fruit Juice and the Soluble Extracts

Lemon Verna fruit (*Citrus limon* (L.) Burm. F. var. Verna) was harvested in the summer season of 2022 from a commercial citrus organic orchard in Orihuela (Alicante), Spain. The fruit was immediately transported to the laboratory. Subsequently, the fruit was washed with distilled water. After drying at room temperature, the fruit was treated with melatonin (purchased from Sigma, Sigma-Aldrich, Madrid, Spain) aqueous solution at 10 mM by immersing for 30 min. Samples were freshly squeezed for lemon juice and then lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) in order to preserve their chemical composition as much as possible until they were used. Afterward, the sample was reduced to a doughy dense mass and preserved in a freezer at −20 °C until further analysis. For oral intervention study in mice via drinking water, we used a concentration

of freeze-dried lemon juice of 38.6, 57.8 and 77.1 mg/100 mL to obtain a dose equivalent to 1, 1.5 and 2 mL, respectively, of lemon juice reconstituted in the final volume of water administered to the mice. From lower to higher juice concentrations, the melatonin dose was 154, 231 and 308 ng, respectively, based on the concentration of melatonin in this proportion of freeze-dried lemon juice. For the second trial, the diet test, we dissolved 8 g of freeze-dried lemon juice in 100 mL of water and added it to 2 kg of normal food. In other words, the concentration of freeze-dried juice in the food is 4 mg/g, which is equivalent to 4 mL of rehydrated lemon juice, in which we can find 9.96 ng of melatonin.

2.2. Phenolic Compounds Profile and Stability of the Aqueous Extract

For identification and quantification of phenolic compounds, we use the extraction described in Gironés-Vilaplana et al. (2012) [17] with slight modifications. Regarding HPLC system, water/formic acid (99:1, *v*/*v*) and acetonitrile were used as the mobile phases A and B, respectively, with a flow rate of 1 mL per min. The injection volume was 20 µL, and chromatograms were recorded at 320 and 360 nm in an Agilent HPLC 1200 Infinity series equipped with a photodiode array detector (Agilent Technologies, Waldbronn, Germany) and a mass detector in series (Bruker Daltonics Ultra HCT-ESI Ion Trap, Bremen, Germany) and a Luna C18 column (250 ×40 mm, 5 µm particle size). The ionization conditions were 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and nitrogen flow rate were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range of *m*/*z* from 100 to 1200. Individual phenolics quantification was performed in duplicate in each sample by using an HPLC-DAD system with the same conditions that were used for phenolics identification. Individual phenolic compounds were identified by their mass in an HPLC-DAD-ESI/MS, their spectra and retention time, using previous bibliography [18]. Moreover, some of them were corroborated using analytical standards. For quantitative analysis, a calibration curve of two standards, 5-*O*-caffeoylquinic acid and 3-luteolin-*O*-rutinoside (Sigma Aldrich, Germany), was used for the quantification of hydroxycinnamic acids and luteolin derivatives at 320 and 360 nm, respectively. The total identified polyphenol concentration was calculated as the sum of the individual phenolic concentrations, and the results were expressed in mg per g of lemon juice.

The stability of the rehydrated lemon juice was evaluated for 5 consecutive days at room temperature. In this study, the aqueous extract was prepared at the same concentration that was provided to the mice in drinking water and analyzed daily through a colorimetric analysis described in another study [19] to visualize if there was degradation of ascorbic acid in the lemon juice. On the other hand, total antioxidant activity was measured by the ABTS-peroxidase system and total phenols by the Folin–Ciocalteu method, both previously analyzed in another study on lemon [20].

2.3. Mice

Forty female mice were used for the drinking study: twenty transgenic (HPV16+/+) and twenty wild-type (WT) (HPV16−/−), aged 18–20 weeks old. For diet test, only twenty mice were used: 10 WT and 10 HPV16+/−, aged 30 weeks old. Cutaneous lesions in this mouse strain begin to proceed from the hyperplastic to the dysplastic stage at the age of 20–22 weeks [21], creating an opportunity to test new strategies to block this progression. The mouse strain [22] was donated by Drs Jeffrey Arbeit and Douglas Hanahan from the University of California through the National Cancer Institute Mouse Repository (Frederick, MD, USA). The animals were genotyped weaning, using tail tip samples by using a polymerase chain reaction technique previously used in our works.

2.4. Experimental Procedures

The experimental procedures were approved by the national authorities (approval number 014139) and carried out at the University of Trás-os-Montes and Alto Douro animal facilities. The animals were kept under controlled experimental conditions. All mice were acclimated for four weeks in a controlled environment (20 ± 2 °C, 12 h light/dark cycle and

relative humidity 50 ±10%) and had free access to food and water. Two experiments were performed. In one of them, we administered freeze-dried lemon through drinking water, and in the other, we administered the preparation through the animals' diet. The first is called "drink test" or "drink assay", and the second one is called "diet test" or "food test". For the diet assay, a commercial rodent feed (certified Mucedola 4RF21, Milan, Italy) was used as the basis for the preparation of modified diets containing melatonin-treated lemon extracts. Lemons that were lyophilized were reconstituted with water to form a juice and incorporated into a modified diet at concentrations of 0.4% (w/w). These concentrations were calculated considering the maximum daily recommendations for melatonin (5 mg/d) for an adult (70 kg). For a 30 g mouse with an average daily intake of 5 g, this corresponds to 19.93 µg. Diets were prepared using an industrial mixer (CPM Europe, model C-300, Zaandam, The Netherlands) and adding 5% (w/w) water to the mixture to form new pellets (4.2 mm diameter). The base diet was prepared following the same method but without the addition of lemon extract. Subsequently, all batches of feed were dried in an oven at 40 °C for 48 h and stored at 4 °C until the feed was ready for use. Throughout the first experiment (drink assay), mice were fed a standard diet (4RF21 GLP, Mucedola, Italy) ad libitum. For the drink assay, animals were divided into eight groups (each with n = 5). Groups 1 to 4 were wild-type (WT), and groups 5 to 8 were transgenic (HPV16): group 1 (G1, WT, control), group 2 (G2, WT, 1 mL lemon), group 3 (G3, WT, 1.5 mL lemon), group 4 (G4, WT, 2 mL lemon), group 5 (G5, HPV16, control), group 6 (G6, HPV16, 1 mL lemon) group 7 (G7, HPV16, 1.5 mL lemon) and group 8 (G8, HPV16, 2 mL lemon). For the diet assay, mice were divided into four groups of 5 animals each: group 1 (G1, WT, control), group 2 (G2, WT, 4 mL lemon), group 3 (G3, HPV16, control) and group 4 (G4, HPV16, 4 mL lemon). Animals' body weight, water and food intake were recorded and monitored every 5 days and were known as "sample dates". At the same time, animals were carefully observed to confirm their well-being through their humane endpoint evaluation. The lemon juice melatonin-enriched extract was administered in drinking water for 30 days at different concentrations and was renewed every 48 h. The diet test also lasted 30 days, and the animal's diet food was added every 72 h. At the end of the 30 days, all animals (both experiments) were sacrificed by intraperitoneal administration of a mixture of xylazine and ketamine, followed by cardiac puncture exsanguination, according to FELASA guidelines, and biological samples of blood and organs (heart, lung, liver, spleen, kidneys, as well as chest and ear skin samples) were collected for analysis.

2.4.1. Determination of Microhematocrit and Total Plasma Proteins (TPP)

The hematocrit was measured using microhematocrit method. Samples were centrifuged at 9000 rpm for 5 min, and the height of the packed red blood cells was measured with a graduated ruler. The results were expressed as the percentage of blood cell volume. For the serum biochemistry, blood samples were allowed to clot and centrifuged at 3000 rpm for 15 min (4° C). To measure the total plasma proteins, the blood samples were allowed to clot and centrifuged at 3000 rpm for 15 min (4° C). The serum concentrations of TPP were determined in an autoanalyzer (Prestige 24i, Cormay PZ, Marynin, Poland).

2.4.2. Histological Analysis

Samples of heart, liver, kidney, lung and spleen were collected and immediately fixed in 10% neutral buffered formalin for at least 24 h. The fixed tissues were then dehydrated through a graded series of ethanol, cleared with xylene and embedded in paraffin wax. The tissue sections were stained with hematoxylin and eosin (H&E) to evaluate the histology of the organs. The H&E staining method is commonly used to visualize the general architecture and cellular details of tissue sections. The stained sections were examined under a light microscope by a trained histopathologist. The evaluation criteria included the presence of inflammation, fibrosis, necrosis, cellular infiltrates and any other histopathological changes that may indicate tissue damage or disease. Lesions were classified as previously described for this mouse strain [23]. The histopathological

analysis was performed in a blinded manner, where the histopathologist was unaware of the treatment groups or experimental conditions. The results of the histopathological analysis were recorded and used to draw conclusions about the effects of the lemon juice treatment on the organs.

2.4.3. Hepatic and Kidney Oxidative Stress

The levels of oxidative stress markers were measured in liver and kidney tissue homogenates. Both organs were homogenized in cold buffer solution (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of $MgCl_2$ and 0.5 mM of phenylmethyl sulfonyl fluoride PMSF, prepared in ethanol to prevent protein degradation, pH 7.4) using a motor-driven Teflon and glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C (Sigma model 3K30, Osterode, Germany), and supernatants were collected for analysis. Superoxide dismutase activity (Cu/Zn-SOD) was determined by the nitroblue tetrazolium (NBT) reduction generated by superoxide radicals generated by xanthine oxidase system at 560 nm [24]. For quantitative analysis, a calibration standard curve constructed by SOD from bovine erythrocytes was used (0–3.75 U mL^{-1}). The activity of catalase (CAT) was determined at 240 nm in accordance with a previously published method [25] and was calculated using bovine catalase as a standard (0–5 U mL^{-1}).

2.5. Statistical Analysis

Statistical analysis was performed using the SPSS program (Statistical Package for Social Sciences, Chicago, IL, USA) version 17. A statistical ANOVA followed by the Bonferroni test was performed, and values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Phenolic Compounds Profile and Stability of the Extract

Table 1 summarizes the chemical composition analyzed present in lemon juice. Ten compounds were detected, the two major ones being hesperidin and eriocitrin (flavanones). On the other hand, some compounds, such as luteolin-7-*O*-rutinoside (flavone) or quercetin 3-*O*-glucoside (flavanol), showed a very low concentration. Gallic acid and cynarin were not detected. Hesperidin was the most abundant compound in the lemon juice, with 69.9 ± 3.9 mg 100 mL^{-1}, followed by eriotricin (19.5± 0.6 mg 100 mL^{-1}). On the other hand, diosmetin 6,8-di-*C*-glucoside and diosmetin 8-*C*-glucoside were the next most important compounds present in lemon juice (13.7± 0.2 and 12.9 ± 0.3 mg 100 mL^{-1}, respectively). Otherwise, the compounds with the lowest concentration in the juice were quercetin 3-*O*-glucoside, caffeic acid (hydroxycinnamic acid) and luteolin-7-*O*-glucoside with an amount of 1.2 ±0.0, 1.5 ±0.0 and 1.5 ± 0.2 mg 100 mL^{-1}, respectively. In terms of the stability of the aqueous extract resulting from redissolving the freeze-dried lemon material in water, it was studied for 96 consecutive hours (4 days) at room temperature, and it was visualized that by day 3 or 72 h, the concentration of phenolic compounds began to decrease (Table 2) in terms of vitamin C and total antioxidant activity. Therefore, the feeding water was maintained up to a maximum of 72 h to avoid degradation of these compounds. At 96 h, significant differences were observed in all parameters compared to 72 h. Vitamin C content remained around 30 mg 100 mL^{-1} until 96 h when it decreased to 22.3 ± 1.6 mg 100 mL^{-1} ($p < 0.05$). As for the content of total phenolic compounds and total antioxidant activity at 96 h, a decrease of 7 and 6 mg 100 g^{-1}, respectively, was observed in relation to the content at 72 h ($p < 0.05$).

Table 1. Phenolic profile obtained by LC-DAD-ESI/MSn of fresh lemon juice with melatonin, expressed in mg 100 mL^{-1} (mean ± standard deviation).

Compound Name	[a] Rt (min)	[b] Molecular Ion [c] (*M*/*z*)	[c] MS/MS (*M*/*z*)	[d] λ max (nm)	Quantification
Cynarin	5	169	124, 78, 124	250,280	nd
Caffeic acid	6	463	300, 270, 300	250,280	1.5 ± 0.0
Gallic acid	8	609	301, 163, 150	288	nd
Diosmetin 6,8-di-*C*-glucoside (lucenin-2,4'-methyl ether)	10	624	608, 590, 530, 506, 488	250,268,342	13.7 ± 0.2
Diosmetin 8-*C*-glucoside (orientin 4'-methyl ether)	17	463	446, 428, 344, 314	250,268,342	12.9 ± 0.3
Eriocitrin (eriodictyol-7-*O*-rutinose)	19.1	179	135, 134, 106	285,325	19.5 ± 0.6
Luteolin-7-*O*-rutinoside	20.4	448	287, 153, 135	254,267	1.5 ± 0.2
Chrysoeriol 8-*C*-glucoside (scoparin)	22	462	446, 428, 314	255,268	12 ± 0.5
Apigenin 6,8-di-*C*-glucoside (vicenin-2)	23	594	560, 476, 458	268,334	11.5 ± 0.2
Hesperidin (hesperetin 7-*O*-rutinoside)	25	595	287, 150, 135	285,332	69.9 ± 3.9
Rutin	27	515	353, 191, 135	285,332	9.8 ± 0.6
Quercetin 3-*O*-glucoside	33.8	608	299, 270, 301	285,332	1.2 ± 0.0
Total phenolic content					153.5 ± 20.1

[a] RT = retention time. [b] Molecular ion = tandem mass spectrometry. [c] MS/MS = molecular mass fragments. [d] λ max = wavelengths of maximum absorption in the visible region. nd = not detected.

Table 2. Evolution of bioactive compounds content and antioxidant capacity at room temperature (22 °C) at 24, 48 and 72 h in lemon juices treated with melatonin.

Functional Parameter	24 h	48 h	72 h	96 h
Vitamin C	32.04 ± 1.8	31.91 ± 2.1	29.22 ± 1.9	22.3 ± 1.6 [1]
Total Phenolic Content	45.09 ± 3.2	43.96 ± 2.9	40.36 ± 3.3	33.14 ± 1.8 [2]
Total Antioxidant Activity	24.63 ± 1.3	24.45 ± 1.6	21.16 ± 1.5	15.58 ± 1.5 [3]

Data are the mean ± standard deviation (SD). Results of vitamin C have been expressed as mg 100 mL^{-1} of fresh weight (FW). Total phenolic content has been expressed as mg 100 g^{-1} of FW. TAA has been expressed as mg 100 g^{-1} of FW. [1, 2, 3]. 96 h statistically different from 72 h ($p < 0.05$).

3.2. Mice Experiments

During the experimental work, the animals showed no signs of behavioral change, nor did we register mortality. Tables 3 and 4 show the animal's body weight variation in both experiments for the different groups under study. In the drink assay (Table 3), we can see a decrease in body weight in groups 2, 3 and 5. Over the sample dates, the values of body weight show statistically significant differences between groups. In the second sample date, we can observe statistically significant differences between wild-type groups 1 and 2 ($p < 0.05$); also, group 2 was statistically different from groups 3 and 4 ($p < 0.05$). In the fourth sample date, the statistically significant differences were presented between group 5 and group 6 ($p < 0.05$) and group 7 and group 8 ($p < 0.05$). There was also a statistically significant difference in the sixth sample date between group 3 and group 2 ($p < 0.05$). Regarding the average weight gain (Figure 1A), the results showed statistically significant differences between the control and WT groups 2 and 3 ($p < 0.05$) and also between the control HPV16 (G5) and HPV16 treated groups (G6, G7 and G8) ($p < 0.05$). In the diet test (Table 2), at the beginning of the trial, the average body weight was between 26.96 and 29.87 g; at the end, these values were 30.28 and 28.45 g. Significant differences were not found between the animal's weight at the beginning and at

the end of the trial. However, the consumption of lemon in group 2 was lower, and there was a lower weight than group 1 during the whole trial. Regarding mean weight gain (Figure 1B), statistically significant differences were found between groups 2 and 4 ($p < 0.05$)—these two groups were exposed to lemon extract.

Table 3. Body weight variation in grams (mean ± SD) in drink test for each group ((G1, WT, control), (G2, WT, 1 mL lemon), (G3, WT, 1.5 mL lemon), (G4, WT, 2 mL), (G5, HPV16, control), (G6, HPV16, 1 mL lemon), (G6, HPV16, 1.5 mL lemon) and (G6, HPV16, 2 mL lemon)).

	G1 (WT, Control)	G2 (WT, 1 mL Lemon)	G3 (WT, 1.5 mL Lemon)	G4 (WT, 2 mL Lemon)	G5 (HPV16, Control)	G6 (HPV16, 1 mL Lemon)	G7 (HPV16, 1.5 mL Lemon)	G8 (HPV16, 2 mL Lemon)
1º sample date	28.05 ± 1.68	24.75 ± 1.91	28.76 ± 3.30	27.49 ± 1.79	27.39 ± 1.05	26.5 ± 1.67	26.88 ± 2.05	28.51 ± 1.09
2º sample date	26.9 ± 0.86 [1]	22.38 ± 1.52 [2]	26.69 ± 2.68	26.68 ± 1.81 [3]	26.95 ± 1.85	23.41 ± 1.59	23.06 ± 3.02	26.41 ± 1.52
3º sample date	27.91 ± 0.86	24.67 ± 1.11	28.52 ± 2.94	27.91 ± 1.43	26.37 ± 1.28	26.07 ± 1.66	26.32 ± 2.54	28.41 ± 1.42
4ºsample date	27.92 ± 0.99	24.57 ± 0.74	27.93 ± 2.84	28.44 ± 1.8	19.87 ± 2.25	24.48 ± 1.89 [4]	24.42 ± 2.14 [5]	28.47 ± 1.24 [6]
5º sample date	27.9 ± 1.85	24.81 ± 1.01	28.14 ± 3.09	28.98 ± 1.63	26.03 ± 1.44	26.39 ± 2.18	26.48 ± 2.05	28.7 ± 2.08
6º sample date	28.07 ± 0.84	24.34 ± 1.65	28.25 ± 2.73 [7]	28.08 ± 1.84	26.7 ± 1.53	26.81 ± 1.47	27.4 ± 1.71	29.08 ± 1.44

[1] G1 was statistically different from G2 ($p < 0.05$). [2] G2 was statistically different from G3 ($p < 0.05$). [3] G2 was statistically different from G4 ($p < 0.05$). [4] G6 was statistically different from G5 ($p < 0.05$). [5] G7 was statistically different from G5 ($p < 0.05$). [6] G8 was statistically different from G5 ($p < 0.05$). [7] G3 was statistically different from G2 ($p < 0.05$).

Table 4. Body weight variation in grams (mean ± SD) in food test for each group ((G1, WT, control), (G2, WT, 4 mL lemon), (G3, HPV16, control) and (G4, HPV16, 4 mL lemon)).

