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Elicitation strategies in preharvest to increase quality of table grape and pomegranate fruit at harvest and during storage

Doctoral Thesis

María Emma García Pastor

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Director: Dr. Pedro Javier Zapata Coll

Co-director: Dr. Antonio Fabián Guillén Arco

Elicitation strategies in preharvest to increase quality of table grape and pomegranate fruit at harvest and during storage

María Emma García Pastor

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Supervisors:

Director: Dr. Pedro Javier Zapata Coll

Co-director: Dr. Antonio Fabián Guillén Arco

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El **Dr. D. Pedro Javier Zapata Coll**, director, y el **Dr. D. Antonio Fabián Guillén Arco**, codirector de la tesis doctoral titulada **"Elicitation strategies in preharvest to increase quality of table grape and pomegranate fruit at harvest and during storage"**,

INFORMA/N:

Que **Dña. María Emma García Pastor** ha realizado bajo nuestra supervisión el trabajo titulado **"Elicitation strategies in preharvest to increase quality of table grape and pomegranate fruit 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.

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Director de la tesis	Codirector de la tesis
Dr. D. *Pedro Javier Zapata Coll*	**Dr. D. *Antonio Fabián Guillén Arco***

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

INFORMA:

Que **Dña. María Emma García Pastor** ha realizado bajo la supervisión de nuestro Programa de Doctorado el trabajo titulado **"Elicitation strategies in preharvest to increase quality of table grape and pomegranate fruit 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.

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Profa. Dra. Dña. Juana Fernández López

Coordinadora del Programa de Doctorado en Recursos y Tecnologías Agrarias, Agroambientales y Alimentarias (ReTos-AAA)

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'Me enseñaron que el camino del progreso no es ni rápido ni fácil'

Marie Curie (1867-1934)

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❤ Emma

Publications Category

This doctoral thesis is classified in a **compendium of publications category** to qualify for Doctor Degree from Miguel Hernández University of Elche. For that purpose, the 8 selected research articles and their quality, in accordance with the 2020 edition of Journal Citation Reports® (JCR®), are shown:

Publication 1

García-Pastor, M.E., Serrano, M., Guillén, F., Castillo, S., Martínez-Romero, D., Valero, D., & Zapata, P.J. (2019). Methyl jasmonate effects on table grape ripening, vine yield, berry quality and bioactive compounds depend on applied concentration. ***Scientia Horticulturae***, 247, 380-389. doi: 10.1016/j.scienta.2018.12.043

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ISSN: 0304-4238
JCR® Category: *Horticulture*
Quartile: Q1
Rank: 4/37
Impact Factor (2020): 3.463 – Impact Factor (5 years): 3.672

Publication 2

García-Pastor, M.E., Serrano, M., Guillén, F., Giménez, M.J., Martínez-Romero, D., Valero, D., & Zapata, P.J. (2020a). Preharvest application of methyl jasmonate increases crop yield, fruit quality and bioactive compounds in pomegranate 'Mollar de Elche' at harvest and during postharvest storage. ***Journal of the Science of Food and Agriculture***, 100(1), 145-153. doi: 10.1002/jsfa.10007

Editors: M. Shepherd and A. Waterhouse.
ISSN: 0022-5142
JCR® Category: *Food Science & Technology*
Quartile: Q1
Rank: 47/144
Impact Factor (2020): 3.638 – Impact Factor (5 years): 3.802

Publication 3

García-Pastor, M.E., Serrano, M., Guillén, F., Zapata, P.J., & Valero, D. (2020b). Preharvest or a combination of preharvest and postharvest treatments with methyl jasmonate reduced chilling injury, by maintaining higher unsaturated fatty acids, and increased aril colour and phenolics content in pomegranate. ***Postharvest Biology and Technology***, 167, 111226. doi: 10.1016/j.postharvbio.2020.111226

Editors: C. Watkins, B. Defilippi, M.I. Gil, S.P. Tian, P. Tonutti, X. Yin and M. Zude Sasse.
ISSN: 0925-5214
JCR® Category: *Food Science & Technology*
Quartile: Q1
Rank: 20/144
Impact Factor (2020): 5.537 – Impact Factor (5 years): 5.821

Publication 4

García-Pastor, M.E., Zapata, P.J., Castillo, S., Martínez-Romero, D., Guillén, F., Valero, D., & Serrano, M. (2020c). The Effects of Salicylic Acid and Its Derivatives on Increasing Pomegranate Fruit Quality and Bioactive Compounds at Harvest and During Storage. ***Frontiers in Plant Science***, 11, 668. doi: 10.3389/fpls.2020.00668

Editor: Y. Zhao.
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JCR® Category: *Plant Sciences*
Quartile: Q1
Rank: 17/235
Impact Factor (2020): 5.753 – Impact Factor (5 years): 6.612

Publication 5

García-Pastor, M.E., Giménez, M.J., Zapata, P.J., Guillén, F., Valverde, J.M., Serrano, M., & Valero, D. (2020d). Preharvest application of methyl salicylate, acetyl salicylic acid and salicylic acid alleviated disease caused by *Botrytis cinerea* through stimulation of antioxidant system in table grapes. ***International Journal of Food Microbiology***, 334, 108807. doi: 10.1016/j.ijfoodmicro.2020.108807

Editors: L. Cocolin, J.F. Ayala-Zavala, J. Chen, B. Kimura, G. LaPointe, A. Mas, M. Suk Rhee, M. Zwietering and N. Magan.
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Publication 6

García-Pastor, M.E., Zapata, P.J., Castillo, S., Martínez-Romero, D., Valero, D., Serrano, M., & Guillén, F. (2020e). Preharvest Salicylate Treatments Enhance Antioxidant Compounds, Color and Crop Yield in Low Pigmented-Table Grape Cultivars and Preserve Quality Traits during Storage. ***Antioxidants***, 9(9), 832. doi: 10.3390/antiox9090832

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Publication 8

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Editor: Y. Zhao.
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DOCTORAL THESIS STRUCTURE

This Doctoral Thesis has been structured following Miguel Hernández University internal regulation for the presentation of Doctoral Thesis as 'Compendium of Publications', this is:

- ***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 8 publications used for this PhD Thesis are presented in the following order (not matching with the publication date):
  - *i.* ***Publication 1.*** Methyl jasmonate effects on table grape ripening, vine yield, berry quality and bioactive compounds depend on applied concentration. *Scientia Horticulturae,* 247, 380-389. doi: 10.1016/j.scienta.2018.12.043
  - *ii.* ***Publication 2.*** Preharvest application of methyl jasmonate increases crop yield, fruit quality and bioactive compounds in pomegranate 'Mollar de Elche' at harvest and during postharvest storage. *Journal of the Science of Food and Agriculture,* 100(1), 145-153. doi: 10.1002/jsfa.10007
  - *iii.* ***Publication 3.*** Preharvest or a combination of preharvest and postharvest treatments with methyl jasmonate reduced chilling injury, by maintaining higher unsaturated fatty acids, and increased aril colour and phenolics content in pomegranate. *Postharvest Biology and Technology,* 167, 111226. doi: 10.1016/j.postharvbio.2020.111226
  - *iv.* ***Publication 4.*** The Effects of Salicylic Acid and Its Derivatives on Increasing Pomegranate Fruit Quality and Bioactive Compounds at Harvest and During Storage. *Frontiers in Plant Science,* 11, 668. doi: 10.3389/fpls.2020.00668

v. ***Publication 5.*** Preharvest Salicylate Treatments Enhance Antioxidant Compounds, Color and Crop Yield in Low Pigmented-Table Grape Cultivars and Preserve Quality Traits during Storage. Antioxidants, 9(9), 832. doi: 10.3390/antiox9090832

vi. ***Publication 6.*** Preharvest application of methyl salicylate, acetyl salicylic acid and salicylic acid alleviated disease caused by *Botrytis cinerea* through stimulation of antioxidant system in table grapes. *International Journal of Food Microbiology,* 334, 108807. doi: 10.1016/j.ijfoodmicro.2020.108807

vii. ***Publication 7.*** Preharvest Application of Oxalic Acid Improved Pomegranate Fruit Yield, Quality, and Bioactive Compounds at Harvest in a Concentration-Dependent Manner. *Agronomy,* 10(10), 1522. doi: 10.3390/agronomy10101522

viii. ***Publication 8.*** Oxalic acid preharvest treatment improves colour and quality of seedless table grape 'Magenta' upregulating on-vine ABA metabolism and relative *Vv*NCED1 gene expression and the antioxidant system in berries. *Frontiers in Plant Science,* 12, 740240. doi: 10.3389/fpls.2021.740240

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.

Future Research Lines: A brief description of future research lines that are already being developed as well as those that can be approached in an early and a long-distance future, both at a scientific and technology transfer levels from the results obtained in the present PhD Thesis, is addressed.

References: Literature used for writing and justifying this PhD Thesis in the complementary sections to '*Publications'* has been referenced.

Abstract - Resumen

ABSTRACT

Pomegranate and table grape are fruit with high acceptation by consumers due to their organoleptic quality and their content in both nutritive and bioactive compounds related to human health. However, the table grape cultivars, 'Crimson' and 'Magenta', and the 'Mollar de Elche' pomegranate show an important lack of red intense colour at harvest and some others alterations that determine their quality losses during postharvest storage. Fungal decay in table grape and in the case of pomegranate its sensitivity to develop chilling injury (CI) when they are stored under cold conditions are key factors which compromise fruit storage, transport and marketing.

In recent years, research has been performed aimed to find preharvest treatments with natural compounds to increase fruit quality at harvest and to maintain it during storage, due to consumers' concerns and legal restrictions regarding the use of postharvest chemical treatments. In this sense, the application of naturally occuring plant compounds as preharvest treatments to delay ripening and senescence, preserving fruit and vegetable quality, has received considerable attention. Therefore, the aim of this PhD Thesis is to provide solutions to the pomegranate and table grape quality problems through preharvest treatments with methyl jasmonate (MeJa), salicylic acid derivatives; salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa), and oxalic acid (OA) to mainly solve the colour problems of these cultivars at harvest as well as their quality losses during postharvest storage, such as the chilling injury incidence and fungal decay in pomegranate fruit and table grape, respectively, increasing their shelf-life.

Treatments were performed by foliar spray application of 1, 5 and 10 mM concentrations in 2016 for both crops, containing 0.5 % Tween 20 as surfactant. Control vines or pomegranate trees were sprayed with 0.5 % Tween 20 aqueous solution. In 2017 season, these concentrations were repeated for pomegranate crop and reduced to 0.01, 0.1 and 1 mM in the case of table grape, since in general, these treatments at 5 and 10 mM decreased vine yield and delayed on vine-ripening process, which could be hastened by lower doses. However, no negative effects caused by OA treatments at these concentrations on table grape crop yield or berry ripening process were observed. Thus, these doses were not reduced in the second season for this treatment. In 2018, the concentrations of treatments were selected for both crops based on these previous experiments carried out in 2016 and 2017 seasons, in which the best results in term of yield and fruit quality attributes were obtained.

The results of this PhD Thesis have shown that MeJa preharvest treatments at 0.1 mM accelerated berry ripening, mainly colour evolution due to increased anthocyanin biosynthesis, leading to earlier harvest and increasing vine yield in both table grape cultivars. In addition, berry quality parameters, such as size, weight, firmness, total soluble solids and bioactive compound content were also enhanced by this treatment, leading to berries with increased health beneficial effects. Respect to pomegranate crop, MeJa at 5 mM could be selected for practical application

purposes in order to get earlier harvest and increase pomegranate crop yield, fruit quality, mainly colour development, and its content in phenolics, anthocyanins and ascorbic acid at harvest and during storage at 10 °C. Combined 5 mM MeJa treatments (Pre- + Postharvest) were also performed and results showed that Pre- + Postharvest MeJa treatments reduced external and internal CI symptoms in pomegranate husk, likely by better maintaining the cell membrane structure through enhancing the unsaturated fatty acids (UFA)/saturated fatty acids (SFA) ratio. However, preharvest MeJa treatment is recommended over Pre- + Postharvest application to increase pomegranate fruit storability during cold storage since no significant differences between both treatments were observed.

On the other hand, it is worth noting the effects of preharvest treatments with salicylates at 10 mM, and especially SA, on increasing total and individual anthocyanin concentration in pomegranate arils which reached a deeper red colour and, in turn, would be more appreciated in the international market. In addition, SA at 10 mM improved pomegranate fruit quality and their content on antioxidant compounds at harvest and during storage at 10 °C. Contradictorily on table grape, considering the overall results, it could be concluded that 0.1 mM MeSa treatment could be a useful tool to increase crop yield and accelerate on-vine ripening process on both cultivars studied, which would lead to improve the economic profit of table grape crop. Furthermore, this treatment was the most effective on enhancing anthocyanin biosynthesis and berry colour in these poorly colored cultivars as well as inducing resistance to *Botrytis cinerea* spoilage, probably due to enhanced levels of phenolic compounds and activity of antioxidant enzymes.

Finally, 10 mM OA treatment led to an early harvest of 'Mollar de Elche' cultivar since tree yield and number of fruits were higher at the first harvest date. This concentration of OA resulted in the greatest effects improving fruit quality parameters, beneficial health effects to consumers and sensory quality properties. Nevertheless, preharvest applications with OA at 5 mM improved the skin colour of table grapes at harvest by the up-regulation of *Vitis vinifera* 9-cis-epoxycarotenoid dioxygenase 1 gene (*Vv*NCED1) and abscisic acid (ABA) homeostasis during berry development and on-vine ripening. Additionally, OA delayed table grape postharvest ripening and senescence processes during storage, which was mainly mediated by the stimulation of enzymatic and non-enzymatic antioxidant systems, as well as by the reduction in the ABA metabolism.

Therefore, it has been demonstrated that preharvest treatments with MeJa, salicylates and OA, applied at the best tested concentrations, could be considered a safe strategy, based on naturally occurring plant compounds, in preharvest to improve fruit quality attributes, mainly colour, at harvest and during storage of table grape and pomegranate fruit. Overall, a solution is provided to a regional problem. The results would help to the horticultural companies since they would provide fruits with higher quality standards at harvest and after their postharvest handling, storage and marketing.

RESUMEN

La granada y la uva de mesa son frutos con una elevada aceptación por parte de los consumidores debido a su calidad organoléptica y al contenido en compuestos tanto nutritivos como bioactivos relacionados con la salud humana. Sin embargo, las variedades de uva de mesa, 'Crimson' y 'Magenta', y la granada 'Mollar de Elche' presentan una importante escasez del color rojo intenso deseado en el momento de la recolección y algunas otras alteraciones que determinan sus pérdidas de calidad durante el almacenamiento postcosecha. La incidencia fúngica en la uva de mesa y en el caso de la granada su sensibilidad a desarrollar daños por frío (DF) cuando se almacenan a bajas temperaturas son factores claves los cuales comprometen el almacenamiento, transporte y comercialización del fruto.

En los últimos años, se han realizado investigaciones encaminadas a encontrar tratamientos precosecha con compuestos naturales 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 postcosecha. En este sentido, la aplicación de compuestos derivados de las plantas y de origen natural como tratamientos precosecha para retrasar la maduración y la senescencia, preservando la calidad de las frutas y hortalizas, ha recibido una atención considerable. Por tanto, el objetivo de esta Tesis Doctoral es dar solución a los problemas de calidad de la granada y la uva de mesa mediante tratamientos precosecha con jasmonato de metilo (JaMe), derivados del ácido salicílico; ácido salicílico (AS), ácido acetilsalicílico (AAS) y salicilato de metilo (SaMe), y ácido oxálico (AO) para solucionar principalmente los problemas de color de estas variedades en el momento de la recolección así como sus pérdidas de calidad durante el almacenamiento postcosecha, como la incidencia de daños por frío y la incidencia fúngica en la granada y la uva de mesa, respectivamente, aumentando su vida útil.

Los tratamientos se realizaron mediante pulverización foliar a las concentraciones de 1, 5 y 10 mM en 2016 para ambos cultivos, conteniendo un 0,5 % de Tween 20 como surfactante. Las parras o granados control fueron pulverizados con una solución acuosa de Tween 20 al 0,5 %. En la campaña de 2017, estas concentraciones se repitieron para el cultivo de granada y se redujeron a 0,01, 0,1 y 1 mM en el caso de la uva de mesa, ya que en general estos tratamientos a 5 y 10 mM disminuyeron el rendimiento de la parra y retrasaron el proceso de maduración en la misma, el cual podría ser acelerado con dosis más bajas. Sin embargo, no se observaron efectos negativos causados por los tratamientos con AO a estas concentraciones sobre el rendimiento del cultivo de uva de mesa o el proceso de maduración de la baya. Por tanto, estas dosis no se redujeron en la segunda campaña para dicho tratamiento. En 2018, las concentraciones de los tratamientos fueron seleccionadas para ambos cultivos en base a estos experimentos previos realizados en las campañas de 2016 y 2017, en las cuales se obtuvieron los mejores resultados en términos de rendimiento y atributos de calidad de fruto.

Los resultados de esta Tesis Doctoral han demostrado que los tratamientos precosecha de JaMe 0,1 mM aceleraron la maduración de la baya, principalmente la evolución del color debido al aumento de la biosíntesis de antocianinas, conduciendo a una cosecha más temprana e incrementando el rendimiento de la parra en ambas variedades de uva de mesa. Además, los parámetros de calidad de las bayas, como el tamaño, el peso, la firmeza, los sólidos solubles totales y el contenido de compuestos bioactivos, también se mejoraron con este tratamiento, lo que condujo a bayas con mayores efectos beneficiosos para la salud. Con respecto al cultivo de granada, JaMe 5 mM podría seleccionarse para fines de aplicación práctica con el fin de obtener una recolección más temprana y aumentar el rendimiento del cultivo de granada, la calidad del fruto, principalmente el desarrollo del color, y su contenido en fenoles, antocianinas y ácido ascórbico en el momento de la recolección y durante el almacenamiento a 10 °C. Tratamientos combinados de JaMe 5 mM (Pre- + Postcosecha) fueron realizados y los resultados mostraron que los tratamientos Pre- + Postcosecha de JaMe redujeron los síntomas de DF externos e internos en la corteza de la granada, probablemente al mantener mejor la estructura de la membrana celular mediante la mejora del ratio de ácidos grasos insaturados (AGI)/ácidos grasos saturados (AGS). Sin embargo, el tratamiento precosecha de JaMe es recomendado sobre la aplicación Pre- + Postcosecha para incrementar la capacidad de almacenamiento de la granada durante el almacenamiento en frío, ya que no se observaron diferencias significativas entre ambos tratamientos.

Por otro lado, cabe destacar los efectos de los tratamientos precosecha con salicilatos 10 mM, y especialmente AS, sobre el incremento de la concentración de antocianinas totales e individuales en los arilos de granada que alcanzan un color rojo más intenso y, a su vez, serían más apreciados en el mercado internacional. Además, el AS 10 mM mejoró la calidad de la granada y su contenido en compuestos antioxidantes en el momento de la recolección y durante el almacenamiento a 10 °C. Contradictoriamente en uva de mesa, considerando los resultados generales, se podría concluir que el tratamiento con SaMe 0,1 mM podría ser una herramienta útil para incrementar el rendimiento del cultivo y acelerar el proceso de maduración en la parra en ambas variedades estudiadas, lo que conduciría a mejorar el beneficio económico del cultivo de uva de mesa. Además, este tratamiento fue el más efectivo mejorando la biosíntesis de antocianinas y el color de la baya en estas variedades con escasez de color, así como induciendo resistencia al crecimiento de *Botrytis cinerea*, probablemente debido a niveles mejorados de compuestos fenólicos y de la actividad de los enzimas antioxidantes.

Finalmente, el tratamiento de AO 10 mM condujo a una cosecha temprana de la variedad 'Mollar de Elche' ya que el rendimiento del árbol y el número de frutos fueron mayores en la primera fecha de recolección. Esta concentración de AO dio lugar a los mayores efectos mejorando los parámetros de calidad del fruto, los efectos beneficiosos para la salud de los consumidores y las propiedades de calidad sensorial. Sin embargo, las aplicaciones precosecha con AO 5 mM mejoraron el color de la piel de la uva de mesa en el momento de la recolección mediante la sobreregulación del gen *Vitis vinifera* 9-cis-epoxycarotenoide dioxygenasa 1

(*Vv*NCED1) y la homeostasis del ácido abscísico (ABA) durante el desarrollo de la baya y la maduración en la parra. Adicionalmente, el AO retrasó los procesos de maduración y senescencia en postcosecha de la uva de mesa durante el almacenamiento, lo que estuvo mediado principalmente por la estimulación de los sistemas antioxidantes enzimáticos y no enzimáticos, así como por la reducción del metabolismo del ABA.

Por lo tanto, se ha demostrado que los tratamientos precosecha con JaMe, salicilatos y AO, aplicados a las mejores concentraciones ensayadas, podrían ser considerados una estrategia segura, basada en compuestos derivados de las plantas con origen natural, en precosecha para mejorar los atributos de calidad del fruto, principalmente el color, en la recolección y durante el almacenamiento de la uva de mesa y la granada. En general, se aporta una solución a un problema regional. Los resultados ayudarían a las empresas hortícolas ya que proporcionarían frutos con estándares de calidad más elevados en el momento de la recolección y tras su manejo postcosecha, almacenamiento y comercialización.

1

Introduction

1. INTRODUCTION

1.1. Scientific background and object

Pomegranate and table grape are fruit with high acceptation by consumers due to their organoleptic quality and their content in both nutritive and bioactive compounds related to human health. However, the table grape cultivars, 'Crimson' and 'Magenta', and the 'Mollar de Elche' pomegranate are very different non-climacteric fruit, but with similar problems that depreciate their quality. These problems are related to the lack of red intense colour at harvest and with some others alterations that determine their quality losses during postharvest storage, such as weight loss, over-ripening, fungal decay, and in the case of pomegranate its sensitivity to develop chilling injury.

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 **'Preharvest treatments with methyl jasmonate and salicylic acid derivatives to increase pomegranate and table grape quality at harvest and during postharvest'**, reference AGL2015-63986-R, 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 combined with the study of the preharvest application of oxalic acid. Besides, María Emma García was funded by a research scholarship (reference number: 1541/16) from Miguel Hernández University of Elche.

The aim of this PhD Thesis is to provide solutions to the pomegranate and table grape quality problems through preharvest treatments with methyl jasmonate (MeJa), oxalic acid (OA), salicylic acid (SA) and its derivatives; acetyl salicylic acid (ASA) and methyl salicylate (MeSa) to mainly solve the colour problems of these fruit at harvest. Specifically, both fruit species do not reach the desired intense red coloration at harvest, mainly due to the absence of low temperatures in the crop areas of the Spanish Southeast (Almería, Murcia and Alicante), where the PhD Thesis is also developed, that would be necessary to stimulate the synthesis of anthocyanins, both on the pomegranate husk and arils and on the skin of table grape. This problem of fruit quality means that agricultural companies have many problems to market these fruits in the national and international markets, despite the fact that their organoleptic, nutritional and functional properties are highly appreciated by consumers.

Figure 1 shows this lack of fruit colour for the different cultivars object of study on this PhD Thesis, when the ripening stage is reached, in which the harvest is carried out for the commercialization. This important problem is intended to be solved by using preharvest treatments with elicitors applied at the appropriate concentration and at key moments of the developmental fruit cycle.

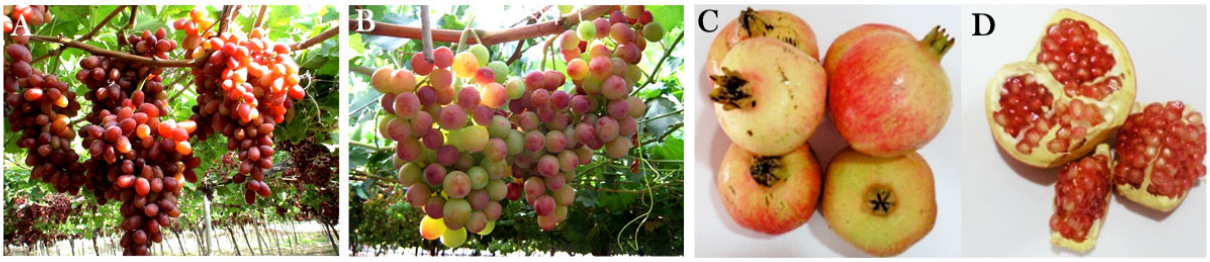


Figure 1: *Fruit showing a common problem of colour lack at harvest.* ***A)*** *'Crimson' table grapes.* ***B)*** *'Magenta' table grapes.* ***C)*** *'Mollar de Elche' pomegranate fruit.* ***D)*** *Arils of 'Mollar de Elche' pomegranate fruit (Adapted from Project AGL2015-63986R).*

On the other hand, quality losses caused by decay, dehydration and over-ripening occur in these fruit species during postharvest storage. In addition, in the case of pomegranate fruit, there is an additional problem, their sensitivity to chilling injury incidence when stored under cold conditions necessary for its storage, transport and marketing (Valero and Serrano, 2010).

This scenario, together with the growing consumer concern about the use of non-natural compounds that may harm their health, forces researches to find sustainable strategies able to increase fruit quality and/or maintain it during postharvest storage. In recent years, research is being carried out with innovative compounds or elicitors that have effects on the process of fruit development in plant and on the evolution of ripening and changes related to quality loss during postharvest storage. Our research group have a wide scientific background within the thematic of the current PhD Thesis and have obtained beneficial effects with the application, either as preharvest or postharvest treatments, of MeJa, SA, ASA, MeSa and OA on increasing quality at harvest and maintaining organoleptic, nutritional and functional quality during storage in fruit such as plum, pomegranate or sweet cherry, and vegetables like artichoke. In previous national projects, satisfactory results have been obtained in terms of quality maintenance of table grapes through postharvest treatments or packaging in MAP and essential oils (AGL2003-03527/ALI), as well as in sweet cherries and plums through preharvest (AGL2012-35402/ALI) and postharvest treatments with jasmonates and salicylates (AGL2006-04359/ALI), or the use of *Aloe vera* applied at preharvest and postharvest in stone fruit, pomegranates and grapes (AGL2009-10857/ALI).

The main hypothesis of this PhD Thesis is that the elicitors cited above could increase the colour of pomegranate and table grape cultivars at harvest as well as increase others quality parameters at harvest and reducing quality losses during postharvest storage. Overall, a solution would be given to a regional problem and a net benefit will be obtained in terms of increased acceptance by consumers and their possibilities for exporting to international markets. The results will help to the horticultural companies since they will provide fruit with higher quality standards at harvest and after postharvest handling, storage and marketing.

1.2. Colour problems and market requirements

The colour highly influences the commercial value of table grape and pomegranate fruit and is the major factor determining consumer acceptance as well as their commercial quality. Besides, fruit grown under warm climate conditions tend to show less colour development (Poole and Gray, 2002; Peppi et al., 2006; Roberto et al., 2012; Ferrara et al., 2015) maybe due to high summer daily temperature together with the narrow day/night temperature range during ripening (Mori et al., 2007; Crupi et al., 2012; Koyama et al., 2018), reducing acceptability for consumers. This phenomenon is attributed to low anthocyanin accumulation in response to high temperature during the ripening process. As we well know, the red colour of pomegranate husk and arils, and berry skin are a consequence of anthocyanin biosynthesis and accumulation in the cells and is directly influenced by the anthocyanin profile and concentration (**Figure 2**).

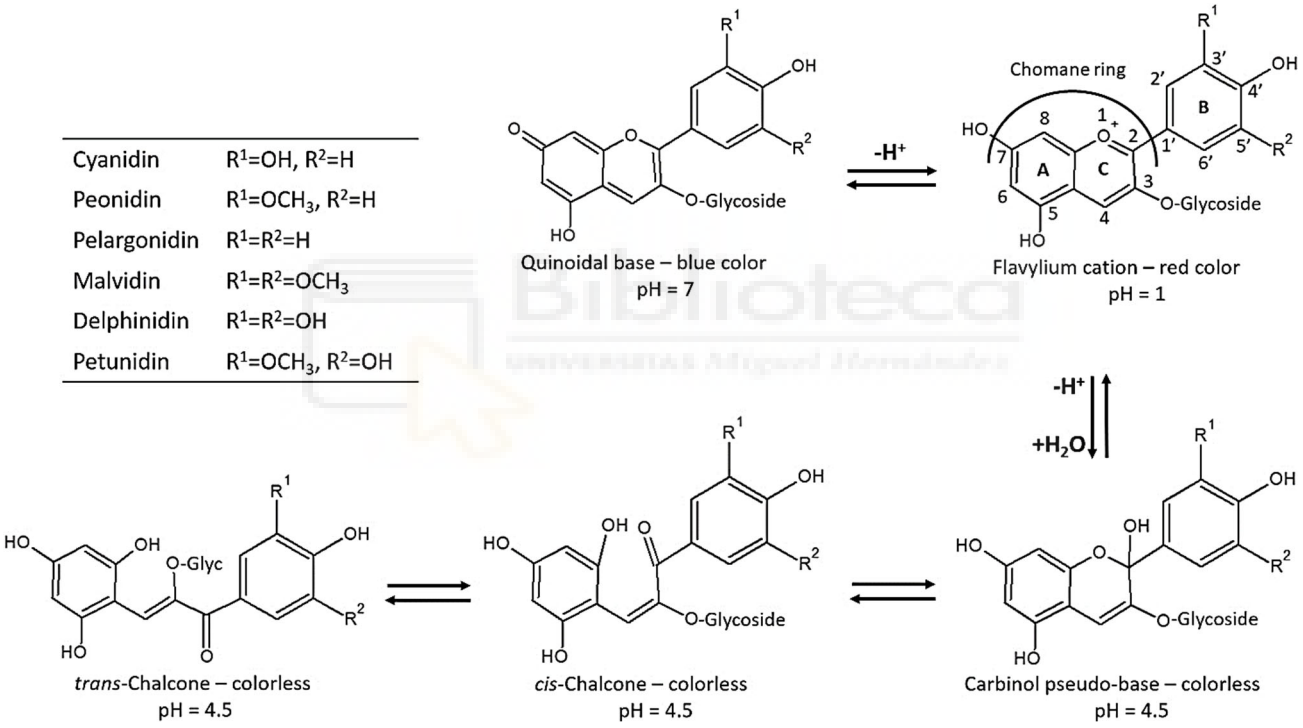


Figure 2: *Anthocyanin skeleton showing the aromatic rings (A and B) and the oxygenated heterocycle (C). Replacing the radicals R_1 and R_2 with those of the table, it is possible to observe the six most common types of anthocyanins (Adapted from Tarone et al., 2020; Based on Hosseinian et al., 2008 and Castañeda-Ovando et al., 2009).*

Anthocyanins are hydrosoluble pigments located in the vacuole that are responsible for the blue, red and purple colour of the fruit and classified as flavonoids with glycosilated derivatives of the 3,5,7,3'-tetrahydroxyflavylium cation. The free aglycones (anthocyanidins) are highly reactive with sugars to form glycosides since all anthocyanins are *O*-glycosilated. The main aglycones found in fruit are pelargonidin, cyanidin, peonidin, delphinidin, petunidin and

malvidin, while the most relevant sugars are *D*-glucose, *L*-rhamnose, *D*-galactose, *D*-xylose and arabinose (Welch et al., 2008). Their structures are composed of two aromatic rings (A and B) linked by three carbons in an oxygenated heterocycle (C), namely a chromane ring with a second aromatic ring (B) attached in position 2 (Prior and Wu, 2006; Hosseinian et al., 2008), as shown in **Figure 2**.

In general, anthocyanin concentration increases during ripening in a range of pink, red and purple colored fruit, both climacteric and non-climacteric ones, although great variations exist in the total anthocyanin content at commercial harvest among fruit species and cultivars and regarding the predominant anthocyanin. For instance, great variations have been found among table grape cultivars (6-200 mg 100 g^{-1}) and even the major anthocyanin (cyanidin 3-glucoside, peonidin 3-glucoside or malvidin 3-glucoside) was different depending on the cultivar (Carreño et al., 1997; Orak, 2007). Accordingly, in fifteen cultivars of pomegranate fruit the quantitative results indicated a wide variability in terms of ellagitannins and anthocyanin content, ranged from 20 to 600 mg L^{-1}, also confirmed by colorimetric analysis. In addition, the pomegranate juices were characterized by the presence of several anthocyanins as the main phenolic components, with strong differences concerning the relative amount of each individual anthocyanin glycoside deriving on a different profile for each cultivar (Balli et al., 2020).

Flavonoids are synthesized along the general phenylpropanoid pathway by the activity of a cytosolic multienzyme complex loosely associated at the cytoplasmic surface of the endoplasmic reticulum, as can be seen in **Figure 3**. This pathway has largely been characterized in different plant species (Winkel-Shirley, 2006), and also in *V. vinifera* where the expression of genes shows significant differences between organs and cultivars, especially for genes involved in anthocyanin synthesis. In red cultivars, such as 'Crimson' or 'Magenta' table grapes, all the genes are expressed in berry skin, although with different temporal patterns. In berry flesh their expression is low, and in particular, phenylalanine ammonia lyase (PAL) and UDP-glucose: flavonoid 3-O-glucosyl transferase (UFGT) genes are not expressed (Boss et al., 1996). These two genes codify for enzymes involved in the first and last steps of the anthocyanin pathway, respectively. PAL allows the hydrolysis of ammonia from phenylalanine, whereas UFGT catalyzes the glycosylation of anthocyanidins for the production of the anthocyanins (coloured and stable products).

Furthermore, it has been evidenced that UFGT is not detectable in white cultivars and the expression of other genes is lower, if compared with the skin of red cultivars. UFGT activity increases in parallel with anthocyanin content, while PAL and chalcone isomerase (CHI) enzyme activity decreases from the beginning of the ripening process. Biosynthesis of proanthocyanidins shares common steps with anthocyanin pathway, but branches off from this after the reduction of leucocyanidin (or cyanidin) to catechin (or epicatechin) by the enzymatic activity of a leucoanthocyanidin reductase (LAR) or anthocyanidin reductase (ANR), respectively (Braidot et al., 2008). However, gene expression and activation of enzymes for

anthocyanin biosynthesis are also influenced by three main factors: climatic conditions, phenological stage and cultural practices.

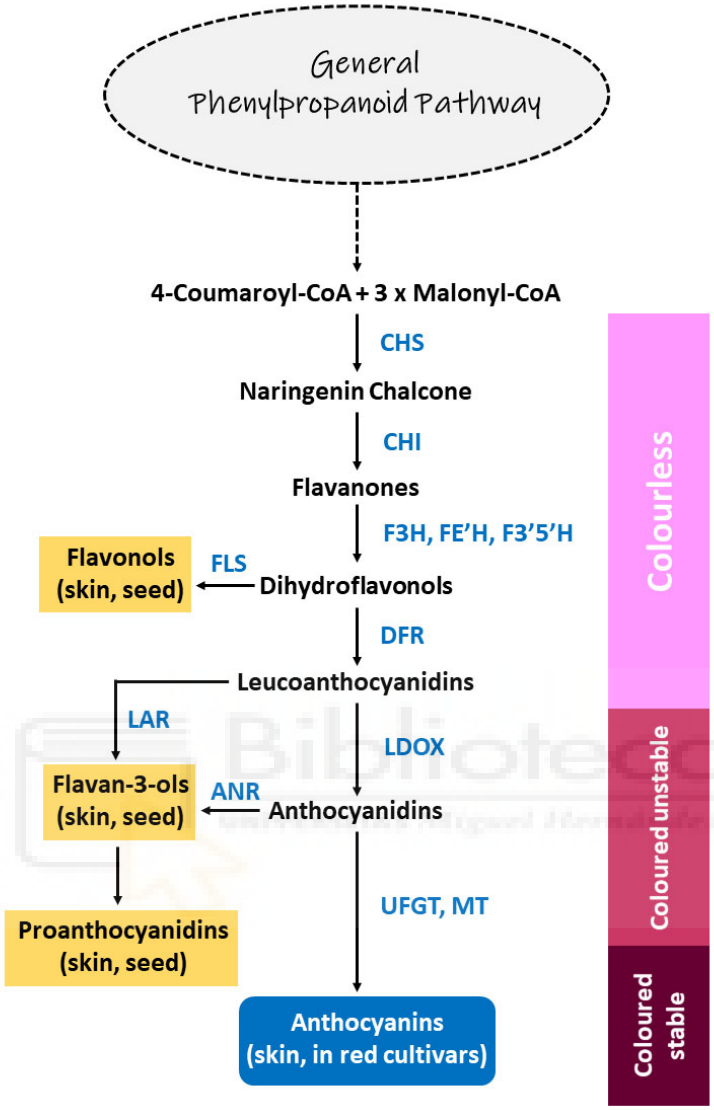


Figure 3: *Scheme of flavonoid biosynthetic pathways in grapevine. Anthocyanins are synthesized by a multienzyme complex loosely associated to the endoplasmic reticulum (CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3'5'-hydroxylase; DFR, dihydroflavonol reductase; LDOX, leucoanthocyanidin oxidase; UFGT, UDP-glucose: flavonoid 3-O-glucosyl transferase; MT, methyltransferase). Flavonol and proanthocyanidin syntheses branch off from the anthocyanin pathway (FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase) (Adapted from Braidot et al., 2008).*

Bibliography mentions several environmental factors that affect the synthesis of anthocyanin compounds, being luminosity and temperature the main ones; the first could be consider a stimulator, and the second at high ranges, a reducer or inhibitor. Since the 1960s, the

effects of temperature and light on the anthocyanin accumulation have been extensively investigated (reviewed by Downey et al., 2006). However, the classic factor in colour development is temperature. There is no information available that relates each pigment to an optimum temperature. In a series of phytotron studies on potted 'Kyoho' grapevines from 2009 to 2011, Shinomiya et al. (2015) have evaluated the impact of temperature and sunlight on the berries skin coloration. Temperature-control experiments in 2009 showed that a low-temperature treatment (25 °C day/20 °C night) resulted in a darker skin coloration than those of high-temperature treatments (35 °C day/30 °C night and 30 °C day/25 °C night). In addition, a low-temperature treatment from 0 to 25 days after veraison resulted in a darker berry skin than a treatment from 26 to 50 days after veraison. In 2010, high temperature treatments (35 °C day/25 °C night and 30 °C day/25 °C night) significantly inhibited the skin coloration, and in the last year, the skin coloration was suppressed by a high night temperature treatment (30 °C).

Therefore, the skin coloration could be recovered by a low night temperature of 20 to 25 °C, as demonstrated by Mori et al. (2005). These observations suggested that the temperature threshold for insufficient skin coloration in table grapes ranges between 25 and 30 °C. Also, in a particular study in 2015, Shinomiya et al. (2015) estimated that the threshold temperature for insufficient skin coloration for commercial table grape production was ~ 27 °C. This is due to the increased deglycosylation of anthocyanins caused by high temperatures leading to colourless compounds and that at the biochemical level decreases the activity of the UFGT enzyme (Cruz-Vargas, 2014). The same pattern on influence of anthocyanin biosynthesis temperature has been reported in pomegranate fruit (Borochov-Neori et al., 2013).

Specifically, Alicante and Murcia, the main crop areas of these fruits, are characterized by a warm climate. In them, during the fruit developmental and ripening cycle, temperatures increase within the range between 25 and 30 °C, which negatively influences the biosynthesis and stability of individual anthocyanins of each fruit species. For example, the maximum average temperatures reached in the summer months, July and August, exceeded 30 °C for both provinces, and in September, they were 29.1 and 30.2 °C in Alicante and Murcia, respectively (**Table 1**).

In table grapes crop, exogenous application of enantiomer *(S)-cis*-abscisic acid (*S*-ABA) or ethephon (an ethylene-releasing compound) at veraison (onset of ripening), when physiological changes start to appear, such as the increase of soluble solids, berry softening and colouring, have been performed to increase berry colouration. Thus, several studies have demonstrated that multiple applications of exogenous *S*-ABA can increase the anthocyanin concentration in grape skin efficiently (Roberto et al., 2012, 2013; Koyama et al., 2014, 2019; Yamamoto et al., 2015; Domingues Neto et al., 2017). However, the application time and concentration of *S*-ABA are critically important for the effective improvement of colour development in grape skin (Peppi et al., 2006, 2007, 2008), apart from the cultivar and location.

In grapes, ABA stimulates transcription of DNA codifying for several enzymes responsible for anthocyanin accumulation, including the UFGT gene (Jeong et al., 2004).

Table 1: *Average, minimum and maximum temperatures estimated for Alicante and Murcia from 1982 to 2012. Source: Climate-Data.org (https://es.climate-data.org). Accesed June 2020.*

		January	February	March	April	May	June	July	August	September	October	November	December
ALICANTE	$T_{average}$ (°C)	10.9	11.8	13.9	16.0	19.1	22.7	25.5	26.1	23.6	19.4	15.5	12.4
	$T_{min.}$ (°C)	6.0	6.6	8.4	10.7	13.6	17.2	19.9	20.6	18.2	14.4	10.5	7.6
	$T_{max.}$ (°C)	15.9	17.1	19.5	21.4	24.6	28.3	31.2	31.6	29.1	24.5	20.5	17.2
MURCIA	$T_{average}$ (°C)	10.3	11.3	14	16.2	19.4	23.7	26.2	26.3	23.8	19.1	14.6	11.7
	$T_{min.}$ (°C)	4.8	5.6	8.1	10.5	13.4	17.3	19.5	19.6	17.4	13.2	9.0	6.5
	$T_{max.}$ (°C)	15.8	17.1	20.0	22.0	25.4	30.1	33.0	33.1	30.2	25.0	20.2	16.9

Data: 1982 - 2012

ABA is a sesquiterpenoid hormone, derived from carotenoids, being involved in several physiological processes, from seed dormancy to senescence, including plant stress responses and the regulation of fruit development (Nambara and Marion-Poll, 2005; Finkelstein, 2013; Leng et al., 2014). ABA has been shown to play a pivotal role in the ripening process of non-climacteric fleshy fruits, modulating colour changes (through modulation of anthocyanin biosynthesis) and sugar accumulation (Kumar et al., 2014; Wang et al., 2015a). Specifically, in 'Crimson Seedless' *S*-ABA and sucrose treatments improved table grape coloration allowing earlier harvest (Ferrara et al., 2015; Olivares et al., 2017), as well as regulated deficit irrigation treatment applied dat postveraison stage, due to increases in anthocyanin concentration in berry skin (Conesa et al., 2016). In this sense, it has been also reported that water restrictions applied in pomegranate fruit in summer (during the linear phase of fruit growth) led to an increase in aril anthocyanin content (Bartual et al., 2015).

However, the high cost of *S*-ABA reduces its practical application and the effects of ethephon on colour development are inconsistent and can cause berry softening. Therefore, more research is needed to find out other compounds with commercial application possibilities in order to meet the fruit colour and quality requirements that the market demands.

1.3. Production facts and crop importance

Table grape and pomegranate fruit are seasonal fruit with great acceptance by consumers which leads to an increase in the production of their crops. Grapes are among the most widespread fruit in the world, with a total production of hundreds million tons per year, partly (~ 30 %) consumed as fresh table grape (Medouni-Adrar et al., 2015). Nowadays, the main table grape producing countries are located in the northern hemisphere, being Spain the 4th world grape producer (FAOSTAT, 2018). In the European Union, the main table grape producing countries are Italy, Spain, Greece and Romania (**Figure 4A**). In Spain, more than 14,000 ha are destined to the table grape crop, being Region de Murcia and Comunidad Valenciana areas the main Spanish producers of table grapes with 66.24 and 28.71 % of the national production, respectively (**Figure 4B**). Likewise, Alicante reached more than 28 % of the national production according to the latest available data (MAPA, 2018).

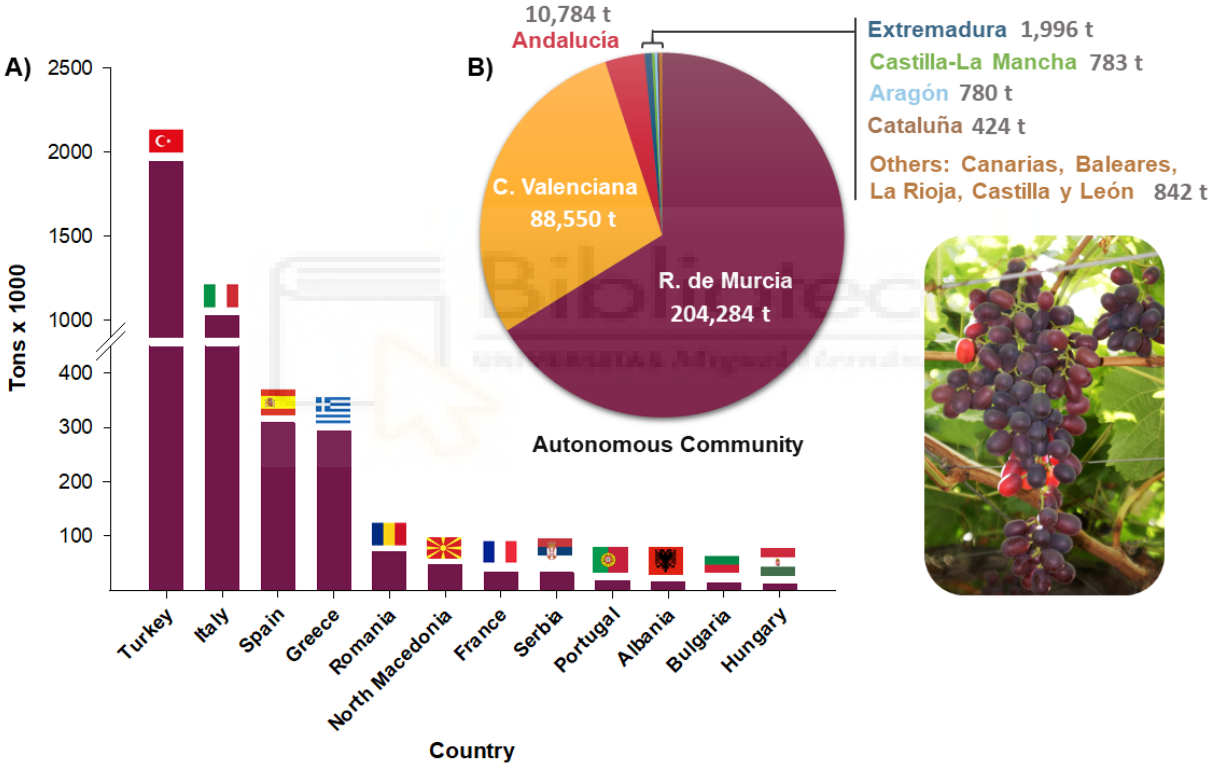


Figure 4: *A) Total table grape production by country. Year 2018. Source: EUROSTAT (https://ec.europa.eu/eurostat). Accesed June 2020. The first top 12 countries are displayed only.* ***B)*** *Total table grape production by Region in Spain. Year 2018. Source: MAPA (https://www.mapa.gob.es/es/). Accesed June 2020.*

On the other hand, pomegranate crop is widely extended in all continents of the world except for the Antarctic (Ward, 2003; Akcaoz et al., 2009). The consumption and production of this highly valuable crop started to increase all over the world. However, the production is still limited mainly due to the physiological disorders, pests, diseases and postharvest problems (Kahramanoglu, 2019). No reliable information is available about the total pomegranate

production in the world due to the rapid increase in their production in the last years. It was estimated to be around 3.8 million tons in 2017. The main pomegranate producing countries are India, Iran, USA, Turkey, Spain and Israel, being Spain one of the main producers in the world and the main producer and exporter in Europe.

The Spanish production of pomegranate in 2018 was 75,673 t (**Figure 5A**), which represents 1.61 % of the total non-citrus fruit trees grown in Spain. The production is concentrated in Comunidad Valenciana (78,61 %; **Figure 5B**), mainly in Alicante province (69.42 %), specifically in three municipalities; Elche, Albatera and Crevillente, in order of importance, which shows their socio-economic value in these regions, representing a very high percentage of their total fruit production and agricultural production (Melgarejo, 2010). Among Spanish cultivars of pomegranate fruit, the most cultivated one is 'Mollar de Elche' which is grown in Elche Region and safeguarded by a Protected Designation of Origin (PDO) since 2016 [R (UE) 2016/83]. Other important cultivars are: 'Wonderful', 'Acco' and 'Smith'.

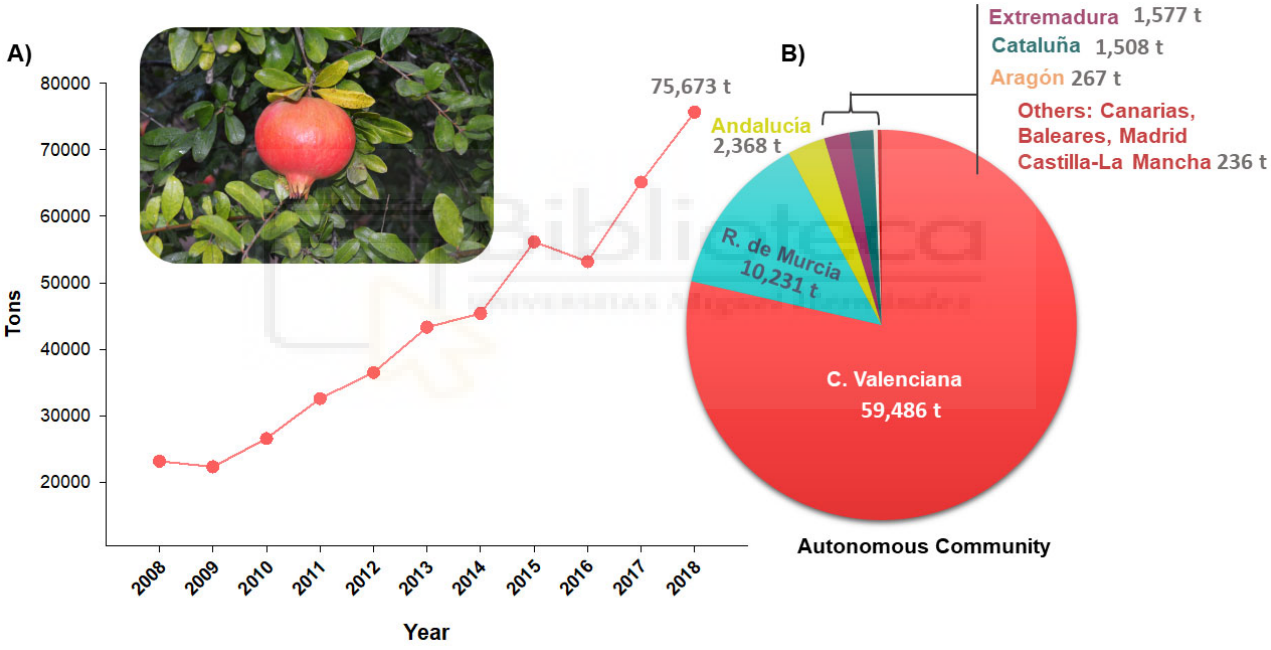


Figure 5: A) *Evolution of total pomegranate fruit production in Spain until 2018. Source: MAPA (https://www.mapa.gob.es/es/). Accesed June 2020. The last 10 years are displayed only.* ***B)*** *Total pomegranate fruit production by Region in Spain. Year 2018. Source: MAPA (https://www.mapa.gob.es/es/). Accesed June 2020.*

Approximately, the 84 % of the total production of pomegranates (75,673 t) is destined for fresh consumption and the rest is for industrialization, mainly intended to the juice industries. Estimated local consumption is about 30 % and the rest is exported, mainly to Europe, but since the competition is growing, Spanish exporters are searching new markets in Middle East and Asia. These data indicate the importance of both crops in Spain, and more specifically in the Autonomous Communities located in the southeast of Spain, where this PhD Thesis is developed.

1.4. Previous research works of Miguel Hernández University' Postharvest group on table grape and pomegranate fruit

The quality losses during postharvest storage of table grape and pomegranate fruit are due mainly to weight loss, colour changes, and accelerated softening. Additionally, deterioration in table grapes is also attributed to high incidence of berry decay, mainly due to *Botrytis cinerea,* and in pomegranate fruit is associated to their high susceptibility to chilling injury incidence under cold storage. Postharvest Group of Fruits and Vegetables of Miguel Hernández University of Elche has a wide research experience studying some techniques, tools or strategies to maintain the postharvest quality or delaying quality losses in these fruit species.

Modified atmosphere packaging (MAP) consists on sealing a certain quantity of fruit or vegetables by the use of plastic films with a particular permeability to gas diffusion. Then, respiration of commodities increases CO_2 concentration and decreases O_2 concentrations inside the package, while transpiration rate increases vapour pressure. Our group has found that MAP maintains quality of table grapes (Martínez-Romero et al., 2003). Specifically, clusters packaged in MAP showed higher firmness, reduced weight losses, lower changes in soluble solids concentration and better sensory attributes. Moreover, the grapes packaged in nonperforated oriented polypropylene (N-OPP) film maintained colour, which could indicate a major concentration of anthocyanins, during 53 days of cold storage.

Despite the fact that MAP can preserve table grape organoleptic quality, the CO_2 concentration inside packages is usually not high enough to exert a fungicide effect. Thus, the use of essential oils, such as eugenol, menthol, thymol or carvacrol in combination with MAP applied in table grape cultivars has been widely studied by our research group as natural antimicrobial compounds for controlling postharvest decay while maintaining fruit quality during postharvest storage (Valverde et al., 2005a; Valero et al., 2006; Guillén et al., 2007; Martínez-Romero et al., 2007). Thereafter, the combined use of these two tools led to maintain table grape quality and safety for longer storage periods, namely 3 additional weeks as compared to controls under MAP only (Valverde et al., 2005a).

In 'Autumn Royal' and 'Aledo' table grapes, changes in L* and a* colour parameters were reduced by the addition of eugenol and especially thymol, the delay being higher with the maximum applied dose (150 µL). Also, the lower percentages of decay were obtained for this dose and the quality losses in terms of functional properties were significantly reduced by both essential oils (Valero et al., 2006; Guillén et al., 2007). On the other hand, the role of carvacrol vapour, applied at 0.05, 0.2, 0.5 and 1 mL L^{-1}, inhibited totally the growth of *Botrytis cinerea* in potato dextrose agar (PDA), while in berries the reduction of decayed fruit was significantly higher as carvacrol concentration increased (Martínez-Romero et al., 2007).

In addition, a novel edible coating based on *Aloe vera* gel was developed by our research group according to SP Patent Filed 200302937, and has been used as a preservation tool to

maintain the quality, safety and functional properties of 'Crimson Seedless' table grapes during cold storage and subsequent shelf life (Valverde et al., 2005b; Serrano et al., 2006). This *Aloe vera* gel showed antifungal efficacy *in vitro* and its use applied as preharvest treatment could inhibit microbial spoilage reducing decay incidence during postharvest storage of table grapes (Castillo et al., 2010).

Heat treatments have been used to extend storability of several fruit. The effect of prestorage heat treatment in pomegranate fruit was investigated by our group in order to maintain nutritive and functional properties during postharvest cold storage. Pomegranate fruit were heat treated (dipped in water at 45 °C for 4 min) and stored at 2 °C for 90 days. Arils from heat-treated pomegranates exhibited higher total antioxidant activity than controls, which was correlated with the high levels of total phenolics and, to a lesser extent, with ascorbic acid and anthocyanin contents. The levels of sugars (glucose and fructose) and organic acids (malic, citric and oxalic acids) also remained higher in arils from treated fruit (Mirdehghan et al., 2006). In addition, a reduction in chilling injury of pomegranate fruit after this heat treatment was reported noted by Mirdehghan et al. (2007a). The higher polyamine levels as well as the maintenance of the unsaturated (UFA)/saturated (SFA) fatty acid ratio in heat-treated pomegranate fruit during storage could account for the maintenance of membrane integrity and fluidity.

Other studies in pomegranate fruit that have been carried out by our postharvest group have been the application of polyamines, because of their antisenescent activity and their radical-scavenging properties. Exogenous application of putrescine or spermidine by pressure infiltration or by immersion induced acclimation of pomegranate to low temperature and, in turn, protected these fruits from chilling injury, by increasing the levels of endogenous putrescine and spermidine, since the normal levels would not be high enough to induce this adaptation to cold storage. In addition, polyamine treatment retarded the ripening process by reducing softening and the increase in the ratio total soluble solids/total acidity, as well as the weight loss. Pressure infiltration of polyamines was the best tool to increase the endogenous polyamines, although immersion also imparted beneficial effects, which could be attributed to the waxy nature of pomegranate husk (Mirdehghan et al., 2007b; 2007c).

In ready-to-eat pomegranate arils, *Aloe vera* gel applied as an edible coating has been found to be effective in quality retention and reduction of microbial spoilage. In this sense, the combination of *Aloe vera* gel at 100 % + ascorbic acid and citric acid at 1 % was the most effective treatment (Martínez-Romero et al., 2013).

In relation to the research line of this PhD Thesis, our research group has a wide experience in the postharvest application of elicitors in pomegranate fruit. Elicitors are compounds capable of activating the plant defence system, which increase the synthesis of secondary metabolites (Peña-Cortés et al., 2005; Gutiérrez-Gamboa et al., 2017; Martínez-Esplá et al., 2017a; Ramírez-Godoy et al., 2018) inducing the expression of enzyme codifying genes

related to plant defence responses and due to the accumulation of secondary metabolites (Baenas et al., 2014; Gorni and Pacheco, 2016), as can be seen in **Figure 6**.

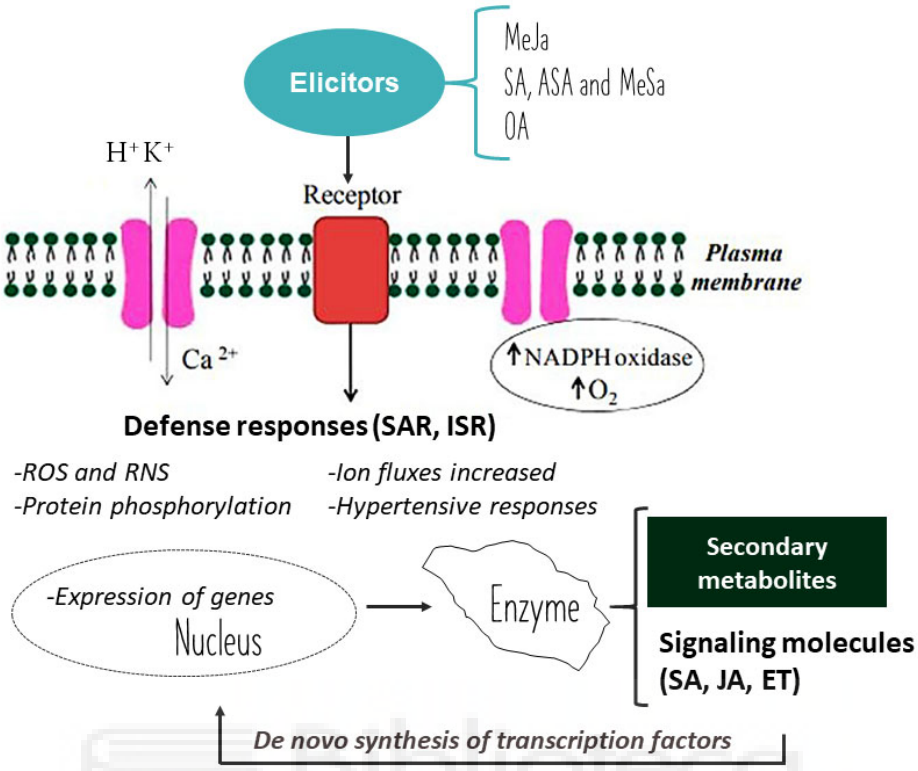


Figure 6: *General mechanism after elicitor perception. Abbreviations: MeJa, methyl jasmonate; SA, salicylic acid; ASA, acetyl salicylic acid; MeSa, methyl salicylate; OA, oxalic acid; SAR, systemic acquired response; ISR, induced systemic resistance; ROS, reactive oxygen species; RNS, reactive nitrogen species; NADPH, nicotinamide adenine dinucleotide phosphate; JA, jasmonic acid; ET, ethylene (Adapted from Baenas et al., 2014).*

Thus, the main effects that elicitors have on plants are the increase in acquired and/or induced systemic resistance (Terry and Joyce, 2004) and the stimulation of secondary metabolism for the synthesis of phytochemicals (Ruiz-García and Gómez-Plaza, 2013). Elicitors can be classified as biotic or abiotic compounds, although some plant hormones should be also considered as elicitors. **Figure 6** shows some examples of elicitors, which can be applied at preharvest or postharvest period: methyl jasmonate (MeJa), salicylic acid (SA), acetyl salicylic acid (ASA), methyl salicylate (MeSa) and oxalic acid (OA). Specific biosynthetic pathways are activated in treated plants depending on the used compound. However, the same elicitor affects differently to each plant species, and then, it is important to consider the type of elicitor to be used in the cultivation process (Rodrigues-Brandão et al., 2014), either applied alone or in combinations and selecting the key moments of application throughout the plant growth and developmental cycle.

Our group found that MeJa and MeSa, as vapour treatments, had potential for postharvest applications in reducing chilling injury, maintaining quality and improving the

health benefits of pomegranate fruit consumption, by increasing the antioxidant capacity (Sayyari et al., 2011a). In this line, a study of the application of genetic algorithm–artificial neural network (GA-ANN) and adaptive neuro fuzzy inference system (ANFIS) to modeling of MeJa effects of pomegranate fruit's quality characteristics revealed that is possible predict quality parameters of pomegranates with a satisfactory correlation coefficient: 0.87 and 0.92 for ANN and ANFIS, respectively (Sayyari et al., 2017b). Similarly, pomegranate fruit were treated with SA at different concentrations (0.7, 1.4 and 2 mmol L^{-1}) and stored at 2 °C for 3 months to investigate chilling injury influence. SA treatments, especially at 2 mmol L^{-1} concentration, were highly effective on reducing chilling injury and electrolyte leakage in the pomegranate husk, as well as ascorbic acid loss (Sayyari et al., 2009; Sayyari et al., 2017a). ASA also reduced chilling injury in pomegranates and could have potential postharvest application on reducing this physiological disorder, maintaining quality and improving the health benefits of pomegranate fruit consumption by increasing the antioxidant activity (Sayyari et al., 2011b).

Finally, OA applied at three concentrations (2, 4 and 6 mmol L^{-1}) by dipping of 'Mollar de Elche' pomegranate fruit significantly reduced chilling injury symptoms, especially at 6 mmol L^{-1} concentration. In addition, the application of OA in postharvest led to lower losses of total phenolics and a significant increase in both ascorbic acid content and antioxidant activity, while in control pomegranates these traits showed a significant reduction during storage (Sayyari et al., 2010).

1.5. Literature review of elicitation strategies in pre- and postharvest

To date, important results have been published by different authors about the effect of these elicitors; MeJa, salicylates (SA, ASA and MeSa) and OA, on some fruits and vegetables. Specifically, **Tables 2, 3 and 4** (**A** and **B** inclusive) show a literature review related to the effect of these compounds regarding the following parameters:

II. Alleviating chilling injury (CI) symptoms, improving chilling tolerance in fruit during cold storage, or fruit browning.
III. Promoting or delaying climacteric and non-climacteric ripening process and/or senescence, maintaining postharvest quality.
IV. Maintaining functional quality during storage and/or increasing bioactive compound content and antioxidant activity.
V. Increasing antioxidant enzymes activity.
VI. Inducing resistance against pathogen and programmed cell death.

In relation to the main objective provided in this PhD Thesis, it could be added that an increase in the fruit **red coloration** mainly due to the elicitation process by these compounds could be associated with an increase in anthocyanin concentration, suggesting that these elicitors could be used as a fruit coloring promotor, including field applications. In most studies, a direct relationship is found between increased anthocyanins and stimulated phenylalanine ammonia-

lyase (PAL) enzyme, which catalyzes the formation of naringenin from phenylalanine, this relationship being linked to the promoter effect of the elicitor (**Tables 2, 3 and 4-A**). Likewise, other secondary objectives of the current PhD Thesis include the effects of these compounds on yield, on the maintenance of quality during postharvest storage, on bioactive compound content and antioxidant activity, and on the incidence of decay or chilling injury.

Contradictory results have been shown regarding whether these treatments, either applied at preharvest or postharvest, promote or delay the ripening process of both climacteric and non-climacteric fruit. Thus, the effect of the elicitor is variable according to the fruit species, cultivars, doses, form and moment of application (**Tables 2, 3 and 4-A**). In addition, it has been observed that these elicitors affect the on tree-ripening process and **yield**, even in a dose-dependent manner. A delay on the ripening process could be due to the inhibition of ethylene biosynthesis; decrease lipoxygenase (LOX) enzyme activity; and enhancing defence system against oxidation damage in fruit under room temperature and cold storage; an induction in cystathionine β-synthase domain-containing protein, which can regulate ethylene precursors; a suppression in the activity of 1-aminocyclopropane-1-carboxylic acid synthase (ACC), regulatory enzyme in the ethylene production; a decrease of polygalacturonase (PG) and pectin-metil esterase (PME) activities; among others. This delay could maintain the **fruit quality during postharvest storage** (**Tables 2, 3 and 4-A**).

In general, the application of these elicitors led to higher bioactive compound content and antioxidant potential at harvest, **maintaining functional quality**, during postharvest storage (**Tables 2, 3 and 4-A**). However, the action mechanism by which these elicitors increased the bioactive compounds and antioxidant properties is not well understood and deserves further research despite of these treatments could be a useful tool to improve the health-beneficial properties on fruit consumption.

As commented above, these compounds play an important role in response to plant stress and induce systemic resistance in plants, improving the resistance of fruit to some diseases. Thus, these treatments could reduce the **postharvest fruit decay** or disease incidence through the induction of pathogenesis-related proteins, delaying fruit ripening or stimulating the antioxidant enzymes (**Tables 2, 3 and 4-B**). Finally, some authors suggested that these elicitors contributed to improve **chilling injury** tolerance in the fruit and membrane integrity maintenance, which might be attributed to enhanced antioxidant capacity and delayed membrane lipid oxidation; maintained higher levels of ATP and energy status; or enhanced activity of the enzymes related to energy metabolism and involved in antioxidative pathways; improved unsaturated/saturated fatty acid ratio; and enhanced content of reducing sugars, glucose and fructose (**Tables 2, 3 and 4-A and B**).

However, molecular and metabolomic studies are needed to better understand the mechanisms of action of these compounds. Therefore, study how some genes, directly or indirectly involved, are affected in all these effects discussed by the applied treatments would be

very useful to fill a knowledge gap that exists nowadays regarding the mechanism of action of these compounds.

Similarly, it would be interesting to measure the endogenous content of plant growth regulators or hormones during the cycle of application after treatments to, likewise, study the endogenous elicitation pathways. In conclusion, and related to the hypothesis proposed in this PhD Thesis, some authors have reported an increase in **colour** or an improvement in the anthocyanin content of some fruits by these elicitors, which in general terms affect their ripening process.

An increase in anthocyanin content has been detected in fruit after MeJa treatments affecting the fruit ripening despite the different doses and application method used. Some experiments have revealed that 0.1-10 mM of MeJa applications can induce changes in the colour and also enhances anthocyanin accumulation in red raspberry (Wang and Zheng, 2005; Chanjirakul et al., 2006; Ghasemnezhad and Javaherdashti, 2008), blackberry (Wang et al., 2008), blueberry (Huang et al., 2015; Ribera-Fonseca et al., 2020), strawberry (Saavedra et al., 2016; Valenzuela-Riffo et al., 2020; Zuñiga et al., 2020), Chinese bayberry (Wang et al., 2009a), grape and grapevine (Ruiz-García et al., 2012; Ruiz-García and Gómez-Plaza, 2013; Fernández-Marín et al., 2014; Flores et al., 2015; Portu et al., 2015, 2016; Gómez-Plaza et al., 2017), blood orange (Habibi et al., 2020), mango (Muengkaew et al., 2016), plum (Karaman et al., 2013; Martínez-Esplá et al., 2014a; Ozturk et al., 2015b) and apple (Kondo et al., 2001; Rudell et al., 2005; Shafiq et al., 2013; Ozturk et al., 2015a). All these studies are included in **Table 2A** and all these previous reports suggest that MeJa could be used as a coloring promotor of fruit, including field applications.

Likewise, **Table 3A** shows some literature references reporting positive effects of salicylate treatments with respect to the improvement of anthocyanin content in fruit, such as sweet cherry (Valero et al., 2011; Giménez et al., 2014, 2016, 2017; Valverde et al., 2015), pomegranate (Sayyari et al., 2011b; Drogoudi et al., 2021), blood orange (Habibi et al., 2020), plum (Martínez-Esplá et al., 2017b, 2018) and table grape (Gomes et al., 2021). Finally, OA has also shown (**Table 4A**), although to a lesser extent than the other elicitors studied, an increase in the content of total anthocyanins with the consequent increase in the colour of sweet cherry (Valero et al., 2011; Martínez-Esplá et al., 2014b) and table grape (Kok and Bal, 2019).

Table 2A: *Literature review of methyl jasmonate (MeJa) applied as pre- or postharvest treatment in some fruits and vegetables.*

Methyl jasmonate (MeJa)							
	I. Alleviating chilling injury (CI) symptoms, improving chilling tolerance in the fruit during cold storage, or fruit browning:		II. Promoting or delaying ripening process and/or senescence, maintaining postharvest quality:		III. Maintaining functional quality during storage and/or increasing bioactive compound content and antioxidant activity:	
Postharvest	Guava fruit	González-Aguilar et al., 2004	Peach, mango, tomato and apple	Peña-Cortés et al., 2005	Apple	Kondo et al., 2001
	Peach	Meng et al., 2009	Plum	Khan and Singh, 2007	Raspberry	Chanjirakul et al., 2006
	Loquat	Cao et al., 2009, 2012	Raspberry	Concha et al., 2013	Blackberry	Wang et al., 2008
	Pomegranate fruit	Sayyari et al., 2011a	Jujube fruit	Dong et al., 2016	Red raspberry	De la Peña et al., 2010
	Cucumber	Li et al., 2012	Eggplant	Fan et al., 2016	Pomegranate fruit	Sayyari et al., 2011a
	Tomato	Zhang et al., 2012, 2016	Apricot	Ezzat et al., 2017	Grape	Flores et al., 2015
	Peach	Jin et al., 2013	Blueberry	Wang et al., 2019b	Blueberry	Huang et al., 2015
	Banana	Zhao et al., 2013	Mandarin	Baswal et al., 2020	Grape	Portu et al., 2015
	Lemon	Siboza et al., 2014	Apricot	Ezzat et al., 2020	Strawberry	Asghari and Hasanlooe, 2016
	Avocado	Sivankalyani et al., 2015	Tomato	Rivero Meza et al., 2021	Strawberry	Spagnuolo et al., 2016
	Cucumber	Liu et al., 2016			Grape	Gómez-Plaza et al., 2017
	Peach	Yu et al., 2016			Queen pineapple	Sangprayoon et al., 2019
	Kiwifruit	Li et al., 2017			Blood orange	Habibi et al., 2020
	Peach	Chen et al., 2019			Kiwifruit	Wei et al., 2021
	Blood orange	Habibi et al., 2019				
	Queen pineapple	Sangprayoon et al., 2019				

Pomegranate fruit	Chen et al., 2020
Green bell pepper	Ma et al., 2020
Pepper fruit	Seo et al., 2020
Litchi	Deshi et al., 2021
Banana fruit	Elbagoury et al., 2021

Preharvest

Apple	Fan et al., 1997	Apple	Rudell et al., 2005
Peach	Janoudi and Flore, 2003	Raspberry	Wang and Zheng, 2005
Apple	Rudell et al., 2005	Raspberry	Ghasemnezhad and Javaherdashti, 2008
Raspberry	Wang and Zheng, 2005	Blackberry	Wang et al., 2008
Blackberry	Wang et al., 2008	Chinese bayberry	Wang et al., 2009a
Peach	Ziosi et al., 2008, 2009	Grapevine	Ruiz-García et al., 2012
Apple	Altuntas et al., 2012	Grapevine	Ruiz-García and Gómez-Plaza, 2013
Peach	Soto et al., 2012	Plum	Karaman et al., 2013
Plum	Karaman et al., 2013	Apple	Shafiq et al., 2013
Peach	Ruiz et al., 2013	Grapevine	Fernández-Marín et al., 2014
Plum	Martínez-Esplá et al., 2014a	Red raspberry	Flores and Ruíz del Castillo, 2014
Plum	Zapata et al., 2014	Plum	Martínez-Esplá et al., 2014a
Orange	Khajehyar et al., 2016	Plum	Zapata et al., 2014
Mango	Muengkaew et al., 2016	Berry	Flores and Ruíz del Castillo, 2015
Strawberry	Saavedra et al., 2016	Apple	Ozturk et al., 2015a
Citrus fruit	Dong et al., 2020	Plum	Ozturk et al., 2015b
Sweet cherry	Faizy et al., 2021	Grape and grapevine	Portu et al., 2015, 2016

	Mango	Muengkaew et al., 2016
	Strawberry	Saavedra et al., 2016
	Artichoke	Martínez-Esplá et al., 2017c
	Lemon	Serna-Escolano et al., 2019
	Pomegranate fruit	Asghari et al., 2020
	Blueberry	Ribera-Fonseca et al., 2020
	Strawberry	Gundogdu et al., 2020
	Strawberry	Valenzuela-Riffo et al., 2020
	Strawberry	Zuñiga et al., 2020

Table 2B: *Literature review of methyl jasmonate (MeJa) applied as pre- or postharvest treatment in some fruits and vegetables.*

Methyl jasmonate (MeJa)				
IV. Increasing antioxidant enzymes activity:			V. Inducing resistance against pathogen and programmed cell death:	
Postharvest	Raspberry	Chanjirakul et al., 2006	Grapefruit	Droby et al., 1999
	Loquat	Cao et al., 2009	Cherry	Yao and Tian, 2005
	Peach	Jin et al., 2009	Loquat	Cao et al., 2008
	Peach	Meng et al., 2009	Tomato	Zhu and Tian, 2012
	Cucumber	Li et al., 2012	Banana	Tang et al., 2013
	Avocado	Sivankalyani et al., 2015	Cherry	Wang et al., 2015b

	Strawberry	Asghari and Hasanlooe, 2016	Grape	Wang et al., 2015c, 2015d
	Jujube fruit	Dong et al., 2016	Apple	He et al., 2020
	Eggplant	Fan et al., 2016	Tomato	Min et al., 2020
	Orange	Khajehyar et al., 2016	Kiwifruit	Pan et al., 2020
	Cucumber	Liu et al., 2016	Blueberry	Wang et al., 2020
	Mango	Muengkaew et al., 2016		
	Tomato	Zhang et al., 2016		
	Blood orange	Habibi et al., 2019		
	Blueberry	Wang et al., 2019b		
	Kiwifruit	Pan et al., 2020		
	Blueberry	Wang et al., 2020		
	Apricot	Cavusoglu et al., 2021		
Preharvest	Plum	Zapata et al., 2014	Cherry	Yao and Tian, 2005
	Lemon	Serna-Escolano et al., 2019	Tomato	Shu et al., 2020
	Tomato	Shu et al., 2020		
	Strawberry	Zuñiga et al., 2020		
	Lemon	Serna-Escolano et al., 2021a		

Table 3A: *Literature review of salicylates (SA, ASA and MeSa) applied as pre- or postharvest treatment in some fruits and vegetables.*

Salicylates (SA, ASA and MeSa)								
	I. Alleviating chilling injury (CI) symptoms, improving chilling tolerance in the fruit during cold storage, or fruit browning:		II. Promoting or delaying ripening process and/or senescence, maintaining postharvest quality:		III. Maintaining functional quality during storage and/or increasing bioactive compound content and antioxidant activity:	
Postharvest	Tomato	Ding et al., 2002	Peach	Han et al., 2003	Pomegranate fruit	Sayyari et al., 2011b
	Banana	Kang et al., 2003	Cherry	Valero et al., 2011	Cherry	Valero et al., 2011
	Sweet pepper	Fung et al., 2004	Kiwifruit	Yin et al., 2013	Apricot	Wang et al., 2015e
	Loquat fruit	Cai et al., 2006	Sweet cherry	Giménez et al., 2016	Nectarine	Bal, 2016
	Mango	Han et al., 2006	Kiwifruit	Huang et al., 2016	Sweet cherry	Giménez et al., 2016
	Pomegranate fruit	Sayyari et al., 2009, 2011b	Plum	Sharma and Sharma, 2016	Queen pineapple	Sangprayoon et al., 2019, 2020
	Peach	Cao et al., 2010	Pear	Zhang et al., 2019	Blood orange	Habibi et al., 2020
	Pineapple	Lu et al., 2010, 2011	Blood orange	Habibi et al., 2020		
	Plum	Luo et al., 2011	Pear	Zhang et al., 2020		
	Tomato	Zhang et al., 2011	Blueberry	Jiang et al., 2022		
	Tomato	Aghdam et al., 2014				
	Avocado	Glowacz et al., 2017				
	Blood orange	Habibi et al., 2019				
	Queen pineapple	Sangprayoon et al., 2019, 2020				
	Pepper	Seo et al., 2020				
	Pear fruit	Adhikary et al., 2021				

Apricot	Batool et al., 2021
Guava	Madhav et al., 2021
Sweet pepper	Rehman et al., 2021

Preharvest

Cucumber seedling	Seydpour and Sayyari, 2016	Sweet cherry	Giménez et al., 2015, 2016	Orange	Huang et al., 2008
Sweet cherry	Giménez et al., 2017	Mango	Vijay Rakesh Reddy et al., 2016	Sweet cherry	Giménez et al., 2014, 2017
Apricot	Fan et al., 2021	Plum	Martínez-Esplá et al., 2018	Sweet cherry	Valverde et al., 2015
				Apple	Supapvanich et al., 2017
				Plum	Martínez-Esplá et al., 2017b, 2018
				Date Palm	Ahmed et al., 2021
				Pomegranate fruit	Drogoudi et al., 2021
				Apple	Gacnik et al., 2021
				Table grape	Gomes et al., 2021

Table 3B: *Literature review of salicylates (SA, ASA and MeSa) applied as pre- or postharvest treatment in some fruits and vegetables.*

Salicylates (SA, ASA and MeSa)				
	IV. Increasing antioxidant enzymes activity:		V. Inducing resistance against pathogen and programmed cell death:	
Postharvest	Peach	Han et al., 2003	Peach	Zhang et al., 2008
	Peach	Tareen et al., 2012	Tomato	Mandal et al., 2009
	Apricot	Wang et al., 2015e	Peach	Panahirad et al., 2012
	Pomegranate fruit	Dokhanieh et al., 2016	Apple	Da Rocha Neto et al., 2016
	Kiwifruit	Li et al., 2017	Avocado	Glowacz et al., 2017
	Blood orange	Habibi et al., 2019	Mango	He et al., 2017
	Banana	Chotikakham et al., 2020	Tomato	Zhang et al., 2017
			Muskmelon	Xue et al., 2020
			Nectarine	Lyousfi et al., 2021
Preharvest	Cherry	Yao and Tian, 2005	Cherry	Yao and Tian, 2005
	Sweet cherry	Valverde et al., 2015	Strawberry	Babalar et al., 2007
	Plum	Martínez-Esplá et al., 2017b	Cucumber	Dong and Hwang, 2017
	Apple	Supapvanich et al., 2017	Eggplant	Ghahremani et al., 2021
	Tomato	El-Hady et al., 2021	Tomato	Prakash et al., 2021
	Strawberry	Roshdy et al., 2021		
	Lemon	Serna-Escolano et al., 2021a		

Table 4A: *Literature review of oxalic acid (OA) applied as pre- or postharvest treatment in some fruits and vegetables.*

Oxalic acid (OA)							
	I. Alleviating chilling injury (CI) symptoms, improving chilling tolerance in the fruit during cold storage, or fruit browning:		II. Promoting or delaying ripening process and/or senescence, maintaining postharvest quality:		III. Maintaining functional quality during storage and/or increasing bioactive compound content and antioxidant activity:	
Postharvest	Litchi	Zheng and Tian, 2006	Peach and mango	Zheng et al., 2007a, 2007b, 2007c	Pomegranate	Sayyari et al., 2010
	Mango	Ding et al., 2007	Jujube fruit	Wang et al., 2009b	Cherry	Valero et al., 2011
	Peach	Zheng et al., 2007a	Pomegranate fruit	Sayyari et al., 2010	Mango	Zheng et al., 2012
	Pomegranate fruit	Sayyari et al., 2010	Cherry	Valero et al., 2011	Rocket and spinach	Cefola and Pace, 2015
	Peach	Jin et al., 2014	Plum	Wu et al., 2011	Muskmelon	Deng et al., 2015
	Mango	Li et al., 2014, 2015	Banana	Huang et al., 2013b	Mango	Razzaq et al., 2015
	Tomato	Li et al., 2016	Artichoke	Ruíz-Jiménez et al., 2014	Tomato	Li et al., 2016
	Apricot	Wang et al., 2016	Rocket and spinach	Cefola and Pace, 2015	Pomegranate fruit	Ehteshami et al., 2021
	Kiwifruit	Liang et al., 2017	Grape	Sabir and Sabir, 2017	Litchi	Hossain et al., 2021
	Melon fruit	Wang et al., 2018, 2019a				
	Litchi	Ali et al., 2021				
	Apricot	Batool et al., 2021				
Preharvest			Kiwifruit	Zhu et al., 2016	Cherry	Martínez-Esplá et al., 2014b
			Artichoke	Martínez-Esplá et al., 2017a	Peach	Razavi and Hajilou, 2016
			Strawberry	Anwar et al., 2018	Kiwifruit	Zhu et al., 2016
			Kiwifruit	Ali et al., 2019	Artichoke	Martínez-Esplá et al., 2017a

			Plum	Martínez-Esplá et al., 2019	Grape	Kok and Bal, 2019
					Plum	Martínez-Esplá et al., 2019
					Lemon	Serna-Escolano et al., 2021b

Table 4B: *Literature review of oxalic acid (OA) applied as pre- or postharvest treatment in some fruits and vegetables.*

Oxalic acid (OA)					
IV. Increasing antioxidant enzymes activity:			V. Inducing resistance against pathogen and programmed cell death:		
Postharvest	Litchi	Zheng and Tian, 2006		Pear	Tian et al., 2006
	Peach and Mango	Zheng et al., 2007a, 2007b, 2012		Mango	Zheng et al., 2007b, 2007c, 2012
	Banana	Huang et al., 2013a		Jujube fruit	Wang et al., 2009b
	Mango	Li et al., 2015		Muskmelon	Deng et al., 2015
	Muskmelon	Deng et al., 2015			
Preharvest	Peach	Razavi and Hajilou, 2016		Kiwifruit	Zhu et al., 2016
	Plum	Martínez-Esplá et al., 2019			
	Lemon	Serna-Escolano et al., 2021b			

2

Aim and Objectives

2. AIM AND OBJECTIVES

In accordance with quality problems of pomegranate fruit and table grape and the strategies and previous research that have been previously commented, we found of the utmost importance addressing the study of preharvest treatments with elicitors, both in 'Mollar de Elche' pomegranate fruit and in table grape cultivars, 'Magenta' and 'Crimson', which are characterized by having a non-uniform pink colour in order to provide solutions to a real problem in the marketing of these fruits, as mentioned above.