	G1 (WT, Control)	G2 (WT, 4 mL Lemon)	G3 (HPV16, Control)	G4 (HPV16, 4 mL Lemon)
1º sample date	29.87 ± 3.08	28.95 ± 1.59	26.96 ± 2.56	26.77 ± 2.66
2º sample date	32.03 ± 3.86	29.53 ± 1.25	28.38 ± 3.04	28.23 ± 2.56
3º sample date	29.72 ± 2.28	28.52 ± 0.69	29.02 ± 3.01	28.83 ± 2.72
4º sample date	29.99 ± 2.44	28.01 ± 0.81	27.32 ± 2.10	27.09 ± 2.61
5º sample date	30.66 ± 2.46	27.94 ± 0.75	27.31 ± 2.50	27.52 ± 2.35
6º sample date	30.28 ± 2.40	28.45 ± 0.53	27.98 ± 2.15	28.51 ± 2.38

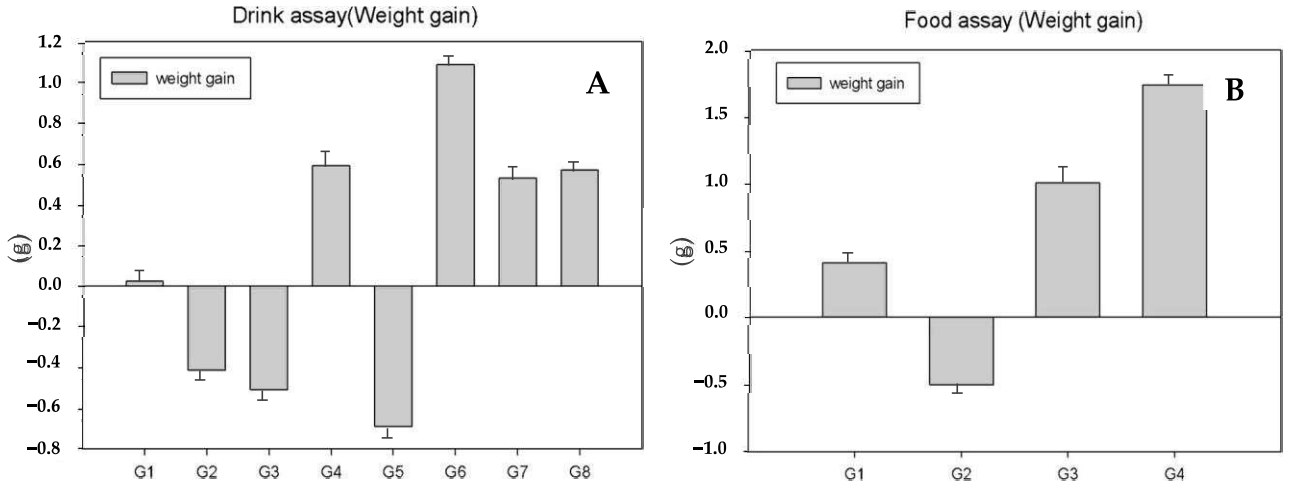

Figure 1. The weight gain in grams (mean ± SD) in drink assay (**A**) for each group ((G1, WT, control), (G2, WT, 1 mL lemon), (G3, WT, 1.5 mL lemon), (G4, WT, 2 mL), (G5, HPV16, control), (G6, HPV16, 1 mL lemon), (G6, HPV16, 1.5 mL lemon) and (G6, HPV16, 2 mL lemon)) and food assay (**B**) for each group ((G1, WT, control), (G2, WT, 4 mL lemon), (G3, HPV 16, control) and (G4, HPV 16, 4 mL lemon)).

The average food and water consumption for the water administration test is represented in Figure 2. We can observe that the transgenic groups (G5, G6, G7 and G8) have a higher consumption of both water and food at the end of the trial. Moreover, in the diet test, the group with the highest water consumption was group 4, as can be seen in Figure 3A. For food consumption, group 1 (WT) had the lowest intake at the end of the trial, and group 5 (HPV16) had the highest intake at both the start and end of the test with an average of 9.75 g per animal at the end of the test (Figure 3B). In general, the groups composed of transgenic animals had higher consumption than wild-type animals.

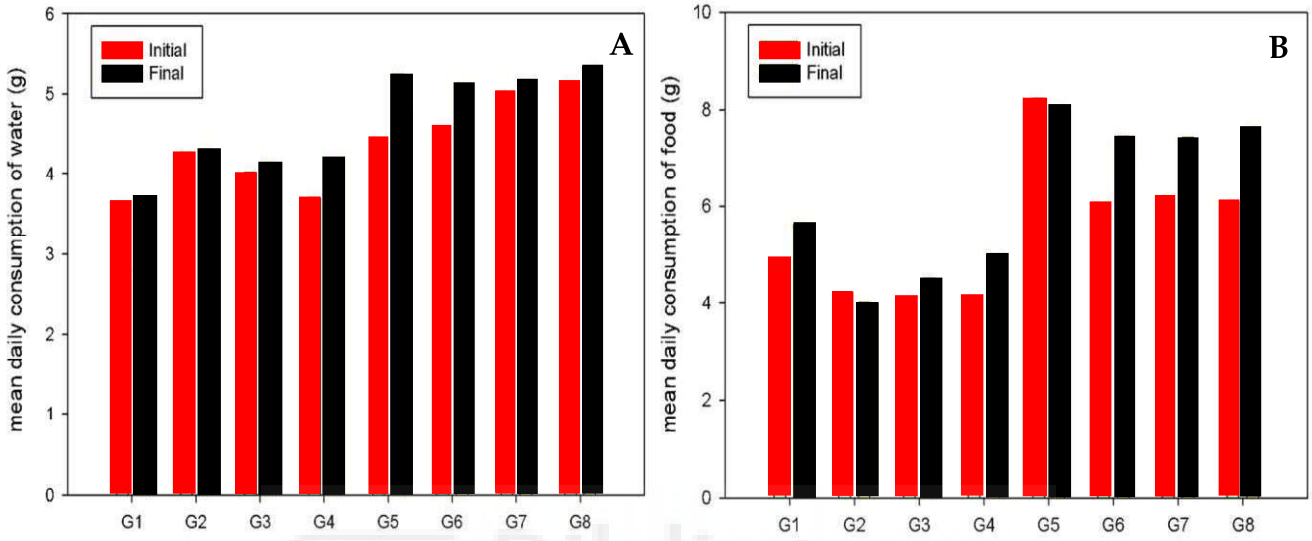

Figure 2. Mean daily consumption (gram) of water (**A**) and food (**B**) per animal in each group ((G1, WT, control), (G2, WT, 1ml lemon), (G3, WT, 1.5 mL lemon), (G4, WT, 2 mL lemon), (G5, HPV16, control), (G6, HPV16, 1 mL lemon), (G6, HPV16, 1.5 mL lemon) and (G6, HPV16, 2 mL lemon)) at the beginning and at the end of drink test.

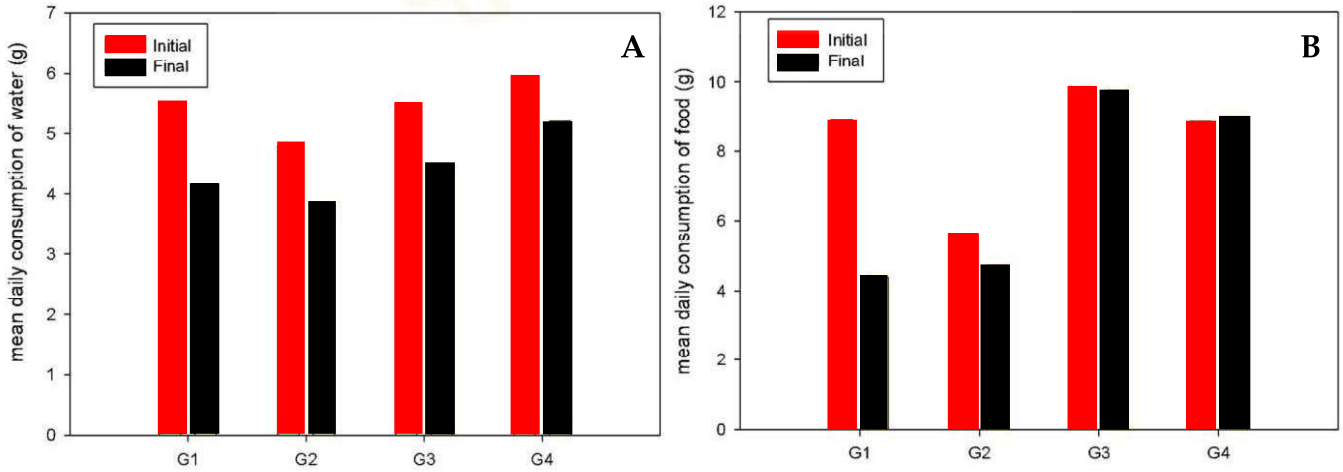

Figure 3. Mean daily consumption (gram) of water (**A**) and food (**B**) per animal in each group ((G1, WT, control), (G2, WT, 4 mL lemon), (G3, HPV 16, control) and (G4, HPV 16, 4 mL lemon)) at the beginning and at the end of diet test. Relative organ weights (grams) of the drinking test (mean ± standard error) and in food assay.

In the drink test, relative organ weights (Table 5) showed significative differences between group 1 and group 5 in the spleen and heart ($p < 0.05$), being that these organs were heaviest in the transgenic group in comparison with the WT group. Also, the liver of WT group 3 presented a smaller size than the liver of HPV16 group 7. Concerning other organ weights, there were no significant differences. In Table 6, the relative organ weights

of the food test were represented. We can observe that there were no significant differences between any of the groups.

Table 5. Relative organ weights (grams) of the drinking test (mean ± SD).

	G1 (WT, Control)	G2 (WT, 1 mL Lemon)	G3 (WT, 1.5 mL Lemon)	G4 (WT, 2 mL Lemon)	G5 (HPV16, Control)	G6 (HPV16, 1 mL Lemon)	G7 (HPV16, 1.5 mL Lemon)	G8 (HPV16, 2 mL Lemon)
Spleen	0.0039 ± 0.0003 [1]	0.0038 ± 0.0004	0.0042 ± 0.0004	0.0041 ± 0.0004	0.0051 ± 0.0005	0.0049 ± 0.0009	0.0046 ± 0.0007	0.0049 ± 0.0006
Heart	0.0041 ± 0.0003 [2]	0.0046 ± 0.0007	0.0044 ± 0.0004	0.0045 ± 0.0004	0.0051 ± 0.0002	0.0048 ± 0.0005	0.0045 ± 0.0002	0.0045 ± 0.0005
Liver	0.0400 ± 0.0022	0.0419 ± 0.0030	0.0417 ± 0.0017 [3]	0.0426 ± 0.0017	0.0473 ± 0.0010	0.0461 ± 0.0028	0.0468 ± 0.0030	0.0470 ± 0.0010
Lung	0.0078 ± 0.0009	0.0073 ± 0.0002	0.0067 ± 0.0004	0.0071 ± 0.0003	0.0080 ± 0.0009	0.0068 ± 0.0004	0.0080 ± 0.0026	0.0072 ± 0.0004
Kidney (left)	0.0054 ± 0.0008	0.0058 ± 0.0005	0.0054 ± 0.0008	0.0050 ± 0.0006	0.0065 ± 0.0002	0.0057 ± 0.0004	0.0060 ± 0.0008	0.0061 ± 0.0004
Kidney (right)	0.0054 ± 0.0005	0.0055 ± 0.0007	0.0054 ± 0.0009	0.0057 ± 0.0007	0.0062 ± 0.0003	0.0054 ± 0.0007	0.0058 ± 0.0002	0.0053 ± 0.0003

[1] Statistically different from G5 ($p < 0.05$). [2] Statistically different from G5 ($p < 0.05$). [3] Statistically different from G7 ($p < 0.05$).

Table 6. Relative organ weights (grams) of the diet assay (mean ± SD).

	G1 (WT, Control)	G2 (WT, 4 mL Lemon)	G3 (HPV16, Control)	G4 (HPV16, 4 mL Lemon)
Spleen	0.0044 ± 0.0009	0.0047 ± 0.0005	0.0050 ± 0.0008	0.0057 ± 0.0015
Heart	0.0049 ± 0.0007	0.0058 ± 0.0009	0.0048 ± 0.0004	0.0048 ± 0.0009
Liver	0.0466 ± 0.0034	0.0495 ± 0.0049	0.0482 ± 0.0053	0.0478 ± 0.0045
Lung	0.0081 ± 0.0005	0.0081 ± 0.0008	0.0069 ± 0.0008	0.0069 ± 0.0008
Kidney (left)	0.0059 ± 0.0006	0.0060 ± 0.0003	0.0055 ± 0.0014	0.0063 ± 0.0006
Kidney (right)	0.0067 ± 0.0012	0.0061 ± 0.0005	0.0061 ± 0.0011	0.0056 ± 0.0006

3.3. Microhematocrit and TPP Values

From Tables 7 and 8, we can observe the microhematocrit and the total plasma protein (PPT) value parameters for the different groups under study for both experiments. In the drink test, wild-type mice show slightly elevated microhematocrit compared with HPV16, in contrast to the PPT value, which is mildly higher in HPV16 compared with wild types. This difference did not reach statistical significance. Moreover, between the treated and untreated mice (wild-type as well as HPV 16), there were no significant differences (Table 7). In the food test, we observed similar results (Table 8).

Table 7. Microhematocrit (Ht) and PPT (mean ± SD) values for the drink assay for each group.

Groups	G1 (WT, Control)	G2 (WT, 1 mL Lemon)	G3 (WT, 1.5 mL Lemon)	G4 (WT, 2 mL lemon)	G5 (HPV16, Control)	G6 (HPV16, 1 mL Lemon)	G7 (HPV16, 1.5 mL Lemon)	G8 (HPV16, 2 mL Lemon)
Ht (%)	46.5	45.6	46.34	45.62	43.76	44.55	45.42	45.04
PPT (g/dL)	4.78 ± 0.25	4.56 ± 0.09	4.94 ± 0.22	4.98 ± 0.28	5.1 ± 0.2	5 ± 0.12	5.32 ± 0.28	5.18 ± 0.53

Table 8. Microhematocrit (Ht) and PPT (mean ± SD) values for the drink assay for each group.

Groups	G1 (WT, Control)	G2 (WT, 4 mL Lemon)	G3 (HPV16, Control)	G4 (HPV16, 4 mL Lemon)
Ht (%)	47.98	48.38	45.8	47.9
PPT (g/dL)	4.95 ± 0.24	5.02 ± 0.39	5.78 ± 0.46	5.36 ± 0.18

3.4. Histology

The results of the heart, lung, liver, spleen and kidney histology tests for the drink test are summarized in Table 9 and Figure 4. There were no significant differences in histological parameters. Transgenic animals treated with lemon juice (G6, G7, G8) showed fewer kidney lesions compared with the control group (G5). The results about chest skin in the "normal" parameter showed that group 1 is significantly higher than group 5 ($p < 0.05$), group 3 is significantly higher than group 7 ($p < 0.05$), and group 4 is statistically higher

than group 8 ($p < 0.05$). The results about the "normal" parameter in ear skin showed that group 1 is significantly higher than group 5 ($p < 0.05$), as well as group 3 with respect to group 7 ($p < 0.05$) or group 4 with respect to group 8 ($p < 0.05$). For the diet test (Table 10), there were significant differences only between group 1 and group 3 ($p < 0.05$) and group 2 and group 4 ($p < 0.05$) in the "normal" parameter of both chest and ear skin.

Table 9. Number of animals (%) with histological lesions in all experimental groups of drink test.

Organs	Lesion	G1 (WT, Control)	G2 (WT, 1 mL Lemon)	G3 (WT, 1.5 mL Lemon)	G4 (WT, 2 mL Lemon)	G5 (HPV16, Control)	G6 (HPV16, 1 mL Lemon)	G7 (HPV16, 1.5 mL Lemon)	G8 (HPV16, 2 mL Lemon)
Liver	Normal	2/5 (40%)	5/5 (100%)	3/5 (60%)	3/5 (60%)	3/5 (60%)	2/5 (40%)	2/5 (40%)	3/5 (60%)
	Hepatitis	3/5 (60%)	0/5 (0%)	2/5 (40%)	2/5 (40%)	2/5 (40%)	2/5 (40%)	3/5 (60%)	2/5 (40%)
Spleen	Normal	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
Kidney	Normal	4/5 (80%)	5/5 (100%)	3/5 (60%)	2/5 (40%)	1/5 (20%)	4/4 (100%)	4/5 (80%)	4/5 (80%)
	Nephritis	1/5 (20%)	0/5 (0%)	2/5 (40%)	3/5 (60%)	4/5 (80%)	0/4 (0%)	1/5 (20%)	1/5 (20%)
Lung	Normal	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
Chest skin	Normal	5/5 [1] (100%)	5/5 (100%)	5/5 [2] (100%)	5/5 [3] (100%)	0/5 (0%)	0/4 (0%)	0/5 (0%)	0/5 (0%)
	Hyper-plasia	0/5 (0%)	0/5 (0%)	0/5 [4] (0%)	0/5 (0%)	2/5 (40%)	3/4 (75%)	5/5 (100%)	4/5 (80%)
	Dysplasia	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	3/5 (60%)	1/4 (25%)	0/5 (0%)	1/5 (20%)
	SCC	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/4 (0%)	0/5 (0%)	0/5 (0%)
Ear skin	Normal	5/5 [5] (100%)	5/5 (100%)	5/5 [6] (100%)	5/5 [7] (100%)	0/5 (0%)	0/4 (0%)	0/5 (0%)	0/5 (0%)
	Hyper-plasia	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	1/4 (25%)	1/5 (25%)	2/5 (40%)
	Dysplasia	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	4/5 (80%)	3/4 (75%)	4/5 (80%)	3/5 (60%)
	SCC	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	1/5 (20%)	0/4 (0%)	0/5 (0%)	0/5 (0%)

[1] Significantly different from G5 ($p < 0.05$). [2] Significantly different from G7 ($p < 0.05$). [3] Significantly different from G8 ($p < 0.05$). [4] Significantly different from G7 ($p < 0.05$). [5] Significantly different from G5 ($p < 0.05$). [6] Significantly different from G7 ($p < 0.05$). [7] Significantly different from G8 ($p < 0.05$).

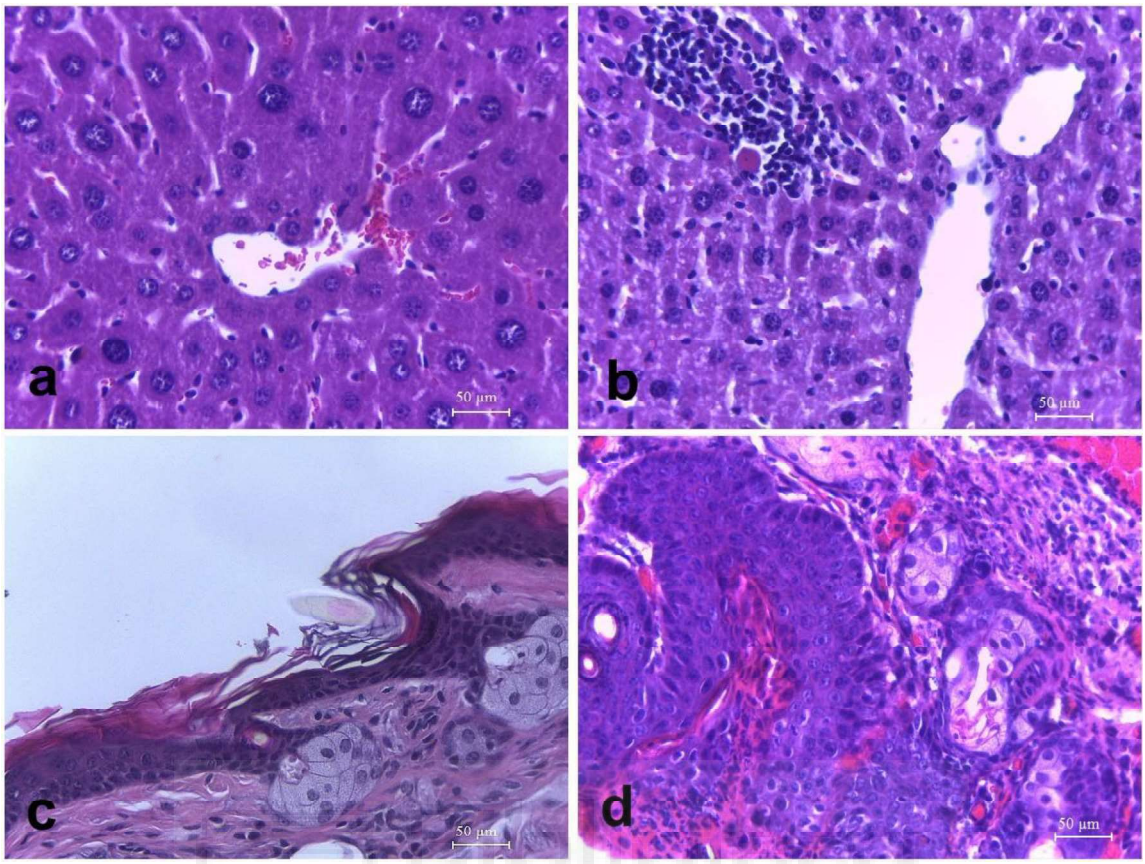

Figure 4. Representative histological images (hematoxylin and eosin, 400×). (**a**) Normal hepatic histology, group 5 mouse (HPV16, control). (**b**) Focal leukocytic infiltration, midzonal to centrilobular, with hepatocellular necrosis, group 4 mouse (WT, 2 mL lemon). (**c**) Normal skin histology, group 1 mouse (WT, control). (**d**) Epidermal dysplasia, group 5 mouse (HPV 16, control).