On the other hand, the chilling injury tolerance and the induction of defence antioxidant systems by these compounds will make it possible to reduce quality losses due to chilling injury and fungal decay during postharvest storage and marketing of these fruits, and to maintain or increase the fruit antioxidant properties, with the consequent benefit for the health of consumers. Nowadays, there is no knowledge about the effect of preharvest application of these compounds in pomegranate fruit, and in grapes there are evidences in some grapevine cultivars and only one study table grape, so this PhD Thesis will fill this scientific knowledge gap.

Therefore, the **overall aim** of the present PhD Thesis was improving colour of pomegranate fruit and table grape by applying preharvest treatments with methyl jasmonate (MeJa), salicylic acid (SA), acetyl salicylic acid (ASA), methyl salicylate (MeSa) and oxalic acid (OA) during the developmental cycle of pomegranate fruit and table grape (**Figure 7**). In addition, some **specific objectives** (**Figure 7**) were established as follow:

I. Increase pomegranate and table grape crop yield by treatments with these elicitors.
II. Increase fruit quality parameters and maintain them during storage.
III. Enhance fruit bioactive compounds from harvest until consumption.
IV. Reduce pomegranate chilling injury (CI) incidence.
V. Reduce postharvest fungal decay, increasing shelf-life throughout the commercialization.

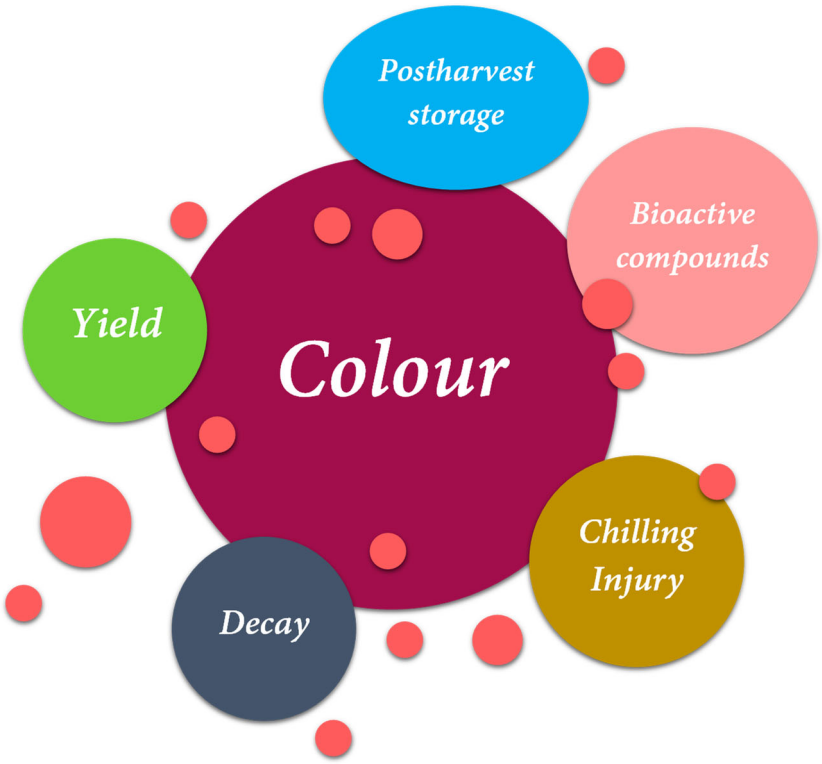
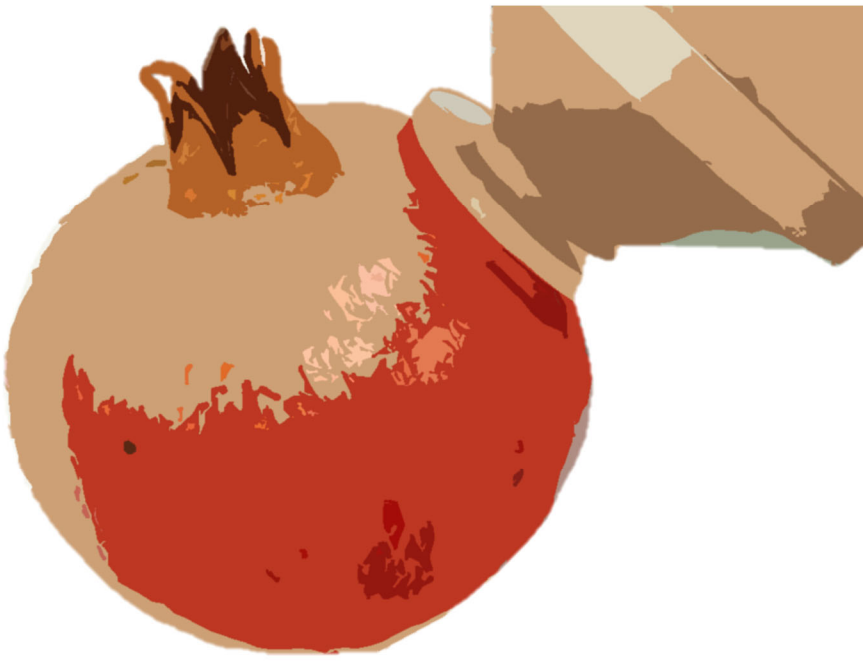


Figure 7. *Diagram showing the aim and objectives of this PhD Thesis.*

3

Materials and Methods



3. MATERIALS AND METHODS

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

3.1. Plant material and experimental design

The studies proposed in this PhD Thesis were performed during three growing seasons (2016, 2017 and 2018) with two different seedless table grape (*Vitis vinifera* L.) cultivars, 'Magenta' and 'Crimson', and 'Mollar de Elche' pomegranate (*Punica granatum* L.) cultivar. Table grape cultivars were 7-8 and 10-11 years old, respectively, and both of them grafted onto Paulsen 1103 rootstocks. Vines were planted 2.5 x 3 m in a sandy soil in a commercial plot of vineyards in Calasparra (Murcia, Spain). On the other hand, pomegranate trees used were bewteen 8 and 11 years-old and planted at 6 x 5 m in a commercial orchard located in Elche, south of Alicante, Spain. Pruning, thinning, fertilization and irrigation were carried out during the experiments according to standard cultural practices for both crops. Completely randomized block design were set up with different number of replicates and vines or trees depending of the treatment and year. Fruit were harvested mainly based on commercial criteria of fruit size, colour and total soluble solids content characteristic of each cultivar.

Elicitor treatments used in the experiments presented in this PhD Thesis were prepared with the same procedure and under the same conditions. All the compounds used: methyl jasmonate (MeJa), salicylic acid (SA), acetyl salicylic acid (ASA), methyl salicylate (MeSa), and oxalic acid (OA), 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. All solutions contained 0.5 % Tween-20 as surfactant. In table grape crop, treatments were applied three times, the first one (T1) when berry volume was ca. 40 % of its final one, the second one (T2) at veraison stage and the third one (T3) 3 days before the first harvest date. However, four treatments were sprayed along the developmental growth cycle of pomegranate crop as follows: the first one (T1) when fruit reached the 30 % of its final size (before the skin and colour changes), the second one (T2) when fruit reached the 50 % of its final size, the third one (T3) 1 month before harvesting, and the fourth one (T4) 4 days before harvesting, that is to say, at 80, 110, 140 and 170 days after full blossom, respectively.

Specifically, the treatments and concentrarions (**Figure 8**) assayed in both fruit were the following:

- ***Control:*** Control vines or pomegranate trees were sprayed with 0.5 % Tween-20 aqueous solution.

- ***Methyl jasmonate (MeJa):*** Treatments were performed in 2016 season at 1, 5 and 10 mM for both fruit species. In 2017, these concentrations were repeated for pomegranate crop, although lower concentrations (0.01, 0.1 and 1 mM) were performed in vine trees due to a delay on the berry ripening process. Based on the experiments carried out in 2016 and 2017 seasons in pomegranate fruit in which the best results in term of yield and fruit quality traits were obtained with 5 mM dose, pomegranate trees were sprayed with 5 mM MeJa solution in 2018 season (Preharvest treatment). In this experiment, MeJa-treated pomegranate fruit were also treated with 5 mM MeJa as postharvest treatment (Pre + Postharvest treatment). Postharvest treatment was performed by dipping the fruit in 15 L of MeJa solution, containing 0.5 % Tween-20, for 15 min, and then they were left to dry at room temperature.

- ***Salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa):*** In table grape, SA, ASA, and MeSa treatments were applied at 1, 5 and 10 mM concentrations in 2016 and at 1, 0.1 and 0.01 mM concentrations in 2017, since in general, salicylates at 5 and 10 mM decreased vine yield and delayed berry ripening process. Based on the results of two growing seasons (2016 and 2017) in terms of yield, berry ripening process on tree, berry quality and bioactive compound content, the best concentration for each treatment and cultivar was chosen. Thus, SA and MeSa were applied at 0.01 and 0.1 mM, respectively, for both cultivars in 2018 season. However, ASA was sprayed at 1 and 0.1 mM for 'Crimson' and 'Magenta' cultivars, respectively. In this last season, harvested grapes were disinfected with 100 ppm of chlorine during 1 min, were injured with a sterile lancet and were inoculated with *B. cinerea* CECT21000 (Spanish collection of type cultures) spore suspension at 7500 CFU mL^{-1} until runoff. On the other hand, SA, ASA, and MeSA treatments were performed at 1, 5 and 10 mM in 2017 season for pomegranate fruit, being 10 mM the concentration repeated in the 2018 experiment for all compounds.

- ***Oxalic acid (OA):*** In 2016 season, OA was applied at 1, 5 and 10 mM in pomegranate crop. The following year, in 2017 experiment, the best concentration selected based on the results of the previous year, OA at 10 mM, was applied following the same process as in the 2016 season. With respect to table grape crop, OA treatments were performed in 2018-growing season with only the 'Magenta' seedless cultivar at 5 mM concentration. This concentration was chosen as the optimum among three concentrations tested (1, 5 and 10 mM) in previous experiments (growing seasons 2016 and 2017). A 5 mM OA treatment was the best in terms of yield, berry maturity-quality and bioactive compounds (data not shown in this PhD Thesis).

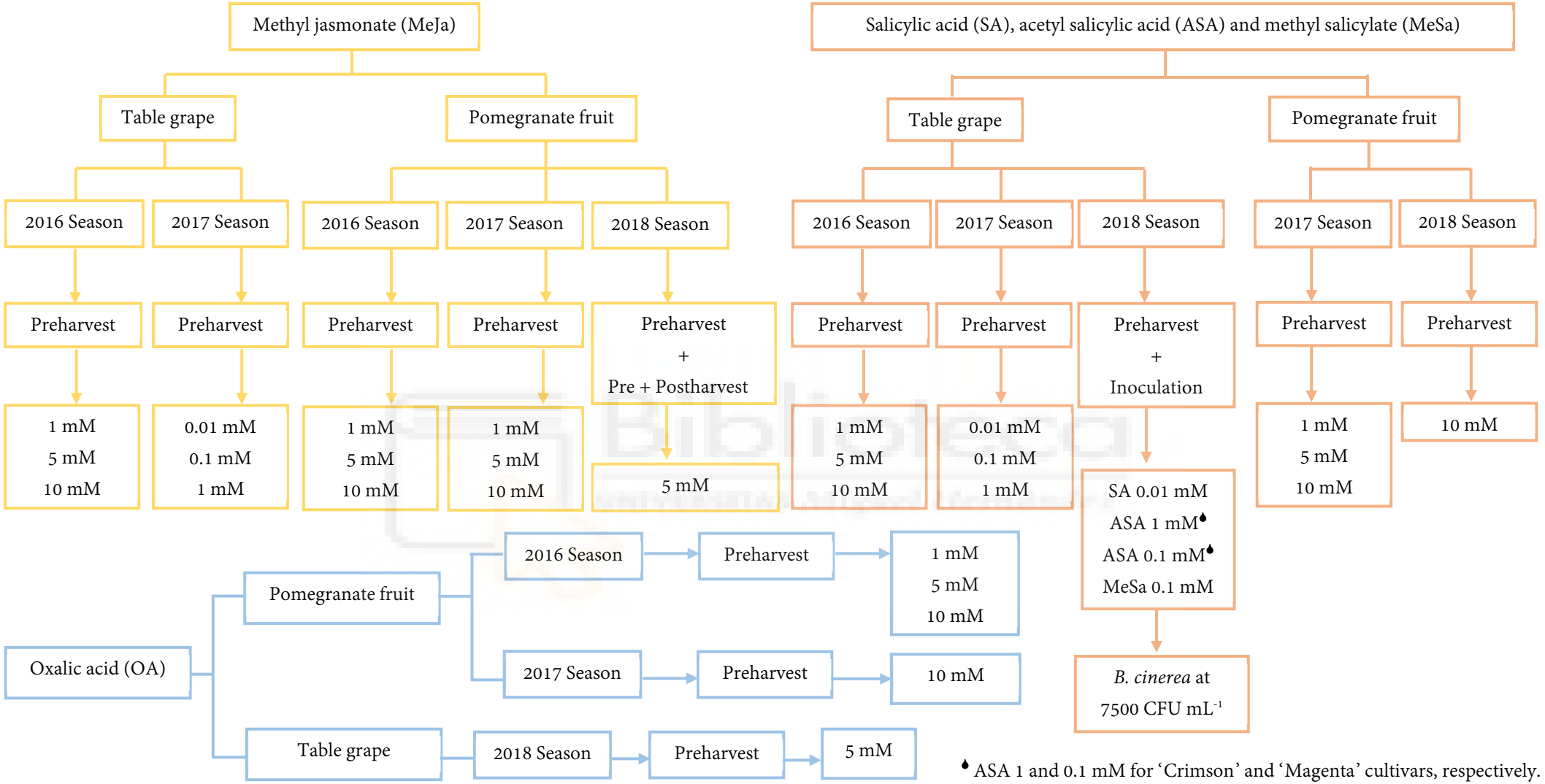


Figure 8: *Experimental design scheme of the different treatments included in this PhD Thesis.*

3.2. Fruit growth

The evolution of pomegranate fruit growth was determined in the labelled fruit from T1 treatment, when fruit reached the 30 % of its final size, by measuring the equatorial diameter.

3.3. Crop yield: kg per tree or vine, number of fruit per tree, percentage of fruit harvested in the first harvest date, fruit weight (g) and berry volume (mm^3)

Due to the heterogeneous fruit on-tree ripening process, two harvests were made in two dates, separated by 20 or 21 days, for pomegranate crop and four harvests were performed for both table grape cultivars, according to ripening commercial criteria. On each harvest date, the yield (kilograms harvested per tree or vine, and number of pomegranate fruit per tree) were determined. Vine production was expressed as accumulated yield (kg $vine^{-1}$) from the first to the last harvest date. In addition, due to the economic profit of the pomegranate fruit from the first harvest date, the first harvest yield and the percentage (%) of fruit harvested at the first picking date were also determined. Then, fruit mass or weight (g) and berry volume (mm^3) were measured at different harvest dates.

3.4. Weight loss

Pomegranate fruit and clusters were weighed at harvest and after each storage period, and weight loss was expressed as percentage (%) with respect to initial weight.

3.5. Respiration rate

To measure pomegranate fruit respiration rate, each fruit lot was hermetically sealed during 30 or 60 min, depending on the experiment, in a 3 L jar. After that, one sample from the holder atmosphere of 1 mL was withdrawn with a syringe and injected into a Shimadzu TM 14A gas chromatograph (Kyoto, Japan) equipped with a thermal conductivity detector, as previously described by Sayyari et al. (2011b).

3.6. External and internal colour

External colour of pomegranate fruit was determined along six points of the equatorial perimeter in each fruit using the CIE Lab system in a colorimeter (CRC200, Minolta Camera Co., Tokyo, Japan). For the internal colour, this parameter was measured on the surface of a Petri dish filled with arils. In table grape, colour was measured individually in each berry by using the same colorimeter. After recording L*, a*, and b* parameters, colour was expressed as hue angle (arctg b*/a*).

In addition, external and internal colour were measured by digital image analysis in some of the experiments. Thus, photographs of the pomegranate fruit were captured using a digital camera (Nikon D3400) in a light box with black background. The setup conditions of the camera were as follows: light provided by two LEDs of colour temperature of 5600 K, flash speed

of 1/5 s, ISO-200, focal opening (f) 20, and length 35 mm. For external and internal colour measure, two images of the front and the back side, and another of the cut surface of pomegranate fruit were captured, saved as JPEG file, and analyzed by using the software ImageJ v1.52a (NIH Image, National Institutes of Health, Bethesda, United States). The CIE Lab model was also used to calculate hue angle according to García et al. (2017).

3.7. Firmness

Fruit firmness was measured individually in each fruit as the force that achieved a 5 and 3 % deformation of the berry and pomegranate fruit diameter, respectively, by using a TX-XT2i Texture Analyzer, Stable Microsystems, Godalming, UK. Results were expressed as the relation between the applied force to achieve the deformation percentage and the distance travelled.

3.8. Chilling injury (CI) index and ion leakage (IL)

Chilling injury (CI) index was individually evaluated internally and externally in each pomegranate fruit based on the percentage of husk surface affected by CI symptoms (dehydration, browning and pitting). Thus, a 6-point hedonic reference scale (**Figure 9**) was used to evaluate external and internal CI index as follows: 0 (no symptoms), 1 (1-20 %), 2 (21-40 %), 3 (41-60 %), 4 (61-80 %) and 5 (> 81 %). This scale was performed with own photographs made by the authors from previous studies and the degree of CI symptoms was assessed according to them. CI index was calculated as:

$$\frac{\Sigma\ (\textbf{Value of hedonic scale})\ \text{x}\ (\textbf{Number of fruit with the corresponding score})}{\textbf{Total number of fruit in the sample}}$$

Figure 9: *Reference scale to evaluate external and internal chilling injury (CI) index.*

Ion leakage was determined by cutting 16 disks (5 mm diameter × 5 mm thickness) with a cork borer from the pomegranate fruit husks and steeped in a glass vial containing 20 mL of 0.3 M mannitol. After 3 h under constant shaking, initial conductivity was measured using a conductivity meter (XS COND51+). Then, the disks were frozen overnight and autoclaved for

900 s at 120 °C. After that, disks were cooled to room temperature and conductivity was again measured. Ion leakage was expressed as percentage (%) of the total and calculated according to Mirdehghan et al. (2007a).

3.9. Visual decay incidence

A visual scale of 6-hedonic points or stages (S0, S1, S2, S3, S4 and S5) was used at fifth day after inoculation of table grape with *Botrytis cinerea* to evaluate the evolution and visual appearance of the fungus growth for each berry. The decay incidence scale was different for each table grape cultivar as can be seen in **Figure 10**. Results were expressed as percentage (%) of spoiled grapes in each stage based on the total number of fruit in each box.

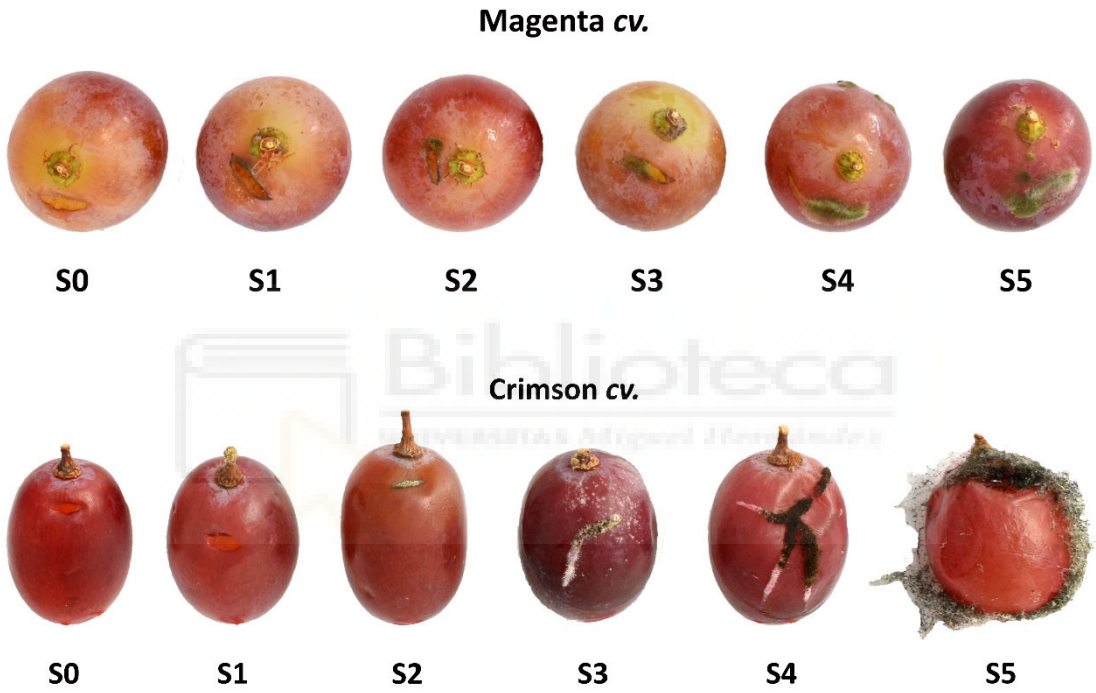


Figure 10: *Decay incidence scale for 'Magenta' and 'Crimson' table grape cultivars. Stages meaning: S0, without damage; S1, wound browning, S2, microbial growth covering 1-2 mm of wound in 'Magenta' or all the wound (6 mm) in 'Crimson'; S3, microbial growth covering 3-4 mm of wound in 'Magenta' or covering a quarter of the berry and showing mycelial growth in 'Crimson'; S4, microbial growth covering 4-5 mm of wound and showing mycelial growth in 'Magenta' or covering the half of the berry and showing mycelial growth and softening in 'Crimson'; S5, all the wound covered (6 mm) with the fungus and mycelium in 'Magenta' or covering the whole berry and showing mycelial growth and softening in 'Crimson'.*

3.10. Total soluble solids (TSS) and titratable acidity (TA)

Pomegranate aril sample and berries of each replicate, treatment and cultivar were ground to obtain a homogeneous juice sample in which total soluble solids (TSS) and titratable

acidity (TA) content were measured in duplicate with a digital refractometer Atago PR-101 (Atago Co. Ltd., Tokyo, Japan) at 20 °C and in 1 mL of the same juice diluted in 25 mL distilled H_2O by automatic titration (785 DMP Titrino, Metrohm, Phyathai, Thailand) with 0.1 N NaOH up to pH 8.1, respectively. Ripening index (RI) was calculated as the ratio between TSS and TA.

3.11. Individual sugars and organic acid content

To measure individual sugars and organic acid content, 5 g of pomegranate arils of each replicate were extracted with 5 mL of water with 0.5 % phosphoric acid and table grape juice was centrifuged at 10,000 *g* for 10 min. Both supernatants were filtered through 0.45 µm Millipore filter and injected in duplicate into a high-performance liquid chromatography (HPLC) system (Hewlett-Packard HPLC series 1100). The elution system consisted of 0.1 % phosphoric acid running isocratically at 0.5 mL min^{-1} through a Supelco column (Supelcogel C-610H, 30 cm 7.8 mm, Supelco Park, Bellefonte, United States). Organic acids were detected at 210 nm of absorbance and sugars by refractive index detector. A standard curve of pure sugars and organic acids purchased from Sigma (Poole, UK) was used for quantification.

3.12. Total phenolics and total anthocyanin quantification

Bioactive compounds were quantified in frozen samples under liquid N_2 which were stored at -80 °C. In pomegranate fruit, the total phenolic and total anthocyanin content were determined in the edible portion, the arils, while in table grape were measured both in the whole fruit (skin and flesh) and in the skin tissue. Total phenolics were extracted as previously reported by Martínez-Esplá et al. (2014b). Thus, 5 g of sample were homogenizing with 10 mL of water: methanol (2:8, v/v) containing 2 mM sodium fluoride (NaF) (to inactivate polyphenol oxidase activity and prevent phenolic degradation). The homogenization process was different depending on the fruit species. For pomegranate fruit, it was carried out during 30 s by using a homogenizer (Ultraturrax, T18 basic, IKA, Berlin, Germany). However, table grape tissues were manually ground in a mortar and pestle. In the case of table grape skin tissue, 1 g of frozen skin was manually ground in a mortar and pestle with 5 mL of water: methanol (2:8) containing 2 mM NaF and then, sonicated in an ultrasonic bath for 60 min. The homogenate of pomegranate and table grape was centrifuged at 10,000 *g* for 10 or 15 min, respectively, at 4 °C, and phenolics were quantified in duplicate in the supernatant by using the Folin-Ciocalteu reagent as previously reported (Sayyari et al., 2011a).

For total anthocyanin extraction, 5 g of arils or 10 g of frozen berry tissues were homogenized with 15 mL of methanol/formic acid/water (25:1:24, v/v/v), as described previously. Anthocyanin extraction from table grape skin was performed using 1 g of frozen skin tissue manually ground into 5 mL of the same extraction solvent. The homogenate of table grape was sonicated in an ultrasonic bath for 60 min. Then, both homogenates were centrifuged at the same conditions described above. The absorbance at 520 nm was read in the supernatant and in duplicate for each extract using a spectrophotometer (UNICAM Helios-α, Artisan Technology

Group, Champaign, IL, USA). Total anthocyanin content (TAC) was quantified taking into account the molar absorption coefficient and molecular weight of the predominant anthocyanin in each fruit species.

3.13. Individual anthocyanin identification and quantification

To identify and quantify individual anthocyanin concentration, the extracts obtained for total anthocyanin measurement described previously were filtered through a 0.45 µm polyvinylidene fluoride (PVDF) filter (Millex HV13, Millipore, Bedford, MA, USA). Identification of anthocyanins 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 (DAD) and a mass detector in series (Agilent Technologies, Waldbronn, Germany), as previously reported by Martínez-Esplá et al. (2014). To quantify individual anthocyanins, 20 µL of two samples from each extract were injected into a HPLC system (Agilent HPLC 1200 Infinity series) working with the chromatographic conditions previously reported (Martínez-Esplá et al., 2014b, 2017a).

Chromatograms were recorded at 520 nm and quantification was performed by using calibration curves carried out with malvidin 3-O-glucoside, cyanidin 3-O-glucoside, cyanidin 3,5-O-di-glucoside, pelargonidin 3-O-glucoside, pelargonidin 3,5-O-di-glucoside and peonidin 3-O-glucoside purchased from Sigma-Aldrich (Darmstadt, Germany). Delphinidin 3-O-glucoside, delphinidin 3,5-O-di-glucoside and petunidin-3-O-glucoside were quantified in some experiments as cyanidin 3-O-glucoside or malvidin 3-O-glucoside equivalents.

3.14. Ascorbic acid (AA), dehydroascorbic acid (DHA) and total vitamin C

Ascorbic (AA) and dehydroascorbic (DHA) acid content was measured in frozen pomegranate arils according to Peña-Estévez et al. (2016). For the extraction, 5 g of sample were homogenized with 5 mL of methanol: water (5:95, v/v) containing 0.1 mM citric acid, 0.05 mM ethylenediamine tetraacetic acid disodium salt, and 4 mM NaF for 30 s on an Ultraturrax, T18 basic, IKA, Berlin, Germany. Then, the extract was filtered through a four-layer cheesecloth, the pH was adjusted to 2.35-2.40 with 2 N ClH, and centrifuged at 10,000 *g* for 15 min at 4 °C. The supernatant was purified through a methanol-activated C18 cartridge (Sep-Pak cartridges C18, Waters, Dublin, Ireland) and filtered through a 0.45 µm PFTE filter.

For DHA derivatization, 750 µL of extract were mixed with 250 µL of 7.7 M 1,2-phenylenediamine in an HPLC amber vial. The mixture was allowed to react for 37 min and then 20 µL of sample were injected onto a Luna (250 mm x 4.6 mm, 5 µm particle size) C18 column (Phenomenex, Macclesfield, United Kingdom) with a C18 security guard (4.0 mm x 3.0 mm) cartridge system (Phenomenex, Macclesfield, United Kingdom) using a HPLC system (Agilent HPLC 1200 Infinity series). The mobile phase was 50 mM KH_2PO_4 containing 5 mM hexadecyl trimethyl ammonium bromide and 5 % methanol (pH 4.59) with isocratic flow of 1 mL min^{-1}.

Absorbance was recorded at 261 nm for AA (Rt = 9.4 min) and at 348 nm for DHA (Rt = 4.5 min) and they were quantified by comparison with AA and DHA standards areas (Sigma-Aldrich, Germany).

3.15. Total antioxidant acitivity quantification

Total antioxidant activity (TAA) was quantified in pomegranate arils and table grape skin according to the procedure described in Sayyari et al. (2011b), 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}.

3.16. Antioxidant enzyme activities

Antioxidant enzymes were measured in berry skin tissue. Crude extracts to determine ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) enzymes were performed by homogenizing 1 g of frozen tissue with 5 mL of phosphate buffer 50 mmol L^{-1}, pH 6.8, containing 1 % (w/v) polyvinylpyrrolidone (PVP) and 1.0 mmol L^{-1} ethylenediamine-tetraacetic acid. Then, the extracts were centrifuged at 10,000 *g* for 30 min at 4 °C and the supernatant was used for the quantification as reported previously (Zapata et al., 2014). Briefly, for APX quantification, the reaction mixture contained 200 µL of extract in 3 mL of 50 mmol L^{-1} potassium phosphate (pH 7.0), 0.5 mmol L^{-1} ascorbic acid and 1.0 mmol L^{-1} H_2O_2, and the decrease of absorbance at 290 nm from time 0 to 60 s was measured. For CAT, 100 µL of extract were added to 3 mL of reaction mixture containing 15 mmol L^{-1} H_2O_2 and 50 mmol L^{-1} phosphate buffer (pH 7.0), and the decrease of absorbance at 240 nm during 1 min was measured. Finally, for POD activity, the reaction mixture contained 200 µL of extract in a final volume of 3 mL of 50 mmol L^{-1} phosphate buffer (pH 7.0), 12 mmol L^{-1} H_2O_2 and 7 mmol L^{-1} guaiacol, and the increase of absorbance at 470 nm during 1 min was measured. Activity of APX, CAT or POD were expressed in terms of units of enzymatic activity, with one enzymatic unit (U) being defined as a decrease or increase, depending on the enzyme, of 0.01 ascorbate min^{-1}.

3.17. Fatty acid profile and quantification

Pomegranate husk fat was directly methylated according to Trigueros and Sendra (2015) by trans-esterification of 25 mg of sample with 2 mL of 0.5 M sodium methoxide. Fatty acid profile and quantification were determined by high resolution gas chromatography, analysing the fatty acid methyl esters obtained. Methyl esters were separated on a Shimadzu GC17A gas chromatography unit coupled to a mass spectrometry detector GC–MS QP5050, Shimadzu (Kyoto, Japan) with a SupraWax-280 column, filled with 100 % polyethylene glycol (Teknokroma S. Co. Ltd., 165 Barcelona, Spain; 30 m length, 0.25 mm internal diameter, 0.25 µm film thickness) and they were identified by comparison of their retention times with Supelco 37-component FAME Mix reference standard (Sigma-Aldrich Co., St. Louis, MO, USA). The chromatography conditions were applied according to Ferrara et al. (2014) and the quantification was carried out in duplicate in each sample using nonadecanoic acid (19:0, C-19) as internal standard.

3.18. Abscisic acid (ABA) and catabolite analyses

Freeze-dried powdered table grape material of whole berry (flesh + skin tissues) was weighed (5.0 ± 0.1 mg) and extracted with 500 µL of precooled (-20 °C) methanol:water:formic acid (60:35:5 v/v/v), as described by Müller and Munné-Bosch (2011) with some modifications. The labelled forms of the compounds; (-)-5,8´8´8´-d_4-abscisic acid (d4-ABA); (+)-4,5,8´,8´,8´-d_5-abscisic acid glucose ester (d5-ABA-GE); (±)-5,8´,8´,8´-d_4-7´-hydroxy-ABA (d4-OH-ABA); (-)-7´,7´,7´-d_3-phaseic acid (d3-PA) and (-)-7´,7´,7´-d_3-dihydrophaseic acid (d3-DPA) were added to the mixture as internal standards. ABA and catabolite content were quantified according to Morris et al. (2018) with slight modifications, by using a LC/MS-MS instrument with an Agilent 1200 series HPLC system (Agilent, Berks., UK) coupled to a Q-Trap 6500 mass spectrometer (AB Sciex, Framingham, USA). The extracts were analysed by injecting 20 µL onto a Phenomenex 3 µm C18 Luna 100 x 2 mm with guard column at 40 °C.

3.19. Relative *Vv*NCED1 gene expression

RNA was extracted from 0.1 g of freeze-dried table grapes, using the whole fruit (flesh + skin tissues), according to the protocol described by Le Provost et al. (2007) with slight modifications. Briefly, freeze-dried samples were manually grounded with liquid nitrogen and mixed with 1 mL of extraction buffer (2.5 % (w/v) CTAB, 2 % (w/v) polyvinylpyrrolidone or PVP K-40, 1.0 M TRIS-HCL at pH = 8.0, 0.5 M EDTA, 5.0 M NaCl) containing β-mercaptoethanol at 2 %, previously heated to 65 °C, and vortexed vigorously during 15 s. The mixture was incubated 10 min in a water bath at 65 °C and mixed by inverting tubes every 3 min. For purification, chloroform-isoamyl-alcohol (24:1) was added and mixed as the same way. Then, the mixture was centrifugated at 10,000 x *g* for 10 min at room temperature. Finally, the supernatant was transferred to a clean Eppendorf tube and mixed, inverted and incubated

overnight at -20 °C with 1/3 volume of LiCl 10 M. Next day, the solution was centrifuged at 10,000 x *g* for 10 min at 4 °C and the supernatant was discarded. The pellet was washed with 500 µL of cold (-20 °C) 80 % ethanol and centrifuged at 10,000 x *g* for 5 min at 4 °C. The supernatant was removed, and when the pellet was dried out, it was re-suspended in 100 µL of RNAse free-water and storage at -80 °C.

A DNase treatment was done on the eluted RNA by using Baseline-ZERO DNase (Epicentre/Lucigen USA) according to the manufacturer's recommendations. RNA quantification was carried out by the spectrophotometric absorbance using a NanoDrop 2000 and a Qubit 2.0 Fluorometer (ThermoFisher Scientific, USA). The expression analysis of *Vv*NCED1 gene was carried out by GenXPro GmbH (Germany). Total RNA (15 - 40 ng per reaction) was used as template for the OneStep qPCR reactions. Reverse transcription and qRT-PCR was performed using the MDX025 Low LOD 1-Step qPCR Mix (Meridian/Bioline, USA/Germany), according to the manufacturer's recommendations. RNA from treatments was used as the template for the qPCR reactions.

Vitis vinifera 9-cis-epoxycarotenoid dioxygenase 1 gene (*Vv*NCED1; LOC100232942) was amplified using the described primers in **Table 5** (Rattanakon et al., 2016). qRT-PCR was performed on a StepOne Thermocycler system (Applied biosystems, Thermo-Fisher), according to Serna-Escolano et al. (2021a). Expression of *Vv*NCED1 gene was normalized with three endogenous control genes; *Vitis vinifera* actin-7 (ACT; LOC100232866), *Vitis vinifera* ubiquitin-60S ribosomal protein L40-2 (UBI; LOC100253716) and *Vitis vinifera* glyceraldehyde-3-phosphate dehydrogenase cytosolic (GAPDH; LOC100233024), on normalization of genes in table grapes (Reid et al., 2006; Pagliarani et al., 2017), as can also be seen in **Table 5**. Relative *Vv*NCED1 gene expression in treated fruit was calculated with respect to control fruit.

Table 5: *Transcriptomic details of primers for the targeted and endogenous control genes.*

Gene		Forward and reverse primers	Amplicon length	NCBI reference sequence
NCED1	**F**	5′-GCAGAGGACGAGAGTGTAAAGGA-3′	130 pb	XM_019216859.1
	R	5′-GCAGAGTAAAAACACATGAAGCTAGTG-3′		
ACT	**F**	5′- GCCCCTCGTCTGTGACAATG-3′	100 pb	XM_002282480.4
	R	5′-CCTTGGCCGACCCACAATA-3′		
UBI	**F**	5′-TCTGAGGCTTCGTGGTGGTA-3′	99 pb	XM_002273532.2
	R	5′-AGGCGTGCATAACATTTGCG-3′		
GAPDH	**F**	5′-CCACAGACTTCATCGGTGACA-3′	70 pb	XM_002263109.3
	R	5′-TTCTCGTTGAGGGCTATTCCA-3′		

3.20. Descriptive sensory evaluation

Harvested pomegranate fruit were preconditioning in cold chambers at 8 °C and at 85-90 % of relative humidity before descriptive sensory evaluation. Ten trained panelists (25 to 55 years; 5 females and 5 males) from the Department of Agro-Food Technology of Miguel Hernández University of Elche were selected to participate in this study. Panelists received two preliminary orientation sessions of 60 min discussing about the main attributes appreciated by consumers in the whole fruit (brightness, colour uniformity and intensy, and firmness) and in the arils (sweetness, sourness, bitterness, astringent, solubility in saliva and seed hardness). Overall linking was also evaluated in treated and untreated samples, which were served to each panelist at a controlled temperature (20 ± 2 °C) in a testing room with a combination of natural and fluorescent light. A numerical scale from 0 to 10 was used, where 0 represented no perceptible intensity, and 10 represented extremely strong intensity.

3.21. Statistical analysis

Results of this PhD Thesis were expressed as mean ± SE of three or five replicates depending of the experimental design in each experiment. 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. 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 cappital 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 for Windows. Some correlations were performed in one study between internal or external CI indexes and IL, and between arils hue angle and their total anthocyanin content.

4

Publications



4. PUBLICATIONS

4.1. Publication 1

PUBLICATION 1 (Literal transcription)

Methyl jasmonate effects on table grape ripening, vine yield, berry quality and bioactive compounds depend on applied concentration

María E. García-Pastor, María Serrano, Fabián Guillén, Salvador Castillo, Domingo Martínez-Romero, Daniel Valero, Pedro J. Zapata

Scientia Horticulturae, **247: 380-389 (2019)**

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Methyl jasmonate effects on table grape ripening, vine yield, berry quality and bioactive compounds depend on applied concentration

María E. García-Pastor[a], María Serrano[b,*], Fabián Guillén[a], Salvador Castillo[a], Domingo Martínez-Romero[a], Daniel Valero[a], Pedro J. Zapata[a]

[a] *Department of Food Technology, EPSO, University Miguel Hernández, Ctra. Beniel km 3.2, 03312, Orihuela, Alicante, Spain*
[b] *Department of Applied Biology, EPSO, University Miguel Hernández, Ctra. Beniel km 3.2, 03312, Orihuela, Alicante, Spain*

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ABSTRACT

In the present research the effect of preharvest methyl jasmonate (MeJA) treatment on the ripening process and fruit quality parameters at harvest was evaluated, for the first time, in two table grape cultivars, 'Magenta' and 'Crimson', during two years, 2016 and 2017. MeJA treatments (applied when berry volume was ca. 40% of its final one, at veraison and 3 days before the first harvest date) affected grape ripening process and vine yield differently depending on applied concentration. Thus, MeJA at 5 and 10 mM delayed berry ripening and decreased berry weight and volume as well as vine yield, in a dose-dependent way, in both cultivars, although the effect on 'Crimson' was more dramatic than in 'Magenta'. However, treatments with MeJA at 1, 0.1 and 0.01 mM accelerated ripening and increased total phenolics and individual anthocyanin concentrations, the major effects being obtained with 0.1 mM concentration. In addition, total soluble solids (TSS) and firmness levels were also increased by these MeJA treatments. These results might have a great agronomic and commercial importance since fruit with higher size and harvested earlier would reach higher prizes at markets and berries with higher firmness and TSS would be more appreciated by consumers. Moreover, MeJA treatments increased the content of antioxidant compounds, such as phenolics and individual anthocyanins, leading to enhance the homogeneous pigmentation of the whole cluster, with additional effects on increasing the health beneficial effects of grape consumption.

1. Introduction

Table grape (*Vitis vinifera* L.) is considered one of the most appreciated fruit around the world, the Spanish production being 399,144 tons in 2017 (MAPA, 2017). Table grape marketing value depends on cluster size and shape as well as on berry size, colour, juiciness, sugar/acidity ratio and aroma. Veraison is a key point of berry development, in which pigmentation of skin starts (due to synthesis of anthocyanins in red cultivars), sugars and aroma compounds increase and acid content and firmness decrease, while berry growths until the end of ripening (Kuhn et al., 2014). However, some seedless red skin cultivars, such as 'Magenta' and 'Crimson' in spite of having very good taste and aroma have a heterogeneous berry pigmentation in the cluster, probably due to the high temperatures in the Southeast of Spain during berry ripening, which prevent proper colour development (Ferrara et al., 2015), leading to diminution of their market quality. In this sense, abscisic acid (ABA) and ethephon (an ethylene- releasing compound) treatments at veraison stage have been shown toincrease skin anthocyanin concentration although most of these studies have been performed with wine grape cultivars (Marzouk and Kassem, 2011; Kuhn et al., 2014). Specifically, in 'Crimson Seedless' ABA and sucrose treatments improved table grape coloration allowing earlier harvest (Ferrara et al., 2015; Olivares et al., 2017), as well as regulated deficit irrigation applied during post-veraison stage, due to increases in anthocyanin concentration in berry skin (Conesa et al., 2016). However, the high cost of ABA reduces its practical application and the effects of ethephon on colour development are inconsistent and can cause berry softening. Therefore, more research is needed to find out other compounds with commercial application possibilities.

Jasmonic acid (JA) and its volatile derivative methyl jasmonate (MeJA), known as jasmonates (JAs), are considered as hormones acting in the regulation of a wide range of physiological processes in plants, including growth, photosynthesis, reproductive development and responses to abiotic and biotic stresses (Creelman and Mullet, 1997; Dar et al., 2015). Nevertheless, the most studied effect of these compounds has been their role as elicitors or signalling agents triggering the plant

* Corresponding author.
E-mail address: m.serrano@umh.es (M. Serrano).

defence responses against herbivores and pathogens' attacks (Wasternack, 2014). In this sense, post- and preharvest MeJA treat- ments of table grape and wine grape cultivars, respectively, primes defence responses, leading to increase disease resistance against *Botrytis cinerea*, the major postharvest disease that limits table grape storage (Jiang et al., 2015; Jia et al., 2016). However, in recent years it has been also reported that JAs, applied as pre- or postharvest treatments, have also effects on fruit ripening and quality parameters at harvest and during storage (Serrano et al., 2018).

Currently, most of the knowledge about MeJA effects on fruit quality attributes and ripening is derived from postharvest treatments. Thus, it has been reported that postharvest MeJA treatments have effects on reducing a wide range of stress-induced injuries during the postharvest period, such as chilling injury (CI), infection by some pathogens, and mechanical damage among others (Peña-Cortés et al., 2005; Sayyari et al., 2011; Wang et al., 2015). In addition, it has been reported that postharvest treatments with MeJA promote climacteric fruit ripening by increasing ethylene production in fruit such as peach, mango, tomato, apple and plum and even in non-climacteric fruit such as strawberry (Peña-Cortés et al., 2005; Serrano et al., 2018). However, papers about the effect of preharvest MeJA treatments on fruit growth and ripening on tree, and on fruit quality parameters at harvest are more limited and different results have been obtained depending on applied concentration, fruit species and developmental stage at which treatments were performed. For instance, in peach, MeJA 0.4 mM applied at S3 stage delayed fruit ripening throughout down-regulation of crucial ripening-related genes (Ziosi et al., 2008), while this process was accelerated with MeJA 10 mM treatments applied at the same development stage (Janoudi and Flore, 2003). MeJA treatments, at 5, 10 and 20 mM, to apple trees at early developmental stage delayed the ripening process, while this process was accelerated when MeJA was applied at the latest developmental stages (Rudell et al., 2005). On the other hand, a delay in the ripening process was found in 'Black Splendor' and 'Royal Rosa' plum cultivars with MeJA treatment at 0.5 mM applied at three key points of fruit development, while no effect was observed when 1 and 2 mM MeJA doses were applied (Martínez- Esplá et al., 2014).

Specifically, in wine grapes, several reports have shown that MeJA treatments to vineyard led to increase phenolic content, mainly anthocyanins, flavonols and stilbenes, on grape and wine although huge differences between growing season and varieties were found (Portu et al., 2015, 2016, 2018a; Gómez-Plaza et al., 2017). Nevertheless, no information is available in these papers regarding the effects of MeJA treatments on grape ripening process. Only in a recent paper, a 10-days delay on the technological maturity (ºBrix and pH) as a consequence of MeJA vineyards treatment has been reported in the wine variety 'Sangiovese' (D'Onofrio et al., 2018).

However, as far as we know, no information is available about the effects of preharvest MeJA treatments on table grape and just in one recent paper the effect of postharvest MeJA treatment has been evaluated, showing an increase in antioxidant activity and total phenolic and anthocyanin concentrations in 'Red Globe' cultivar (Flores et al., 2015). Thus, the aim of this research was to evaluate the effects of MeJA treatments on two table grape cultivars, 'Magenta' and 'Crimson', mainly focused to increase anthocyanin content in these poor-coloured cultivars.

2. Materials and methods

2.1. Plant material and experimental design

This study was performed during two growing seasons (2016 and 2017) with two different *Vitis vinifera* L. seedless table grape cultivars, 'Magenta' and 'Crimson', which were 7 and 10 years old, respectively and planted 2.5 x 3 m in a sandy soil in a commercial orchard in Calasparra (Murcia, Spain). MeJA (purchased from Sigma-Aldrich, Madrid Spain, CAS Number 39924-52-2) treatments were performed by foliar spray application of 1.5 L per vine of 1, 5 and 10 mM MeJA in 2016 and 0.01, 0.1 and 1 mM MeJA in 2017, containing 0.5% Tween 20 as surfactant. Treatments were made early in the morning and during favourable weather conditions where rainfall or winds were not forecasted for the following 24 h. Control vines were sprayed with 0.5% Tween 20 aqueous solution. MeJA treatments were applied three times, the first one when berry volume was ca. 40% of its final one, the second one at veraison stage and the third one 3 days before the first harvest date (Table 1). Pruning, thinning, fertilization and irrigation were carried out during the experiments according to standard cultural practices for table grape. A completely randomized block design with three replicates of three vines for each treatment, cultivar and year was set up.

Table 1
Dates of methyl jasmonate (MeJA) treatments (T1, T2 and T3) of 'Magenta' and 'Crimson' cultivars.

	'Magenta'		'Crimson'	
Treatment	2016	2017	2016	2017
T1	24th June	27th June	23rd June	26th June
T2	8th July	18th July	9th July	12th July
T3	18th July	21st July	25th July	28th July

2.2. Determination of vine yield

Clusters were harvested when berries reached the characteristic size, colour and soluble solid content (°Brix) of each cultivar in order to pick up full mature grapes, so that for both cultivars and years four harvests were performed and production of each vine (kg vine^{-1}) was measured for each harvest date. Vine production was expressed as accumulated yield (kg vine^{-1}) until the last harvest date (mean ± SE of three replicates of three vines).

2.3. Fruit quality parameters

Berry volume and weight were measured in three replicates of 60 berries (20 berries from each vine) taken at random from the clusters harvested at dates indicated in Fig. 4 legend. After that, 30 berries from each replicate (10 berries from each vine) were used to measure individually in each one colour (by using a Minolta colorimeter, CRC200, Minolta Camera Co., Japan, and expressed as L*, a* and b* parameters) and firmness (by using a TX-XT2i Texture Analyzer, Stable Microsystems, Godalming, UK, to measure the force that achieved a 5% deformation of the berry diameter and expressed as N mm^{-1}). Data of colour and firmness are the mean ± SE of three replicates of 30 berries. Then, the 30 berries of each replicate were cut and ground to obtain a homogeneous juice sample in which total soluble solids (TSS) were determined in duplicated with a digital refractometer Atago PR-101 (Atago Co. Ltd., Tokyo, Japan) at 20 °C, and expressed as g 100 g^{-1} (mean ± SE). Total acidity was determined also in duplicated in the same juice by automatic titration (785 DMP Titrino, Metrohm) with 0.1 N NaOH up to pH 8.1 and results (mean ± SE) expressed as g tartaric acid equivalent 100 g^{-1} fresh weight.

2.4. Total phenolics and individual anthocyanin quantification

The remaining 30 berries from each replicate (10 berries from each vine) were cut in small pieces and ground under liquid N_2 and stored at −80 °C until total phenolics and individual anthocyanins were quantified. Total phenolics were extracted as previously reported (Martínez-Esplá et al., 2014) by using 5 g of berry tissues and 10 mL of water:-methanol (2:8) containing 2 mM NaF (to inactivate polyphenol oxidase activity and prevent phenolic degradation) and after centrifugation at

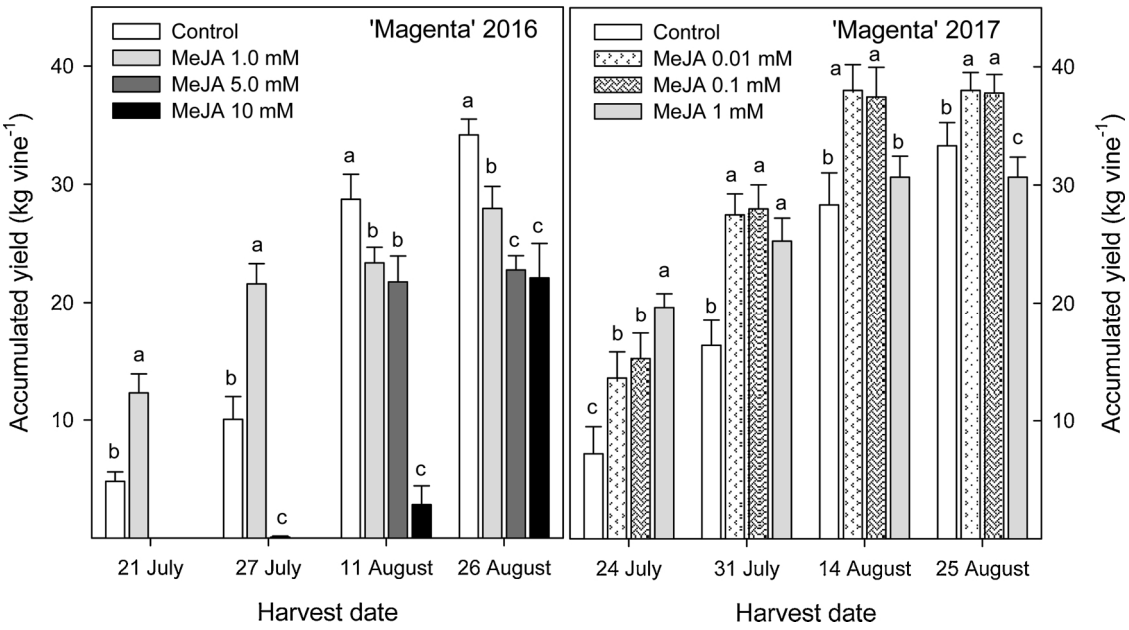

Fig. 1. Accumulated yield in 'Magenta' control and MeJA treated vines in 2016 and 2017 experiments. Data are the mean ± SE of three replicates of three vines for each treatment and cultivar. Different letters show significant differences ($P < 0.05$) among treatments for each harvest date.

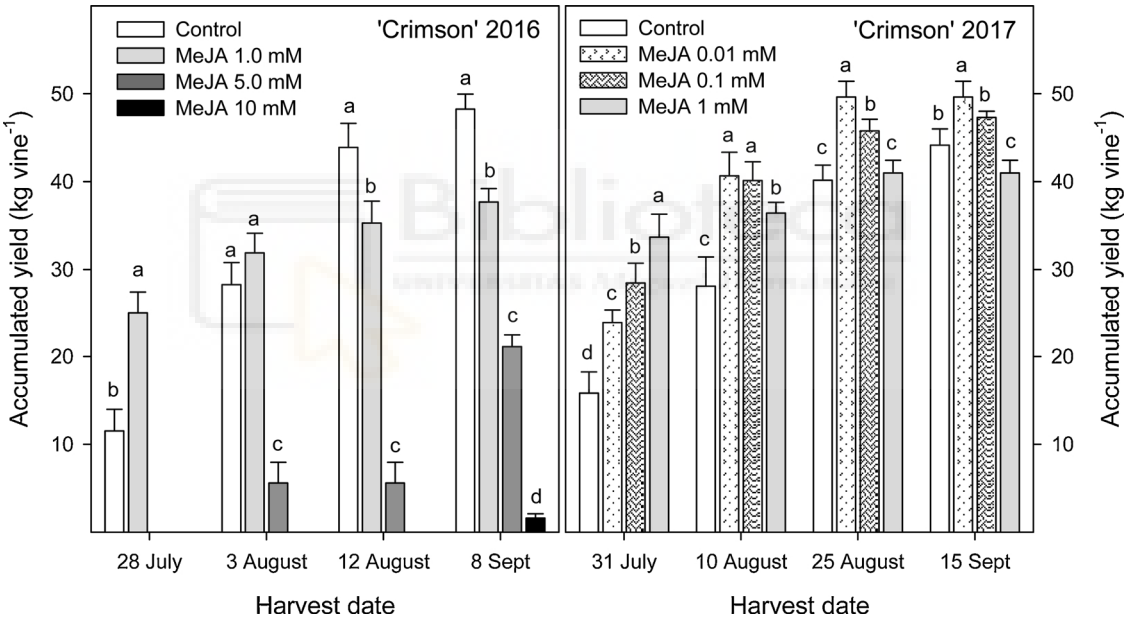

Fig. 2. Accumulated yield in 'Crimson' control and MeJA treated vines in 2016 and 2017 experiments. Data are the mean ± SE of three replicates of three vines for each treatment and cultivar. Different letters show significant differences ($P < 0.05$) among treatments for each harvest date.

10,000 g for 15 min phenolics were quantified in the supernatant using the Folin-Ciocalteu reagent and results (mean ± SE) were expressed as mg gallic acid equivalent 100 g^{-1} fresh weight.

To extract anthocyanins 10 g of frozen berry tissues and 15 mL of methanol/formic acid/water (25:1:24, v/v/v) were manually ground in a mortar and pestle and then sonicated in an ultrasonic bath for 60 min and after that centrifuged at 10,000g for 15 min. The supernatant was filtered through a 0.45 µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and used for individual anthocyanin quantification by HPLC analysis as previously reported (Martínez-Esplá et al., 2014). Chromatograms were recorded at 520 nm. Anthocyanin standards were: malvidin 3-glucoside purchased from Sigma-Aldrich, Germany and cyanidin 3-rutinoside and pelargonidin 3-rutinoside purchased from Polyphenols SA (Sandnes, Norway) and results were mean ±SE.

2.5. *Statistical analysis*

A one-way analysis of variance (ANOVA) was performed with data from analytical determinations for each cultivar and year by using the SPSS software package v. 12.0 for Windows. Mean comparisons were performed using HSD Duncan's test to examine if differences were significant at $P < 0.05$

3. Results and discussion

3.1. *Grape ripening and vine yield*

MeJA treatments affected grape ripening process and vine yield differently depending on applied concentration. Clusters were

Fig. 3. Photographs showing the visual aspect of 10 mM MeJA treated table grapes at the first harvest date in 2016 experiment, the 21st of July and 28th July for 'Magenta' and 'Crimson' cultivars, respectively.

harvested when grapes reached the size, colour and soluble solid content characteristic of each cultivar, according to commercial practices. Thus, in the experiment performed in 2016, MeJA 1 mM treatment accelerated the berry ripening process in both cultivars, since accumulated vine yield was higher than in control vines at the first and second harvest dates, while a delay in the ripening process was observed for grapes treated with MeJA 5 and 10 mM, this effect being dose dependent (Figs. 1 and 2). For instance, in 'Magenta' treated with MeJA 10 mM the ripening process was delayed for three weeks, since the first grapes reaching the commercial ripening stage (just 2.6 kg vine^{-1}) were harvested at August the 11st while in control vines ca. 29 kg vine^{-1} had ripened and had been harvested by this date. However, the effect of MeJA 10 mM on inhibiting the ripening process was even higher in 'Crimson' cultivar, since the first clusters were harvested at the last harvest date, that is, five weeks later than control grapes (Fig. 2). In Fig. 3 it can be observed the green colour of 'Magenta' and 'Crimson' 10 mM treated grapes at the first harvest date while some of the cluster from control and 1 mM treated vines of both cultivars had reached the red colour characteristic of commercial harvest. In addition, MeJA treatments at 1, 5 and 10 mM decreased total yield in both cultivars, the effect being dose-dependent and higher in 'Crimson' than in 'Magenta' cultivar. Thus, total yield in control vines was 34.18 ± 1.33 and 48.25 ± 1.68 kg vine^{-1} in 'Magenta' and 'Crimson' cultivars, respectively, and significantly lower, 22.09 ± 2.9 and 1.59 ± 0.51 kg vine^{-1}, in those treated with MeJA 10 mM.

In the view of these results, treatments with MeJA at 1, 0.1 and 0.01 mM were applied in 2017 and all of them accelerated grape ripening since at the first harvest date more kg of grapes were harvested from all MeJA-treated vines with respect to controls, the effect being significantly higher as increased MeJA concentration from 0.01 to 1 mM. Nevertheless, as in 2016 experiment, MeJA 1 mM decreased vine total yield. However, concentrations of 0.01 and 0.1 mM increased total yield with respect to control vines, without significant differences between both doses (Figs. 1 and 2). Not only MeJA treatments affected vine yield but also berry size and weight since both were reduced by 1, 5 and 10 mM MeJA treatments, in both cultivars, and in a dose

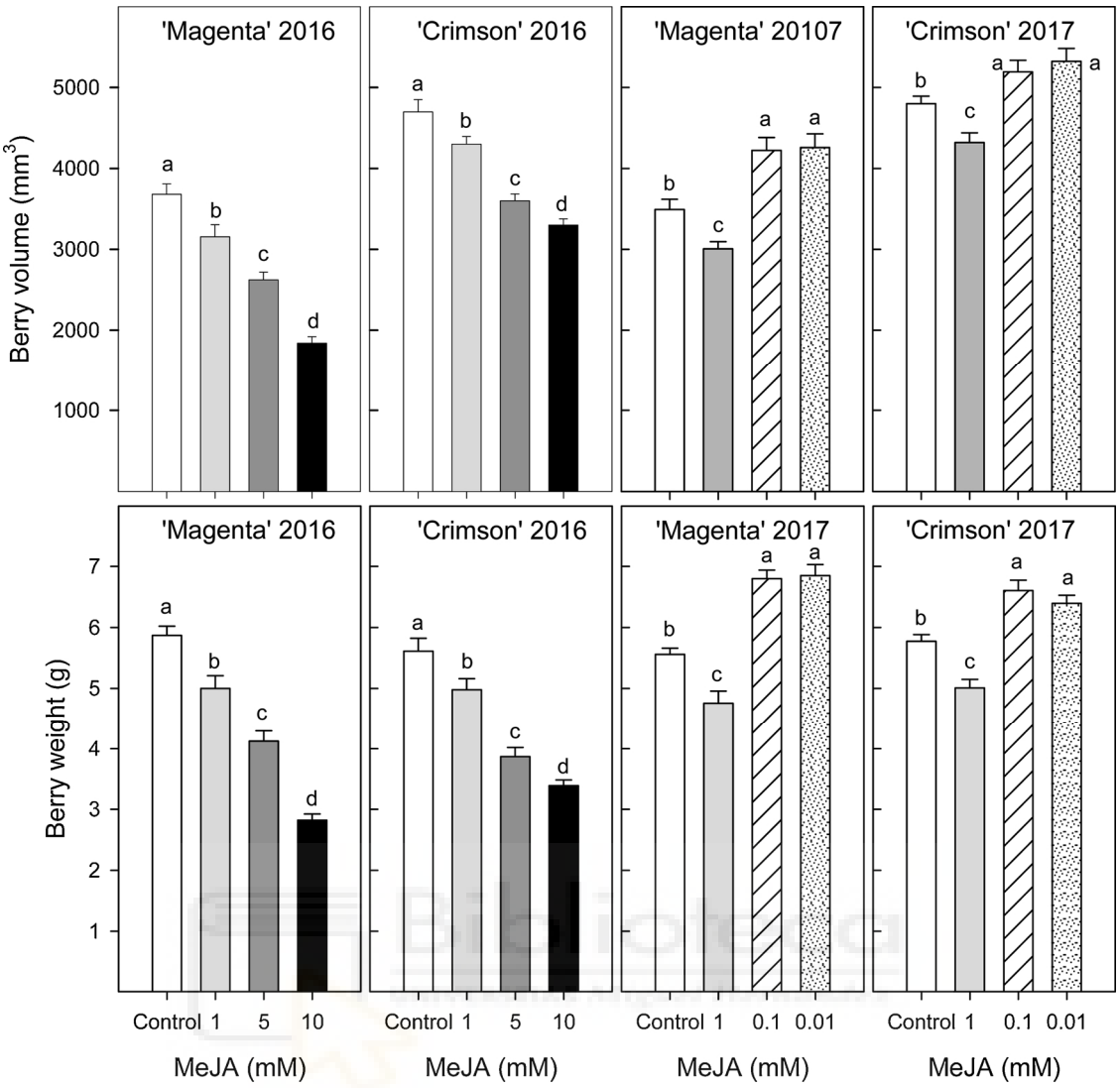

Fig. 4. Effect of vine MeJA treatments on berry volume and weight. Data are the mean ± SE of three replicates of 60 berries (20 berries from each vine) from the 1st harvest for control and MeJA 1 mM and from the 3rd harvest from MeJA 5 and 10 mM for 'Magenta' 2016. For 'Crimson' 2016 data are mean ± SE of three replicates of 60 berries (20 berries from each vine) for the 1st harvest for control and MeJA 1 mM, from the 2nd harvest for MeJA 5 mM and from the 4th harvest for MeJA 10 mM. For 2017 experiment, data are the mean ± SE of three replicates of 60 berries (20 berries from each vine) from the first harvest date for both cultivars and all treatments. Different letters show significant differences ($P < 0.05$) among treatments.

dependent way while increases in size and weight were obtained with 0.1 and 0.01 mM treatments (Fig. 4). Thus, the effects of MeJA treatments on vine yield were due to their effects on berry size which were different depending on applied concentration, without affecting the number of berries per cluster. In fact, cluster thinning was performed according to cultural practices before the MeJA treatments started, so that all clusters were left with similar size and number of berries and no shattering was observed during grape development on vine in control or treated clusters.

The effect of MeJA on fruit size has been published in a limited number of papers and contradictory results have been observed depending on fruit species, applied doses and fruit development stage. Thus, in agreement with the present results, MeJA treatments at 5, 10 or 20 mM of 'Fuji' apples trees at 48 days after full blossom reduced fruit size and weight due to inhibition of cell expansion or elongation (Rudell et al., 2005). However, by using lower concentration, increases in fruit weight and volume were obtained by preharvest 0.5 mM MeJA treatments in 'Black Splendor' and 'Royal Rosa' plum (Martínez-Esplá et al., 2014). On the other hand, a single treatment of peach with 0.8 mM MeJA at 56 DAFB (S2 stage) or 0.2 mM applied at S3 stage did not affect fruit diameter or weight at harvest (Ziosi et al., 2008; Ruiz et al., 2013).

In a similar way, the effect of MeJA treatments on table grape ripening was different depending on the applied concentration, that is, strong inhibition at 10 mM and acceleration at lower doses, mainly at 1 and 0.1 mM. These differences on berry colour between control and 0.1 mM MeJA treated grapes for both cultivars can be observed in Fig. 5. Accordingly, 0.01 and 0.1 mM MeJA treatments of 'Fujiminon' wine grape cultivar induced berry colouring, softening and synthesis of aroma compounds and, in turn, acceleration of the ripening process by increasing the expression level of a series of fruit colouring, cell-wall hydrolysis and aroma metabolism-related genes (Jia et al., 2016). On the other hand, (D'Onofrio et al., 2018) have recently reported that the application of MeJA 10 mM to 'Sangiovese' wine grape cultivar at the lag phase (EL 34) and 5 and 10 days later (EL 35 or veraison) slowed down the berry ripening process delaying by 10 days the technological maturity. However, this effect has not found in several wine cultivars such as 'Tempranillo', 'Monsatrell', Syrah', 'Merlot' or 'Graciano', treated with 10 MeJA at veraison and 3 and/or 6 days later (Portu et al., 2015, 2018a, 2018b; Gómez-Plaza et al., 2017). In all these previously

A: 'Magenta' control at the first harvest date (24th July, 2017)

B: 'Magenta' 0.1 mM MeJA at the first harvest date (24th July, 2017)

C: 'Crimson' control at the first harvest date (31st July, 2017)

D: 'Crimson' 0.1 mM MeJA at the first harvest date (31st July, 2017)

Fig. 5. Photographs showing the visual aspect of control and 0.1 mM MeJA treated table grapes at the first harvest date in 2017 experiment, the 24th of July and 31st July for 'Magenta' and 'Crimson' cultivars, respectively.

published papers, experiments were performed with wine grapes cultivars while the present results show the effect of MeJA treatment on grape ripening for the first time in table grape cultivars. Nevertheless, it is interesting to note that 10 mM MeJA concentration applied before veraison reduced the grape ripening process (Figs. 1–3) while when applied at veraison this effect was not observed in the previous paper commented above. Moreover, this process can be accelerated by applying lower concentration (Figs. 1 and 2). However, the molecular mechanism involved in these effects deserves further research.

3.2. *Grape quality at harvest*

Berry were harvested when reached their commercial ripening stage, mainly assessed by skin colour, and thus, no significant differences were obtained on L*, a* or b* colour parameters between control and MeJA treated berries (Table 2). Samples from MeJA 5 and 10 mM treatments were not analysed based on yield data. However, for both cultivars and years, firmness and TSS were significantly higher in treated than in control berries, the major effects being observed for MeJA 0.1 mM concentration. On the other hand, TA levels were not

Table 2
Colour (L*, a* and b* parameters), total soluble solids (TSS, g 100 g^{-1}), total acidity (TA, g 100 g^{-1}) and firmness (N mm^{-1}) of 'Magenta' and 'Crimson' table grapes at harvest as affected by methyl jasmonate (MeJA) preharvest treatments. *.

Treatments	L*	a*	b*	Firmness	TSS	TA
	'Magenta' 2016					
Control	31.2 ± 0.3^{a}	9.8 ± 0.2^{a}	1.9 ± 0.2^{a}	2.20 ± 0.01^{a}	18.1 ± 0.4^{a}	0.64 ± 0.02^{a}
MeJA 1 mM	31.0 ± 0.3^{a}	10.1 ± 0.2^{a}	2.2 ± 0.2^{a}	2.31 ± 0.03^{b}	19.9 ± 0.1^{b}	0.55 ± 0.01^{b}
	'Magenta' 2017					
Control	33.8 ± 0.5^{a}	10.0 ± 0.4^{a}	1.7 ± 0.3^{a}	1.86 ± 0.03^{a}	18.0 ± 0.1^{a}	0.99 ± 0.01^{a}
MeJA 1 mM	32.3 ± 0.5^{a}	9.4 ± 0.4^{a}	1.8 ± 0.4^{a}	2.03 ± 0.04^{b}	19.1 ± 0.1^{b}	0.89 ± 0.02^{b}
MeJA 0.1 mM	34.7 ± 0.9^{a}	10.7 ± 0.6^{a}	2.3 ± 0.3^{a}	2.23 ± 0.04^{c}	19.2 ± 0.2^{b}	0.95 ± 0.02^{ab}
MeJA 0.01 mM	33.9 ± 0.5^{a}	11.2 ± 0.4^{a}	2.5 ± 0.5^{a}	2.01 ± 0.03^{b}	19.0 ± 0.2^{b}	1.00 ± 0.01^{a}
	'Crimson' 2016					
Control	30.8 ± 0.3^{a}	5.4 ± 0.2^{a}	0.6 ± 0.4^{a}	2.30 ± 0.04^{a}	18.6 ± 0.3^{a}	0.76 ± 0.01^{a}
MeJA 1 mM	32.7 ± 0.4^{a}	6.8 ± 0.4^{a}	1.0 ± 0.3^{a}	2.62 ± 0.02^{b}	19.7 ± 0.2^{b}	0.79 ± 0.02^{a}
	'Crimson' 2017					
Control	27.7 ± 0.6^{a}	9.8 ± 0.3^{a}	2.3 ± 0.3^{a}	3.51 ± 0.09^{a}	17.6 ± 0.1^{a}	1.16 ± 0.01^{a}
MeJA 1 mM	26.9 ± 0.7^{a}	9.2 ± 0.4^{a}	1.9 ± 0.2^{a}	3.84 ± 0.08^{b}	18.85 ± 0.2^{b}	1.17 ± 0.03^{a}
MeJA 0.1 mM	28.8 ± 0.5^{a}	10.6 ± 0.5^{a}	2.9 ± 0.3^{a}	4.58 ± 0.09^{c}	19.4 ± 0.1^{c}	1.20 ± 0.01^{a}
MeJA 0.01 mM	28.2 ± 0.5^{a}	10.1 ± 0.4^{a}	2.1 ± 0.4^{a}	3.93 ± 0.11^{b}	18.9 ± 0.1^{b}	1.21 ± 0.03^{a}

* Different letters show significant differences ($P < 0.05$) between treatments.

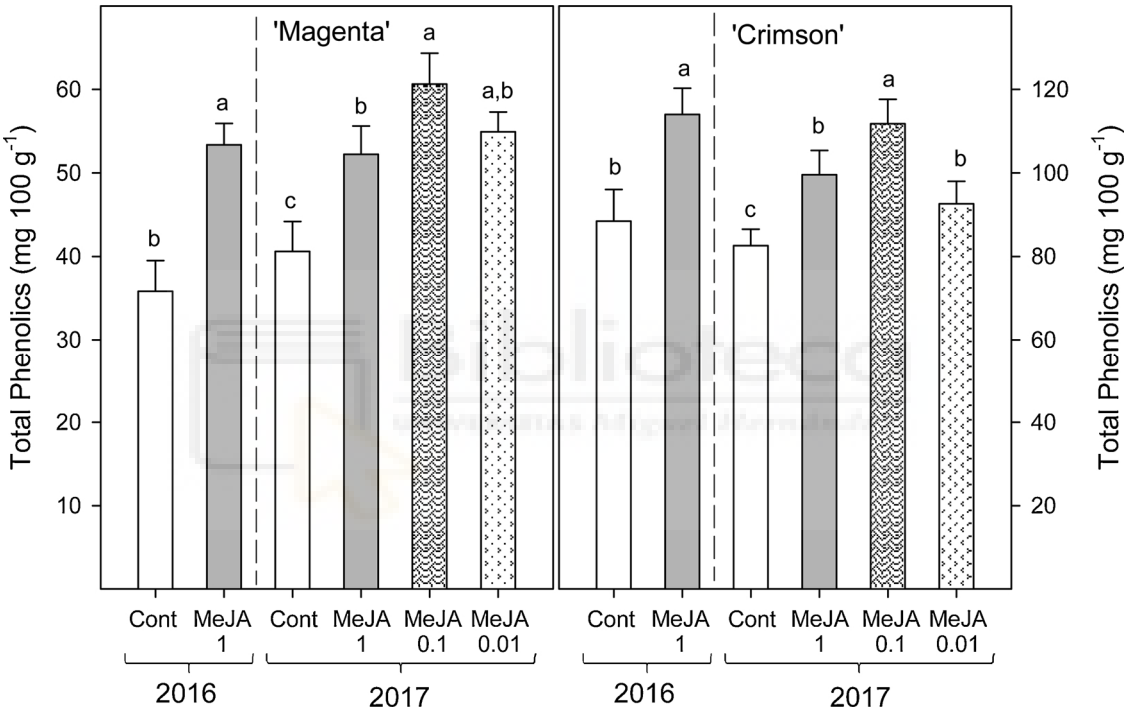

Fig. 6. Total phenolic concentration at harvest in control (Cont) and MeJA-treated (1, 0.1 and 0.01 mM) 'Magenta' and 'Crimson' table grapes. Data are the mean ± SE of three replicates of 20 berries from the first harvest date for both cultivars and all treatments. Different letters show significant differences ($P < 0.05$) among treatments.

affected in 'Crimson' cultivar while a significant reduction was found in 1 mM treated 'Magenta' berries. Thus, preharvest MeJA treatments led to increase table grape organoleptic quality parameters, such as size, weight, firmness and total soluble solids. Accordingly, the application of MeJA at 0.01 or 0.1 mM in blackberry and raspberry cultivars increased the content of TSS, the effect being proportional to the applied concentration (Wang and Zheng, 2005; Wang et al., 2008). In mango, preharvest MeJA treatment led to fruit with higher firmness levels at harvest as well as to increased concentration of glucose, fructose and sucrose (Muengkaew et al., 2016). The effect of MeJA treatments on enhancing fruit TSS and sugar content could be due to an increase of both the net photosynthetic rate of vine and the sink strength of berry cells which would lead to increase sugar accumulation, leading to enhance berry volume and weight. In this sense, it has been reported that MeJA at 1.0 mM stimulated dry matter accumulation in cauliflower seedlings by promoting synthesis of chlorophyll and increasing the net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration (Wu et al., 2012). In turn, MeJA treatment would increase available photoassimilates to support fruit growth. From the agronomic and commercial point of view, the obtained results would have a great importance, since fruit with higher size and harvested earlier would reach higher prizes at markets and berries with higher firmness and TSS would be more appreciated by consumers.

3.3. *Phenolic and anthocyanin concentration*

Table grapes are rich in bioactive compounds, such as phenolics including anthocyanins, flavonoids and resveratrol, which have been reported to have health beneficial effects preventing cardiovascular diseases and having anti-inflammatory, anticancer and anti-diabetic

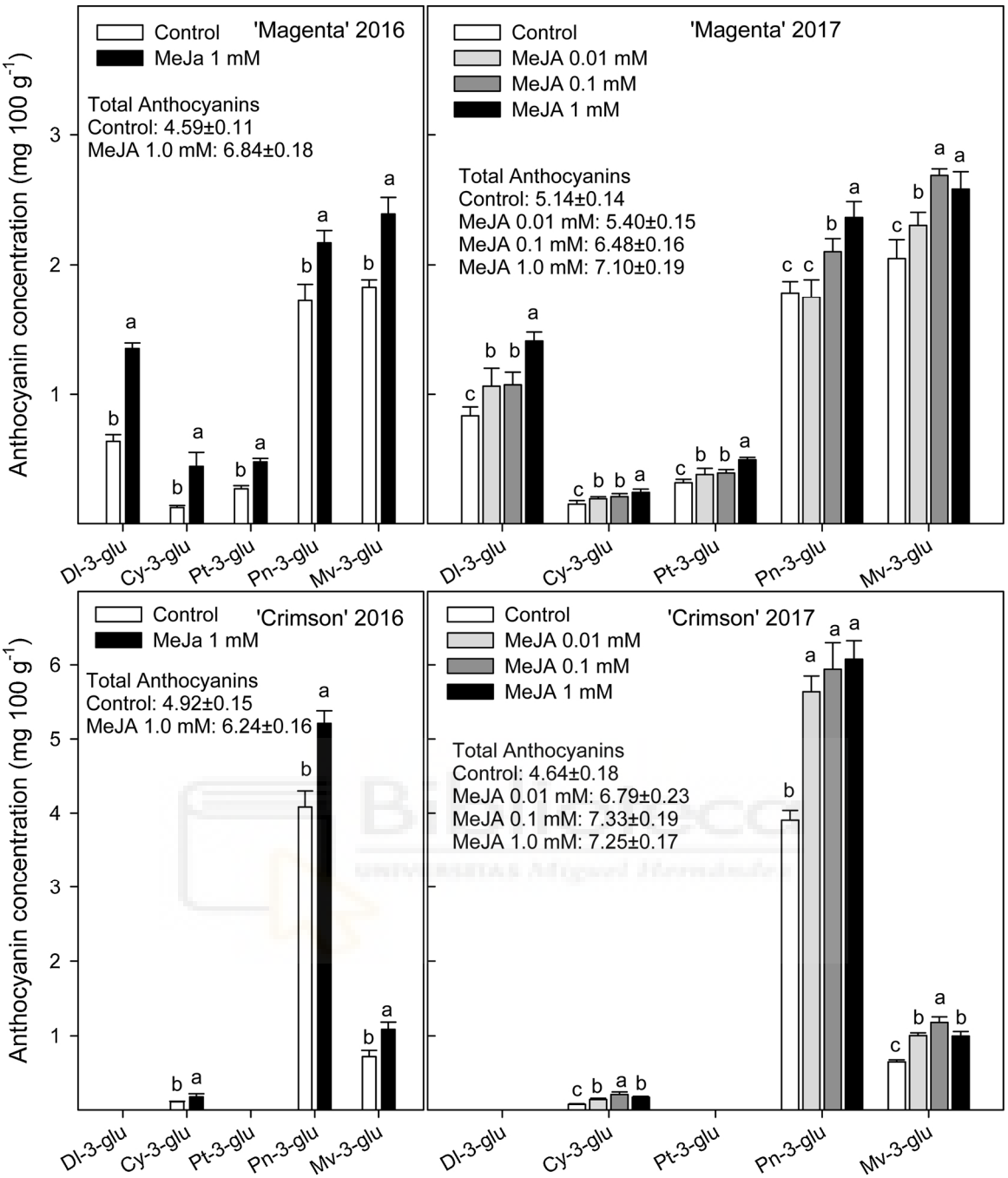

Fig. 7. Individual anthocyanin concentration at harvest in control (Cont) and MeJA-treated (1, 0.1 and 0.01 mM) 'Magenta' and 'Crimson' table grapes. Data are the mean ± SE of three replicates of 20 berries from the first harvest date for both cultivars and all treatments. Different letters show significant differences ($P < 0.05$) among treatments.

activities (Flamini et al., 2013; Doshi et al., 2015) which could depend on the gut microbiota composition (Espín et al., 2017). Previous reports have shown that quality of fruit and vegetable products could be greatly improved by MeJA treatment by inducing accumulation of bioactive compounds, such as glucosinolates in broccoli and radish sprouts (Baenas et al., 2016), phenolics in artichoke (Martínez-Esplá et al., 2017), anthocyanins and other polyphenol compounds (hydrophilic antioxidants) in plums (Martínez-Esplá et al., 2014), black currants (Flores and Ruiz Del Castillo, 2016), raspsberries (Flores and Ruiz Del Castillo, 2015), mangos (Muengkaew et al., 2016) and apples (Ozturk et al., 2015), as well as in grapevine and wines (Ruiz-García et al., 2013; Portu et al., 2015, 2016; Gil-Muñoz et al., 2017), as a result of enhanced phenylalanine ammonialyase (PAL) activity. Accordingly, the present results show that total phenolic concentration at harvest was increased 1.3 and 1.5-fold by MeJA 1 mM treatments in 'Crimson' and 'Magenta' cultivars, respectively, in 2016 experiment. Similarly, in 2017 experiments, an increase in phenolic concentration was found as a consequence of MeJA treatments, the most effective concentration being 0.1 mM in both cultivars (Fig. 6).

On the other hand, five anthocyanins were identified and quantified in 'Magenta' cultivar for the first time, the main ones being peonidin 3-glucoside (Pn-3-glu) and malvidin 3-glucoside (Mv-3-glu), with concentrations of 1.7–2.0 mg 100 g^{-1}, in control berries of 2016 and 2017 experiments, respectively, followed by delphinidin 3-glucoside (Dl-3-glu, 0.6–0.8 mg 100 g^{-1}), while cyaniding 3-glusocide (Cy-3-glu) and petunidin 3-glucoside (Pt-3-glu) were found at lower concentrations

(Fig. 7). However, in 'Crimson' table grapes just three anthocyanins were found, the major one being Pn-3-glu with concentration ca. 4 mg 100 g^{-1} in control grapes followed by Mv-3-glu (0.6–0.7 mg 100 g^{-1} in control grapes) and just traces of Cy-3-glu were found (Fig. 7). Ac- cordingly, Olivares et al. (2017) reported that Pn-3-glu accounted for 69% of 'Crimson' table grape anthocyanin content followed by Mv-3- glu with 15% and Cy-3-glu with 11%, although they also reported minor concentrations of Dl-3-glu and Pt-3-glu. However, Baiano and Terracone (2011) only found two anthocyanins in this cultivar, Pn-3- glu as major one and Cy-3-glu as minor one as well as Ferrara et al. (2015) who reported that Pn-3-glu composed around 85% and Cy-3-glu ca. 5% of total anthocyanins. Thus, total and individual anthocyanin concentration, as well as their profile, depend not only on genetic factors but also on environmental conditions and viticulture practices. In this sense, it has been recently reported that cluster thinning and girdling can influence profile and concentration of individual antho- cyanins in 'Sugrathirteen' table grape (Basile et al., 2018). Nevertheless, it is worth noting that MeJA treatments increased total and individual anthocyanin concentration, in both cultivars and years, the higher ef- fects being found for 1 and 0.1 mM MeJA treatments (Fig. 7). Taking into account that both cultivars were growth under similar environ- mental and agronomic conditions, these differences are due to MeJA treatments.

No previous reports are available in the literature regarding the effect of MeJA treatment on anthocyanin content in table grape although in grapevine the foliar application of MeJA increased anthocyanins in grape and wine, the magnitude of these effects being affected by growing season and variety (Portu et al., 2015, 2018a, 2018b; Gómez-Plaza et al., 2017). For instance, two applications of MeJA 10 mM, at veraison and one week later, or three applications, at veraison and three and six days later, increased total anthocyanin concentration in 'Garnacha' (Portu et al., 2017), 'Tempranillo' (Portu et al., 2015, 2016, 2018b), 'Monastrell' and 'Merlot' (Ruiz-García et al., 2013; Gómez-Plaza et al., 2017) but not effect was found in 'Graciano' (Portu et al., 2018a) or 'Syrah' (Gómez-Plaza et al., 2017). Accordingly, treatment with MeJA at 0.05 and 0.1 mM thirty days before harvest promoted anthocyanin biosynthesis in peach by increasing the expression of genes codifying enzymes involved in anthocyanin biosynthesis pathway (Wei et al., 2017). Moreover, in apple fruit this effect was dose-dependent since 1 and 0.1 MeJA mM postharvest treatments promoted anthocyanin accumulation by up-regulating these genes while 10 mM inhibited anthocyanin biosynthesis (Feng et al., 2017), which is in accordance with the present results (Figs. 1–3). In addition, it has been also reported that MeJA treatment increases some lipophilic antioxidant compounds such as carotenoids and vitamin E, which together with the hydrophilic ones previously commented would lead to improve quality and health properties of fresh fruit and vegetable consumption (Wang et al., 2008; Muengkaew et al., 2016; Reyes-Díaz et al., 2016; Serrano et al., 2018).

4. Conclusion

Taking into account that MeJA is already classified by the U.S. Food and Drug Administration (FDA) as "Generally Recognised as Safe" (GRAS, FDA-EPA, 2013), results from the present research show that it could be considered as a useful tool to increase economic profit of table grape growers. In fact, MeJA treatments at 0.1 mM applied at key points of berry development accelerated berry ripening, mainly colour evolution due to increased anthocyanin biosynthesis, leading to earlier harvest and increased vine yield in both table grape cultivars. In addition, berry quality parameters, such as size, weight, firmness and TSS, were also enhanced by these treatments. Finally, it is worth noting that higher concentrations of bioactive compounds with antioxidant activity, such as phenolics and individual anthocyanins, were found in grapes from treated vines than in controls, for both cultivars and years, leading to berries with increased health properties. Nevertheless, the possibility of reducing the number of MeJA treatments deserves further research.

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4.2. Publication 2

PUBLICATION 2 (Literal transcription)

Preharvest application of methyl jasmonate increases crop yield, fruit quality and bioactive compounds in pomegranate 'Mollar de Elche' at harvest and during postharvest storage

María E. García-Pastor, María Serrano, Fabián Guillén, María J. Giménez, Domingo Martínez-Romero, Daniel Valero, Pedro J. Zapata

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Preharvest application of methyl jasmonate increases crop yield, fruit quality and bioactive compounds in pomegranate 'Mollar de Elche' at harvest and during postharvest storage

María E García-Pastor,[a] María Serrano,[b] Fabián Guillén,[a] María J Giménez,[a] Domingo Martínez-Romero,[a] Daniel Valero[a] and Pedro J Zapata[a*]

Abstract

BACKGROUND: Previous reports have addressed the effectiveness of postharvest methyl jasmonate (MeJA) treatments on maintaining quality properties of pomegranate fruit during storage. However, there is no literature regarding the effects of preharvest MeJA treatments on pomegranate 'Mollar de Elche' crop yield, fruit ripening, quality attributes and bioactive compounds content (at harvest or after long-term storage), which were evaluated in this research.

RESULTS: Preharvest MeJA treatments (1, 5, and 10 mmol L^{-1}) increased pomegranate crop yield. MeJA at 1 and 5 mmol L^{-1} accelerated the on-tree ripening process, while it was delayed with 10 mmol L^{-1}. Losses in fruit weight, firmness and organic acids during storage at 10 °C were delayed in MeJA treated fruit, leading to quality maintenance. In addition, MeJA treatments improved arils colour due to increased concentration of total and individual anthocyanins, at harvest and during storage. Total phenolic and ascorbic acid contents and total antioxidant activity [hydrophilic (H-TAA) and lipophilic (L-TAA) fractions] were also higher in arils from treated pomegranate fruits than in controls.

CONCLUSION: Preharvest treatments with MeJA could be a promising tool to improve pomegranate crop yield, fruit quality and its content in bioactive compounds at harvest and during storage. The higher effects were obtained with MeJA at 5 mmol L^{-1} dose, which could be the selected treatment for practical application purposes.

Keywords: ripening process; colour; organic acids; anthocyanin; antioxidant activity

INTRODUCTION

Pomegranate fruit (*Punica granatum* L.) is one of the oldest known edible fruit which is usually consumed as fresh fruit, although it is also used to elaborate juices, jams, liqueurs and other processed products.[1,2] Pomegranate is highly appreciated by consumers due to its high organoleptic properties and content on a wide range of phytochemical compounds with antioxidant potential, mainly phenolics, including anthocyanins and other complex flavanoids and hydrolysable tannins (ellagitannins, gallic acid and ellagic acid). These compounds are responsible for the health beneficial properties of pomegranate fruit consumption namely protection against some degenerative diseases, such as cardiovascular diseases, inflammations, infarct brain ischemia, Alzheimer, diabetes and cancers, among others.[3–5] There have been described more than 1000 ornamental and edible cultivars of pomegranate worldwide, the last ones being further divided into sour, sweet – sour and sweet cultivars according to their titratable acidity content and juice taste.[2,6] Among Spanish cultivars, the most cultivated one is 'Mollar de Elche', which is very much appreciated by consumers due to its high concentration of sugars and low acidity and its barely discernible seeds, since they are very small and soft and can be easily eaten.[7–9] Production of this cultivar is safeguarded by a Protected Designation of Origin (PDO) since 2016.[10] However, its arils have a pale pink colour because they have lower anthocyanin content than other Spanish cultivars, such as 'White', 'CG8', 'Katirbasi' or 'Wonderful'[9,11] or other Tunisian, Turkish or Iranian cultivars[12–14] which depreciates its value in international markets. In recent years, research has been performed aimed to find preharvest treatments with natural compounds to increase fruit quality at harvest and to maintain it during storage, due to consumers' concerns and legal restrictions regarding the use of postharvest chemical treatments. Methyl jasmonate (MeJA) is an endogenous plant hormone derived from the jasmonic acid (JA) and both of them play important roles in plant development, mainly

* *Correspondence to: PJ Zapata, Department of Food Technology, EPSO, University Miguel Hernández, Ctra. de Beniel km 3,2 03312, Orihuela, Alicante, Spain. E-mail: pedrojzapata@umh.es*

a *Department of Food Technology, EPSO, University Miguel Hernández, Orihuela, Alicante, Spain*

b *Department of Applied Biology, EPSO, University Miguel Hernández, Orihuela, Alicante, Spain*

inducing defence mechanisms against pathogens and abiotic stresses, although they are also involved in the regulation of fruit growth and ripening.[15,16] Currently, most of the knowledge about MeJA effects on fruit quality attributes and ripening is derived from postharvest treatments, which have been focused on reducing a number of stress-induced injuries during the postharvest period such as chilling injury, infection by some pathogens and mechanical stress among others.[16] In addition, MeJA treatments applied as foliar spray during fruit development on tree, either in climacteric or non-climacteric fruits, have been reported to increase phenolic and anthocyanin concentrations in table grapes,[17] plums,[18] black currants[19] and apples[20] as well as phenolic concentration in juice and flavedo of lemon fruits.[21] Similar effects of preharvest MeJA treatments on increasing phenolic compound have been achieved on artichoke heads.[22] Moreover, it is interesting to point our that MeJA has been classified as 'generally recognized as safe' (GRAS) substance by the US Food and Drug Administration (FDA).[23] Thus, MeJA can be considered as an alternative to improve quality and heath beneficial properties of fruits and vegetables.[16,24]

Specifically, in pomegranate literature about the effect of preharvest or postharvest MeJA treatments is scarce. In fact, only one paper is available regarding postharvest treatment with MeJA in which MeJA was applied to 'Mollar de Elche' pomegranate as vapour treatment at 0.1 and 0.01 mmol L^{-1} in sealed containers for 16 h at 20 °C. Results showed that MeJA reduced chilling injury symptoms and delayed the postharvest ripening process during storage at 2 °C, manifested by lower acidity and firmness losses, with the additional benefit of maintaining higher levels of antioxidant compounds such as total phenolics and anthocyanins.[25] However, preharvest MeJA treatment of pomegranate has been assayed only in a recent paper on 'Malas' cultivar, in which MeJA was applied at 1 and 2 mmol L^{-1} 15 days before harvesting and results showed a significant effect on reducing chilling injury damage during storage at 4 °C as well as on increasing total anthocyanin and flavonoid concentrations at harvest and during storage.[26] However, in this previous paper the effects of preharvest MeJA treatment on pomegranate crop yield, fruit growth and ripening on tree were not reported which are studied for the first time in the present experiment by using a sweet pomegranate cultivar, 'Mollar de Elche', and applying MeJA treatments at monthly intervals. In addition, the effects of preharvest MeJA treatments on quality attributes of the whole fruit and arils, individual sugars and acids content, total phenolics, total and individual anthocyanin concentration and antioxidant activity at harvest and during 60 days of storage at 10 °C (a non-chilling temperature) are new goals addressed in the present research.

MATERIAL AND METHODS

Plant material and experimental design

The experiment was performed in a commercial orchard located in Elche, south of Alicante, Spain, in 2016, and repeated in 2017 season, by using 8 years-old trees (planted at 6 m × 5 m) of the cultivar 'Mollar de Elche'. For each treatment (control and 1, 5 and 10 mmol L^{-1} MeJA), three blocks of two trees each one were selected. Each block or replicate for the four treatments was set in a row, leaving an untreated tree between each block and an untreated row between each treated row in order to avoid treatment cross effects. In addition, at least one tree without treatment was left in each row to avoid edge effect. Treatments were performed by applying 3 L of freshly prepared MeJA (Sigma Aldrich, Madrid, Spain) at 1, 5 or 10 mmol L^{-1}, containing 1 mL L^{-1} Tween-20, to each tree at monthly intervals (94, 64, 34 and 4 days before harvesting). Control trees were sprayed with distilled water containing 1 mL L^{-1} Tween-20. The concentrations and dates of application were selected based on previous experiments with other fruits such as table grape,[17] plum[18] and lemon[21] and taking into account the harvest dates of this cultivar in similar growing conditions in previous seasons, so that the first treatment was applied before the skin and colour changes and the last one 4 days before the first harvest. Pruning, thinning, fertilization [nitrogen/phosphorus/potassium (N/P/K) 160/80/160] and irrigation by using 4 L per hour-water pipes (April: two watering cycles of 1 h per week, May, June, July, August and September: two watering cycles of 2 h per week and October: one watering of 1 h) were carried out during the experiments according to standard cultural practices for pomegranate. The fruits were harvested mainly based on commercial criteria of fruit size, colour and total soluble solids (TSS) content characteristic of this cultivar. Due to the heterogeneous fruit on-tree ripening process, two harvests were made in two dates, separated by 21 days. In both harvest dates, the yield of each tree was measured (kilograms per tree and number of fruits per tree) and with these data the average fruit mass was calculated. Taking into account that most of the fruits were harvested at the first harvest date, fruits from the first harvesting were used to measure fruit quality properties at harvest and to perform the storage experiment as follow. From the fruits harvested from each tree, 25 fruits were taken at random and the fruits from the two trees of each block were mixed so that 50 fruits were taken for each replicate and transported to the laboratory immediately. This procedure ensures that a homogeneous sample containing fruits from the different locations around the tree canopy was taken for each replicate which was representative of the commercial harvest. Then, three lots of ten fruits, homogenous in size and colour and without visual defects, were made for each replicate and treatment. One lot of each replicate and treatment was used to measure fruit properties at harvest (day 0) and the remaining were stored at 10 °C and relative air humidity of 85 to 90%. After 30 and 60 days of storage one lot of each treatment and replicate was taken at random in which fruit properties during storage were measured.

Respiration rate and quality parameters

For each sampling date during storage each lot was weighed, and weight loss was expressed as percentage with respect to weight at day 0. For respiration rate, five fruits of each replicate were hermetically sealed in a 3 L jar for 60 min. After that, 1 mL from the holder atmosphere was withdrawn and used for carbon dioxide (CO_2) quantification in a gas chromatograph with thermal conductivity detector and the chromatographic conditions previously described.[27] Respiration rate was expressed as μmol kg^{-1} s^{-1}. The determination of fruit firmness was carried out individually in each of the ten fruits of each replicate by using a TX-XT2i Texture Analyzer (Stable Mycrosystems, Godalming, UK) which applied a force to achieve a 3% deformation of the fruit diameter. Results are expressed as the relation between the applied force and the distance travelled (N mm^{-1}) and are the mean ± standard error (SE). Finally, external colour was determined along six points of the equatorial perimeter in each of the ten fruits from each replicate, using the CIE Lab system in a colorimeter (CRC200, Minolta Camera Co., Tokyo, Japan). After recording L^*, a^*, and b^* parameters, colour was expressed as hue angle (arctg b^*/a^*). After that, fruits were peeled and the arils of the ten fruits of each replicate were combined to obtain a homogeneous sample for each replicate. The

aril colour was measured with the same colorimeter on the surface of a Petri dish filled with arils.

Individual sugars and organic acids content

For sugars and organic acids determinations, 5 g of the aril sample of each replicate were extracted with 5 mL of water and the supernatant was filtered through 0.45 µm Millipore filter and injected into a high-performance liquid chromatography (HPLC) system (Hewlett-Packard HPLC series 1100). The elution system consisted of 0.1% phosphoric acid running isocratically with a flow rate of 0.5 mL min^{-1} through a Supelco column (Supelcogel C-610H, 30 cm 7.8 mm, Supelco Park, Bellefonte, PA, USA). Organic acids were detected by absorbance at 210 nm and sugars by refractive index detector. Results were expressed as g kg^{-1}. A standard curve of pure sugars and organic acids purchased from Sigma (Poole, UK) was used for quantification. Results are the mean ± SE of three replicates.

Total phenolics, total anthocyanin and antioxidant activity quantification

Phenolic extraction was performed by homogenizing 5 g of aril sample with 10 mL of water/methanol (2:8) containing 2 mmol L^{-1} sodium fluoride (NaF) (to inactivate polyphenol oxidase activity and prevent phenolic degradation). The extracts were centrifuged at 10 000 × *g* for 10 min at 4 °C and the total phenolics in the supernatant were quantified using the Folin– Ciocalteu reagent as previously described by Sayyari *et al.*[25] The results were expressed as g gallic acid equivalent (GAE) kg^{-1} and are the mean ± SE of three replicates. To extract anthocyanins, 5 g of arils were homogenized in 15 mL of methanol/formic acid/water (25:1:24, *v*/*v*/*v*) and then, centrifuged at 10 000 × *g* for 10 min at 4 °C. Total anthocyanin content (TAC) was quantified spectrophotometrically at 520 nm and expressed as g kg^{-1} of cyanidin 3-*O*-glucoside equivalents (Cy 3-glu, molar absorption coefficient of 23 900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}). Total antioxidant activity (TAA) was quantified according to the procedure described in Sayyari *et al.*,[25] which enables to determine TAA due to both hydrophilic (H-TAA) and lipophilic (L-TAA) compounds in the same extraction. In brief, for each sample, 5 g of arils were homogenized in 10 mL of 50 mmol L^{-1} phosphate buffer pH 7.8 and 5 mL of ethyl acetate, and then centrifuged at 10 000 × *g* for 15 min at 4 °C. 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 extract was proportional to the 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, and results are expressed as grams of Trolox equivalent (TE) per kilogram and are the mean ± SE of three replicates.

Individual anthocyanin quantification

To identify and quantify individual anthocyanin concentration, the extracts obtained for total anthocyanin measurement described previously were filtered through a 0.45 µm polyvinylidene fluoride (PVDF) filter (Millex HV13, Millipore, Bedford, MA, USA). Identification of anthocyanins 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 (DAD) and a mass detector in series (Agilent Technologies, Waldbronn, Germany) working as described in Martínez-Esplá *et al.*[18] For anthocyanin quantification, extracts were injected into a HPLC system (Agilent HPLC 1200 Infinity series) with the chromatographic conditions previously reported.[18] Chromatograms were recorded at 520 nm and quantification was performed by using calibration curves carried out with Cy 3-glu, cyanidin 3,5-*O*-di-glucoside (Cy 3,5-di-glu), pelargonidin 3-*O*-glucoside (Pl 3-glu) and pelargonidin 3,5-*O*-di-glucoside (Pl 3,5-di-glu) purchased from Sigma-Aldrich (Darmstadt, Ger- many). Delphinidin 3-*O*-glucoside (Dp 3-glu) and delphinidin 3,5-*O*-di-glucoside (Dp 3,5-di-glu) were quantified as Cy 3-gluc equivalents. Results were expressed as mg kg^{-1} (mean ± SE of three replicates).

Statistical analysis

Results are expressed as mean ± SE of three replicates. Data for the analytical determinations were subjected to analysis of variance (ANOVA). Sources of variation were storage time and treatment. Mean comparisons were performed using HSD Tukey's test to examine if differences between control and treated fruit were significant at $P < 0.05$. All analyses were performed with SPSS software package v. 17.0 for Windows.

RESULTS AND DISCUSSION

Crop yield and on-tree fruit ripening

The effect of MeJA treatments on pomegranate crop yield and fruit ripening and quality attributed was assayed in 2016 and 2017 seasons, and similar results were obtained. Thus, just the results of the 2016 season are provided. Pomegranate fruit were harvested at commercial ripening stage, based on size, skin colour and TSS content (*ca* 16 °Brix) characteristic for 'Mollar de Elche' cultivar, according to commercial practices, and two harvests were made separated 3 weeks apart. Total yield (in kilograms per tree) was 37.75 ± 2.08 in control trees and significantly higher ($P < 0.05$), 32.8%, 38.1%, and 21.8%, in 1, 5 and 10 mmol L^{-1} MeJA treated-trees, respectively. This increase was due to the number of fruit that were harvested per tree which was significantly increased by 1, 5 and 10 mmol L^{-1} MeJA treatments while no significant effects were observed in fruit mass attributed to MeJA treatments (Fig. 1). In addition, it is worth noting that tree yield at the first harvest date was significantly higher ($P < 0.05$) in 1 and 5 mmol L^{-1} MeJA-treated trees (42.24 ± 0.67 and 43.79 ± 3.06 kg per tree, respectively) than in controls (28.65 ± 2.16 kg per tree), while a significantly lower yield was obtained with the highest MeJA concentration (19.19 ± 1.27 kg per tree) in this first harvest date (Fig. 1). These results show that the on-tree growth and ripening processes were accelerated by 1 and 5 mmol L^{-1} MeJA treatments since more fruits had reached their commercial harvesting attributes at the first harvest date, while the contrary occurred with 10 mml L^{-1} MeJA treatment. Similar effects of preharvest MeJA treatments have been found in wines and table grapes, although with different MeJA concentrations. Thus, preharvest 0.1 and 1 mmol L^{-1} MeJA treatments accelerated the ripening process in 'Magenta' and 'Crimson' table grapes, manifested by higher berry growth and anthocyanin accumulation in skin, while it was delayed by 5 and 10 mmol L^{-1} MeJA treatments and even many berries failed to ripen properly with the highest concentration, due to failure to synthesize anthocyanins, this effect being higher

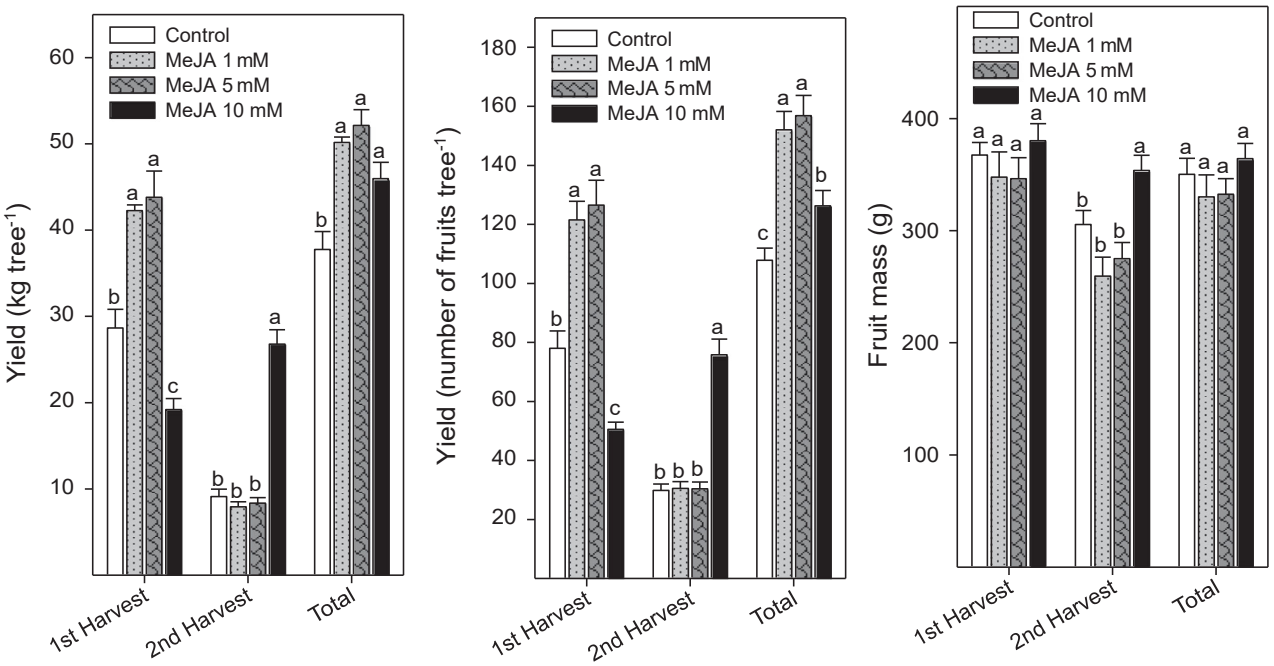

Figure 1. Effects of methyl jasmonate (MeJA) treatments on crop yield (kilograms per tree and number of fruit per tree) and fruit weight at the two harvest dates and at total harvest. Data are the mean ± standard error of three replicates. Different letters show significant differences at $P < 0.05$.

in 'Crimson' than in 'Magenta'.[17] Accordingly, D'Onofrio *et al.*[28] reported that the application of MeJA 10 mmol L^{-1} to 'Sangiovese' wine grape cultivar slowed down the berry ripening process and delayed by 10 days the technological maturity, while acceleration of the ripening process has been reported in 'Fujimino' wine grape cultivar by 0.01 and 0.1 mmol L^{-1} MeJA preharvest treatments by increasing the expression of genes involved in fruit colouring, cell-wall hydrolysis and aroma compound biosynthesis.[29] The effect of 1 and 5 mmol L^{-1} MeJA treatments on advancing pomegranate fruit ripening has important economic implications since early harvested fruit usually reach higher prices in the market. However, a limited number of papers are available regarding the effect of MeJA on vegetable product size and crop yield, and contradictory results have been reported. Thus, preharvest treatments of plum trees with several concentrations of MeJA (0.5, 1, and 2 mmol L^{-1}) increased crop yield, fruit size and weight in 'Black Splendor' and 'Royal Rosa' cultivars, the most effective concentration being dependent on cultivar while no effect was observed on number of fruit per tree.[18] In a similar way, 0.01 and 0.1 mmol L^{-1} preharvest MeJA treatments increased berry size and total yield in 'Magenta' and 'Crimson' table grape cultivars,[17] while MeJA application to lemon trees, from 0.1 to 1.0 mmol L^{-1} did not affect yield or fruit weight.[21] In the present experiments, the increase in yield was due to an increase in the number of harvested fruits showing that MeJA applied at these concentrations could have reduced the abscission process during fruit growth and ripening on tree together with an increment in the plant photoassimilates available to support fruit growth. In this sense, a net increase in chlorophyll content and photosynthesis rate has been reported in cauliflower seedlings after 1.0 mmol L^{-1} MeJA treatment.[29]

Respiration rate and quality parameters at harvest and during storage

The respiration rate of pomegranate fruit was significantly lower ($P < 0.05$) in fruit from MeJA-treated trees than in those from control trees at harvest. During postharvest storage at 10 °C, respiration rate decreased in both, control and treated fruit, although it remained significantly lower in fruits from MeJA-treated trees than in controls (Table 1). MeJA 5 mmol L^{-1} was the treatment that led to the lowest values of respiration rate, at harvest and after 30 days of storage (1.2 and 1.6-fold decrease, respectively, with respect to controls). Similar effects on reducing the respiration rate during postharvest storage were observed on preharvest MeJA-treated plums by Zapata *et al.*[27] which were related with a reduced cell metabolism rate and a delay on senescence process during storage.

Firmness values at harvest were not significantly affected by MeJA treatments (Table 1). However, all MeJA treatments significantly ($P < 0.05$) decreased firmness losses of pomegranate fruit during storage, this effect being still evident after 60 days of storage in 5 mmol L^{-1} MeJA treated fruit. Accordingly, Sayyari *et al.*[25] showed that postharvest MeJA treatment delayed softening of 'Mollar de Elche' pomegranate fruit during storage at 2 °C, which was attributed to an effect of MeJA on reducing pectinmethylesterase (PME) activity leading to decreased de-esterification of pectin and in turn, to maintain fruit firm- ness. Furthermore, the increase in weight loss during storage was delayed in MeJA treated fruits, with final values of 8.78 ± 0.58% in control fruits and significantly ($P < 0.05$) lower values in all MeJA treated fruit (Table 1). With respect to skin colour, no significant differences were observed in hue angle between control and MeJA treated fruit at harvest (Table 1) as expected because pomegranate fruits were harvested when their commercial ripening stage was reached, mainly assessed by skin colour and size. During storage, hue angle increased in fruit from control and treated trees, although it was significantly lower in fruit from MeJA treated trees (Table 1), showing a delay in the skin browning process that occurred during storage as a consequence of MeJA treatments. However, the arils hue angle at harvest was significantly lower ($P < 0.05$) in fruit from 1 (30.60 ± 1.13), 5 (28.55 ±0.82) and

Table 1. Respiration rate (RR, µmol kg^{-1} s^{-1}), firmness (N mm^{-1}), weight loss (%) and colour (hue angle) of pomegranate fruits from control and methyl jasmonate (MeJA, 1, 5, and 10 mmol L^{-1}) treated trees at harvest and after 30 and 60 days of postharvest storage at 10 °C

Parameter	Days	Control	MeJA (1 mmol L^{-1})	MeJA (5 mmol L^{-1})	MeJA (10 mmol L^{-1})
RR	0	0.151 ± 0.002^{aA}	0.134 ± 0.003^{bcA}	0.122 ± 0.006^{cA}	0.143 ± 0.002^{bA}
	30	0.115 ± 0.008^{aB}	0.088 ± 0.006^{bB}	0.072 ± 0.002^{cB}	0.092 ± 0.003^{bB}
	60	0.081 ± 0.007^{aC}	0.055 ± 0.002^{bC}	0.054 ± 0.002^{bC}	0.057 ± 0.001^{bC}
Firmness	0	16.79 ± 0.74^{aA}	17.56 ± 0.67^{aA}	18.51 ± 0.45^{aA}	16.99 ± 0.34^{aA}
	30	10.70 ± 0.51^{bB}	16.00 ± 0.54^{aA}	16.45 ± 0.74^{aA}	15.35 ± 0.67^{aA}
	60	9.90 ± 0.99^{bB}	11.77 ± 0.61^{bB}	12.82 ± 0.31^{aB}	10.89 ± 0.41^{bB}
Weight loss	0	–	–	–	–
	30	6.14 ± 0.46^{aA}	4.86 ± 0.25^{bA}	3.44 ± 0.30^{cA}	4.79 ± 0.30^{bA}
	60	8.78 ± 0.38^{aB}	6.45 ± 0.36^{bB}	4.90 ± 0.25^{cB}	6.02 ± 0.32^{aB}
Skin hue angle	0	46.78 ± 1.47^{aA}	44.23 ± 1.33^{aA}	46.49 ± 1.57^{aA}	50.45 ± 1.87^{aA}
	30	57.11 ± 1.09^{aB}	49.22 ± 1.72^{bA}	48.14 ± 1.88^{bA}	51.65 ± 1.43^{bA}
	60	64.40 ± 1.81^{aC}	56.48 ± 1.96^{bB}	55.90 ± 2.01^{bB}	58.61 ± 1.37^{bB}
Arils hue angle	0	39.91 ± 1.68^{aA}	30.60 ± 1.13^{bcA}	28.55 ± 0.82^{bA}	32.55 ± 1.30^{cA}
	30	34.51 ± 1.21^{aB}	26.32 ± 1.20^{bB}	24.09 ± 0.65^{bB}	27.49 ± 0.88^{bB}
	60	32.02 ± 1.29^{aB}	24.49 ± 0.47^{bB}	21.23 ± 0.85^{cC}	24.78 ± 1.13^{bB}

Data are the mean ± standard error of three replicates. For each parameter, different lowercase letters within a row show significant differences at $P < 0.05$ among treatments and different capital letters within a column show significant differences at $P < 0.05$ during storage.

10 (32.55 ± 1.30) mmol L^{-1} MeJA-treated trees than in controls (39.91 ± 1.68), showing a deeper red colour in the arils of MeJA treated fruits, especially for 5 mmol L^{-1} MeJA dose in which the lowest hue value was obtained. During storage, hue angle decreased in arils from control and treated fruit, with values being significantly lower ($P < 0.05$) in arils from treated fruit than in controls after 60 days of storage. Thus, preharvest MeJA treatments led to improved red colouration of pomegranate arils, which is an important quality trait for this cultivar, due to increased anthocyanin concentration as will be addressed in the next section.

'Mollar de Elche' pomegranate is a sweet cultivar very much appreciated by consumers due to its balanced sugar and acid content, with TSS of 15 to 16 °Brix and titratable acidity of 0.2 to 0.3 g 100 g^{-1}.[8,9,30] Fructose was the major sugar in pomegranate arils, with concentrations ranging from 115 to 120 g kg^{-1}, without significant differences attributed to treatments or storage (Table 2). Glucose was the second major sugar, with concentration of 41.72 ± 1.10 g kg^{-1} in control fruits at harvest and significantly higher ($P < 0.05$) in those from 5 and 10 mmol L^{-1} MeJA treatments. Finally, sucrose was found as minor sugar (*ca* 0.5–06 g kg^{-1}) and

Table 2. Sugar (g kg^{-1}) and organic acid (g kg^{-1}) concentrations in pomegranate arils from control and methyl jasmonate (MeJA, 1, 5 and 10 mmol L^{-1}) treated trees at harvest and after 60 days of storage at 10 °C

Parameter	Days	Control	MeJA (1 mmol L^{-1})	MeJA (5 mmol L^{-1})	MeJA (10 mmol L^{-1})
Fructose	0	116.12 ± 3.32^{aA}	112.30 ± 2.30^{aA}	120.62 ± 1.59^{aA}	119.03 ± 2.68^{aA}
	60	119.40 ± 4.30^{aA}	115.72 ± 3.97^{aA}	122.91 ± 2.44^{aA}	120.25 ± 2.81^{aA}
Glucose	0	41.72 ± 1.10^{bA}	41.61 ± 1.84^{bA}	48.71 ± 1.71^{aA}	47.74 ± 1.23^{aA}
	60	43.11 ± 1.20^{bA}	42.63 ± 1.98^{bA}	49.41 ± 1.60^{aA}	48.50 ± 1.39^{aA}
Sucrose	0	0.60 ± 0.11^{aA}	0.57 ± 0.12^{aA}	0.56 ± 0.13^{aA}	0.59 ± 0.10^{aA}
	60	0.51 ± 0.12^{aA}	0.50 ± 0.10^{aA}	0.50 ± 0.13^{aA}	0.51 ± 0.09^{aA}
Malic acid	0	3.21 ± 0.20^{bA}	3.62 ± 0.24^{bA}	3.93 ± 0.17^{aA}	3.71 ± 0.12^{bA}
	60	2.61 ± 0.11^{bB}	3.43 ± 0.13^{bA}	3.72 ± 0.12^{aA}	3.23 ± 0.20^{bA}
Succinic acid	0	1.72 ± 0.05^{aA}	1.82 ± 0.10^{aA}	2.03 ± 0.13^{aA}	1.90 ± 0.12^{aA}
	60	1.45 ± 0.08^{cB}	1.79 ± 0.14^{abA}	2.01 ± 0.14^{aA}	1.61 ± 0.11^{bcA}
Citric acid	0	0.92 ± 0.03^{aA}	0.61 ± 0.05^{aA}	0.74 ± 0.08^{aA}	0.81 ± 0.06^{aA}
	60	0.78 ± 0.02^{aB}	0.55 ± 0.06^{aA}	0.70 ± 0.04^{aA}	0.67 ± 0.05^{aA}
Fumaric acid	0	0.34 ± 0.01^{aA}	0.32 ± 0.04^{aA}	0.33 ± 0.03^{aA}	0.34 ± 0.02^{aA}
	60	0.27 ± 0.02^{aB}	0.31 ± 0.03^{aA}	0.30 ± 0.02^{aA}	0.28 ± 0.02^{aA}
Oxalic acid	0	0.11 ± 0.01^{aA}	0.12 ± 0.02^{aA}	0.31 ± 0.01^{aA}	0.27 ± 0.01^{aA}
	60	0.10 ± 0.01^{aA}	0.11 ± 0.01^{aA}	0.30 ± 0.02^{aA}	0.26 ± 0.01^{aA}
Ascorbic acid	0	0.39 ± 0.03^{cA}	0.48 ± 0.02^{bA}	0.52 ± 0.05^{aA}	$0.50 \pm 0.04b^{A}$
	60	$0.31 \pm 0.02c^{B}$	0.44 ± 0.03^{bA}	0.49 ± 0.02^{aA}	0.46 ± 0.03^{bA}

Data are the mean ± standard error of three replicates. For each parameter, different lowercase letters within a row show significant differences at $P < 0.05$ among treatments and different capital letters within a column show significant differences at $P < 0.05$ during storage.

without significant differences attributed to treatments. In addition, no significant changes in sugar concentrations were observed during storage either in control or in treated pomegranates. This sugar profile is in agreement with previous reports on other pomegranate cultivars.[7,8,31,32] The major organic acid was malic acid, with concentration of 3.21 ± 0.20 g kg^{-1} in control fruit at harvest and significantly ($P < 0.05$) higher in 5 mmol L^{-1} MeJA treated ones (3.93 ± 0.17 g kg^{-1}), followed by succinic and citric acids while fumaric and oxalic acids were found at lower concentrations and without significant differences among control and treated fruit (Table 2). Generally, all organic acids decreased in the arils of control fruit after 60 days of storage, while no significant changes occurred in treated fruit (Table 2). Accordingly, postharvest MeJA treatment reduced titratable acidity losses during storage,[25] which could be due to the lower respiration rate found in treated fruit with respect to controls and in turn to the lower consumption of these respiratory substrates. Concentration and profile of organic acid depend on ripening stage and cultivar. Thus, in sour cultivars the major organic acid is citric acid with concentration higher than 20 g L^{-1} at commercial ripening stage, while in sweet cultivars the major one is malic acid with concentration even ten-fold lower.[7,8]

Bioactive compounds and total antioxidant activity

It is well known that pomegranates are rich in bioactive compounds with antioxidant activity, such as phenolic compounds, including anthocyanins and ascorbic acid. Although the concentration at time of harvest is affected by several factors, such as cultivars, ripening stage and environmental conditions.[13,14,33–35] In pomegranate arils, loss of ascorbic acid occurred during storage (Table 2) according to previous reports in 'Ganesh',[31] 'Wonderful'[36] and 'Malas'[26] cultivars. However, it is worth noting that the concentration of ascorbic acid was significantly higher in fruit treated with MeJA compared to controls ($P < 0.05$, Table 2). The dose of 5 mmol L^{-1} was the most effective on increasing the ascorbic acid content which led to 33 and 58% higher concentrations at harvest and after 60 days of storage, respectively, with respect to control fruits. Ascorbic acid was also significantly increased by postharvest MeJA treatments on mango fruit[37] which was attributed to the enhanced transcription of the genes involved in the *de novo* biosynthesis of ascorbic acid previously reported in plant cell suspension.[38]

Total phenolic and anthocyanin concentrations at harvest were 0.842 ± 0.015 and 0.135 ± 0.006 g kg^{-1}, respectively in arils of control fruit, which were in the range of data reported for this cultivar in previous papers,[11,25,30] although lower than those reported for other cultivars with more coloured arils.[9,12,26,33,39] Indeed, differences in total and individual phenolic and anthocyanin concentrations have been reported due to ripening stage, agricultural practices and environmental conditions, apart from those attributed to cultivars.[13,30,33,34] For both group of compounds, concentrations increased during storage and were significantly higher ($P < 0.05$) in fruits treated with MeJA than in controls at harvest and during storage, the highest concentrations being found with 5 mmol L^{-1} dose (Fig. 2). Accordingly, MeJA treatments led to accumulation of anthocyanins and other polyphenol compounds in plums,[18] black currants,[19] mangos,[40] apples,[20] table grape,[17]

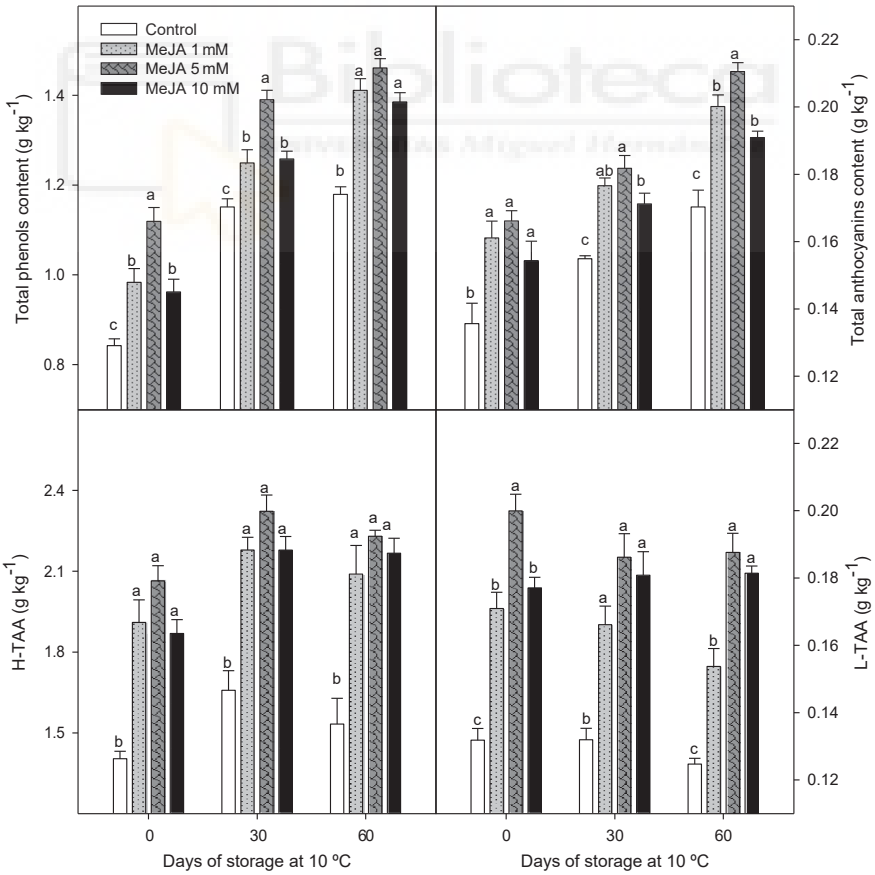

Figure 2. Total phenolics, total anthocyanins and total hydrophilic (H-TAA) and lipophilic (L-TAA) antioxidant activity in pomegranate arils from control and methyl jasmonate (MeJA, 1, 5 and 10 mmol L^{-1}) treated trees at harvest and during postharvest storage at 10 °C. Data are the mean ± standard error of three replicates. Different letters show significant differences at $P < 0.05$.

grapevine and wines[41] and artichoke heads[22] as well as in juice and flavedo of lemon fruits,[21] as a result of enhanced phenylalanine ammonialyase (PAL) activity and increased expression of genes codifying enzymes involved in anthocyanin biosynthesis pathway.[42,43] Then, MeJA treatments would lead to improved quality and health properties of pomegranate consumption, given the role of phenolics, including anthocyanins, and ascorbic acid in these properties.[3,16,23,44]

Antioxidant activity was measured in hydrophilic (H-TAA) and lipophilic extracts (L-TAA) of pomegranate arils and both were significantly increased ($P < 0.05$) as a consequence of MeJA treatments, either at harvest or during storage (Fig. 2). H-TAA was ten-fold higher than L-TAA showing that hydrophilic compounds are the major contributors to antioxidant capacity of pomegranate arils, according to previous reports in the C25 sour – sweet cultivar[45] and in the sweet cultivar 'Gobsi'[35] as well as in eight Iranian cultivars[12] and in a wide range of fresh fruits.[46] Moreover, significant correlations were obtained between H-TAA and total phenolic and total anthocyanin concentrations, $r^2 = 0.625$ and 0.613, respectively, showing that phenolic and anthocyanin compounds are the major compounds responsible for antioxidant properties of pomegranate arils. Ellagitanins has been reported as the predominant phenolic compounds in arils of 'Mollar de Elche' pomegranate, followed by anthocyanins, while proanthocyanidins accounted for just 2%,[47] all of them having great antioxidant potential and being responsible for the health beneficial effects of pomegranate consumption.[4,5] In addition, the increase in L-TAA found in the arils of MeJA treated fruit could be attributed to stimulation of carotenoids and vitamin E biosynthesis, as previously reported[16,23] which would lead to enhance the health properties of pomegranate since these compounds have also antioxidant properties.[46]

Individual anthocyanins profile and concentration

Six anthocyanins were identified and quantified in 'Mollar de Elche' cv. (Fig. 3), the main ones being Cy 3-glu and Cy 3,5-di-glu, followed by Pl 3-glu and Dp 3-glu, while 3Pl 3,5-di-glu and Dp 3,5-di-glu were found at lower concentrations (Fig. 3). Similar anthocyanin profile has been previously reported in arils of 'Mollar de Elche' cultivar, although concentrations of each individual anthocyanin were slightly different[11,30] showing changes due to cultural practices and environmental conditions. Cy 3-glu has also been described as the predominant anthocyanin in 'Wonderful' pomegranate[48] while Dp 3-glu was the major one for some Croatian cultivars,[39] Pl 3,5-di-glu for Iranian cultivars[12] and Cy 3,5-di-glu for Tunisian cultivars[6] providing further evidence of variation in anthocyanin content among pomegranate accession.

However, it is worth noting that 1, 5 and 10 mmol L^{-1} MeJA treatments significantly increased ($P < 0.05$) the concentrations of the major anthocyanins, Cy 3-glu, Cy 3,5-di-glu and Dp 3-glu at harvest with respect to those found in the arils of control fruit and these effects were still evident after 60 days of storage, especially in arils from 5 mmol L^{-1} treatment (Fig. 3). These results are in agreement with the increase in total anthocyanin content found in the arils of MeJA treated fruit (Fig. 2). Accordingly, an increase on total anthocyanin concentration was found in 'Malas' pomegranate at harvest as a consequence of preharvest MeJA treatment.[26] However, no previous reports are available in the literature regarding the effect of MeJA treatment on individual anthocyanin content in pomegranate fruit although some evidence exists for other fruits. Thus, treatment with MeJA at 0.05 and 0.1 mmol L^{-1} 30 days before harvest promoted anthocyanin biosynthesis in peach by increasing the expression of genes codifying enzymes involved in anthocyanin biosynthesis pathway.[43] Moreover, in apple fruit this effect was dose-dependent since MeJA postharvest treatments promoted anthocyanin accumulation by up-regulating the expression of these genes the effect being higher for 1 than for 0.1 mmol L^{-1} doses.[49]

CONCLUSIONS

Preharvest treatments with MeJA at 1, 5, and 10 mmol L^{-1} increased crop yield. In addition, the on-tree fruit ripening process was accelerated by 1 and 5 mmol L^{-1} doses. Quality parameters after 30 and 60 days of storage at 10 °C were maintained at higher levels in MeJA treated fruit, manifested by reduced weight loss, respiration rate and losses of firmness and titratable acidity. Moreover, MeJA

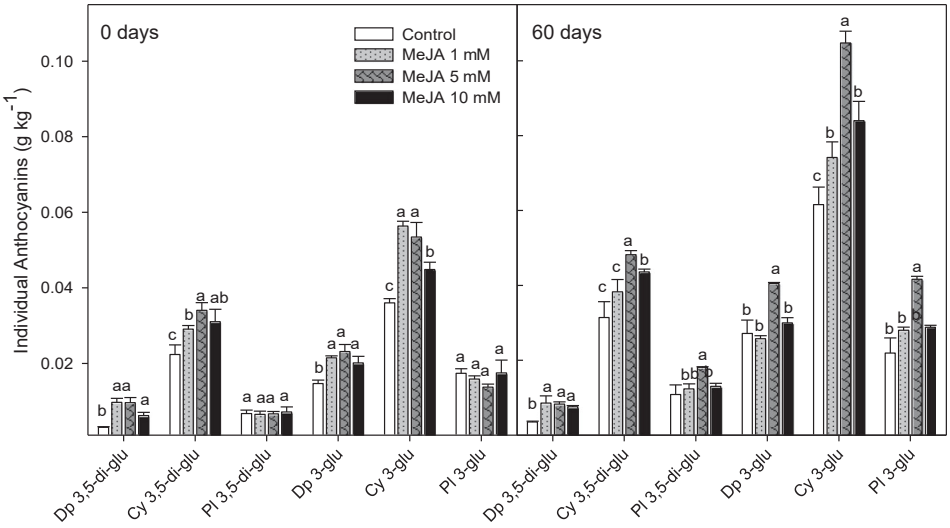

Figure 3. Individual anthocyanin concentration in pomegranate arils from control and methyl jasmonate (MeJA, 1, 5 and 10 mmol L^{-1}) treated trees at harvest and after 60 days of postharvest storage at 10 °C. Data are the mean ± standard error of three replicates. For each individual anthocyanin different letters show significant differences at $P < 0.05$.

treatments improved arils colour and their content in bioactive compounds (phenolics, anthocyanins and ascorbic acid), these effects being maintained during storage. Among the assayed doses, the highest effects were found with MeJA at 5 mmol L^{-1} which could be selected for practical application purposes in order to get earlier harvest and increase pomegranate crop yield, fruit quality and its content in bioactive compounds with health beneficial effects at harvest and during storage.

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4.3. Publication 3

PUBLICATION 3 (Literal transcription)

Preharvest or a combination of preharvest and postharvest treatments with methyl jasmonate reduced chilling injury, by maintaining higher unsaturated fatty acids, and increased aril colour and phenolics content in pomegranate

María E. García-Pastor, María Serrano, Fabián Guillén, Pedro J. Zapata, Daniel Valero

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Preharvest or a combination of preharvest and postharvest treatments with methyl jasmonate reduced chilling injury, by maintaining higher unsaturated fatty acids, and increased aril colour and phenolics content in pomegranate

María E. García-Pastor[a], María Serrano[b], Fabián Guillén[a], Pedro J. Zapata[a], Daniel Valero[a,*]

[a] *Department of Food Technology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Orihuela, Alicante, Spain*
[b] *Department of Applied Biology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Orihuela, Alicante, Spain*

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ABSTRACT

In the present research the effects of preharvest 5 mM methyl jasmonate (MeJa) treatments, alone (Pre) or in combination with postharvest 5 mM MeJa treatment (Pre + Post), on reducing chilling injury (CI) of pomegranate during 90 d of storage at 2 °C plus 3 d at 20 °C and its relationship with changes in fatty acid composition of cell membranes were assayed. In addition, fruit and aril quality traits, total content in phenolics and anthocyanins and antioxidant activity of the arils were evaluated. Both, external and internal CI symptoms and the increase in ion leakage (IL) were reduced by Pre and Pre + Post MeJa treatments. The major fatty acids in pomegranate husk were palmitic, oleic, linoleic and linolenic acids. MeJa treatments led to higher concentration of unsaturated fatty acids (UFA) at harvest, which was maintained at higher levels during storage, while saturated fatty acid (SFA) concentration was lower in treated fruit than in controls. The concentration of total phenolics and anthocyanins were lower in the arils from control fruit than in arils of Pre and Pre + Post treated fruit during the whole storage period. In general, there were no significant differences between Pre and Pre + Post MeJa treatments on their effects on reducing CI, maintaining membrane stability and bioactive compounds with antioxidant activity. Thus, preharvest MeJa treatments may be sufficient to increase the cold storage potential of pomegranate fruit by reducing CI symptoms and enhancing the content bioactive compounds with antioxidant activity.

1. Introduction

Pomegranate fruit (*Punica granatum* L.) is one of the oldest known edible fruit and very appreciated by consumers, due to its high organoleptic properties. Also, the content of a wide range of phytochemical compounds with antioxidant potential has been associated with health beneficial properties, including protection against cardiovascular diseases, inflammations, infarct brain ischemia, Alzheimer, diabetes and cancers, among others (Faria and Calhau, 2011; Asgary et al., 2017; Panth et al., 2017). There are more than 1000 ornamental and edible cultivars of pomegranate described worldwide (Hasnaoui et al., 2011; Pareek et al., 2015). Among these, the Spanish cultivar 'Mollar de Elche' has high quality attributes, such as high sugar and low acid concentration, small and soft seeds and pleasant flavour (Melgarejo et al., 2000; Nuncio-Jáuregui et al., 2014; Fernandes et al., 2017). Recently, the 'Mollar de Elche' cultivar has been safeguarded by a Protected Designation of Origin (DOP) since 2016 [R (UE), 2016/83].

Important quality losses including husk desiccation and browning, decay and loss of firmness occur during postharvest storage of pomegranate fruit. In addition, decreases in ascorbic acid, acidity and colour of the arils led to reduction of consumers' acceptability in terms of freshness, juiciness and taste (Pareek et al., 2015). In order to avoid these undesirable changes and to prolong storability the best postharvest tool has been cold storage. However, pomegranate fruit is sensitive to chilling injury (CI) when stored at temperatures below 5 °C, the main symptoms being skin desiccation, browning and pitting, depression of the fruit surface and higher susceptibility to decay. Thus, recent studies are focused to find out postharvest treatments to be applied in combination with cold storage in order to reduce CI and increase storability. In this sense, positive results have been obtained by postharvest treatments of pomegranates with polyamines (Mirdehghan et al., 2007a), oxalic acid (Sayyari et al., 2010), salicylic acid (Sayyari

* Corresponding author.
E-mail address: daniel.valero@umh.es (D. Valero).

et al., 2009, 2017), acetyl salicylic acid (Sayyari et al., 2011b), methyl salicylate (Sayyari et al., 2011a), and heat treatments (Mirdehghan et al., 2007b). More recently, carboxymethyl cellulose and chitosan edible coatings combined with oxalic or malic acids (Ehteshami et al., 2019), salicyloyl chitosan treatment (Sayyari et al., 2016) and controlled atmosphere storage (Sidhu et al., 2019) were also effective on reducing pomegranate CI.

On the other hand, methyl jasmonate (MeJa), an endogenous plant hormone derived from the jasmonic acid (JA) playing important roles in plant development, such as inducing systemic acquired resistance, providing plant tolerance against different kinds of stress, flowering, seedling germination and regulation of fruit growth and ripening (Wasternack and Strnad, 2016; Serrano et al., 2018) has been reported to reduce CI in a wide range of fruit (Aghdam and Bodbodak, 2013; Glowacz and Ree, 2016). For instance, postharvest MeJa treatment before storage at chilling temperature reduced CI in cherry tomato (Zhang et al., 2012), carambola (Mustafa et al., 2016), kiwifruit (Li et al., 2017), papaya (González-Aguilar et al., 2003), peach (Meng et al., 2009), and mangosteen (Mustafa et al., 2018). Specifically, in pomegranate, only in one previous paper the effect of postharvest MeJa treatment on reducing CI has been reported, with additional effects on delaying the postharvest ripening process and maintaining higher levels of antioxidant compounds (Sayyari et al., 2011a). Accordingly, in a recent paper, it has been reported that MeJa treatments (at 1, 5 and 10 mM) applied during on-tree 'Mollar de Elche' pomegranate fruit growth delayed the postharvest ripening during storage at 10 °C, manifested by lower losses in fruit weight, firmness and organic acids, leading to fruit quality maintenance. In addition, the concentration of antioxidant compounds (phenolics, individual anthocyanins and ascorbic acid) and total antioxidant activity were higher in arils from pomegranate treated fruit than in controls, at harvest and during storage at 10 °C, a non-chilling temperature (García-Pastor et al., 2020). The effects of preharvest MeJa treatment on reducing CI of pomegranate has been assayed only in a recent paper on 'Malas' cultivar (Koushesh Saba and Zarei, 2019), although the mechanism involved in this effect has not been elucidated yet and deserves further research. Thus, the main goal of the present research was to evaluate for the first time the effects of preharvest MeJa treatments, alone or in combination with postharvest MeJa treatment, on reducing CI of pomegranate and its relationship with changes in fatty acid composition of cell membranes during storage. In addition, pomegranate and arils quality and their content in bioactive compounds were also evaluated.

2. Materials and methods

2.1. Plant material and experimental design

The experiment was performed in a commercial orchard located in Elche (south of Alicante, Spain), in 2018 by using 9 years-old trees (planted at 6 x 5 m) of the cultivar 'Mollar de Elche'. Three blocks or replicates of 3 trees each one were randomly selected for 5 mM MeJa treatment and control. Trees were sprayed with 3 L of a freshly prepared 5 mM MeJa (Sigma Aldrich, Madrid) solution, containing Tween-20 (1 mL L^{-1}), or distilled water containing 1 mL L^{-1} Tween-20 at 80, 110, 140 and 170 days after full blossom, the last one being performed 4 d before harvesting. The concentration of MeJa and dates of application were selected based on previous experiments carried out in 2016 and 2017 seasons on this cultivar, in which 1, 5 and 10 mM MeJa doses were applied and the best results in term of yield and fruit quality attributes, at harvest and during storage at 10 °C, were obtained with 5 mM dose (García-Pastor et al., 2020). The fruit were harvested at commercial ripening stage characteristic of this cultivar based on fruit size (≈360 g) and total soluble solids (≈16 g L^{-1}) content. Immediately after harvest about 20 fruit were taken from each tree, that is, 60 fruit from each block or replicate and were transferred to the laboratory. Pomegranates with defects (sunburn, crack, bruise and cut in the husk) were discarded.

The following scheme was used for experimental design and sampling dates: All fruit were stored and analysed after 0, 30, 60, and 90 d of storage at 2 °C (85–90 % RH) plus 3 d at 20 °C (55–60 % RH). From each replicate of control trees, 4 lots of 15 fruit (5 per replicate) were taken, while from MeJa-treated trees 8 lots of 15 fruit, 4 of them being stored as indicated above for control fruit (Pre treatment) and the remaining being treated with 5 mM MeJa as postharvest treatment (Pre + Post treatment). Postharvest treatment was performed by dipping the fruit in 15 L of MeJa solution containing 1 mL L^{-1} Tween-20 for 15 min, and then they were left to dry at room temperature. The following parameters were measured for all fruit: respiration rate, fruit firmness, weight loss and external chilling injury (CI) index were assayed in the whole fruit. Then, each fruit was carefully cut at the equatorial zone with sharpened knives and the arils were manually extracted. One half of the skin was used to determine internal CI index individually and thereafter to measure ion leakage (IL). The other half was immediately cut in slices of 1 x 0.5 cm to obtain a homogeneous skin sample from fruit of each replicate. These samples were frozen in liquid N_2 and freeze-dried in an Alpha 2–4 freeze drier (Christ Alpha 2–4; Braum Biotech) for 1 d under reduced pressure, 2.2 MPa. The temperature in the drying chamber was −25 °C, while the heating plate reached 15 °C. Later, samples were milled until reach a fine powder and vacuum-packed to be used for measuring fatty acid composition. The arils from the 5 fruit of each replicate were also combined. Samples of 50 g were used to measure colour, total soluble solids (TSS) and titratable acidity (TA) and another 100 g sample of each replicate was frozen in liquid N_2, milled and stored at -20 °C for total phenolics, total anthocyanins and total antioxidant activity determinations. For these determinations results were expressed on a fresh weight basis.

2.2. Respiration rate and quality parameters

To measure fruit respiration rate, the 5 fruit of each replicate were hermetically sealed in a 3 L jar for 3600 s. After that, 1 mL from the holder atmosphere was withdrawn and used for CO_2 quantification in a gas chromatograph, equipped with thermal conductivity detector and under the chromatographic conditions previously described (Sayyari et al., 2011b). Respiration rate was expressed as μg kg^{-1} s^{-1} CO_2 and was the mean ± SE. The determination of fruit firmness was carried out individually in each of the 5 fruit of each replicate by using a TX-XT2i Texture Analyzer (Stable Mycrosystems, Godalming, UK) which applied a force to achieve a 5% deformation of the fruit diameter. Results were expressed as the relation between the applied force and the distance travelled (kN m^{-1}) and were the mean ± SE. Fruit were weighed at harvest and at each sampling date during storage and weight loss was expressed as percentage (%) with respect to the initial weight. TSS were determined in duplicate in the juice obtained from the aril sample of each replicate by using a digital refractometer Atago PR-101 (Atago Co. Ltd., Japan) at 20 °C, and results were the mean ± SE, expressed as g L^{-1}. TA was determined in duplicate in 1 mL of the same juice diluted in 25 mL distilled H_2O by potentiometric titration with 0.1 N NaOH up to pH 8.1, and results were the mean ± SE expressed as g of malic acid equivalent L^{-1}.

2.3. Chilling injury (CI) index and ion leakage

External and internal chilling injury index (CI) were individually evaluated in each fruit according to a 6-point hedonic scale (Fig. 1) based on the percentage of husk surface affected by CI symptoms (dehydration, browning and pitting): 0 (no symptoms), 1 (1–20 %), 2 (21–40 %), 3 (41–60 %), 4 (61–80 %) and 5 (> 81%). These scales were performed with own photographs made by the authors from previous studies and the degree of CI symptoms was assessed according to them. CI index was calculated as: Σ (value of hedonic scale) x (number of fruit with the corresponding score) / (total number of fruit in the sample).

Fig. 1. Reference scale to evaluate external e internal chilling injury (CI) index of 'Mollar de Elche' pomegranate during storage at 2 °C + 3 d at 20 °C.

Results were expressed as the mean ± SE of three replicates of 5 fruit. To measure ion leakage, 16 disks (5 mm diameter ×5 mm thick- ness) were cut with a cork borer from the husks of the 5 fruit of each replicate and steeped in a glass vial containing 0.3 M mannitol. Conductivity was measured after 3 h under constant shaking (initial conductivity), using a conductivity meter (XS COND51+). After that, the disks were frozen overnight, autoclaved for 900 s at 120 °C and cooled to room temperature, and then, conductivity was again measured. Ion leakage was expressed as percentage of the total and calculated as follow (Mirdehghan et al., 2007a):

(Initial conductivity × 100)/(total conductivity)

2.4. Fatty acid profile and quantification

Pomegranate husk fat was directly methylated according to Trigueros and Sendra (2015). Fatty acid profile and quantification were determined by high resolution gas chromatography, analysing the fatty acid methyl esters obtained by trans-esterification of 25 mg of sample with 2 mL of 0.5 M sodium methoxide. Methyl esters were separated on a Shimadzu GC17A gas chromatography unit coupled to a mass spectrometry detector GC–MS QP5050, Shimadzu (Kyoto, Japan) with a SupraWax-280 column, filled with 100% polyethylene glycol (Teknokroma S. Co. Ltd., 165 Barcelona, Spain; 30 m length, 0.25 mm internal diameter, 0.25 µm film thickness). The following conditions were applied (Ferrara et al., 2014): Helium was used as carrier gas, injector and FID-detector temperatures were 220 and 250 °C, respectively, and oven temperatures were 140 °C for 2 min, which increased to 165 °C at 6 °C/min and thereafter from 165 °C to 225 °C at 2.8 °C/min and was held at 225 °C for 25 min. Volume of injected sample was 1 µL with split 1:20. Fatty acid methyl esters were identified by comparison of their retention times with Supelco 37-component FAME Mix reference standard (Sigma-Aldrich Co., St. Louis, MO, USA). Quantification was carried out in duplicate in each sample on the basis of peak areas using nonadecanoic acid (19:0 c-19) as internal standard and results (mean ± SE) were expressed as milligram nonadecanoic acid equivalent per kg on a dry weight basis (mg kg^{-1}).

2.5. Aril colour, total anthocyanins, total phenolics and total antioxidant activity determination

The arils colour was measured on the surface of a Petri dish filled with arils, using the CIE Lab system in a colorimeter (CRC200, Minolta Camera Co., Tokyo, Japan). After recording L*, a* and b* parameters, arils colour was expressed as Hue angle (arctg b*/a*). For total anthocyanin quantification, 5 g of arils were homogenised in 15 mL of methanol/formic acid/water (25:1:24, v/v/v) and then, centrifuged at 10,000 g for 10 min at 4 °C. Total anthocyanin content (TAC) was quantified in the supernatant by reading absorbance at 520 nm and was expressed as mg kg^{-1} of cyanidin 3-O-glucoside equivalents (Cy 3-glu, molar absorption coefficient of 23,900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}).

Total phenolics were extracted by homogenizing 5 g of aril sample with 10 mL of water: methanol (2:8) containing 2 mM NaF (to inactivate polyphenol oxidase activity and prevent phenolic degradation). The extracts were centrifuged at 10,000 g for 10 min at 4 °C and total phenolic concentration was quantified in the supernatant by using the Folin-Ciocalteu reagent as previously described by Sayyari et al. (2011a). Results were expressed as g gallic acid equivalent (GAE) kg^{-1} and are the mean ± SE of three replicates. Total antioxidant activity (TAA) was measured according to Sayyari et al. (2011b), which enables to determine hydrophilic (H-TAA) and lipophilic (L-TAA) antioxidant activity in the same extraction. Briefly, for each sample, 5 g of arils were homogenised in 10 mL of 50 mM phosphate buffer pH = 7.8 and 5 mL of ethyl acetate, and then centrifuged at 10,000 g for 15 min at 4 °C. The upper fraction was used to quantify L-TAA and the lower to quantify H-TAA, respectively. In both cases, TAA was determined in a reaction mixture containing 2,20-azino-bis-(3- ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), horse radish peroxidase enzyme and its oxidant substrate (hydrogen peroxide), in which $ABTS^{+\cdot}$ radicals are generated and monitored at 730 nm. Then, pomegranate extract was added and the decrease in absorbance after 90 s was calculated, which 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 are expressed as g of trolox equivalent (TE) kg^{-1} and are the mean ± SE of three replicates.

2.6. Statistical analysis

Results are expressed as mean ± SE of three replicates. Data for the analytical determinations were subjected to analysis of variance (ANOVA). Sources of variation were storage time and treatment. Mean comparisons were performed using HSD Tukey's test to examine if differences between control and treated fruit were significant at $P < 0.05$. All analyses were performed with SPSS software package v. 17.0 for Windows. Correlations were performed between internal or external CI indexes and IL and between arils Hue angle and their content in total anthocyanins.

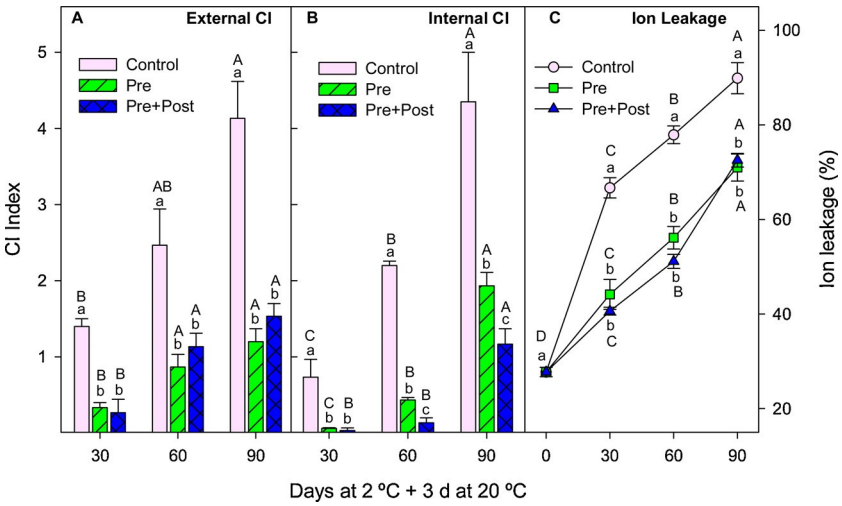

Fig. 2. External (A) and internal (B) chilling injury (CI) index and ion leakage (C) in the husk of control, preharvest (Pre) and preharvest plus postharvest (Pre + Post) 5 mM methyl jasmonate treated 'Mollar de Elche' pomegranate fruit during storage at 2 °C + 3 d at 20 °C. Data are the mean ± SE of three replicates of five fruit. Different capital letters show significant differences for each treatment during storage and different lowercase letters show significant differences among treatments for each sampling date at $P < 0.05$.

3. Results

3.1. Chilling injury and ion leakage

External chilling (CI) injury symptoms were manifested by depression of the fruit surface, browning and pitting, while internal CI symptoms appeared as brown spots in internal husk surface and locular septa (Fig. 1). Both, external and internal CI index increased during storage reaching final values of 4.13 ± 0.48 and 4.35 ± 0.65, respectively, in control fruit (Fig. 2A and B). However, in Pre and Pre + Post MeJa treated fruit the increases in external and internal CI indexes were significantly lower ($P < 0.05$) than in controls. However, differences between Pre and Pre+Post treatments were only significant for internal CI after 60 and 90 days of storage. Ion leakage (IL) in husk tissue also increased during storage in control and treated fruit, although this increase was significantly ($P < 0.05$) delayed in treated fruit with respect to controls, without significant differences between Pre and Pre+Post treatments (Fig. 2C).

3.2. Changes in fatty acid composition

Twenty-five fatty acids were identified and quantified in pomegranate skin, the major ones being C16:0 (palmitic), C18:1 (oleic), C18:2 (linoleic) and C18:3 (linolenic) acids, followed by C10:0 (capric), C14:0 (myristic), and C18:0 (stearic) acids (Fig. S1). In addition, other seventeen minor fatty acids were also identified, twelve of them for the first time in pomegranate: myristoleic acid (C14:1), heneicosanoic acid (C21:0), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), margaric acid (C17:0), margaroleic acid (C17:1), vaccenic acid (C18:1), arachidic acid (C20:0), methyl linolenate (C18:3), eicosadienoic acid (C20:2), behenic acid (C22:0), lignoceric acid (C24:0) and nervonic acid (C24:1). This fatty acid profile was similar in control and treated fruit, either at harvest or during postharvest storage, and thus chromatogram from control fruit at harvest is shown (Fig. S1).

Palmitic acid concentration in the skin of control fruit at harvest was significantly ($P < 0.05$) higher (191.6 ± 12.4 mg kg^{-1}) than in those of Pre and Pre + Post MeJa treated ones (160.9 ± 10.5 and 14.34 ± 0.63 mg kg^{-1}, respectively). These differences were observed until the last sampling date in spite of the fact that palmitic acid decreased in skin of control and treated fruit during storage (Fig. 4). C10:0 and C18:0 concentrations also decreased during storage although no significant differences were observed between control and treated fruit, while no changes were observed in C14:0 either during storage or attributed to MeJa treatments (Fig. 3). All the UFA fatty acids were found at significant ($P < 0.05$) higher concentration in treated fruit than in controls at harvest, the highest enhancement being found in linolenic

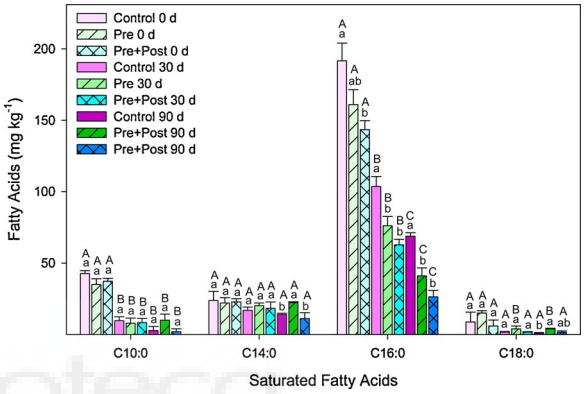

Fig. 3. Effect of preharvest (Pre) and preharvest plus postharvest (Pre + Post) 5 mM methyl jasmonate treatments on saturated fatty acid content in pomegranate husk at harvest (0 d) and after 30 and 90 d of storage at 2 °C + 3 d at 20 °C. Data are the mean ± SE of three replicates of five fruit. For each saturated fatty acid, different capital letters show significant differences for each treatment during storage and different lowercase letters show significant dif- ferences among treatments for each sampling date at $P < 0.05$.