Table 10. Number of animals (%) with histological lesions in all experimental groups of diet test.

Organs	Lesion	G1 (WT, Control)	G2 (WT, 4 mL Lemon)	G3 (HPV16, Control)	G4 (HPV16, 4 mL Lemon)
Liver	Normal	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
	Hepatitis	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Spleen	Normal	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
Kidney	Normal	5/5 (100%)	2/5 (20%)	4/5 (80%)	4/5 (80%)
	Nephritis	0/5 (0%)	3/5 (60%)	1/5 (20%)	1/5 (20%)
Lung	Normal	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)

Table 10. *Cont.*

Organs	Lesion	G1 (WT, Control)	G2 (WT, 4 mL Lemon)	G3 (HPV16, Control)	G4 (HPV16, 4 mL Lemon)
Chest skin	Normal	5/5 [1] (100%)	5/5 [2] (100%)	0/5 (0%)	0/5 (0%)
	Hyperplasia	0/5 (0%)	0/5 (0%)	4/5 (80%)	4/5 (80%)
	Dysplasia	0/5 (0%)	0/5 (0%)	1/5 (20%)	1/5 (20%)
	SCC	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Ear skin	Normal	5/5 [3] (100%)	5/5 [4] (100%)	0/5 (0%)	0/5 (0%)
	Hyperplasia	0/5 (0%)	0/5 (0%)	1/5 (20%)	0/5 (0%)
	Dysplasia	0/5 (0%)	0/5 (0%)	3/5 (60%)	5/5 (100%)
	SCC	0/5 (0%)	0/5 (0%)	1/5 (20%)	0/5 (0%)

[1] Significantly different from G3 ($p < 0.05$). [2] Significantly different from G4 ($p < 0.05$). [3] Significantly different from G3 ($p < 0.05$). [4] Significantly different from G4 ($p < 0.05$).

3.5. Oxidative Stress

Concerning hepatic oxidative stress analyses, significant differences were observed between groups for two of the markers included in this study. The results of the drink test presented in Table 11 showed that wild-type (WT) treated groups (G2, G3 and G4), when compared with the wild-type control group (G1), have a slight increase in all enzymatic activities ($p < 0.05$). Thus, there were significant differences between groups 3 and 4 and group 1 ($p < 0.05$) in SOD activity. Moreover, there were also significant differences between the WT treated groups ($p < 0.05$), being that group 4 was the one with the highest amount of SOD (42.87 ± 7.67 U $min^{-1}mg^{-1}$). In CAT activity, G4 presented significant differences between untreated WT (G1) and treated groups 2 and 3. On the other hand, HPV16 groups also showed significant differences between treated (G6 and G7) and control group (G5) ($p < 0.05$), both in SOD and CAT activity. Group 6 presented the lowest activity (SOD: 19.34 ± 4.98 U $min^{-1}mg^{-1}$/CAT: 7.65 ± 1.03 mmol H_2O_2 $min^{-1}mg^{-1}$)). In the diet test, no significant differences were observed between groups for any of the enzymatic activity markers (Table 12).

Table 11. Oxidative stress parameters evaluated in the liver of the drink test mice (mean ± SD).

Groups	G1 (WT, Control)	G2 (WT, 1 mL Lemon)	G3 (WT, 1.5 mL Lemon)	G4 (WT, 2 mL Lemon)	G5 (HPV16, Control)	G6 (HPV16, 1 mL Lemon)	G7 (HPV16, 1.5 mL Lemon)	G8 (HPV16, 2 mL Lemon)
SOD	20.75 ± 3.03	27.31 ± 7.93	27.21 ± 3.1 [1]	42.87 ± 7.67 [2,3]	35.21 ± 6.52	19.34 ± 4.98 [6,7]	27.18 ± 1.63 [8]	26.74 ± 3.52
CAT	9.35 ± 2.23	11.51 ± 1.36	12.06 ± 0.85	14.69 ± 1.07 [4,5]	13.94 ± 1.43	7.65 ± 1.03 [9,10]	10.14 ± 1.55 [11]	11.49 ± 2.16

Data are the mean ± DS of five independent experiments performed in duplicate. Results of SOD activity have been expressed as U min^{-1} mg^{-1}. CAT activity content has been expressed as mmol H_2O_2 $min^{-1}mg^{-1}$). [1,2] G1 was significantly different from G3 and G4 ($p < 0.05$). [3] G4 was significantly different from G2 and G3 ($p < 0.05$). [4] G4 was significantly different from the G1 ($p < 0.05$). [5] G4 was significantly different from G2 and G3 ($p < 0.05$). [6,8,9,11] G5 was significantly different from G6 and G7 ($p < 0.05$). [7,10] G6 was significantly different from G7 and G8 ($p < 0.05$).

Table 12. Oxidative stress parameters evaluated in the liver of diet test mice (mean ± SD).

Groups	G1 (WT, Control)	G2 (WT, 4 mL Lemon)	G3 (HPV16, Control)	G4 (HPV16, 4 mL Lemon)
SOD	27.81 ± 6.04	28.24 ± 4.67	35.66 ± 0.29	33.58 ± 2.58
CAT	7.65 ± 1.54	8.26 ± 1.02	12.68 ± 0.91	13.29 ± 1.17

Data are the mean ± DS of five independent experiments performed in duplicate. Results of SOD activity have been expressed as U min^{-1} mg^{-1}. CAT activity content has been expressed as mmol H_2O_2 $min^{-1}mg^{-1}$).

4. Discussion

Traditionally, fruits and vegetables have been used for medicinal purposes, providing the treatment of various diseases. These foods have contributed greatly to the development of new therapeutic strategies thanks to their secondary metabolites or bioactive compounds, which interact with cellular targets. Lemon contains numerous beneficial bioactive compositions, including phenolic compounds (mainly flavonoids), vitamins, carotenoids, essential oils, minerals and dietary fiber [26] with anti-inflammatory, antimicrobial and antitumor activities [27]. However, this fruit has a reduced shelf life, and one of the ways to maintain these compounds for a longer time is the application of post-harvest treatments such as melatonin, which has been shown to have several effects on quality maintenance [8,9]. The profile of phenolic compounds identified in lemon juice extract presented the flavanones hesperidin and eriocitrin as the predominant compounds above the flavones diosmetin-6,8-di-*C*-glucoside and diosmetin-8-*C*-glucoside, which is consistent with Gonzalez-Molina et al.'s (2009) previous studies [26] in which the most abundant compounds were hesperidin and eriocitrin, although with the difference that, in this study, the most abundant compound was eriocitrin instead of hesperidin, and the predominance was not in equal proportion (3:6 vs. 1:5). Hesperidin accounts for almost 50% of the total phenolic compounds in our lemon phenolic compounds' composition, as reported other authors [28]. Hesperidin has shown numerous positive effects on human health; however, in this work, one of the most important effects is restricting virus replication and progression [29,30]. Other compounds found with a considerable content were scoparin and vicenin-2 (12 ± 0.5 and 11.5 ± 0.2 mg g^{-1}, respectively). This content was higher than that found in other works, as well as the total content of phenolic compounds [26,31]. We can also highlight the presence of other compounds in very low concentrations (luteolin-7-O-rutinoside, quercetin-3-O-glucoside, caffeic acid or rutin) but which may have an important and significant role in decreasing intracellular ROS concentration and in protecting lipid, DNA and mitochondrial functionality from the damage induced by free radicals [32]. Other works [28,33] show different profiles of polyphenolic compounds from ours. This occurs, for example, in the work of Gonzalez-Molina et al., 2009 [26], in which they mention the presence of diosmin among the most notable compounds in lemon Verna, while in our work, we did not find this compound. This could be due to factors such as the variety, the ripening stage at which the fruit is harvested, the water content or the part of the fruit analyzed, as well as the type of analysis carried out.

The observation of a decrease in the concentration of phenolic compounds, as well as vitamin C and total antioxidant activity, over 72 h of storage, is consistent with studies demonstrating the sensitivity of these compounds to adverse environmental conditions [34]. These findings highlight the importance of considering appropriate preservation strategies to maintain the stability of bioactive compounds in lemon juice during storage and processing [35].

To reach the proposed goals, two experimental tests were carried out in parallel to assess how the consumption of melatonin-treated lemon affects both healthy (WT) and sick (HPV16) animals. It is known that in experimental laboratory work, it is important to observe the animals regarding their well-being, and whenever there is discomfort on the part of the animals, they must be sacrificed in advance [36]. However, the humane endpoints evaluated and recorded weekly never reached the sum of 4, a value from which the animals would have to be sacrificed before the scheduled date for the end of the test.

It is therefore concluded that, in accordance with our results, the exposure of animals to *C. limon* juice treated with melatonin appears to be safe. We can, therefore, conclude that fruits treated with compounds such as melatonin (which is a natural origin elicitor) at these doses do not have any toxicity for animals and humans, as has already been proven in other studies [37], so it is advisable to consume them in the doses studied, which extrapolated to a human with an average weight of 70 kg would correspond to 5 L of lemon juice.

In the drinking test, fluctuations in body weight and weight gain of mice for the different groups were observed throughout the study, with significant differences between groups. These differences in body weight may indicate possible effects of lemon juice with melatonin on the metabolism and physiology of the mice, as were found in other research in which the chemical components present in lemon, such as hesperidin, may have effects on metabolism and physiology [28,38]. In addition, Saini R.K. et al. (2022) support the idea that lemon juice may affect the physiology of mice [28]. In the diet test, although no significant differences in body weight were observed between the beginning and end of the study in either group, differences in mean weight gain were observed between the groups exposed to lemon juice with melatonin. The lemon extract did not cause dose-dependent changes in body weight or weight gain. However, it is worth noting that untreated HPV16-transgenic mice showed weight loss during the experimental period, while all transgenic mice treated with lemon extract showed mild weight gains. These findings suggest that the administration of melatonin-treated lemon may have different effects on weight gain in WT and HPV16 animals, showing that healthy animals tend to lose weight while sick animals tend to gain weight. These statistical differences support the hypothesis that the extract is safe under these experimental conditions and may have a favorable impact on the animal's health status. The average water consumption for each group was constant throughout the tests, which agrees with the results published in a study that evaluated the safety of green tea ingestion in ICR mice, where it was concluded that the average consumption of both food and drink did not vary regardless of tea concentration [39]. These results also imply that the extract was palatable enough not to impair the animal's drinking behavior, which was a limitation in our study because the administration of lemon juice in other studies is commonly carried out by intragastric administration or via oral gavage [40,41]. In addition, it was also found that in both tests, the transgenic animals showed a higher consumption of water. This observation is reported in other studies and explained by the fact that transgenic animals develop skin lesions, thus losing their barrier functions in controlling hydration and, therefore, they need to ingest more water in order to reach balance [42]. Concerning the relative masses of organs, no statistical differences were found between treated and untreated groups in both experiments; however, some differences were found between the WT and HPV16 groups, which is consistent with other studies [43]. In agreement with the observations in water consumption, transgenic mice showed higher concentrations of total plasmatic proteins in blood samples, suggesting mild dehydration. The hematocrit was lower in HPV16 animals compared with WT mice, and the lemon extract improved the hematocrit values, further suggesting it may have a positive effect on these animals' health. Furthermore, histological analyses did not reveal lesions associated with the extract. Transgenic animals showed hepatic lesions typical of their strain, regardless of the treatment in accordance with other works [44] although it should be noted that the lemon extract showed fewer kidney lesions in HPV16 treated groups compared to the control. Antioxidant enzymes, such as SOD and CAT, represent the defense response system to excess ROS. In our study, treatment with lemon juice aqueous extract significantly decreased SOD and CAT activity in HPV-16 groups. The increased SOD activity reflects the possible activation of a compensatory mechanism to counteract free radicals in the liver, so lemon juice treatment in HPV16 prevented ROS accumulation by decreasing SOD and CA activity. These results were similar to other studies [40], showing that the extract has a favorable toxicological profile. The liver of K14HPV16 mice is particularly prone to inflammation, and therefore, this animal model is useful to test the potential hepatotoxicity of natural compounds.

5. Conclusions

The administration of *Citrus limon* (L.) Burm. F. var. Verna treated with melatonin had no negative effects on the welfare of the animals both when it was administered in the drinking water and when it was administered in the food, so we can conclude that the consumption of this compound, under these circumstances, is not toxic. Lemon juice showed positive results in modifying weight gain, which means that it can have an effect on the metabolism and physiology of wild-type and transgenic mice. It was observed that the transgenic animals that were exposed to lemon extract in both tests had a higher water consumption than the other animals, which is not related to the extract consumption. The animals treated with lemon extract showed a trend toward a reduction in the incidence of histological lesions. For the other parameters analyzed, we observed that the consumption of lemon extract could improve the antioxidant activity in HPV16 mice. Further studies are needed to understand how the composition of the lemon juice extract influences the development of health status and treatment in this animal model and if different doses of this extract would cause some different effects.

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Institutional Review Board Statement: The experimental procedures were approved by the relevant Portuguese authorities (approval number 014139) and were carried out in the animal facilities of the University of Trás-os-Montes and Alto Douro.

Data Availability Statement: Data is contained within the article.

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4.5. PUBLICATION 5

PUBLICATION 5 (Literal trascription)

Melatonin as an Efficient and Eco-Friendly Tool to Increase Yield and to Maintain Quality Atributes during Lemon Storage

Badiche-El Hilali, F., García-Pastor, M.E., Valverde, J.M., Castillo, S., Valero, D., Serrano, M.

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Melatonin as an Efficient and Eco-Friendly Tool to Increase Yield and to Maintain Quality Attributes during Lemon Storage

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Abstract: Lemon fruit (*Citrus limon* (L.) Burm.) is highly appreciated by consumers due to its antioxidant properties and health benefits. However, its shelf life can be limited by various factors, reducing the economy, and thereafter, new strategies to maintain the quality of lemons are necessary. Melatonin is a derivative of tryptamine, which is ubiquitously found in plants and has a wide range of functions regulating numerous physiological processes in plants. During two consecutive harvests, we evaluated the effect of preharvest treatments with melatonin on crop yield and on quality and functional properties of fruit of lemon cv. Verna at harvest and weekly after storage up to 28 days at 2 and 10 °C plus 2 days at 20 °C. Melatonin was applied as foliar spray treatments at dosages of 0.1, 0.3, and 0.5 mM and at three different stages of fruit development. The results showed that melatonin treatment had a positive impact on crop yield as well as in fruit quality parameters, such as firmness, content of bioactive compounds, and antioxidant activity, especially for a 0.5 mM dose. Taking all these effects into account, the application of melatonin along the growth cycle of fruit development could be considered a non-contaminant and eco-friendly tool for improving crop yield and quality of 'Verna' lemons at harvest and during postharvest storage.

Keywords: *Citrus*; yield; quality; phenolic compounds; antioxidant activity; preharvest; postharvest

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1. Introduction

Originating in Asia, lemon fruit (*Citrus limon* (L.) Burm.) is traditionally consumed for its pleasant taste and perfume and also for its beneficial effects for health, the most prominent ones being antioxidant, anticancer, anti-inflammatory, and antidiabetic, along with neuroprotection and cardiovascular defense [1,2]. These effects are primarily attributed to its rich concentration of bioactive compounds such as phenolic compounds and ascorbic acid [3]. For all these reasons, the lemon has always been valued by consumers, and within the *Citrus*, a genus of the plant family *Rutaceae*, it is the third most produced and consumed species worldwide [4]. Spain is the 7th main lemon fruit producer country around the world, with 863.2 thousand tons in 2022 and 52,600 ha cultivated [5]. This production is mainly located in the eastern regions of Spain (Valencian community and the region of Murcia), in which the lemon crop is a significant source of employment and economic income, with an average trade balance in the last years of ca. 3.2 million euros [6] for the nation. The Verna lemon is among the most widely grown in these regions, mainly due to the great adaptability of this cultivar to the climate of these Spanish areas [7].

While the production and trade of lemons have become increasingly important within the agricultural-food sector, driven by the rising demand from domestic and global markets,

substantial difficulties persist concerning the quality and management of this citrus fruit. Challenges include improving production efficiency and sustainability, enhancing the growth and maturation processes, and refining postharvest practices. The progressive loss of quality in fruits and vegetables during transportation and commercialization translates into significant economic losses, posing significant challenges to the industry. The most commonly used method to mitigate the rapid deterioration of these foodstuffs is cold storage [8]. Important quality losses, including a series of depressions in the skin, usually presented in brown color, husk desiccation or decomposition, and firmness loss, occur during post-harvest cold preservation of lemon [9]. Furthermore, titratable acidity, ascorbic acid, and phenolic compounds of the fruit also decrease, leading to a reduction in consumer acceptability in terms of freshness, juiciness, and flavor [10]. Lately, to prevent these undesirable changes and extend shelf life, different preharvest strategies have begun to be developed to improve the quality attributes of lemons at the time of harvesting and maintain them for a longer period during cold storage.

In this scenario, the utilization of several natural elicitor moieties represents a more sustainable alternative to chemicals [11]. Elicitors offer a feasible option to foster a model of modern sustainable agriculture by replacing the reliance on agrochemicals for food. Thus far, there have been no reports suggesting that the use of elicitors, whether of biotic or abiotic origin, causes adverse effects on plants, human health, or the environment [12]. Moreover, fruit crops are highly susceptible to a variety of abiotic stressors, which have been exacerbated by abnormal shifts in climate [13]. Among these, the rise in atmospheric temperature stands as a critical issue, influencing crop yields, food quality, security, availability, and nutrient profiles [14]. In response, it is imperative to implement measures to mitigate climate change effects and pursue effective adaptation strategies. Despite the limitations in the research, the application of natural elicitors, such as γ-aminobutyric acid, has demonstrated potential in mitigating the adverse impacts of climate change on the lemon crop [15].

Melatonin (MEL) was discovered in 1995 in plants belonging to both monocotyledonous and dicotyledonous species and is mainly recognized in animals for its function in controlling sleep [16]. Melatonin plays crucial roles in plants, acting as a growth regulator, antioxidant, and defense mediator. It stimulates seed germination and the growth of roots and shoots, as observed in rice seeds [17]. Additionally, it protects against oxidative stress by enhancing the activity of antioxidant enzymes and reducing reactive oxygen species, aiding plants in tolerating adverse conditions such as salinity [18]. Melatonin additionally regulates the expression of defense-related genes in Arabidopsis plants, preparing them to face pathogens and abiotic stress [19]. Furthermore, it may influence the regulation of circadian rhythms and photoperiods in plants [20]. Overall, MEL is a multifunctional molecule of great interest for biotechnology and agriculture due to its roles in growth, protection, and potential regulation of plants internal biological rhythms.

Moreover, recent studies reported an important role of MEL in controlling the fruit ripening process after preharvest treatment application. Therefore, the use of 0.1 mM melatonin in cherry tomatoes at different growth stages by spraying systems improves postharvest disease resistance [21]. Foliar spray application of melatonin on apricot trees resulted in higher crop yield and increased weight of the fruits [22], as well as increased pigmentation of red pear and sweet cherry by activating the anthocyanin biosynthesis [23,24]. In addition, preharvest 0.1 mM MEL treatment of apricot trees led to fruit with higher-quality attributes during cold or room storage temperatures due to a delay of the ripening process in this climacteric fruit species [25]. Accordingly, postharvest melatonin treatment delayed postharvest ripening in other climacteric fruits, such as cherimoya [26], mango [27], banana [28], and pear [29], throughout the reduction of ethylene production due to decreased ACC-synthase (ACS) and ACC-oxidase (ACO) activities and the expression of their codifying genes. Moreover, pre- and postharvest melatonin treatments have been reported to delay the ripening process in non-climacteric fruit [30,31]. For instance, sweet cherry tree treatment with melatonin, as well as postharvest treatments, let to fruit with delayed

ripening during cold storage, which was attributed to enhanced activity of antioxidant systems, both enzymatic and non-enzymatic ones [32,33]. However, the effect of MEL before harvest on the yield and quality characteristics of lemon fruits at harvest or during storage has not been evaluated. Thus, the aim of this research was to evaluate the effect of MEL on lemon quality at harvest and during cold storage. By addressing these issues, results would encourage the development of more sustainable farming methods to increase the value of lemon fruit, benefiting the environment, producers, and consumers.

2. Results

2.1. Crop Yield

Crop productivity and yield were evaluated through three parameters: kilograms of fruit per tree (kg tree^{-1}) (Figure 1), the number of lemons per tree (N° fruit tree^{-1}) (Figure 2), and the average fruit weight in grams (Figure S1). 'Verna' lemon cultivar is usually harvested between May and July at three different dates. For each picking date, two fruit categories were established: commercial and waste (non-commercial) lemons, according to the marketing procedures. In Figure 1A, it can be observed that in the first harvest, the MEL treatments significantly increased ($p < 0.001$) the yield in kg tree^{-1} of commercial fruit and decreased (MEL 0.3 and 0.5 mM) that of waste (Table S1). In the second harvest (Figure 1B), a similar effect was observed ($p < 0.05$). However, in the third harvest (Figure 1C), there was no significant ($p \geq 0.05$) difference between the production of the treated trees and the controls (Table S1). In both the first and second harvests, there was a significant interaction ($p < 0.001$ and $p < 0.01$, respectively) between the MEL treatment and the type of fruit harvested on the production in kg (Table S1).

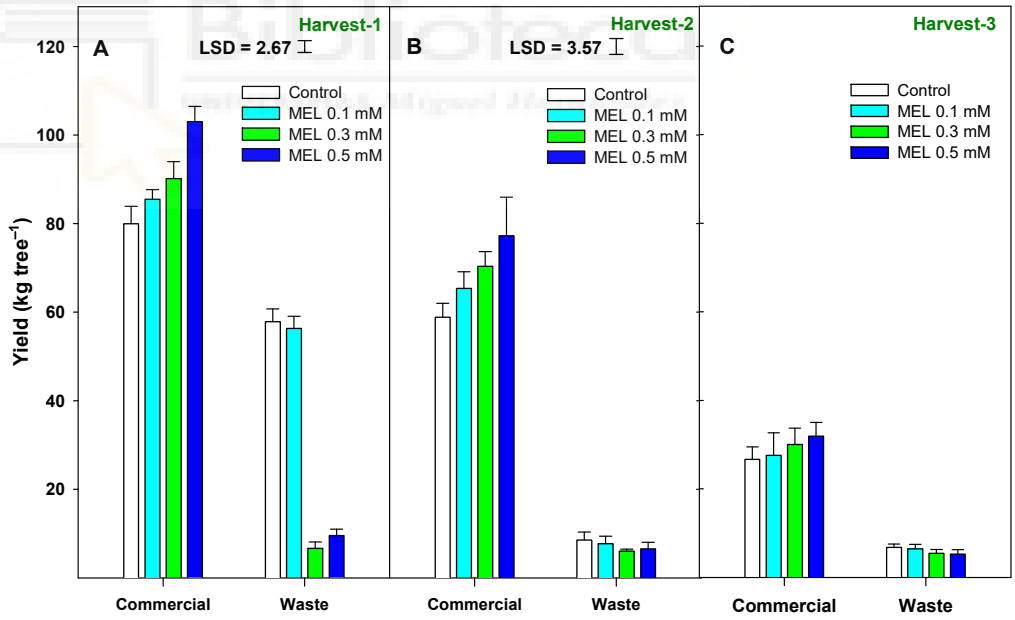

Figure 1. Yield (kg tree^{-1}) as affected by preharvest melatonin (MEL) application at three harvesting dates and the two established categories: commercial and waste. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment and fruit categories are shown when such interaction was significant.

Next, it was observed that the number of fruits per tree showed the same trend as the crop yield evaluated in kg per tree (Table S1). There was a significant increase ($p < 0.05$) in the number of fruits of MEL-treated trees with respect to control trees (Figure 2A,B). On the other hand, the production type factor was also significant ($p < 0.001$) in the number of fruits per tree, being lower in the proportion of waste fruit in lemon trees treated with MEL 0.3 and 0.5 mM than in controls. In Harvest-3 (Table S1), the same effect as in the two previous

harvests for two of the factors studied (treatment and production type) was found but with a higher significance level ($p < 0.001$) in the treatment. In both Figures 1 and 2, the most effective treatment was the MEL 0.5 mM dose. Finally, in Figure S1A–C, which represents the average weight of the fruits, it can be observed that in general, the treatments with MEL had no significant effect ($p \geq 0.05$) on fruit weight either for commercial or waste fruit, and there wasn't a significant interaction between both factors.

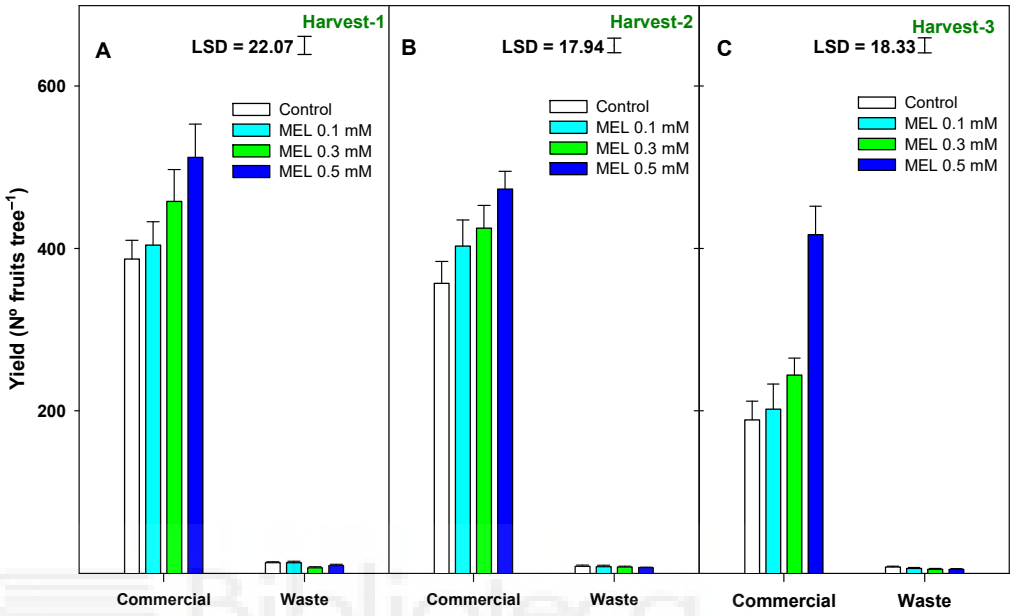

Figure 2. Yield (number of fruits tree^{-1}) as affected by preharvest melatonin (MEL) application at three harvesting dates and the two established categories: commercial and waste. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment and fruit categories (commercial or waste) are shown.

2.2. Fruit Quality Parameters

For each preharvest application (control and MEL at 0.1, 0.3, and 0.5 mM), lemons from Harvest-1 and Harvest-2 were submitted to postharvest storage for one month at two temperature regimes: 2 and 10 °C plus a further period of 2 days at 20 °C (shelf-life, SL). The following quality traits were determined at harvest (day 0) and after 7, 14, 21, and 28 days plus SL: weight loss, respiration rate, fruit firmness, total soluble solids (TSSs), and total acidity (TA).

Table S2 shows that weight loss was significantly lower ($p < 0.001$) in the treated fruits (Mel at 0.1, 0.3, and 0.5 mM) compared to the control fruits for both harvests and both temperatures (Figure 3A–D). It was also observed that throughout the storage period, weight losses increased significantly ($p < 0.001$), independently of the treatment and storage temperature, although at 2 °C the losses were lower (Table S2) than at 10 °C. The MEL 0.5 mM treatment was the most effective dose in reducing weight losses in both harvests at 2 °C (4.3 ± 0.2% for Harvest-1 and 2, respectively). Furthermore, the interaction between treatment effect and storage time significantly influenced (Table S2) the weight losses in the four conditions studied, although more so in Harvest-1 at 10 °C and Harvest-2 at 2 °C ($p < 0.001$), according to the LSD values in Figure 3A–D.

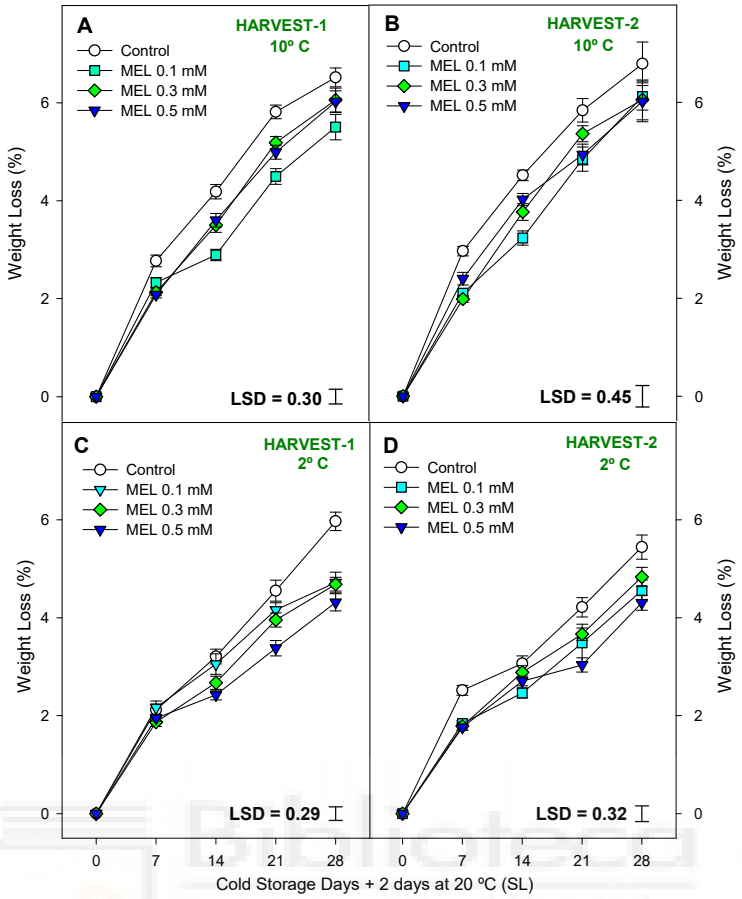

Figure 3. Weight loss (%) during 28 days of storage + shelf-life (SL) at two temperatures (2 and 10 °C) from two harvest dates (HARVEST-1 and HARVEST-2) in lemon fruit as affected by melatonin (MEL) treatment. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment and storage time are shown.

The next parameter studied was firmness. In Table S2, it can be observed that the MEL-treated lemons had higher firmness than the control fruits, being significantly higher at $p < 0.05$ in Figure 4A,C,D and significantly higher at $p < 0.01$ in Figure 4B. Overall, the most effective treatment for this parameter was MEL 0.5 mM. Firmness decreased significantly ($p < 0.001$) throughout storage, both at 2 and 10 °C, reaching losses of 30–40% compared to harvest time. No significant differences ($p \geq 0.05$; Table S2) in the effect of the interaction between treatment and storage time were observed, so the LSD is not represented. Regarding the respiration rate, it can be observed in Table S2 and Figure 5A–D how all the factors, treatment and storage time and the interaction of both, showed significant differences with respect to the controls ($p < 0.001$), being the respiration rate of treated fruits lower than the control and with a decelerating evolution from the beginning to the end of cold storage in the four conditions of the study. In relation to total acidity (Figure S2) and total soluble solids (Figure S3), significant ($p < 0.001$, except harvest-2 at 10 °C with $p < 0.05$) effects of MEL treatments were observed (Table S2), with TA and TSS content showing higher values in fruit from MEL-treated trees with respect to control ones at the end of the cold storage in both harvests and temperatures. TSS showed a stable trend throughout storage, although the storage variable was significant ($p < 0.001$, except harvest-1 10 °C with $p < 0.01$) (Table S2). A similar behavior was observed with TA ($p < 0.001$; Table S2). Finally, it can be highlighted that in the TSS, the LSD, represented as the interaction between treatment and storage time, was significant ($p < 0.001$).

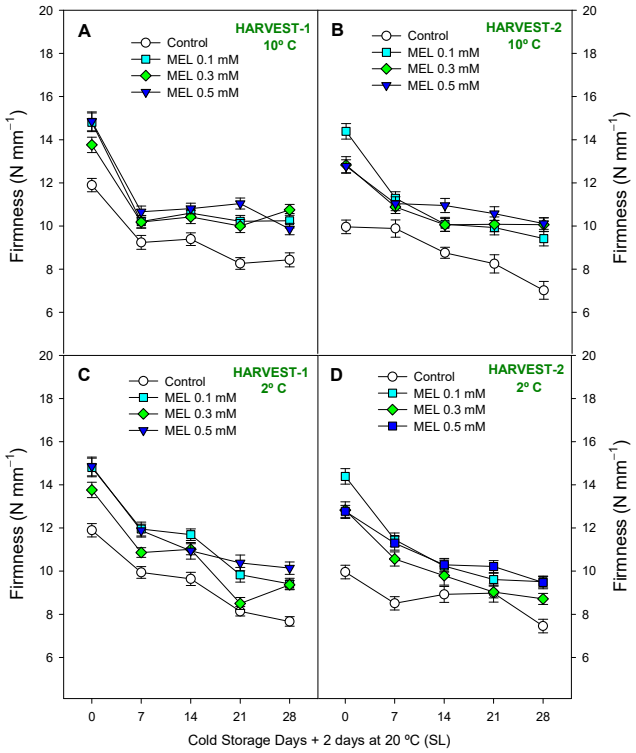

Figure 4. Firmness (N mm^{-1}) during 28 days of storage + shelf-life (SL) at two temperatures (2 and 10 °C) from two harvest dates (HARVEST-1 and HARVEST-2) in lemon fruit as affected by melatonin (MEL) treatment. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment and storage time are not shown because these interactions were not significant.

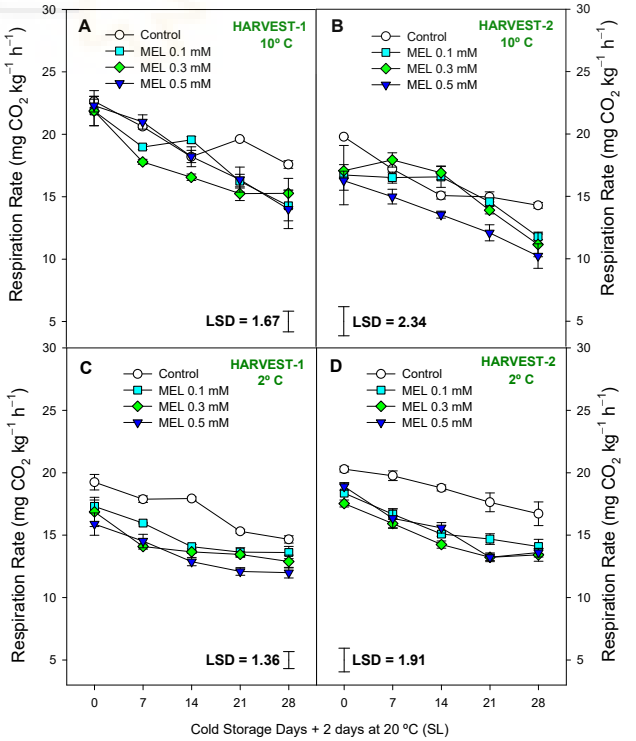

Figure 5. Respiration rate (mg CO_2 kg^{-1} h^{-1}) during 28 days of storage + shelf-life (SL) at two temperatures (2 and 10 °C) from two harvest dates (HARVEST-1 and HARVEST-2) in lemon fruit as affected by melatonin (MEL) treatment. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment and storage time are shown.

2.3. Fruit Functional Compounds and Antioxidant Activity

In lemons, total phenolic content (TPC) was analyzed in both peel (Figure 6) and juice (Figure 7), and concentration was significantly higher in peel (≈200–250 mg 100 g^{-1}) than in juice (20–40 mg 100 g^{-1}) at the end of the preservation experiment at both harvests and temperatures. Also, they showed different behavior, with an increase during 28 days of storage in the peel and a decrease in the juice. However, either in TPC in peel or TPC in juice, it was shown a significant effect of melatonin treatment with respect to the control (Table S3). As for phenols of the peel, it can be observed that in both harvests and temperatures (Figure 6A–D), MEL at 0.3 and 0.5 mM led to significantly higher ($p < 0.05$) phenolic content than in control, in general, throughout the entire duration of the storage period. In turn, at 2 °C it was possible to observe (Table S3) higher significant differences ($p < 0.01$) between MEL-treatment and control. At 10 °C, in both harvest dates (Figure 7A,B), MEL-treated fruits had a significantly ($p < 0.001$; Table S3) higher TPC in juice than controls. At 2 °C, in both harvests (Figure 7C,D), significant differences were also observed ($p < 0.05$) with higher content in MEL-treated fruits. MEL 0.5 mM was the dose that led to the highest phenolic content in lemon juice at the end of storage at 10 °C, both in the first and second harvest, being 40% higher than the control at the same time. A drastic drop in the phenol content of the juice was observed during storage at both temperatures (Figure 7A–D), so we can say that storage time significantly influenced the loss of phenols from the juice ($p < 0.001$; Table S3). In Figure 8 and Table S3, we observe that the total antioxidant activity was significantly influenced ($p < 0.05$) by the treatment and storage time factors analyzed.

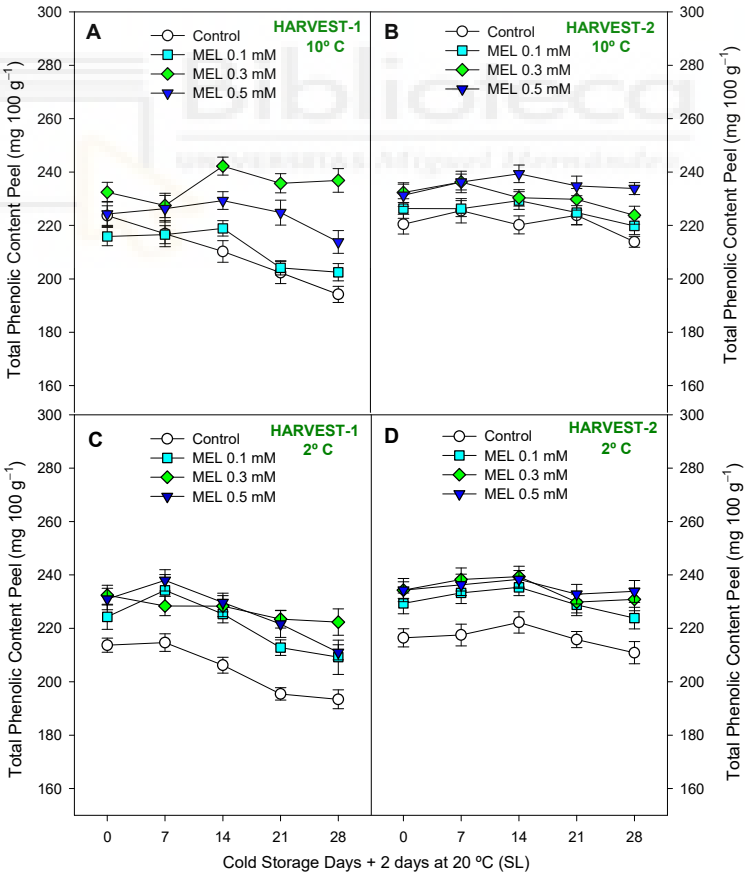

Figure 6. Total phenolic content in peel (mg·100 g^{-1}) during 28 days of storage + shelf-life (SL) at two temperatures (2 and 10 °C) from two harvest dates (HARVEST-1 and HARVEST-2) in lemon fruit as affected by melatonin (MEL) treatment. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment and storage time point are not shown because such interaction were not significant.