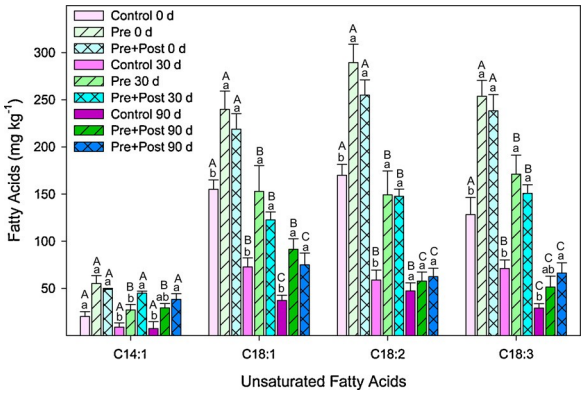

Fig. 4. Effect of preharvest (Pre) and preharvest plus postharvest (Pre + Post) 5 mM methyl jasmonate treatments on unsaturated fatty acid content in pomegranate husk at harvest (0 d) and after 30 and 90 d of storage at 2 °C + 3 d at 20 °C. Data are the mean ± SE of three replicates of five fruit. For each unsaturated fatty acid, different capital letters show significant differences for each treatment during storage and different lowercase letters show significant differences among treatments for each sampling date at $P < 0.05$.

Table 1
Respiration rate (RR) and quality parameters, firmness, weight loss, total soluble solids (TSS) and titratable acidity (TA) in pomegranate fruits from control and preharvest (Pre) and pre- plus postharvest methyl jasmonate (MeJA)-treated (Pre + Post) fruit during postharvest storage at 2 °C + 3 d at 20 °C.

Parameter	Days	Control	MeJA 5 mM	
			Pre	Pre + Post
RR (μg kg^{-1} s^{-1})	0	5.96 ± 0.41 ^{aA}	5.14 ± 0.43 ^{bA}	5.26 ± 0.82 ^{bA}
	30	6.61 ± 0.61 ^{aB}	6.00 ± 0.58 ^{bB}	5.77 ± 0.64 ^{bB}
	60	6.14 ± 0.60 ^{aAB}	5.61 ± 0.34 ^{bB}	5.36 ± 0.41 ^{bAB}
	90	4.48 ± 0.39 ^{aC}	4.06 ± 0.29 ^{bC}	3.68 ± 0.75 ^{bC}
Firmnes (kN m^{-1})	0	26.10 ± 0.41 ^{bA}	28.08 ± 0.62 ^{aA}	27.93 ± 0.55 ^{aA}
	30	18.94 ± 0.46 ^{bB}	25.19 ± 0.83 ^{aB}	24.95 ± 0.76 ^{aB}
	60	18.54 ± 0.72 ^{bB}	21.83 ± 0.89 ^{aC}	21.48 ± 0.69 ^{aC}
	90	18.01 ± 0.52 ^{bB}	19.98 ± 0.48 ^{aC}	19.77 ± 0.39 ^{aC}
Weight loss (%)	0	–	–	–
	30	10.28 ± 0.40 ^{aA}	7.13 ± 0.13 ^{bA}	7.27 ± 0.29 ^{bA}
	60	17.39 ± 0.55 ^{aB}	12.57 ± 0.54 ^{bB}	13.13 ± 0.24 ^{bB}
	90	18.98 ± 0.27 ^{aC}	16.04 ± 0.40 ^{bC}	16.44 ± 0.55 ^{bC}
TSS (g L^{-1})	0	157.70 ± 2.10 ^{aA}	166.80 ± 3.70 ^{aA}	169.50 ± 4.20 ^{aA}
	30	167.00 ± 2.20 ^{aB}	170.70 ± 1.90 ^{aA}	171.20 ± 2.70 ^{aA}
	60	168.30 ± 1.40 ^{aB}	171.5 ± 1.40 ^{aA}	172.20 ± 2.00 ^{aA}
	90	169.50 ± 1.30 ^{aB}	172.3 ± 1.70 ^{aA}	172.50 ± 1.40 ^{aA}
TA (g L^{-1})	0	4.10 ± 0.30 ^{aA}	4.30 ± 0.20 ^{aA}	4.20 ± 0.20 ^{aA}
	30	5.30 ± 0.40 ^{aAC}	5.60 ± 0.30 ^{aB}	5.50 ± 0.40 ^{aB}
	60	8.40 ± 0.50 ^{aB}	8.90 ± 0.50 ^{aC}	8.80 ± 0.60 ^{aC}
	90	6.50 ± 0.30 ^{aC}	6.60 ± 0.20 ^{aD}	6.60 ± 0.30 ^{aB}

*Data are the mean ± SE. Different capital letters show significant differences for each treatment during storage and different lowercase letters show significant differences among treatments for each sampling date at $P < 0.05$.

acid, 1.98 and 1.86-fold increases in Pre and Pre + Post MeJa treated fruit, respectively, with respect to control fruit (Fig. 4). All the UFA decreased during storage, although their concentrations were maintained at significant ($P < 0.05$) higher levels in treated than in control fruit, without significant differences between Pre and Pre + Post treatments.

3.3. Respiration rate, fruit and aril quality parameters, bioactive compounds and total antioxidant activity

Pre and Pre + Post MeJa treatment significantly inhibited the fruit respiration rate ($P < 0.05$) at harvest and during storage, although no significant differences were found between both treatments (Table 1). Fruit firmness decreased during storage in control and treated fruit, but were significantly ($P < 0.05$) delayed in Pre and Pre+Post MeJa treated fruit, without significant differences between both treatments. Fruit weight loss increased during storage reaching final values 18.98 ± 0.27 % in control fruit and significantly ($P < 0.05$) lower (16.04 ± 0.40 %) in treated ones. The major effects of MeJa treatments on delaying weight losses and fruit softening were found after 30 and 60 d of storage (Table 1). However, TSS and TA were not affected by MeJa treatments, with values of TSS ranging between 160 and 170 g L^{-1} and TA increasing from 4.0 to 6.5 g L^{-1} during the whole storage period (Table 1). On the other hand, colour Hue angle at 0 d was significantly ($P < 0.05$) higher in control fruit than in treated ones and these differences were maintained during storage, although decreases during storage occurred in all fruit (Fig. 5A). On the contrary, total anthocyanin content was increased by MeJa treatments, either at harvest and during storage, the major effect being found in Pre + Post MeJa treated fruit (Fig. 5B). Similarly, total phenolic concentration and antioxidant activity of the hydrophilic extracts (H-TAA) were significantly ($P < 0.05$) higher in treated than in control fruit from harvest until the last sampling date (Fig. 5C and D).

4. Discussion

The first cell structures affected by CI are cell membranes, which change from a flexible liquid-crystalline phase to a solid-gel structure when fruit are exposed to chilling temperatures, leading to losses of semi-permeability and functionality of cytoplasmic and intracellular cell membranes (Rui et al., 2010; Valero and Serrano, 2010). These impairments on cell membrane structure and function leads to decompartmentalization of the substrates and enzymes in fruit organelles resulting in enzymatic oxidation of phenols to *o*-quinones by peroxidase and polyphenol oxidase (Zhang et al., 2015) which could be responsible for the brown spots in external and internal pomegranate busk surfaces. Then, Pre and Pre + Post MeJa treatments would have an effect on maintaining membrane structure since lower CI symptoms and IL values were observed in treated fruit in comparison to the control (Figs. 1 and 2). It was found that both, external and internal CI indexes were correlated with IL ($r^2 = 0.823$ and 0.733, respectively) by taking into account data of control and treated fruit for all sampling dates. Thus, IL could be used as an indicator of CI and cell membrane integrity, according to previous reports in a wide range of fruit species including pomegranate (Sayyari et al., 2011a; Ehteshami et al., 2019). However, significant differences between Pre and Pre + Post MeJa treatments were observed only in internal CI while external CI and ion leakage were similar in both treatments. Thus, for practical purposes in order to reduce CI, pre-harvest MeJa treatments could be sufficient, with similar effects on reducing CI than the postharvest treatments previously reported (Sayyari el al., 2011a).

It is well known that damage of the membrane structure and subsequent changes in lipid constituents is correlated with the occurrence of CI. These changes in lipid composition show mainly decrease in the ratio of unsaturated to saturated fatty acids. This could be affecting the phase transition of membrane lipids from a liquid-crystalline to a solid-gel state, and in turn leading to membrane peroxidation and damage with accelerating the occurrence of CI (Wongsheree et al., 2009).

Fatty acid profile in 'Mollar de Elche' pomegranate husk addressed in the present results agrees with previous published papers. Thus, Mirdehghan et al. (2007b) identified and quantified 10 fatty acids in this pomegranate cultivar, 5 saturated (C10:0, C12:0, C14:0, C16:0 and C18:0), 2 mono-unsaturated (C16:1 and C18:1) and 3 poly-unsaturated (C18:2 *cis*, C18:2 *trans* and C18:3). Sayyari et al. (2017) also identified C20:1 (gadoleic acid) and C22:1 (erucic acid). However, twelve new fatty acids (Fig. S1) were identified and quantified for the first time in pomegranate husk in the present research. Among these new fatty acids, myristoleic acid (C14:1) accounted for ca. 2 % of the total fatty acid content, while the remaining fatty acids, heneicosanoic acid (C21:0), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), margaric acid (C17:0), margaroleic acid (C17:1), vaccenic acid (C18:1), arachidic acid (C20:0), methyl linolenate (C18:3), eicosadienoic acid (C20:2), behenic acid (C22:0), lignoceric acid (C24:0) and nervonic acid (C24:1) were found at very low concentrations, accounting for 0.5–0.1% of total fatty acid composition. In addition, in these previous papers, it was reported that heat (Mirdehghan et al., 2007b) or salicylic acid (Sayyari et al., 2017) treatments decreased CI damages and delayed the losses in the major SFA and UFA occurring during storage at chilling temperatures. Accordingly, 'Mallas Saveh' pomegranate fruit treated with salicyloyl chitosan showed reduced CI symptoms and exhibited higher membrane UFA/SFA ratio (Sayyari et al., 2016). The destructive process of cellular membranes during fruit storage at chilling temperatures has been ascribed to phospholipids hydrolysis into free fatty acids and peroxidation of UFA due to the coordinated action of lipid metabolizing enzymes, such as phospholipase, lipase and lipoxygenase (Wang et al., 2018; Zhang et al., 2018; Lin et al., 2017). Accordingly, glycine betaine treatment reduced CI in peach fruit, throughout reduction of lipoxygenase, phospholipase D and lipase activities and their gene expression and increasing the expression of genes related to fatty acid biosynthesis and desaturation (Wang et al., 2019).

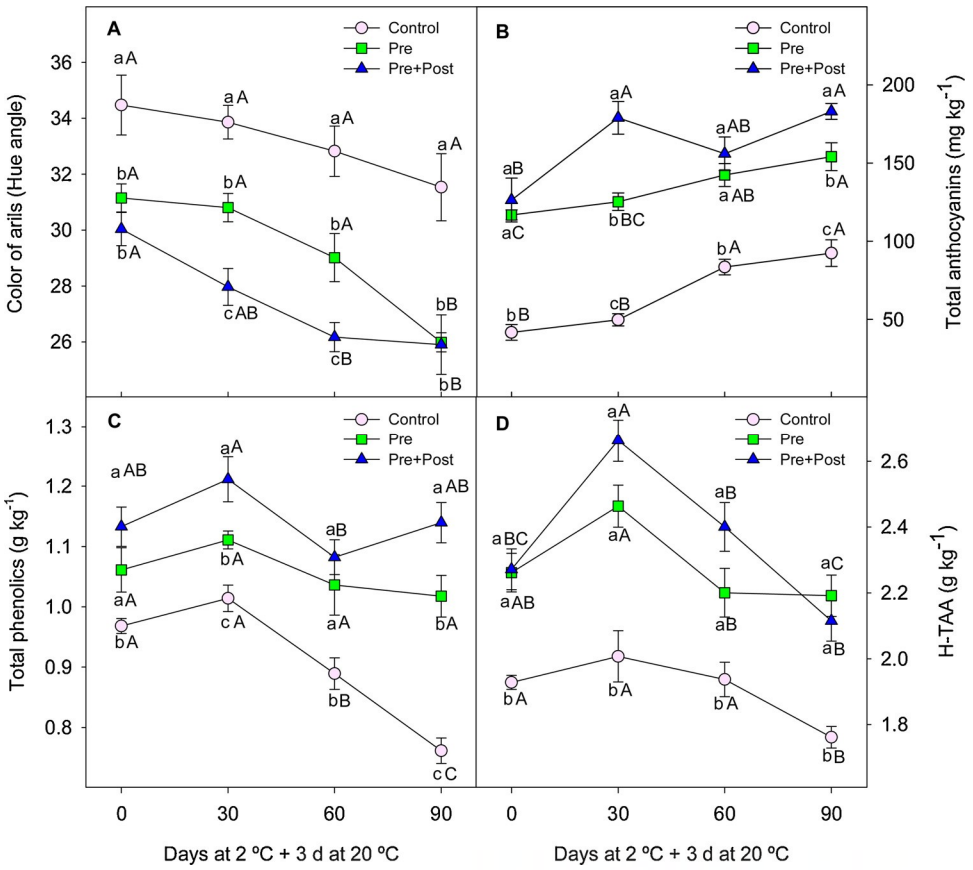

Fig. 5. Colour (A), total anthocyanin (B), total phenolics (C) and hydrophilic total antioxidant activity (H-TAA, D) in the arils of control, preharvest (Pre) and preharvest plus post- harvest (Pre + Post) 5 mM methyl jasmonate treated 'Mollar de Elche' pomegranate fruit during storage at 2 °C + 3 d at 20 °C. Data are the mean ± SE of three replicates of five fruit. Different capital letters show significant dif- ferences for each treatment during storage and different lowercase letters show significant differences among treatments for each sam- pling date at $P <$ 0.05.

These effects resulted in higher levels of unsaturated/saturated fatty acid ratio and maintenance of normal cell membrane structure and function. Thus, MeJa treatments might reduce peroxidation of the cell membrane lipids leading to maintenance of high fatty acid content and particularly, higher UFA/SFA ratio during storage and in turn, higher membrane integrity and lower CI and IL.

The effect of MeJa treatment on decreasing CI in pomegranate has been reported in a recently published paper in 'Malas' cultivar (Koushesh Saba and Zarei, 2019), although fatty acids were not evaluated in this study. Moreover, to the best of our knowledge, just in one previous paper the effect of MeJa treatment on fatty acid composition of cell membranes has been reported. In this study, Cao et al. (2009) showed that postharvest treatment with MeJa of loquat fruit significantly reduced CI and maintained higher UFA/SFA ratio during storage, suggesting that MeJa induced CI tolerance in fruit tissues by reducing losses in UFA and maintaining a high UFA/SFA ratio. The results of the present study are in agreement with this previous report, and show that MeJa reduced CI throughout maintenance of cell membrane stability. Thus, the sharp decrease in individual and total UFA concentration observed in control fruit from 0 to 30 d of storage were significantly ($P <$ 0.05) delayed in all MeJa treated fruit (Figs. 4 and 2S). Moreover, UFA/SFA ratio was significantly ($P <$ 0.05) increased as a consequence of Pre and Pre + Post MeJa treatments at 0 d and maintained at higher levels during the whole storage period, the effect being significantly ($P <$ 0.05) higher when MeJa was applied as Pre +Post treatment than as Pre treatment alone (Fig. 3S). These effects could be described as a mechanism of acclimation to low temperature and would account for maintenance of membrane semi-permeability, leading to lower losses of intracellular water, ions and metabolites and, in turn, being responsible for the lower weight loss, IL and CI index scores found in treated fruit.

Fruit and aril quality parameters and antioxidant compounds were increased in pomegranate fruit as a consequence of Pre and Pre + Post MeJa treatments. Thus, weight loss and softening were delayed in treated fruit, without significant differences between Pre and Pre + Post treatments, as commented above for reduction of CI damages and IL. Both treatments were also effective on decreasing colour Hue angle of the arils showing they had a deeper red colour, due to an increase in the biosynthesis of anthocyanins whose concentration was higher in treated than in control fruit from 0 d until the last sampling date, especially in the arils of fruit receiving Pre + Post MeJa treatments. In fact, a negative correlation (y= -14.4x+553, $r^2 = 0.879$) was found between arils Hue angle and their content in anthocyanins considering data of all treatments and sampling dates. With respect to total phenolics and H-TAA, significant ($P <$ 0.05) higher levels were also found in treated than in control fruit during the whole storage period. These results are supported by previous experiments in which concentration of anthocyanins and other phenolic compounds were increased by preharvest MeJa treatments in plums (Martínez-Esplá et al., 2014), blackcurrants (Flores and Ruiz Del Castillo, 2016), mangoes (Muengkaew et al., 2016), apples (Ozturk et al., 2015), table grape (García-Pastor et al., 2019) or lemon (Serna-Escolano et al., 2019), as a consequence of stimulation of the expression of genes involved in their biosynthesis (Jia et al., 2016; Wei et al., 2017). Total phenolic contents were also maintained at higher levels in MeJa treated peaches due to increases in PAL and SOD activities and decreases in PPO and POD activities, which were related to reduction of CI symptoms (Jin et al., 2009). On the contrary, carambola exposed to postharvest MeJa treatments led to a reduction in both total phenolics and antioxidant activity (Mustafa et al., 2016). Increases in phenolic content as a consequence of preharvest MeJa treatment in pomegranates stored at 10 °C have been also reported in our previous paper (García-Pastor et al., 2020). In the present paper, these effects of preharvest MeJa treatment were also observed during storage at 2 °C, confirming a positive role of MeJa treatments on increasing health properties of pomegranate fruit, which have been attributed to anthocyanins and other phenolic

compounds (Reyes-Díaz et al., 2016; Asgary et al., 2017; Serrano et al., 2018; Bassiri-Jahromi, 2018). It is worth noting that total phenolic and anthocyanin concentrations were higher in Pre + Post MeJa treated fruit than in Pre-treated ones during the whole storage time, showing an accumulative effect of Pre + Post MeJa treatments. In this sense, it has been reported in apple fruit that the effect of postharvest MeJa treatments on promoting anthocyanin accumulation was dose-dependent since higher up-regulation in the expression of the genes involved in anthocyanin biosynthesis occurred for 1 than for 0.1 mM doses (Feng et al., 2017).

5. Conclusions

The results from this study demonstrate that Pre and Pre + Post MeJa treatments reduced external and internal CI symptoms in pomegranate husk, likely by better maintaining the cell membrane structure through reducing UFA losses and enhancing the UFA/SFA ratio. We identify twelve new fatty acids in the pomegranate husk. In addition, fruit and aril quality parameters and their content in antioxidant compounds were increased in pomegranate fruit as a consequence of Pre and Pre + Post MeJa treatments. For most of the analysed parameters, there were no significant differences between Pre and Pre + Post MeJa treatments. For this reason, preharvest MeJa treatments is recommended over postharvest application to increase storability of pomegranate fruit during cold storage, with reduction of CI symptoms and increasing quality traits and bioactive compounds with antioxidant potential, and in turn its health beneficial effects.

Declaration of Competing Interest

The authors declare that the study was conducted in absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2020.111226.

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Supplementary figure legends

Figure S1. Fatty acid profile in pomegranate in control husk at 0 d. C10:0 (capric acid), C12:0 (lauric acid), C14:0 (myristic acid), C14:1 (myristoleic acid), C15:0 (pentadecanoic acid), $C15:1_{c10}$ (pentadecenoic acid), C16:0 (palmitic acid), $C16:1_{c9}$ (palmitoleic acid), C17:0 (margaric acid), $C17:1_{c10}$ (margaroleic acid), C18:0 (stearic acid), $C18:1_{c7}$ (vaccenic acid), $C18:1_{c9}$ (oleic acid), $C18:2_{t9,12}$ (linoelaidic acid), $C18:2_{c9,12}$ (linoleic acid), C20:0 (arachidic acid), $C18:3_{c6,9,12}$ (methyl linolenate), $C20:1_{c11}$ (gadoleic acid), $C18:3_{c9,12,15}$ (linolenic acid), C21:0 (heneicosanoic acid), $C20:2_{c11,14}$ (eicosadienoic acid), C22:0 (behenic acid), $C22:1_{c13}$ (erucic acid), C24:0 (lignoceric acid) and $C24:1_{c15}$ (nervonic acid).

Figure S2. Total unsaturated (UFA) and saturated fatty acids (SFA) in husk of pomegranates as affected by preharvest (Pre) and preharvest plus postharvest (Pre+Post) 5 mM methyl jasmonate treatments at harvest (0 d) and after 30 and 90 d of storage at 2 ºC + 3 d at 20 ºC. Data are the mean ± SE of three replicates of five fruit. Different capital letters show significant differences for each treatment during storage and different lowercase letters show significant differences among treatments for each sampling date at $P < 0.05$.

Figure S3. Total unsaturated (UFA) and saturated fatty acid (SFA) ratio in husk of pomegranates as affected by preharvest (Pre) and preharvest plus postharvest (Pre+Post) 5 mM methyl jasmonate treatments at harvest (0 d) and after 30 and 90 d of storage at 2 ºC + 3 d at 20 ºC. Data are the mean ± SE of three replicates of five fruit. Different capital letters show significant differences for each treatment during storage and different lowercase letters show significant differences among treatments for each sampling date at $P < 0.05$.

Supplementary material

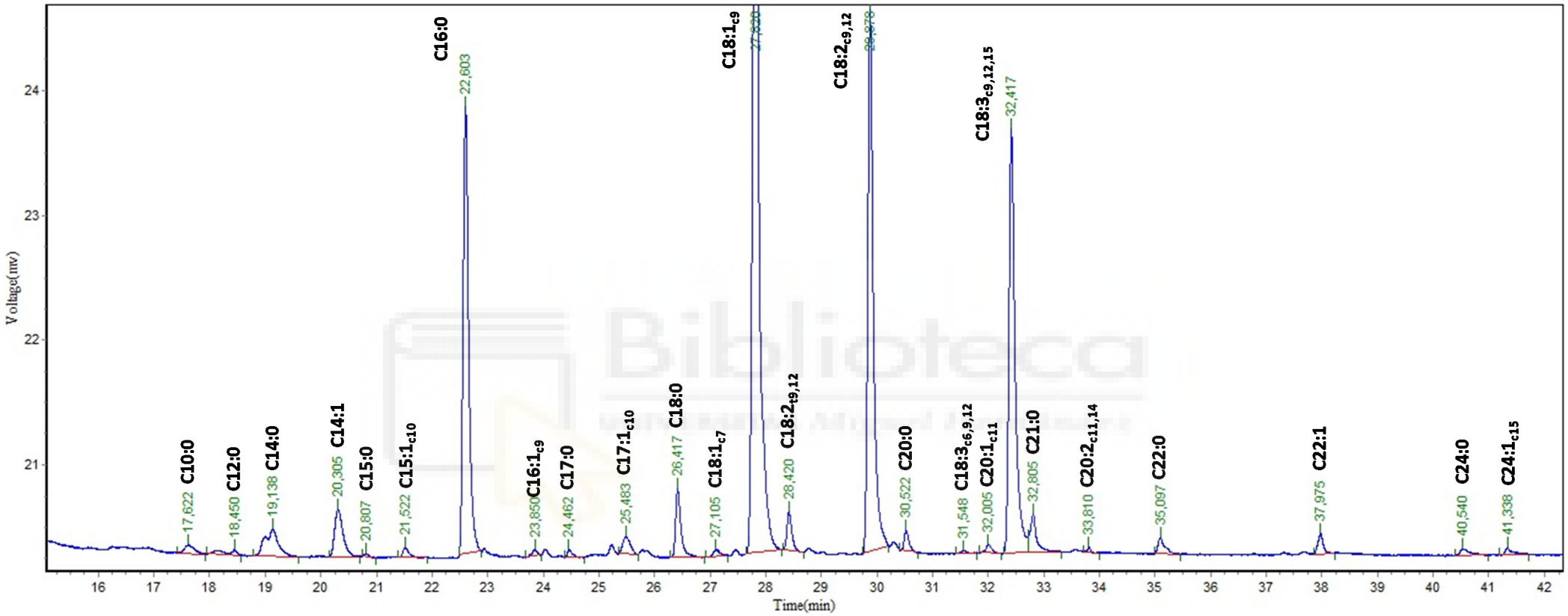

Figure S1.

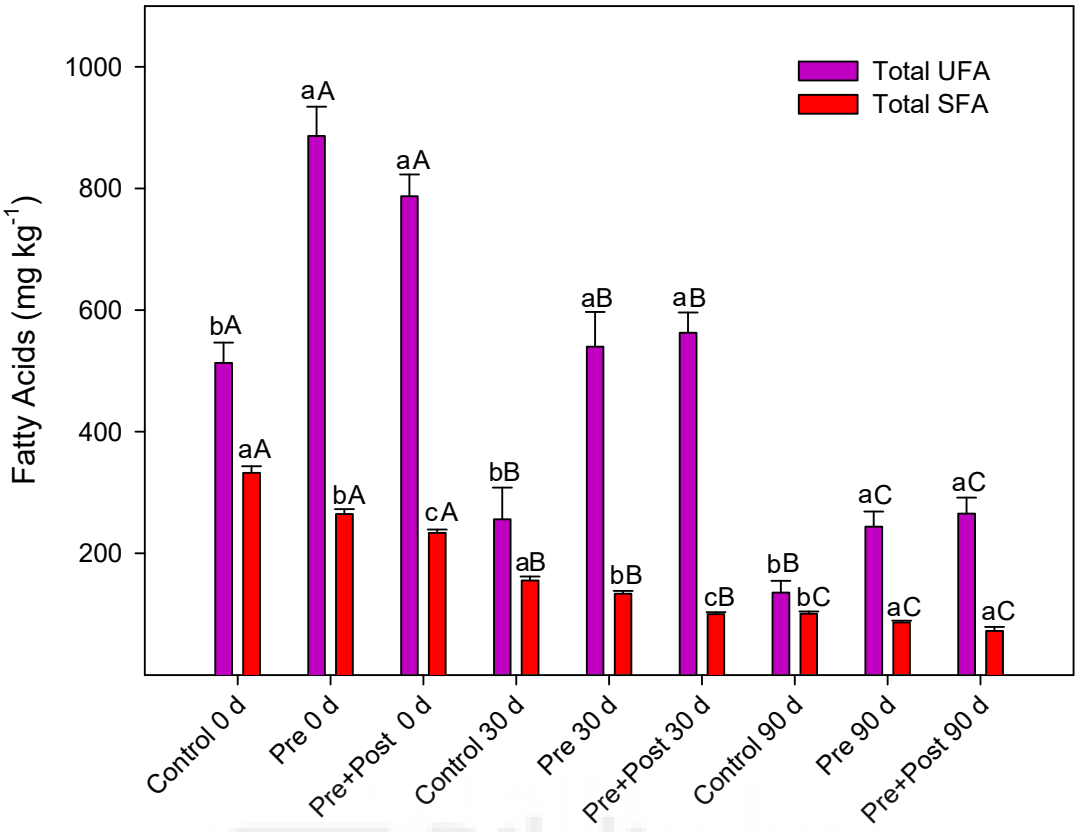

Figure S2.

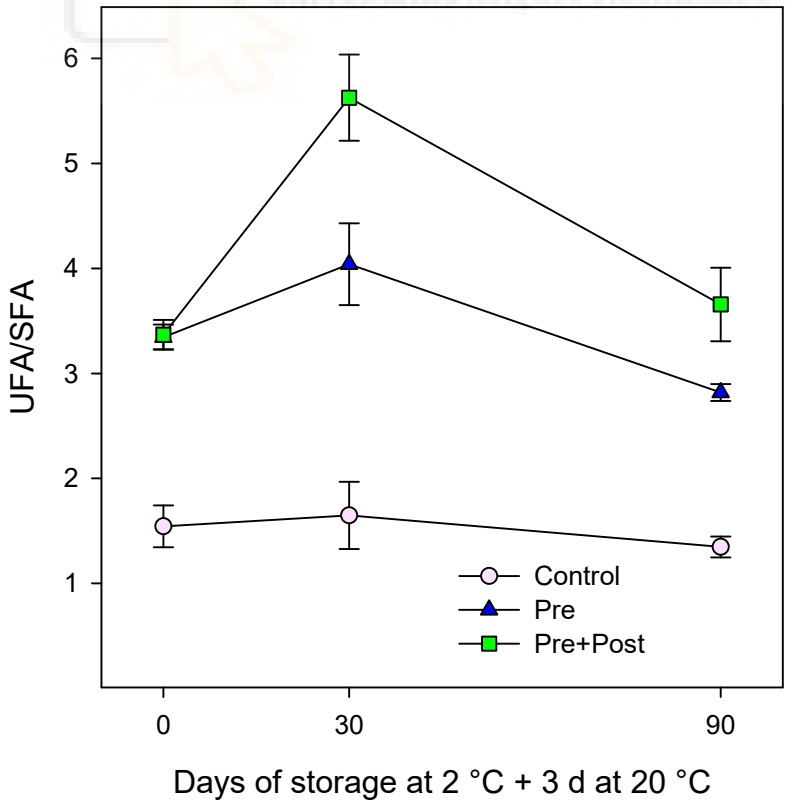

Figure S3.

4.4. Publication 4

PUBLICATION 4 (Open access)

The effects of salicylic acid and its derivatives on increasing pomegranate fruit quality and bioactive compounds at harvest and during storage

María E. García-Pastor, Pedro J. Zapata, Salvador Castillo, Domingo Martínez-Romero, Fabián Guillén, Daniel Valero, María Serrano

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frontiers in Plant Science

The Effects of Salicylic Acid and Its Derivatives on Increasing Pomegranate Fruit Quality and Bioactive Compounds at Harvest and During Storage

*María E. García-Pastor[1], Pedro J. Zapata[1], Salvador Castillo[1], Domingo Martínez-Romero[1], Fabián Guillén[1], Daniel Valero[1] and María Serrano[2]**

[1] Department of Agro-Food Technology, University Miguel Hernández, Orihuela, Spain, [2] Department of Applied Biology, University Miguel Hernández, Orihuela, Spain

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Edited by:
Cai-Zhong Jiang, Crops Pathology and Genetics Research Unit, USDA-ARS, United States

Reviewed by:
Francisco Garcia-Sanchez, Spanish National Research Council, Spain
Valerio Cristofori, University of Tuscia, Italy

***Correspondence:**
María Serrano
m.serrano@umh.es

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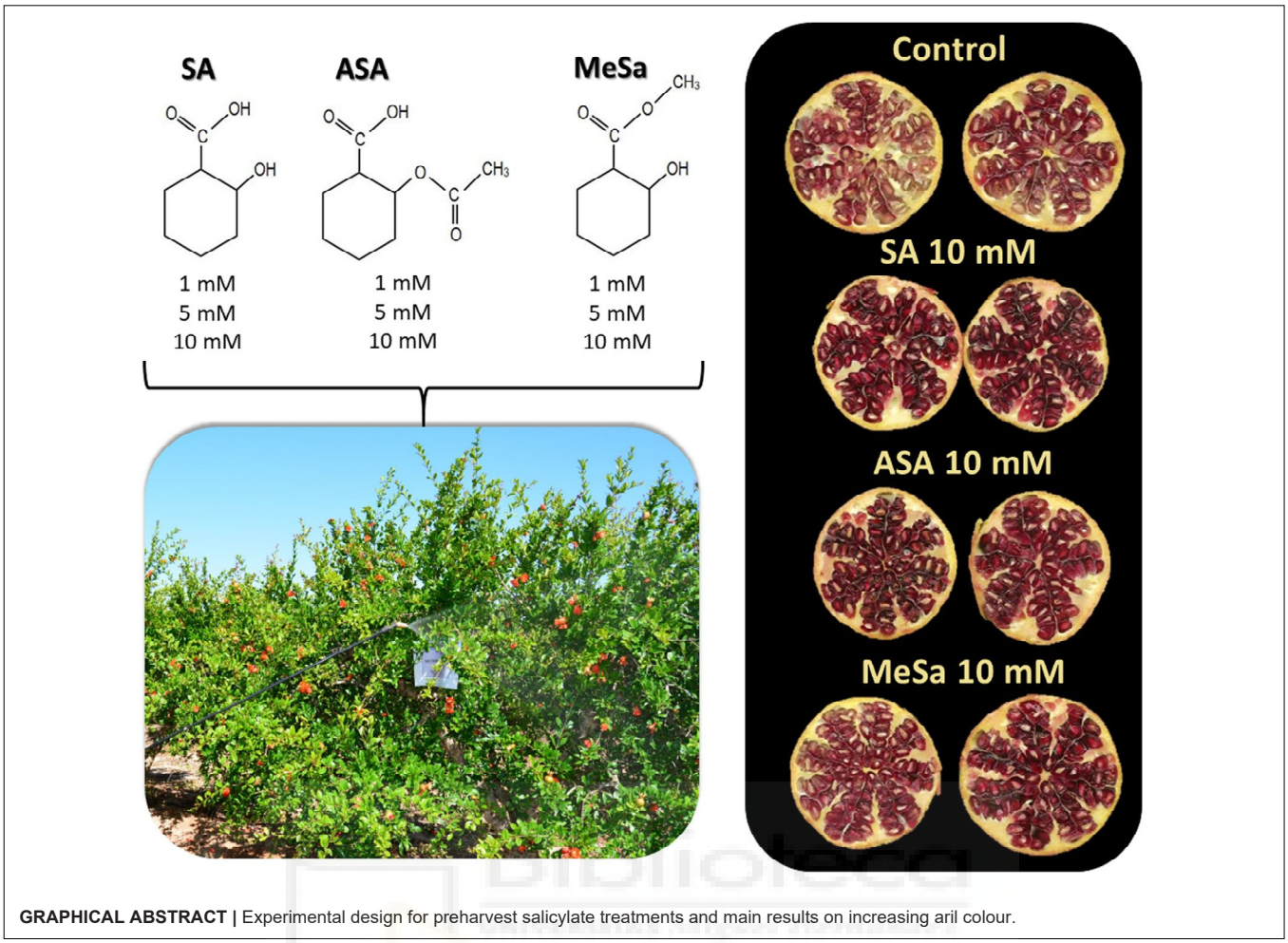
In the present research two experiments were performed to evaluate the effect of pre-harvest salicylic acid (SA), acetyl salicylic acid (ASA), and methyl salicylate (MeSa), applied as a foliar spray to pomegranate "Mollar de Elche," on crop yield, fruit quality parameters, and bioactive compounds at harvest and during storage. In the 2017 experiment, trees were treated with SA, ASA, and MeSa at 1, 5, and 10 mM and a higher crop yield (kg tree^{-1} and number of harvested fruit tree^{-1}) and quality parameters (firmness, aril color, and individual sugars and organic acids) at harvest were obtained, as well as a higher concentration of phenolics, anthocyanins, and ascorbic acid. The best results were achieved with 10 mM dose of the three assayed compounds, which was chosen for the 2018 experiment, and results for crop yield and fruit quality attributes were confirmed. These quality traits and the concentration of phenolics, anthocyanins, and ascorbic acid were maintained at higher levels in pomegranate fruit from treated trees than in controls during prolonged storage at 10°C. In addition, the effects of salicylate treatments on increasing total and individual anthocyanin concentration in pomegranate arils led to arils with a deeper red color **(Graphical Abstract)** and, in turn, fruit that would be more appreciated in the international market. This fact, together with the increased crop yield, would contribute to the increased profit of this crop. Thus, pre-harvest treatment with salicylates, and especially SA at 10 mM concentration, could be a safe, natural, and new tool to improve fruit quality and its content on antioxidant compounds with health beneficial effects (namely, ascorbic acid, phenolics, and anthocyanins) at harvest and during storage.

Keywords: ***Punica granatum*** **L, acetyl salicylic acid, methyl salicylate, anthocyanins, phenolics, ascorbic acid, sugars, organic acids**



GRAPHICAL ABSTRACT | Experimental design for preharvest salicylate treatments and main results on increasing aril colour.

INTRODUCTION

Pomegranate is one of the oldest known edible fruits, associated with ancient civilizations in the Middle East. It was originated in the area nowadays occupied by Iran and Afghanistan and from this area it was spread to India, China, Turkey, Egypt, Tunisia, Morocco, and Spain (Pareek et al., 2015). Spain is the major producer and exporting country of pomegranate fruit in the European Union, with a production area of 3,567 ha and a production of 65,165 t in 2017 (MAGRAMA, 2017). The edible portion of pomegranate are the arils, which consist of around 80% juice and 20% seed (Cristofori et al., 2011) and the fruit can be consumed as fresh fruit or used to prepare juices, canned beverages, jellies, jams, and flavorings and colorings for drinks (Valero et al., 2014).

Pomegranate fruit quality depends largely on fruit size, skin color, and absence of visual defects, such as sunburn, growth cracks, cuts, bruises, and decay, as well as on aril color, sugar and acid content, and the presence of small and soft seeds. In Spain, "Mollar de Elche" is the most cultivated pomegranate cultivar which is very appreciated by consumers due to its high concentration of sugars, low acidity, and its barely discernible seeds, since they are very small and soft and can be easily eaten (Nuncio-Jáuregui et al., 2014). On the other hand, pomegranate has been used in the folk medicine of many countries from ancient times, and its beneficial effects against several diseases, such as atherosclerosis, inflammatory and infective-mediated diseases, Alzheimer, diabetes, infarct brain ischemia, and several types of cancer, have been reported recently and attributed to its phenolic compounds and anthocyanins (Faria and Calhau, 2011; Ismail et al., 2012; Asgary et al., 2017; Panth et al., 2017). Nevertheless, the phytochemical composition of pomegranate fruit is affected by several factors, such as genotype, area of cultivation, environmental conditions, and agronomic management, among others. Thus, six different anthocyanins, namely, cyanidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, delphinidin-3,5-diglucoside, cyanidin-3-glucoside, pelargonidin-3-glucoside, and delphinidin-3-glucoside, were identified in a wide range of Italian pomegranate cultivars, although their concentrations were different depending on cultivars, as well as other phenolic compounds (Fanali et al., 2016; Russo et al., 2018). Great variations on total and individual phenolic and anthocyanin concentrations were also found by Zaouay et al. (2012) in thirteen Tunisian cultivars. However, "Mollar de Elche" cultivar does not have a high anthocyanin content as compared with other worldwide-known cultivars such as "Wonderful" and,

in turn, its arils are only slightly red-colored and the skin has a cream-pink color (Li et al., 2015; Pareek et al., 2015; Cano-Lamadrid et al., 2018). This fact makes it difficult for this cultivar to reach international markets. Thus, treatments to increase the coloration of the skin and arils of "Mollar de Elche" pomegranate would lead to its increased commercialization in international markets as well as its antioxidant properties and health beneficial effects. In this sense, it has been reported that water restrictions applied in summer (during the linear phase of fruit growth) led to an increase in aril anthocyanin content (Bartual et al., 2015), as well as treatment with methyl jasmonate during on-tree pomegranate fruit development (García-Pastor et al., 2020).

Salicylic acid (SA) and its derivatives, acetyl salicylic acid (ASA) and methyl salicylate (MeSa), are plant hormones that play important roles in a wide range of physiological processes, from seed germination to flowering and fruit ripening, although the most studied roles have been their effects on inducing plant defense systems against different biotic and abiotic stresses (Tiwari et al., 2017; Koo et al., 2020). It has been reported that postharvest treatments with salicylates reduce decay and chilling injury in a wide range of fruits, and improve other quality properties, such as appearance, texture, and nutritional compounds (Asghari and Aghdam, 2010; Glowacz and Ree, 2015). Thus, SA, ASA, or MeSa applied as postharvest treatments resulted in higher quality attributes in apricots (Wang et al., 2015) and increased the content on anthocyanins and other bioactive compounds in blood oranges (Habibi et al., 2020), sweet cherries (Valero et al., 2011), kiwifruit (Zhang et al., 2003), mango (Ding et al., 2007), sugar apples (Mo et al., 2008), and peaches (Tareen et al., 2012), with additional effects on delaying the postharvest ripening process. On the other hand, the application of these salicylates during fruit development on trees has been reported to improve fruit quality parameters at harvest. Thus, SA treatment at 0.1 and 0.2 mM of vine (at veraison stage) increased anthocyanin content in berries (Oraei et al., 2019) and SA, ASA, or MeSa treatments (0.5, 1, and 2 mM) applied at three key points of fruit development enhanced sugars, organic acids, and antioxidant compounds in plums at harvest and after storage (Martínez-Esplá et al., 2017, 2018) as well as in sweet cherries (Giménez et al., 2014, 2015, 2017; Valverde et al., 2015). In these previous papers, a delay on the postharvest ripening process was also observed, which was attributed to the increased concentration of antioxidant compounds and the activity of antioxidant enzymes.

Specifically, in pomegranate SA, ASA, and MeSa postharvest treatments reduced chilling injury (CI) and maintained fruit quality and higher levels of total antioxidant compounds, such as anthocyanins, phenolics, or ascorbic acid (Sayyari et al., 2009, 2011a,b). Accordingly, postharvest 2 mM SA treatment in combination with a controlled atmosphere storage delayed quality losses and extended the storage life of "Hicazna" pomegranates (Koyuncu et al., 2019). In addition, 0.25 mM ASA treatment of pomegranate arils reduced browning and maintained higher concentrations of phenolics and anthocyanins during storage (Dokhanieh et al., 2016). As a preharvest treatment, just one previous paper is available in which 1 mM SA treatment reduced bacterial blight, a devastating pomegranate disease caused by *Xanthomonas axonopodis pv. Punicae* (Lalithya et al., 2017). However, there are no previous reports regarding the effect of pre-harvest treatments of pomegranate trees with SA, ASA, or MeSa on fruit growth and ripening, as well as on fruit quality attributes at harvest, which was the main goal of the present research. In addition, it was hypothesized that an increase on anthocyanin content would occur as a consequence of these treatments, according to results on other fruit species obtained in previous reports, as commented above.

MATERIALS AND METHODS

Plant Material and Experimental Design

The experiments (2017 and 2018) were performed in a commercial orchard of 'Mollar de Elche' pomegranate trees (10,11 years-old), planted at 6 × 5 m, located in Elche, south of Alicante, Spain (UTMX: 694006.000 UTMY: 4234860.000). Climatic conditions in the crop field were: a semi-arid Mediterranean climate, with mean annual temperatures of 19.28 and 18.97°C for 2017 and 2018, respectively; maximum temperatures in summer, from June to September, of 31.62 and 31.42 for 2017 and 2018, respectively; and accumulated rainfall of 238.32 and 270.19 mm for 2017 and 2018, respectively[1]. Soil was composed of sand, silt, and clay at 30, 34, and 36%, respectively, and had a pH of 7.8. Irrigation was carried out by using a drip irrigation system with eight emitters per tree, each delivering 4 L h^{-1} as follows. April: two watering cycles of 1 h per week; May, June, July, August, and September: two watering cycles of 2 h per week; and October: one watering cycle of 1 h. The irrigation water used had an electrical conductivity ca. 3 dS m^{-1}. Fertilization was applied in the irrigation system at 160/80/160 kg ha^{-1} nitrogen/phosphorus/potassium (N/P/K) ratio. Pruning and thinning were carried out during the experiments according to standard cultural practices for pomegranate. In 2017 experiment, three blocks or replicates (of three trees each) were selected, totally at random, for treatments: SA, ASA, and MeSA at 1, 5, and 10 mM and control. Treatments were performed by foliar spray application of 3 L of freshly prepared SA, ASA, or MeSa (Sigma Aldrich, Madrid) solutions, containing 1 mL L^{-1} Tween-20 (a polyoxyethylene sorbitol ester, acting as a non-ionic detergent). Control trees were sprayed with distilled water containing 1 mL L^{-1} Tween-20. Dates of treatments were set by taking into account the harvest dates of this cultivar in similar growing conditions in previous seasons, so that the first treatment was applied before the skin color changes and the last one 4 days before the first harvest, according to previous reports (García-Pastor et al., 2020). Dates of treatments in 2017 were: 3rd July, 2nd August, 1st September, and 2nd October. In the 2018 experiment, treatments were SA, ASA, or MeSa at 10 mM concentration and control, which were applied as in the 2017 experiment but instead using five trees for each of the three blocks or replicates. Dates of treatments were: 10th July, 10th August, 11th September, and 11th October. For both

[1] http://riegos.ivia.es/datos-meteorologicos

years, each block or replicate for each treatment was set in a row, leaving an untreated tree between each block and an untreated row between each treated row in order to avoid treatment cross effects. In addition, at least one tree without treatment was left in each row to avoid edge effect.

Fruits were harvested according to commercial criteria based on fruit size (8.5–9.0 cm of diameter and weight of 325–350 g), skin color (cream-light pink), and total soluble solids (TSS) content characteristic of this cultivar (more than 15°Brix). In both years, fruits were picked on two dates, separated by 20 days, due to the heterogeneous fruit on-tree ripening process. On both harvest dates, the yield (kg tree^{-1} and number of fruits tree^{-1}) were determined. In the 2017 experiment, five fruits from the first picking, homogenous in size and color and without visual defects, were selected from each replicate and treatment and immediately transferred to the laboratory for analytical determinations. In the 2018 experiment, four lots of five fruits also homogenous in size and color and without visual defects were chosen at random for each replicate and treatment, immediately transferred to the laboratory, and stored for 0 (day 0), 30, 60, and 90 days at 10°C and at a relative humidity of 85–90%. For each sampling date during storage, one lot was taken at random for each replicate and treatment.

Fruit Yield, Respiration Rate and Qualitative Traits

Yield, expressed as kg tree^{-1} and number of fruit tree^{-1}, was measured at two harvest dates and growing seasons. Then, fruit mass (g) and the percentage of fruits harvested at the first picking were calculated. Results were expressed as the mean ± SE. The weight of each pomegranate lot was measured at day 0 and after each storage period, and weight loss was expressed as a percentage with respect to initial weight. To quantify respiration rate, each fruit lot was hermetically sealed in a 3 L jar for 30 min. After that, 1 mL from the holder atmosphere was withdrawn with a syringe and injected into a Shimadzu TM 14A gas chromatograph (Kyoto, Japan) equipped with a thermal conductivity detector under the chromatographic conditions previously described (Sayyari et al., 2011b).Respiration rate was expressed as g of CO_2 released by kg^{-1} s^{-1}.

Fruit firmness was measured individually in each of the five fruits of each replicate by using a TX-XT2i Texture Analyzer (Stable Mycrosystems, Godalming, United Kingdom) which applied a force to achieve a 3% deformation of the fruit diameter. Results were expressed as the relation between the applied force and the traveled distance (kN m^{-1}) and are the mean ±SE.

Skin and aril color were measured by digital image analysis. Photographs of the pomegranates were captured using a digital camera (Nikon D3400) in a light box with black background. The setup conditions of the camera were as follows: light provided by two LEDs of color temperature of 5600 K, flash speed of 1/5 s, ISO-200, focal opening (f) 20, and length 35 mm. For skin color measure, one image of the front and another of the back side of the five fruits of each of the three replicates for each treatment were captured, saved as JPEG file, and analyzed by using the software ImageJ v1.52a (NIH Image, National Institutes of Health, Bethesda, United States). The CIELab model was used to calculate Hue angle (arctg b*/a*) according to García et al. (2017). Pomegranate fruits were cut by the equatorial plane and aril color was measured by taking photographs of the cut surface as indicated above. After that, arils of the five pomegranates of each replicate were combined to obtain a homogeneous sample for each replicate in which the following parameters were measured. Titratable acidity (TA) was determined in duplicate in each sample, by using 1 mL of diluted juice (in 25 mL distilled H_2O), obtained from 30 g of pomegranate arils, which was automatically titrated (785 DMP Titrino, Metrohm) with 0.1 N NaOH up to pH 8.1, and the results were expressed as g malic acid equivalent kg^{-1} in fresh weight basis. Total soluble solids (TSS) were measured in duplicate in the same juice by using a digital refractometer (Atago PR-101, Atago Co., Ltd., Tokyo, Japan) at 20°C and expressed as g kg^{-1} in fresh weight basis. After that, the aril samples were stored at –25°C until the following determinations were performed.

Total Phenolics, Total Anthocyanin, and Total Antioxidant Activity Quantification

To extract phenolic compounds, 5 g of arils were homogenized with 10 mL of water: methanol (2:8, v/v) containing 2 mM NaF by using a homogenizer (Ultraturrax, T18 basic, IKA, Berlin, Germany) for 30 s. The extracts were centrifuged at 10,000 *g* for 10 min at 4°C and the supernatant was used to quantify total phenolics (in duplicate in each extract) by using the Folin-Ciocalteu reagent as previously described by Sayyari et al. (2011a). The results were expressed as g gallic acid equivalent (GAE) kg^{-1} and are the mean±SE of three replicates. For anthocyanin extraction, 5 g of arils were homogenized as above in 15 mL of methanol: formic acid: water (79:1:20, v/v/v) and then centrifuged at 10,000 *g* for 10 min at 4°C. The absorbance at 520 nm was measured in the supernatant (in duplicate for each sample) and total anthocyanin content (TAC) was expressed as g kg^{-1} of cyanidin 3-*O*-glucoside equivalents (Cyn 3-gluc, molar absorption coefficient of 23,900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}). To measure total antioxidant activity (TAA), 5 g of arils were homogenized in 10 mL of 50 mM phosphate buffer pH = 7.8 and 5 mL of ethyl acetate as indicated above. The homogenate was centrifuged at 10,000 *g* for 15 min at 4°C and the upper and lower fractions were used to quantify lipophilic (L-TAA) and hydrophilic total antioxidant activity (H-TAA), respectively. H-TAA and L-TAA were determined in duplicate in each extract as previously described (Sayyari et al., 2011a), in a reaction mixture containing 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 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 extract was proportional to TAA of the sample which was calculated by using a calibration curve made with Trolox [(R)-(+)-6-hydroxy- 2, 5, 7, 8-tetramethyl-croman-2-carboxylic acid] (0–20 nmol) from Sigma Aldrich (Madrid, Spain), and results are expressed as g of Trolox Equivalent (TE) kg^{-1} and are the mean ± SE of three replicates.

Individual Anthocyanin Quantification

The extracts obtained for total anthocyanin quantification described previously were filtered through a 0.45 µm PVDF filter (Millex HV13, Millipore, Bedford, MA, United States) and then individual anthocyanins were identified by liquid chromatography coupled to mass spectrometry (HPLC- DAD-ESI/MSn) by using an Agilent HPLC1100 series machine equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany), as previously reported (Martínez-Esplá et al., 2014). To quantify individual anthocyanins, two samples of each extract were injected into a HPLC system (Agilent HPLC 1200 Infinity series) working with the chromatographic conditions previously reported (Martínez-Esplá et al., 2014). Chromatograms were recorded at 520 nm and quantification was performed by using calibration curves carried out with cyanidin 3-*O*-glucoside (Cyn 3-gluc), cyanidin 3,5-*O*-di-glucoside (Cyn 3,5-di-gluc), pelargonidin 3-*O*-glucoside (Plg 3-gluc), and pelargonidin 3,5-*O*-di-glucoside (Plg 3,5-di- gluc) (Sigma-Aldrich, Germany). Delphinidin 3-*O*-glucoside (Dlp 3-gluc) and delphinidin 3,5-*O*-di-glucoside (Dlp 3,5- di-gluc) were quantified as Cyn 3-gluc equivalents. Results were expressed as mg kg^{-1} fresh weight (mean ± SE of three replicates).

Ascorbic Acid (AA), Dehydroascorbic Acid (DHA), and Total Vitamin C Quantification

Ascorbic (AA) and dehydroascorbic (DHA) acids were measured according to Peña-Estévez et al. (2016). Briefly, 5 g of frozen arils were homogenized with 5 mL of methanol: water (5:95) containing 0.1 mM citric acid, 0.05 mM ethylenediamine tetraacetic acid disodium salt, and 4 mM NaF for 30 s on an Ultraturrax, T18 basic, IKA, Berlin, Germany. Then, the extract was filtered through a four-layer cheesecloth, the pH was adjusted to 2.35–2.40 with 2 N ClH, and centrifuged at 10,000 *g* for 15 min at 4°C. The supernatant was purified through a methanol-activated C18 cartridge (Sep-Pak cartridges C18, Waters, Dublin, Ireland) and filtered through a 0.45 µm PFTE filter. For DHA derivatization, 750 µL of extract were mixed with 250 µL of 7.7 M 1,2-phenylenediamine in an HPLC amber vial. The mixture was allowed to react for 37 min and then 20 µL were injected onto a Luna (250 mm 4 x 6 mm, 5 µm particle size) C18 column (Phenomenex, Macclesfield, United Kingdom) with a C18 security guard (4.0 mm x 3.0 mm) cartridge system (Phenomenex, Macclesfield, United Kingdom) using a HPLC system (Agilent HPLC 1200 Infinity series). The mobile phase was 50 mM KH_2PO_4 containing 5 mM hexadecyl trimethyl ammonium bromide and 5% methanol (pH 4.59) with isocratic flow of 1 mL min^{-1}. Absorbance was recorded at 261 nm for AA (Rt = 9.4 min) and at 348 nm for DHA (Rt = 4.5 min) and they were quantified by comparison with AA and DHA standards areas (Sigma-Aldrich, Germany). The results (mean ±SE) were expressed as g kg^{-1} fresh weight.

Individual Sugars and Organic Acids Content

To measure sugars and organic acids, 5 g of the aril sample of each replicate were extracted with 5 mL of 0.5% phosphoric acid and the supernatant was filtered through 0.45 µm Millipore filter and injected in duplicate into a HPLC system (Hewlett-Packard HPLC series 1100). The elution system consisted of 0.1% phosphoric acid running isocratically at 0.5 mL min^{-1} through a Supelco column (Supelcogel C-610H, 30 cm 7.8 mm, Supelco Park, Bellefonte, United States). Organic acids were detected by absorbance at 210 nm and sugars by refractive index detector and quantified by using standard curves of pure sugars and organic acids (Sigma-Aldrich, Germany). Results were expressed as g kg^{-1} fresh weight and are the mean ± SE of three replicates.

Statistical Analysis

Results are expressed as mean ± SE of three replicates. Data for the analytical determinations were subjected to analysis of variance (ANOVA) being sources of variation treatment for the 2017 experiment and storage time and treatment for the 2018 experiment. Mean comparisons were performed using HSD Tukey's test to examine if differences between control and treated fruit were significant at $P < 0.05$. All analyses were performed with SPSS software package v. 17.0 for Windows.

RESULTS

Crop Yield

SA treatments of pomegranate trees during the development of pomegranate fruit increased crop yield in a dose-dependent way, the effect being significant ($P < 0.05$) with 5 and 10 mM. Thus, the yield in the 2017 experiment, expressed as kg $tree^{-1}$, was 37.75 ±3.28 in control trees and 51.08 ± 6.52 in those treated with 10 mM SA. Yield was also increased by MeSa treatments although no significant differences were observed among 1, 5, and 10 mM doses. These increases were due to the higher number of fruits harvested from each tree, while fruit mass was not affected by treatment. However, ASA treatments did not have a significant effect ($P < 0.05$) on crop yield, neither on kg $tree^{-1}$ nor on number of fruit $tree^{-1}$ (**Table 1**). These results were confirmed in the 2018 experiment, in which 10 mM dose was applied for SA, ASA, and MeSa treatments (**Table 1**). The percentage of fruits that were harvested in the first picking date was 55.32 ± 3.18% in control fruits which was increased, in a dependent-concentration manner, by SA and ASA treatments, with ca 90% of total fruit being harvested at the first picking date on SA and ASA 10 mM treated fruits. However, the percentage of fruits picked at the first picking date on trees treated with MeSa, either at 1, 5, or 10 mM concentration, was similar to those of non-treated trees (**Table 1**).

Fruit Quality Parameters and Bioactive Compounds at Harvest

Fruit quality parameters, such as firmness, TA, and skin and aril color, at harvest, were improved by all salicylate treatments in the 2017 experiment. Thus, fruit firmness and TA were significantly

TABLE 1 | Effects of preharvest salicylic acid (SA at 1, 5, and 10 mM), acetyl salicylic acid (ASA at 1, 5, and 10 mM), and methyl salicylate (MeSa at 1, 5, and 10 mM) treatments on pomegranate crop yield (kg tree^{-1} and number of fruit tree^{-1}), fruit mass, and% of fruits harvested in the first picking date in the 2017 and 2018 experiments.

	kg tree^{-1}	Fruits tree^{-1}	Fruit mass (g)	% first pick
2017 experiment				
Control	37.54 ± 3.28 a	110.96 ± 7.17 a	338.3 ± 9.54 a	55.32 ± 3.18 a
SA 1	41.45 ± 3.14 ab	123.8 ± 5.2 ab	334.8 ± 9.27 a	49.61 ± 2.65 a
SA 5	45.59 ± 4.29 bc	135.6 ± 6.1 bc	336.2 ± 7.48 a	81.87 ± 2.77 c
SA 10	51.08 ± 3.52 c	141.8 ± 5.7 c	352.9 ± 11.8 a	88.48 ± 3.18 d
ASA 1	42.62 ± 3.56 ab	127.8 ± 4.6 b	333.5 ± 12.8 a	63.17 ± 1.11 b
ASA 5	39.70 ± 3.58 a	117.21 ± 6.6 a	338.7 ± 10.3 a	84.67 ± 4.14 cd
ASA 10	42.39 ± 2.08 ab	124.9 ± 3.74 ab	339.2 ± 12.1 a	88.84 ± 3.64 d
MeSa 1	50.90 ± 3.78 c	142.0 ± 6.4 c	358.0 ± 9.89 a	54.63 ± 5.51 a
MeSa 5	50.43 ± 2.74 c	152.6 ± 9.57 c	330.5 ± 15.3 a	50.28 ± 4.70 a
MeSa 10	50.39 ± 2.66 c	151.0 ± 7.8 c	333.8 ± 16.2 a	49.69 ± 4.61 a
2018 experiment				
Control	43.42 ± 4.06 a	119.6 ± 9.28 a	362.9 ± 11.1 a	55.21 ± 4.22 a
SA 10	51.39 ± 2.90 b	145.7 ± 8.82 b	352.8 ± 9.81 a	65.67 ± 3.70 b
ASA 10	41.41 ± 3.99 a	118.7 ± 8.03 a	348.9 ± 13.3 a	64.59 ± 3.80 b
MeSa 10	55.96 ± 4.70 b	150.0 ± 13.6 b	372.9 ± 7.75 a	47.91 ± 3.96 a

Within a column, different letters show significant differences ($P < 0.05$) among treatments.

higher ($P < 0.05$) in all SA, ASA, and MeSa treated fruits than in controls, the effects being, in general, dose-dependent and higher for SA and ASA treatments than for MeSa (**Figures 1A,C**). On the contrary, Hue angle in skin and arils was significantly ($P < 0.05$) lower in all treated fruits with respect to control ones (**Figures 1B,D**), which show a deep red color of both skin and arils as a consequence of treatments, although no significant differences were observed among treatments. TSS at harvest were 158.5 ±1.0 g kg^{-1} in arils control fruits and significantly ($P < 0.05$) higher in 10 mM SA, ASA, and MeSa treated ones, 173.8 ±0.7, 168.9 ± 0.9, and 169.7 ±1.2 g kg^{-1}, respectively, while no significant effects were observed for salicylate treatments at 1 and 5 mM (data not shown).

In 2017, SA, ASA, and MeSa treatments also induced a significant ($P < 0.05$) increase in total phenolics and anthocyanin concentrations in pomegranate arils, which was higher as increased the applied dose (**Figures 2A,B**). A similar trend was observed for H-TAA, the highest increase being observed on arils of 10 mM SA treated fruits (**Figure 2C**). However, for L-TAA the effects of salicylate treatments were not so evident, since it was significantly ($P < 0.05$) increased only by SA and ASA 10 mM treatments (**Figure 2D**). Taking into account the results obtained in the 2017 experiment, SA, ASA, and MeSa treatments were applied at 10 mM in 2018 and fruits were used for the storage experiment.

Evolution of Pomegranate Quality Parameters and Respiration Rate During Storage

Weight loss increased along storage time, reaching final values of 9.42 ± 0.43% in control fruits and significantly ($P < 0.05$) lower 5.90 ± 0.49, 7.02 ± 0.36, and 7.88 ± 0.58% in fruits from SA, ASA, and MeSa treated ones, respectively (**Supplementary Figure S1A**). Respiration rate at harvest was 86.36 ±1.14 g kg^{-1} s^{-1} in control fruit and this was significantly ($P < 0.05$) higher than those from SA treated trees (76.30 ± 2.52 g kg^{-1} s^{-1}). However, no significant differences were observed between control and ASA or MeSa treated fruit. Respiration rate decreased sharply during storage from day 0 to day 30 and then more slowly after that, and it was lower in SA treated fruits than in controls during the whole storage time (**Supplementary Figure S1B**).

Fruit firmness at harvest was significantly ($P < 0.05$) increased by SA and ASA treatments, from values of 15.89 ± 0.74 kN m^{-1} in control fruit to 18.46 ± 0.46 and 18.88 ± 0.74 kN m^{-1} in those treated with SA and ASA, respectively, confirming the results obtained in the 2017 experiment. During storage, fruit firmness decreased in control and treated fruits, although final firmness levels after 90 days of storage at 10°C remained significantly higher ($P < 0.05$) in fruits from treated trees than in controls (**Supplementary Figure S2A**). With respect to arils color, lower values of Hue angle were measured at harvest in fruits from SA, ASA, and MeSa treated trees. Hue angle of arils decreased during storage, although values of control fruits were always higher than those of treated ones (**Supplementary Figure S2B**). These results indicated that the aril red color increased as a consequence of salicylate treatments, either at harvest or during storage for 90 days, as can be observed in **Supplementary Figure S3**. Fructose was the major sugar in pomegranate arils, followed by glucose, and both sugars were significantly ($P < 0.05$) increased by salicylate treatments, while sucrose was found at a very low concentration without significant differences attributed to treatments (**Figure 3A**). Organic acids were also increased by salicylate treatments, the highest increase

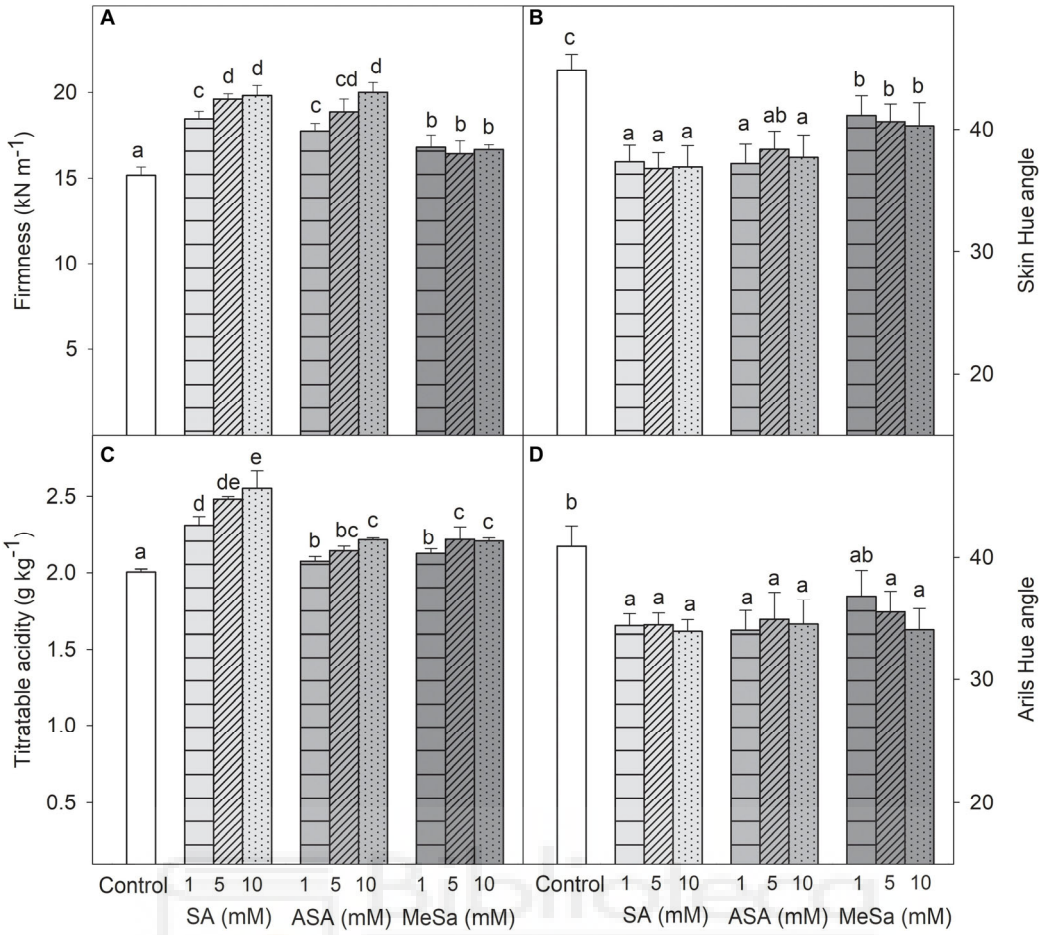


FIGURE 1 | Fruit firmness **(A)**, skin color (Hue angle) **(B)**, titratable acidity **(C)**, and arils color (Hue angle) **(D)** in pomegranate from control and salicylic acid acetyl salicylic acid (ASA), and methyl salicylate (MeSa) treated fruits at harvest, in the 2017 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments.

being found in the major organic acid, malic acid, by SA treatment (**Figure 3B**).

Bioactive Compounds and Antioxidant Activity During Storage

In the 2018 experiment, SA, ASA, and MeSa treatments at 10 mM led to arils with increased concentrations of total phenolics and total anthocyanins at harvest time (**Figures 4A,B**), in agreement with results from the 2017 experiment. Total phenolic concentration increased during storage in pomegranate arils for all treatments, the major increases being found during the first month of storage. At the last sampling date, phenolic concentration was 1.22 ± 0.04 g kg^{-1} in arils from control fruit, and significantly higher ($P < 0.05$), in those from SA, ASA, and MeSa treated ones, at 1.54 ± 0.04, 1.42 ± 0.03, and 1.30 ± 0.04 g kg^{-1}, respectively (**Figure 4A**). For total anthocyanin concentration, values at harvest were significantly higher ($P < 0.05$) in arils from treated fruits than in those from controls, without significant differences among SA, ASA, or MeSa treatments. Anthocyanin concentration also increased during the whole storage period and was higher in arils from all treated fruits than in controls, the highest increase being found in arils of SA and ASA treated fruits (**Figure 4B**). Individual anthocyanin concentration was measured at day 0 in the arils from control and treated fruits. In all aril samples a similar anthocyanin profile was found, Cyn 3-gluc being the major anthocyanin (62.29 ±4.58 mg kg^{-1} in control fruit), followed by Dlp 3-gluc, Plg 3-gluc, Cyn 3,5-di-gluc, and Dlp 3,5-di-gluc while Plg 3,5-di-gluc was found at a very low concentration (**Figure 5**). It is worth noting that all individual anthocyanins, except those found at very low concentrations, were increased as a consequence of salicylate treatments, although no significant differences were observed between SA, ASA, and MeSa treatments.

Similar trends than those for phenolic and anthocyanin content was observed for H-TAA in the arils, which increased during storage, especially in fruits from SA treated trees, while similar increases were found in arils from ASA and MeSa treatments, although they were significantly higher than in arils from control fruits (**Figure 6A**). However, L-TAA showed very

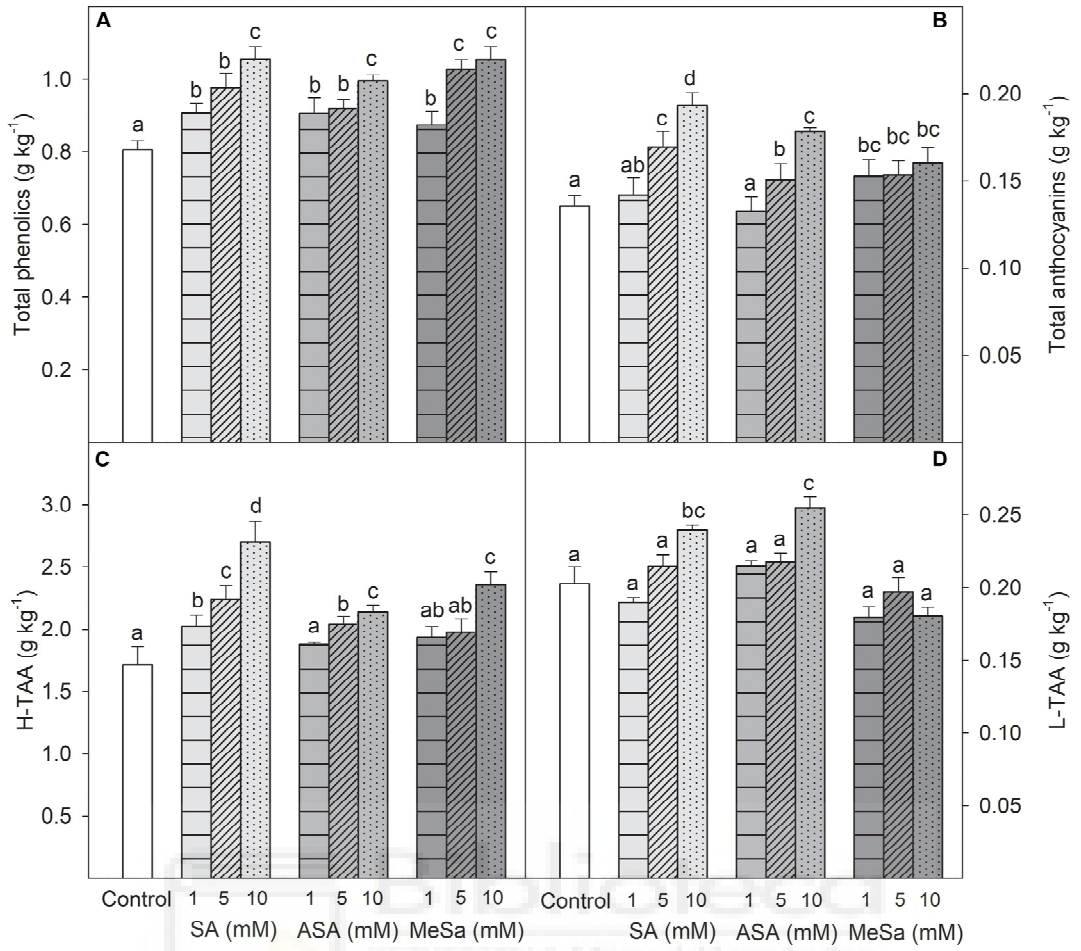


FIGURE 2 | Total phenolic concentration **(A)**, total anthocyanin concentration **(B)**, total hydrophilic antioxidant activity (H-TAA) **(C)**, and total lipophilic antioxidant activity (L-TAA) **(D)** in arils of pomegranate from control and salicylic acid (SA), acetyl salicylic acid (ASA), and methyl salicylate (MeSa) treated fruits at harvest, in the 2017 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments.

low values as compared with H-TAA, its increase during storage was small and differences between control and treated fruits were only significant at the last two sampling dates (**Figure 6B**).

Ascorbic acid content was significantly ($P < 0.05$) increased due to salicylate treatments, with values at harvest of 0.055 ± 0.008 g kg^{-1} in arils of control fruit, 0.086 ± 0.009 g kg^{-1} in those treated with MeSa, and 0.130 ± 0.008 and 0.123 ± 0.003 g kg^{-1} in arils of SA and ASA treated fruits. During storage, AA acid concentration decreased in all treatments, although higher values were found in arils of all treated fruits than in controls during the whole storage time, with final values of 0.019 ± 0.003 g kg^{-1} in control fruit and between 0.067 and 0.082 g kg^{-1} in treated ones (**Figure 7A**). DHA concentration at harvest was also significantly increased ($P < 0.05$) by salicylate treatments and maintained at higher levels during storage, although the effects were not as pronounced as in ascorbic acid (**Figure 7B**). Total vitamin C, calculated as the sum of AA plus DHA, followed a similar trend, with significantly higher values ($P < 0.05$) in treated than in control fruits, at harvest and along the whole storage time (**Figure 7C**).

DISCUSSION

Crop management, and especially water availability, during pomegranate fruit development has been reported to affect fruit ripening, quality, and phytochemical content (Galindo et al., 2014). In the present experiments, climatic conditions were similar for 2017 and 2018 and control and salicylate treated trees were under similar climatic and agronomic conditions. Thus, differences among control and treated trees on yield, fruit ripening, and quality attributes would be due just to the effects of treatments. Results showed that SA, ASA, and MeSa treatments increased crop yield due to an increase in the number of fruits that were harvested from each tree, which had a similar mass, independently of the applied treatment. The increase in fruit number by salicylate treatments could be due to: (i) an increased flowering rate, (ii) an increased rate

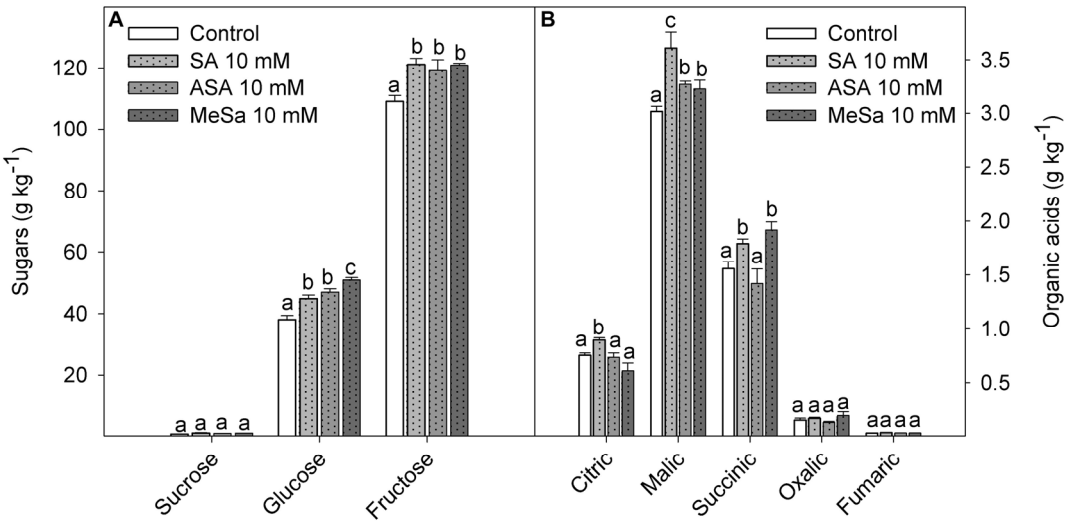


FIGURE 3 | Concentration of major individual sugars **(A)** and organic acids **(B)** in arils of pomegranate from control and salicylic acid (SA), acetyl salicylic acid and methyl salicylate (MeSa) treated fruits at harvest, in the 2018 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments for each individual sugar and organic acid.