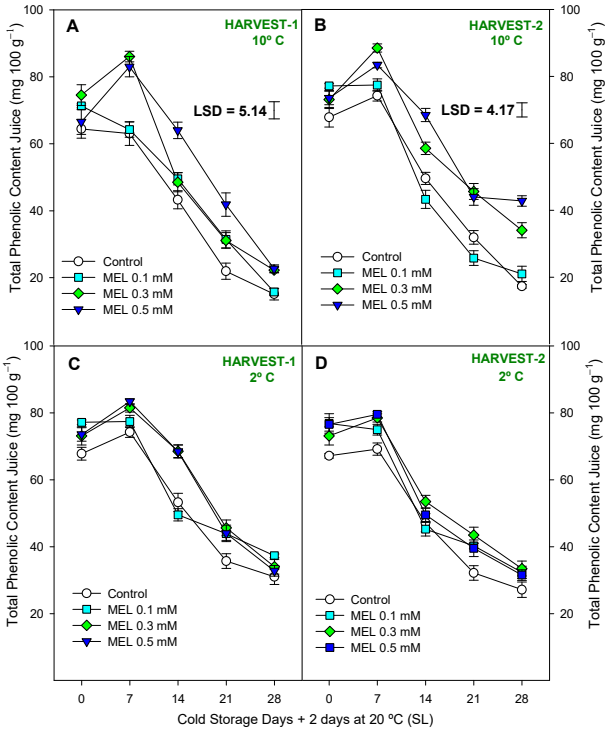

Figure 7. Total phenolic content in juice (mg 100 g^{-1}) during 28 days of storage + shelf-life (SL) at two temperatures (2 and 10 °C) from two harvest dates (HARVEST-1 and HARVEST-2) in lemon fruit. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment and storage time are shown when such interaction was significant.

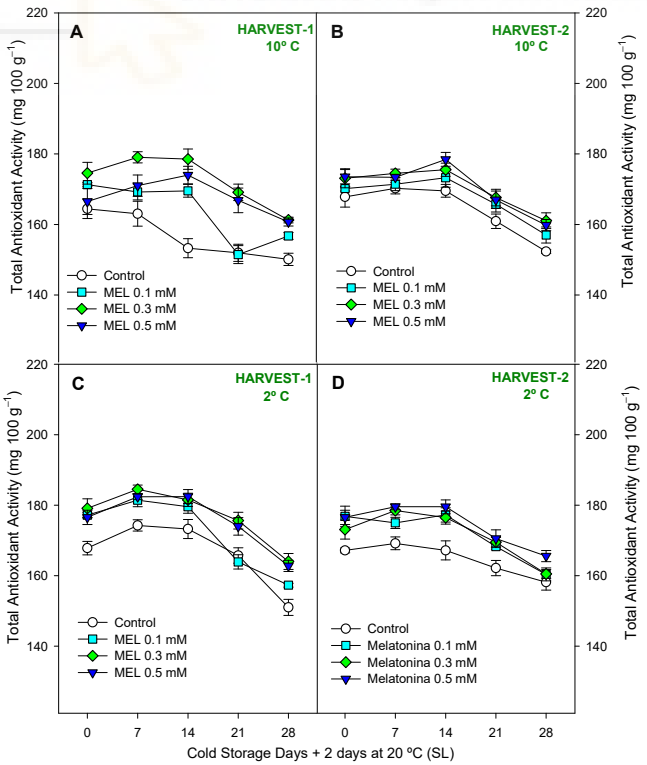

Figure 8. Total Antioxidant Activity (mg·100 g^{-1}) during 28 days of storage + shelf-life (SL) at two temperatures (2 and 10 °C) from two harvest dates in lemon fruit. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment and storage time are shown when such interaction was significant.

3. Discussion

Since the first pioneer report of preharvest MEL on pomegranate by García-Pastor et al. [34], few studies have addressed the role of MEL on crop yield, and results were contradictory depending on several factors, such as the number of applications, the stage of fruit development, and the fruit species, among others. To the best of our knowledge, this is the first evidence showing the efficacy of MEL on lemons during on-tree growth and ripening, resulting in enhancement of the yield performance of 'Verna' lemons, improving quality attributes at harvest, and maintenance after storage for 28 days at two temperatures (2 and 10 °C). Results of the present study clearly showed that MEL treatments of lemon trees produced increases in crop yield performance based on kg per tree and nº of fruits per tree. The highest effect was observed for the 0.5 mM dose, where the total yield improved by about 30% for the 1st and 2nd harvest dates compared to the control trees. In agreement with these results, MEL-treated sweet cherries, plums, or pomegranates with the same concentrations also increased crop yield [24,34,35] by enhancing the average fruit weight. Similarly, apricots treated with MEL at 0.1 mM showed a net increase in fruit yield at the time of harvest [25]. On the other hand, the application of MEL at other doses than those used in this study also showed positive effects on enhancing crop yield in strawberries and grape berries [36,37]. This yield potential could be related to two factors: the increase in the average fruit weight and the higher fruit number. In pomegranates, the increase in production was due to both factors [34]. The larger fruit size and weight could be the result of a higher accumulation of endogenous melatonin [37] or, alternatively, of a high photosynthetic efficiency and a delay in leaf senescence [38] and increases in net photosynthesis of the trees due to higher total chlorophyll and leaf area [25]. However, in the present study, the increase in yield was related to the higher number of fruits, since fruit weight did not show significant differences among treatments. Since treatments were applied at the exponential phase of the growth cycle, the observed higher fruit number in treated trees suggests a key role of MEL in reducing the fruitlet abscission or the "June-drop" occurring in citrus fruits, mainly due to environmental factors. In this sense, the role of MEL in counteracting the negative damage of both abiotic and biotic stressors has been reported [39–41]. It is well known that secondary metabolites such as phenolic compounds accumulate in fruit when plants are under abiotic stress conditions [42]. Then, taking into account that melatonin treatments increase total phenolic content in lemon fruit, fruit would be more tolerant to environmental stresses, such as wind, rain, or drought, leading to a reduction in the normal fruit drop during fruit development and to an increase in crop yield due to enhanced number of fruit harvest by tree. Therefore, MEL is thought to have influence on signal transduction as well as regulating plant physiological and biological processes, and thus considered as a biological plant growth regulator that improves a plant production capacity [43].

Appearance, together with internal quality, are important factors in determining the economic value of fruits and consumer preferences. Internal quality includes indicators of firmness, weight loss, respiration rate, TSS, and TA, among others. The results suggest that MEL application induced a retardation of the lemon ripening during the postharvest storage (at both temperatures), based on the delayed changes in respiration rate, weight loss, and fruit firmness by the action of preharvest MEL treatments. There is no scientific evidence regarding the role of preharvest MEL treatments on the behavior of these quality traits in lemon fruit. After 28 days of storage, control samples (untreated lemons) lost significantly more weight than the MEL-treated lemon fruits in both harvest and both temperatures. It is true that these losses were lower at 2 °C than at 10 °C. Postharvest weight loss in fruits and vegetables is caused by enhanced respiration and transpiration rates [44]. Weight loss increased as did the progression of storage [45], independently of the treatment, although, as expected, the 2 °C provoked lower physiological weight loss than 10 °C, which was attributed to the lower respiration rate and transpiration occurring at 2 °C [46,47]. These findings suggest that foliar application of MEL could improve preservation through a process involving reduction of the respiration rate [48], which agrees with the results of the

present experiment, in which MEL treatments produced a reduction in respiration rate with respect to control fruits. Similar results were observed by Bal [49] on plum, who reported the least amount of weight loss in MEL treatments throughout storage as well as in 'Balady Banzahir' limes during shelf life [50].

Citrus fruits are susceptible to chilling injury (CI) when stored at low (non-optimal) temperature, although their susceptibility depends on fruit species and cultivar, apart from other agronomic or environmental factors during growth [51]. However, it is interesting to note that in the present experiment, no CI damage was found on the lemon peel surface until the last sampling date either in control, or in MEL-treated fruit. Accordingly, it has been reported that for the 'Verna' cultivar, the visual fruit appearance was good after four weeks of storage at 2 °C plus 2 days at 20 °C without external symptoms of CI damage [15]. However, in this previous paper, increases in ion leakage, which is an index used to measure membrane damage associated with CI, were reported, showing that membrane damage had started to occur, although it was not high enough to be manifested externally.

In terms of respiration rate, research in sweet cherries indicated that preharvest spraying with MEL diminished respiration rate at harvest time and during storage [52]. Regarding firmness, MEL treatments induced an important effect on delaying firmness losses at the end of storage for both temperatures and harvest dates. In several studies in which MEL was applied as a preharvest treatment, it was observed that ambiguous results depended on the dose and fruit species [24]. However, related to the postharvest of MEL-treated fruits, more results coinciding with the present ones were reported. For instance, the postharvest application of MEL in "Newhall" navel orange inhibited the firmness loss and kept it longer during storage [53], and similar effects were observed in citric "kiyomi tangor" [54].

For consumers, the contents of TSS and TA are considered essential quality traits related to acceptability. For this reason, MEL-treated lemons could be considered highly valued by consumers in comparison with control fruits. There are some reports evidencing that postharvest MEL could induce the suppression in the content of TSS or TA [31], although in tomato, MEL application stimulated the accumulation of TSS and particularly citric acid [55]. Consequently, it could be postulated that the reduction of the degradation rate of some nutritional compounds like TSS and TA, as well as the reduction of fruit softening, could be due to the delay of the normal postharvest maturation and senescence processes as a result of preharvest MEL treatments, leading to maintenance of lemon fruit quality attributes for longer periods. Similar findings were observed for guavas [52], oranges [53], and peaches [56] after postharvest MEL treatments. Moreover, postharvest MEL treatments of 'Fino' lemon fruit have been recently reported to have positive effects on reducing fruit weight loss, softening, color changes, and total acidity losses [57]. In this sense, Tijero et al. [58] found that the inhibition of postharvest ripening of melatonin-treated sweet cherry, could be related to the increased levels of cytokinins, suggesting a crosstalk between both plant hormones. However, this is not a single example since it has been reported in recent reviews that MEL could interact with other plant hormones, such as auxins, gibberellins, ABA, jasmonic acid and salicylic acid, as well as in other physiological processes involved in fruit ripening, such as degradation of cell wall components, carbohydrates and pigments, energy metabolism, and antioxidant systems, among others [41,59].

Citrus fruits are an excellent source of bioactive compounds considered as potent antioxidants, such as vitamin C and E, polyphenols, flavonoids, and carotenoids [3]. As can be seen in the obtained results, the flavedo of both the control and MEL-treated fruits has a higher concentration of phenolic compounds than the juice, showing that the peel of lemons and other citrus fruits, as an important source of phenolic compounds [60–63], could be very interesting as by-products to be revalorized in the food industry as a natural source of antioxidant compounds. The peel phenolic levels of control 'Verna' lemons found in this study were higher than those measured in peels of other lemon cultivars [64], showing genetic differences among lemon cultivars. On the other hand, our results agree with those obtained with limes where total phenolic content in the pulp decreased during

storage [52], which could be attributed to the action of the enzyme polyphenol oxidase involved in the degradation of the phenolic compounds during the ripening process [61]. In similar studies on cherries and pomegranates, authors reported that total phenolic content increased after MEL treatment at 0.3 and 0.5 mM [24,37]. They found that MEL treatment enhanced glucose-6-phosphate dehydrogenase (G6H), shikimate dehydrogenase (SD), and phenylalanine ammonia lyase (PAL) activities but inhibited polyphenol oxidase (PPO) and peroxidase (POD) activities, which would lead to phenolics accumulation. On the other hand, an increase in phenolic content was also observed in MEL-treated oranges, suggesting that the capacity of resistance to oxidative stress was efficiently activated by MEL treatment through the enhancement of the ascorbate-glutathione cycle (AsA-GSH) [53]. Some studies showed a correlation between the increase in phenol content and the increase in the total antioxidant activity of the fruit [25,36] which is in line with the present results. Therefore, the higher antioxidant activity of the lemons treated with melatonin would be a consequence of the increase in phenolic compounds with antioxidant activity.

4. Materials and Methods

4.1. Plant Material, Experimental Design and Storage Conditions

The experiment was conducted in the growing season 2020–2021, in a commercial plot located in Orihuela (Alicante, Spain, 38°7′49.09″ N, 0°59′54.58″ W), under Mediterranean climate conditions (with ≈19 °C mean annual temperature and accumulated rainfall of 319 mm in 2021) and standard growing conditions for organic lemons. 'Verna' 15-year-old lemon trees grafted on *Citrus macrophylla* and planted at 7 × 5 m were randomly selected for each MEL concentration treatment. Melatonin (Sigma-Aldrich, Madrid, Spain) treatments were carried out by using 5 L per tree, applied with a foliar pulverization machine, of newly brewed melatonin solutions at 0.1, 0.3, and 0.5 mM containing 1 mL L^{-1} Tween 20. In the same way, 5 L of distilled water with 1 mL L^{-1} Tween 20 were applied to control trees. Each treatment utilized three replicates consisting of two trees. Every treatment was applied monthly, starting after the end of the physiological fruit drop stage until 3 days before the first harvest (1 February, 1 March, 31 March, and 30 April 2021). The fruits were harvested at the yellow commercial ripening stage, and since the on-tree maturation process of lemon fruit is not homogenous for all fruit, three harvests were performed: the first one on 3 May 2021(Harvest-1), the second on 31 May 2021 (Harvest-2), and the third one on 9 July 2021 (Harvest-3). In addition, treatments were repeated again 3 days before Harvest-2 and Harvest-3. For each harvest date, total yield per tree of commercial and non-commercial fruit was recorded as kg $tree^{-1}$ and as number of fruits per tree (n° fruits $tree^{-1}$). Non-commercial fruit or waste were separated according to commercial practices consisting of small fruit and fruit showing symptoms of fungal or pest attack and mechanical damages due to rubbing against branches or hail impact. In addition, a sample of 100 fruit per tree, for both commercial and waste fruit, was taken at random and weighed to obtain data on fruit weight average. For Harvest-1 and Harvets-2, these samples of 100 commercial fruit for each replicate were mixed and transported to the laboratory immediately. Then, 10 lots of 30 fruits, homogeneous in size and color, were selected for each of the three field replicates for each treatment. Four lots of each replicate were stored at 2 °C and 4 lots at 10 °C, with a relative humidity of 85–90%. At 7, 14, 21, and 28 days of storage, one lot of 30 fruit for each replicate and treatment was taken at random and left at 20 °C for two days to simulate commercial conditions after cold storage, in which the following analytical determinations were performed.

4.2. Physiological and Quality Parameters

After placing five fruits in a 0.5 L plastic bottle for 60 min, the respiration rate was measured at room temperature. Subsequently, 1 mL of the carrier atmosphere was taken and CO_2 was quantified in a gas chromatograph (Shimadzu 14B-GC, Manchester, UK) coupled to a thermal conductivity detector [64], and the respiration rate was expressed as mg CO_2 kg^{-1} h^{-1}. Weight loss was expressed as a percentage (%) after calculating the

difference between initial weight and weight after storage. The TX-XT2i texture analyzer (Stable Microsystems, Godalming, UK) coupled to a steel plate applied a force causing a deformation of 5% of the diameter of the fruit to measure the firmness, expressing the results in N mm^{-1}. After these parameters were measured in each replicate, results were expressed as the mean ± SE. Lemon juice samples were then taken from each of the fruits comprising each replicate and combined, and the following parameters were measured: total soluble solids (TSS) using a digital refractometer (Hanna Instruments, RI, USA) and titratable acidity (TA) using an automatic titrator (785 DMP Titrino; Metrohm, Herisau, Switzerland), by titration of 0.5 mL of juice with 0.1 mM NaOH to pH 8.1; the results (mean ± SE) were expressed in % (g·100 mL^{-1}).

4.3. Total Phenolics Content and Total Antioxidant Activity

Total phenols were measured in both peel (flavedo) and juice. For peel extraction, 2 g of flavedo were homogenized in 15 mL of water: methanol (2:8, *v*/*v*) containing 2.0 mM NaF. The extracts were centrifuged at 10,000× *g* for 15 min at 4 °C. For phenolic quantification in lemon juice, the extraction was similar. A total of 2 mL of juice was used and the centrifuge conditions were 8000× *g* for 7 min at 4 °C. Folin-Ciocalteau reagent was used to measure in duplicate the total phenolic content (TPC) in each sample, as previously reported [64]. Results (mean ± SE) were expressed as mg gallic acid equivalent 100 g^{-1} FW. The ABTS method was used to determine the total antioxidant activity. Briefly, 5 g of pulp were homogenized with 7.5 mL phosphate buffer (pH = 6.8) and 10 mL ethyl acetate for 2 min and centrifuged at 10,000× *g* at 4 °C for 20 min. After separation of the phases (hydrophilic and lipophilic), each extract was measured in duplicate with an ABTS peroxidase system and then summed and expressed as a total antioxidant activity in mg 100 g^{-1} Trolox equivalents.

4.4. Statistical Analysis

For the field experiments, a randomized design of three replicates of two trees per treatment was used. Fruit samples were taken from each replicate and used for the storage experiment. The experimental data for each cultivar were independently subjected to ANOVA analysis. For yield analysis, the factors of variation were treatment and fruit type. For the storage experiment, the factors of variation were treatment and storage temperature. All analyses were performed with the SPSS v. 22.0 software package for Windows (SPSS, 2011). Finally, least significant differences (LSD) were calculated at $p < 0.05$ for the interaction between the analyzed factors, and the values are shown in each figure.

5. Conclusions

The results of this study showed that MEL, applied as preharvest treatment in 'Verna' lemon trees along the growth cycle, was effective on improving the crop performance and yield since a higher percentage of commercial lemons and fewer wasted fruits were obtained, the best concentration being 0.5 mM. In addition, quality traits at harvest (fruit firmness and the content of TSS and TA) were higher in MEL-treated 'Verna' lemons in the two harvests (harvest-1 and harvest-2) in which the yield and quality of fruit were analyzed, lasting during storage. Moreover, MEL-treated trees showed a higher concentration of total phenolic compounds in juice as well as in the peel of lemons, especially for the 0.5 mM of MEL. Considering all quality traits, we can conclude that MEL induced a certain delay in the postharvest senescence at 2 °C (two weeks) and 10 °C (one week). For this reason, we demonstrated preharvest treatments with MEL may be effective in increasing crop yield to obtain higher-quality lemons. This can be regarded as a natural and environmentally friendly strategy and could alleviate the negative effects of climate change and, more generally, of abiotic stresses. Nevertheless, the physiological mechanisms involved in the effects of MEL preharvest treatments on increasing lemon tree yield and retarding fruit quality losses during storage deserve future research.

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Melatonin as an Efficient and Eco-Friendly Tool to Increase Yield and to Maintain Quality Attributes during Lemon Storage

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Table S1. Analyses of variance (ANOVA) of crop parameters (kg tree^{-1}, fruit number tree^{-1} and fruit weight) for different harvest dates (1st, 2nd and 3rd harvest) as according to 2 categories: commercial and waste in 'Verna' lemon using the treatment, the production type and the interaction between the two as factors.

Parameter	Harvest-Nº	Treatment	Production type	Treatment * Production type	Reference
Yield (kg tree^{-1})	Harvest-1	32.98***	948.06***	97.06***	Fig. 1A
Yield (kg tree^{-1})	Harvest-2	3.39*	1075.85***	5.56**	Fig. 1B
Yield (kg tree^{-1})	Harvest-3	0.26 (NS)	216.41***	0.97 (NS)	Fig. 1C
Fruit number tree^{-1}	Harvest-1	3.44*	854.58***	4.00*	Fig. 2A
Fruit number tree^{-1}	Harvest-2	3.87*	1141.27***	4.07*	Fig. 2B
Fruit number tree^{-1}	Harvest-3	18.62***	457.00***	19.32***	Fig. 2C
Fruit weight (g)	Harvest-1	0.50 (NS)	1.71 (NS)	1.03 (NS)	Fig. S1A
Fruit weight (g)	Harvest-2	0.99 (NS)	2.66 (NS)	1.52 (NS)	Fig. S1B
Fruit weight (g)	Harvest-3	0.70 (NS)	1.80 (NS)	1.33 (NS)	Fig. S1C

[1] NS = not significant; *, ** and *** significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; data were previously tested for normality test. Production type is classified as: commercial or waste

Table S2. Analyses of variance (ANOVA) of quality parameters [weight loss, respiration rate (RR), firmness, total soluble solids (TSS) and total acidity (TA)] for different harvest dates (1st and 2nd) in 'Verna' lemon during 28 days of storage + shelf-life at 2 temperatures (2 and 10 °C) using the treatment, the storage time and the interaction between the two as factors.

Parameter	Harvest-Nº	Temperature	Treatment	Storage time	Treatment * Storage time	Reference
Weight Loss (%)	Harvest-1	10 °C	49.15***	1915.78***	5.74***	Fig. 3A
Weight Loss (%)	Harvest-2	10 °C	15.80***	701.75***	1.99*	Fig. 3B
Weight Loss (%)	Harvest-1	2 °C	13.72***	359.69***	2.92**	Fig. 3C
Weight Loss (%)	Harvest-2	2 °C	28.12***	709.56***	3.59***	Fig. 3D
Firmness (N mm^{-1})	Harvest-1	10 °C	3.58*	10.93***	0.58 (NS)	Fig. 4A
Firmness (N mm^{-1})	Harvest-2	10 °C	4.17**	7.43***	0.69 (NS)	Fig. 4B
Firmness (N mm^{-1})	Harvest-1	2 °C	3.73*	8.07***	0.56 (NS)	Fig. 4C
Firmness (N mm^{-1})	Harvest-2	2 °C	3.82*	7.60***	0.59 (NS)	Fig. 4D
RR (mg CO_2 kg^{-1} h^{-1})	Harvest-1	10 °C	25.37***	125.16***	14.22***	Fig. 5A
RR (mg CO_2 kg^{-1} h^{-1})	Harvest-2	10 °C	7.06***	7.33***	8.32***	Fig. 5B
RR (mg CO_2 kg^{-1} h^{-1})	Harvest-1	2 °C	5.97***	93.66***	10.75***	Fig. 5C
RR (mg CO_2 kg^{-1} h^{-1})	Harvest-2	2 °C	2.08 (NS)	35.33***	11.87***	Fig. 5D
TSS (g 100 g^{-1})	Harvest-1	10 °C	32.58***	4.88**	8.71***	Fig. S2A
TSS (g 100 g^{-1})	Harvest-2	10 °C	9.66***	9.76***	9.34***	Fig. S2B
TSS (g 100 g^{-1})	Harvest-1	2 °C	59.43***	7.39***	4.98***	Fig. S2C
TSS (g 100 g^{-1})	Harvest-2	2 °C	52.58***	53.24***	13.54***	Fig. S2D
TA (%)	Harvest-1	10 °C	7.55***	11.99***	0.915 (NS)	Fig. S3A
TA (%)	Harvest-2	10 °C	3.88*	10.47***	0.90 (NS)	Fig. S3B
TA (%)	Harvest-1	2 °C	6.81***	25.52***	1.29 (NS)	Fig. S3C
TA (%)	Harvest-2	2 °C	13.95***	22.31***	1.51 (NS)	Fig. S3D

[1] NS = not significant; *, ** and *** significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; data were previously tested for normality test.

Table S3. Analyses of variance (ANOVA) of functional parameters [total phenolic content in peel (TPC peel), total phenolic content in juice (TPC juice) and total antioxidant activity (TAA)] for different harvest dates (1st and 2nd) in 'Verna' lemon during 28 days of storage + shelf-life at 2 temperatures (2 and 10 °C) using the treatment, the storage time and the interaction between the two as factors.

Parameter	Harvest-Nº	Temperature	Treatment	Storage time	Treatment * Storage time	Reference
TPC Peel (mg 100 g^{-1})	Harvest-1	10 °C	3.43*	18.68***	0.24 (NS)	Fig. S4A
TPC Peel (mg 100 g^{-1})	Harvest-2	10 °C	3.08*	17.51***	0.25 (NS)	Fig. S4B
TPC Peel (mg 100 g^{-1})	Harvest-1	2 °C	10.14**	20.08***	0.31 (NS)	Fig. S4C
TPC Peel (mg 100 g^{-1})	Harvest-2	2 °C	10.04**	17.13***	0.27 (NS)	Fig. S4D
TPC Juice (mg 100 g^{-1})	Harvest-1	10 °C	23.92***	486.32***	5.64***	Fig. 6A
TPC Juice (mg 100 g^{-1})	Harvest-2	10 °C	13.14***	243.32***	1.87*	Fig. 6B
TPC Juice (mg 100 g^{-1})	Harvest-1	2 °C	3.18*	186.19***	1.76 (NS)	Fig. 6C
TPC Juice (mg 100 g^{-1})	Harvest-2	2 °C	2.73*	213.66***	0.90 (NS)	Fig. 6D

TAA (mg 100 g^{-1})	Harvest-1	10 °C	3.18*	20.12***	0.36 (NS)	Fig. S5A
TAA (mg 100 g^{-1})	Harvest-2	10 °C	3.25*	21.70***	0.25 (NS)	Fig. S5B
TAA (mg 100 g^{-1})	Harvest-1	2 °C	3.73*	23.79***	0.26 (NS)	Fig. S5C
TAA (mg 100 g^{-1})	Harvest-2	2 °C	3.12*	20.88***	0.32 (NS)	Fig. S5D

[1] NS = not significant; *, ** and *** significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; data were previously tested for normality test.

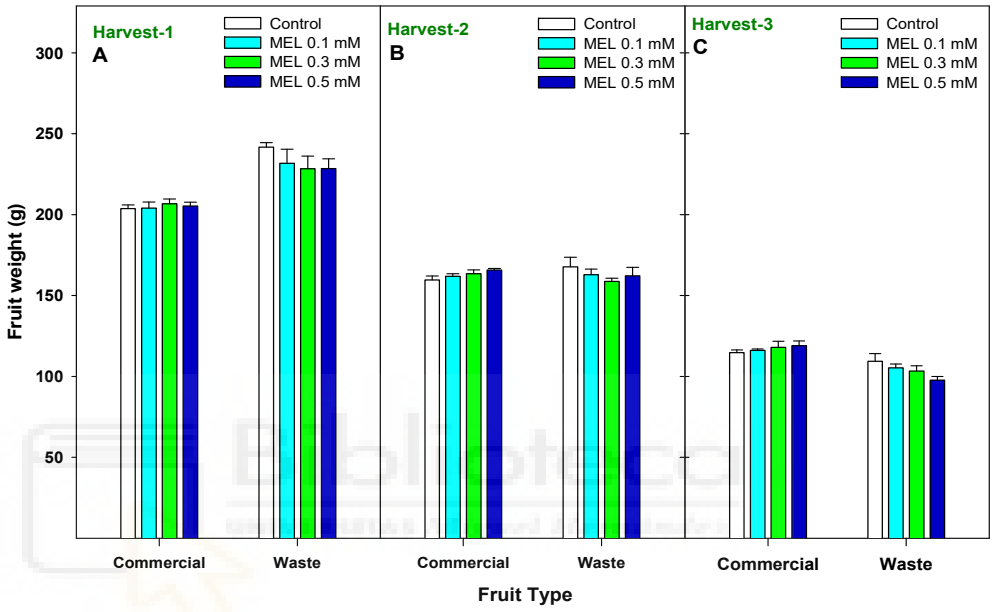

Figure S1. Influence of Melatonin preharvest treatment on yield (average fruit weight) in 3 harvest dates as according to 2 categories: commercial and waste. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment*production type point is only shown when such interaction was significant.

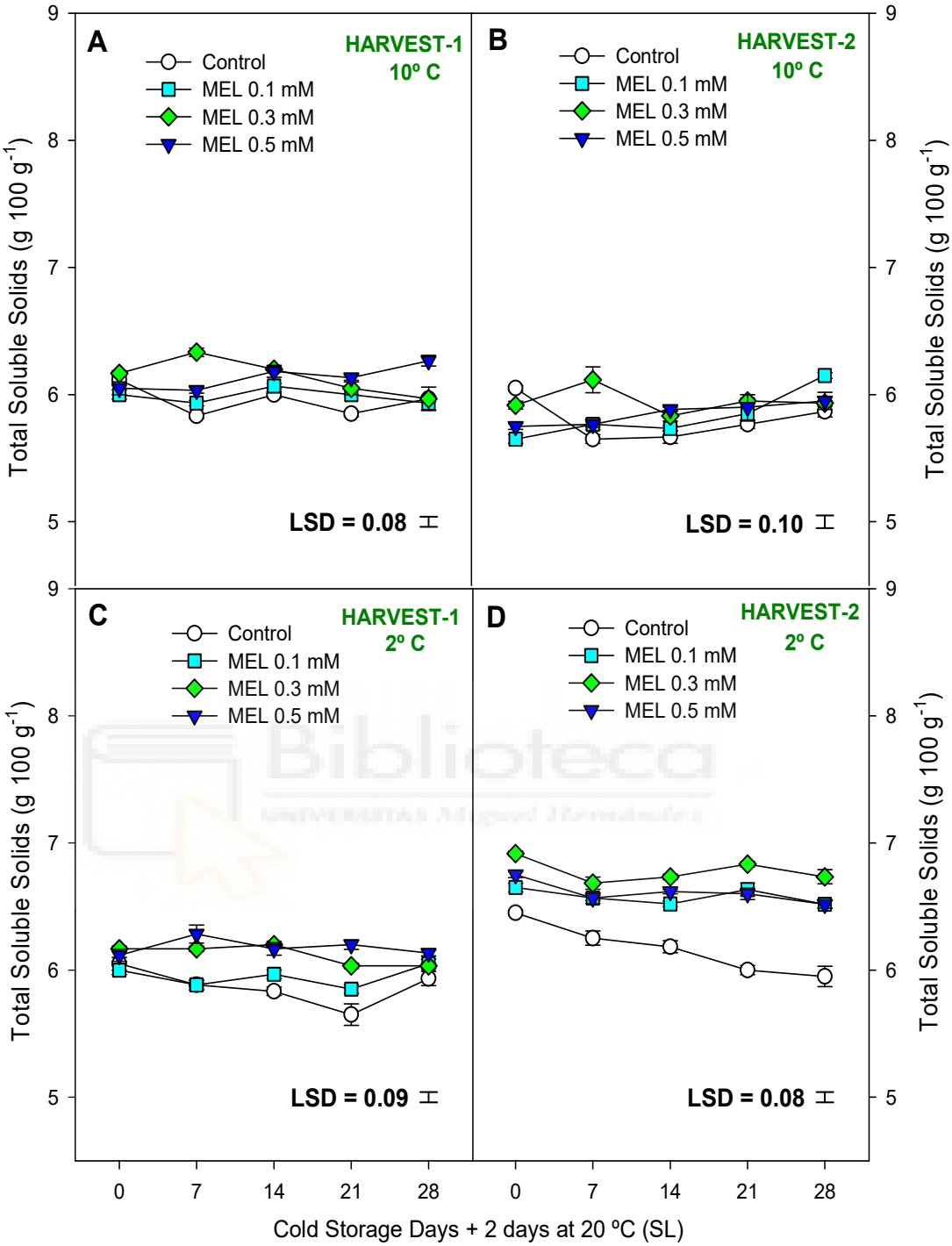

Figure S2. Total Soluble Solids (g 100 g^{-1}) during 28 days of storage + shelf-life (SL) at 2 temperatures (2 and 10 °C) from 2 harvest dates in lemon fruit. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment*storage time point is only shown when such interaction was significant.

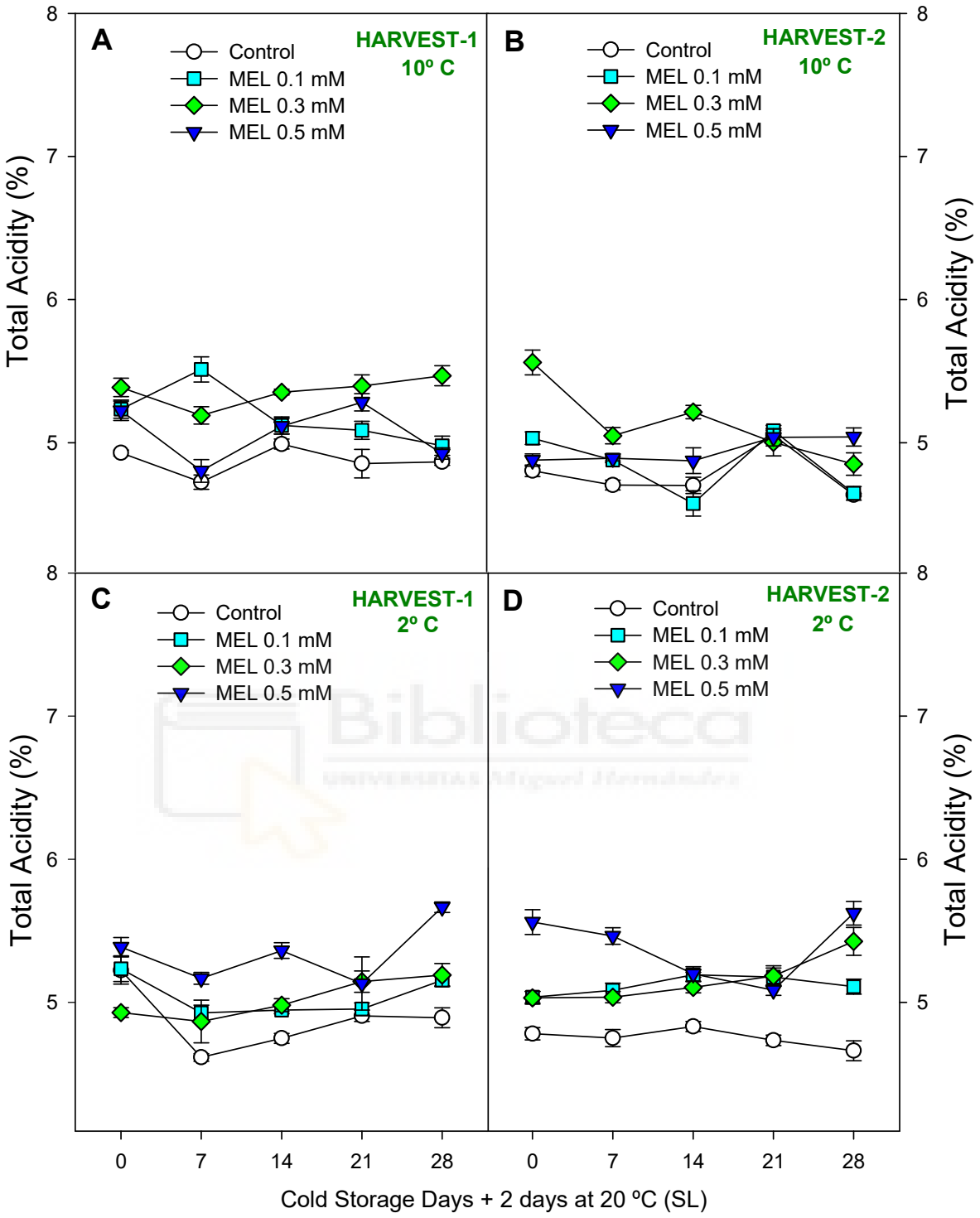

Figure S3. Total Acidity (%) during 28 days of storage + shelf-life (SL) at 2 temperatures (2 and 10 °C) from 2 harvest dates in lemon fruit. Data are the mean ± SE. LSD at $p < 0.05$ for the interaction treatment*storage time point is only shown when such interaction was significant.

5 Results and Discussion

5. RESULTS AND DISCUSSION

Lemon fruits (*Citrus limon* L.) belong to a very important crop in the Mediterranean region that has been growing steadily for several decades. This is because lemon is highly appreciated by consumers all around the world due to their organoleptic properties and phytochemical compounds content, being the most relevant carotenoids, ascorbic acid and phenolic compounds, mainly flavonoids (eriocitrin and hesperidin), which determine their biological activity with antioxidant potential and health benefits (Saini et al., 2022). Visual appearance, freshness and firmness are key factors defining the external quality of lemons, while the main internal quality parameters are acidity and antioxidant potential, which together largely define consumer acceptance and purchase decision (Lado et al., 2018). Another very important characteristic is the flavour, which depends mainly on the ratio between sugars and organic acids, while the aroma depends on the volatile compounds present in the essential oils (monoterpenes and sesquiterpenes), accumulated in the oil glands of the lemon peel (Asencio et al., 2018). However, growers faced a challenge year by year in order to obtain yields with enough quality. The crop has to develop optimally and provide good size and high-quality fruit at harvest time, with the aim of improve crop yields. Complex processes driven by different factors, control the quality of the plant product both pre-harvest and during subsequent post-harvest storage. Fruit and vegetable quality are the result of the interaction between factors such as crop management, climatology and plantation location, but the genotype determines to a large degree the ripening process, senescence and modulates the response to stress situations (Valero and Serrano, 2010). In addition, various abiotic and biotic agents can compromise yields and shelf life of harvested plant products. Plant tissues are able to cope with these agents to some extent through a cascade of biochemical signalling pathways. However, the better the condition of the plant, the more efficient the natural defence system in the fruit and other plant organs will be, and therefore the more tolerant it will be to suboptimal conditions. Once the fruit has been harvested, an energy stress is triggered by the ripening and senescence of the tissues.

Elicitors are molecules that are naturally synthesised in plants and whose regulatory function is very important, as they mediate the physiological, biochemical and molecular response that the plant produces when exposed to abiotic and biotic stresses. They induce the biosynthesis of a wide variety of metabolites, including a wide range of bioactive compounds (Ruíz-García and Gómez-Plaza, 2013), which serve not only as functional molecules that assist in plant adaptation, but can also be beneficial to human health if these fruits and vegetables are consumed (Klimek-Szczykutowicz et al., 2020). Recent studies show how the use of chemical elicitors during pre-harvest can contribute to disease control during post-harvest (Valero and Serrano, 2010). In recent years, different compounds have been applied as pre-harvest and post-harvest treatments to increase quality attributes in oranges and mandarins at harvest, the main effects being increased size, weight, firmness, colour and

antioxidant potential (Garmendia et al., 2019). Some of the most commonly used have been methyl jasmonate (MeJa), salicylic acid (SA), acetyl salicylic acid (ASA), methyl salicylate (MeSa) and oxalic acid (OA). Our research group has widely experience with these elicitors, applying them to different fruits, before and after harvesting; pomegranates, plum, sweet cherry or blood orange, are a few examples (García-Pastor et al., 2020; Zapata et al., 2014; Giménez et al., 2014; Habibi et al., 2019) how the application of these compounds can improve some issues such as chilling injury, promoting or delaying climacteric and non-climacteric ripening process or increase antioxidant enzymes activity after the treatments. However, for lemon there are few works that have studied the effect of the use of elicitors in pre-harvest or post-harvest (Siboza et al., 2014; Serna-Escolano et al., 2019b; Serna-Escolano et al., 2021) and none for the elicitors gamma (γ)-aminobutiric acid (GABA) and melatonin (MEL).

Therefore, we started the study of the application of MEL and GABA during pre-harvest and post-harvest in lemon in order to improve the fruit quality at harvest and during cold storage and consequently, its shelf life, by increasing and preserving the bioactive compounds and their antioxidant systems. GABA and MEL are organic compounds that occur naturally in plants and are involved in numerous of their physiological processes. GABA is a very important non-protein amino acid of the free amino acid pool of living organisms, whose metabolic role regulates plant growth and development (Ramos-Ruiz et al., 2019). On the other hand, melatonin regulates seed germination, plant growth, defence systems and the ripening and senescence process of fruits (Arnao & Hernández-Ruiz ,2006). On already harvested mangoes that were treated with GABA (Rastegar et al., 2020) and MEL (Liu et al., 2020), in independent studies, related its effect to the activation of the antioxidant systems of the fruit through the increase of phenolic compounds and flavonoids in the case of GABA, while in MEL the effect was found to be delay in ripening and softening. In cherry and pomegranate, three independent studies showed that pre-harvest application of MEL and GABA independently increased crop yields (Carrión-Antolí et al., 2021; Medina-Santamarina et al., 2021 & Lorente-Mento et al., 2023). In both fruits, on the other hand, an increase of bioactive compounds and the activity of antioxidant systems was observed after being treated with both compounds separately (Lorente-Mento et al., 2021 & Carrión-Antolí et al., 2023). In addition, in different fruits treated with GABA and MEL post-harvest, higher levels of ascorbic acid and ATP were maintained (Yang et al., 2011; Zhou et al., 2022 & Leon et al., 2004). Most of the published results on fruits were in post-harvest treatments with GABA and MEL, as the aim was to improve their resistance to biotic stresses during the storage period, increasing the systemic acquired resistance, which at the same time also reduced the incidence of rots caused by attack of plant pathogenic fungi. (Guillen et al., 2022; Medina-Santamarina et al., 2023; Asgarian et al., 2022 & Palma et al., 2019).