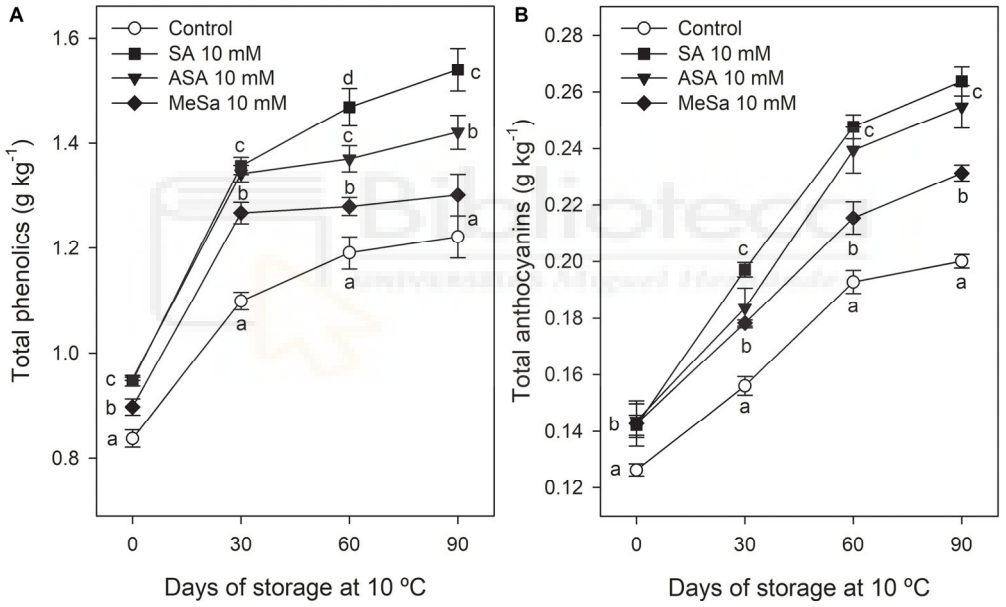


FIGURE 4 | Total phenolic **(A)** and anthocyanin **(B)** concentration in arils of pomegranate from control and salicylic acid (SA), acetyl salicylic acid (ASA), and salicylate (MeSa) treated fruits during storage at 10◦C, in the 2018 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments for each sampling date.

of set fruits, or (iii) a decrease in fruit abscission. However, in our experiments, treatments were performed when fruit had reached ca 30% of their final size so that flowering or fruit set were not affected and the increase in fruit number was due to the reduction of fruit abscission that naturally occurs during the fruit developmental process. Accordingly, an increase in plum tree yield has been recently reported as a consequence of salicylate treatments, although it was due to increased fruit mass but not fruit number (Martínez-Esplá et al., 2018). Moreover, significant increases in total yield and cluster weight of 'Flame Seedless' table grapes were observed as a consequence of vine treatment with a foliar spray of 1.5 or 2.0 mM SA (Champa et al., 2015). These results prove that treatment with salicylates increases net photo-assimilate production in plants and/or the sink strength of developing fruits. In fact, foliar application of SA to ginger plants increased leaves' chlorophyll content,

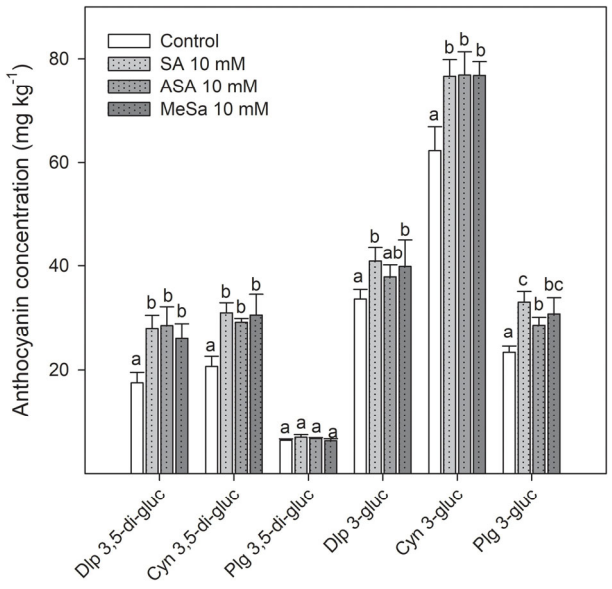


FIGURE 5 | Concentration of delphinidin 3,5-di-*O*-glucoside (Dlp 3,5-di-gluc), cyanidin 3,5-di-*O*-glucoside (Cyn 3,5-di-gluc), pelargonidin 3,5-di-*O*-glucoside (Plg 3,5-di-gluc), delphinidin 3-*O*-glucoside (Dlp 3-gluc), cyanidin 3-*O*-glucoside (Cyn 3-gluc), and pelargonidin 3-*O*-glucoside (Plg 3-gluc) in arils of pomegranate from control and salicylic acid (SA), acetyl salicylic acid (ASA), and methyl salicylate (MeSa) treated fruits at harvest, in the 2018 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments for each individual anthocyanin.

photosynthetic rate, and total dry weight (Ghasemzadeh and Jaafar, 2013). Moreover, an increase in Rubisco activity and total yield by SA treatment were also reported in maize and mustard plants (Fariduddin et al., 2003; Elgamaal and Maswada, 2013). However, the recent results also show an effect on reducing the abscission of pomegranate fruit. Accordingly, Helaly et al. (2018) found a higher percentage of fruit retention and crop yield on two mango cultivars for three consecutive years as a consequence of SA treatments. These effects were attributed to the role of SA activating growth and the nutritional state of trees due to an increase in fresh and dry weight and chlorophyll, carotenoid, and sugar concentration in leaves, showing an effect on enhancing net photosynthesis on tree. In addition, given the pivotal role of SA on increasing tree tolerance to environmental stresses (Tiwari et al., 2017; Koo et al., 2020), its effect allowing pomegranate tress to overcome drought and high temperature stresses during summer seasons in the Spain Southeast cannot be discarded. In fact, maximum mean temperatures in summer, from June to September, were very high in the field crop, ca. 31.5°C in both years. On the other hand, it is worth noting that the percentage of fruits harvested in the first picking date was higher in 5 and 10 mM treated trees and in all ASA treated ones than in controls (**Table 1**). These results show that the on-tree ripening process was accelerated by SA and ASA treatments, especially when applied at 10 mM concentration since all trees (control and treated) were located on the same farm and under similar agronomic and environmental conditions, and pomegranate fruits were harvested upon reaching their commercial ripening stage, according to their size and skin color. This effect could be attributed to an increase of net photosynthesis and/or sink strength induced by treatment with salicylates previously commented (Fariduddin et al., 2003; Elgamaal and Maswada, 2013; Ghasemzadeh and Jaafar, 2013). This is important to point out the rule showing an inverse relationship between tree fruit

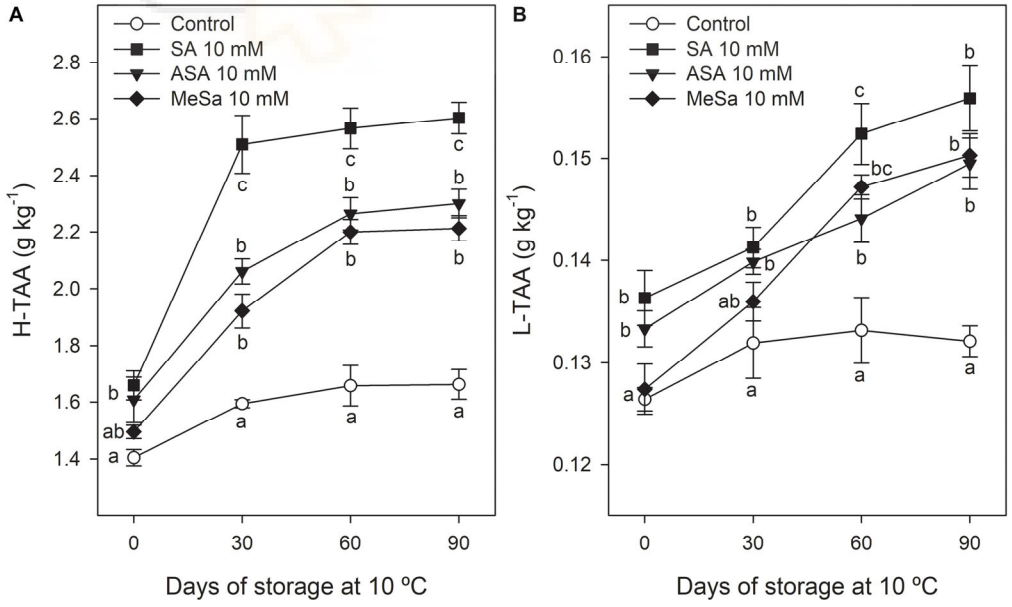


FIGURE 6 | Total hydrophilic antioxidant activity (H-TAA) **(A)** and total lipophilic antioxidant activity (L-TAA) **(B)** in arils of pomegranate from control and salicylic (SA), acetyl salicylic acid (ASA), and methyl salicylate (MeSa) treated fruits during storage at 10◦C, in the 2018 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments for each sampling date.

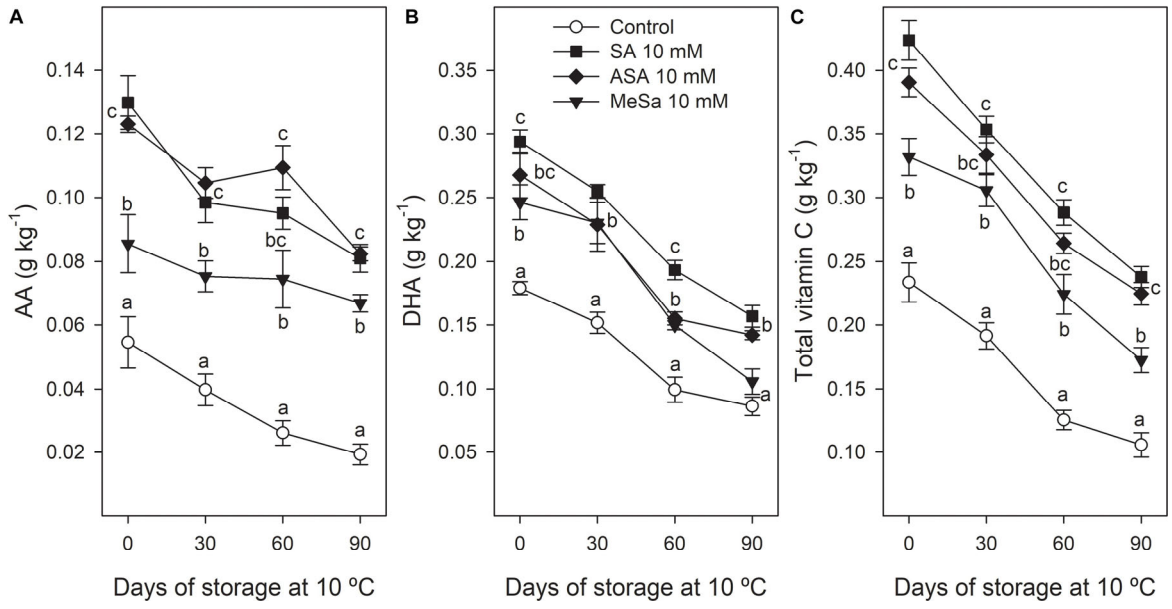


FIGURE 7 | Ascorbic acid (AA) **(A)**, dehydroascorbic acid (DHA) **(B)**, and vitamin C **(C)** in arils of pomegranate from control and salicylic acid (SA), acetyl acid (ASA), and methyl salicylate (MeSa) treated fruits during storage at 10◦C, in the 2018 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments for each sampling date.

load and fruit quality. In this experiment, despite that, the number of fruits was higher in treated trees but the fruit quality was not decreased.

Fruit firmness, TA, TSS, and skin and aril color are indicators of pomegranate fruit quality (Nuncio-Jáuregui et al., 2014; Valero et al., 2014; Pareek et al., 2015). Then, the higher firmness, TSS, and TA and the lower skin and aril Hue angle values found at harvest in the pomegranates of treated trees show that they had higher quality attributes than controls. It is worth noting that the major sugars, fructose and glucose, and the major organic acid, malic acid, were found at higher concentrations in arils of treated fruit than in control (**Figure 3A**). This sugar profile is in agreement with previous reports on 'Mollar de Elche' and other sweet pomegranate cultivars, while in sour cultivars the major organic acid is citric acid (Nuncio-Jáuregui et al., 2014; Cano-Lamadrid et al., 2018). In addition, increases in weight losses (**Supplementary Figure S1**) and decreases in firmness (**Supplementary Figure S2A**) and arils Hue angle (**Supplementary Figure S2B**) show the normal evolution of the postharvest ripening process in "Mollar de Elche" and other pomegranate cultivars (Sayyari et al., 2011a, 2016; García-Pastor et al., 2020), which were delayed by pre-harvest salicylate treatments. Accordingly, the evolution of the postharvest ripening process was delayed in two sweet cherry cultivars by pre-harvest treatments with SA, ASA, and MeSa leading to maintenance of fruit quality parameters (Valverde et al., 2015; Giménez et al., 2017). In this non-climacteric fruit species, as well as in plum (Martínez-Esplá et al., 2017), which is a climacteric fruit, the effect of salicylate preharvest treatments on delaying the postharvest ripening process was attributed to an increase on the concentration of antioxidant compounds and the activity of the antioxidant enzymes. The enhance of these antioxidant systems could lead to efficient scavenging of reactive oxygen species (ROS) which are generated during fruit ripening, a process considered as a functionally modified protracted form of senescence (Hodges et al., 2004).

Pomegranate fruits are a rich source of bioactive compounds, such as phenolics, including anthocyanins and other complex flavonoids and hydrolyzable tannins, and ascorbic acid, as compared with other fruits of the Mediterranean diet, although they are found at different concentrations depending on cultivar, cultural practices, and environmental conditions (Mphahlele et al., 2014; Li et al., 2015; Attanayake et al., 2018). These bioactive compounds have antioxidant properties which are responsible for the beneficial health effects attributed to pomegranate fruit consumption (Faria and Calhau, 2011; Asgary et al., 2017; Panth et al., 2017). Results of the present research show that pre-harvest treatments with salicylates increased total phenolic compound and total and individual anthocyanin concentrations, as well as ascorbic acid, leading to increases in H-TAA. These effects of salicylate treatments on increasing antioxidant compounds were significant at harvest and were maintained during long term storage (**Figures 2A,B,C**, **4A,B**, **5**, **6A**, **7A**) showing that pre-harvest treatment with salicylates would provide the fruit with increased beneficial health effects for human consumption. In fact, it has been claimed that these hydrophilic compounds are responsible for the antioxidant properties of a wide range of fresh fruit species (Valero and Serrano, 2010; Valverde et al., 2015; Martínez-Esplá et al., 2018). This statement is supported by the present results which show that H-TAA was 10-fold higher than L-TAA and strongly correlated $r^2 = 0.827$ ($y = 1.726x–0.120$) and $r^2 = 0.753$ ($y = 7.929x + 0.439$) with total phenolic and total

anthocyanin content, respectively, by taking into account data of all treatment and sampling dates of the 2018 storage experiment.

On the other hand, it is worth noting that AA concentration in pomegranate arils decreased during storage, which is a general trend previously reported in other fruit species such as kiwifruit (Yang et al., 2016), peaches (Falagán et al., 2016) and even in "Hicaznar" (Koyuncu et al., 2019) and "Mridula" (Barman et al., 2014) pomegranate cultivars. However, AA concentration was higher on arils of pomegranates from treated trees than in controls, at harvest and along the storage process. Accordingly, postharvest SA treatment at 2 mM delayed the decrease in AA during storage at 2°C + 2 days at 20°C in "Malas Saveh" pomegranate (Sayyari et al., 2009), as well as in "Malase Yazd" pomegranate, especially if SA dipping treatment was applied as a hot solution (Dokhanieh et al., 2016). This effect could be attributed to an increase in the GR/APX system activity, to lower activity of ascorbic acid oxidase activity (AAO), and/or to higher reducing sugar (glucose and fructose) accumulation. It has been reported that AA is oxidized to DHA by AAO during storage, leading to increases in DHA in a wide range of fresh fruits (Mazurek and Pankiewicz, 2012), including kiwifruit (Yang et al., 2016). However, in the present experiment, DHA decreased in pomegranate arils during storage (**Figure 7B**), as has been reported to occur in peaches (Falagán et al., 2016). Nevertheless, DHA concentration was maintained at higher levels in salicylate treated fruits than in controls, leading to higher concentration in vitamin C (**Figure 7C**), which is an important effect of salicylate treatments on the nutritional value of pomegranate, along with the enhancing of its antioxidant properties.

No previous reports are available in the literature regarding the effect of pre-harvest salicylate treatments on increasing phenolic and anthocyanin content in pomegranate fruit, although some papers exist on other fruits for comparative purposes. In this sense, SA, ASA, and MeSA applied as pre-harvest treatments on sweet cherry and plum trees increased fruit total phenolic and anthocyanin concentrations at harvest with respect to controls, and these differences were maintained during cold storage (Giménez et al., 2014, 2015, 2017; Martínez-Esplá et al., 2018). Accordingly, SA pre-harvest treatments of vine led to higher levels of these bioactive compounds in table grape "Flame Seedless" at harvest and during postharvest storage (Champa et al., 2015). On pomegranate fruits, postharvest treatments with SA, ASA, or MeSA have been reported to maintain total phenolics, anthocyanins, and antioxidant activity at higher levels than in control fruit during cold storage (Sayyari et al., 2011a,b) as well as SA and ASA on sweet cherry (Valero et al., 2011), and SA on cornelian cherry fruit (Dokhanieh et al., 2013) and apricot (Wang et al., 2015). These enhancements were attributed to an increase in the activity of phenylalanine ammonia lyase (PAL), which is the main enzyme involved in the biosynthetic phenolic pathway. Accordingly, dipping of pomegranate arils with SA maintained higher anthocyanin and phenolic concentrations during storage, the effect being higher if SA dipping was performed at 45 than
at 25°C and also attributed to the enhanced activity of PAL (Dokhanieh et al., 2016).

CONCLUSION

Overall results show that, in the 2017 and 2018 experiments, salicylate pre-harvest treatments of pomegranate trees increased crop yield (kg tree^{-1} and number of harvested fruit tree^{-1}) and fruit quality parameters at harvest, such as firmness, aril color, and individual sugar and organic acid contents. Moreover, aril content on bioactive compounds, such as phenolics, anthocyanins, and ascorbic acid, was also increased by salicylate treatments. The quality traits and the concentration of bioactive compounds were maintained at higher levels in pomegranate fruit from treated trees than in controls during prolonged storage at 10°C. It is worth noting the effects of these treatments on increasing total and individual anthocyanin concentration in pomegranate arils which reached a deeper red color and, in turn, would be more appreciated in the international market, all these effects contributing to increasing the profit of this crop. Thus, pre-harvest treatment with salicylates, and especially SA at 10 mM, could be a safe and natural new tool to improve pomegranate fruit quality and their content on antioxidant compounds with beneficial health effects, at harvest and during storage.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DV and MS conceived and designed the work in association with other authors. SC, PZ, and DM-R performed the field treatments. MG-P performed most of the analytical determination in collaboration with the other authors. MS and DV analyzed the data and wrote the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00668/full#supplementary-material

FIGURE S1 | Weight loss **(A)** and respiration rate **(B)** in arils of pomegranate from control and salicylic acid (SA), acetyl salicylic acid (ASA), and methyl salicylate (MeSa) treated fruits during storage at 10◦C, in the 2018 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments for each sampling date.

FIGURE S2 | Fruit firmness **(A)** and aril color (Hue angle) **(B)** in pomegranate from control and salicylic acid (SA), acetyl salicylic acid (ASA), and methyl salicylate (MeSa) treated fruits during storage at 10◦C, in the 2018 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments for each sampling date.

FIGURE S3 | Pictures of the cut surface of pomegranate from control and salicylic acid (SA), acetyl salicylic acid (ASA), and methyl salicylate (MeSa) treated fruits at harvest **(A)** and after 90 days of storage at 10◦C **(B)**, in the 2018 experiment. Pictures represent one of the three replicates for each treatment.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary figure legends

Figure S1. Weight loss (A) and respiration rate (B) in arils of pomegranate from control and salicylic acid (SA), acetyl salicylic acid (ASA), and methyl salicylate (MeSa) treated fruits during storage at 10 ºC, in the 2018 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments for each sampling date.

Figure S2. Fruit firmness (A) and aril color (Hue angle) (B) in pomegranate from control and salicylic acid (SA), acetyl salicylic acid (ASA), and methyl salicylate (MeSa) treated fruits during storage at 10 ºC, in the 2018 experiment. Data are the mean ± SE. Different letters show significant differences ($P < 0.05$) among treatments for each sampling date.

Figure S3. Pictures of the cut surface of pomegranate from control and salicylic acid (SA), acetyl salicylic acid (ASA), and methyl salicylate (MeSa) treated fruits at harvest (A) and after 90 days of storage at 10 ºC (B), in the 2018 experiment. Pictures represent one of the three replicates for each treatment.

Supplementary material

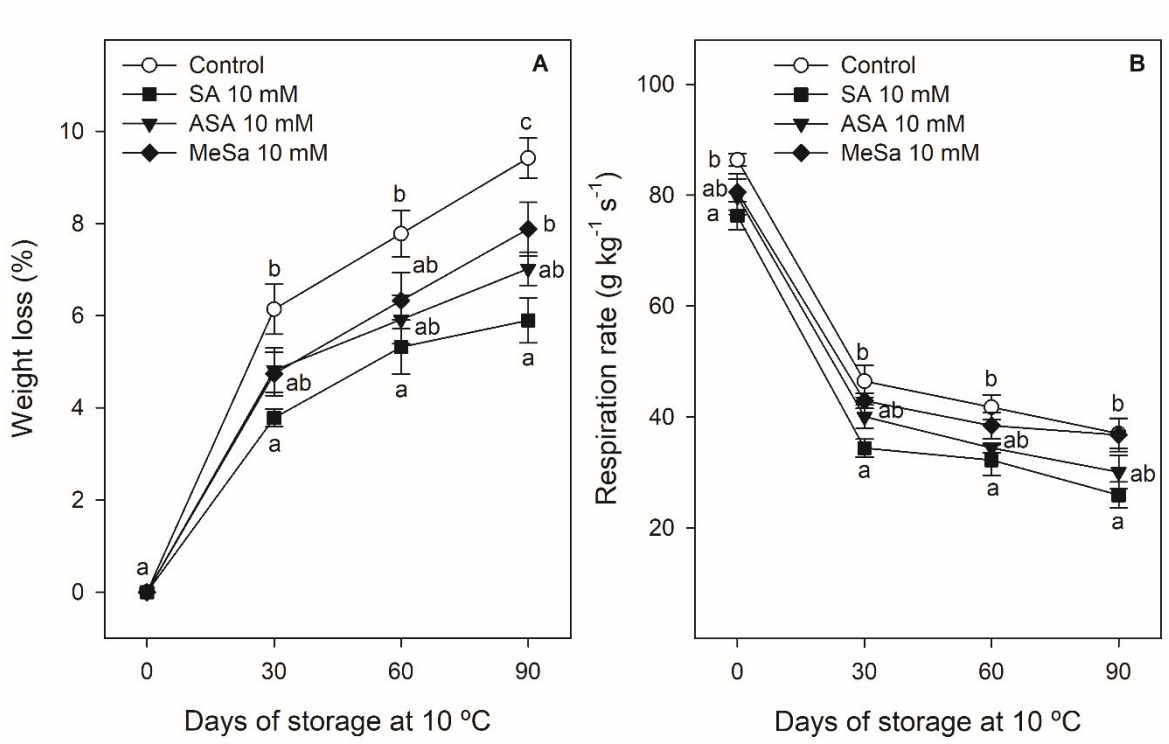

Figure S1.

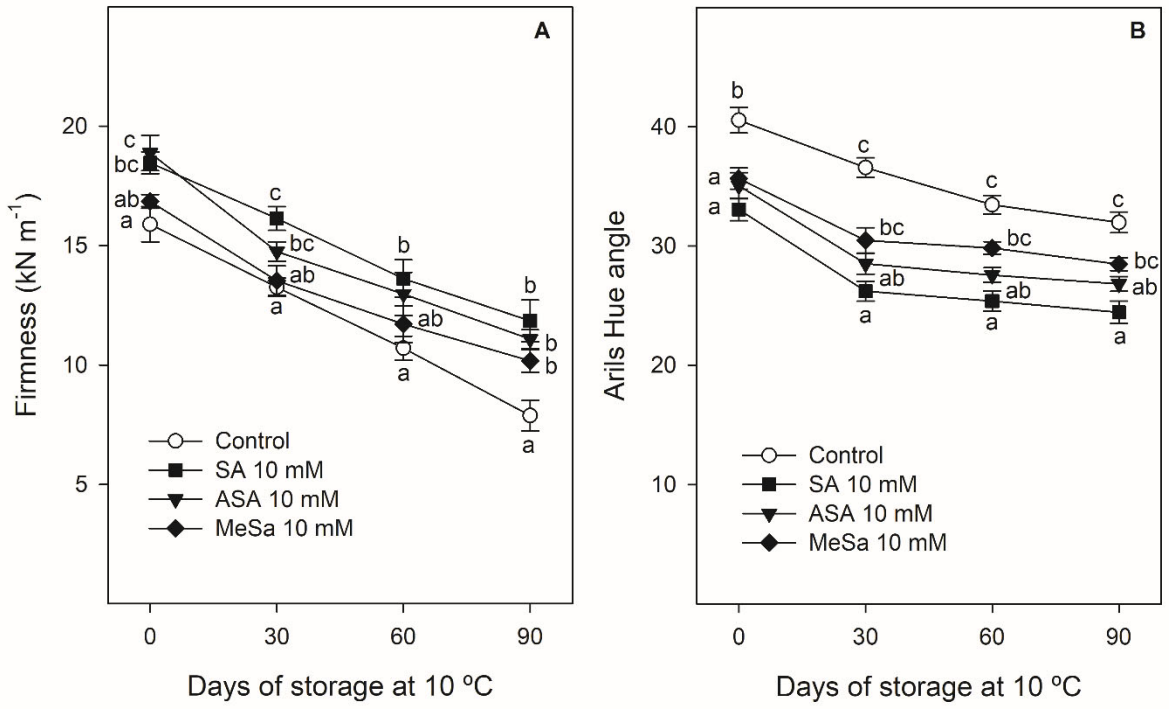

Figure S2.

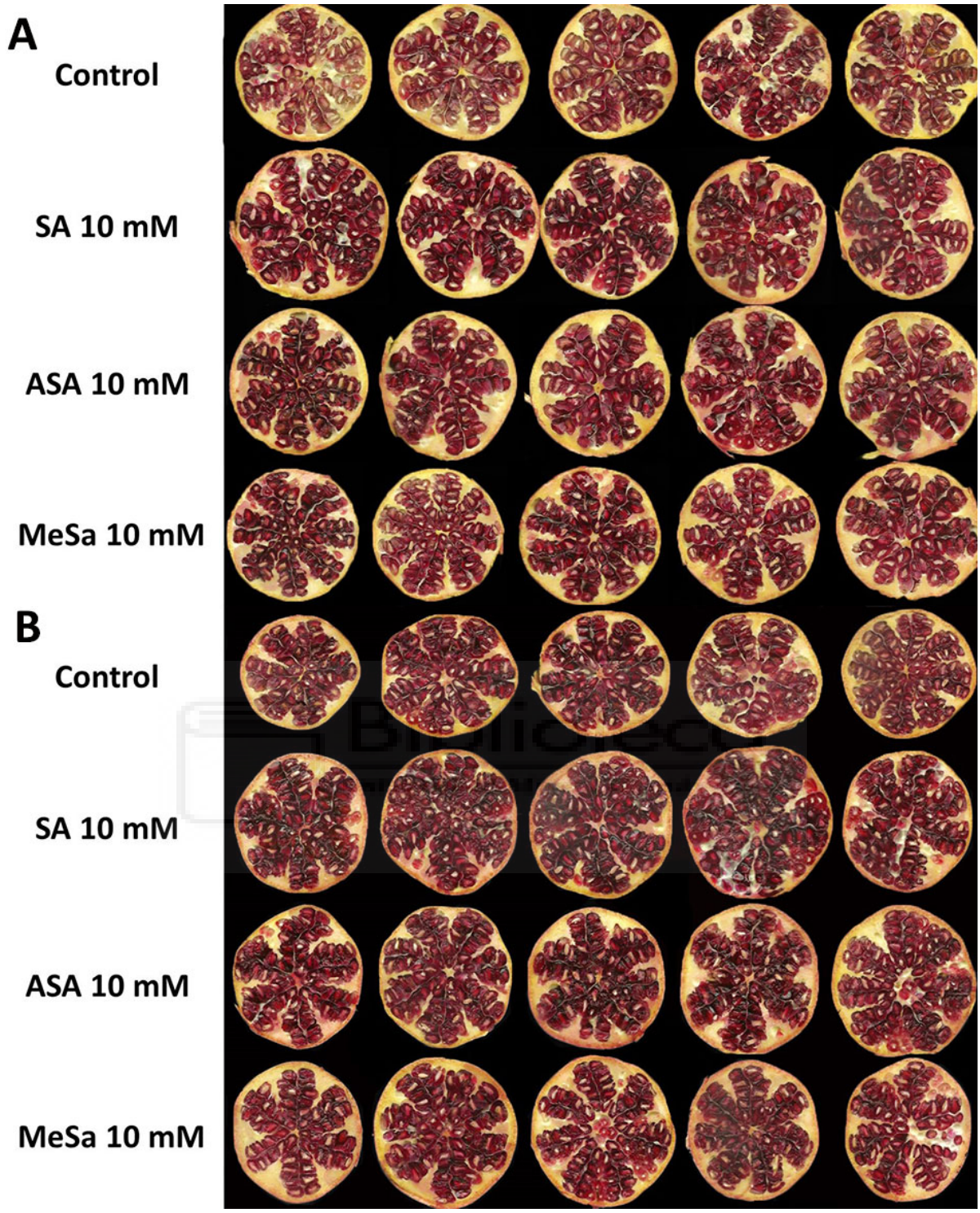
A
Control
SA 10 mM
ASA 10 mM
MeSa 10 mM
B
Control
SA 10 mM
ASA 10 mM
MeSa 10 mM

Figure S3.

4.5. Publication 5

PUBLICATION 5 (Open access)

Preharvest salicylate treatments enhance antioxidant compounds, color and crop yield in low pigmented-table grape cultivars and preserve quality traits during storage

María E. García-Pastor, Pedro J. Zapata, Salvador Castillo, Domingo Martínez-Romero, Daniel Valero, María Serrano, Fabián Guillén

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Article

Preharvest Salicylate Treatments Enhance Antioxidant Compounds, Color and Crop Yield in Low Pigmented-Table Grape Cultivars and Preserve Quality Traits during Storage

María E. García-Pastor [1], Pedro J. Zapata [1], Salvador Castillo [1], Domingo Martínez-Romero[1], Daniel Valero [1], María Serrano [2,*] and Fabián Guillén [1]

[1] Department of Food Technology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Orihuela, Alicante, Spain; m.garciap@umh.es (M.E.G.-P.); pedrojzapata@umh.es (P.J.Z.); scastillo@umh.es (S.C.); dmromero@umh.es (D.M.-R.); daniel.valero@umh.es (D.V.); fabian.guillen@umh.es (F.G.)

[2] Department of Applied Biology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Orihuela, Alicante, Spain

* Correspondence: m.serrano@umh.es; Tel.:+34-966-749-616

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Abstract: Previous reports reported on the effectiveness of preharvest salicylic acid (SA) treatment on increasing fruit quality properties although no information is available about acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments. Thus, SA, ASA and MeSa were applied at 1, 5, and 10 mM in 2016 and at 1, 0.1 and 0.01 mM in 2017 to vines of 'Magenta' and 'Crimson' table grapes. Preharvest salicylate treatments at high concentration, 5 and 10 mM, delayed berry ripening and reduced crop yield, while ripening was accelerated and yield increased at lower concentrations. In addition, SA, ASA, and MeSa treatments, at 1, 0.1, and 0.01 mM, improved berry color due to increased concentration of total and individual anthocyanins, for both cultivars. Quality parameters, and especially, antioxidant bioactive compounds, such as total phenolics and total and individual anthocyanins, were found at higher levels in treated berries at harvest and during prolonged cold storage, the highest effects being found in 0.1 mM MeSa treated table grapes. Overall, it could be concluded that MeSa treatment at 0.1 mM could be the most useful tool to increase bioactive compounds with antioxidant properties in table grape and in turn, their health beneficial properties, with additional effects on increasing crop yield, accelerating on-vine ripening process and maintaining quality traits during prolonged storage.

Keywords: anthocyanins; ripening; *Vitis vinifera*; postharvest; firmness

1. Introduction

Table grape quality depends mainly on cluster size and shape, berry size, sugar/acidity ratio, aroma, and color. During grape development, veraison is considered the most important stage since most of the changes associated with maturation usually start, such as sugar accumulation, acidity reduction, onset of pigment occurrence and synthesis of volatile aroma compounds. These quality traits are going on until berry reaches full maturity [1]. In addition, grapes contain bioactive antioxidant compounds, such as vitamins and phenolic compounds, which have beneficial effects on human health, namely anti-inflammatory, anticancer, and anti-diabetic effects as well as effect on preventing cardiovascular diseases [2–4], which could depend on the gut microbiota composition [5]. Among phenolics, anthocyanins have special importance since they are responsible for color of all red, purple, and dark-purple *Vitis vinifera* L. cultivars, either destined to fresh consumption or

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winemaking [1,4]. However, 'Magenta' and 'Crimson' are red and purple skin table grape cultivars, respectively, with low color development. Moreover, berry pigmentation in the cluster of these cultivars is heterogeneous, which leads to depreciation of their market value. These problems are attributed to high temperatures in the Southeast of Spain during berry ripening, which do not allow proper color development [6].

To improve berry coloration, some attempts were performed in recent years. Thus, ethephon (an ethylene- releasing compound) and abscisic acid (ABA) treatments at veraison stage increased skin anthocyanin concentration although most of these studies were performed with wine grape cultivars [1]. In particular, in 'Crimson Seedless', treatments of vines with ABA or sucrose improved berry coloration [6,7] as well as regulated deficit irrigation applied at post-veraison stage [8]. However, the effects of ethephon on color development are inconsistent and can cause berry softening and the high cost of ABA reduces its practical application. Thus, new research are needed to find out other cost-efficient preharvest treatments able to induce anthocyanin biosynthesis in table grape. Recently, it was reported that Methyl jasmonate (MeJa) treatments at 0.1 mM, applied at key points of berry development, accelerated color evolution due to increased anthocyanin biosynthesis in 'Magenta' and 'Crimson' table grape cultivars [9].

Salicylic acid (SA) and its derivatives, acetylsalicylic acid (ASA) and methyl salicylate (MeSa) are presently considered hormonal compounds with a wide range of physiological effects on plant tissues, from germination to flowering, although the most studied ones are the induction of systemic acquired resistance and resistance against abiotic stresses [10]. In this sense, postharvest treatments with salicylates reduced chilling injury and decay (by increasing fruit resistance to diseases) in a wide range of commodities, with also positive effects on improving fruit quality properties, such as appearance, texture, and nutritional content [10–12]. In addition, in recent years, preharvest treatments with salicylates were reported to improve fruit quality attributes and fruit resistance to pathogen attacks. Thus, foliar spray treatments of jujube plants with 2 mM SA decreased decay caused by *Alternaria alternata* and *Monilinia fructicola*, either at harvest or during cold storage [13]. SA treatment of wax apple fruit, 24 h before harvesting, maintained fruit firmness and visual appearance during storage and higher levels of phenolic content and antioxidant enzyme activities [14]. Moreover, SA, ASA, or MeSa treatments of sweet cherry trees, at 0.5, 1 and 2 mM concentrations, applied at key points of fruit development increased fruit quality attributes at harvest, such as weight, firmness and content of bioactive compounds namely phenolics, including anthocyanins, which were maintained during storage [15–17]. Similar effects of salicylate preharvest treatments were reported in plum [18,19]. In addition, SA, ASA and MeSA are natural compounds in plants which are recognized as GRAS (generally recognized as safe) for the United States Food and Drug Administration (FDA) and previous reports showed that fruit treatments with salicylates do not impart taste or off-flavor to fruit, although some sensory attributes, such as sweetness and firmness, increased [15–19].

In table grape, there are a few recent reports regarding the effects of SA preharvest treatments on berry quality. Thus, Champa et al. [20] showed that 1.5 and 2 mM SA treatments of 'Flame Seedless' cultivar maintained berry color, firmness, phenolic content, and organoleptic properties during cold storage. Similar results were reported by Lo'ay and EL-Boray in SA-treated table grapes of this cultivar during storage at 28 °C [21]. Accordingly, preharvest SA treatments reduced berry weight loss and softening during storage at ambient temperature in 'Thompson' table grape cultivar [22]. In the white seeded 'El-Bayadi' cultivar, preharvest SA treatment led to grapes with higher total antioxidants (TA) and total phenolic and flavonoids content at harvest, which was attributed to a delay of the on-plant ripening process [23]. Moreover, SA treatment at pre-veraison stage of 'Sahebi' grapes led to increases not only in total phenolics and flavonoids at harvest but also in anthocyanin concentration, especially in malvidin-3-glucoside, the major anthocyanin in this cultivar [24]. According to these previous reports, we hypothesized that anthocyanin content would be increased by salicylate preharvest treatments in 'Magenta' and 'Crimson' table grapes cultivars, improving their color development and in turn, their market value as well as their antioxidant properties. Thus, the effects of SA, ASA, and MeSa

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preharvest treatments on berry quality properties at harvest and during cold storage were evaluated in these two table grape cultivars over two growing seasons, with special emphasis in color and anthocyanin content. This is worth noting that as far as we know, there are not previous reports regarding ASA and MeSa treatments on any grape cultivar either applied as pre- or post-harvest treatment.

2. Materials and Methods

2.1. Plant Material, Treatments and Experimental Design

This study was performed with 'Magenta' and 'Crimson', two different *Vitis vinifera* L. seedless table grape cultivars, both of them grafted onto Paulsen 1103 rootstocks, during two growing seasons (2016 and 2017). Vines were planted in a sandy soil, at 2.5 × 3 m, in a commercial orchard in Calasparra (Murcia, Spain). The training system consisted on an overhead trellis at a height of 1.9 m above ground level which was upper and laterally covered with a thread warp net to protect the vines from hail, birds, and insects. Vines were irrigated according to their water requirements along the growth cycle by using a programmer drip irrigation system consisting on a drip irrigation line per row with three emitters per plant. Fertilizers were applied in the irrigation system and pruning and thinning were carried out according to local cultural practices for table grape. SA, ASA, and MeSa treatments were applied at 1, 5 and 10 mM concentrations in 2016 and at 1, 0.1 and 0.01 mM concentration in 2017, since in general, salicylates at 5 and 10 mM decreased vine yield and delayed berry ripening process, as commented in Result section. Treatments were performed by spraying 1.5 L per vine of freshly SA, ASA, or MeSa (purchased from Sigma-Aldrich, Darmstadt, Germany) prepared solutions containing 0.5% Tween 20 as surfactant. The treatments were carried out at early morning and under favorable weather conditions in which no rain or winds were forecast for the next 24 h. An aqueous solution of 0.5% Tween was used to spray control vines. Three treatments were applied for each compound: when berries had ca 40% of their final volume (=1650 and 2100 mm^3 for 'Crimson' and 'Magenta', respectively), at veraison stage and 3 days before the first harvest date (Table 1). Cultural practices during the experiments, such as pruning, irrigation and fertilization, followed standard procedures for table grape crop. A completely randomized block design by using three replicates of three vines for each treatment, cultivar and year was set up.

Table 1. Dates of salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments (T1, T2 and T3) of 'Magenta' and 'Crimson' cultivars.

Cultivar	'Magenta'		'Crimson'	
Treatment	**2016**	**2017**	**2016**	**2017**
T1	23rd June	27th June	24th June	28th June
T2	8th July	12th July	9th July	15th July
T3	18th July	21st July	25th July	28th July

2.2. Vine Yield Determination and Storage Experiment

Clusters were harvested when berries reached the commercial ripening stage according to size, color and total soluble solid content of these cultivars, 170–180 g·100 g^{-1}. Then, four harvests were performed for both cultivars and years, and kg harvested from each vine was measured for each harvest date. Production for each vine was expressed as accumulated yield (kg·$vine^{-1}$) for the first to the last harvest date (mean ± SE of three replicates of three vines).

In 2016, storage experiment was performed with table grapes from control and 1 mM SA, ASA and MeSa treated vines of the second harvest date (27th July and 3rd August for 'Magenta' and 'Crimson', respectively), while in 2017, storage experiment was performed with table grapes from control and SA, ASA, MeSa treated table grapes at 1, 0.1 and 0.01 mM of the second harvest date (31st July and 10th August for 'Magenta' and 'Crimson', respectively). Storage experiments were performed when enough number of clusters were harvested which was at the second harvest date for both years and

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cultivars. In both years, 8 clusters from each replicate and cultivar of control and treated vines were immediately transported to the laboratory and stored at 2 °C and 90% RH for 0, 15, 30 and 45 days. For each sampling date, two clusters were taken at random from each replicate (three biological replicates each of one consisting on two clusters) in which the following parameters were measured.

2.3. Quality Parameters

Clusters were weighed at day 0 and after each storage period, and weight loss was expressed in percentage with respect to weight at harvest. Color L*, a* and b* parameters were measured individually in 30 berries from each replicate (15 berries from each of the two clusters per replicate) by using a Minolta colorimeter (CRC200, Minolta Camera Co., Osaka, Japan), and color was expressed as Hue angle (arctan b/a). Firmness was measured as the force that achieved a 5% deformation of the berry diameter by using a Texture Analyzer (TX-XT2i, Stable Microsystems, Godalming, UK), and was expressed as $N \cdot mm^{-1}$. Data of color and firmness are the mean ± SE of three replicates of 30 berries. After that these 30 berries of each replicate were cut in small pieces and ground to obtain a homogeneous juice sample. Total soluble solids (TSS) were measured in duplicate in each juice sample by using a digital refractometer (Atago PR-101, Atago Co. Ltd., Tokyo, Japan) at 20 °C, and expressed as $g \cdot kg^{-1}$ on a fresh weight (FW) basis (mean ± SE). Total acidity was measured, also in duplicate, in the same juice by automatic titration with 0.1 N NaOH up to pH 8.1 (785 DMP Titrino, Metrohm, Phyathai, Thailand) and results (mean ± SE) are expressed as g tartaric acid equivalent kg^{-1} FW.

2.4. Total Phenolics and Total and Individual Anthocyanin Quantifications

Another sample of 30 berries from each replicate was taken as above, cut in small pieces, ground under liquid N_2 and stored at −80 °C until quantification of total phenolics and total and individual anthocyanins were performed. Phenolic compounds were extracted by homogenizing 5 g of grape sample with 10 mL of water: methanol (2:8, *v*/*v*) containing 2 mM NaF for 30 s by using a homogenizer (Ultraturrax, T18 basic, IKA, Berlin, Germany). The extracts were centrifuged at 10,000× *g* for 10 min at 4 °C and total phenolics were quantified in the supernatant (in duplicate in each extract) by using the Folin-Ciocalteu reagent according to previous report [19]. Anthocyanins were extracted by homogenizing manually 10 g of frozen berry tissues with 15 mL of methanol/formic acid/water (25:1:24, *v*/*v*/*v*) by using a mortar and pestle. Then, the homogenate was sonicated in an ultrasonic bath for 60 min and then centrifuged at 10,000× *g* for 15 min. To measure total anthocyanin concentration in the supernatant (in duplicate) the absorbance at 520 nm was read in an spectrophotometer (UNICAM Helios-α, Artisan Technology Group, Champaign, IL, USA) and results were expressed as g of malvidin 3-glucoside equivalent (molar absorption coefficient of 27,000 $L \cdot cm^{-1} \cdot mol^{-1}$ and molecular weight of 493.4 $g \cdot mol^{-1}$) per kg FW (mean ± SE). For individual anthocyanin quantification, the supernatant was filtered through a 0.45 µm fluoruro de polivinilideno (PVDF) filter (Millex HV13, Millipore, Bedford, MA, USA) and 20 µL were injected into a high-performance liquid chromatography (HPLC) system (Agilent HPLC1200 Infinity series, Agilent Technologies Inc., Waldbronn, Germany) working as previously reported [19]. Chromatograms were recorded at 520 nm. Anthocyanin standards were: malvidin 3-glucoside and peonidin 3-glucoside (purchased from Sigma-Aldrich, Darmstadt, Germany) and cyanidin 3-rutinoside (purchased from Polyphenols SA, Sandnes, Norway) and results ($g \cdot kg^{-1}$ FW) were mean ± SE. Delphinidin 3-glucoside and petunidin 3-glucoside were expressed as malvidin 3-glucoside equivalents.

2.5. Statistical Analysis

Data were subjected to ANOVA analysis, with treatments and storage time as sources of variation. Tukey's test was used to examine whether mean differences were significant at $p < 0.05$. All analyses were performed by using SPSS software package v. 11.0 for Windows (SPSS, 2001, IBM Corporation, Armonk, NY, USA). In addition, a Student *t*' test was performed when comparing data of two sampling dates or years.

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3. Results

3.1. Vine Yield and Berry Ripening Process

Clusters were harvested when berries reached their commercial ripening stage based on skin color (homogeneous purple color of the berries in a cluster) and TSS content characteristic of these cultivars (170–180 g·100 g^{-1}). Given the heterogeneous ripening of the clusters within a vine, four harvests were performed for both table grape cultivars and years. Total yield of vines of 'Magenta' cultivar treated with SA or ASA at 5 and 10 mM was significantly decreased ($p < 0.05$) with respect to yield of control vines in 2016 experiment. Thus, total yield was 34.18 ± 1.34 kg·$vine^{-1}$ in control vines, 26.40 ± 4.66 and 18.90 ± 4.85 kg·$vine^{-1}$ in those treated with SA at 5 and 10 mM, respectively, and 18.91 ± 4.89 and 20.77 ± 1.60 kg·$vine^{-1}$ in 5 and 10 mM ASA treated ones, respectively, while no significant differences were observed between 1 mM SA or ASA treatments and controls (Figure S1A,C). However, for MeSa treatments different results were obtained since total yield was not significantly ($p > 0.05$) affected by any of the MeSa doses. In addition, for the first, second and third harvest dates, more kg·$vine^{-1}$ were harvested from MeSa treated vines than from controls, especially for 1 mM dose (Figure S1E). Thus, given the results obtained in year 2016, vines of 'Magenta' cultivar were treated with SA and its derivatives at 1, 0.1 and 0.01 mM concentrations for 2017 experiment. Results from salicylate treatments at 1 mM concentration confirmed those of 2016 experiment. However, SA and ASA treatments at 0.1 and 0.01 mM increased significantly ($p < 0.05$) the accumulated vine yield for all harvest dates, as well as MeSa at 0.1 and 1 mM (Figure S1B,D,F), showing an effect on accelerating the on-vine berry ripening process.

In 'Crimson' cultivar results were different in some extension because the delay of the on-vine ripening process and the reduction on total yield was only evident and significant ($p < 0.05$) for SA and ASA at 10 mM but not for 5 mM as occurred in 'Magenta' cultivar. Thus, total yield was 43.77 ± 4.36 kg·$vine^{-1}$ in controls and 35.42 ± 2.07 and 27.56 ± 4.29 kg·$vine^{-1}$ in 10 mM SA and ASA treated ones, respectively (Figure S2A,C). With respect to MeSa treatments, 10 mM dose significantly ($p < 0.05$) reduced total yield which was 37.48 ± 3.16 kg·$vine^{-1}$, (14.37% less than control) (Figure S2E). For 2017 experiment 1, 0.1 and 0.01 mM of SA, ASA and MeSa were applied. Total yield was not significantly affected by any of the salicylate treatments, although more kg·$vine^{-1}$ were harvested the first and second harvest dates from vines treated with 1 mM of SA and ASA (Figure S2B,D) confirming the results of 2016 experiment. For MeSa treatments at 1, 0.1 and 0.01 mM concentrations the amount of kg harvested the first and second harvest dates was significantly higher ($p < 0.05$) in treated vines than in controls, the highest effect being found with 0.1 mM concentration (Figure S2F), showing that the on-vine ripening process was accelerated.

3.2. Bioactive Compounds: Anthocyanins and Phenolics

In 2016 experiment, total anthocyanin concentration at harvest was significantly higher ($p < 0.05$) in grapes from 1 mM SA, ASA and MeSa treated vines than in controls in both cultivars and increases occurred during storage in all of them, although anthocyanin concentration was maintained at higher levels in treated berries, the highest increases being found for 1 mM MeSa treatment in both cultivars (Figure 1A,B).

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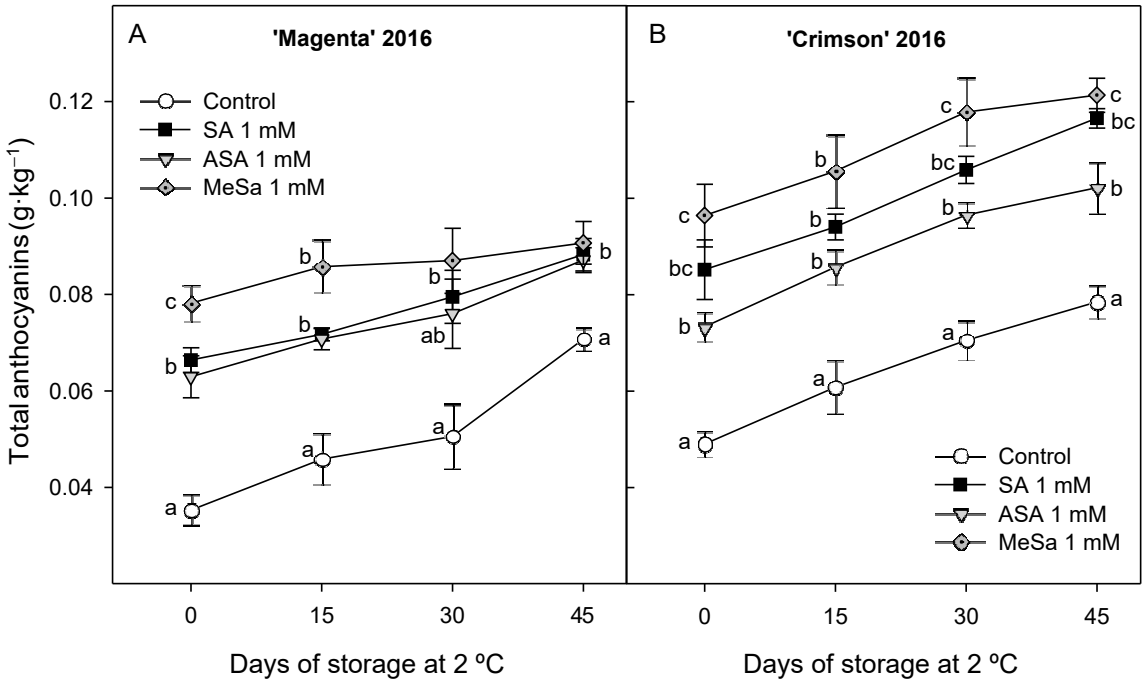

Figure 1. Effects of preharvest 1 mM salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments on total anthocyanin concentration on 'Magenta' (**A**) and 'Crimson' (**B**) cultivars during storage at 2 °C in 2016 experiment. Data are the mean ± SE of quantifications made in duplicate in three replicates. Different letters show significant differences ($p < 0.05$) among treatments for each sampling date.

Total anthocyanin concentration at harvest was also significantly ($p < 0.05$) increased by preharvest salicylate treatments in 2017 experiment in both cultivars, and the highest concentrations were found for 0.01 mM SA, 0.1 mM ASA and 0.1 mM MeSa treatments in 'Magenta' (Figure 2A) and for 0.01 mM SA, 1 mM ASA and 0.1 mM MeSa in 'Crimson' (Figure 2B). Thus, total anthocyanin content during storage was measured in table grapes from these treatments and results showed increases during storage, as occurred in 2016 experiment, with higher concentrations in berries from treated vines than in those from control ones until the last sampling date (Figure 2C,D).

Individual anthocyanins were quantified in grape samples from control and 1 mM SA, ASA and MeSa treated vines in 2016 experiment and in samples from control and vines treated with the best concentration of SA, ASA, and MeSa in terms of their effects on increasing total anthocyanin concentration in 2017 experiment. Thus, five individual anthocyanins were identified and quantified in 'Magenta' cultivar in both years, the major ones being peonidin 3-*O*-glucoside (Pn-3-glu) and malvidin 3-*O*-glucoside (Mv-3-glu), with concentrations between 0.015 and 0.017 $g \cdot kg^{-1}$, followed by delphinidin 3-*O*-glucoside (Dlp-3-glu), ca. 0.006 $g \cdot kg^{-1}$, and petunidin 3-*O*-glucoside (Pt-3-glu) and cyanidin 3-*O*-glucoside (Cy-3-glu) at very low concentrations, between 0.002 and 0.003 $g \cdot kg^{-1}$ in table grapes from control vines (Figure 3A,C). For 'Crimson' table grapes, only three anthocyanins were detected, the major one being Pn-3-glu, followed by Mv-3-glu and Cy-3-glu, with concentrations ca. 0.04–0.042, 0.005–0.006 and 0.001 $g \cdot kg^{-1}$, respectively in control berries (Figure 3B,D). In 2016 experiments, salicylate treatments led to berries with significant ($p < 0.05$) higher concentration in all the individual anthocyanins, especially in the major ones, and the highest increases were found for 1 mM MeSa treatment in both cultivars (Figure 3A,B). Similar effects of salicylate treatments on increasing individual anthocyanin concentration were observed in both table grape cultivars in 2017 experiment, the major increase being found for 0.1 mM MeSa treatment (Figure 3C,D).

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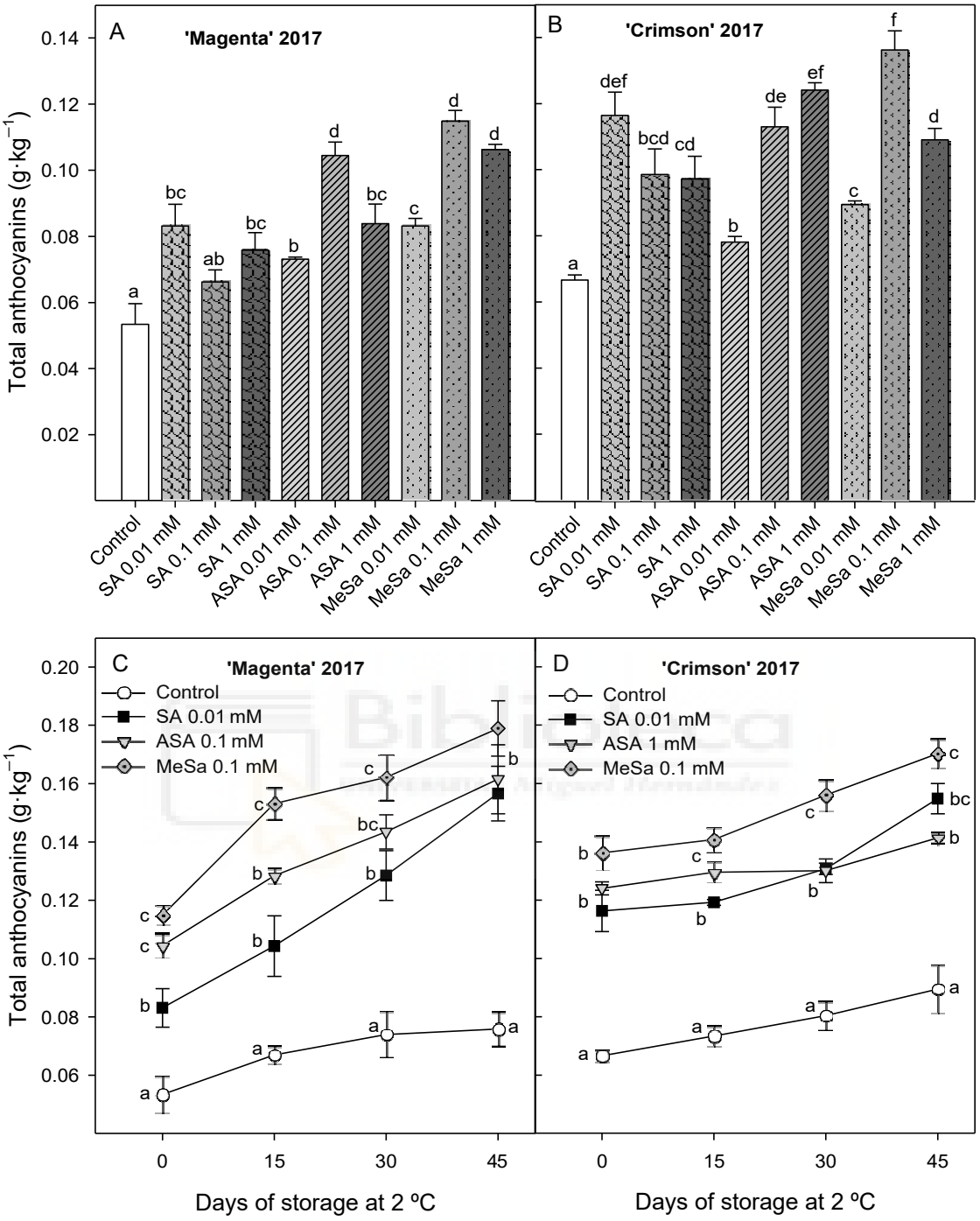

Figure 2. Total anthocyanin concentration at harvest in control and salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treated berries of 'Magenta' (**A**) and 'Crimson' (**B**) cultivars and during storage (**C**,**D**) at 2 °C in 2017 experiment. Data are the mean ± SE of quantifications made in duplicate in three replicates. Different letters show significant differences ($p < 0.05$) among treatments for each sampling date.

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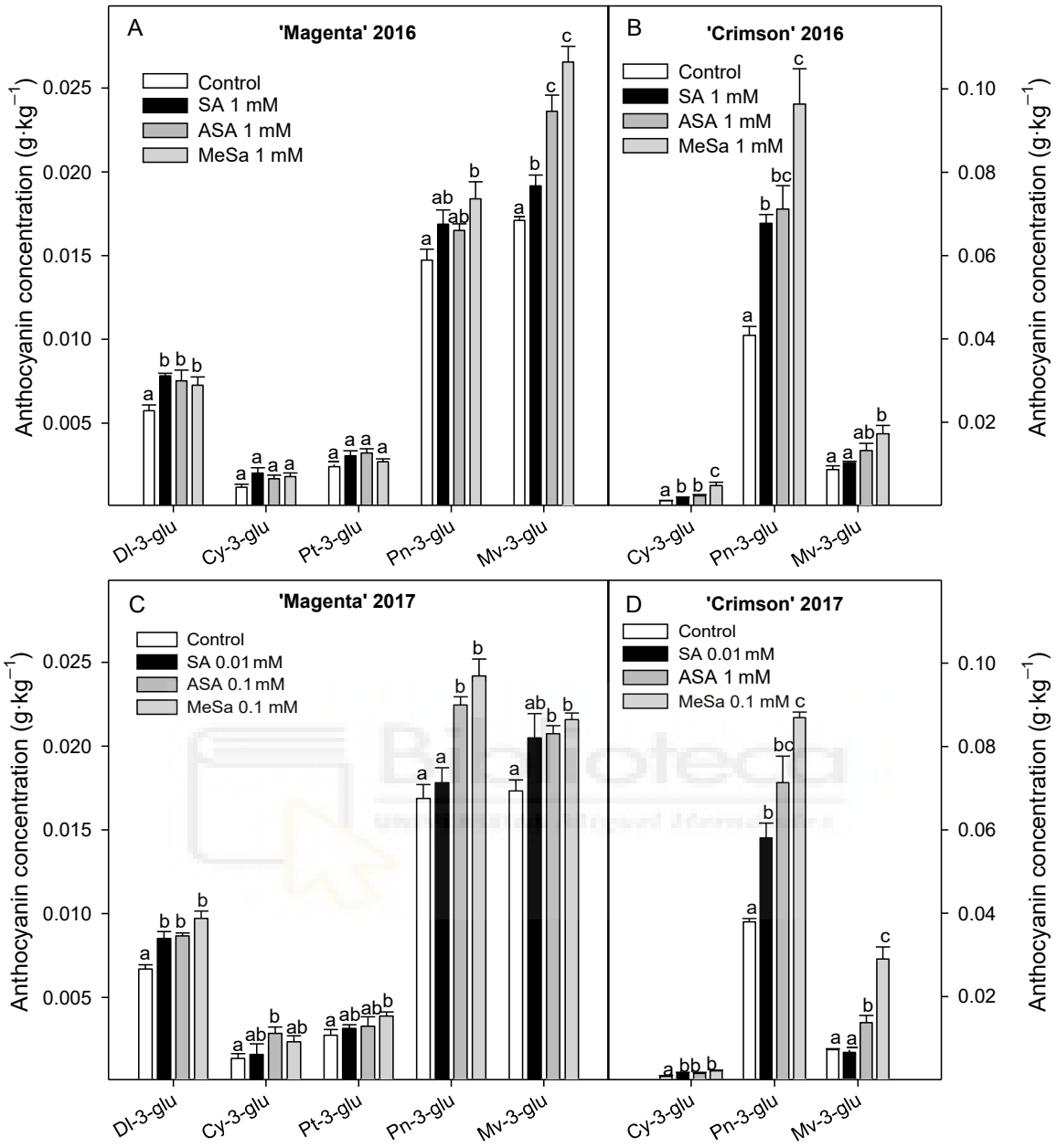

Figure 3. Individual anthocyanin concentration in 'Magenta' and 'Crimson' table grapes as affected by salicylic acid (SA), acetyl salicylic acid (SA) and methyl salicylate (MeSa) treatments at harvest in 2016 (**A**,**B**) and 2017 (**C**,**D**) experiments. Data are the mean ± SE of quantifications made in duplicate in three replicates. Different letters show significant differences ($p < 0.05$) among treatments for each individual anthocyanin.

Total phenolic concentration at harvest in 2016 was significantly higher ($p < 0.05$) in berries from salicylate treated vines than in controls, the effect being higher for MeSa treatment, followed by ASA and SA ones in 'Magenta' (Figure 4A), while no significant differences were found among salicylate treatments in 'Crimson' (Figure 4B). Phenolic concentration increased during storage although they were maintained at higher levels ($p < 0.05$) in treated than in control berries, the higher effects being found in MeSa treatment for both cultivars. Similarly, higher total phenolic concentrations were found in treated than in control berries at harvest in 2017 experiment, especially for 0.1 mM MeSa in 'Magenta' (Figure 4C) and 0.01 mM SA in 'Crimson' (Figure 4D).

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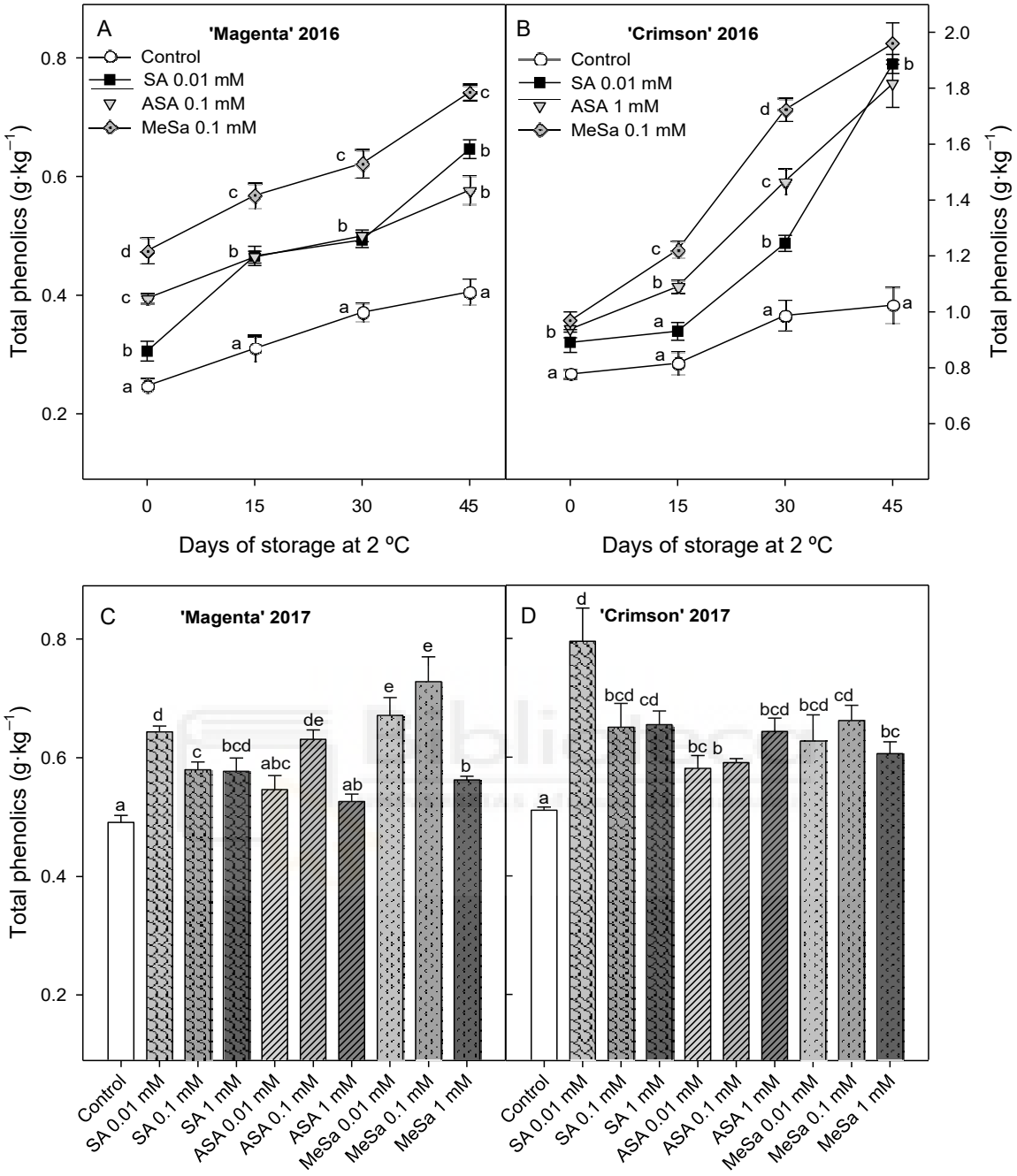

Figure 4. Total phenolic concentration at harvest and during storage at 2 °C in 2016 experiment (**A**,**B**) and at harvest in 2017 experiment (**C**,**D**) in control and salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treated berries of 'Magenta' and 'Crimson' cultivars. Data are the mean ± SE of quantifications made in duplicate in three replicates. Different letters show significant differences ($p < 0.05$) among treatments for each sampling date.

Total phenolic concentration increased during storage in berries from control and treated vines, as was found in 2016 experiment, although values were significantly ($p < 0.05$) lower in control berries than in treated ones (Figure 5A,B). The highest effect on increasing total phenolic content during the whole storage period was found for 0.1 mM Mesa and 0.01 mM SA treatments in 'Magenta' and 'Crimson' cultivars, respectively.

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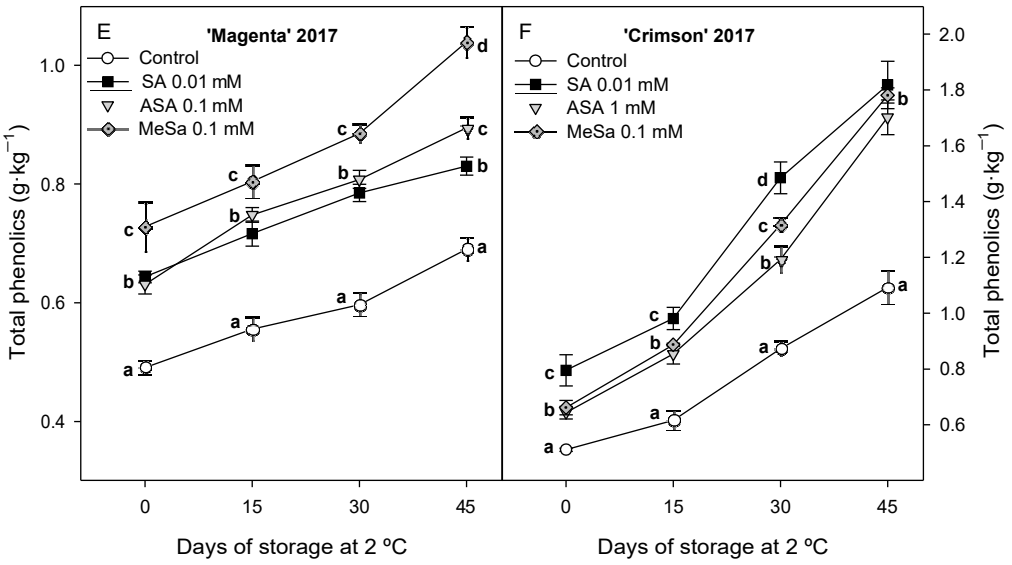

Figure 5. Total phenolic concentration at harvest and during storage at 2 °C in 2017 experiment in control and salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treated berries of 'Magenta' (**A**) and 'Crimson' (**B**) cultivars. Data are the mean ± SE of quantifications made in duplicate in three replicates. Different letters show significant differences ($p < 0.05$) among treatments for each sampling date.

3.3. Quality Parameters

Quality parameters of table grapes, such as color, TSS, TA, firmness and weight loss were measured at harvest and during storage. Values of Hue angle colour were significantly lower ($p < 0.05$) in 1 mM salicylate treated grapes than in controls at harvest in 2016 experiment (Figure 6A,B), showing that treated berries had a deeper purple colour than control ones, the highest effect being found in 1 mM MeSa treated berries for both cultivars. Values of Hue angle decreased during storage in berries from control and treated vines for both cultivars, although they were always higher in control than in treated ones. In 2017 experiment, when SA, ASA and MeSa were applied at 1, 0.1 and 0.01 mM concentrations, 'Magenta' and 'Crimson' berries had also significant ($p < 0.05$) lower values of Hue angle colour than control ones at harvest and decreased occurred during storage (Figure S3). The highest effect on decreasing Hue angle among the three assayed concentration was found at 0.01 mM for SA treatments (Figure S3A,D) and for 0.1 mM for MeSa treatments (Figure S3C,F) in both cultivars, while for ASA treatments, the highest effect was found for 0.1 mM concentration in 'Magenta' (Figure S3B) and for 1 mM in 'Crimson' (Figure S3E).

Concentrations of TSS at harvest were 195.5 ± 1.0 and 180.2 ± 2.2 g·kg^{-1} in 'Magenta' berries and 183.0 ± 2.5 and 175.8 ± 1.2 g·kg^{-1} in 'Crimson' for 2016 and 2017, respectively and they generally increased during storage. However, TSS were significantly ($p < 0.05$) higher in 1 mM SA, ASA and MeSa treated berries than in controls, at harvest and after 45 days of cold storage, while no significant differences were found for 0.1 and 0.01 mM salicylate treatments (Table 2). On the contrary, TA values decreased during storage in control and treated table grapes of both cultivars, although values were higher in treated than in controls, the effects being significant ($p < 0.05$) for all the applied doses (Table 3). Berry firmness also decreased during storage and values were significantly ($p < 0.05$) higher in salicylate treated table grapes than in control, at harvest and during cold storage for both cultivars and years. In 2016 experiment, no significant differences were observed among 1 mM SA, ASA and MeSa treatments on 'Magenta' while in 'Crimson' the highest firmness values were observed for 1 mM SA treated berries (Figure S4). In 2017 experiment, the highest values of firmness, either at harvest or for each sampling date during storage, were observed for 0.01 mM SA dose and 0.1 mM MeSa dose in both cultivars and for 0.1 and 1 mM ASA doses for 'Magenta' and 'Crimson' cultivars,

respectively (Figure S5). Finally, cluster weight losses increased during storage reaching final values of 7.13 ± 0.24%and 9.09 ± 0.36% in 'Crimson' and 'Magenta' control table grapes, respectively, in 2016 experiment and significantly ($p < 0.05$) lower values in grapes from salicylate treated vines (Table S1). Accordingly, weight losses during storage were also reduced in 2017 experiment as a consequence of preharvest salicylate treatments, the highest effects being found for 0.1 mM MeSa treatment in both cultivars (Table S1).

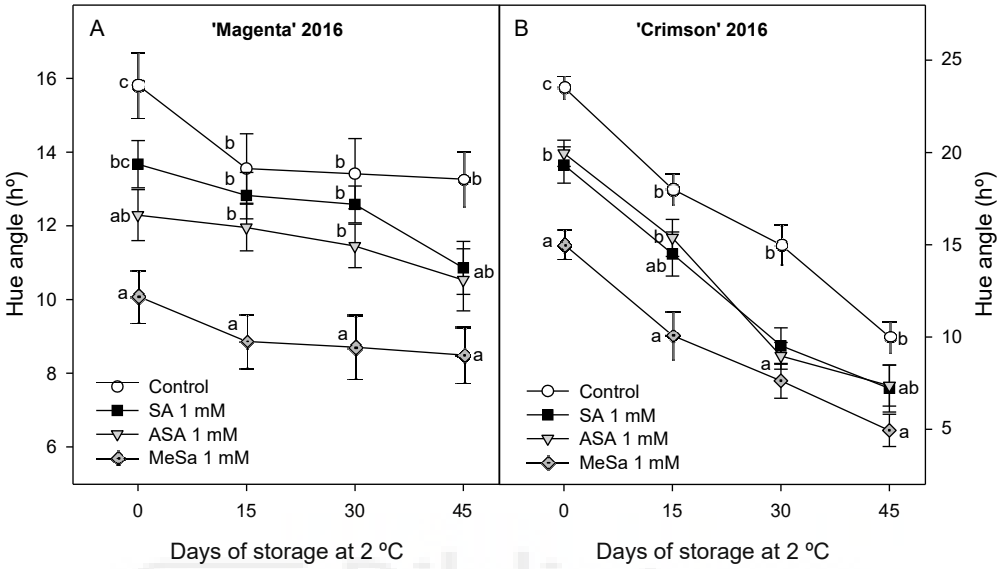

Figure 6. Effects of preharvest 1 mM salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments on Hue angle colour evolution of 'Magenta' (**A**) and 'Crimson' (**B**) table grapes during storage at 2 °C in 2016 experiment. Data are the mean ±SE of measures made on 30 berries for each of the three replicates. Different letters show significant differences ($p < 0.05$) among treatments for each sampling date.

Table 2. Total soluble solids ($g \cdot kg^{-1}$) at harvest (Day 0) and after 45 days of storage at 2 °C (Day 45) in 'Magenta' and 'Crimson' table grapes as affected by preharvest salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments in 2016 and 2017 experiment.

Cultivar	'Magenta' 2016		'Crimson' 2016	
	Day 0	Day 45	Day 0	Day 45
Control	195.5 ± 1.0 ^{aA}	204.5 ± 3.2 ^{aB}	186.3 ± 1.5 ^{aA}	204.3 ±2.1 ^{aB}
SA 1 mM	206.1 ± 3.1 ^{bA}	214.2 ±1.5 ^{bA}	195.5 ± 2.0 ^{bA}	218.2 ±3.7 ^{bB}
ASA 1 mM	207.8 ± 2.9 ^{bA}	218.9 ±3.7 ^{bA}	198.2 ± 1.6 ^{bA}	223.0 ±2.3 ^{bB}
MeSa 1 mM	207.2 ±2.7 ^{bA}	216.8 ± 2.9 ^{bA}	196.4 ± 1.3 ^{bA}	217.3 ±1.7 ^{bB}
	'Magenta' 2017		'Crimson' 2017	
	Day 0	Day 45	Day 0	Day 45
Control	180.2 ± 2.2 ^{aA}	191.9 ± 2.5 ^{aB}	175.8 ±1.2 ^{aA}	199.0 ±2.3 ^{aB}
SA 0.01 mM	185.5 ± 6.4 ^{abA}	198.0 ± 0.5 ^{aA}	178.7 ±0.5 ^{abA}	200.3 ±1.4 ^{aB}
SA 0.1 mM	186.5 ± 5.2 ^{abA}	201.2 ±3.2 ^{aA}	179.9 ± 2.6 ^{abA}	204.0 ±1.3 ^{aB}
SA 1 mM	199.0 ±2.6 ^{bA}	206.8 ± 2.1 ^{bA}	181.3 ± 0.9 ^{bA}	210.3 ±1.2 ^{bB}
ASA 0.01 mM	186.5 ± 5.6 ^{abA}	198.8 ± 1.6 ^{aA}	179.5 ± 1.0 ^{abA}	193.7 ±2.4 ^{aB}
ASA 0.1	189.3 ±3.2 ^{abA}	191.8 ±0.9 ^{aA}	178.7 ± 3.8 ^{abA}	199.7 ±1.8 ^{aB}
ASA 1 mM	205.7 ± 6.9 ^{bA}	214.7 ± 3.1 ^{bA}	190.3 ± 2.1 ^{cA}	214.0 ±1.6 ^{bB}
MeSa 0.01 mM	190.7 ± 4.8 ^{abA}	196.7 ± 1.4 ^{aA}	178.7 ± 2.0 ^{abA}	192.2 ±1.4 ^{aB}
MeSa 0.1 mM	187.2 ± 3.4 ^{abA}	195.2 ± 1.4 ^{aA}	174.2 ± 5.5 ^{abA}	191.5 ±1.5 ^{aB}
MeSa 1 mM	197.8 ± 5.4 ^{bA}	207.7 ± 4.2 ^{bA}	195.2 ± 2.6 ^{cA}	212.5 ±2.5 ^{bB}

Different capital letters show significant differences for each treatment during storage (according to Student *t*' test) and different lowercase letters show significant differences among treatments for each sampling date (according to ANOVA analysis) at $p < 0.05$.

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Table 3. Total acidity ($g \cdot kg^{-1}$) at harvest (Day 0) and after 45 days of storage at 2 °C (Day 45) in 'Magenta' and 'Crimson' table grapes as affected by preharvest salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments in 2016 and 2017 experiment.

	'Magenta' 2016		'Crimson' 2016	
	Day 0	**Day 45**	**Day 0**	**Day 45**
Control	7.5 ± 0.2 ^{aA}	6.1 ± 0.2 ^{aB}	9.0 ± 0.1 ^{aA}	6.6 ± 0.2 ^{aB}
SA 1 mM	8.6 ± 0.3 ^{bA}	7.0 ± 0.2 ^{bB}	11.8 ± 0.4 ^{bA}	7.7 ± 0.2 ^{bB}
ASA 1 mM	8.5 ± 0.2 ^{bA}	6.9 ± 0.1 ^{bB}	10.8 ± 0.4 ^{bA}	7.6 ± 0.3 ^{bB}
MeSa 1 mM	8.7 ± 0.1 ^{bA}	7.2 ± 0.3 ^{bB}	12.4 ± 0.1 ^{bA}	7.8 ± 0.3 ^{bB}
	'Magenta' 2017		'Crimson' 2017	
	Day 0	Day 45	Day 0	Day 45
Control	8.6 ± 0.2 ^{aA}	7.3 ± 0.3 ^{aB}	10.2 ± 0.1 ^{aA}	8.1 ± 0.2 ^{aB}
SA 0.01 mM	11.3 ± 0.1 ^{cA}	9.6 ± 0.2 ^{cdB}	14.1 ± 0.2 ^{cA}	12.3 ± 0.3 ^{cB}
SA 0.1 mM	10.2 ± 0.4 ^{bA}	8.5 ± 0.2 ^{bB}	12.9 ± 0.3 ^{bA}	10.8 ± 0.1 ^{bB}
SA 1 mM	10.3 ± 0.3 ^{bA}	8.6 ± 0.3 ^{bB}	13.0 ± 0.2 ^{bA}	10.9 ± 0.3 ^{bB}
ASA 0.01 mM	10.6 ± 0.3 ^{bcA}	9.3 ± 0.4 ^{bcB}	14.3 ± 0.2 ^{cdA}	11.0 ± 0.4 ^{bB}
ASA 0.1	14.5 ± 0.7 ^{dA}	11.1 ± 0.5 ^{dB}	14.2 ± 0.5 ^{bcdA}	11.1 ± 0.2 ^{bB}
ASA 1 mM	11.0 ± 0.1 ^{bcA}	9.1 ± 0.2 ^{bcB}	15.0 ± 0.2 ^{dA}	12.7 ± 0.4 ^{cB}
MeSa 0.01 mM	11.1 ± 0.3 ^{bcA}	9.0 ± 0.3 ^{bcB}	14.3 ± 0.3 ^{cdA}	10.7 ± 0.3 ^{bB}
MeSa 0.1 mM	11.4 ± 0.2 ^{cA}	9.9 ± 0.2 ^{cdB}	14.9 ± 0.2 ^{dA}	12.5 ± 0.2 ^{cB}
MeSa 1 mM	11.0 ± 0.2 ^{bcA}	8.9 ± 0.2 ^{bcB}	13.7 ± 0.4 ^{bcA}	10.6 ± 0.1 ^{bB}

Different capital letters show significant differences for each treatment during storage (according to Student *t*' test) and different lowercase letters show significant differences among treatments for each sampling date (according to ANOVA analysis) at $p < 0.05$.

4. Discussion

Results demonstrated that treatments with salicylates affected vine yield and grape ripening depending on the applied compound, concentration, and cultivar. Grapevine treatment with SA or ASA at 5 and 10 mM led to a significant decrease ($p < 0.05$) on total yield of 'Magenta' while these effects only were significant for 10 mM dose on 'Crimson' in 2016 experiment. No berry drop was observed as a consequence of salicylate treatments and morphological traits of clusters was similar in control and treated vines. Thus, the effects of salicylate treatments at high doses on reducing total yield were attributed to a delay or inhibition on the ripening process since many berries failed to ripen properly and some clusters did not reach the requested commercial quality and were discarded. However, when lower doses of SA and ASA were applied, the ripening process was accelerated, as was observed for SA and ASA at 0.1 and 0.01 mM in 'Magenta' and at 1 mM in 'Crimson'. On the contrary, all the applied MeSa concentration, except 0.01 mM, accelerated the ripening process in 'Magenta' and except 10 mM in 'Crimson', although for both cultivars the highest effect was found for 0.1 mM dose. Thus, to accelerate the ripening process and achieve higher prices at market the most appropriate concentration of each salicylate should be established for each particular table grape cultivar. In fact, different effects of preharvest SA treatments on grape ripening were reported depending on cultivar and applied concentration. Thus, in 'Flame Seedless' preharvest treatments with 1.5 and 2 mM of SA hastened berry ripening but 1 mM has not effect [20], while ripening was delayed by 0.72 mM SA in 'Thompson' grapes [22]. Accordingly, ripening also was delayed in the wine grape cultivar 'Syrah' by 0.72 and 3.6 mM SA foliar spray treatments at veraison stage [25]. Similar results were obtained by preharvest treatment with 4 mM SA on the white table grape cultivar 'El-Bayadi' [23] and by 7.2 mM SA injected into berries before veraison [26], the delay being attributed to the antagonist effects of SA on ABA biosynthesis, which is the main hormone implied in the ripening of this non-climacteric fruit. In general, these results and the present ones show that preharvest salicylate treatments at high concentration led to a delay the ripening process in grapes while it could be hastened by lower doses.

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Accordingly, in other non-climacteric fruit, such as sweet cherry, SA and ASA at 2 mM delayed the on-tree ripening process while not effect was observed for 0.5 and 1 mM concentrations [15].

On the other hand, increases in productivity by SA treatments were reported in other crops due to enhanced leaf area, photosynthetic pigments concentration in leaves and photosynthesis rate [27]. However, these effects depend on applied dose. For instance, treatment of cucumber plants with 0.075, 0.1 and 0.15 mM led to increased fruit weight and crop yield while they were reduced by 0.25 and 0.5 mM SA treatments [28]. Accordingly, the present results show different effects of salicylate treatments on crop yield depending not only on applied doses but also on cultivar. Thus, in 'Magenta' cultivar, SA, and ASA at 0.1 and 0.01 mM and MeSa at 0.1 mM increased total yield significantly ($p < 0.05$) while it decreased by 5 and 10 mM SA and ASA doses. However, in 'Crimson' total yield was significantly reduced by SA and ASA at 10 mM but not significantly enhanced by any of the salicylate treatments (Figures S1 and S2). The increase in yield in 'Magenta' was due to an increase on berry volume of 5%, 7.6% and 13% as a consequence of 0.01 mM SA, 0.1 ASA and 0.1 MeSa treatments, respectively. Accordingly, previous reports showed an increase on fruit size on sweet cherry and plum after SA, ASA, or MeSa preharvest treatments [15,16,18] and on pepper fruit from plants treated with MeSa [29], which were attributed to an increase on sugar translocation from leaves to fruit. It is worth noting that as clusters with higher berry size are more appreciated by consumers and reach higher prices at markets than small ones, these treatments could increase economic benefit of this crop, apart from their effects on increasing total yield. In other table grape cultivars, it was reported that 1, 1.5, and 2 mM SA increased cluster size and yield on 'Flame Seedless' [20] as well as 0.72 mM SA in 'Thompson' [22]. However, results of the present research show that lower SA concentration could be even more effective to increase yield in the 'Magenta' cultivar, although it is not applicable to all table grape cultivars because no significant increases on yield were observed in 'Crimson' for the wide range of SA doses assayed.

Results of color hues show that salicylate treatments improved color in 'Magenta' and 'Crimson' table grapes, since the lower values of Hue angle obtained in treated berries, either at harvest or during storage, show deeper red and purple colors which were due to increases in anthocyanin biosynthesis. In fact, highly negative correlations were found between anthocyanin concentration and color Hue values for both table grape cultivars and years taking into account data of control and treated berries for all sampling dates during storage ('Magenta' 2016: $y = -0.0068x + 0.1515$, $r^2 = 0.9720$; 'Crimson' 2016: $y = -0.0033x + 0.1338$, $r^2 = 0.8567$; 'Magenta' 2017: $y = -0.0103x + 0.2283$, $r^2 = 0.9059$; 'Crimson' 2017: $y = -0.0085x + 0.2137$, $r^2 = 0.9257$). Thus, salicylate treatments would lead to improve the market quality of these cultivars, usually depreciated by their lack of proper coloration. The effects of SA, ASA, and MeSa preharvest treatments on increasing anthocyanin content was reported in sweet cherry [15–17] and plum [18,19]. In grape no previous reports about ASA or MeSa treatments are available in the literature, although a few ones regarding SA treatments were reported. Oraei et al. [24] reported that SA spraying treatment, at concentrations from 50 to 200 mM on "Sahebi" cultivar, at pre-veraison stage, led to an increase in phenolic and anthocyanin content. The authors explained these results as the effect of an activation of phenylalanine ammonia lyase (PAL) activity in the vine. Chen et al. [30] reported that in vivo infiltration of 150 µM SA into entire 'Cabernet Sauvignon' berries after harvest activated PAL by enhancing the accumulation of PAL mRNA and the synthesis of a new PAL protein as well as the enzyme activity. In addition, it was reported in Chinese cabbage that SA increases the expression of genes codifying by enzymes such as chalcone synthase (CHS) and chalcone isomerase (CHI), which are involved further downstream in the pathway of flavonoids [31]. These effects of salicylate treatment on increasing PAL activity would be also responsible for the enhanced total phenolic concentration found in berries from treated vines. Thus, salicylate preharvest treatments would lead to increase antioxidant properties and health beneficial effects of table grape consumption given the recognized role of phenolic including anthocyanins in health beneficial properties [2–4,25,31]. These effects would be even higher after prolonged cold storage since, in general, the highest differences among control and treated berries

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in total phenolic and total anthocyanin concentrations were found at the last sampling date. As a general trend, 1.5–2 folds' increases were found in total phenolic and anthocyanin concentration from harvest to day 45 of cold storage, which cannot be attribute to concentration of the compounds in berry tissues due to weight losses because were lower than 10% in both cultivars and years. Increases in total phenolic and anthocyanin concentrations during cold storage were reported in other table grape cultivars, such as 'Flame Seedless' and 'Red Globe' after 45 and 60 days of storage, respectively, in which weight losses were ca. 1% [32,33].

On the other hand, previous studies showed that climatic conditions, especially high temperatures, have a detrimental effect on color and anthocyanin accumulation in grapes from veraison to ripening [34,35]. In our experimental conditions, medium and maximum temperatures from July to September, when veraison and ripening occurred in both table grape cultivars, were similar for 2016 and 2017 (Table S2). However, minimum temperatures of July, August and September were lower in 2017 than in 2016 (Table S2) which was related to a higher content on total anthocyanins in control and 1 mM salicylate-treated berries for both cultivars in 2017 experiment (Table S3). These results could be attributed to both lower expression levels of anthocyanin biosynthetic genes and lower activities of anthocyanin biosynthetic enzymes, particularly UDP-glucose flavonoid 3-*O*-glucosyltransferase (UFGT, a key enzyme in anthocyanin biosynthesis), as reported by Mori et al. [34]. With respect to total phenolic concentration at harvest, higher values were found in control and 1 mM salicylate-treated berries in 2016 than in 2017 for 'Crimson' cultivar, while the contrary occurred in 'Magenta' (Table S3) and in turn a clear relationship with the temperatures from version to ripening cannot been found. In fact, in a recent study with wine grape cultivars, it was reported that the impact of climatic variables on phenolic content is very complex, since maximum, minimum and medium temperatures as well as rain and water stress along the growing cycle have a different impact on individual phenolic compounds [36] and then further research is required to better understand these relationships.

With respect to quality parameters, increases in weight loss and TSS and decreases in TA and firmness occurred during cold storage in all berries which are related to the postharvest ripening process in fresh fruit, including table grape, and lead to quality deterioration and losses of their marketable value [37–39]. However, the evolution of these quality parameters was delayed in salicylate-treated grapes of both cultivars. Accordingly, SA preharvest treatment of 'Thompson' (at 0.72 mM, at pea and veraison stages) reduced softening and weight loss during storage at 20 °C [22], as well as 2 and 4 mM SA treatments (at veraison stage and 14 days before harvesting) reduced cluster water loss, rachis browning index and softening during storage at 28 °C in 'Flame Seedless' grapes, due to a reduced activity of the enzymes polygalacturonase, xylanase and cellulase [21]. The preservation of quality parameters and organoleptic properties during cold storage were also reported by Champa et al. [20] in 'Flame Seedless' cultivar as a result of preharvest treatments with SA (1.5 and 2.0 mM) at pea stage and at veraison. As postharvest treatment, SA (1, 2, and 4 mM) improved berry and cluster appearance during storage for up to 45 days at 0 °C, followed by 2 days at 20 °C, on 'Bidaneh Ghermez' grapes [40]. Moreover, in 'Flame Seedless' table grape cultivar, it was reported that the combination of pre- and post-harvest SA treatment was even more effective on maintaining grape quality than pre- or postharvest treatment alone, since higher firmness, lower weight loss and better appearance of berries as well as of rachis were observed after long storage time [41]. According to the present results and the commented previous ones, it is clear that preharvest salicylate treatments could be considered an effective tool to maintain table grape quality during storage throughout delaying the postharvest ripening process. However, the mechanism involved is still unclear although it could be related to the increase of the SA endogenous levels induced by salicylate treatment. In this sense, Zhang et al. [42] showed that kiwifruit ripening process was correlated with a decrease in SA endogenous concentration, while ASA treatment increased endogenous levels of SA and delayed ripening and senescence, manifested by lower softening, lipoxygenase activity and reactive oxygen species (ROS) production. Moreover, in plum and sweet cherry, it was reported that the activity of the antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and

catalase (CAT) increased by preharvest treatments with SA, ASA, and MeSa [17,18,43]. Taking into account that ROS production in increased during the postharvest ripening process, the increase in the activity of these antioxidant enzymes, together with enhanced concentrations of antioxidants compounds such as anthocyanins and phenolics, would lead to a more efficient system of ROS cleaning and in turn, to delay berry ripening and senescence processes, being responsible for the maintenance of berry quality attributes during prolonged cold storage found in table grapes from salicylate-treated vines.

5. Conclusions

Results show that the effect of SA, ASA, and MeSa preharvest treatments on yield and quality attributes of table grape at harvest and during storage depends on applied compound, concentration, and cultivar. However, considering the overall results, it could be concluded that 0.1 mM MeSa treatment could be a useful tool to increase crop yield and accelerate on-vine ripening process on both cultivars, which would lead to improve the economic profit of this crop. In addition, this treatment was the most effective on enhancing anthocyanin biosynthesis and berry color in these poorly colored cultivars. Moreover, quality parameters of grapes from treated vines were maintained during cold storage at higher levels as compared with those from controls. It is worth noting that total anthocyanins and phenolics, which have antioxidant properties, were found at higher concentrations at harvest and during prolonged cold storage in treated berries, which would lead to increase the health beneficial effects of table grape consumption. In addition, SA, ASA, and MeSa are natural compound, present in almost all plant tissues that have always been consumed by humans so it does not imply adverse effects on human health.

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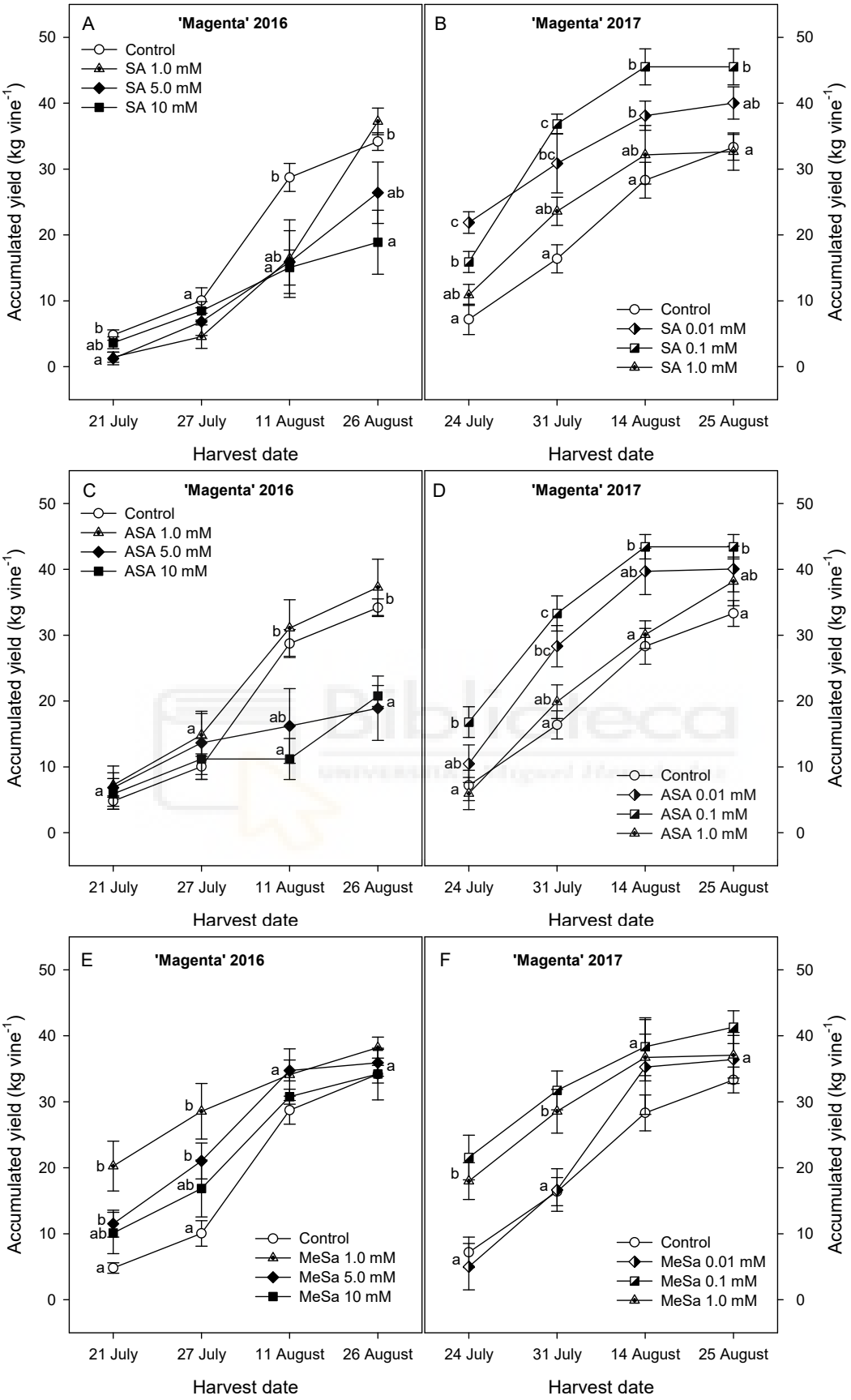

Figure S1: Accumulated yield of 'Magenta' table grape as affected by salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments in 2016 (A, C, E, respectively) and 2017 (B, D, F, respectively) experiments. Data are the mean ± SE of three replicates of three vines. Different letters show significant differences (P<0.05) among treatments for each harvest date.

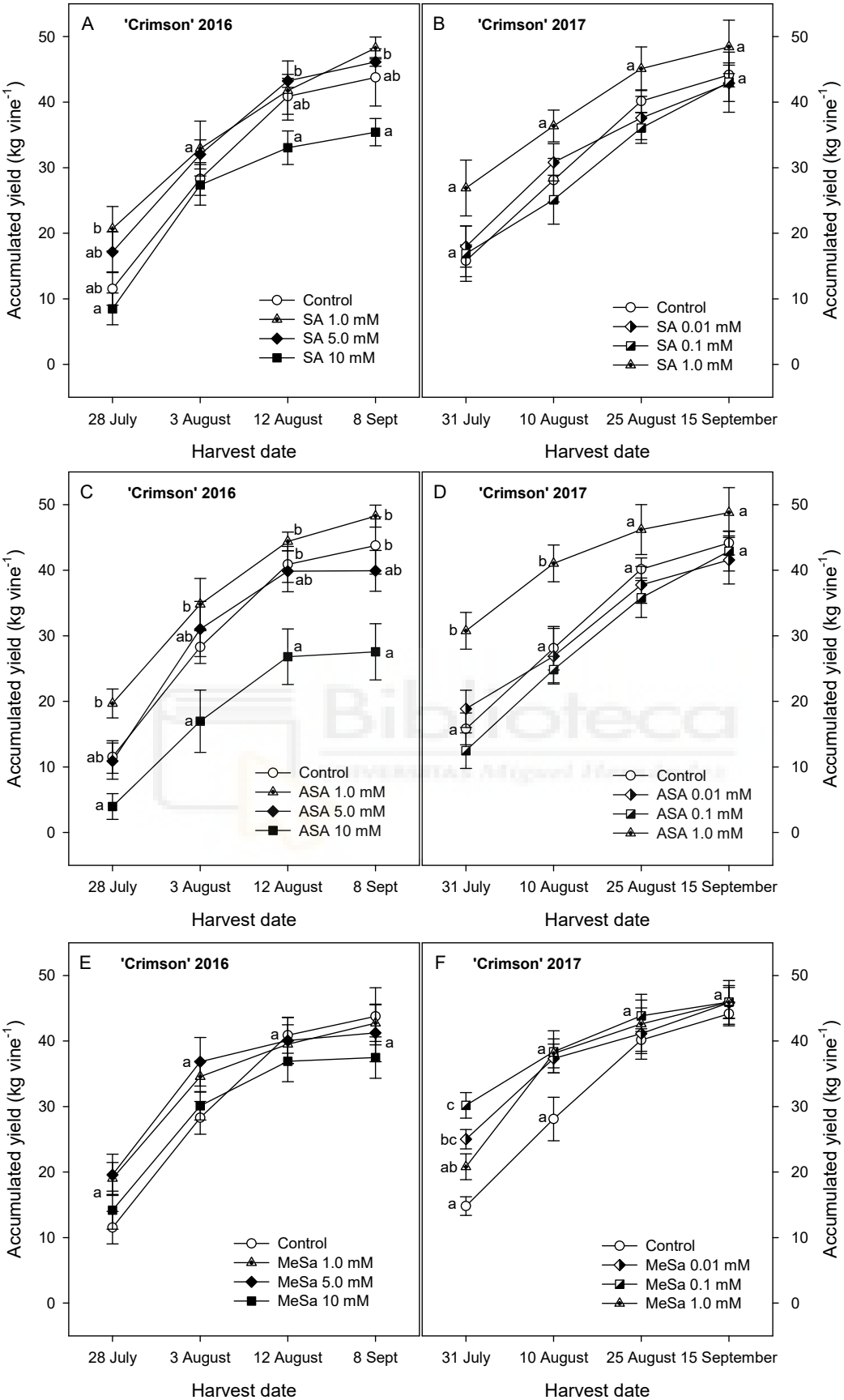

Figure S2: Accumulated yield of 'Crimson' table grape as affected by salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments in 2016 (A, C, E, respectively) and 2017 (B, D, F, respectively) experiments. Data are the mean ± SE of three replicates of three vines. Different letters show significant differences (P<0.05) among treatments for each harvest date.

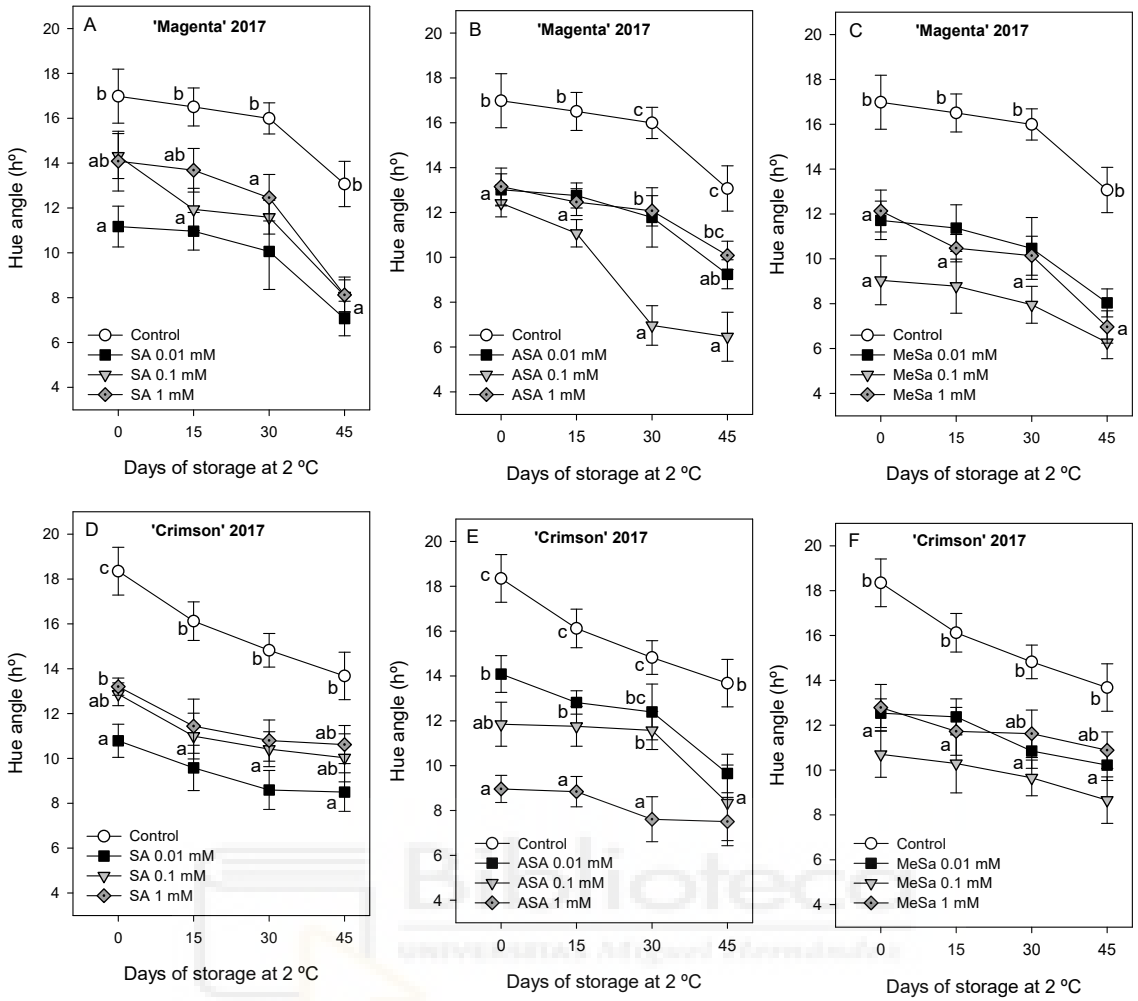

Figure S3: Effects of preharvest salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments on Hue angle colour evolution during storage at 2 °C of 'Magenta' (A, B and C) and 'Crimson' (D, E and F) table grapes in 2017 experiment. Data are the mean ± SE of measures made in three replicates of 30 berries. Different letters show significant differences (P<0.05) among treatments for each sampling date.

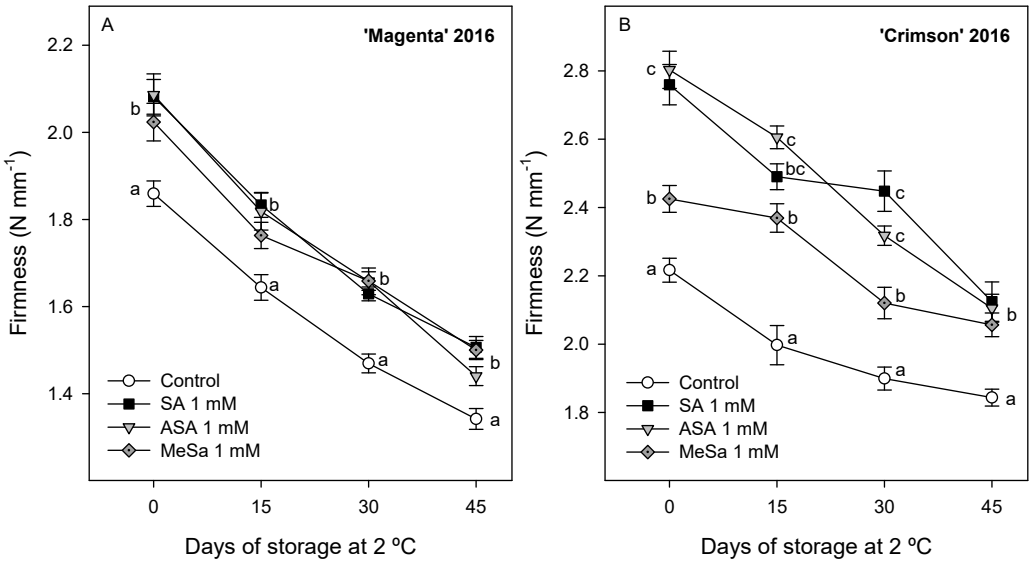

Figure S4: Effects of preharvest salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments on fruit firmness evolution during storage at 2 °C of 'Magenta' (A) and 'Crimson' (B) table grapes in 2016 experiment. Data are the mean ± SE of measures made in three replicates of 30 berries. Different letters show significant differences (P<0.05) among treatments for each sampling date.

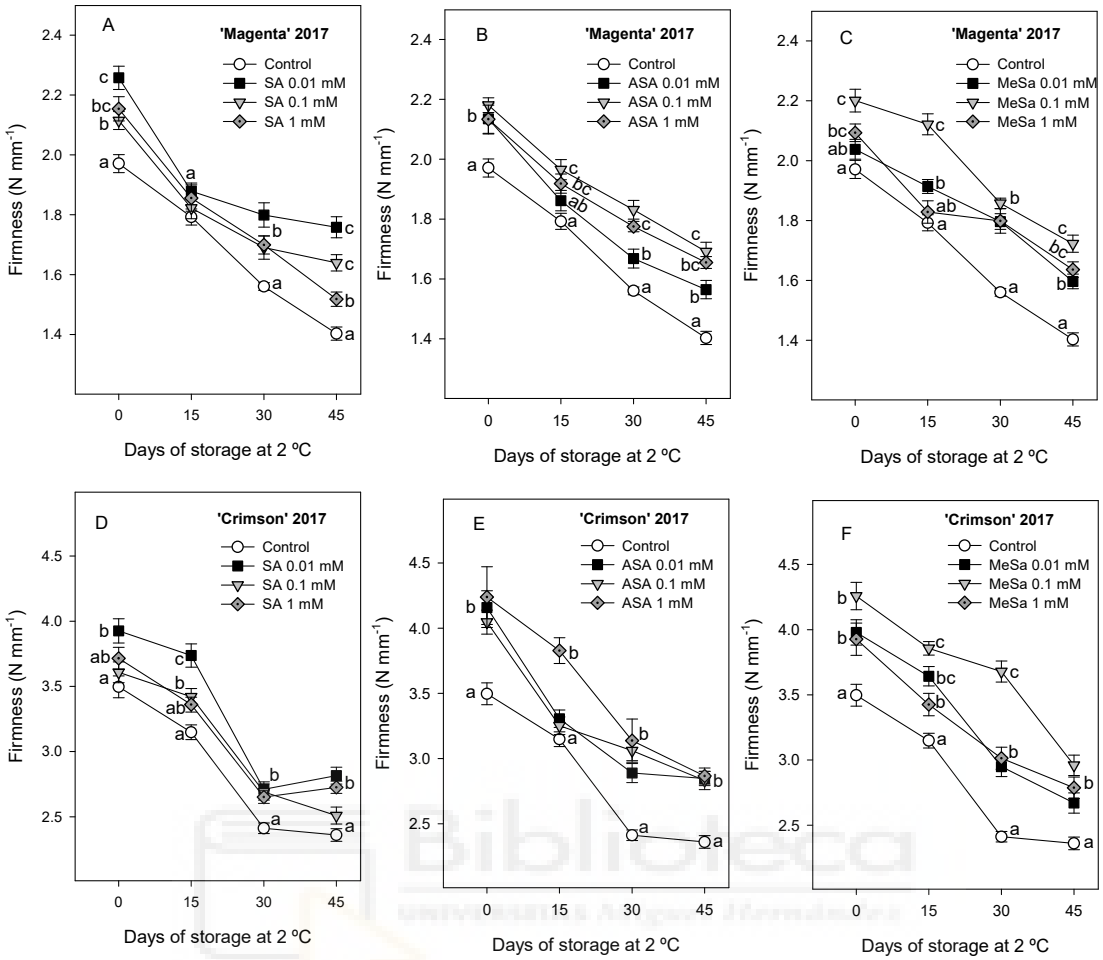

Figure S5: Effects of preharvest salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treatments on fruit firmness evolution during storage at 2 °C of 'Magenta' (A, B and C) and 'Crimson' (D, E and F) table grapes in 2017 experiment. Data are the mean ± SE of measures made in three replicates of 30 berries. Different letters show significant differences (P<0.05) among treatments for each sampling date.

Table S1. Weight loss (%) during storage in cluster of `Crimson` and `Magenta table grapes from control and salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treated vines in 2016 and 2017 experiments.

	Days of storage at 2 °C		
	15	30	45
2016 Experiment		'Crimson'	
Control	2.74 ± 0.29abA	4.47 ± 0.42aB	7.13 ± 0.24bC
SA 1 mM	2.66 ± 0.04aA	4.33 ± 0.17aB	5.85 ± 0.16aC
ASA 1 mM	2.88 ± 0.07bA	4.59 ± 0.32aB	6.04 ± 0.39abC
MeSa 1 mM	2.74 ± 0.11abA	4.14 ± 0.19aB	5.48 ± 0.25aC
2016 Experiment		`Magenta'	
Control	3.74 ± 0.35aA	6.69 ± 0.41cB	9.09 ± 0.36bC
SA 1 mM	3.55 ± 0.21aA	6.09 ± 0.48bcB	7.42 ± 0.48aB
ASA 1 mM	3.32 ± 0.33aA	5.38 ± 0.21bB	7.51 ± 0.42aC
MeSa 1 mM	2.81 ± 0.21aA	4.51 ± 0.24aB	6.76 ± 0.38aC
2017 Experiment		'Crimson'	
Control	3.61 ± 0.09bA	5.68 ± 0.11bB	8.26 ± 0.46cC
SA 0.01 mM	2.59 ± 0.10aA	5.56 ± 0.16bB	6.33 ± 0.24aC
SA 0.1 mM	2.40 ± 0.07aA	5.75 ± 0.17bB	6.98 ± 0.04bC
SA 1 mM	2.75 ± 0.26aA	4.77 ± 0.44abB	7.07 ± 0.33abcC
ASA 0.01 mM	2.66 ± 0.30aA	4.38 ± 0.23aB	7.32 ± 0.20bcC
ASA 0.1 mM	2.50 ± 0.15aA	4.85 ± 0.39abB	6.93 ± 0.25abcC
ASA 1 mM	2.59 ± 0.23aA	4.00 ± 0.22aB	6.63 ± 0.09aC
MeSa 0.01 mM	2.91 ± 0.33abA	4.56 ± 0.52abB	7.01 ± 0.30abcC
MeSa 0.1 mM	2.07 ± 0.42aA	4.31 ± 0.81abB	6.14 ± 0.26aB
MeSa 1 mM	2.09 ± 0.39aA	4.38 ± 0.51abB	6.87 ± 0.25abC
2017 Experiment		`Magenta'	
Control	2.92 ± 0.11bA	6.73 ± 0.54cB	8.75 ± 0.28dC
SA 0.01 mM	2.29 ± 0.26abA	5.36 ± 0.62abcB	6.32 ± 0.42abcB
SA 0.1 mM	3.02 ± 0.16bA	5.37 ± 0.30abcB	7.12 ± 0.12bcC
SA 1 mM	2.86 ± 0.04bA	5.82 ± 0.41bcB	6.76 ± 0.29abcB
ASA 0.01 mM	2.50 ± 0.12aA	5.06 ± 0.32abB	7.41 ± 0.20bcC
ASA 0.1 mM	2.41 ± 0.06aA	4.87 ± 0.36abB	6.82 ± 0.12bC
ASA 1 mM	2.44 ± 0.12aA	5.54 ± 0.25bcB	7.50 ± 0.21cC
MeSa 0.01 mM	2.78 ± 0.28abA	5.24 ± 0.06bB	7.18 ± 0.26bcC
MeSa 0.1 mM	2.19 ± 0.35abA	4.60 ± 0.17aB	6.36 ± 0.07aC

MeSa 1 mM	2.76 ± 0.28abA	4.68 ± 0.14aB	6.96 ± 0.30abcC

Data are the mean ± SE of three replicates. Different capital letters show significant differences for each treatment during storage and different lowercase letters show significant differences among treatments for each sampling date at $P< 0.05$.

Table S2. Data of monthly media maximum, minimum and medium temperatures (°C) in the experimental field for 2016 and 2017 experiments. Data were recorded by an automatic weather station located close to the experimental field. *

	May		June		July		August		September	
Temperature	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
Maximum	22.12	22.37	29.24	28.44	30.63	29.61	28.76	30.52	25.80	25.56
Minimum	14.62	17.13	21.25	21.06	23.23	20.9	22.96	20.47	19.02	17.71
Medium	18.51	20.01	23.91	25.13	26.41	26.89	25.82	26.34	23.28	22.84

* Sistema de Información Agrario de Murcia. Instituto Murciano de Investigación y Desarrollo Agrario de la Región de Murcia. http://siam.imida.es/

Table S3. Comparative values of total anthocyanin concentration and total phenolic concentration on 'Crimson' and 'Magenta' table grapes from control and salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa) treated vines on 2016 and 2017 experiments.

		'Crimson'		'Magenta'	
		2016	2017	2016	2017
Total anthocyanins (g kg^{-1})	Control	0.049±0.003a	0.067±0.002b	0.035±0.003a	0.053±0.006b
	SA 1 mM	0.085±0.006a	0.097±0.007a	0.066±0.003a	0.076±0.005a
	ASA 1 mM	0.073±0.003a	0.124±0.002b	0.063±0.004a	0.084±0.006b
	MeSa 1 mM	0.096±0.007a	0.109±0.003a	0.078±0.004a	0.106±0.002b
Total phenolics (g kg^{-1})	Control	0.777±0.016b	0.510±0.006a	0.247±0.013a	0.491±0.012b
	SA 1 mM	0.891±0.036b	0.655±0.023a	0.306±0.017a	0.577±0.023b
	ASA 1 mM	0.938±0.031b	0.643±0.023a	0.395±0.008a	0.526±0.012b
	MeSa 1 mM	0.968±0.032b	0.606±0.020a	0.475±0.022a	0.563±0.006b

* Different letters show significant differences according to Student t' test between the two growing seasons for each parameter at $P< 0.05$.

4.6. Publication 6

PUBLICATION 6 (Literal transcription)

Preharvest application of methyl salicylate, acetyl salicylic acid and salicylic acid alleviated disease caused by *Botrytis cinerea* through stimulation of antioxidant system in table grapes

María E. García-Pastor, María J. Giménez, Pedro J. Zapata, Fabián Guillén, Juan M. Valverde, María Serrano, Daniel Valero

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Preharvest application of methyl salicylate, acetyl salicylic acid and salicylic acid alleviated disease caused by *Botrytis cinerea* through stimulation of antioxidant system in table grapes

María Emma García-Pastor[a], María José Giménez[a], Pedro J. Zapata[a], Fabián Guillén[a], Juan Miguel Valverde[a], María Serrano[b], Daniel Valero[a,*]

[a] *Department of Food Technology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Orihuela, Alicante, Spain*
[b] *Department of Applied Biology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Orihuela, Alicante, Spain*

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ABSTRACT

The main goal of this study was to describe impact of preharvest application of methyl salicylate (MeSA), acetyl salicylic acid (ASA) and salicylic acid (SA) on the reduction of disease caused by *Botrytis cinerea* in two table grape cultivars ('Crimson' and 'Magenta'). Based on previous studies, MeSA and SA were applied at 0.1 and 0.01 mM for both cultivars, while ASA was applied at 1 mM in 'Crimson' and 0.1 mM in 'Magenta'. At time of harvest, berry maturity-quality attributes, bioactive compounds and antioxidant enzymes were determined. In addition, grapes were artificially inoculated with *B. cinerea* spores, and the berries were ranked for visual decay incidence after 5 days of inoculation. Salicylates preharvest treatments led to higher total acidity, content of bioactive compounds and activity of antioxidant enzymes in treated than in control berries. The application of salicylate derivatives induced resistance to *B. cinerea* spoilage, since higher percentage of berries with no symptoms was observed and on the contrary, the highest percentages of berries were obtained in control grapes. All preharvest treatments with SA, ASA and MeSA alleviated postharvest disease caused by *B. cinerea* probably due to increasing levels of phenolic compounds and activity of antioxidant enzymes, although the best results were obtained with MeSA at 0.1 mM. Also, for this treatment and dose, higher quality properties, such as higher concentrations of ascorbic, succinic and fumaric acids, were observed compared with no treated-grapes.

1. Introduction

The grape (*Vitis vinifera* L.) commercialisation chain is very demanding in terms of preserving fruit quality. Table grape is subjected to long storage periods before reaching its final destination, and there are risks of various postharvest losses (Champa, 2015), representing up to 25% and 50% of total production in industrialized and developing countries. Grey mould caused by *Botrytis cinerea* has been reported as a major postharvest disease of grapes (Martínez-Romero et al., 2007; Saito et al., 2019), which has a negative impact on the quality of fresh grapes such as weight loss, colour fading, accelerated softening, and reduction of shelf life, all of these causing severe economic losses. The control of this disease is very difficult since postharvest treatments with synthetic fungicides or SO_2 are not allowed in several countries due to their adverse effects on food safety and the environment (Youssef et al., 2015).

Salicylic acid (SA) and its derivatives, acetyl salicylic acid (ASA) and methyl salicylate (MeSA), are naturally occurring compounds ubiquitously distributed in the whole plant kingdom and classified under the group of plant hormones having diverse regulatory roles in the metabolism of plants (Hayat and Ahmad, 2007). SA has emerged as a key plant defence hormone with critical roles in different aspects of plant immunity, and is involved in systemic acquired resistance (SAR) demonstrated in several plant tissues, including fruits (Zhang and Li, 2019). Accumulation of SA plays a significant role in stimulation of local defence at initial infection site as well as in the distant tissues that are infection free for induction of SAR, while MeSA serves as a long-distance SAR signal which occurs via phloem or throughout other tissues and even in the outer parts of the plant due to its volatile nature (Nazar et al., 2017). Recently, enhanced disease resistance upon exogenous SA application has been reported in different fruits species including, tomato, pepper, orange and banana, among others (Koo et al., 2020).

As postharvest treatments several approaches have been reported to

* Corresponding author.
E-mail address: daniel.valero@umh.es (D. Valero).

reduce the incidence and severity of *B. cinerea* in table grapes. Thus, essential oils, such as eugenol, thymol, or menthol added to modified atmosphere packages (MAP) improved the storability of table grapes and delayed rates of rachis deterioration and berry decay (Serrano et al., 2008; Valverde et al., 2005a). These essential oils maintained higher total phenolics and total antioxidant activity in both skin and flesh of the berries (Valero et al., 2006). Carvacrol vapour treatment of grape inoculated with *B. cinerea* inhibited fungal growth in a dose-dependent manner, suggesting that carvacrol could be used as alternative to the use of synthetic fungicides or SO_2 (Martínez-Romero et al., 2007). Other essential oils, such as sage oil, lavender oil, mint oil, and tea tree oil can effectively inhibit the growth of *B. cinerea* and the antifungal effects are dose dependent, the best antifungal effect being found from mint oil (Xueuan et al., 2018). These authors also reported that volatile vapour worked better than direct contact, suggesting that essential oils cause changes in membrane permeability and cell wall damage. On the other hand, edible coatings have shown efficacy in controlling fruit decay. For instance, *Aloe vera* gel was able to reduce microbial counts for both mesophilic aerobic and yeast and moulds on table grapes over storage with benefits in reducing berry decay (Valverde et al., 2005b).

As preharvest treatments, synthetic fungicide sprays may provide an alternative to the control of postharvest grey mould, although fungicide resistance in *B. cinerea* can result in the failure of disease control (Saito et al., 2019), apart from safety regulations. Preharvest application with several compounds has shown benefits in terms of improving quality on table grapes. For instance, methyl jasmonate (MeJA) has recently reported to affect the ripening process on table grape depending on concentration, since 5 and 10 mM delayed ripening while 1, 0.1 and 0.01 mM accelerated the maturation (García-Pastor et al., 2019). With respect to salicylates, their preharvest application improved the quality and enhanced the nutritive and bioactive compounds parameters at harvest and during storage in several fruit commodities, such plum (Martínez-Esplá et al., 2017, 2018) and sweet cherry (Giménez et al., 2015, 2017). In sweet cherry, an effect of preharvest SA treatments on reducing decay during storage was also reported (Yao and Tian, 2005) as well as in pears (Cao et al., 2006). In apricot, it has been recently reported that SA treatment, 7 and 2 days before harvesting decreased decay rate attributed to *Alternaria alternata* during cold storage, due to increased phenolic content, antioxidant capacity and activity of peroxidase (POD) and phenylalanine ammonia lyase (PAL) activities (Cui et al., 2020). Specifically in table grape, just in one previous paper the effects of preharvest SA treatments on reducing berry decay incidence during storage has been reported in 'Flame Seedless' cultivar (Champa et al., 2015).

However, as far as we know, there are no reports on the effect of salicylates applied as preharvest treatments in reducing the incidence and severity of the disease caused by *B. cinerea*. The aim of this study was to apply SA, ASA and MeSA as preharvest treatments and find out their effects on the incidence and severity of decay on table grapes inoculated with *B. cinerea*, as well the possible mechanism of action involved in the alleviation of this fungal disease.

2. Materials and methods

2.1. Plant material and field experimental design

The experiments were carried out in 2018 in a commercial plot of vineyards in Calasparra (Murcia, Spain) using two seedless table grape (*Vitis vinifera* L.) cultivars 'Crimson' (11-years old vine) and 'Magenta' (8-years old vine). Before the onset of veraison, the vineyards were preharvest treated with distilled water (control), salicylic acid (SA), acetyl salicylic acid (ASA) or methyl salicylate (MeSA) (purchased from Sigma-Aldrich, Madrid, Spain). For 'Crimson', three treatments were performed on June 26th (T1), on July 13th at veraison (T2) and on August 6th (T3). The applied doses were 0.01 mM SA, 1 mM ASA and 0.1 mM MeSA. For 'Magenta', three treatments were also performed on June 22nd (T1), on July 10th at veraison (T2) and on July 6th (T3). For this cultivar, the applied doses were 0.01 mM SA, 0.1 mM ASA and 0.1 mM MeSA. These concentrations were chosen based on previous experiments in two growing seasons (2016 and 2017), in which the best results for these treatments in terms of yield, berry maturity-quality and bioactive compounds were obtained. All treatments were performed by foliar spray application of 1 L per vine, containing 0.5% Tween 20 as surfactant. Treatments were made at sunrise and during favourable weather conditions, where rainfall or winds were not forecasted for the following 24 h. Pruning, thinning, fertilization and irrigation were carried out during the experiments according to local cultural practices for table grape without any use of fungicides. A completely randomized block design with five replicates of three vines for each cultivar and treatment was established. Clusters were harvested when berries reached the characteristic size, colour and soluble solid content (°Brix) of each cultivar in order to pick up full mature grapes. Fig. 1S shows a scheme of the experimental design.

2.2. Soluble solids content, titratable acidity and ripening index

A first set of bunches was used to determine the berry maturity-quality characteristics. Ten berries were sampled from each replicate (5 bunches, total 50 berries) and cultivar, and then rachis and peduncles were separated, cut and ground to obtain a homogeneous juice sample, in which total soluble solids (TSS) content were determined in duplicate with a digital refractometer Atago PR-101 (Atago Co. Ltd., Tokyo, Japan) at 20 °C, and expressed as g 100 g^{-1} (mean ± SE). Total acidity (TA) was determined also in duplicated in the same juice by automatic titration (785 DMP Titrino, Metrohm) with 0.1 N NaOH up to pH 8.1 and results (mean ± SE) expressed as g tartaric acid equivalent 100 g^{-1} fresh weight (FW). Ripening index (RI) was calculated as the ratio between TSS and TA. Data were the mean ± SE of five replicates.

2.3. Individual sugars and organic acids

The juice used for TSS and TA determination was centrifuged at 10,000 ×*g* for 10 min and the supernatant was filtered through a 0.45 μm Millipore filter and then injected into a high-performance liquid chromatography (HPLC) system (Hewlett-Packard HPLC series 1100) to quantify individual sugars and organic acids. The elution system consisted of 0.1% phosphoric acid running isocratically with a flow rate of 0.5 mL min^{-1} through a Supelco column (Supelcogel Ce610H, 30 cm 7.8 mm, Supelco Park, Bellefonte, PA, USA). Organic acids were detected by absorbance at 210 nm and sugars by refractive index detector. Results were expressed as g 100 g^{-1} at harvest. A standard curve of pure sugars and organic acids purchased from Sigma (Poole, UK) was used for quantification. Results were the mean ± SE of five replicates.

2.4. Skin bioactive compounds, antioxidant activity and antioxidant enzymes

Fourth set of 30 berries from each replicate were used for the determination of phenolics, anthocyanins (total and individual), antioxidant capacity and antioxidant enzymes activity. The berries were separated from the rachis, as well as the peduncle, and were peeled to separate the skin from the flesh. Both tissues were immediately frozen in liquid N_2, milled with mortar and pestle and kept at −80 °C until analysis. To extract total phenolics 1 g of skin tissue was manually ground in a mortar and pestle with 5 mL of water: methanol (2:8) containing 2 mM NaF (to inactivate polyphenol oxidase activity and prevent phenolic degradation) and then, sonicated in an ultrasonic bath for 60 min. The homogenate was centrifuged at 10,000*g* for 15 min, and phenolics were quantified in the supernatant using the Folin-Ciocalteu reagent as previously reported (Martínez-Esplá et al., 2017). Results

(mean ± SE) were expressed as mg gallic acid equivalent 100 g^{-1} FW. To extract total anthocyanins, 1 g of frozen skin tissue and 5 mL of methanol: formic acid: water (25:1:24, v/v/v) were manually ground, as same as previously, and then sonicated in an ultrasonic bath for 60 min and after that centrifuged at 10,000*g* for 15 min. Total anthocyanin concentration was measured by reading absorbance at 520 nm in an UNICAM Helios-α spectrophotometer (Cambridge, UK), and expressed as mg of malvidin 3-glucoside equivalent (molar absorption coefficient of 27,000 M^{-1} cm^{-1} and molecular weight of 493.4 g mol^{-1}) per 100 g FW (mean ± SE). The supernatant was filtered through a 0.45 µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and used for individual anthocyanin quantification by HPLC analysis as previously reported (Martínez-Esplá et al., 2017). Chromatograms were recorded at 520 nm. Anthocyanin standards were: malvidin 3-glucoside for 'Magenta' and peonidin 3-glucoside for 'Crimson' cultivar (purchased from Sigma-Aldrich, Germany). Results are the mean ± SE of five replicates.

To measure total antioxidant activity (TAA), 1 g of skin tissue were manually homogenized in a mortar with 5 mL of 50 mM phosphate buffer pH = 7.8 and 5 mL of ethyl acetate. The homogenate was centrifuged at 10,000*g* for 15 min at 4 °C and the upper and lower fractions were used to quantify lipophilic (L-TAA) and hydrophilic total antioxidant activity (H-TAA), respectively. As previously described (Sayyari et al., 2011a), H-TAA and L-TAA were determined in duplicate in each extract using a reaction mixture containing 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 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 grapes extract was proportional to TAA of the sample which was calculated by using a calibration curve made 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 mg of Trolox Equivalent (TE) 100 g^{-1} and were the mean ± SE of five replicates.

Crude extracts to measure peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) enzymes in berry skin were performed by homogenizing 1 g of frozen tissue with 5 mL of phosphate buffer 50 mmol L^{-1}, pH 6.8, containing 1% (w/v) polyvinylpyrrolidone and 1.0 mmol L^{-1} ethylenediamine-tetraacetic acid. Then, the extracts were centrifuged at 10,000*g* for 30 min at 4 °C and the supernatant was used for the quantification as reported previously (Zapata et al., 2017). Briefly, for POD activity, the reaction mixture contained 200 µL of extract in a final volume of 3 mL of 50 mmol L^{-1} phosphate buffer (pH 7.0), 12 mmol L^{-1} H_2O_2 and 7 mmol L^{-1} guaiacol. The increase of absorbance at 470 nm during 1 min was measured and POD activity was expressed as U min^{-1} g^{-1} (where U was defined as an increase of 0.01 absorbance min^{-1}). For CAT, 100 µL of extract were added to 3 mL of reaction mixture containing 15 mmol L^{-1} H_2O_2 and 50 mmol L^{-1} phosphate buffer (pH 7.0). The decrease of absorbance at 240 nm during 1 min was measured and CAT activity was expressed as U min^{-1} g^{-1} (where U was defined as a decrease of 0.01 absorbance min^{-1}). Finally, for APX quantification, the reaction mixture contained 200 µL of extract in 3 mL of 50 mmol L^{-1} potassium phosphate (pH 7.0), 0.5 mmol L^{-1} ascorbic acid and 1.0 mmol L^{-1} H_2O_2. The decrease of absorbance at 290 nm from time 0 to 60 s was measured and APX activity was expressed in terms of units of enzymatic activity (U min^{-1} g^{-1}), with one enzymatic unit (U) being defined as a decrease of 0.01 ascorbate min^{-1}. Results are the mean ± SE of five replicates.

2.5. Experimental design of berry inoculation

One set of 120 berries from each of the five replicates were used for the inoculation experiment with *Botrytis cinerea*. Berries of the clusters were separated from the rachis by individually cutting them with scissors, without damaging them and maintaining the peduncle. Once selected the most homogeneous samples, these grapes were disinfected in a water bath with 100 ppm of chlorine during 1 min and allowed to dry spread on filter papers. Once dry, the 120 berries from each replicate, treatment and cultivar were placed into a plastic box with lid (30 × 15 × 5 cm) to inoculate them. Previously to inoculation process, these grapes were injured with a sterile lancet inside a laminar flow hood. Berry wound was always made on the right side attached to the peduncle and was 6 mm in deep (Fig. 1S). The fungus used in this study was *B. cinerea* CECT21000 (Spanish collection of type cultures) and routinely cultured on potato dextrose agar (PDA). The spores of *B. cinerea* were collected and diluted with sterile water until reaching the concentration of 7500 CFU mL^{-1} and used as stock. Five replicates of 120 single berries per treatment were inoculated by spraying them with this spore suspension of *B. cinerea* until runoff. Each berry received 900 spores, then air dried for 30 min, and boxes were closed slightly, allowing the evaporation of water excess and the oxygen and CO_2 exchange, and incubated for 5 days at 25 ± 1 °C with 80–85% relative humidity.

2.6. Visual decay incidence

Grapes were inspected at fifth day after inoculation and considered spoiled based on a visual scale of six hedonic points named as stages: S0, S1, S2, S3, S4 and S5. The evolution and visual appearance of the fungus growth was different for each table grape cultivars, and in turn the meaning of the S0 to S5 decay incidence scale was established as follow (photographs at the bottom of Figs. 1 and 2). In 'Magenta' cultivar the decay incidence scale was: S0, without damage; S1, wound browning; S2, microbial growth covering 1–2 mm of wound; S3, microbial growth covering 3–4 mm of wound; S4, microbial growth covering 4–5 mm of wound and even showing mycelial growth; S5, all the wound covered (6 mm) with the fungus and mycelium was observed. For 'Crimson' cultivar, this scale was: S0, without damage; S1, wound browning; S2, microbial growth covering the wound (6 mm); S3, microbial growth, covering a quarter of the berry and showing mycelial growth; S4, microbial growth, covering the half of the berry and

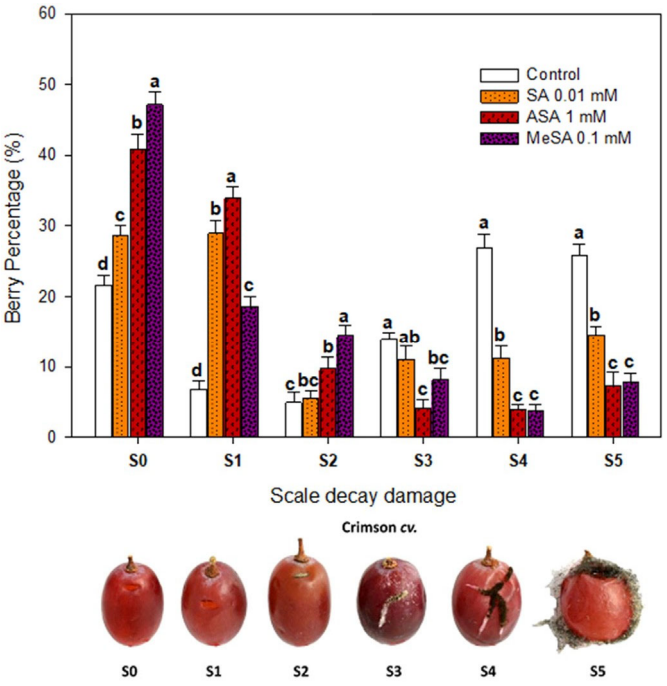

Fig. 1. Percentage of decayed berries according to the scale of visual aspect scale (S0–S5) of decay incidence in 'Crimson' table grape as affected preharvest treatments with control, salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSA). Data are the mean ± SE. Data are the mean ± SE. Different letters show significantly differences ($P < 0.05$) among treatments.

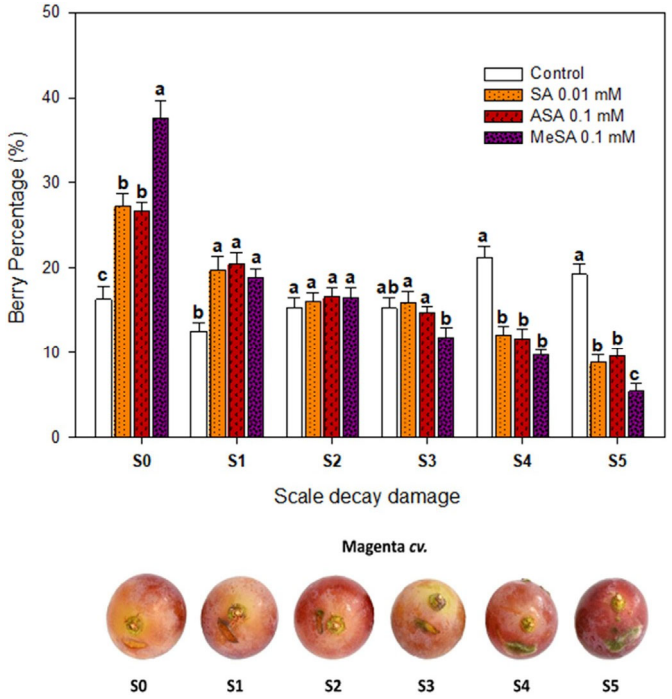

Fig. 2. Percentage of decayed berries according to the scale of visual aspect scale (S0–S5) of decay incidence in 'Magenta' table grape as affected preharvest treatments with control, salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSA). Data are the mean ± SE. Data are the mean ± SE. Different letters show significantly differences ($P < 0.05$) among treatments.

showing mycelial growth and softening; S5, microbial growth, covering the whole berry and showing mycelial growth and softening. Results were expressed as percentage of spoiled grapes in each stage based on the total number of fruits per box (mean ± SE of five replicates).

2.7. Statistical analysis

All statistical analyses were performed using the SPSS software package v. 12.0 for Windows. A one-way analysis of variance (ANOVA) was also performed to determine the significance of mean differences among treatments and cultivars, using HSD Duncan's test to examine if differences were significant at $P < 0.05$. Differences were indicated using different letter designations.

3. Results and discussion

3.1. Effect of SA, ASA and MeSA on Botrytis cinerea disease

Recently harvested grape berries, from both control and preharvest salicylate treated vines, were artificially injured and inoculated by spraying them with 7500 UFC mL^{-1} of *B. cinerea* suspension. After 5 days of inoculation, the disease incidence and severity were visually ranked in a six hedonic scale, and results are shown in Fig. 1 ('Crimson') and Fig. 2 ('Magenta'). For both cultivars similar results were obtained. The lowest decay incidence ($P < 0.05$) was obtained in those berries treated with MeSA, since the percentages of berries with absence of damage (S0) were 47 and 38%, for 'Crimson' and 'Magenta', respectively. On the other hand, this treatment showed the lowest percentage of berries with severe disease (S4 and S5) with values of 3–7% and 5–9%, respectively. The ASA treatment also showed good control of *B. cinerea* disease, but with lower performance than in MeSA-treated grapes. The most severe symptoms were shown in control grapes, with percentages of S4-S5 stages of 26–27 and 20–21% for 'Crimson' and 'Magenta', respectively. Moreover, in berries from control vines the lowest percentage at S0 stage (absence of symptoms), 26 and 16% for 'Crimson' and 'Magenta', respectively was obtained.

These results clearly demonstrated that preharvest treatment with salicylates, and especially MeSA, were able to induce resistance of table grape to be colonized by *B. cinerea*. There is no literature about the effect of preharvest application of ASA or MeSA on inducing fruit resistance to pathogen attack for comparative purposes, although a few reports are available about preharvest treatments with SA. Thus, preharvest treatments with SA delayed decay during storage in sweet cherry (Yao and Tian, 2005), pears (Cao et al., 2006) and apricot (Cui et al., 2020), in the last commodity the effect being attributed to increases in POD and PAL activities. In grapes, just in one paper has been reported the effect of preharvest SA treatments on reducing decay during storage in 'Flame Seedless' cultivar (Champa et al., 2015). On the other hand, many studies have shown that postharvest treatment with SA or MeSA at appropriate concentrations could enhance resistance to pathogens in postharvest of fruits and vegetables such as mango, sweet cherry and pomegranate (Sayyari et al., 2011a; Valverde et al., 2015; Zheng et al., 2006). Accordingly, disease development in tomato fruit caused by *B. cinerea* was effectively suppressed by MeSA treatment in terms of percentage of disease incidence and the lesion area (Min et al., 2018). In apples, SA was totally effective in controlling blue mould caused *Penicillium expansum* as well as on maintaining the fruit quality characteristics related to weight loss, TSSS and TA (da Rocha Neto et al., 2015, 2016). These authors proposed as mechanism of action to the fact that SA caused leakage of the pathogen's proteins to the medium, measured by lipid damage, and intracellular disorganization.

3.2. Bioactive compounds and antioxidant enzymes

Grape polyphenols are characterised by a large range of chemical structures and can be found in the skin, flesh and seed of the berry. Grape skin contains flavanols, flavonols, anthocyanins, and stilbenes, which concentration of these phenolic compounds is affected by cultivar and environmental factors during the growth and development. The interest in these compounds is based on their beneficial effects for human health mainly due to their well-known antioxidant activity and capacity to scavenge free radicals (Doshi et al., 2015; Flamini et al., 2013).

In our study, all preharvest treatment enhanced the concentration of total phenolics, total anthocyanins and total antioxidant activity (TAA) due to hydrophilic (H-TAA) and lipophilic compounds in the grape skin for both cultivars (Fig. 3). Total phenolics in control grapes were 177 ± 10 and 167 ± 9 mg 100 g^{-1}, for 'Crimson' and 'Magenta', respectively, this concentration being significantly ($P < 0.05$) increased in SA, ASA and MeSA-treated berries, up to ~270 and 310 mg 100 g^{-1} for 'Crimson' and 'Magenta', respectively. It is worth noting that no significant differences ($P < 0.05$) were observed among phenolic content in SA, ASA and MeSA treated berries. Total antioxidant activity (TAA) was determined separately in hydrophilic (H-TAA) and lipophilic (L-TAA) extracts, although values were higher for H-TAA than L-TAA. However, both parameters were enhanced in all treated ($P < 0.05$) grapes (1.5-fold) with respect to controls for both cultivars.

Both cultivars are red seedless table grapes, although 'Crimson' has a purple while 'Magenta' has a light-red colour due to the occurrence of anthocyanins, which are responsible for the pigmentation of these cultivars. As shown in Fig. 3, the concentration of total anthocyanins was significantly higher in 'Crimson' (~70 mg 100 g^{-1}) than in 'Magenta' (~30 mg 100 g^{-1}) and thus it reflects the differences in colour. Similar to the other bioactive compounds, the concentration of anthocyanins was significantly enhanced ($P < 0.05$) in treated grapes with respect to controls. Moreover, the individual profile of anthocyanins by HPLC was analysed (Table 1) and 5 anthocyanins were identified in both cultivars: Delphinidin-3 glucoside (Dl-3 gluc), Cyanidin-3 glucoside (Cy-3 gluc), Petunidin-3 glucoside (Pt-3 gluc), Peonidin-3

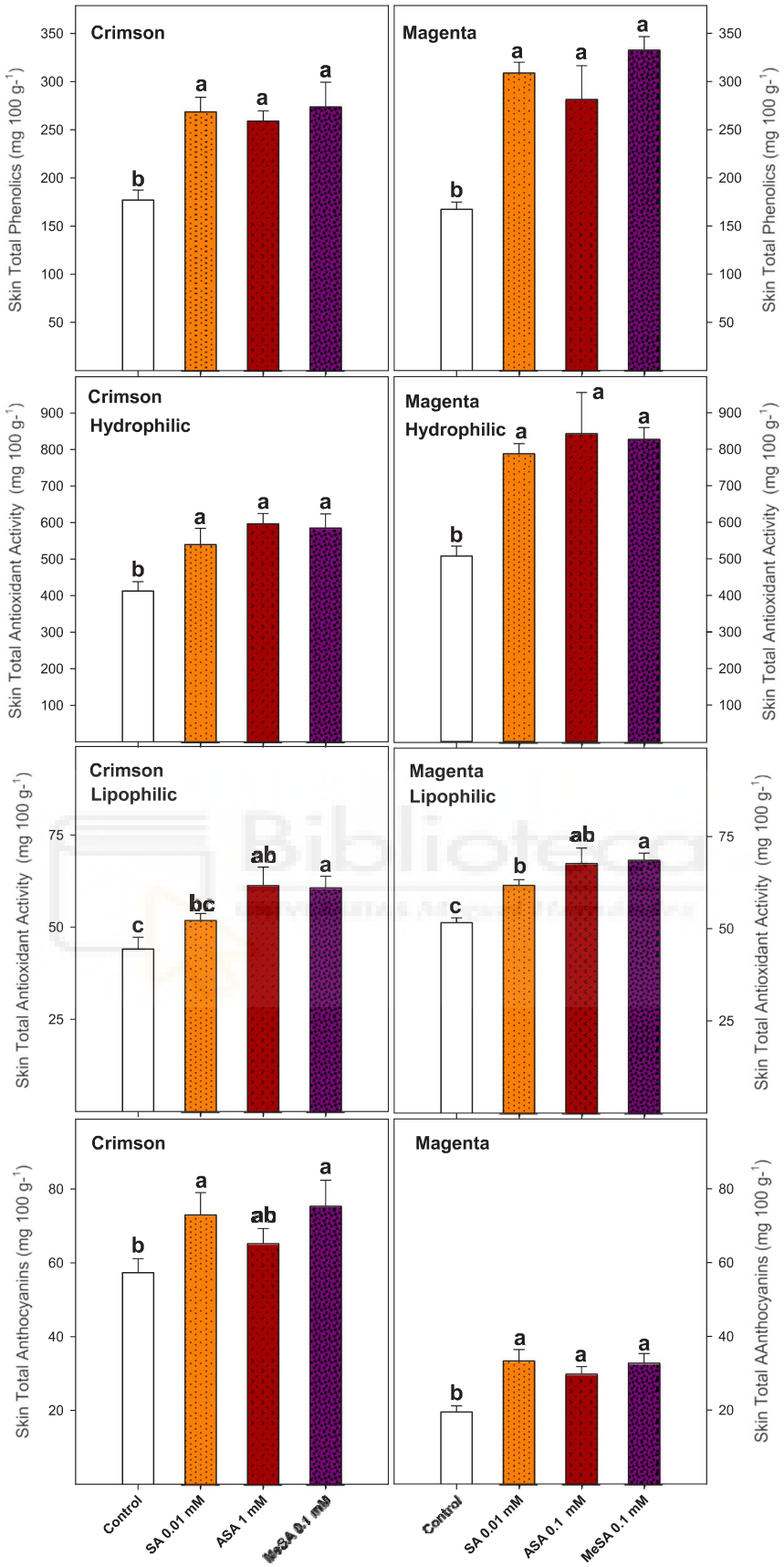

Fig. 3. Bioactive compounds the skin of two grape cultivars 'Crimson' and 'Magenta' as affected preharvest treatments with control, salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSA). Data are the mean ± SE. Different letters show significantly differences ($P < 0.05$) among treatments.

Table 1
Levels of individual anthocyanins (mg 100 g^{-1}) in the skin of two table grape cultivars at harvest as affected by treatment. Delphinidin-3 glucoside (Dl-3 gluc), Cyanidin-3 glucoside (Cy-3 gluc), Petunidin-3 glucoside (Pt-3 gluc), Peonidin-3 glucoside (Pn-3 gluc) and malvdin-3 glucoside (Mv-3 gluc).[a]

	Dl-3 gluc	Cy-3 gluc	Pt-3 gluc	Pn-3 gluc	Mv-3 gluc
'Crimson'					
Control	1.16 ± 0.28 a	0.61 ± 0.17 a	0.96 ± 0.23 a	18.21 ± 1.25 b	6.08 ± 0.61 b
SA 0.01 mM	0.78 ± 0.14 ab	0.46 ± 0.13 a	0.69 ± 0.14 a	26.94 ± 2.12 a	9.35 ± 0.78 a
ASA 1 mM	0.37 ± 0.14 b	0.16 ± 0.03 b	0.31 ± 0.08 b	22.34 ± 1.95 ab	7.77 ± 0.61 ab
MeSA 0.1 mM	0.96 ± 0.16 a	0.72 ± 0.17 a	0.91 ± 0.17 a	26.35 ± 2.51 a	8.88 ± 1.07 a
'Magenta'					
Control	1.35 ± 0.49 a	0.22 ± 0.07 a	0.76 ± 0.19 a	4.02 ± 0.75 a	5.18 ± 0.53 b
SA 0.01 mM	1.78 ± 0.51 a	0.23 ± 0.09 a	0.90 ± 0.26 a	5.53 ± 1.31 a	7.75 ± 0.91 a
ASA 0.1 mM	2.20 ± 0.48 a	0.34 ± 0.11 a	1.01 ± 0.18 a	5.15 ± 0.35 a	7.26 ± 0.43 a
MeSA 0.1 mM	2.29 ± 0.41 a	0.36 ± 0.09 a	1.15 ± 0.19 a	5.35 ± 0.86 a	7.35 ± 1.08 ab

[a] For each cultivar and parameter different letter following the mean are significantly different ($P < 0.05$) among treatments.

Table 2
Enzyme activity (U min^{-1} g^{-1}) of ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) in the skin of two grape cultivars at harvest as affected by treatments.[a]

	APX	CAT	POD
'Crimson'			
Control	247 ± 9 b	471 ± 44 c	119 ± 4 c
SA 0.01 mM	268 ± 8 b	666 ± 42 b	134 ± 7 bc
ASA 1 mM	267 ± 9 b	580 ± 33 bc	139 ± 4 b
MeSA 0.1 mM	296 ± 6 a	829 ± 58 a	159 ± 7 a
'Magenta'			
Control	192 ± 9 c	380 ± 23 c	78 ± 2 c
SA 0.01 mM	254 ± 8 b	543 ± 21 b	87 ± 4 bc
ASA 0.1 mM	244 ± 9 b	680 ± 44 a	94 ± 2 b
MeSA 0.1 mM	278 ± 7 a	719 ± 35 a	113 ± 5 a

[a] For each cultivar and parameter different letter following the mean are significantly different ($P < 0.05$) among treatments.

glucoside (Pn-3 gluc) and malvdin-3 glucoside (Mv-3 gluc). In 'Crimson', the major anthocyanin was Pn-3 gluc followed by Mv-3 gluc while in 'Magenta' Mv-3 gluc and Pn-3 glu were the major anthocyanins and found at similar concentrations. As expected, all treated grapes showed significant ($P < 0.05$) higher concentration in these major individual anthocyanins than controls.

On the other hand, the activity of the antioxidant enzymes ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) in the skin of the two grape cultivars at harvest was significantly ($P < 0.05$) enhanced due to all salicylate treatments. This effect was especially important for MeSA-treated berries which had the highest levels of APX, CAT and POD while control grapes showed the lowest activities (Table 2).

There are a number of mechanisms involved in improving the resistance of fresh produce to diseases by postharvest treatments with salicylates, e.g. changes in phenolic biochemistry, increased accumulation of H_2O_2, increased activity of antioxidant enzymes, induction of pathogenesis-related (PR) proteins and defence-related enzymes (Nazar et al., 2017; Zhang and Li, 2019). In the case of table grapes two of the above mechanisms of action are supported by data, that is the increase of phenolic compounds, including anthocyanins, and the greater activity of the antioxidant enzymes.

The increase in total phenolic compounds has been regarded as an indicator for plant defence response since they are able to scavenge free radicals (Beckman, 2000). Reactive oxygen species (ROS) are accumulated in plants under pathological and senescence conditions, leading to oxidative injury. It is well-known that APX, CAT, and POD are the key enzymes involved in ROS elimination, and the decrease in them may lead to high levels of ROS, as has been shown in control grapes. Therefore, the higher levels of antioxidant enzymes in treated berries could be involved in fruit resistance against *B. cinerea*. Accordingly, postharvest treatment with MeSA at 0.05 mM was effective in reducing disease incidence and severity of *B. cinerea* inoculated on tomato (Zhang et al., 2017). In addition, this treatment also enhanced the antioxidant enzymes CAT and POD as well as led to higher content of total phenolics, which agrees with our results on table grape. Chilling injury is another physiological disorder due to abiotic stress by low temperature storage, and salicylates have proved their efficacy on alleviating the chilling injury associated symptoms, such as skin browning, spots and weight loss. Thus, blood oranges treated with MeSA induced an accumulation of APX and CAT and the chilling injury was reduced (Habibi et al., 2019).

3.3. *Effect of SA, ASA and MeSA on berry maturity-quality parameters*

Among the flavour metabolites, sugar and organic acid compositions, which are measured through total soluble solids (TSS) and titratable acidity (TA), are most commonly associated with the taste of

Table 3
Levels of total soluble solids (g 100 g^{-1}), total acidity (g 100 g^{-1}), TSS/TA ratio (ripening index), glucose (g 100 g^{-1}), and fructose (g 100 g^{-1}) in two table grape cultivars at harvest as affected by treatments.[a]

	TSS (g 100 g^{-1})	TA (g 100 g^{-1})	TSS/TA ratio	Glucose (g 100 g^{-1})	Fructose (g 100 g^{-1})
'Crimson'					
Control	19.44 ± 0.28 b	0.56 ± 0.02 b	34.78 ± 0.63 a	9.02 ± 0.08 b	7.33 ± 0.08 c
SA 0.01 mM	19.87 ± 0.13 b	0.58 ± 0.01 b	34.25 ± 0.47 ab	9.16 ± 0.06 b	7.71 ± 0.08 b
ASA 1 mM	18.32 ± 0.25 c	0.64 ± 0.02 a	28.63 ± 0.73 c	8.62 ± 0.15 c	6.66 ± 0.15 d
MeSA 0.1 mM	20.63 ± 0.17 a	0.63 ± 0.01 a	32.74 ± 0.63 b	9.94 ± 0.06 a	8.50 ± 0.04 a
'Magenta'					
Control	16.70 ± 0.18 b	0.68 ± 0.02 b	24.55 ± 0.76 a	8.31 ± 0.12 b	7.01 ± 0.11 b
SA 0.01 mM	16.76 ± 0.13 b	0.64 ± 0.01 b	26.19 ± 0.70 a	8.07 ± 0.10 b	7.07 ± 0.08 b
ASA 0.1 mM	15.27 ± 0.09 c	0.78 ± 0.03 a	19.57 ± 0.79 c	7.63 ± 0.09 c	5.29 ± 0.05 c
MeSA 0.1 mM	17.93 ± 0.18 a	0.83 ± 0.02 a	21.61 ± 0.41 b	9.18 ± 0.09 a	7.34 ± 0.07 a

[a] For each cultivar and parameter different letter following the mean are significantly different ($P < 0.05$) among treatments.

Table 4
Concentration of individual organic acids two table grape cultivars at harvest as affected by treatment. Major organic acids (tartaric, citric and malic acids were expressed in g 100 g^{-1}) while minor organic acids (ascorbic, succinic and fumaric acids were expressed in mg 100 g^{-1}).[a]

	Tartaric acid	Malic acid	Citric acid	Ascorbic acid	Succinic acid	Fumaric acid
'Crimson'						
Control	0.27 ± 0.03 b	0.15 ± 0.01 b	0.09 ± 0.02 a	15.4 ± 0.11 c	0.08 ± 0.02 b	0.53 ± 0.04 b
SA 0.01 mM	0.35 ± 0.02 a	0.15 ± 0.01 b	0.08 ± 0.01 a	15.5 ± 0.19 c	0.12 ± 0.01 b	0.45 ± 0.02 b
ASA 1 mM	0.34 ± 0.01 a	0.17 ± 0.01 ab	0.04 ± 0.01 b	16.4 ± 0.24 b	0.16 ± 0.11 b	0.45 ± 0.02 b
MeSA 0.1 mM	0.38 ± 0.02 a	0.19 ± 0.01 a	0.07 ± 0.01 ab	22.6 ± 0.12 a	3.33 ± 0.06 a	0.74 ± 0.04 a
'Magenta'						
Control	0.32 ± 0.02 c	0.22 ± 0.03 a	0.07 ± 0.02 a	15.7 ± 0.09 b	4.85 ± 0.05 c	0.54 ± 0.02 b
SA 0.01 mM	0.41 ± 0.02 b	0.24 ± 0.02 a	0.11 ± 0.02 a	15.9 ± 0.22 b	5.64 ± 0.11 b	0.63 ± 0.05 ab
ASA 0.1 mM	0.44 ± 0.03 ab	0.24 ± 0.01 a	0.10 ± 0.01 a	15.7 ± 0.16 b	5.63 ± 0.19 b	0.47 ± 0.07 b
MeSA 0.1 mM	0.49 ± 0.02 a	0.26 ± 0.01 a	0.08 ± 0.01 a	19.3 ± 0.21 a	8.16 ± 0.12 a	0.84 ± 0.09 a

[a] For each cultivar and parameter different letter following the mean are significantly different ($P < 0.05$) among treatments.

fruits, including table grapes. Clusters were harvested when berries reached the characteristic size, colour and TSS (°Brix) of each cultivar in order to pick up full mature grapes. However, preharvest treatment significantly affected ($P < 0.05$) the content of TSS and TA at harvest (Table 3). For both cultivars, MeSA at 0.1 mM significantly increased the content of both TSS and TA with respect to control grapes, while a reduction in TSS was observed for 'Crimson' and 'Magenta' treated with ASA 1 and 0.1 mM, respectively. However, preharvest treatment with SA did not show significant differences comparing with control berries. Similarly, the ripening index or ratio TSS/TA revealed that ASA treatment induced a delay of ripening process. Between cultivars, 'Crimson' showed higher TSS (18–20 g 100 g^{-1}) than 'Magenta' (16–17 g 100 g^{-1}), while total acidity was lower in 'Crimson' (0.5–0.6 g 100 g^{-1}) than on 'Magenta' (0.6–0.8 g 100 g^{-1}). It is well known that different cultivars had different levels of TSS and TA. In a survey of 129 grape cultivars from Europe, North America and Japan, the content of TSS differed with average values of 16.5, 16.7 and 17.2, respectively (Shiraishi et al., 2010). Table grape cultivars are classified into two groups on the basis of their sugar composition: hexose accumulators (Type 1), which accumulate fructose, glucose, and trace amounts of sucrose, and sucrose accumulators (Type 2), which accumulate fructose, glucose, and a large amount of sucrose. Accordingly, both 'Crimson' and 'Magenta' belong to Type 1 cultivars. In fact, as shown in Table 3, only glucose and fructose were detected by HPLC-RI, and sucrose was not detected. Similarly to TSS, glucose and fructose were enhanced in grapes treated with MeSA and reduced in those treated with ASA, showing a high correlation between TSS and the content of sugars ($R^2 = 0.896$).

With respect to organic acids (Table 4), tartaric acid was found at the highest concentration followed by citric and malic acids, while ascorbic, succinic and fumaric acids were considered as minor. The concentration of organic acids was different depending on treatment and cultivar. All treated grapes with salicylates showed the maximum concentration of tartaric acid. Tartaric acid is synthesized in many plants, but accumulates in high quantities in the fruit of only a few genera, most significantly members of the *Vitaceae* family (Valero and Serrano, 2010), and thus considering a characteristic organic acid of grapes. With respect to ascorbic, succinic and fumaric acids, only grapes treated with MeSA at 0.1 mM had significant ($P < 0.05$) higher concentrations compared with controls and other treated grapes. Interestingly, from these results it can be highlight the increase of ascorbic acid or vitamin C. Many studies have investigated the effect of salicylates treatments on soluble solids, sugar content and acidity in fresh produce, most of them being applied as postharvest application. Generally, higher content of soluble solids, sugars and organic acids were found during storage of salicylate-treated fruits (Giménez et al., 2017; Habibi et al., 2019) and may be associated with lower metabolism, as occurred in MeSA-treated grapes. On the contrary, postharvest treatment with SA or ASA in pomegranate did not change the content of TSS or TA (Sayyari et al., 2011b), while in sweet cherry was reduced (Valero et al., 2011).