Therefore, the aim of this PhD Thesis is to provide solutions to the lemon quality problems discussed above through preharvest treatments with gamma (γ)-

aminobutiric acid and melatonin to mainly solve the pathogenic problems of these fruits at harvest as well as the alterations that determine their quality losses during postharvest storage, such as the loss of firmness or fungal decay in lemon.

5.1. Effect of pre-harvest applications of GABA and MEL in Fino and Verna crop yield

GABA and MEL treatments affected crop yield differently depending on the concentration used in the two studied cultivars. **Publication 1** showed that in the Fino-95 variety, during 2 consecutive production cycles (2019-2020 and 2020-2021), the treatment with GABA 100 mM was the most effective dose in increasing the yield of the lemon tree (kg tree^{-1}), in addition to the increase in the number of fruits per tree (nº fruits tree^{-1}), since the production was lower in the control on both harvest dates and in both vegetative cycles. Fruits were divided in two categories (green and yellow) according to market requirements and total yield was enhanced with the GABA 100 mM by 23 and 56% with respect to control trees, for green and yellow and first and second harvest, respectively in 2019-2020 growing season. In 2020-2021 growing season, for green (harvest-1) and yellow (harvest-2) lemons the percentage was 15 and 14%, respectively. The other used doses of GABA also showed significant increases in crop yields although it is worthy to point out that the effects for both crop yield parameters were not dose-dependent, since GABA at 10 mM showed higher proficiency than at 50 mM. In light of these results, the same study was carried out on the Verna cultivar (**Publication 2**). The results showed a similar effect on increasing the yield of this crop in both kg tree^{-1} and number of fruits tree^{-1} during the 2020-2021 growing season. However, the dose that showed the best effect was 50 mM in the first and second harvest. The most effective dose increased the crop yield by 33 and 70% for the first and second harvest respectively. As well as in Fino lemons, is interesting to highlight that the improvement of crop yield was not dose dependent, since preharvest GABA at 10 mM showed better proficiency than 100 mM. None of the two cultivars presented an increase in the weight of the treated fruit observed with respect to the control, which indicates that this parameter did not affect the increase in yield but it was the number of fruits per tree.

With regard to pre-harvest melatonin treatments, in this thesis we have only been able to present the results relating to the application on Verna lemons (**Publication 5**), and the results showed a positive effect of the treatment on yield. During 2020-2021 growing season the MEL treatments showed an increase in yield in kg tree-^{1} with respect to the control trees. This was due to an increase in the number of fruits per tree^{-1} as the parameter fruit weight (g) showed no significant difference with the control. The best dose was 0.5 mM in all the harvests, increasing yield compared to the control by 28% and 31% in first and second harvest, respectively. On the other hand, no dose-dependence was observed, as the results varied according to the dose, regardless of whether it was higher or lower concentration.

Table 2. Comparative percentage analysis of the fold increase between the effect of the pre-harvest treatments tested in this Doctoral Thesis on the total yield (kg tree^{-1}) of lemon. The most effective treatments have been selected in each work. The results have been calculated as a total of the harvests and growth periods studied.

Crop yield in kg tree^{-1} evaluated at harvest

Publication	Cultivar	Best Dose of treatment	Growing season	Yield (treatment vs control)
1	Fino-95	GABA 100 mM	2019-2020/ 2020-2021	⬆ 20%
2	Verna	GABA 50 mM	2020-2021	⬆ 50%
5	Verna	MEL 0.5 mM	2020-2021	⬆ 30%

In general, the effects of the elicitation treatments on the yield of lemon trees were due to their effect on the number of fruits, which differed according to the compound applied and the concentration, without affecting the weight of the fruits. The increase in the number of fruits by the treatments applied could be due to: an increase in the flowering rate, an increase in the fruit set rate, or a decrease in fruit abscission. Since in our experiments, the treatments were carried out when the fruits had reached their active growth phase, flowering or fruit set were not affected and the increase in fruit number was possibly due to a reduction in fruit abscission that occurs naturally during the fruit development process. It is clear that elicitor treatments increased the fruit-branch union strength, leading to reduced normal fruit drop occurring during fruit development, especially in the last phase of fruit maturation, which is attributed to environmental factors. In this sense, the effect of melatonin in reducing both biotic and abiotic stress in plants has previously been described (Kolodziejczyk & Posmyk, 2016; Arnao & Hernández-Ruiz, 2019). For other hand, some researchs shown that GABA increases plant stress tolerance by improving photosynthesis. The effects of GABA on increasing photosynthesis rate have been mainly studied in plants under stress condition (Li et al., 2021) such as 'Mollar de Elche' pomegranate (Lorente-Mento et al., 2023) and cherry trees (Carrión-Antolí et al., 2021).

The works related with the mechanism of action of GABA preharvest application over crop yield are very limited, although some evidence exist in seeds and vegetables as well as in some fruits studied by our group. The application of GABA can affect different growth stages and morphological attributes of plants. In consequence of foliar treatment with GABA under field conditions the yield of white gourds was significantly influenced. Furthermore, pre-yield factors such as the number of female flowers and especially the number of male flowers per plant increased as a function of the applied product dose (Ali et al., 2010). The dipping of pak-choi seeds in GABA 5 mmol L^{-1} significantly improved the growth and quality

of the crop and had an effect on the nitrate metabolism of the plants. GABA increased the activities of nitrate reductase（NR）and nitrite reductase（NiR), glutamic acid decarboxylase（GAD), and gene and protein expression in leaves (Jing-Rui et al., 2016). Coincidentally, in several of these studies, different physiological and biochemical mechanisms, such as net photosynthetic rate, leaf chlorophyll content, antioxidant enzymes and enzymes of nitrogen metabolism, increased, suggesting that these parameters positively influenced yield increase under the influence of GABA (Ramos-Ruiz et al., 2019). These observations suggest that exogenous GABA has beneficial effects on plant development in both non-stressed and, in particular, stressed plants at various stages, showing that GABA increases plant stress tolerance by improving photosynthesis (Li et al., 2021) which was related with GABA-induced increase of the activity of antioxidant enzymes and reduction of ROS damage to photosynthetic tissues. In onion, the application of GABA increased the bulb yield and other morphological characters, the highest effect being reported for GABA at 1.0 mg L^{-1} (Islam et al., 2002). In line with this report, foliar application of GABA at 0.5, 1, and 2 mM ameliorated drought stress and improved the yield of snap bean (Abd El-Gawad et al., 2021). In addition, in 'Mollar de Elche' pomegranate trees, GABA preharvest application increased crop yield in a dose-dependent way (Lorente-Mento et al., 2019) being the 10 and 100 mM the most effective doses. Related with our experiments, *Citrus* seeds, treated with GABA, improved the adaptation of the seeds to the saline conditions indicated by a higher germination rate and longer radicles (Ziogas et al., 2017). On the contrary, other studies about the preharvest GABA treatments didn't shown any significative change on crop yield (Al Shoffe et al., 2021).

On the other hand, previously published articles have shown that foliar treatment with melatonin affects crop yields. In the case of tomato plants, it has been shown, in different studies, that pre-harvest applications of melatonin are able to increase the tolerance to acid rain stress and also the stress endured by the plant under water deficit conditions (Debnath et al., 2019; Ibrahim et al., 2020). In addition, prior imbibition of tomato seeds in melatonin solutions, or application of melatonin by irrigation, has been effective in increasing tomato yields under stress (Liu et al., 2016). These effects were attributed to an increase in leaf chlorophyll content and increased photosynthetic rate. Therefore, and given the semi-arid climatic conditions of southern Spain where the pre-harvest studies of this PhD thesis were conducted, melatonin treatment could be increasing the net photosynthetic rate and allowing the proper growth of a higher number of fruit. This would improve the trees tolerance to heat and drought stress. In addition, these treatments could stimulate the sink strength of the fruits, leading to accumulate more sugars and develop a larger size at harvest. This effect was proposed by Meng et al. (2015) when they observed that grape berries treated with melatonin at pre-veraison exhibited higher endogenous melatonin accumulation, in addition to larger berry size and weight. Indeed, a similar effect was recently observed after pre-harvest melatonin treatment of apricots and cherries (Abd El-Naby et al., 2019; Carrión-Antolí et al., 2022b). However, if this effect existed it

was not very high since melatonin treatment over lemon tree did not lead to increased fruit size.

5.2. Effect of elicitation strategies on improving fruit quality parameters at harvest and maintain them during storage

Some indicators of fruit quality are weight, firmness, colour or total soluble solids (TSS) and total acid (TA) contents (Agustí-Fonfría, 2010 & Aguilar-Hernández et al., 2021). In the case of lemons, the international market requires bright, attractive and yellow fruit. However, in some varieties it is common for fruits that have already reached physiological maturity and have not yet undergone a degradation of their chlorophylls and therefore have not yet reached commercial maturity, is to apply ethylene to produce degreening.

Respect to fruit quality traits, all pre-harvest and post-harvest treatments included in the present PhD Thesis affected these parameters both, at harvest (preharvest treatments) and during postharvest storage (pre and postharvest treatments) experiments for both fruit varieties. Specifically, preharvest GABA treatments in Fino-95 lemons (**Publication 1**) did not affect negatively organoleptic quality parameters, such as firmness, TSS or TA at harvest time. In the colour parameter, an improvement was observed during the second harvest of the second growing season period (2020-2021). No significant differences were found between the control and GABA-treated trees for the 10- and 50-mM, although the preharvest application of GABA at 100 mM showed fruits with larger size. For the first time, our results have reported that GABA treatments (at 10, 50 and 100 mM) applied during on-tree Verna lemon fruit growth delayed the postharvest ripening during storage at 2 and 10 °C, manifested by lower weight losses in fruit and higher firmness, compared with control, leading to fruit quality maintenance (**Publication 2**). In both harvest dates and both storage temperatures, TSS and TA were not affected by GABA treatments.

In relation to melatonin, pre-harvest treatments on Verna lemons showed significant effects on quality parameters (**Publication 5**). Thus, the results showed a positive effect of MEL treatments on all the parameters studied (respiration rate, weight loss, firmness, TSS and TA) in the two harvests and the two storage temperatures. It is worth noting that the best results were shown for weight loss and firmness, where the treatment of 0.5 mM produced a decrease in weight loss over the control of 38% (harvest-1, 2 °C), 26% (harvest-2, 2 °C), 8% (harvest-1, 10 °C) and 12% (harvest-2, 10 °C) and an increase of firmness of 32% and 27 % at 2 °C in harvest-1 and harvest-2, respectively. Also 16% and 44% at 10 °C in harvest-1 and harvest-2, respectively. The MEL concentration that showed the best results was MEL 0.5 mM. In **Publication 3**, concerning leafy and non-leafy lemon treated after harvest with melatonin at different doses, it was shown that the 1 mM dose produced the greatest effect compared to the control. MEL treatment over Fino lemon, reduced the respiration rate during storage in comparison to untreated fruit. It also reduced

weight losses and firmness losses, as well as cell integrity as observed when assessing electrolyte leakage. Colour, TSS and TA were not affected by the treatments.

The effects of pre-harvest GABA applications on the different lemon varieties studied in this PhD thesis were different. The only similarity between Fino and Verna lemons was that GABA treatments did not negatively affect (or maintained during storage) TSS and TA, independently of the applied concentrations. The internal quality of citrus fruits is determined by acids, sugars and amino acids, which determine the taste of the fruit (Tadeo et al., 2008). The major organic acid in endocarp of lemon is citric acid, which accounts for 70 to 90 % of the total acidity of the fruit (Serna-Escolano, 2021). In general, a higher content or slower degradation rate of organic acids may contribute to a better postharvest storage performance of citrus fruits. As well as in our research, in pomegranates treated with pre-harvest GABA, it was observed that the treatments did not affect the TSS and TA (Lorente-Mento et al., 2023). Moreover, in oranges treated with GABA at post-harvest, the degradation rate of acids and sugars during storage was slower (Sheng et al., 2017). On the contrary, other research has shown that pre-harvest GABA application reduced the acidity content, increased the sugar content and increased the sugar content (Cheng et al., 2023; Zarbakhsh & Shahsavar, 2023). This could suggest that GABA plays an important role in the regulation of organic acid metabolism in citrus fruits (Sun et al., 2013). Many studies have shown that GABA shunting is the main pathway for citrate degradation (Cercós et al., 2006, Etienne et al., 2013), and glutamate decarboxylase (GAD) is a key enzyme in this pathway, which is closely related to citrate utilisation (Liu et al., 2014). On the other hand, with respect to external quality of lemon, firmness and weight losses are two important parameters that can determine its shelf life (Valero et al., 1998) and generally, firmness decreases and weight loss increases with prolonged storage time. Fruits lose their weight due to dehydration or biological changes such as the increase of respiration and transpiration rate (Valero & Serrano, 2010). Conversely, the decrease in firmness is mainly attributed to the degradation of cellulose, hemicellulose and pectin in the cell wall (Ortiz et al., 2018), whose chains could form interlocking networks through hydrogen bonds (Park & Cosgrove, 2012). In pre-harvest GABA treated sweet cherries, the dose with the best effect was 50 mM showing an improvement in firmness and decreased weight loss (Carrión-Antolí et al., 2023) as in the case of Verna lemons. GABA treatment could maintain the integrity of cell membranes and reduce metabolic activity during storage period (Wang et al., 2014). Yan et al. (2024) showed that GABA treatment increased starch content, suggesting that GABA treatment effectively retarded starch degradation into soluble sugars and preserved fruit firmness leading to fruit quality maintenance after harvest. The results of other studies showed that GABA application might inhibit membrane lipid peroxidation by altering the activity of lipoxygenase enzyme leading to higher cell wall stability (Yu et al., 2014). Also, many reports have shown that there are several GABA transporters in the plasma membrane, which have the ability to concentrate GABA in cells (Batushansky et al., 2015; Lang et al., 2016). Therefore, GABA might affect weight

loss by affecting fruit firmness. It seems that GABA reduced the weight loss of pistachio through reducing respiration rate and integrity maintenance of cell plasma membrane (Saeedi et al., 2022).

Several studies have evaluated the properties of melatonin as pre- and post-harvest technology. These have reported that each species needs to be studied independently, as the treatment conditions for one plant species do not correspond to those observed in other species, especially with regard to the optimal melatonin concentration to achieve a favourable effect. The effect of pre- and post-harvest applications of MEL on cv Fino-95 and Verna lemons (**Publication 3** and **Publication 5**) had a clear effect on weight losses. In leafy fruits (Publication 3), the transpiration of the leaves contributes additionally to weight losses and particularly to a more accelerated loss of aesthetic quality. The positive effects on weight loss reduction produced by exogenous melatonin applications have been previously described in a wide variety of fruits (Li et al., 2022; El-Beltagi et al., 2023). For instance, MEL treatment decreased weight loss in sweet cherries during storage at 0 °C (Wang et al., 2019). In accordance with our results, the postharvest application of MEL at 1 mmol L^{-1} in strawberries notably reduced the weight loss of fruit (Liu et al., 2018). However, in contrast to our study, the dose of 0.01 mmol L^{-1} showed positive effects in relation to weight losses in peach (Gao et al., 2016) while in pineapple postharvest 0.1 mM MEL treatment did not affect weight loss (Guillén et al., 2022). Weight losses are mainly due to transpiration across the fruit surface. The reduction of weight losses in fruit from melatonin-treated trees could be attributed to the effect of melatonin increasing cuticle thickness that has been previously described in different postharvest studies on mangoes (Rastegar et al., 2020) and nectarines (Bal, 2021), as well as to the lower respiration observed previously. In accordance with these results, the respiration rate was reduced in MEL-treated fruit compared to the controls (**Publication 3** and **5**). This positive effect observed on respiration in MEL-treated fruit was maintained during post-harvest storage regardless of storage temperature. Michailidis et al. (2021) observed that cherries treated with MEL at pre-harvest with melatonin showed a lower respiration at harvest, in agreement with our results. In other studies, reduced respiration after post-harvest application of melatonin has been observed in different climacteric and non-climacteric fruits (Gao et al., 2016; Zhai et al., 2018; Liu et al., 2020; Wang et al., 2019). This effect on decreasing the respiration rate during storage would indicate an effect of MEL on reducing the cell metabolism rate on fruit. In addition, Hu et al. (2017) and Onik et al. (2021) suggest that MEL may play a role in reducing the expression of certain genes responsible for ethylene synthesis, contributing to a delay in the fruit senescence process. Finally, the increase in cuticle thickness, as in weight loss, could also explain the observed reduction in weight loss (Lara et al., 2014).

As well as on weight loss and respiration rate, MEL applied as pre-harvest and post-harvest treatment had positive effects on firmness by reducing losses during storage in both varieties. There are several studies in which the application of MEL at, pre- and post-harvest, is in line with our results (Carrión-Antolí et al., 2022b;

Lorente-Mento et al 2021; Medina-Santamarina et al., 2021; Guillén et al., 2022). The reduced firmness loss of the fruits could be related to a decrease in cell wall degradation through the effect of melatonin on the inhibition of cell wall hydrolases such as polygalacturonase, pectin methyl esterase and β-galactosidase which are related to cell wall changes (Qu et al., 2022). Moreover, in different plant species, melatonin applications have been observed to stimulate the regulation of genes related to cell structure, improving membrane structure and fluidity (Sun et al., 2016; Zhai et al., 2018), by increasing the concentration of unsaturated fatty acids (Wang et al., 2020; Kong et al., 2020). The increased firmness, together with the maintenance of the energy balance that allows for proper cell function, could be preventing cell death, in addition to structural damage. At cellular level, we were able to observe in the skin of lemons (**Publication 3**), as cell compartmentalisation was maintained to a higher degree in melatonin-treated fruit by assessing electrolyte leakage. This cellular collapse leads to oxidation of phenolic compounds and the appearance of browning in the different fruits (Wang et al., 2020). In relation to TSS and TA we can observe different results depending on the moment of application of the treatment, since our results show a significant effect in Verna lemon fruits treated pre-harvest with MEL and not significant in Fino lemon treated post-harvest, as it happened in the treatments with GABA pre-harvest. TSS and TA concentration is a key factor to judge the fruit quality. In general, during the storage process, it is normal for climacteric fruits to increase their sugar content and reduce their acidity content (Bal, 2021). However, in non-climacteric fruits, and specifically in lemon, no more significant changes in the composition of the fruit after harvesting (Agustí et al., 2003). Thus, pre-harvest melatonin treatments delayed acid and sugar losses, but without significantly increasing or decreasing their content. There are numerous studies in which MEL treatments affected the soluble solids or acidity content, either increasing or decreasing it, coinciding with our results in Verna lemons (Liu et al., 2018; Liu et al., 2016; Bal, 2021; Medina-Santamarina et al., 2021). Soluble solids, including organic acids and sugars, play a important role in the overall regulation of cellular metabolism and maintaining osmotic equilibrium (Sivakumar et al., 2002) as well as, while also extending the shelf life of fresh fruits and processed products (Basha et al., 2012). They also can contribute to the protection of membranes or macromolecules and act as control agents for oxidative compounds (Mahajan et al., 2005). Consequently, by reducing the degradation rate of nutrients (such as TSS and TA), post-harvest senescence is ultimately delayed and fruit quality is maintained.

In recent years, the relationship between the reduction in cellular metabolism in melatonin-treated fruit and its action in stimulating the GABA bypass pathway has been demonstrated. This route will provide additional energy substrates such as GABA itself, which are key in situations of stress with greater metabolic demand. They can also contribute to the protection of membranes or macromolecules and act as control agents for oxidative compounds (Aghdam et al., 2018). Thus, by stimulating this pathway, melatonin would stabilise the energy balance and thus ensure that cells function more efficiently for longer.