4. Conclusions

In this report we demonstrated for the first time that preharvest application of SA, ASA and MeSA induced resistance of table grapes to be colonized with *B. cinerea*. The mechanism of action involved in this effect could be the increased levels of phenolic compounds and the activity of antioxidant enzymes APX, CAT and POD, although the best results were obtained with MeSA at 0.1 mM in both table grape cultivars. These preharvest treatments also showed benefits in term of table grape quality, such as higher TSS, TA, sugars and organic acids, especially tartaric and ascorbic acid. In addition, bioactive compounds and antioxidant activity are also enhanced by preharvest salicylate treatments.

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Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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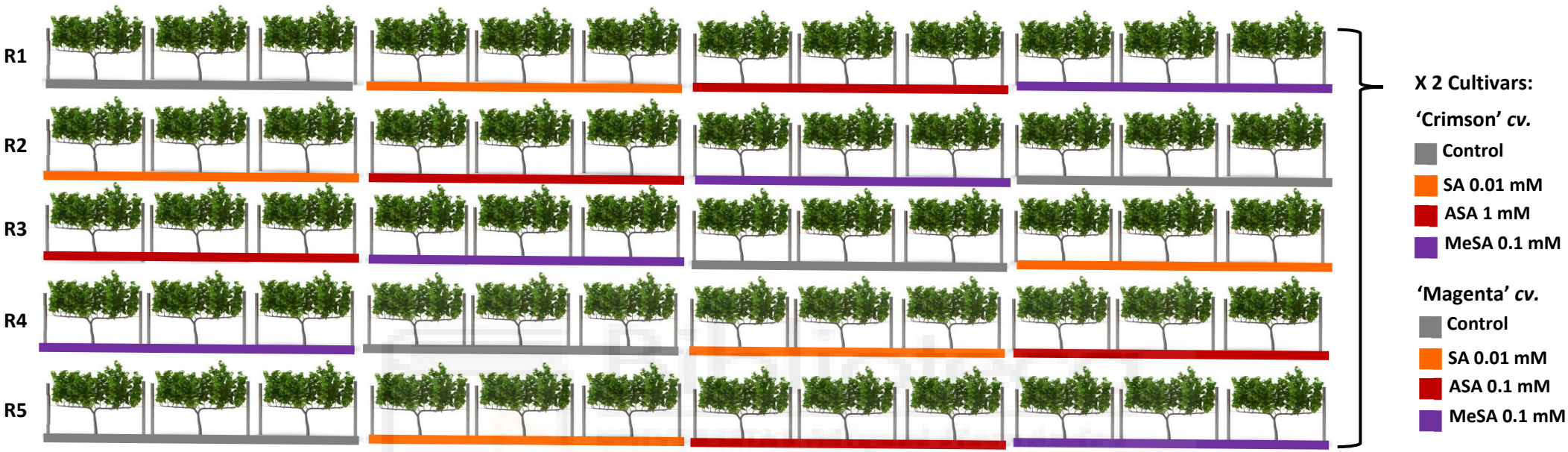
Supplementary figure legends

Figure 1S. Scheme of the experimental design with description of field treatments and the inoculation experiments with *Botrytis cinerea*.

Supplementary material



Inoculation experiments with *Botrytis cinerea*

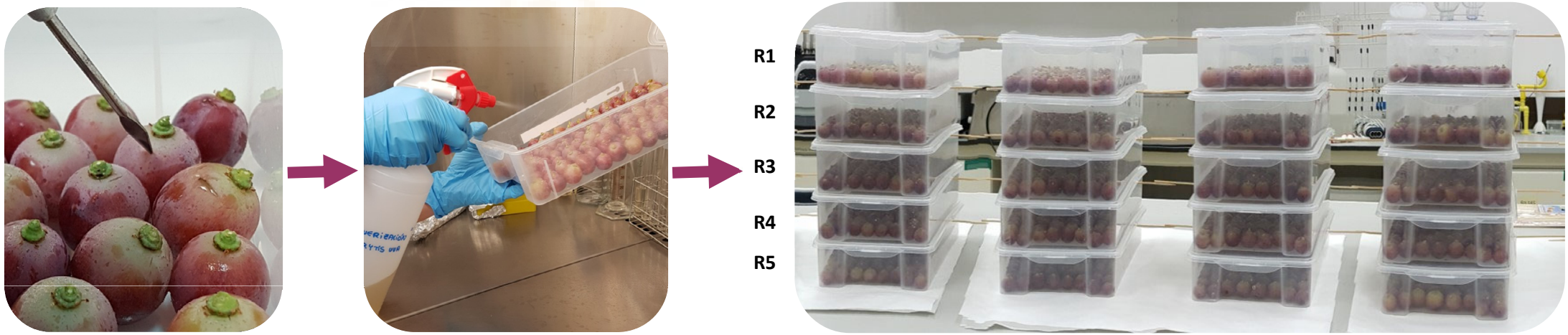


Figure 1S.

4.7. Publication 7

PUBLICATION 7 (Open access)

Preharvest application of oxalic acid improved pomegranate fruit yield, quality, and bioactive compounds at harvest in a concentration-dependent manner

María E. García-Pastor, María J. Giménez, Juan M. Valverde, Fabián Guillén, Salvador Castillo, Domingo Martínez-Romero, María Serrano, Daniel Valero, Pedro J. Zapata

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Preharvest Application of Oxalic Acid Improved Pomegranate Fruit Yield, Quality, and Bioactive Compounds at Harvest in a Concentration-Dependent Manner

María E. García-Pastor [1], María J. Giménez [1], Juan M. Valverde [1], Fabián Guillén [1], Salvador Castillo [1], Domingo Martínez-Romero [1], María Serrano [2], Daniel Valero [1] and Pedro J. Zapata [1,*]

[1] Department of Food Technology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Alicante, Spain; m.garciap@umh.es (M.E.G.-P.); maria.gimenezt@umh.es (M.J.G.); jm.valverde@umh.es (J.M.V.); fabian.guillen@umh.es (F.G.); scastillo@umh.es (S.C.); dmromero@umh.es (D.M.-R.); daniel.valero@umh.es (D.V.)

[2] Department of Applied Biology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Alicante, Spain; m.serrano@umh.es

* Correspondence: pedrojzapata@umh.es; Tel.:+34-966749789

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Abstract: The effects of oxalic acid (OA) applied in preharvest on pomegranate crop yield and quality properties have not yet been investigated. Pomegranate trees were treated with OA at 1, 5, and 10 mM along the developmental growth cycle during 2016, from full blossom until harvest, and the fruit diameter was recorded monthly. The yield was significantly higher in OA-treated pomegranate trees, the on-tree ripening process being promoted in a concentration-dependent manner. With respect to quality traits, OA at 10 mM showed better results in terms of firmness, external color due to a red color enhancement, and respiration rate and sensory attributes. Then, a second-year experiment was performed with OA at 10 mM, and crop yield and fruit quality results were confirmed. In addition, an increase in glucose and total acidity, specifically malic and ascorbic acids, was observed in OA 10 mM-treated pomegranate fruit. Bioactive compounds were also higher in OA-treated fruit than control, the 10 mM concentration being the most effective in increasing total phenolics, total anthocyanins, and total antioxidant activity. Overall, results suggest that OA applied at 10 mM as preharvest treatment could be an effective tool to enhance pomegranate crop and quality and to improve the content of health-related compounds.

Keywords: anthocyanins; color; fruit size; phenolics; *Punica granatum*

1. Introduction

Pomegranate (*Punica granatum* L.) is a widely grown horticulture crop in many tropical and subtropical countries. The Romans received it from Carthage, hence the name of the genus Punica. During growth, pomegranate increased fresh weight continuously. The edible parts of pomegranate fruit represented 52% of the total weight, comprising 78% juice and 22% seeds [1].

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Shulman et al. studied the growth curve of fruit and showed a single sigmoid curve [2]. Juice, total soluble solids, and anthocyanin content increased continuously during maturation while acidity decreased [3]. Pomegranate, being a non-climacteric fruit, should be picked when fully ripe, and fruit are ready for harvest in 5 to 6 months after the appearance of blossoms [4]. On the other hand, harvest maturity is also known to influence quality and could have a negative impact during storage, since early harvest may affect the development of the characteristic color, taste, and aroma of pomegranates, while late-harvested fruit exhibit a reduced shelf life [5]. Proper harvest of pomegranates also optimizes the health-promoting compounds in the fruit and juice. Its therapeutic properties have been used to treat different conditions (cardiovascular, neurological, diabetes, and cancer) for hundreds of years, and currently numerous in vitro and in vivo studies have shown the antioxidant, anti-inflammatory, and anti-tumoral properties of pomegranate [6]. During ripening of pomegranate, an accumulation of anthocyanins occurs in arils, these pigments being the responsible for the pink-red color. Total phenol concentration decreases rapidly in early development and more slowly as the fruit mature. The final concentration can range from 0.90 to 2.10 g kg^{-1}, depending on cultivar and horticultural practices [3,7,8]. Pomegranate is considered as a highly nutritious fruit in which the edible parts of the fruit (arils) contain considerable amount of proteins, carbohydrates, minerals, sugars, vitamins, polysaccharides, and bioactive compounds such as anthocyanins and polyphenols [9].

Pomegranate fruit quality is determined by parameters such as fruit size, skin and aril color, sugar and acid content, and the presence of small and soft seeds. The landrace and best-known Spanish cultivar is "Mollar de Elche", which is characterized by presenting a high content of total soluble solids, low titratable acidity, small and soft seeds which can be easily eaten, and pleasant flavor [10]. These characteristics make it a cultivar highly appreciated by consumers in the markets. The external color is pale pink and the arils are pink-red, and this cultivar ripens in the months of October and November [11,12]. The Mollar de Elche cultivar has been safeguarded by a Protected Designation of Origin (PDO) since 2016 (R (UE) 2016/83) [13]. The external color of pomegranate fruit is one of the most important market requirements, but this cultivar present a lower pigmentation color in the husk [14] compared to other very important cultivars in economic terms, such as "Acco" or "Wonderful". Moreover, the early harvest is another key factor that influences the market prices [15]. Thus, increasing the harvest precocity of pomegranate fruit that reach the market with proper color could increase the economic profit for farmers.

Very few studies have been performed with preharvest treatments with naturally occurring compounds with the aim to increase fruit quality at the time of harvest, and even less with oxalic acid (OA). OA is a natural organic acid having many physiological functions mainly related to the induction of systemic resistance against fungal and viral diseases, through an increase in antioxidant enzymatic activities and phenolic compounds [16]. Recently, it has been classified as a generally recognized safe (GRAS) compound. The antioxidant capacity of this compound has been demonstrated, and its role as a natural antioxidant compound in plant systems has been proposed [17]. Moreover, postharvest OA application has shown benefits by retarding the postharvest ripening of climacteric fruits, but was also effective in non-climacteric fruits such as sweet cherry by extending the storability life during longer periods [18]. Specifically, in pomegranate fruit, the effect of OA treatments on pomegranate fruit quality has been assayed only in a previous paper, in which postharvest treatments, at 2, 4, and 6 mM, were performed before storage at 2 °C, and results showed that CI symptoms were reduced and fruit quality properties maintained as compared with control fruits [19]. However, no previous reports are available in the literature regarding the effect of preharvest OA treatment on fruit growth and ripening and fruit quality properties at harvest and during storage, although a few studies have been published in other fruit species.

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As preharvest treatment, OA has been reported to increase yield and/or improve nutritional quality of sweet cherry [20], peach [21], kiwi [22], artichoke [23], strawberry [24], and plum [25], but its effect on pomegranate has not been investigated yet. Martínez–Esplá et al. treated "Sweet Heart" and "Sweet Late" sweet cherry cultivars with OA at 0.5, 1.0, and 2.0 mM at 98, 112, and 126 days after full blossom (DAFB) by foliar spraying, and reported that fruit treated with 2 mM OA showed the higher fruit size and antioxidant potential at harvest [20]. Similarly, 2 mM OA applied with a mechanical mist sprayer at 63, 77, and 98 DAFB enhanced crop yield (kg per tree) and fruit weight of plum, although the on-tree ripening process was delayed [25]. However, no significant effect was found in the developmental process of artichoke plants sprayed with 2 mM OA solution at 45, 24, and 3 days before harvesting, although the treatment lead to a higher number of first-class artichokes with higher hydrophilic total antioxidant activity at harvest and during cold storage [23]. On the other hand, different OA concentrations (1, 3, and 5 mM) were applied on peach trees by preharvest spray at 15 days before harvest and results showed that OA, especially at 5 mM, enhanced fruit antioxidant capacity during storage [21]. Furthermore, Zhu et al. reported that the same concentration of OA applied on kiwifruit plants at 130, 137, and 144 DAFB increased the postharvest quality and induced disease resistance of the fruit against *Penicillium expansum* [22]. Finally, field-grown strawberry plants were sprayed with 1 and 2 mM OA at the flowering stage to enhance plant growth parameters due to an enhanced concentration of the primary macronutrients [24]. These previous reports show that the effects of OA treatment depend on several factors, such as plant species, cultivar, applied concentration, or key stage of application during the growth developmental and ripening cycle. However, as mentioned above, its effects as a preharvest treatment in pomegranate fruit are unknown.

Thus, the main aim of the present study was to evaluate the effects of preharvest OA treatments at three concentrations (1, 5, and 10 mM) to pomegranate trees on fruit size, crop yield (in terms of early harvest), respiration rate, and fruit quality characteristics, namely physico-chemical and sensory traits and individual sugars and organic acids content, as well as on bioactive compound content and antioxidant activity.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The experiment was performed in a commercial orchard located in Elche, south of Alicante, Spain (UTMX: 694006.000 UTMY: 4234860.000) by using 10-year-old pomegranate trees of Mollar de Elche cultivar, planted at 6 × 5 m, and trained on a single-stem training system. In 2016, three OA concentrations were applied, 1, 5, and 10 mM, and the best results were obtained with the 10 mM concentration. Thus, the 10 mM OA concentration was used in 2017 in order to confirm the 2016 results. For both experiments, 2016 and 2017, a drip irrigation system with eight emitters per tree delivering 4 L h^{-1} was used, as follows: (i) Two watering cycles of 1 h per week in April, (ii) two watering cycles of 2 h per week in May, June, July, August, and September, (iii) and one watering cycle of 1 h in October. Soil was composed of sand (30%), silt (34%), and clay (36%), and had a pH of 7.80. Climatic conditions in the crop field were a semi-arid Mediterranean climate, characterized by mean annual temperatures of 19.07 and 19.28 °C for 2016 and 2017, respectively, and maximum temperatures from June to September of 31.45 and 31.62 °C for 2016 and 2017, respectively. In addition, accumulated rainfall of 251.15 and 238.32 mm were registered for 2016 and 2017, respectively. In 2016, 3 blocks or replicates of five trees each (n = 15 trees per treatment) were selected at random for the following treatments: Control and OA at 1, 5, and 10 mM. Treatments were carried out by foliar spraying (3 L per tree) of freshly prepared OA (Sigma–Aldrich, Madrid, Spain) solutions, containing 1 mL L^{-1} Tween-20, along the developmental growth cycle during 2016 year in different key moments from full blossom until harvest. Specifically, four treatments were sprayed as follows: T1 (when fruit reached 30% of its final size), T2 (when fruit reached 50% of its final size), T3 (1 month before harvesting), and T4 (4 days before harvesting) at 80, 110, 140, and 170 days after full blossom, respectively, which started in March (Julian days = 70 and

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69, for 2016 and 2017, respectively). The last treatment was performed 4 days before harvesting in order to induce the systemic defense in the fruit by the treatments and to maintain this effect close to postharvest. Control trees were sprayed with distilled water containing 1 mL L^{-1} Tween-20. One week before T1 treatment, 10 fruit were labeled on the tree around the equatorial perimeter for each block or replicate of treatment and concentration (n = 30 fruit in total, 2 fruit per tree), in which fruit growth was followed by measuring cheek diameter (mm) monthly. The following year, in the 2017 experiment, treatments were performed with OA at 10 mM, the best concentration selected based on the results of the previous year, and the control, which were applied following the same process described in the 2016 year, although using ten different trees in the same plot for each of the 3 blocks or replicates (n = 30 trees per treatment).

Fruit were harvested at the commercial ripening stage in accordance with commercial criteria based on parameters characteristic of this cultivar such as fruit size (diameter of 9.0 cm, approximately, and fruit weight of ≈ 360 g), husk color (pale pink), and total soluble solids (TSS) content (≥15°Brix). In both experiments, fruit were harvested on two harvest dates due to the heterogeneous fruit ripening process occurred on tree. On both harvest dates, separated by 20 days, the total yield (kg $tree^{-1}$ and number of fruits $tree^{-1}$) was determined for each tree and, with these data, the average fruit weight was calculated. In addition, since the first harvest is the most important for commercial profit, the yield of the first harvest (kg $tree^{-1}$, number of fruits $tree^{-1}$, and fruit weight) was also performed.

In the 2016 experiment, from the fruit harvested from each tree, 2 fruit, homogenous in size and color and without visual defects, were taken at random and the fruit from the five trees of each block were mixed so that 10 fruit were taken for each block or replicate (n = 3) with a total of 30 fruit per treatment and concentration, and were transported to the laboratory immediately. However, in the 2017 experiment and in order to confirm the results, a greater number of fruits were transferred to the laboratory. Thus, 20 fruit of each block or replicate were harvested from the three blocks or replicates in order to obtain a homogeneous and randomized sample of 60 fruit for each treatment. In the same way as the 2016 experiment, the quality parameters were determined from 30 fruit of each treatment (10 fruit from 3 replicates). In addition, another 30 fruit of each treatment, previously selected, were used for descriptive sensory evaluation.

2.2. Fruit Growth, Crop Yield, and Respiration Rate

The evolution of fruit growth was recorded monthly in the labeled fruit from the T1 treatment by measuring the equatorial diameter in the 2016 experiment, and results were expressed in mm. Two harvest dates were performed in both growing seasons. Total yield, expressed as kg $tree^{-1}$ and number of fruits $tree^{-1}$, was measured and expressed as the result of both harvest dates. In addition, due to the economic profit of the fruit from the first harvest date, yield, expressed as kg $tree^{-1}$ and number of fruits $tree^{-1}$, was determined. Then, fruit weight (g) was calculated at two harvest dates. Results were expressed as the mean ± SE.

To quantify fruit respiration rate at harvest, 5 fruit of each replicate were hermetically sealed for 60 min in a 3-L jar. Thus, there were 2 sealed jars of 5 fruit each for each replicate (n = 6 jars per treatment and concentration). After that, one sample from the holder atmosphere of 1 mL was withdrawn with a syringe and injected in a gas chromatograph, as previously described by Sayyari et al. [26]. Respiration rate results were expressed as mg CO_2 kg^{-1} h^{-1} and was the mean ± SE.

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2.3. Physico-Chemical Quality Parameters: Color, Firmness, Total Soluble Solids, Titratable Acidity, Ripening Index, and Individual Sugars and Organic Acids

External color was determined using the CIE Lab system in a colorimeter (CRC200, Minolta Camera Co., Tokyo, Japan) along of the equatorial perimeter in six points of each of the ten fruit from each block or replicate. After recording L*, a*, and b* parameters, color was expressed as hue angle (h°), calculated as arctg b*/a*. Fruit firmness was measured by using a TX-XT2i Texture Analyzer (Stable microsystems, Godalming, United Kingdom) and was determined individually in each of the ten fruit of each block or replicate. The percentage of deformation was a 3% of the fruit diameter. Results were expressed as N mm^{-1}, the relation between the applied force to achieve the deformation percentage and the distance traveled, and were the mean ± SE.

Pomegranate fruit were cut by the equatorial plane and peeled, and the arils of the ten fruit of each replicate were combined to obtain a homogeneous sample for each replicate. Total soluble solids (TSS) were measured in duplicate in the juice obtained from 30 g of pomegranate arils, by using a digital refractometer (Atago PR-101, Atago Co., Ltd., Tokyo, Japan) at 20 °C and were expressed as g kg^{-1} in fresh weight basis (FW). Titratable acidity (TA) was also determined in duplicate in each sample, by using 1 mL of diluted same juice (in 25 mL distilled H_2O), which was automatically titrated (785 DMP Titrino, Metrohm) with 0.1 N NaOH up to pH 8.1, and the results were expressed as g malic acid equivalent kg^{-1} of FW basis. Ripening index (RI) was then calculated as the ratio of TSS/TA.

For sugars and organic acids quantification, 5 g of the aril sample of each replicate were extracted with 5 mL of water of 0.5% phosphoric acid and the supernatant was filtered through 0.45 µm Millipore filter and injected into a HPLC system (Hewlett–Packard HPLC series 1100). In an isocratic system, the elution consisted of phosphoric acid at 0.1% running with a flow rate of 0.5 mL min^{-1} through a Supelco column (Supelcogel C-610H, 30 cm, 7.8 mm, Supelco Park, Bellefonte, PA, USA). Organic acids were detected at 210 nm of absorbance and sugars by detector of refractive index and quantified by using standard curves of pure sugars and organic acids (Sigma–Aldrich, Germany). Results were expressed as g kg^{-1} FW. Results were the mean ± SE of three replicates. For the following determinations, the aril samples were stored at −20 °C.

2.4. Descriptive Sensory Evaluation

A descriptive sensory analysis was performed in the 2017 experiment with trained panelists after a preconditioning of the harvested pomegranate fruit in cold chambers at 8 °C and at 85–90% of relative humidity. Ten panelists, aged between 25 and 55 years, 50% female, from the Department of Agro-Food Technology of Miguel Hernández University of Elche (Orihuela, Alicante, Spain) were selected to participate in this study. Panelists received two preliminary orientation sessions of 60 min on sensory evaluation of pomegranate fruit in order to discuss the main attributes appreciated by consumers. Samples of 15 fruits per treatment were used to evaluate external fruit characteristics and 50 g of arils, obtained from a mixture of 15 fruits per treatment, and were served to each panelist at a controlled temperature of 20 ± 2 °C in a testing room with a combination of natural and fluorescent light.

To quantify the intensity of the pomegranate fruit attributes, the panel used a numerical scale from 0 to 10; where 0 represented no perceptible intensity and 10 extremely strong intensity. The panel evaluated the following attributes: Brightness, color uniformity and intensity, firmness, and sweetness in the whole fruit, and sourness, bitterness, astringence, solubility in saliva (related to the fiber content of the seeds and, therefore, to their ease of eating), seed hardness, and overall liking in the arils. Overall liking was the result of the panelists satisfaction with respect to all the previous parameters.

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2.5. Bioactive Compounds and Total Antioxidant Activity Quantification

The bioactive compounds determined were the total phenolic and total anthocyanins content. Phenolic extraction was performed by homogenizing 5 g of the aril sample with 10 mL of methanol/water (8:2) containing 2 mM NaF by using a homogenizer (Ultraturrax, T18 basic, IKA, Berlin, Germany) for 30 s. As previously described by Sayyari et al. [26], the extracts were centrifuged at 10,000× *g* for 10 min at 4 °C and the supernatant was used to quantify total phenolics compounds in duplicate by using the Folin–Ciocalteu reagent. The results were expressed as g of gallic acid equivalent (GAE) per kg^{-1} of FW and were the mean ± SE of three replicates.

To extract total anthocyanins, 5 g of arils were homogenized in 15 mL of methanol/formic acid/water (25:1:24, *v*/*v*/*v*) and then, centrifuged at 10,000× *g* for 10 min at 4 °C. The absorbance was measured in the supernatant at 520 nm and in duplicate for each extract according to García–Pastor et al. [27]. Total anthocyanin content (TAC) was expressed as g of cyanidin 3-O-glucoside equivalents per kg^{-1} of FW and were the mean ± SE of three replicates.

Total antioxidant activity (TAA) was determined according to Sayyari et al. [26], which determines hydrophilic (H-TAA) and lipophilic (L-TAA) antioxidant activity in the same extraction. 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 the sum of the antioxidant compounds present in both phases in g of Trolox equivalent (TE) per kg^{-1} of FW and were the mean ± SE of three replicates.

2.6. Statistical Analysis

Results were expressed as mean ± SE of three replicates. Data for the analytical determinations of the 2016 experiment were subjected to analysis of variance (ANOVA) using treatment as the factor. Mean comparisons were performed using a multiple range test (Tukey's test) to examine if differences among treatments were significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. For data of the 2017 experiment, a Student's *t*-test was performed in order to detect significant differences between treatments at $p < 0.05$. All analyses were performed with SPSS software package v. 17.0 for Windows.

3. Results

3.1. Fruit Growth, Crop Yield, and Respiration Rate

Pomegranate fruit growth was evaluated by recording the diameter (mm) along the growth cycle in the 2016 season (Figure 1). As expected, a continuous increase in fruit weight was observed with a clear simple Sigmoid growth curve. Pomegranate trees were sprayed 4 times (from T1 to T4; Figure 1) along the fruit developmental cycle, and results showed that all concentrations of OA treatment stimulated fruit growth from the second application (T2; Figure 1), mainly in August, resulting in pomegranate fruit with higher size ($p \leq 0.05$) at this developmental stage (≈63 mm for control fruit and 66–68 mm for OA-treated pomegranate fruit), without significant differences ($p > 0.05$) among the concentrations of OA. Despite the fruit size being promoted by OA treatments at this key moment of the fruit developmental cycle, no significant differences ($p > 0.05$) were observed at harvest among treatments for this growth parameter (Figure 1).

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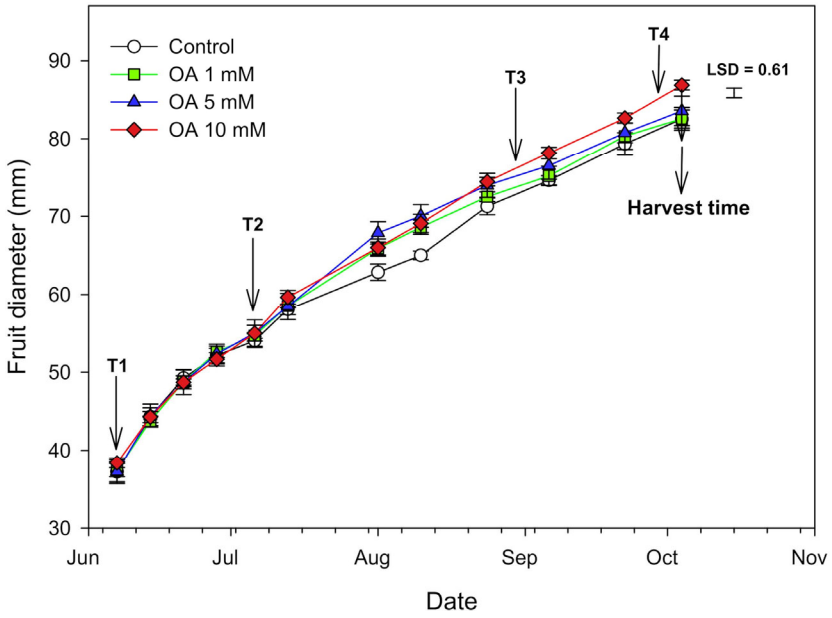

Figure 1. Pomegranate fruit diameter (mm) during on-tree fruit development as affected by oxalic acid (OA) treatment at 1, 5, and 10 mM in the 2016 experiment. Data are the mean ± SE. $LSD_{0.05}$ bar among treatments is shown. Arrows indicate the different key moments of treatments (T1, T2, T3, and T4) and the harvest time.

Two picking dates were performed for both years (2016 and 2017). For both, yield, expressed as kg tree^{-1} and number of fruits tree^{-1}, was significantly higher in OA-treated fruit than controls (Figure 2). Total yield of control fruit in 2016 was 37.75 ± 3.28 kg tree^{-1}, and significantly higher ($p \leq 0.05$) in 1, 5, and 10 mM OA; 47.88 ± 5.17, 46.04 ± 8.06, and 50.68 ± 4.76 kg tree^{-1}, respectively. In the 2017 season, 10 mM OA as the concentration selected in the first season based on crop yield and fruit quality results was repeated and similar results on total yield were obtained compared with non-treated fruit. In addition, a significant increase on yield in the first harvest was observed by the OA treatment in a concentration-dependent manner ($p \leq 0.05$), the higher concentration being the most effective, improving the kilograms of fruit tree^{-1} in terms of early harvest (Figure 2). The next year, similar results were obtained at the first harvest, where 10 mM OA-treated trees produced 37.12 ± 3.13 kg tree^{-1} compared to 24.52 ± 1.61 kg tree^{-1} from controls. A similar trend was shown in relation to the number of fruits tree^{-1} (Figure 2), for which all treatments were effective ($p \leq 0.05$) on increasing this crop parameter (≈139, 132, and 138 fruit for 1, 5, and 10 mM OA, respectively) compared to controls (≈108 fruit) in the first season. No significant differences were found between the different OA treatments ($p > 0.05$). However, the number of fruits tree^{-1} harvested in the first harvest date was significantly higher ($p \leq 0.05$) only in the higher concentrations, OA at 5 and 10 mM, without differences ($p > 0.05$) between 1 mM OA-treated trees and control trees. In the second season, 10 mM OA significantly increased ($p < 0.05$) the number of fruit tree^{-1} of the total and first harvest production than non-treated trees by 20.68 and 82.06%, respectively. For both seasons, fruit weight did not show significant differences ($p \geq 0.05$) among treatments (Figure 2).

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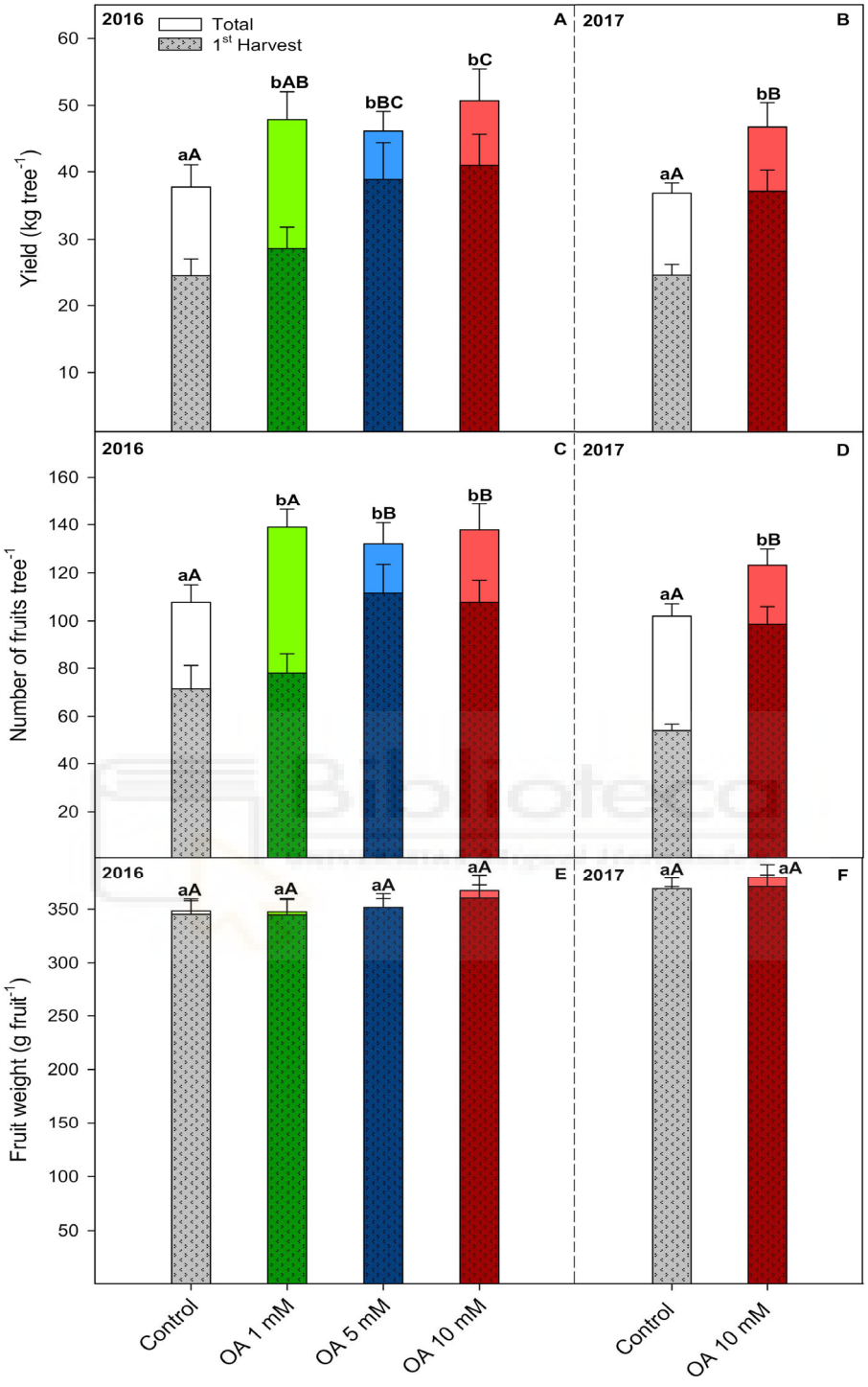

Figure 2. Effects of oxalic acid (OA) treatments on crop yield (kilograms per tree; **A** and **B**), number of fruits per tree (**C** and **D**) and fruit weight (grams per fruit; **E** and **F**) at the first harvest date (dotted bars) and at total harvest (open bars) for 2016 and 2017 years. Data are the mean ± SE. Different lowercase letters show significant differences among treatments for total yield values and different capital letters show significant differences for results of the first harvest date at $p < 0.05$.

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On the other hand, the respiration rate of pomegranate fruit was significantly lower ($p \leq 0.001$) in fruit from 5 and 10 mM OA-treated trees than in those from 1 mM OA-treated trees and control trees at harvest (Table 1). In the 2017 experiment, the results were confirmed for this parameter (Table 1) and pomegranate fruit treated with 10 mM OA showed a significantly lower ($p \leq 0.001$) respiration rate than control ones (19.22 ± 0.25 and 22.44 ± 0.33 mg CO_2 kg^{-1} h^{-1}, respectively).

Table 1. Effects of preharvest oxalic acid (OA) treatments (1, 5, and 10 mM) on pomegranate respiration rate and quality parameters; external color (L*, a* parameter and hue angle), firmness, total soluble solids (TSS), total acidity (TA), and ripening index (RI) in the first picking date in 2016 and 2017 experiments. Data are the mean ± SE.

2016 Experiment					
Parameter		**ANOVA** †	**Control**	**OA 1 mM**	**OA 5 mM**	**OA 10 mM**
Respiration rate (mg CO_2 kg^{-1} h^{-1})		***	23.99 ± 0.32bB	22.49 ± 0.24b	19.35 ± 0.47a	20.36 ± 0.80aA
External color	L*	**	64.22 ± 0.76bA	62.05 ± 0.87ab	60.03 ± 0.95a	59.80 ± 0.92aA
	a*	*	32.23 ± 1.16aB	34.57 ± 1.32ab	36.56 ± 1.18ab	37.85 ± 1.24bB
	h°	*	44.20 ± 1.52bA	41.66 ± 1.62ab	39.38 ± 1.45ab	38.59 ± 1.41aA
Firmness (N mm^{-1})		*	16.79 ± 0.74aA	16.56 ± 0.44a	17.26 ± 0.32ab	18.81 ± 0.37bA
TSS (g kg^{-1})		***	168.40 ± 0.50aA	170.40 ± 0.50a	169.70 ± 0.40a	181.30 ± 0.80bA
TA (g kg^{-1})		***	4.20 ± 0.10aA	4.60 ± 0.10bc	4.40 ± 0.10ab	4.80 ± 0.10cA
RI		NS	40.26 ± 1.22A	37.06 ± 1.01	38.48 ± 1.04	37.73 ± 1.21A
2017 Experiment						
Parameter		**Student's *t*-Test** ¥	**Control**	**OA 1 mM**	**OA 5 mM**	**OA 10 mM**
Respiration rate (mg CO_2 kg^{-1} h^{-1})		*	22.44 ± 0.33A	-	-	19.22 ± 0.25A
External color	L*	*	73.29 ± 0.95B	-	-	67.54 ± 0.89B
	A*	*	15.48 ± 1.31A	-	-	25.44 ± 1.80A
	h°	*	67.60 ± 1.85B	-	-	55.45 ± 2.27B
Firmness (N mm^{-1})		*	25.69 ± 0.62B	-	-	27.71 ± 0.76B
TSS (g kg^{-1})		*	169.10 ± 0.50A	-	-	180.80 ± 0.70A
TA (g kg^{-1})		*	4.10 ± 0.10A	-	-	5.00 ± 0.10A
RI		NS	40.83 ± 1.51A	-	-	37.11 ± 1.37A

† NS = not significant; *, **, and *** significant at $p \leq 0.05$, 0.01, and 0.001, respectively; ¥ values (mean of three replicates) followed by different lowercase letters, within the same row, show significantly differences among treatments, according to the Tukey's multiple range test. ¥ NS = not significant; * significant at $p < 0.05$, according to the Student's *t*-test. Different capital letters in the same column show significant differences among years for each treatment at $p < 0.05$.

3.2. *Physico-Chemical Quality Parameters: Color, Firmness, Total Soluble Solids, Titratable Acidity, Ripening Index, Individual Sugars and Organic Acids, and Sensory Evaluation*

With respect to external color, significant differences were observed on luminosity or L* ($p \leq 0.01$), a* color parameter ($p \leq 0.05$) and hue angle or h° ($p \leq 0.05$) among treatments (Table 1). In a concentration-dependent manner, lower values of luminosity and higher a* values were measured at harvest in fruit from OA-treated trees. Hue angle of pomegranate husk was lower in fruit from 1 (41.66 ± 1.62), 5 (39.38 ± 1.45), and 10 (38.59 ± 1.41) mM OA-treated trees than in controls (44.20 ± 1.52), showing a deeper red external color of OA-treated fruit, especially for the 10 mM concentration in which the lowest hue value was obtained, as can be seen in Figure S1. These differences were significantly increased ($p < 0.05$) between 10 mM OA-treated fruit and the control in the 2017 season for all the color parameters mentioned above (Table 1).

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Fruit firmness at harvest was significantly ($p \leq 0.05$) increased by 10 mM OA treatment (Table 1) with fruit treated with 10 mM OA having 2 units greater firmness in both seasons. Figure 3 shows the sensory profiles of 10-mM-treated and non-treated pomegranate fruit and the list of the main sensory attributes used in the description of the fruit. Significant differences were found in 8 out the total 12 sensory attributes evaluated. This profile was characterized by high values in appearance and firmness attributes from 10 mM OA-treated pomegranate fruit such as size (8.0), brightness (8.0), color uniformity (7.0), color intensity (9.0), and firmness (7.0), which are consistent with the physico-chemical values obtained. However, trained panelists detected that fruit size was higher in 10 mM OA-treated fruit compared to untreated fruit, although no significant differences were observed for this parameter at harvest in 2017 when fruit weight was calculated as the total fruit production by tree (kg) and number of harvested fruit per tree.

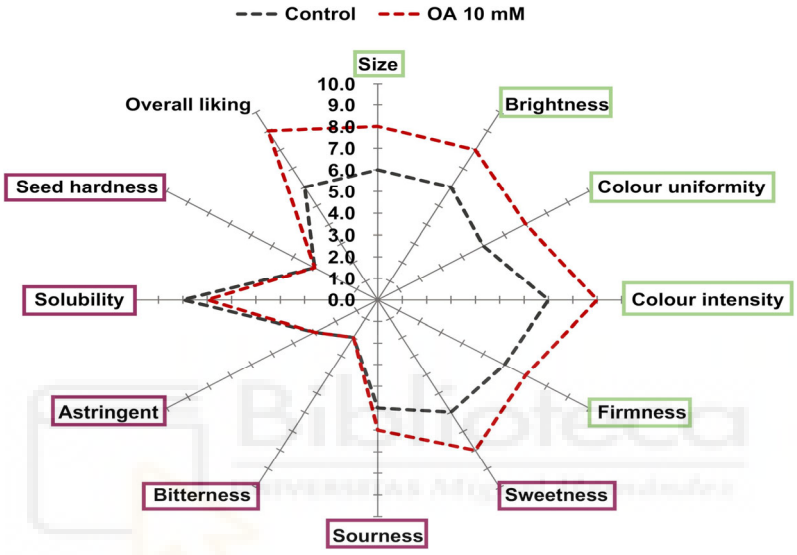

Figure 3. Descriptive sensory analysis of oxalic acid (OA) 10-mM-treated and non-treated (control) pomegranate fruit in the 2017 experiment. Size, brightness, color uniformity and intensity, firmness, sweetness, sourness, bitterness, astringence, solubility, seed hardness, and overall liking were evaluated by trained panelists in a hedonic scale from 0 to 10 points. Parameters highlighted by a green and purple box were evaluated in the whole fruit and in the arils, respectively.

Table 1 shows that TSS at harvest were 168.40 ± 0.50 g kg^{-1} in arils from control, and significantly higher ($p \leq 0.001$) in 10 mM OA-treated ones, with 181.30 ± 0.80 g kg^{-1} in the 2016 experiment, while no significant effects were observed for OA treatments at 1 and 5 mM. This significant difference ($p < 0.05$) between 10 mM OA and control arils were confirmed in the 2017 experiment (Table 1). Similarly, TA showed significant differences, especially for 10 mM OA concentration ($p \leq 0.001$; Table 1), whereas 1 mM OA concentration also increased its total acidity, with an increase for both concentrations of 1.14- and 1.10-fold higher than the control fruit. In the second year, arils from 10 mM OA-treated fruit showed 1.22-fold higher values of total acidity than arils from non-treated pomegranate fruit ($p < 0.05$; Table 1). However, the RI was not affected by OA treatments ($p \geq 0.05$), with values ranging between 37 and 41 for both years (Table 1). Figure 3 showed that sweetness (8.0) and sourness (6.0) were higher in 10 mM OA-treated fruit. Besides, overall linking (9.0) was also found higher in OA-treated pomegranate fruit. However, no significant differences were found between treatments related to bitterness, astringence, solubility in saliva, or seed hardness attributes. On the other hand, significant differences were observed for respiration rate, external color, and firmness, among years (Table 1).

Fructose was the major sugar in pomegranate arils, followed by glucose, the last one being significantly ($p \leq 0.05$) increased by 10 mM OA treatment, reaching 69.04 ± 0.71 g kg^{-1}, while sucrose was found at very low concentration without significant differences ($p > 0.05$) attributed to treatments

(Table 2). Oxalic, malic, and ascorbic organic acids were also significantly increased ($p \leq 0.05$; $p \leq 0.01$; $p \leq 0.01$, respectively) by 10 mM OA treatment, the highest increase being found in the major organic acid, malic acid. Moreover, ascorbic acid increased 1.17-fold higher in 10 mM OA-treated pomegranate than in the control fruit (Table 2).

Table 2. Effects of preharvest oxalic acid (OA) treatments (1, 5, and 10 mM) on individual sugar and organic acid concentration (g kg^{-1}) in the first picking date in 2016 experiment. Data are the mean ± SE.

Parameter		ANOVA †	Control	OA 1 mM	OA 5 mM	OA 10 mM
Individual sugars	Sucrose	NS	0.58 ± 0.01	0.54 ± 0.02	0.59 ± 0.02	0.62 ± 0.01
	Glucose	*	61.04 ± 0.59a	64.71 ± 1.71ab	64.04 ± 1.69ab	69.04 ± 0.71b
	Fructose	NS	106.48 ± 2.01	108.53 ± 1.60	107.28 ± 2.42	115.51 ±2.21
Organic acids	Oxalic acid	*	0.14 ± 0.01a	0.17 ± 0.01ab	0.18 ± 0.01ab	0.21 ± 0.01b
	Citric acid	NS	0.67 ± 0.05	0.66 ± 0.05	0.65 ± 0.02	0.75 ± 0.06
	Malic acid	**	3.06 ± 0.10a	3.39 ± 0.05a	3.28 ± 0.11a	3.93 ± 0.05b
	Ascorbic acid	**	0.47 ± 0.02a	0.50 ± 0.01ab	0.51 ± 0.01ab	0.55 ± 0.01b
	Succinic acid	NS	1.45 ± 0.19	1.59 ± 0.15	1.50 ± 0.06	1.94 ± 0.04

† NS = not significant; *, **, and *** significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Values (mean of three replicates) followed by different letters, within the same row, show significantly differences ($p < 0.05$) among treatments, according to the Tukey's multiple range test.

3.3. Bioactive Compounds and Total Antioxidant Activity

In the 2016 experiment, OA treatment at 5 and 10 mM led to arils with increased concentrations of total phenolics and total anthocyanins compared to control fruit at harvest time ($p \leq 0.001$). For the total anthocyanin concentration, values at harvest were significantly higher ($p \leq 0.001$) in arils from 10 mM OA-treated fruit than in those from 5 mM OA concentration, without significant differences ($p > 0.05$) among both concentrations of OA on phenolic content (Table 3). This increase on bioactive compounds content was in agreement with results from the 2017 experiment, where OA-treated pomegranate at 10 mM showed a 1.10- and 1.27-fold higher content of total phenolic ($p \leq 0.01$) and total anthocyanins ($p \leq 0.05$) than non-treated fruit, respectively (Table 3). With respect to total antioxidant activity (TAA) in the arils, all OA treatments showed a significant increase in a concentration-dependent manner (Table 3). Therefore, 10 mM OA treatment was the most effective at increasing the total antioxidant activity of pomegranate fruit, and this effect was confirmed in the 2017 experiment, although the significant difference was slightly lower in this season ($p < 0.05$; Table 3). Significant differences were observed for the bioactive compound content and TAA among years (Table 3).

Table 3. Effects of preharvest oxalic acid (OA) treatments (1, 5, and 10 mM) on bioactive compound concentration, total phenolics and total anthocyanins, and total antioxidant activity (TAA; g kg^{-1}) in the first picking date in 2016 and 2017 experiments. Data are the mean ± SE.

Parameter	ANOVA †	Control	OA 1 mM	OA 5 mM	OA 10 mM
2016 Experiment					
Total phenolics	***	0.868 ± 0.016aA	0.852 ± 0.017a	0.958 ± 0.015b	0.993 ± 0.023bA
Total anthocyanins	***	0.136 ± 0.006aB	0.145 ± 0.006a	0.168 ± 0.007b	0.198 ± 0.002cB
TAA	***	2.005 ± 0.059aB	2.276 ± 0.032b	2.238 ± 0.030b	2.566 ± 0.037cB
2017 Experiment					
Parameter	**Student *t* test ¥**	**Control**	**OA 1 mM**	**OA 5 mM**	**OA 10 mM**
Total phenolics	*	0.846 ± 0.017A	-	-	0.934 ± 0.016A
Total anthocyanins	*	0.109 ± 0.004A	-	-	0.138 ± 0.009A
TAA	*	1.750 ± 0.034A	-	-	1.946 ± 0.056A

† NS = not significant; *, **, and *** significant at $p \leq 0.05$, 0.01, and 0.001, respectively; ¥ values (mean of three replicates) followed by different lowercase letters, within the same row, show significantly differences among treatments, according to the Tukey's multiple range test. ¥ NS = not significant; * significant at $p < 0.05$, according to the Student's *t*-test. Different capital letters in the same column show significant differences among years for each treatment at $p < 0.05$.

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4. Discussion

4.1. Fruit Growth, Crop Yield, and Respiration Rate

Pomegranate fruit grows continuously from fruit set until the commercial harvest time, enlarging almost to its final size through cell enlargement. During this process, the sepals form a crown, the dry stamen being inside, and the phenological stage of fruit growth lasts around 90 days [28]. The pattern of fruit growth for the Mollar de Elche cultivar shows a simple sigmoid curve, according to previous findings of Shulman et al. [2]. Results showed that OA treatments increased total crop yield due to an increase in the number of fruits that were harvested from each tree, which had a similar mass, independently of the applied concentration. However, in both experiments, treatments were performed when fruit had reached ca 30% of their final size so that flowering or fruit set were not affected and the increase in fruit number was due to the reduction of fruit abscission that naturally occurs during the fruit developmental cycle due to wind or other environmental factors. In addition, results of the yield on the first harvest date showed that OA treatment hastened the time of harvest in a concentration-dependent manner since the largest number of fruits were harvested in the first harvest, leading also to a greater yield in kg tree^{-1} than non-treated trees.

An early harvest has important implications for marketing Mollar de Elche pomegranate fruit. Harvesting of Mollar de Elche pomegranate fruit is based on fruit skin coloration, and increase in red skin color will allow early harvest. All OA concentrations used in the trials promoted the on-tree ripening process and improved early maturation and early harvest of this cultivar, 10 mM OA being the most effective. This effect could have important implications for the yield value since the first fruit reaching the market have, in general, higher commercial prices [15]. Supermarket prices represent the upper price segment (premium red pomegranates, large size) that involves indirectly more fruit quality. Consumer prices in markets are significantly lower during the production season, so the early harvest could lead to an increase in the economic profit for the farmer.

As far as we know, no literature is available about the effect of preharvest application of OA on pomegranate crop yield, and contradictory results have been obtained when this preharvest treatment was applied on different plant species. For instance, preharvest foliar application of OA at low concentrations (1 and 2 mM) at the flowering stage in strawberry plants led to an increased number of fruits per plant, 1 mM OA being the best treatment to favor vegetative growth and enhance yield [24]. Similarly, Martínez–Esplá et al. [25] reported that preharvest OA treatment at 2 mM increased crop yield (kg per tree^{-1}) in "Black Splendor" and "Royal Rosa" plum trees, although the on-tree ripening process was delayed. In addition, an effect of preharvest OA treatment on increasing yield and fruit weight has been found in sweet cherries [20] and plums [25], and was attributed to an increase in net photosynthesis in OA-treated trees and/or to an increase in the sink force of the growing fruit. In this sense, Wang et al. [29] reported that postharvest OA treatment of jujube fruit increased the abundance of RuBisCO activase, RuBisCO large subunit-binding protein subunit β, and PSII oxygen-evolving complex protein, which could lead to an increase in photosynthesis rate as a consequence of OA treatment. Contradictorily, Martínez–Esplá et al. [23] reported that 2 mM OA treatment did not affect the on-plant artichoke developmental process or plant yield. According to our results, preharvest treatment with OA could be a good tool to increase crop yield and promote early harvest of pomegranate fruit, which could be related with an effect of OA on increasing the abundance of RuBisco activase enzyme. However, metabolomic studies are needed to fill this knowledge gap.

In non-climacteric fruit, such as pomegranate fruit, a decrease in respiration is normally observed along the tree-developmental cycle [30]. Our results suggest that OA reduced the respiration rate at the time of harvest in comparison to untreated fruit. Similar results have been reported in mango [31] and plum [25]. This effect on decreasing the respiration rate at harvest would indicate an effect of OA on reducing the cell metabolism rate on tree, which, in turn, could be attributed to a lower metabolic activity induced by the treatment. The reduction in respiration rate by OA exogenous application

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may be associated with the corresponding reduction in ethylene production through inhibition of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity, according to Razzaq et al. [31].

4.2. Physico-Chemical Quality Parameters: Color, Firmness, Total Soluble Solids, Titratable Acidity, Ripening Index, Individual Sugars and Organic Acids, and Sensory Evaluation

Results indicated that the husk red color increased as a consequence of OA treatments. Thus, preharvest OA treatments led to improved external red coloration of pomegranate fruit, which is an important quality trait for this cultivar in terms of market acceptance and early harvest. As we well know, the red color of pomegranate husk and arils are a consequence of anthocyanin biosynthesis and accumulation in the cells and is directly influenced by the amount and composition of anthocyanins presented. Martínez–Esplá et al. [20] reported an increase of color by OA treatments at 0.5, 1.0, and 2.0 mM applied on trees of "Sweet Heart" and "Sweet Late" sweet cherry cultivars. However, in another study, no significant differences were found in skin color hue at harvest between plums treated in preharvest with 2 mM OA than controls from both picking dates [25]. It has been suggested that OA promoted the accumulation of phenylpropanoid metabolites including phenolics, flavonoids, and lignins [22,32] through the activation of phenylalanine ammonia-lyase (PAL). However, these findings were not consistent with the report of Zheng et al. [33], where no stimulation of the PAL enzyme was observed by the OA treatment.

On the other hand, fruit firmness is one of the most common physical parameters used to assess the progress of fruit softening and ripening. During fruit ripening, modifications in the cell wall occur due to which fruit loses its firmness. Accordingly, Razzaq et al. [31] showed that higher OA concentration (0, 1, 3, and 5 mM assayed) maintained the highest mango fruit firmness compared to the control fruit during ripening. The higher firmness associated with the treatment may be attributed to the decreased polygalacturonase (PG) and pectin methyl esterase (PME) enzymes activities; that is, the retardation of pectin solubilization/degradation, the reinforcement of the wall structure of mesocarp cells, or the reduction of the hydrolysis of cell wall components during on-tree ripening might have occurred in the OA-treated fruit [25,34].

Skin color, fruit firmness, TSS, and TA are indicators of pomegranate fruit quality [10,35]. Our data indicated that the OA treatment increased TSS content and TA without delaying or promoting the RI of pomegranate fruit at harvest for both seasons. Our physico-chemical data and sensory evaluation proved that pomegranate fruit treated with OA at 10 mM had appropriate sensory characteristics and higher overall quality than the control fruit, which would lead to better consumer acceptance and improving fruit market potential. Taking into account all these parameters, OA treatment at 10 mM could be a useful tool to improve pomegranate quality traits at harvest. The significant differences observed among years for physico-chemical and functional parameters could be attributed to horticultural practices or climatic conditions [3,7,8], although the differences among treatments were maintained in both years.

The sugar and organic acid profile are in agreement with previous reports on Mollar de Elche and other sweet pomegranate cultivars [10,11,36]. On the other hand, exogenous OA treatment increased endogenous OA content, with 10 mM OA reaching the highest level, according to Wang et al. [29]. In addition, previous reports have found that pre- or postharvest application of OA resulted in fruit with higher ascorbic acid content at harvest and lower losses of this compound during storage [19,24,31,37–39]. OA has been reported as a natural antioxidant by suppressing lipid peroxidation in vitro in a concentration-dependent manner and reducing the ascorbic acid oxidation [40]. However, further investigations need to address the effects of preharvest OA treatment on ascorbic acid metabolism including the de novo biosynthesis, degradation, and recycling to increase ascorbic acid levels in pomegranate fruit during developmental stages and at harvest.

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4.3. Bioactive Compounds and Total Antioxidant Activity

As we well know, bioactive compounds have antioxidant properties which are responsible for the beneficial health effects attributed to pomegranate fruit consumption [41]. Our results show that preharvest treatments with OA, especially applied at 10 mM, increased the total phenolics and total anthocyanins content, as well as ascorbic acid content, as commented above, leading to increases in the hydrophilic total antioxidant activity (H-TAA). In addition, OA would provide the pomegranate fruit with increased beneficial health effects for human consumption. Previous reports have recently demonstrated that OA treatment enhanced bioactive compounds and increased or preserved antioxidant activity in sweet cherry [18], pomegranate [19], artichoke [23], plum [25], mango [31], muskmelon [32], and grape [42].

The mechanism by which OA increased the bioactive compounds and antioxidant properties is not well understood [31] and deserves further research, although OA has been reported as a natural available antioxidant through suppressing lipid peroxidation in vitro and reducing ascorbic acid oxidation [40]. Results reported by Zhu et al. showed for the first time that preharvest application of OA by spraying on kiwifruit plants increased the postharvest fruit quality and induced fruit disease resistance against *P. expansum* [22]. These effects were attributed to increased activity of defense-related enzymes and resistance-related substance concentrations. In the present study, preharvest OA treatment led to fruit with higher ascorbic acid content at harvest and during postharvest storage, which could reduce patulin production, resulting in a partial degradation of this mycotoxin produced by *P. expansum*. Similarly, preharvest OA treatment efficiently improved the functional properties of sweet cherries [20]. As a postharvest treatment, results suggested that priming defense responses are involved in the suppressed postharvest decay on muskmelon OA-treated fruit [32] and in pear and mango fruit inoculated with fungal pathogens [16,33,43,44]. It was also reported by these authors that the effect of OA treatment reducing decay incidence could be associated with the enhancement of defense-related enzyme activities, accumulation of phenolic compounds, and delay of fruit ripening [16,32,33,43,44]. However, for better understanding of the possible mode of action, molecular, metabolomic, or proteomic studies are needed.

5. Conclusions

Preharvest treatments with OA at 1, 5, and 10 mM increased crop yield. In addition, the on-tree fruit ripening process was accelerated in a concentration-dependent manner and 10 mM OA led to the early harvest of Mollar de Elche cultivar, the production and number of fruits being higher in the first harvest date. Among the assayed concentrations, the highest effects were found with OA at 10 mM which could be selected for practical application purposes in order to get earlier harvest, increase pomegranate crop yield, and improve fruit quality. In addition, OA application increased the bioactive compound content, which could provide fruit with health beneficial effects to consumers. Moreover, those fruits treated with OA at 10 mM showed higher sensory quality properties which could promote their marketability in the national and international markets. However, more studies are needed in order to elucidate the effect of OA application on the flowering biology of trees that could affect yield over the year.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/10/1522/s1, Figure S1: Photography data of one replicate (n = 5 fruit) of different treatments; control and oxalic acid (OA) at 1, 5 and 10 mM concentrations in the 2016 experiment.

Author Contributions: P.J.Z., M.S. and D.V. conceived and designed the work in association with other authors. S.C., J.M.V., F.G. and D.M.-R. performed the field treatments. M.E.G.-P. performed most of the analytical determination in collaboration with M.J.G. Finally, P.J.Z. 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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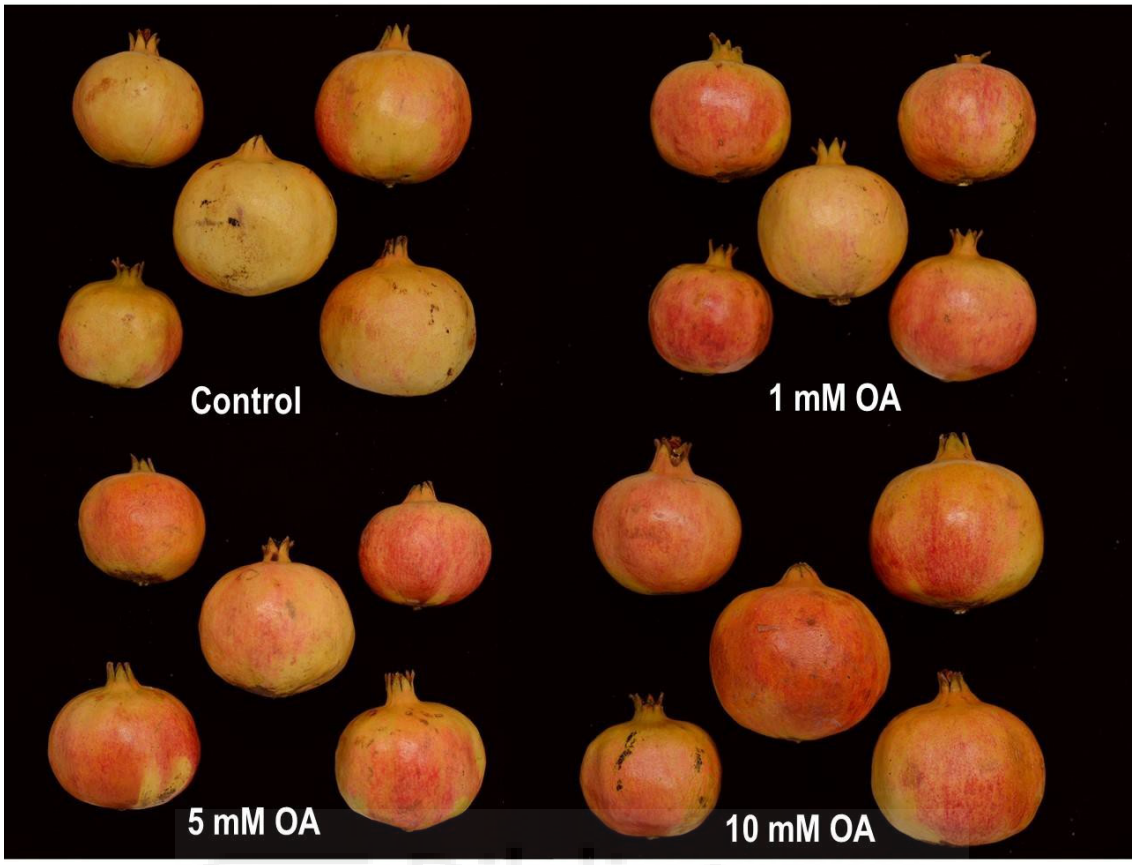

Figure S1. Photography data of one replicate (5 fruits) of different treatments; control and oxalic acid (OA) at 1, 5 and 10 mM concentrations in the 2016 experiment.

4.8. Publication 8

PUBLICATION 8 (Open access)

Oxalic acid preharvest treatment improves colour and quality of seedless table grape 'Magenta' upregulating on-vine ABA metabolism and relative *Vv*NCED1 gene expression and the antioxidant system in berries

María E. García-Pastor, María J. Giménez, Vicente Serna-Escolano, Fabián Guillén, Daniel Valero, María Serrano, Santiago García Martínez, Leon A. Terry, M. Carmen Alamar, Pedro J. Zapata

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Oxalic acid preharvest treatment improves colour and quality of seedless table grape 'Magenta' upregulating on-vine ABA metabolism and relative *Vv*NCED1 gene expression and the antioxidant system in berries

María E. García-Pastor[1], María J. Giménez[1], Vicente Serna-Escolano[1], Fabián Guillén[1], Daniel Valero[1], María Serrano[2], Santiago García-Martínez[2], Leon A. Terry[3], M. Carmen Alamar[3], Pedro J. Zapata[1*]

[1]Department of Food Technology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Orihuela, Alicante, Spain

[2]Department of Applied Biology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Orihuela, Alicante, Spain

[3]Plant Science Laboratory, Cranfield University, Bedfordshire MK43 0AL, UK

*** Correspondence:**
Pedro J. Zapata
pedrojzapata@umh.es

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Abstract

The effect of oxalic acid (OA) in determining poorly coloured table grape quality remains relatively unknown. Some red cultivars, such as seedless table grape 'Magenta', are characterized by a poor berry colour, an attribute highly demanded by the consumer. The aim of this research was to elucidate the effect of a preharvest OA treatment (5 mM) on table grape berry colour and quality by investigating its role on berry development, on-vine ripening, and postharvest senescence. We found that OA significantly increased abscisic acid (ABA) and ABA glucose ester (ABA-GE) content in treated berries. This increase was mediated by changes in the ABA biosynthetic pathway, specifically by the upregulation of *Vv*NCED1 (9-*cis*-epoxycarotenoid dioxygenase) gene. The accumulation of ABA in treated berries resulted in a colour improvement and onto higher individual and total anthocyanins content at harvest compared to control; whilst at harvest, OA treated table grapes showed a significantly lower glucose and fructose content, higher content of tartaric, ascorbic and succinic acids. Furthermore, antioxidant enzyme activity was increased during berry development in OA treated berries. On the other hand, those berries treated with OA showed a delay in loss of firmness and colour during cold storage, as well as less susceptibility to postharvest decay incidence. This effect of OA delaying the senescence process was also related to enzymatic antioxidant system stimulation. For the first time, the role of OA on increasing quality, mainly colour, in table grape was elucidated highlighting that this treatment upregulated the ABA metabolism, relative *Vv*NCED1 gene expression and antioxidant system, delaying postharvest berry senescence.

1 Introduction

Oxalic acid (OA) is a natural compound which can induce systemic resistance against fungal, bacterial and viral diseases, through an increase in defence-related enzyme activities and secondary metabolites (Tian et al., 2006; Zheng et al., 2012). Both pre- and postharvest OA treatments for extending shelf-

life and maintaining quality of postharvest fruits and vegetables have been investigated and developed for commercial use (Razavi and Hajilou, 2016). Recently, the application of natural and eco-friendly compounds as preharvest treatments has received considerable attention (Martínez-Esplá et al., 2017). In those studies, a delay on ripening and senescence as well as a preservation of fruit and vegetable quality were observed. OA has demonstrated an important role in delaying senescence in harvested fruits (Wu et al., 2011) and its effect has been recently reported for sweet cherry (Martínez-Esplá et al., 2014), peach (Razavi and Hajilou, 2016), kiwifruit (Zhu et al., 2016), plum (Martínez-Esplá et al., 2019) and pomegranate fruit (García-Pastor et al., 2020a). However, there is a paucity of research about the effect of OA pre- (Kok and Bal, 2019) and postharvest (Sabir and Sabir, 2017; Hazarika and Marak, 2021) applications on table grape quality.

Grape berries are non-climacteric fruits displaying a double sigmoidal growth curve in which the ripening onset is considered as veraison time (Castellarin et al., 2007). Veraison can be identified as the beginning of grape ripening, which is followed by berry softening and increases on skin colour and sugar content. Both, ethylene and abscisic acid (ABA), as well as their crosstalk, are likely to be required to initiate berry ripening (Sun et al., 2010). Thus, many studies have shown that ABA concentration increased at the onset of ripening (Wheeler et al., 2009; Sun et al., 2010; Pilati et al., 2017), and trace endogenous ethylene induced the expression of *Vv*NCED1 gene, which is involved in ABA biosynthesis (Sun et al., 2010). 9-*cis*-epoxycarotenoid dioxygenase (NCED) is the enzyme catalysing the first step of this reaction which produces xanthoxin, the direct C15 precursor of ABA, from the cleavage of 9-*cis*-violaxanthin or 9-*cis*-neoxanthin. Grapevine NCED genes (*Vv*NCED1, *Vv*NCED2 and *Vv*NCED3) encode for enzymes that cleave carotenoids to form the phytohormone ABA. NCED genes have been proposed to be implicated on ABA signaling during ripening initiation in grape berries (Young et al., 2012). Nevertheless, a lack of correlation between free-ABA and NCED codifying genes in table grape berries suggests that compounds derived from the ABA catabolism/conjugation could also be involved in berry ripening (Wheeler et al., 2009; Castellarin et al., 2016).

Skin colour is a key quality attribute for table grapes. Consumers demand high and homogeneously coloured grapes, which in turn reach premium prices at market and result in higher returns for growers (Peppi et al., 2006). Nevertheless, some red cultivars, such as the seedless table grape 'Magenta', produced through stenospermocarpy mechanism which leads parthenocarpy in the grapes, are characterized by a poor berry colour and a non-uniform colour along clusters, which depreciates their commercial value (Peppi et al., 2006; García-Pastor et al., 2019; 2020b). Abscisic acid (ABA) and ethephon (2-chloroethylphosphonic acid - an ethylene-releasing compound) have been used are commercially used in viticulture to improve colour homogeneity (Peppi et al., 2006; Alenazi et al., 2019; Koyama et al., 2019). However, the effects of ethephon on colour are inconsistent and can lead to berry softening (Peppi et al., 2006) and reduced shelf-life (Szyjewicz et al., 1984). In addition, the application time and concentration of ABA is a critical factor for the effective improvement of grape skin colour, which may vary depending on cultivar and area of application. On the other hand, the high cost of ABA has precluded the development of practical applications (Peppi et al., 2006). Pigment accumulation can be influenced by sugar and hormonal crosstalk (Li et al., 2016). A combined ABA (400 µL L^{-1}) and sucrose (90 µL L^{-1}) treatment applied close to veraison in 'Crimson Seedless' grapes was more effective in accelerating the pigmentation process by significantly increasing anthocyanin levels than when ABA or sucrose treatments were applied independently (Olivares et al., 2017). Unfortunately, these treatments also induced berry softening, which is an undesirable attribute for fresh grapes because softer berries are more susceptible to grey mould caused by *Botrytis cinerea*. Recently, our previous works with some natural and eco-friendly compounds, such as salicylic acid (SA), acetyl salicylic acid (ASA), methyl salicylate (MeSa), and methyl jasmonate (MeJa), applied at preharvest on

'Magenta' and 'Crimson' table grapes improved berry colour and induced fruit resistance against grey mould (García-Pastor et al., 2019; García-Pastor et al., 2020b; 2020c). Thus, the aim of the present study was to assess the potential effect of OA preharvest treatment, applied at three key points of table grape development, on quality parameters, including berry colour, of 'Magenta' poorly coloured table grape at harvest and during postharvest cold storage. This effect will be elucidated based on a metabolomic (ABA, ABA catabolites and antioxidant enzymes) approach and throughout the study of the relative *Vv*NCED1 gene expression about the action or role of OA during berry development, on-vine ripening and postharvest senescence.

2 Materials and Methods

2.1 Preharvest and postharvest experimental design

This study was performed in 2018-growing season with 'Magenta' seedless table grape (*Vitis vinifera* L.) cultivar in a commercial vineyard in Calasparra (Murcia, Spain). Vines were 8-years old, planted in a sandy soil, at 2.5 x 3 m spacing, and grafted onto Paulsen 1103 rootstocks. A programmed irrigation system consisting of a drip irrigation line per row with three emitters per plant was used; vine water requirements and fertilisers along the growth cycle were supplied through this system. Pruning and thinning were carried out according to standard procedures for table grape crop and vines received no fungicide treatment. Oxalic acid (OA; Sigma-Aldrich, Madrid, Spain, CAS No. 144-62-7) treatments were performed by foliar spray application (1-L per vine at 5 mM, containing 0.5 % Tween 20 as surfactant) onto the whole vine canopy, including leaves and clusters. The sprays were carried out with a 15-L backpack sprayer until runoff. This concentration was chosen as the optimum among three concentrations tested (1, 5 and 10 mM) in previous experiments (growing seasons 2016 and 2017). A 5 mM OA treatment was the best in terms of yield, berry maturity-quality and bioactive compounds (data not shown). Control vines were treated with an aqueous solution of 0.5 % Tween 20. A completely randomized block design with five replicates of three vines (15 vines) for each treatment was followed. Treatments were applied three times on the same vines, previously labelled with the type of treatment, during the fruit growth and ripening cycle (viz. T1 on June 22nd: before the onset of veraison, when berry volume was ca. 40 % of its final one (≅ 2100 mm^3), T2 on July 10th: at veraison stage, and T3 on July 26th: 3 days before the first harvest date). Treatments were performed during favourable weather conditions where rainfall or winds were not forecasted for the following 24 h. From those 5 replicates, fifteen berries (5 berries per vine) were taken from each replicate (75 berries) and treatment at 3 days after each treatment (3DAT1, 3DAT2 and 3DAT3). Relative 9-*cis*-epoxycarotenoid dioxygenase (*Vv*NCED1) gene expression, ABA metabolic profile and content and antioxidant enzyme activity in the three different key stages of berry growth and ripening cycle were analysed.

Fifteen homogeneous clusters (5 clusters per vine) were harvested from each replicate (5 replicates; 75 clusters) and treatment at commercial ripening stage according to the characteristic size, colour and total soluble solid content of this cultivar (160-180 g kg^{-1}) at harvest or 3DAT3 stage. The harvest date was the same for the different treatments (3DAT3; on July 29th). These harvested clusters were immediately transported to the laboratory where a first set of twenty-five clusters per treatment (5 clusters per replicate) were used to determine the bioactive compound content, total antioxidant activity, individual sugars, and organic acids concentration. Another set of clusters were stored at 2 ºC and 90 % of relative humidity (RH) for 0, 15, 30 and 45 days (10 clusters per sampling date; 40 clusters per treatment). Firmness, colour, ABA and catabolite content and antioxidant enzyme activity evolution during postharvest storage were determined. In order to test the effect of OA preharvest

application on the postharvest decay caused by *Botrytis cinerea*, berries were wounded at harvest with a sterile lancet (6 mm in depth) and inoculated according to García-Pastor et al. (2020c), using one set of 120 berries from two clusters of each of the five replicates (10 clusters per treatment). Disease incidence or severity was assessed after 5 days at 25 ± 1 ºC and 80-85 % of RH.

2.2 Relative *Vv*NCED1 gene expression

RNA was extracted from 0.1 g of freeze-dried table grapes, using the whole fruit (flesh + skin tissues), according to the protocol described by Le Provost et al. (2007) with slightly modifications. Briefly, freeze-dried samples were manually grounded with liquid nitrogen and mixed with 1 mL of extraction buffer (2.5 % (w/v) CTAB, 2 % (w/v) polyvinylpyrrolidone or PVP K-40, 1.0 M TRIS-HCl at pH = 8.0, 0.5 M EDTA, 5.0 M NaCl) containing β-mercaptoethanol at 2 %, previously heated to 65 ºC, and vortexed vigorously during 15 s. The mixture was incubated 10 min in a water bath at 65 ºC and mixed by inverting tubes every 3 min. For purification, chloroform-isoamyl-alcohol (24:1) was added and mixed as the same way. Then, the mixture was centrifugated at 10,000 x *g* for 10 min at room temperature. Finally, the supernatant was transferred to a clean Eppendorf tube and mixed, inverted and incubated overnight at -20 ºC with 1/3 volume of LiCl 10 M. Next day, the solution was centrifuged at 10,000 x *g* for 10 min at 4 ºC and the supernatant was discarded. The pellet was washed with 500 µL of cold (-20 ºC) 80 % ethanol and centrifuged at 10,000 x *g* for 5 min at 4 ºC. The supernatant was removed, and when the pellet was dried out, it was re-suspended in 100 µL of RNAse free-water and storage at -80 ºC.

According to the manufacturer's recommendations, A DNase treatment was done by using Baseline-ZERO DNase (Epicentre/Lucigen USA) on the eluted RNA. RNA quantification was carried out by the spectrophotometric absorbance using a NanoDrop 2000 and a Qubit 2.0 Fluorometer (ThermoFisher Scientific, USA). The expression analysis of *Vv*NCED1 gene was carried out by GenXPro GmbH (Germany). Total RNA (15 - 40 ng per reaction) was used as template for the OneStep qPCR reactions. Reverse transcription and qRT-PCR was performed using the MDX025 Low LOD 1-Step qPCR Mix (Meridian/Bioline, USA/Germany), according to the manufacturer's recommendations. All reactions were made in a volume of 12 µL including 6.0 µL Low LOD 1-Step RT-qPCR Reaction Mix (2x), 0.15 µL EvaGreen from Jena Bioscience [100 µM], 0.20 µL Primer (fwd and rev dilution of 10 µM each in 0.5x TE buffer) and 0.12 µL MMLV-RT (100x – from MDX025 Low LOD 1-Step qPCR Mix). RNA from 5 biological replicates and treatments was used as the template for the qPCR reactions.

Vitis vinifera 9-*cis*-epoxycarotenoid dioxygenase 1 gene (*Vv*NCED1; LOC100232942) was amplified using the described primers in Table 1 (Rattanakon et al., 2016). qRT-PCR was performed on a StepOne Thermocycler system (Applied biosystems, Thermo-Fisher). Thermocycler parameters were 10 min at 50 °C as RT-Step, followed by 2 min at 95 °C for Taq polymerase activation, then 36 cycles of 5 s at 95 °C were programmed for denaturation and 30 s at 62 °C for annealing and extension according to Serna-Escolano et al. (2021). Additionally, the quality of amplicons was controlled by a Melt Curve Analysis step showing no side products as can be seen in Table 1. Expression of *Vv*NCED1 gene was normalized with three endogenous control genes; *Vitis vinifera* actin-7 (ACT; LOC100232866), *Vitis vinifera* ubiquitin-60S ribosomal protein L40-2 (UBI; LOC100253716) and *Vitis vinifera* glyceraldehyde-3-phosphate dehydrogenase cytosolic (GAPDH; LOC100233024), on normalization of genes in table grapes (Reid et al., 2006; Pagliarani et al., 2017), as can also be seen in Table 1. Relative *Vv*NCED1 gene expression in treated fruit was calculated with respect to control fruit, using 5 biological replicates.

2.3 Abscisic acid (ABA) and catabolite analyses

Freeze-dried powdered table grape material of whole berry (flesh + skin tissues) was weighed (5.0 ± 0.1 mg) and extracted with 500 µL of precooled (-20 ºC) methanol:water:formic acid (60:35:5 v/v/v), as described by Müller and Munné-Bosch (2011) with some modifications. The labelled forms of the compounds; (-)-5,8´8´8´-d4-abscisic acid (d4-ABA); (+)-4,5,8´,8´,8´-d5-abscisic acid glucose ester (d5-ABA-GE); (±)-5,8´,8´,8´-d4-7´-hydroxy-ABA (d4-OH-ABA); (-)-7´,7´,7´-d3-phaseic acid (d3-PA) and (-)-7´,7´,7´-d3-dihydrophaseic acid (d3-DPA) were added to the mixture as internal standards. ABA and catabolite content were quantified according to Morris et al. (2018) with slight modifications, by using a LC/MS-MS instrument with an Agilent 1200 series HPLC system (Agilent, Berks., UK) coupled to a Q-Trap 6500 mass spectrometer (AB Sciex, Framingham, USA). The extracts were analysed by injecting 20 µL onto a Phenomenex 3 µm C18 Luna 100 x 2 mm with guard column at 40 ºC. The mobile phases were: (A) 2 % acetonitrile in 2 mM ammonium formate, and (B) 95 % acetonitrile in water with 0.1 % formic acid, using an increasing gradient of B (2 % for 4 min, 16 % at 20 min and 34.5 % at 25 min) at a flow rate of 200 µL min^{-1}. Deuterated and non-deuterated ABA metabolites: (-)-DPA, (+)-ABA-GE, (-)-PA and (±)-7´-hydroxy-ABA were obtained from the National Research Council of Canada-Plant Biotechnology Institute (Saskatoon, Canada); and (±)-ABA was purchased from Sigma-Aldrich (Darmstadt, Germany). A 10-point calibration curve ranging from 0.5 to 3,000 µg L^{-1} was used for quantification. Phytohormone concentration was expressed in nmol or pmol g^{-1} dry weight (DW) and was the mean ± SE of 5 replicates.