Table 3. Comparative percentage analysis (%) between the mean values determined in the fruits treated pre- and postharvest with GABA and MEL at the best doses the control fruits in two production cycles and/or two harvests during subsequent storage at optimal and sub-optimal temperatures.

Parameters evaluated after storage for 28 (Publication 2 and 5) and 21 days (Publication 3) at 2 and 10 °C

	Publication 2 Verna, GABA 50 mM		Publication 3 Fino, MEL 1 mM	Publication 5 Verna, MEL 0.5 mM	
Storage Temperature	2 °C	10 °C	2 °C	2 °C	10 °C
Respiration Rate	-	-	↓ 7	↓ 23	↓ 31
Weight Loss	↓ 40	↓ 37	↓ 12	↓ 32	↓ 10
Firmness	↑ 7	↑ 27	↑ 17	↑ 29	↑ 34
Total Acidity	↓ 11	↓ 3	↓ 1	↓ 18	↓ 1
Total Soluble Solids	↓ 5	↓ 6	<1	↓ 8	↓ 3
Ion Leakage	-	-	↓ 26	-	-
Total phenols in peel	↑ 13	↑ 7	↑ 10	↑ 10	↑ 15
Total phenols in juice	↑ 19	↑ 15	↑ 15	↑ 13	↑ 23
Total Antioxidant Activity	↑ 32	↑ 23	-	↑ 6	↑ 6

5.3. Effect of elicitation strategies on improving total phenolic content and total antioxidant activity at harvest and during storage

Damage to the antioxidant system is one of the main problems associated with quality maintenance and shelf-life extension during postharvest storage of fruits (Dong et al., 2022) and the accumulation of reactive oxygen species (ROS) was always considered as the main contributor (Tian et al., 2013). The results obtained in this PhD thesis indicated the importance of bioactive compounds and antioxidant systems in the shelf life of the fruit during post-harvest. According to previous studies, phenols, as natural secondary metabolites, have been found to effectively

scavenge free radicals, achieving an antioxidant effect (Pereira et al., 2009). Flavonoid accumulations in fruits change seasonally in response to many factors, including temperature, sunlight and water levels (Zhu et al., 2017). Due to global warming, climatic influences on the production of these compounds in plants must be considered.

It is worth noting that GABA and MEL treatments increased total phenolic concentration at harvest and maintained them over time during storage, irrespective of temperature, in both lemon cultivars. In preharvest GABA-treated Verna lemons the higher effects was found for 50 mM, in the first and second harvest date (**Publication 2**), for the total phenolic content (TPC) and total antioxidant activity (TAA). The MEL preharvest treatments increased the TPC and TAA of Verna cultivars being the most effective dose 0.5 mM (**Publication 5**). Este efecto se produjo tanto en la primera como en la segunda recolección. Finally, in the **Publication 3**, the leafy and non-leafy lemons showed better values of phenolic content and antioxidant activity at the end of storage compared to the control, after being treated with MEL after harvesting. In the in vivo mouse assay (**Publication 4**), we observed that the major phenolic compounds in lemon juice, after treatment with MEL, were hesperidin (hesperetin-7-O-rutinoside) and eriocitrin (eriodictyol-7-O-rutinose). In relation with this research, we also observed that phenolic compounds and total antioxidant activity begin to decrease in lemon juice after 72h at room temperature.

Plant cells have antioxidant systems capable of scavenging reactive oxygen species (ROS) and repairing oxidative damage, including antioxidant compounds (in particular ascorbic acid, phenolic compounds, tocopherols and carotenoids) and antioxidant enzymes such as peroxidase (POD), catalase (CAT). γ-aminobutyric acid (GABA), is considered to be the intersection node of carbon and nitrogen metabolism, and its contribution to flavonoid metabolism in plant growth and development are still unclear. However, correlations between GABA and environmental factors have been extensively investigated (Zhu et al., 2019; Ramesh et al., 2017). Exogenous application of GABA on tea leaves increased the anthocyanin content (Liao et al., 2021), which are compounds belonging to the same family as phenols. In pomegranate and cherry crops, the harvested fruit had a higher content of polyphenolic compounds than the control after being treated with GABA at pre-harvest (Lorente-Mento et al., 2023; Carrión-Antolí et al., 2024). In postharvest, numerous studies have also shown a positive effect on the content of phenolic compounds after GABA application (Asgarian et al., 2022; Habibi et al., 2020; Aghdam et al., 2019). These effects were attributed to increased activity of phenylalanine ammonium-lyase (PAL), the key enzyme in phenolic biosynthesis, and reduced activity of polyphenol oxidase (PPO), the main enzyme involved in phenolic degradation (Mekontso et al., 2021; Ali et al., 2022). Similarly, phenolics and flavonoids content increased in blueberries during storage due to postharvest GABA treatments due to increased activity of PAL, cinnamate-4-hydroxylase (C4H) and 4-coumarate/coenzyme A ligase (4CL), which are key enzymes involved in the

phenylpropanoid pathway in plants (Ge et al., 2018). In pomegranates, Nazoori et al. (2020) reported that GABA dip treatments prior to storage maintained higher levels of total phenolics and anthocyanins after 90 days of storage. On the other hand, it is well known that phenolic compounds are highly correlated with antioxidant activity in fruits (Valero & Serrano, 2010). Thus, in banana, a correlation was found between the total phenol content and a higher antioxidant capacity than the control fruit during storage after GABA treatment (Wang et al., 2014). ROS accumulate during fruit ripening and senescence, causing damage to proteins or DNA, accelerating the senescence process (Hodges et al., 2004). In addition to inducing the accumulation of phenolic compounds, GABA may also induce the upregulation of key antioxidant enzymes (Li et al., 2019; Ren et al., 2021) like POD, CAT, ascorbate peroxidase (APX) or superoxide dismutase (SOD), which would result in higher total antioxidant activity.

Melatonin (MEL), as a strong induction factor, could regulate multiple metabolic pathways in plants and fruits, such as the biosynthesis of both phenolics and endogenous melatonin (Madebo et al., 2021). Like phenols, MEL may also contribute to ROS scavenging (Debnath et al., 2019) and delay postharvest fruit senescence (Gao et al., 2016). Based on our results, the delay of senescence in lemons after pre- and post-harvest treatment with melatonin could be related to the higher concentration of polyphenolic compounds. Zhang et al. (2018) found that MEL functioned not only as an antioxidant but also as an anti-senescence signalling molecule by inhibiting enzymatic and non-enzymatic reactions. Therefore, the promoted accumulation or delayed degradation of phenols by exogenous MEL in this study provided additional evidence of the longer lasting commercial value of the fruits compared to that of the controls. These findings were in line with previous studies exploring the mRNA levels of genes involved in phenolic biosynthesis (Pang et al., 2023). It was shown that the *PaPAL, PaC4H, Pa4CL, PaCHS, PaF3H, PaF3'H, PaDFR, PaANS, PaUFGH* expression was positively regulated under exogenous MEL treatment, responding to the accumulation of phenolic content suggested above. (Zhang et al., 2016). In addition, differential proteins involved in phenolic biosynthesis were also identified in MEL-treated fruits and all of them exhibited increased levels, revealing the positive regulatory role of MEL in phenolic accumulation at the protein level (Sun et al., 2016). In relation to these results, a lower activity of the enzyme PPO has been observed in apricots treated with melatonin in postharvest (Koushesh et al., 2012). Therefore, the higher content of phenolic compounds in apricots treated with melatonin could be related to a lower activity of this enzyme. Jannatizadeh et al. (2019), observed this relationship also in post-harvest treated pomegranates. Another possible explanation is related to amino acids. Amino acids play several roles in the life process of plants. In addition to protein constituents, amino acids are involved in many physiological processes, such as the synthesis of secondary metabolites and stress response (Hildebrandt et al., 2015). In cherries treated with 100 µM MEL, an accumulation or delayed degradation of most amino acids, such as tryptophan (Try) and phenylalanine (Phe), was observed. This

conclusion was in line with the results of a previous study, where higher amino acid concentrations were obtained under exogenous MEL treatment than in untreated plants (Zhang et al., 2021). Try and Phe would act as precursors for phenolic metabolism, leading to an increased precursor content that provides ample substrate for phenolic synthesis, further supporting and demonstrating the regulation of exogenous MEL in phenolic metabolism (Pang et al., 2023). Finally, as we said at the beginning, studies have found that phenolics, as natural secondary metabolites, effectively eliminate free radicals, achieving an antioxidant effect (Pereira et al., 2009). In addition, it has been reported that MEL itself could also directly scavenge radicals (Arnao & Hernández-Ruiz, 2015). Therefore, we hypothesise that the possible explanation for the increase in antioxidant capacity observed in our results.

5.4. Health effects of lemon with natural elicitation strategies

The content of phenolic compounds in lemons has attracted increasing interest in recent years due to their antioxidant properties responsible for their positive impact on human health, in particular by reducing the risk of degenerative diseases. The phytochemicals present in all parts of *C. limon* have an anti-cancer, anti-tumour and anti-inflammatory nature (among many other activities), with immense therapeutic potential (González-Molina et al., 2010) and have therefore traditionally served as an important ingredient in the formulation of various ethnic herbal medicines. These properties are due to the presence of different vitamins and nutrients in citrus fruits (Singh et al., 2021). Lemon has been used in various drugs connected to its antioxidant, anti-ulcer, anthelmintic, insecticidal or antimicrobial, anticancer, cytotoxic and oestrogenic activities. Other functions of the chemical constituents of citrus fruits are hepatoprotective and antihyperglycaemic properties (Klimek-Szczykutowicz et al., 2020).

In this doctoral thesis, the effect of lemon juice, after being treated with 10 mM melatonin, on the diet of healthy and sick mice (with human papillomavirus) was investigated in order to find out what the possible consequences on the health of live animals were. The results of this work (**Publication 4**) showed that the post-harvest administration of MEL-treated lemon juice had no toxic effect on the health of the animals studied. This could mean that, at the doses used in this study, the concentration of melatonin present in the fruit would have no harmful effect on humans. This is in line with recent studies that have evaluated the effect of pre-harvest melatonin on fruit and vegetables, demonstrating that it is a safe and non-toxic substance for humans and that it has a positive effect on the shelf life and quality of food (Cortés-Montaña et al., 2023). On the other hand, some effects on weight gain related to the administration of this compound were also observed, showing that in healthy animals treated with lemon juice with melatonin there was a decrease in weight gain. These differences in body weight may indicate possible effects of lemon juice with melatonin on the metabolism and physiology of mice, as found in other research where chemical compounds in lemon, such as hesperidin, may have effects on metabolism and physiology (Saini et al., 2022; Ávila-Gálvez et al., 2021). It

should be noted that the phenol profile of the treated lemon juice administered to the mice shows hesperidin and eriocitrin as the major compounds. Finally, less oxidative damage was also observed in the liver and kidney cell tissues of diseased animals that had consumed lemon juice in the diet for 30 days. These results were similar to other studies (Zhou et al., 2017), showing that the extract has a favourable toxicological profile.

5.5. Comparative price analysis

For growers, the cost of their plantations is crucial, as depending on this factor, they will obtain a profit or another after the sale of their products. In this doctoral thesis, results have shown that pre-harvest treatments are able to increase crop yields by up to 50% (**Table 2**). Both GABA and MEL have shown a binding effect between the fruit and the tree, which allows the treated trees to present a higher number of kg of fruit per tree at harvest time. The effect of treatments with 10, 50 and 100 mM GABA and 0.1, 0.3 and 0.5 mM MEL increased the yield of lemon fruits which has important economic implications, since a higher number of kg harvested will produce a higher economic benefit. Therefore, the use of these compounds in pre-harvest could increase the economic benefit to growers. The cost of a 100 g bottle of GABA is 62.80 € while a 5 g bottle of MEL costs 419 €. Given that in our studies the trees were treated at three key moments in the evolution of the fruit and each tree was given a dose of 5L, we can estimate that the total price for each dose during the whole trial would be as shown in the table below **Table 4**.

Table 4. Price analysis (expressed in euros) of GABA and MEL treatments at the doses applied. The values were calculated for 5L and 3 applications per tree, which were those carried out in our studies.

Treatment	Price
GABA 10 mM	9.7
GABA 50 mM	48.6
GABA 100 mM	97.1
MEL 0.1 mM	29.2
MEL 0.3 mM	87.6
MEL 0.5 mM	146

The cost of MEL treatments is higher than the cost of GABA treatments. However, it should be noted that, as this was a pilot trial, we used products with a very high purity (≥98%), which would be different in a commercial trial, as GABA and MEL with a lower purity could be used.

Overall, all the results published in this doctoral thesis offer us useful tools to solve the problems presented by the lemon cultivars studied without having a very high cost. Mainly, the treatments are effective to improve the crop yield, as well as to maintain the fruit during post-harvest storage. Moreover, these treatments could be considered as a safe, natural and effective solution to increase the economic profit of

farmers and, in turn, be more appreciated by consumers due to their higher sensory quality traits and antioxidant properties.

6 Conclusions / Conclusiones

6. CONCLUSIONS/CONCLUSIONES

6.1. Conclusions

This Doctoral Thesis has investigated the effect produced after the application of GABA and MEL, compounds of natural origin, as a pre- and post-harvest strategy capable of improving the quality of lemon fruits both at harvest and during post-harvest storage. These new strategies, which are based on changing traditional compounds by new compounds of natural origin, showing a positive impact on fruit quality, meet the demands of both consumers and producers. Therefore, after analysing the results obtained after applications with both compounds, either before or after fruit harvesting, these strategies could constitute a novel alternative of natural origin, capable of improving important issues in fruit quality, given the general conclusions detailed below.

- The treatments increased yield in kg per tree, mainly due to a higher number of fruits per tree in the crops, since the average weight of the fruits is not affected in the crops after the treatments, so this technology could be of significant economic benefit to producers..

- Elicitor treatments stimulated the accumulation of bioactive compounds and maintained the functional quality during post-harvest storage, both in the skin and in the juice, thus increasing the antioxidant activity.

- Quality was maintained for longer, by maintaining the ratio between acidity and soluble solids (ripening index). Also by reducing weight loss and respiration, as well as increasing firmness at the time of harvesting and at the end of storage.

In addition, some of the specific conclusions obtained in this doctoral thesis are shown below.

1. Pre-harvest treatments with GABA at the tested doses of 10, 50 and 100 mM increased total crop yield for both Fino and Verna varieties. The doses with the best effects were GABA 50 mM in Verna and GABA 100 mM in Fino. Also, pre-harvest application with MEL at the tested concentrations of 0.1, 0.3 and 0.5 mM increased total crop yield in the Verna variety. The dose with the best effect was MEL 0.5 mM.

2. At harvest time, fruit from trees treated pre-harvest with GABA and MEL affected lemon quality by increasing firmness or decreasing fruit respiration rate. However, the same effect did not occur in GABA-treated Fino lemons. In general, total soluble solids and total acidity were not or minimally affected.

3. Lemons treated with MEL and GABA, both pre- and post-harvest, showed an increase in the shelf life of the fruit during storage at optimal and suboptimal temperatures, by delaying the reduction of their metabolic activity and the evolution of ripening and senescence parameters. The best doses were GABA

50 and 100 mM and MEL 0.5 mM in pre-harvest and MEL 1 mM in post-harvest.

4. Both the flavedo and albedo of fruit from trees treated pre-harvest and fruit treated post-harvest with GABA and MEL increased both total phenol content and total antioxidant activity. It was observed that this effect was maintained during the days of cold storage.

5. Post-harvest treatment with MEL in leafy and leafless lemons showed that the best effect was with the 1 mM dose, and that leafy lemons showed in general that they could maintain better quality attributes than leafless lemons, possibly due to better melatonin absorption through the leaf.

6. MEL-treated lemon consumption was shown to have no toxic effect on the health of the mice and to improve some issues related to mouse health, such as weight gain.

7. Finally, we can conclude that pre-harvest treatments could be more recommendable for practical application because they could be incorporated into the tanks of other phytosanitary treatments without additional application costs.

6.2. Conclusiones

En esta Tesis Doctoral se ha investigado el efecto que se produce tras la aplicación de GABA y MEL, compuestos de origen natural, como una estrategia pre y post-cosecha capaz de mejorar la calidad de los frutos de limón tanto en la cosecha como durante su almacenamiento postcosecha. Estas nuevas estrategias que se fundamentan en cambiar los compuestos tradicionales por nuevos compuestos de origen natural, muestran un impacto positivo en la calidad de los frutos y satisfacen las demandas, tanto de consumidores como de productores. De manera que, tras analizar los resultados obtenidos después de las aplicaciones de ambos compuestos, bien antes o bien después de la recolección del fruto, estas estrategias podrían constituir una novedosa alternativa de origen natural, capaz de mejorar importantes aspectos en la calidad de los frutos dadas las conclusiones generales que se detallan a continuación.

- Los tratamientos incrementaron la producción en kg por árbol, principalmente debido a un mayor número de frutos por árbol en los cultivos puesto que, el peso medio de los frutos no se ve afectado en los cultivos tras los tratamientos. por lo que esta tecnología podría suponer un importante beneficio económico para los productores.

- Los tratamientos con elicitores estimularon la acumulación de compuestos bioactivos y mantuvieron la calidad funcional durante el almacenamiento en post-cosecha., tanto en la piel como en el zumo, aumentando, en consecuencia, la actividad antioxidante.

- Se mantiene durante más tiempo la calidad, manteniendo la relación entre la acidez y los sólidos solubles (índice de maduración). También se reducen pérdidas de peso y la tasa de respiración, mientras aumenta la firmeza en el momento de la recolección y al final de la conservación.

Además, a continuación, se muestran algunas de las conclusiones específicas obtenidas en esta tesis doctoral.

1. Los tratamientos pre-cosecha con GABA a las dosis ensayadas de 10, 50 y 100 mM aumentaron la producción total del cultivo tanto para la variedad Fino como para la variedad Verna. Las dosis con mejores efectos fueron GABA 50 mM en Verna y GABA 100 mM en Fino. También, la aplicación pre-cosecha con MEL a las concentraciones ensayadas de 0.1, 0.3 y 0.5 mM aumentaron la producción total del cultivo en la variedad Verna. La dosis con mejor efecto fue MEL 0.5 mM.

2. Los tratamientos pre-cosecha con GABA y MEL afectaron a la calidad de limón, al aumentar la firmeza ydisminuir la tasa de respiración de los frutos. Sin embargo, en el limón Fino tratado con GABA no se produce el mismo

efecto. En general, los sólidos solubles totales y la acidez total no se vieron afectados o lo fueron mínimamente.

3. Los limones tratados con MEL y GABA, tanto en pre como en post-cosecha mostraron un aumento de la vida útil del fruto durante el almacenamiento a temperaturas óptimas y subóptimas, al retrasar y reducir su actividad metabólica y la evolución de los parámetros de maduración y senescencia. Siendo las mejores dosis GABA 50 y 100 mM y MEL 0.5 mM en pre-cosecha y MEL 1 mM en post-cosecha.

4. Tanto en el flavedo como en el zumo de los frutos de árboles tratados en pre-recolección y de frutos tratados en post-recolección con GABA y MEL, incrementaron tanto el contenido de fenoles totales como la actividad antioxidante total. Se observó, que este efecto se mantuvo durante los días que duró el almacenamiento en frío.

5. El tratamiento post-cosecha con MEL en limones con hoja y sin hoja, mostró que el mejor efecto fue con la dosis de 1 mM, y que los limones con hoja mostraron en general que podían mantener mejores atributos de calidad con respecto a los limones sin hoja, posiblemente debido a una mejor absorción de melatonina a través de la hoja.

6. El consumo de limón tratado con MEL mostró no tener ningún efecto tóxico para la salud de los ratones y mejorar algunos aspectos relacionados con la salud de los mismos, como la ganancia de peso.

7. Finalmente, podemos concluir que los tratamientos precosecha, podrían ser mas recomendables para su aplicación práctica porque podrían incorporarse en las cubas de otros tratamientos fitosanitarios sin un gasto adicional de aplicación.

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