2.4 Antioxidant enzymes activity

Ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) enzyme activities were measured in the whole berry extracts (flesh + skin tissues) obtained by homogenizing 1 g of frozen tissue with 5 mL of phosphate buffer 50 mM, pH 6.8, containing 1 % (w/v) of polyvinylpyrrolidone (PVP) and ethylenediamine-tetraacetic acid 1 mM. After centrifugation at 10,000 x *g* for 30 min at 4 °C, the supernatant was used for quantification of each replicate in duplicate as reported elsewhere (García-Pastor et al., 2020c). Antioxidant enzyme activities were expressed as units of enzymatic activity (U min^{-1} g^{-1}) of fresh weight (FW) with one enzymatic unit (U) being defined as a 0.01 decrease of ascorbate at 290 and 240 nm min^{-1} for APX and CAT, respectively, and a 0.01 increase of absorbance at 470 nm min^{-1} for POD. Results were the mean ± SE of 5 replicates.

2.5 Bioactive compound content, total antioxidant activity and berry quality parameters at harvest

From the twenty-five clusters (5 clusters per replicate) per treatment, berries from ten clusters (2 clusters per replicate) were peeled to separate the skin from the flesh and those from the others ten replicates were not peeled. Both sample types (whole berry and skin tissue) were frozen in liquid nitrogen, ground and kept at −80 °C until bioactive compound evaluation and total antioxidant activity analysis, which were carried out manually ground in a mortar and pestle. Ten berries were ground from each replicate (5 bunches; 50 berries) and treatment to obtain a homogeneous juice sample for the quantification of total soluble solids (TSS), total acidity (TA), individual sugars and organic acids. All results were the mean ± SE of 5 replicates at harvest.

Anthocyanins were extracted from 10 and 1 g of frozen berry and skin tissue with 15 and 5 mL of methanol: formic acid: water (25:1:24, v/v/v), respectively. Then, samples were sonicated in an ultrasonic bath for 60 min and centrifuged at 10,000 x *g* for 15 min. Total anthocyanin concentration

was measured and expressed as García-Pastor et al. (2020b). The supernatant was filtered through a 0.45 µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and used for individual anthocyanin quantification by injecting 20 µL of extracts into a high-performance liquid chromatography (HPLC) system (Agilent HPLC1200 Infinity series, Agilent Technologies Inc., Waldbronn, Germany), as previously reported (García-Pastor et al., 2020b). Total and individual anthocyanins results were expressed in mg 100 g^{-1} FW.

Total phenolics were extracted by using 5 and 1 g of frozen berry and skin tissue with 10 and 5 mL of water:methanol (2:8) containing 2 mM NaF (to inactivate polyphenol oxidase activity and prevent phenolic degradation), respectively, and then, phenolics were quantified in the supernatant using the Folin-Ciocalteu reagent, as previously reported (García-Pastor et al., 2020b). Results were expressed as mg gallic acid equivalent 100 g^{-1} FW.

To measure total antioxidant activity (TAA), 5 and 1 g of frozen berry and skin tissue were homogenized with 5 mL of 50 mM phosphate buffer pH = 7.8 and 5 mL of ethyl acetate. As previously described by García-Pastor et al. (2020a), hydrophilic (H-TAA) and lipophilic (L-TAA) TAA were determined and measured in duplicate in each extract using a reaction mixture in which $ABTS^{+}$ radicals are generated and monitored at 730 nm. Results were expressed as mg of Trolox Equivalent (TE) 100 g^{-1} FW.

Total soluble solids (TSS) content was determined in duplicate with a digital refractometer Atago PR-101 (Atago Co. Ltd., Tokyo, Japan) at 20 °C, and expressed as g 100 g^{-1}. Total acidity (TA) was determined also in duplicated in the same juice by automatic titration (785 DMP Titrino, Metrohm) with 0.1 N NaOH up to pH 8.1 and results were expressed as g tartaric acid equivalent 100 g^{-1} FW. The homogeneous juice was centrifuged at 10,000 x *g* for 10 min and the supernatant was filtered through a 0.45 µm Millipore filter and then injected into a HPLC system (Hewlett-Packard HPLC series 1100) to quantify individual sugars and organic acids, according to García-Pastor et al. (2020c). Individual sugars were detected by refractive index detector and organic acids by absorbance at 210 nm. Results were expressed as g 100 g^{-1} FW. For quantification, a standard curve of pure sugars and organic acids purchased from Sigma-Aldrich (Poole, UK) was used.

2.6 Quality parameters and visual decay incidence during storage

Table grapes firmness and colour were measured at harvest and during postharvest storage. Firmness (N mm^{-1}) was measured in 50 berries (10 berries per replicate) per treatment as the force that achieved a 5 % deformation of the berry diameter, using a Texture Analyzer (TX-XT2i, Stable Microsystems, Godalming, Surrey, UK). Colour parameters (viz. L*, a* b*, Hue angle [arctan b/a]) were measured individually in another 50 berries from each treatment using a Minolta colorimeter (CRC200, Minolta Camera Co., Osaka, Japan).

Decay incidence was evaluated 5 days after being inoculated by *Botrytis cinerea* and considered spoiled based on a visual scale of six hedonic points named as stages: S0, S1, S2, S3, S4 and S5, according to García-Pastor et al. (2020c), where: S0, without damage; S1, wound browning; S2, microbial growth covering 1-2 mm of wound; S3, microbial growth covering 3-4 mm of wound; S4, microbial growth covering 4-5 mm of wound and even showing mycelial growth; S5, all the wound covered (6 mm) with the fungus and mycelium was observed. Results were expressed as percentage of spoiled grapes in each stage based on the total number of fruits per box (mean ± SE of 5 replicates). Mean values ± SE per replicate were used for further statistical analysis.

2.7 Statistical analysis

A Student's t-test ($p < 0.05$) was performed to detect significant differences between control and treatment (5 mM OA) samples at each given point during pre- and postharvest stages. One-way analysis of variance (ANOVA) was used to determine the significance of mean differences among the three key developmental stages in preharvest (3DAT1, 3DAT2 and 3DAT3), postharvest storage time (0, 15, 30 and 45 days) and decay stages (S0 to S5). HSD Duncan's test was further used to examine if these differences were significant at $p < 0.05$. All statistical analyses were performed with SPSS software package v. 17.0 for Windows.

3 Results

3.1 Effect of oxalic acid preharvest treatment on ABA, relative *Vv*NCED1 gene expression, ABA catabolites and antioxidant enzyme activities during berry development

Free ABA content peaked at veraison stage (3DAT2) and decreased thereafter in control table grapes (Figure 1A). OA preharvest application caused a significant 2.7-fold increase in ABA content in treated berries at 3DAT1 stage compared to control berries. Significant differences on ABA content between both treatments were also observed at 3DAT2 stage yet to a lesser extent (Figure 1A). The relative expression level of *Vv*NCED1 was significantly up-regulated by OA at 3DAT1 and 3DAT2 stages, but especially in the last one (veraison stage) with values 7-fold higher than in non-treated table grapes (Figure 1B), where molecular data did not proportionally support the biochemical data.

In relation to changes in ABA metabolism, the inactive glucose ester (ABA-GE) and three catabolites (7-OH-ABA, PA and DPA) showed a significantly decreasing trend along the tree growth and ripening stages in both control and OA treated berries, except for PA where no significant changes occurred in the last two stages (Figures 1C, D, E and F). Endogenous ABA-GE content was 49 and 18 % higher in OA treated table grapes than in control grapes at 3DAT1 and 3DAT2 stages, respectively (Figure 1C). 7-OH-ABA was the predominant ABA catabolite in grape berries (Figure 1D), followed by PA and DPA which were considerably lower (Figures 1E and F). PA content in OA treated table grapes was 1.5, 1.7 and 1.8-fold higher than in non-treated berries at 3DAT1, 3DAT2 and 3DAT3, respectively (Figure 1E).

The antioxidant enzyme activity evolution during table grape development was dependent on the targeted enzyme (Figure 2). Thus, APX activity increased from 3DAT1 to 3DAT2 in control berries and then showed a stable behaviour. However, APX activity increased until harvest date in OA treated berries. The greatest effect of OA treatment on stimulating APX enzyme was observed at 3DAT3 stage, its activity being 2.5-fold higher than non-treated berries (Figure 2A). Enzymatic activity of CAT was maintained during fruit development in non-treated berries, but an enzymatic peak of 1.4-fold higher activity was observed at 3DAT2 or veraison stage in OA treated table grapes (Figure 2B). Table grapes treated with OA showed 2-fold increase on POD activity than in non-treated berries (Figure 2C).

3.2 Effect of oxalic acid preharvest treatment on bioactive compound content, total antioxidant activity and berry quality parameters at harvest

Berries treated with OA showed a significantly higher content of total anthocyanins and total phenols than non-treated berries at harvest (Table 2). Specifically, those treated berries presented ca. 2-fold higher content of total anthocyanins in the whole fruit and skin tissue compared with control berries. In addition, both H-TAA and L-TAA significantly increased with OA treatment (Table 2).

Individual anthocyanin content was also significantly higher in OA treated table grapes in both tissues, except delphinidin 3-glucoside content which only increased in the skin tissue (Table 2). All the functional parameters were significantly higher in the skin tissue rather than in the whole fruit (flesh + skin). The major anthocyanins of 'Magenta' table grapes were malvidin 3-glucoside and peonidin 3-glucoside in the same proportion, whose contents in the whole fruit and skin tissue were 1.6 and 1.7-fold higher in OA treated table grapes than in control ones, respectively.

TSS and TA showed significant differences between both treatments at harvest (Table 2). OA treated berries had 0.94-fold lower TSS and 1.10-fold higher TA than untreated grapes. Glucose and fructose content was significantly lower (by 6 %) in OA treated table grapes (Table 2), whilst those grapes had a significantly higher content of tartaric (by 13 %), ascorbic (by 5 %) and succinic acid (by 6.5 %) than non-treated berries (Table 2).

3.3 Effect of oxalic acid preharvest treatment on quality parameters, berry decay, ABA and catabolite content and antioxidant enzyme activity during postharvest storage

Table grapes treated with OA were 1.1-fold firmer than controls at harvest and differences between both treatments were maintained during cold storage (Figure 3A). Berry colour was also increased in OA treated table grapes, since significantly lower values of hue angle were observed in OA-treated berries than in control ones at harvest and during cold storage. Hue angle values were 1.3-fold lower for OA treated berries than control ones at the end of postharvest storage (Figure 3B), showing that treated berries had a deeper purple colour than untreated berries.

OA preharvest treatment significantly reduced grey mould disease incidence in treated table grapes (Figure 3C). Thus, the percentage of berries without decay damage (S0) or with mild symptoms (S1, S2 and S3) was significantly higher in those berries treated with OA and these treated berries showed a lower percentage of berries showing severe incidence (S4 and S5) than controls (Figure 4C).

OA treated berries showed a significant ca. 0.6 to 0.7-fold lower ABA content than control berries during 45 days of storage at 2 ºC (Figure 4 and Supplementary Table 1). Treated berries showed a significant 33, 23 and 58 % lower ABA-GE content at the three sampling points, respectively, compared with non-treated berries (Figure 4 and Supplementary Table 1).

APX activity significantly increased by ca. 1.6-2-fold during postharvest storage by OA treatment (Figure 5A). The mean of CAT enzymatic activity from control berries was 57.21 ± 3.02 U min^{-1} g^{-1}, while for OA treated berries was significantly higher, reaching an average of 96.14 ± 2.59 U min^{-1} g^{-1} (Figure 5B). POD enzyme showed an activity increase of 28, 11 and 13 % at 15, 30 and 45 days, respectively, in OA treated table grapes compared to controls (Figure 5C).

4 Discussion

4.1 Oxalic acid preharvest treatment upregulates ABA metabolism, relative *Vv*NCED1 gene expression and antioxidant enzyme activity during berry development

ABA plays a key role in regulating a number of genes at the onset of veraison, including those involved in both anthocyanin signalling and biosynthesis pathway (Gambetta et al., 2010). Skin anthocyanin biosynthesis and accumulation starts from veraison, as the onset of ripening, and continues until harvest (Costantini et al., 2015). The ABA accumulation was also accompanied by sugar accumulation and berry softening, which supports its role controlling this hormone several ripening-associated processes in grapes (Wheeler et al., 2009; Fortes et al., 2015; Pilati et al., 2017). These processes are mainly

under genetic control. Gene expression and activation of the biosynthetic enzymes are also influenced by climatic conditions and cultural practices, including the use of exogenous plant growth regulators (PGRs) (Yamamoto et al., 2015; Basile et al., 2018). The endogenous free ABA content is determined by the dynamic balance between biosynthesis and catabolism/conjugation. In our study, free ABA content of non-treated 'Magenta' seedless table grapes increased at 3DAT2 or veraison stage following a decrease (Figure 1A), as reported by Torres et al. (2018). The OA treatment application significantly increased the ABA content at 3DAT1 stage compared to control grapes (Figure 1A), whilst its effect was maintained at 3DTA2 or veraison stage, but to a lesser extent (Figure 1A). These biochemical changes may be supported by the up-regulation of *Vv*NCED1 gene by OA treatment at 3DAT1 and 3DAT2 stages (Figure 1B). However, the 2.7-fold increase of ABA content in OA treated berries at 3DAT1 showed a lack of correlation with the 2-fold up-regulation of *Vv*NCED1 expression and the ABA-GE or other catabolites content at this developmental stage. In contrast to the results obtained at 3DAT1, the relative *Vv*NCED1 gene expression showed a concomitant increment (≈ 7-fold increase) in OA treated berries at the onset of ripening or 3DAT2 stage (Figure 1B). Thus, we suggest that the greatest ABA content in the OA treated grapes at 3DAT1 (Figure 1A) began prior to the highest up-regulation of endogenous ABA biosynthesis observed at 3DAT2 and mediated by the relative expression of the rate-limiting *Vv*NCED1 gene (Figure 1B). In this sense, this increment on ABA levels could be supported by an ABA-translocation from leaves to berries, as a physiological plant response to OA preharvest treatment.

Table grapes treated with OA 5 mM showed a significantly higher content of ABA-GE at 3DAT1 than non-treated berries (Figures 1C). ABA and ABA-GE were the dominant compounds in the ABA metabolic pool and our results revealed that their concentration was significantly increased by OA 5 mM, particularly at 3DAT1 and 3DAT2 (Figures 1A and C). This suggests that the initial increases at 3DAT1 on ABA content (Figure 1A) by OA treatment may largely result from the increase mediated by ABA biosynthesis (Figure 1B). However, ABA concentrations reached near peak levels prior to the highest up-regulation of the *Vv*NCED1 gene observed at 3DAT2 or at veraison stage, being also possible an ABA translocation from other parts of vine as was previously reported by others authors (Shiozaki et al., 1999; Keller et al., 2015). Specifically, Shiozaki et al. (1999) suggested that the transport of ABA from the leaves via phloem contributes to its increase in the grape berry during ripening. These authors reported that ABA accumulation in the grape berry during ripening was inhibited by vine defoliation at the veraison stage. On the contrary, free ABA in the phloem was not exported from berry to leaves. Moreover, demand-driven rise in phloem inflow at the beginning of table grape ripening was necessary and sufficient to reverse a stress-induced berry shrinkage and enable sugar accumulation and berry growth under drought stress conditions (Keller et al., 2015). OA treatment was applied by foliar spray on vine, and maybe the *Vv*NCED1 gene expression of leaves could be also stimulated, leading to increases in ABA content at 3DAT1 which could be transported via the phloem to the berries as exogenous import (Castellarin et al., 2016). Applications with ABA caused an increase in ABA content in both leaves and berries of 'Malbec' grapevine plants (Murcia et al., 2017), down-regulating the relative expression level of *Vv*NCED1 in ABA treated leaves, likely due to an ABA homeostasis. In this sense, Castellarin et al. (2016) have reported the increases in ABA occurred earlier in 'Zinfandel' grapes during their softening and prior to sharp increases in sugars and anthocyanins. In addition, these authors also observed an absence of significant expression of the *V. vinifera* 9-cis-epoxycarotenoid dioxygenases during the accumulation in ABA content. However, these increases were coincident with decline in the DPA catabolite, indicating that initial increases in ABA may result from lower catabolism and/or exogenous import. In relation to colour development,

previous studies (Castellarin et al., 2016) showed that a disruption of phloem transport to berry clusters (induced through girdling), prior to the onset of ripening, completely inhibited colour development.

On the other hand, the 7-fold increase on relative *Vv*NCED1 gene expression of OA treated berries at 3DAT2 stage (Figure 1B) was not proportionally correlated with the 10 % increase on ABA content at this developmental stage. Thus, values of free ABA at 3DAT2 developmental stage could be regulated by different metabolic pathways: 1) higher conjugation of ABA with glucose in treated berries, increasing ABA-GE content which acts as a reservoir of ABA (Figure 1C); and 2) higher ABA catabolism at the position 8′, leading to a higher PA content, which is biologically active and serves as a bona fide ABA-like phytohormone. However, very similar levels of ABA-GE and ABA catabolites were observed at 3DAT2 between both treatments. Thus, although there is no available information reporting ABA translocation from table grape berries to other parts of the vine, including the leaves, it is highly probable according to the reported metabolomic and molecular data that OA preharvest treatment leads to a dynamic balance or redistribution of berry-derived ABA through the phloem into the aerial parts of the vine. Increasing evidence indicates that various plant membrane transport systems play a significant role in adaptation to stress conditions, such as drought. The role of various transport systems in ABA translocation, stomatal, cuticular, and root responses, as well as osmotic adjustment has been summarized by Jarzyniak and Jasiński (2014). In contrast, other reports have showed that *Vv*NCED1 was up-regulated by ABA and ethephon treatments (Pilati et al., 2017; Suehiro et al., 2019), explaining the intracellular ABA level measured by Pilati et al. (2017).

Our results describing the content of ABA catabolites (Figures 1C-F) showed a decreasing trend in 7-OH-ABA, PA and DPA during grape ripening, which was in agreement with those published by Torres et al. (2018). The catabolism mediated by the hydroxylation of 7’ and 8’-positions was induced in OA-treated table grapes at 3DAT3 or harvest. The OA treatment applied during berry development and ripening on-vine resulted in an accumulation of ABA content at 3DAT1 stage which was further supported at molecular level by the up-regulation of the *Vv*NCED1 gene expression. Similarly, OA also contributed to increasing levels of ABA-GE and PA catabolites during the growth and ripening cycle, which have been highlighted as key molecules in grapevine development and in its physiological responses to environmental stresses (Castellarin et al., 2016).

During the stress response, plant cells exhibit defense mechanisms to detoxify the synthesized ROS including enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX). SOD converts $O2^{\bullet-}$ to H_2O_2, which could be finally eliminated by CAT, POD and APX activities (Hodges et al., 2004). In general, these antioxidant enzymes showed higher activity at three studied stages in table grapes from OA-treated vines than in controls. Increases in the activity of antioxidant enzymes have been also obtained after OA postharvest treatment in climacteric fruits (Zheng et al., 2007a; Deng et al., 2015; Razzaq et al., 2015) and, recently, as preharvest treatment (Martínez-Esplá et al., 2019). The imbalance between the production and elimination of ROS, due to a decline in the activity of antioxidant enzymes, and an increase on lipoxygenase activity, may partly be responsible for initiating the senescence in fruits (Hodges et al., 2004; Razavi and Hajilou, 2016). Our results suggest that preharvest application of OA 5 mM could delay the senescence of treated table grapes in postharvest throughout the upregulation of these antioxidant systems during the developmental cycle and their maintenance during storage as addressed below.

4.2 Oxalic acid preharvest treatment modulates bioactive compound content, total antioxidant activity and berry quality parameters at harvest

In 2016 and 2017, OA was applied at 1, 5 and 10 mM concentrations and cluster were harvested when berries reached colour, size and TSS content characteristic of this cultivar, according to commercial criteria. Given the fact that the ripening process is heterogeneous in the clusters within a vine, harvesting was performed in four different dates (Supplementary Figure 1A and 1B). For both growing cycles, 5 mM OA accelerated the on-vine berry ripening process since higher amount of cluster were harvested from these OA-treated vines at the first harvest date, while no significant effects were observed for 1 or 10 mM concentrations. In addition, it is worth noting that accumulated yield at the last harvesting date was significantly higher in 5 mM OA treated vines with respect to controls, while yield was not affected by 1 mM OA treatment and was reduced by 10 mM OA treatment. Thus, OA 5 mM was chosen as the best dose for the 2018 experiment, and similar results were obtained in term of accelerating the on-vine ripening and increasing vine yield (Supplementary Figure 1C). The effects of 5 mM OA treatment on increasing vine-yield were due to an enhanced berry volume and the reduction in yield observed in 10 mM OA treatment to a lower berry size. In addition, in 'Magenta' cultivar, the ripening process of the berries within a cluster is also heterogeneous, and then, berries not fully coloured are cut and discarded after harvesting by operators in the field. These poorly coloured berries were reduced in clusters from 5 mM treated vines, which also contributed to increase vine yield, apart from the effect on increasing berry size.

On the other hand, our results showed that 5 mM OA preharvest treatment significantly increased the berry volume (mm^3) by 11 %, 16 % and 13.5 % in 2016, 2017 and 2018 seasons, respectively, than control berries (Supplementary Figure 2). The effect of OA preharvest treatment increasing berry size has also been reported in sweet cherry cultivars, 'Sweet Heart' and 'Sweet Late', by Martínez-Esplá et al. (2014), manifested by higher fruit volume and weight in cherries from treated trees than from controls, the higher effect being found with 2 mM OA. Berry size is widely acknowledged to affect berry quality. For instance, a negative relationship between must total soluble solid concentration (TSS, °Brix) and berry size has been described in *Vitis vinifera* L. cv. Syrah (clone SH1A) (Barbagallo et al., 2011). These authors observed that TSS decreased from the smallest to largest berries divided in four berry size categories. This effect was also found by Roby et al. (2004). However, no correlation was found between berry size and juice titratable acidity (TA). Our results showed a similar effect since 5 mM OA-treated berries had significantly higher volume than non-treated berries with the lowest total soluble solids content (Supplementary Figure 2 and Table 2).

In general, our results show that the 5 mM OA-treated table grapes are berries with high bioactive molecules content and antioxidant potential at commercial harvest. Preharvest treatment of vines with OA at 5 mM led to significant increases on bioactive compound content such as total anthocyanins, total phenols, ascorbic acid and total antioxidant activity (H-TAA and L-TAA) at harvest in both whole fruit and skin tissue (Table 2). Treated table grapes also had higher individual anthocyanins content than non-treated berries in both tissues (Table 2). In general, all these functional parameters were mainly improved in the skin tissue. Similar results inducing accumulation of phenolic and anthocyanins at harvest have also been found in other climacterics and non-climacteric fruits when OA was applied at pre- or postharvest stages (Valero et al., 2011; Martínez-Esplá et al., 2014; 2019; Deng et al., 2015; Razzaq et al., 2015; Razavi and Hajilou, 2016; Zhu et al., 2016; Kok and Bal, 2019; García-Pastor et al., 2020a), leading to fruits with higher antioxidant capacity. The mechanism by which OA increased the bioactive compounds and antioxidant properties is not well understood, although it could be attributed to the activation of phenylalanine ammonia-lyase (PAL) activity, the key enzyme in the phenylpropanoid pathway involved in phenolic biosynthesis (Deng et al., 2015; Razavi and Hajilou, 2016; Martínez-Esplá et al., 2019). The higher content of total and individual anthocyanins in table

grapes treated with OA could be related with endogenous ABA accumulation (Figure 1A), as reported by Keller et al. (2015) and Shahab et al. (2020). As expected, bioactive compound content, mainly individual anthocyanins, was higher in skin than the whole berry (Table 2). Anthocyanins are located in the berry skin, being responsible for skin colouration. Therefore, the content of these bioactive compounds is diluted when we analysed the whole berry (flesh + skin).

Finally, table grapes treated with OA 5 mM showed a significant lower TSS content, mediated by lower glucose and fructose content, as well as higher tartaric, ascorbic and succinic acid content, leading to higher total acidity as compared with untreated grapes (Table 2). Thus, it seems that OA 5 mM applied in preharvest delayed the ripening process of 'Magenta' table grapes leading to berries with lower individual sugar concentration and higher acid content. Nevertheless, total and individual anthocyanin concentrations, responsible for berry colour and important parameters related to ripening, were found at higher levels in grapes from OA treated vines than in controls, which would indicate a more advanced ripening stage. In fact, the effect of OA treatment on berry ripening parameters was higher for colour and anthocyanin concentration than for TSS or sugar and acid content. As commented previously, clusters were harvested when berries reached their commercial ripening stage according to commercial criteria and, apparently, they were in a similar ripening stage. However, analytical results show differences in some parameters related to ripening among control and treated berries, although in a different way depending of the considered parameter. Thus, ripening is a complex process and its evolution cannot be followed by a single parameter, being the differences on TSS content possible related with the higher berry volume observed in OA treated berries. On the other hand, OA preharvest treatment increased ABA accumulation at 3DAT1 and 3DAT2, which would lead to colour changes at veraison, as previously was reported when 'Carménère' clusters were sprayed with 50 and 100 µL L^{-1} ABA during pre-veraison (Villalobos et al., 2016), and contributing to accelerate berry on-vine ripening and increase anthocyanin content.

4.3 Oxalic acid preharvest treatment influences quality parameters, decay berry percentage, ABA metabolism and antioxidant enzyme activity during postharvest storage

Berry firmness at harvest was significantly higher in OA-treated table grapes than in controls (Figure 3A). Significant decreases occurred in firmness values during storage at 2 °C, although the effect of OA delaying firmness losses was maintained during postharvest storage (Figure 3A). This OA long-lasting effect has also been reported elsewhere delayed postharvest ripening by retarding softening in some climacteric fruits (Razavi and Hajilou, 2016; Zhu et al., 2016; Martínez-Esplá et al., 2019). Nevertheless, the effects of OA on delaying fruit softening process during storage could also be attributed to a reduction in the activity of cell wall hydrolytic enzymes, as has been reported for exo-polygalacturonase (exo-PG) and pectin methylesterase (PE) in OA postharvest-treated mango and plum fruits, respectively (Wu et al., 2011; Razzaq et al., 2015). In this sense, we hypothesize that this possible reduction on these two-cell wall hydrolytic enzymes, exo-PG and PE, could be in turn related with the higher activity of antioxidant enzymes mediated by OA treatment, which could delay the senescence process in the fruit (Figures 2A, B and C). Furthermore, the formation of oxalate-pectin as a result of OA treatment (Razzaq et al., 2015), leading to reinforcing wall structure of mesocarp cells and slowing down the softening process, could be associated to the effect of this treatment.

On the other hand, berry colour, expressed as hue angle, was improved at harvest and during postharvest cold storage in OA-treated table grapes (Figure 3B). This fact is related with the effect observed at Table 2 on the increase of total and individual anthocyanins content in both whole fruit and skin tissue. An increase of colour by OA treatments was also reported in climacteric and non-climacteric fruits (Martínez-Esplá et al., 2014; García-Pastor et al., 2020a). As previously commented,

it has been suggested that OA promoted the accumulation of phenylpropanoid metabolites through the activation of PAL enzyme. Contradictorily, no stimulation of this enzyme was observed by the OA treatment in a previous report (Zheng et al., 2012). According to our results, we hypothesize that OA could improve the berry colour throughout the ABA metabolomic stimulation, mediated at the same time by the homeostasis of its catabolites and transcribed by the *Vv*NCED1 gene.

Finally, a significantly lower incidence of *Botrytis cinerea* was observed in berries from OA-treated vines than non-treated vines (Figure 3C). OA contributes to induce systemic resistance in plants, and that this may be due to both an increase in peroxidase (POD) activity and synthesis of new POD isoforms, upregulating defence-related enzymes (Tian et al., 2006). Our results show that OA treatment resulted in significant changes in activities of defence enzymes such as POD (Figure 2C and 6C), as well as other antioxidant enzymes (Figure 2A and B; Figure 5A and B) during preharvest, at harvest and during postharvest periods. On the other hand, disease resistance in fruit decreases during postharvest ripening as physiological and biochemical changes increases fruit susceptibility to pathogen infection, and the linkage between postharvest fruit ripening and increasing disease susceptibility is very strong. Thus, the effect of OA on decreasing fruit decay incidence might also be attributed to the delay of berry senescence, as well as has been reported by Zheng et al. (2007b). Therefore, it should be highlighted that in order to elucidate this hypothesis we measured the endogenous ABA and catabolite content during 15, 30 and 45 days of postharvest storage at 2 °C (Figure 4 and Supplementary Table 1), and a significantly lower ABA and catabolite content in OA 5 mM-treated berries than in controls were found. OA treated berries showed a less advanced stage of senescence than non-treated table grapes. Therefore, preharvest application of OA proved to be an effective method to delay quality deterioration and extend the storage shelf-life of table grapes, as was observed when it was applied as postharvest treatment (Zheng et al., 2012).

Other plant growth regulators, such as MeJa and salicylate derivatives (SA, ASA and MeSa), applied as preharvest treatments, have been recently reported to affect quality traits of ‘Magenta’ and ‘Crimson’ seedless table grape cultivars, although differently depending on concentration (García-Pastor et al., 2019; 2020b). Thus, MeJa and salicylate derivatives applied at 5 and 10 mM delayed berry ripening process and reduced crop yield in both cultivars, while ripening was accelerated and yield increased when these compounds were applied at 1, 0.1 and 0.01 mM concentrations. The effects of these treatments at high doses on reducing total yield were attributed to a delay or inhibition on the ripening process since many berries failed to ripen properly and some clusters did not reach the requested commercial quality. However, the concentration that showed better results in terms of berry maturity-quality and bioactive compounds in ‘Magenta’ seedless cultivar depended on the applied compound. Thus, 0.1 mM MeJa, 0.01 mM SA, 0.1 mM ASA and 0.01 mM MeSa treatments, applied at key points of berry development, accelerated berry ripening, mainly colour evolution due to increased anthocyanin biosynthesis, as well as enhanced berry size, weight, firmness, TSS and antioxidant bioactive compound content (phenolics and individual anthocyanins). Moreover, preharvest application of SA, ASA and MeSA induced resistance of table grapes to be colonized with *B. cinerea*, which was attributed to the increased levels of phenolic compounds and the activity of antioxidant enzymes APX, CAT and POD, (García-Pastor et al., 2020c). With respect to OA, in previous experiments performed on growing seasons 2016 and 2017, OA treatments were applied at 1, 5 and 10 mM and the 5 mM concentration was chosen as the optimum among the three concentrations tested, in terms of yield, berry maturity-quality and bioactive compounds (data not shown), to be applied in the present experiment (2018 season). In this study, OA preharvest treatment at 5 mM have shown similar effects in ‘Magenta’ table grape as the other plant growth regulators tested, improving

its quality traits and inducing berry resistance to Botrytis cinerea incidence. Specifically, in terms of colour, which is the main aim of those research works, a significantly 1.3-fold increase on total anthocyanins content in the whole fruit (flesh + skin tissues) was observed by MeJa and SA treatments than untreated berries. However, greater results with 1.7 and 2-fold increases on this content was achieved by the application of ASA and MeSa or OA, respectively. Although in terms of improving total anthocyanins content in the skin tissue, where these coloured pigments are mainly synthesized and concentrated, MeSa preharvest treatment showed a significantly lower effectiveness increasing this content than OA (1.5-fold increase vs. 2.4-fold increase in MeSa and OA-treated berries skin, respectively, than control berries skin). Thus, OA preharvest treatment could be a more useful tool compared to other plant growth regulators tested (MeJa, SA, ASA or MeSa) in improving quality traits, mainly in terms of skin colour, of 'Magenta' poor-coloured table grape.

5 Conclusions

As an important signalling molecule, a preharvest application of OA at 5 mM improved the skin colour of table grapes at harvest by the up-regulation of *Vv*NCED1 and ABA homeostasis during berry development and on-vine ripening. Additionally, OA delayed table grape postharvest ripening and senescence processes during storage, which was mainly mediated by the stimulation of enzymatic and non-enzymatic antioxidant systems, as well as by the reduction in the ABA metabolism. In conclusion, the preharvest application of 5 mM OA could be a useful tool to improve colour and quality of poor-coloured table grape cultivars at harvest and during postharvest storage.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

DV, MS, LAT, MCA and PJZ conceived and designed the work in association with other authors. FG and PZ performed the field treatments. MEG-P performed most of the analytical determination in collaboration with the other authors (MJG and VS-E). MEG-P and SG-M analyzed the data. MEG-P wrote the manuscript under the supervision of PJZ and MCA. All authors approved the final version of the manuscript.

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780 **11 Figure Legends**

781 **Figure 1.** Evolution of ABA berry content (nmol g^{-1}) **(A)**, relative *Vv*NCED1 gene expression **(B)**, and
782 ABA catabolites (ABA-GE **(C)**, 7-OH-ABA **(D)**, PA **(E)** and DPA **(F)**) content (nmol or pmol g^{-1})
783 measured in control and 5 mM oxalic acid (OA)-treated 'Magenta' table grapes during three key
784 growth and ripening stages in preharvest: 3DAT1 (3 days after the first treatment when berries reached
785 ca. 40 % of its final size), 3DAT2 (3 days after the second treatment when veraison stage started) and
786 3DAT3 (3 days after the third treatment, at harvest). Data are the mean ± SE. Significant differences

($p < 0.05$ according to Student's t-test) between control and OA-treated berries were expressed as * symbol placed in the OA bar for each stage and parameter. Different lowercase letters show significant differences ($p < 0.05$ according to HSD Duncan's test) among the three stages during berry development. *Vv*NCED1: *Vitis vinifera* 9-*cis*-epoxycarotenoid dioxygenase 1; ABA: abscisic acid; ABA-GE: ABA glucose ester; 7-OH-ABA: 7- hydroxy-ABA; PA: phaseic acid; DPA: dihydrophaseic acid.

Figure 2. Evolution of ascorbate peroxidase (APX **(A)**), catalase (CAT **(B)**) and peroxidase (POD **(C)**) activities (U min^{-1} g^{-1}) measured in control and 5 mM oxalic acid (OA)-treated 'Magenta' table grapes during three key growth and ripening stages in preharvest: 3DAT1 (3 days after the first treatment when berries reached ca. 40 % of its final size), 3ADT2 (3 days after the second treatment when veraison stage started) and 3DAT3 (3 days after the third treatment, at harvest). Data are the mean ± SE. Significant differences ($p < 0.05$ according to Student's t-test) between control and OA-treated berries were expressed as * symbol placed in the OA bar for each stage and parameter. Different lowercase letters show significant differences ($p < 0.05$ according to HSD Duncan's test) among the three stages during berry development.

Figure 3. Evolution of firmness (N mm^{-1} **(A)**) and hue angle colour (hº **(B)**) during 45 days of postharvest storage at 2 ºC of control and 5 mM OA-treated table grapes. Percentage of decayed berries (% **(C)**) according to the visual aspect scale (S0-S5) of *Botrytis cinerea* decay incidence in 'Magenta' table grapes as affected by preharvest treatments with control and oxalic acid (OA). Data are the mean ± SE. Significant differences ($p < 0.05$ according to Student's t-test) between control and OA-treated berries were expressed as * symbol placed in the OA line or bar for each storage day or decay damage stage and parameter. Different lowercase letters show significant differences ($p < 0.05$ according to HSD Duncan's test) among the storage days and decay damage stages in postharvest.

Figure 4. Berry content evolution of ABA and its catabolites content measured in control and 5 mM oxalic acid (OA)-treated 'Magenta' table grapes during 15, 30 and 45 days of postharvest storage at 2 ºC. Data are the mean ± SE. Colours in the diagram represent the low or high concentration, ranging from red to green, respectively. Significant differences ($p < 0.05$ according to Student's t-test) were expressed as * symbol placed in each column showing differences between treatments for each storage day at 2 ºC. Different letters show significant differences ($p < 0.05$ according to HSD Duncan's test) among storage days for each treatment. ABA: abscisic acid; ABA-GE: ABA glucose ester; 7-OH-ABA: 7- hydroxy-ABA; PA: phaseic acid; DPA: dihydrophaseic acid.

Figure 5. Evolution of ascorbate peroxidase (APX **(A)**), catalase (CAT **(B)**) and peroxidase (POD **(C)**) activities (U min^{-1} g^{-1}) measured in control and 5 mM oxalic acid (OA)-treated 'Magenta' table grapes during 15, 30 and 45 days of postharvest storage at 2 ºC. Data are the mean ± SE. Significant differences ($p < 0.05$ according to Student's t-test) between control and OA-treated berries were expressed as * symbol placed in the OA bar for each storage day and antioxidant enzyme. Different lowercase letters show significant differences ($p < 0.05$ according to HSD Duncan's test) among the three storage days in postharvest.

829 **Table 1.** Transcriptomic details of primers for the targeted and endogenous control genes.

Gene		Forward and reverse primers	Amplicon length	NCBI reference sequence
NCED1	**F**	5′-GCAGAGGACGAGAGTGTAAAGGA-3′	130 pb	XM_019216859.1
	R	5′-GCAGAGTAAAAACACATGAAGCTAGTG-3′		
ACT	**F**	5′- GCCCCTCGTCTGTGACAATG-3′	100 pb	XM_002282480.4
	R	5′-CCTTGGCCGACCCACAATA-3′		
UBI	**F**	5′-TCTGAGGCTTCGTGGTGGTA-3′	99 pb	XM_002273532.2
	R	5′-AGGCGTGCATAACATTTGCG-3′		
GAPDH	**F**	5′-CCACAGACTTCATCGGTGACA-3′	70 pb	XM_002263109.3
	R	5′-TTCTCGTTGAGGGCTATTCCA-3′		

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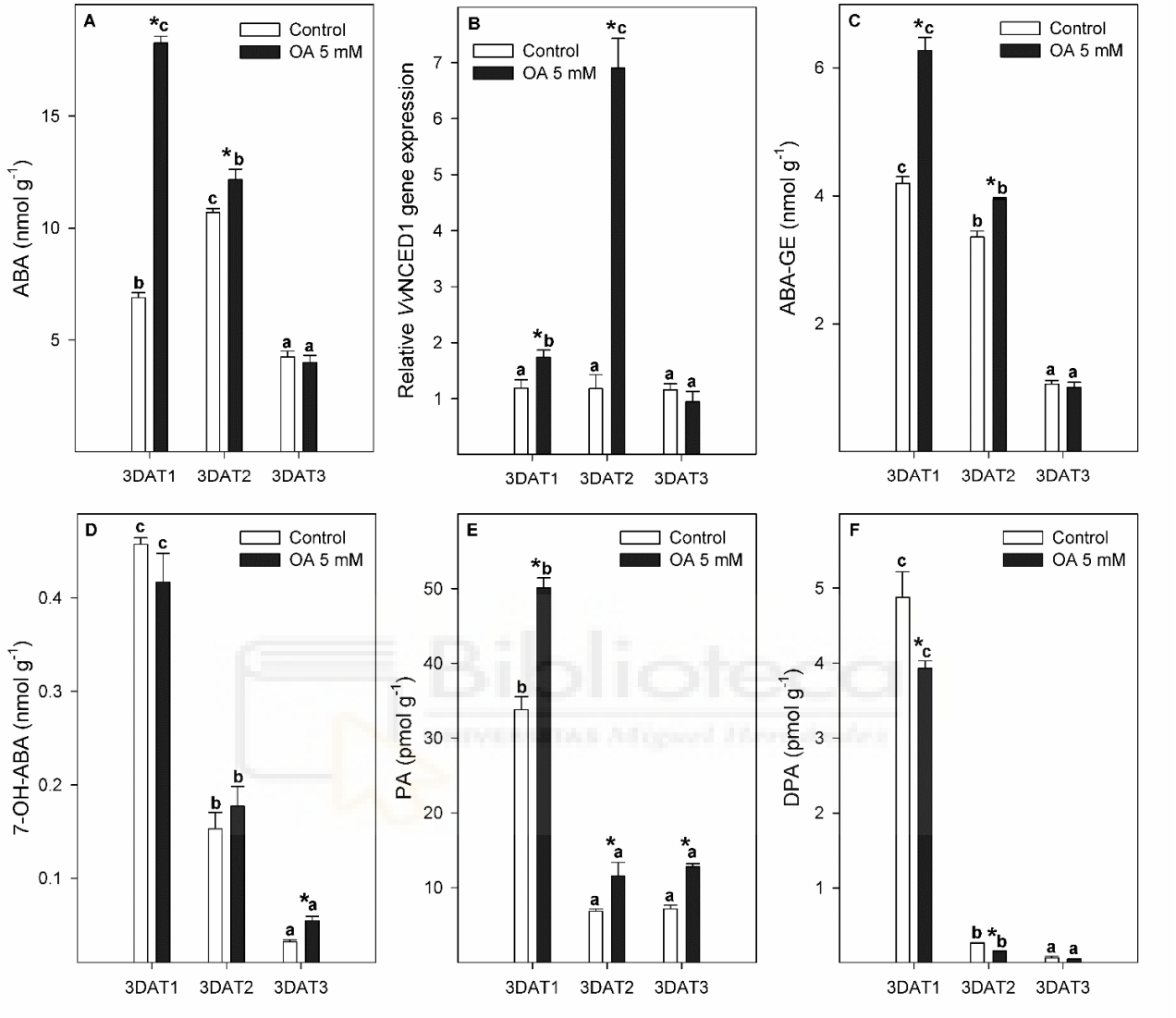
Table 2. Bioactive compound content (total anthocyanins and total phenols), hydrophilic (H-TAA) and lipophilic (L-TAA) total antioxidant activity, individual anthocyanin content (dp-3-gluc; delphinidin 3-glucoside, cy-3-gluc; cyanidin 3-glucoside, pt-3-gluc; petunidin 3-glucoside, pn-3-gluc; peonidin 3-glucoside and mv-3-gluc; malvidin 3-glucoside), total soluble solids (TSS), total acidity (TA) and individual sugar (glucose and fructose) and organic acid content (tartaric acid, malic acid, citric acid, ascorbic acid, succinic acid and fumaric acid) in whole fruit (flesh + skin) or skin tissue of control and 5 mM OA-treated table grapes at harvest. Significant differences ($p < 0.05$ according to Student's t-test) were expressed as * and ♦ symbols placed in each column or row showing differences between both treatments and tissues, respectively, for each parameter at harvest.

	Treatments	Flesh + Skin	Skin
Total Anthocyanins (mg 100 g^{-1})	**Control**	4.76 ± 0.38*♦	16.69 ± 0.69*♦
	OA 5 mM	9.48 ± 0.54*♦	40.72 ± 1.96*♦
Total Phenols (mg 100 g^{-1})	**Control**	33.60 ± 1.23*♦	158.18 ± 6.40*♦
	OA 5 mM	43.84 ± 1.42*♦	336.14 ± 25.35*♦
TAA-Hydrophilic (mg 100 g^{-1})	**Control**	179.11 ± 31.53*♦	478.03 ± 25.11*♦
	OA 5 mM	308.60 ± 13.00*♦	962.96 ± 80.62*♦
TAA-Lipophilic (mg 100 g^{-1})	**Control**	31.80 ± 1.27*♦	51.12 ± 1.30*♦
	OA 5 mM	41.20 ± 1.42*♦	71.37 ± 2.75*♦
Dp-3-gluc (mg 100 g^{-1})	**Control**	0.80 ± 0.07♦	1.35 ± 0.19*♦
	OA 5 mM	0.93 ± 0.09♦	3.46 ± 0.22*♦
Cy-3-gluc (mg 100 g^{-1})	**Control**	0.16 ± 0.02*♦	0.22 ± 0.07*♦
	OA 5 mM	0.26 ± 0.06*♦	0.57 ± 0.16*♦
Pt-3-gluc (mg 100 g^{-1})	**Control**	0.32 ± 0.04*♦	0.76 ± 0.19*♦
	OA 5 mM	0.42 ± 0.06*♦	1.55 ± 0.14*♦
Pn-3-gluc (mg 100 g^{-1})	**Control**	1.78 ± 0.09*♦	4.02 ± 0.56*♦

Mv-3-gluc (mg 100 g^{-1})	OA 5 mM	2.87 ± 0.07$^{*\blacklozenge}$	7.14 ± 0.63$^{*\blacklozenge}$
	Control	2.05 ± 0.05$^{*\blacklozenge}$	5.18 ± 0.54$^{*\blacklozenge}$
	OA 5 mM	3.19 ± 0.07$^{*\blacklozenge}$	8.80 ± 0.64$^{*\blacklozenge}$
Total Soluble Solids (TSS) (g 100 g^{-1})	Control	16.90 ± 0.08*	-
	OA 5 mM	15.82 ± 0.06*	-
Total Acidity (TA) (g 100 g^{-1})	Control	0.67 ± 0.01*	-
	OA 5 mM	0.73 ± 0.02*	-
Glucose (g 100 g^{-1})	Control	8.30 ± 0.13*	-
	OA 5 mM	7.80 ± 0.13*	-
Fructose (g 100 g^{-1})	Control	6.80 ± 0.11*	-
	OA 5 mM	6.39 ± 0.11*	-
Tartaric acid (g 100 g^{-1})	Control	0.34 ± 0.002*	-
	OA 5 mM	0.39 ± 0.010*	-
Malic acid (g 100 g^{-1})	Control	0.26 ± 0.007	-
	OA 5 mM	0.24 ± 0.013	-
Citric acid (g 100 g^{-1})	Control	0.07 ± 0.007	-
	OA 5 mM	0.06 ± 0.011	-
Ascorbic acid (g 100 g^{-1})	Control	0.0157 ± 0.0002*	-
	OA 5 mM	0.0166 ± 0.0002*	-
Succinic acid (g 100 g^{-1})	Control	0.00486 ± 0.00004*	-

	OA 5 mM	0.00520 ± 0.00001*	-
	Control	0.00054 ± 0.00002	-
Fumaric acid (g 100 g^{-1})	**OA 5 mM**	0.00051 ± 0.00008	-

840



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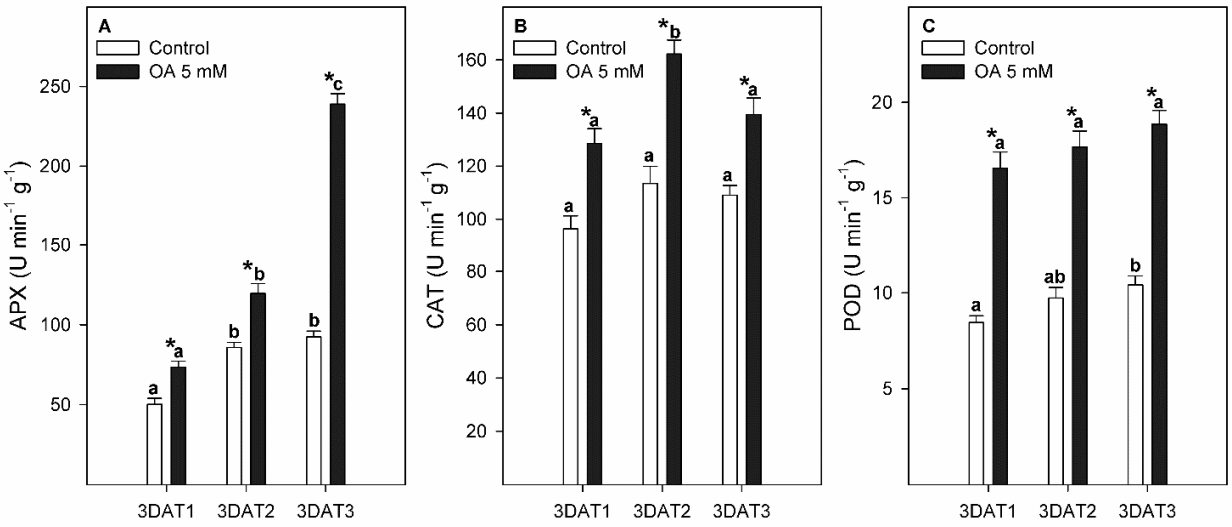
842 **Figure 1.**



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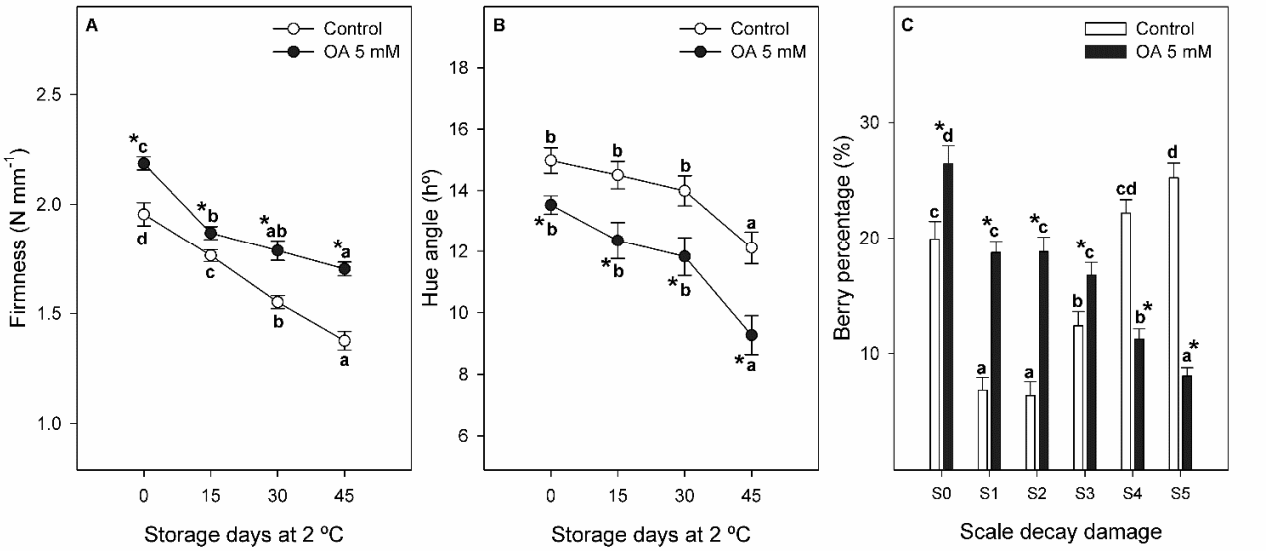
844 **Figure 2.**



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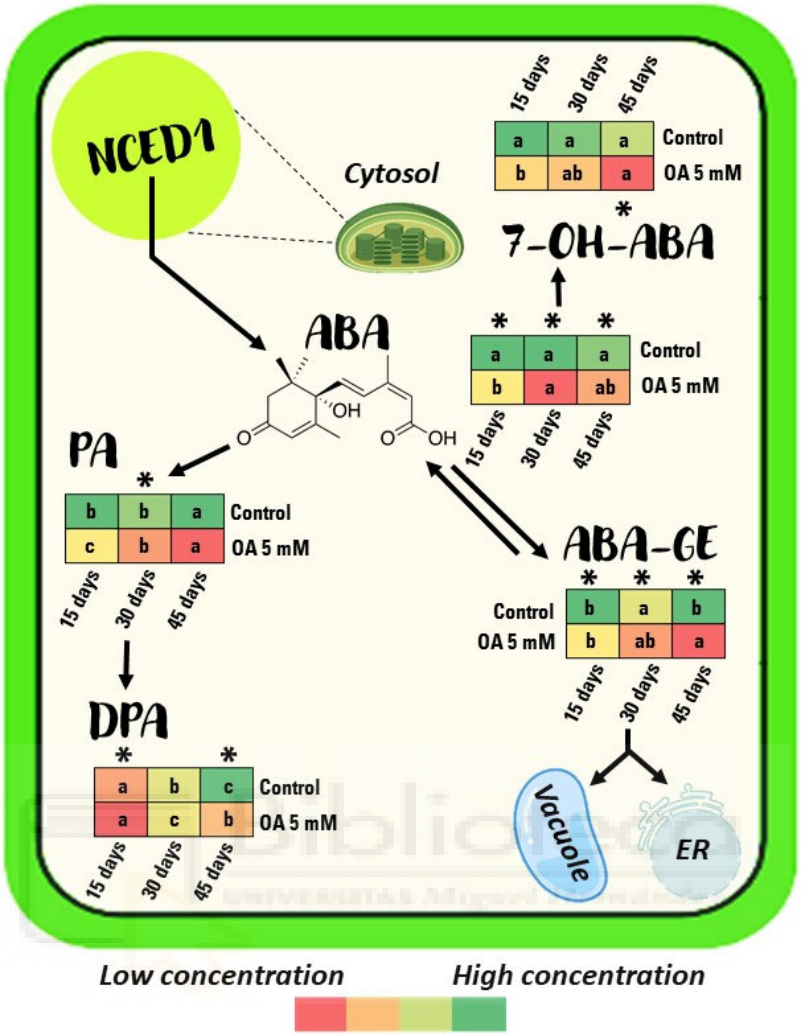
846 **Figure 3.**



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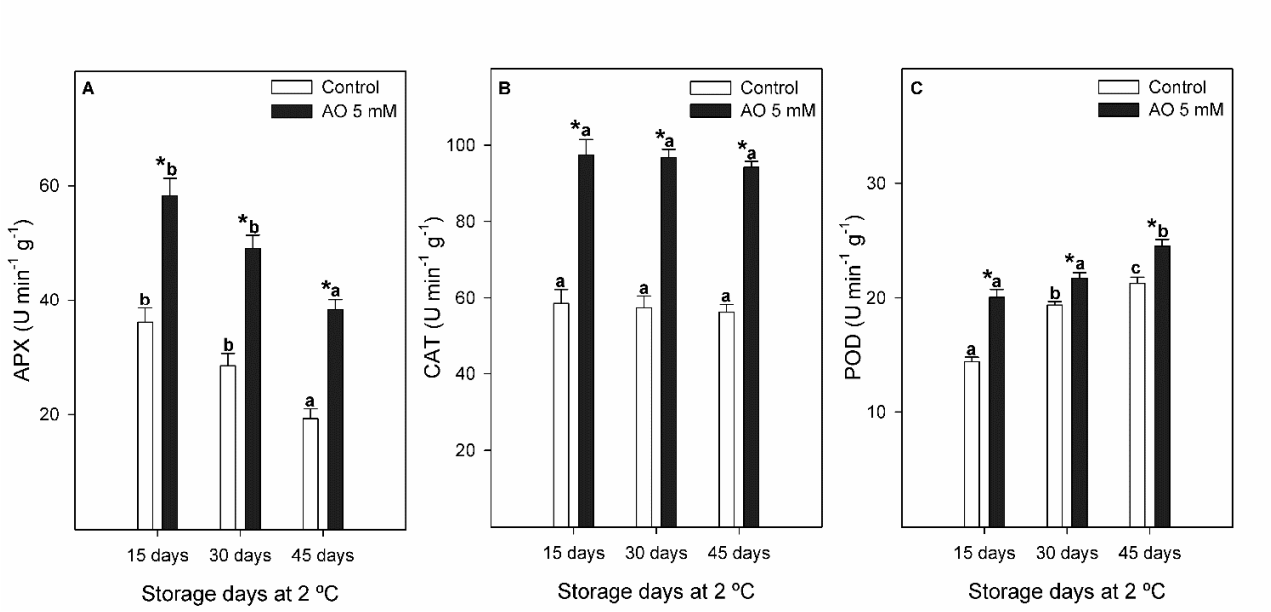
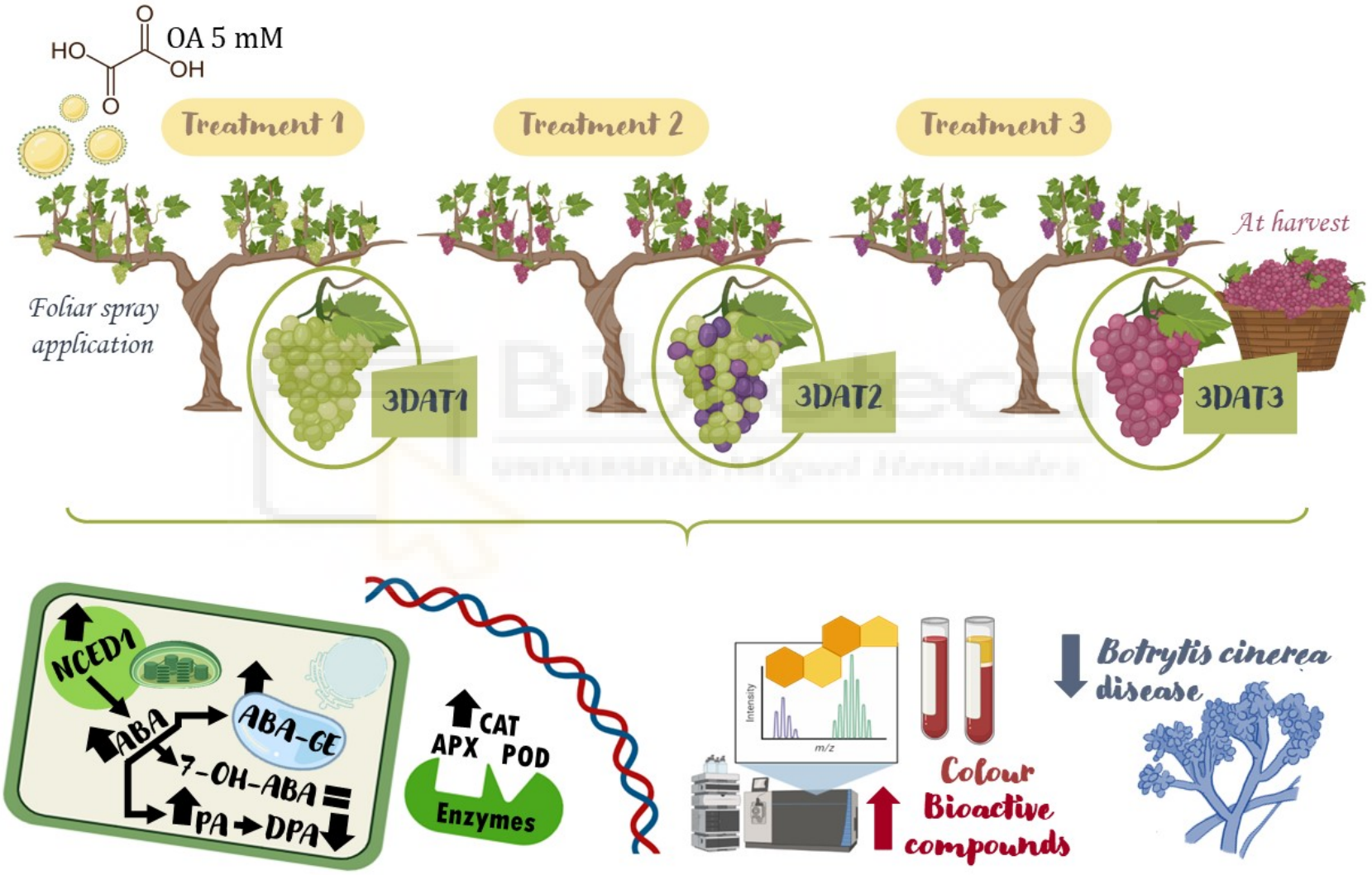


Figure 5.

866 **Graphical Abstract.**



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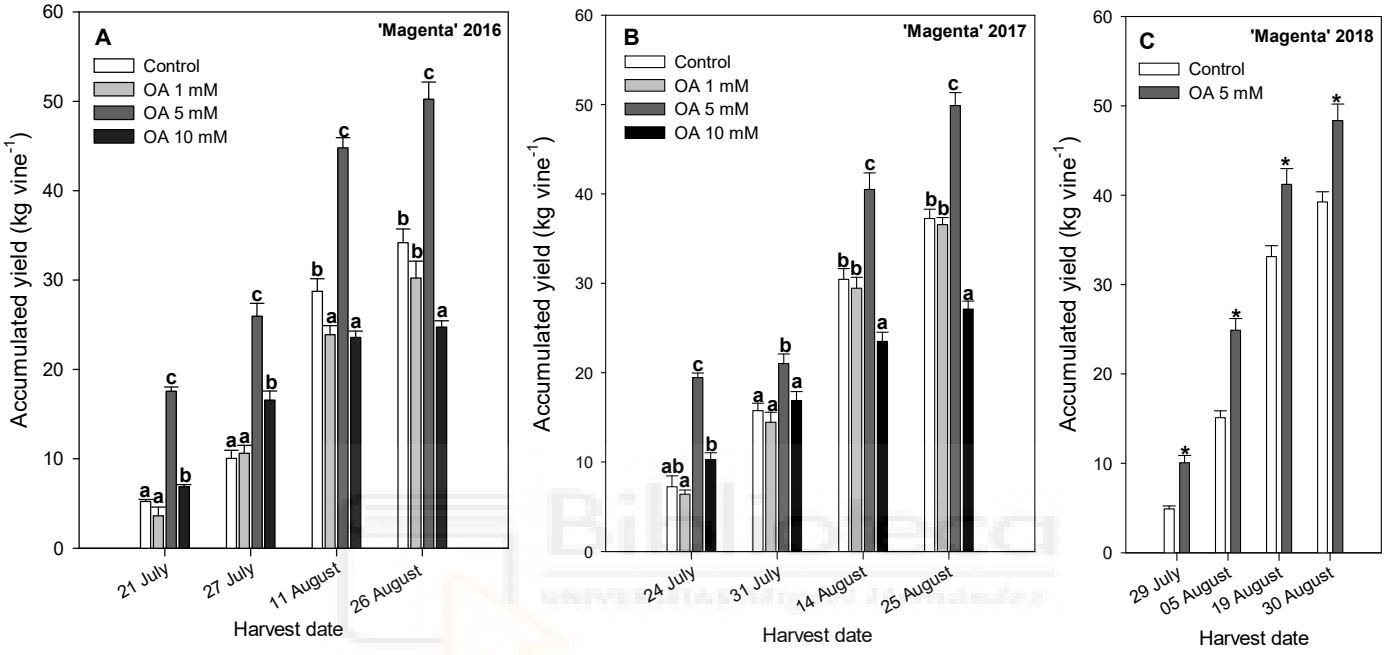
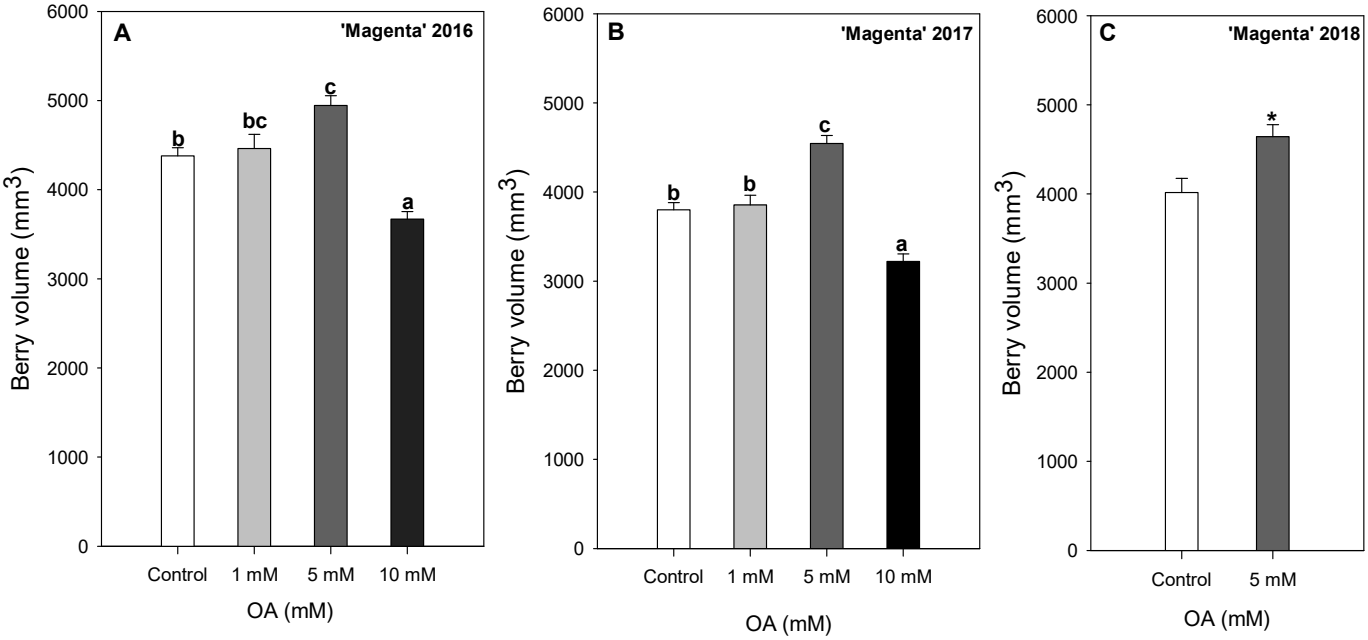
Supplementary Material

1 Supplementary Figures and Tables



Supplementary Figure 1. Accumulated yield (kg vine^{-1}) in control and OA treated vines in 2016 **(A)**, 2017 **(B)** and 2018 **(C)** experiments. Data are the mean ± SE of five replicates of three vines (15 vines) for each treatment in 'Magenta' cultivar. Different lowercase letters show significant differences ($p < 0.05$ according to HSD Duncan's test) among the treatments for each harvest date in 2016 and 2017 seasons. Significant differences ($p < 0.05$ according to Student's *t*-test) between control and OA-treated berries were expressed as * symbol placed in the OA bar for each harvest date in 2018 season.



Supplementary Figure 2. Effect of vine OA treatments on berry volume (mm^3) in 2016 **(A)**, 2017 **(B)** and 2018 **(C)** experiments. Data are the mean ± SE of five replicates of 30 berries (10 berries from each vine) from the first harvest date for both treatments in 'Magenta' cultivar. Different lowercase letters show significant differences ($p < 0.05$ according to HSD Duncan's test) among the treatments for 2016 and 2017 seasons. Significant differences ($p < 0.05$ according to Student's *t*-test) between control and OA-treated berries were expressed as * symbol placed in the OA bar for 2018 season.

Supplementary Table 1. Berry content evolution's supplementary data of ABA and its catabolites content (nmol or pmol g^{-1}) measured in control and 5 mM oxalic acid (OA)-treated 'Magenta' table grapes during 15, 30 and 45 days of postharvest storage at 2 ºC. Data are the mean ± SE. Significant differences between treatments for each storage day at 2 ºC ($p < 0.05$ according to Student's t-test) were highlighted in **Figure 4**. ABA: abscisic acid; ABA-GE: ABA glucose ester; 7-OH-ABA: 7-hydroxy-ABA; PA: phaseic acid; DPA: dihydrophaseic acid.

		ABA (nmol g^{-1})	ABA-GE (nmol g^{-1})	7-OH-ABA (nmol g^{-1})	PA (pmol g^{-1})	DPA (pmol g^{-1})
Control	**15 days**	1.96 ± 0.11	1.13 ± 0.04	0.03 ± 0.002	0.01 ± 0.001	0.06 ± 0.004
	30 days	1.73 ± 0.09	0.86 ± 0.06	0.03 ± 0.002	0.009 ± 0.001	0.10 ± 0.007
	45 days	1.81 ± 0.15	1.38 ± 0.10	0.02 ± 0.001	0.005 ± 0.001	0.20 ± 0.015
OA 5 mM	**15 days**	1.40 ± 0.07	0.76 ± 0.05	0.02 ± 0.002	0.008 ± 0.001	0.04 ± 0.003
	30 days	0.99 ± 0.09	0.66 ± 0.02	0.02 ± 0.002	0.006 ± 0.0004	0.09 ± 0.005
	45 days	1.20 ± 0.09	0.58 ± 0.04	0.01 ± 0.002	0.004 ± 0.0004	0.06 ± 0.005

5

Results and Discussion

5. RESULTS AND DISCUSSION

Table grape (*Vitis vinifera* L.) and pomegranate fruit (*Punica granatum* L.) are highly appreciated by consumers due to their high organoleptic properties and phytochemical compounds content, mainly phenolics compounds and anthocyanins, with antioxidant potential and health beneficial properties. However, some seedless red skin table grapes cultivars, such as 'Magenta' and 'Crimson', despite of having very good taste and aroma have a heterogeneous berry pigmentation in the cluster, leading to diminution of their market quality. Table grape marketing value depends on cluster size and shape as well as on berry size, colour, juiciness, sugar/acidity ratio and aroma. Both cultivars are red seedless table grapes, although 'Crimson' has a purple colour while 'Magenta' has a light-red colour, due to the occurrence of anthocyanins, which are responsible for the pigmentation of these cultivars. In the present PhD Thesis, five anthocyanins were identified and quantified in 'Magenta' cultivar for the first time, the main ones being peonidin 3-*O*-glucoside (Pn-3-glu) and malvidin 3-*O*-glucoside (Mv-3-glu), with concentrations of 1.7-2.0 mg 100 g^{-1} in control berries, followed by delphinidin 3-*O*-glucoside (Dl-3-glu, 0.6-0.8 mg 100 g^{-1}), while cyanidin 3-*O*-glucoside (Cy-3-glu) and petunidin 3-*O*-glucoside (Pt-3-glu) were found at lower concentrations. However, in 'Crimson' table grapes just three anthocyanins were found, the major one being Pn-3-glu with concentrations ca. 4 mg 100 g^{-1} in control grapes followed by Mv-3-glu (0.6-0.7 mg 100 g^{-1}) and just traces of Cy-3-glu were found. Comparing both cultivars, the concentration of total anthocyanins in the skin tissue was significantly higher in 'Crimson' (~70 mg 100 g^{-1}) than in 'Magenta' (~30 mg 100 g^{-1}) and thus it reflects the differences in colour.

This fruit colour problem is also common in some pomegranate fruit cultivars. Specifically, in 'Mollar de Elche', which is the most cultivated Spanish cultivar and safeguarded by a Protected Designation of Origin (PDO) since 2016 [R (UE) 2016/83]. This cultivar is very appreciated by consumers due to its high concentration of sugars and low acidity, its barely discernible seeds, since they are very small and soft and can be easily eaten, and its pleasant flavour (Melgarejo et al., 2000; Nuncio-Jáuregui et al., 2014; Fernandes et al., 2017). However, its arils are only slightly red-coloured and the skin has a cream-pink colour because they have lower anthocyanin content than other Spanish cultivars, such as 'White', 'CG8', 'Katirbasi' or 'Wonderful' (Mena et al., 2012; Fernandes et al., 2017) and other Tunisian, Turkish or Iranian cultivars (Mousavinejad et al., 2009; Mphahlele et al., 2014; Mottaghipisheh et al., 2018). This fact makes it difficult for this cultivar to reach international markets. In fact, pomegranate fruit quality depends largely on fruit size, skin colour, and absence of visual defects, such as sunburn, growth cracks, cuts, bruises, and decay, as well as on aril colour, sugar and acid content, and the presence of small and soft seeds. As individual anthocyanins profile of 'Mollar de Elche' cultivar, six anthocyanins were identified and quantified in the present PhD Thesis. The main ones were cyanidin 3-*O*-glucoside (Cy-3-glu) and cyanidin 3,5-*O*-di-glucoside (Cy-3,5-di-glu), followed by pelargonidin 3-*O*-glucoside (Pl-3-glu) and delphinidin 3-*O*-glucoside (Dp-3-glu), while pelargonidin 3,5-*O*-

di-glucoside (Pl-3,5-di-glu) and delphinidin 3,5-*O*-di-glucoside (Dp-3,5-di-glu) were found at lower concentrations. Moreover, the early harvest is another key factor that influences the market prices (Laribi et al., 2013). Therefore, increasing the harvest precocity of pomegranate fruit that reach the market with the proper colour could increase the economic profit for growers.

Both table grapes and pomegranate fruit are non-climacteric fruits. Grape berries display a double sigmoidal growth curve in which the onset of berries ripening is considered as the time of veraison (Castellarin et al., 2007). Therefore, veraison is a key point of berry development, in which pigmentation of skin starts (due to synthesis of anthocyanins in red cultivars), sugars and aroma compounds increase and acid content and firmness decrease while berry growths until the end of ripening (Kuhn et al., 2014). Both ethylene and abscisic acid (ABA) are likely to be important and their interplaying may be required to start the process of berry ripening (Sun et al., 2010). Thus, many studies have shown that ABA concentration increases at the onset of ripening (Wheeler et al., 2009; Sun et al., 2010; Pilati et al., 2017), and trace endogenous ethylene induces the expression of *Vv*NCED1 gene, which is involved in ABA biosynthesis (Sun et al., 2010). 9-Cis-epoxycarotenoid dioxygenase (NCED) is the enzyme catalyzing the first step of this reaction which produces xanthoxin, the direct C15 precursor of ABA, from the cleavage of 9-cis-violaxanthin or 9-cis-neoxanthin, since the step on ABA biosynthesis is rate-limiting. Grapevine NCED genes (*Vv*NCED1, *Vv*NCED2 and *Vv*NCED3) encode for enzymes that cleave carotenoids to form the phytohormone ABA. Moreover, berry ABA concentration was highly correlated with *Vv*NCED1 transcript abundance and a role of NCED genes in ABA signaling during ripening initiation has been described (Young et al., 2012). Nevertheless, a lack of correlation between free-ABA and NCED codifying genes in berries suggests that compounds derived from ABA catabolism/conjugation could also be involved in berry ripening (Wheeler et al., 2009; Castellarin et al., 2016).

In this sense, exogenous treatments with ABA and an ethylene-releasing compound, such as 2-chloroethylphosphonic acid (ethephon), at veraison stage, have been shown to increase skin anthocyanin concentration although most of these studies have been performed with wine grape cultivars (Marzouk and Kassem, 2011; Kuhn et al., 2014). However, the high cost of ABA has precluded the development of practical application and the effects of ethephon on colour development are inconsistent and can cause berry softening (Peppi et al., 2006) as well as deterioration of quality and reduction of grapes shelf-life (Szyjewicz et al., 1984). Therefore, more research is needed to find out other compounds with commercial application possibilities to increase grape colour. On the other hand, an accumulation of anthocyanins also occurs in pomegrante arils during ripening, these pigments being the responsible for their pink-red colour. The scarce colour acquisition in both type of fruits could be partly mediated by the high temperatures in the Southeast of Spain, where these cultivars are grown, during their ripening, which do not allow proper colour development (Ferrara et al., 2015).

Furthermore, pomegranate fruit displays important quality losses during postharvest storage including husk desiccation and browning, decay, loss of firmness, decreases in ascorbic acid, acidity and arils colour which led to reduction of consumers' acceptability (Pareek et al., 2015). In order to avoid these undesirable changes and to prolong storability, the best postharvest tool has been cold storage. However, pomegranate fruit is sensitive to chilling injury (CI) when stored at temperatures below 5 °C, the main symptoms being skin desiccation, browning and pitting, depression of the fruit surface and higher susceptibility to decay. In addition, the grape supply chain is very demanding in terms of preserving fruit quality since table grape is subjected to long storage periods before reaching its final destination. Thus, there are risks of various postharvest losses (Champa, 2015), being reported grey mould caused by *Botrytis cinerea* as the major postharvest disease of grapes (Martínez-Romero et al., 2007; Saito et al., 2019). This disease has a negative impact on quality properties of fresh grapes, such as weight loss, colour fading, accelerated softening, and reduction of shelf-life, all of these causing severe economic losses. The control of this mould is very difficult since postharvest treatments with synthetic fungicides or SO_2 are not allowed in several countries due to their adverse effects on food safety and the environment (Youssef et al., 2015).

In recent years, research has been performed aimed to find preharvest treatments with natural compounds to increase fruit quality at harvest and to maintain it during storage, due to consumers' concerns and legal restrictions regarding the use of postharvest chemical treatments. In this sense, the application of naturally occurring plant compounds as preharvest treatments to delay ripening and senescence and preserve fruit and vegetable quality has received considerable attention (Martínez-Esplá et al., 2017a). Therefore, the aim of this PhD Thesis is to provide solutions to the pomegranate and table grape quality problems discussed above through preharvest treatments with methyl jasmonate (MeJa), salicylic acid derivatives; salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa), and oxalic acid (OA) to mainly solve the colour problems of these fruits at harvest as well as the others alterations that determine their quality losses during postharvest storage, such as the chilling injury incidence and fungal decay in pomegranate fruit and table grape, respectively.

The results obtained about the effects of MeJa preharvest treatment on table grape and pomegranate fruit have been presented as Publications 1, 2 and 3 in the present PhD Thesis. The first two publications were performed during two growing seasons (2016 and 2017) with two different seedless table grape cultivars, 'Magenta' and 'Crimson' (**Publication 1**), and with 'Mollar de Elche' pomegranate cultivar (**Publication 2**). MeJa treatments were performed by foliar spray applications of 1, 5 and 10 mM MeJa in 2016. In 2017 season, these concentrations were repeated for pomegranate crop and reduced to 0.01, 0.1 and 1 mM MeJa in the case of table grape. In 2018, the concentration of MeJa and dates of application were selected in pomegranate fruit based on these previous experiments carried out in 2016 and 2017 seasons on these fruit species, in which the best results in term of yield and fruit quality attributes were obtained with 5 mM dose both at harvest and during storage at 10 °C (**Publication 3**).

Within the incipient research of different eco-friendly alternatives proposed in the present PhD Thesis, we also find the study of salicylic acid derivatives; salicylic acid (SA), acetyl salicylic acid (ASA) and methyl salicylate (MeSa), as possible tools to apply on pomegranate fruit and table grape crops with the aim of solving the mentioned problems. Results obtained about the effects of these preharvest treatments on pomegranate fruit and table grape have been presented as Publications 4, 5 and 6 in the present PhD Thesis. The first publication was performed during two growing seasons (2017 and 2018) with 'Mollar de Elche' pomegranate cultivar (**Publication 4**), and the two last publications were carried out in 'Magenta' and 'Crimson' table grape cultivars during three growing seasons: 2016 and 2017 (**Publication 5**) and, based on the results obtained in this experiment, repeated in 2018 (**Publication 6**).

Finally, we evaluated the effects of OA preharvest treatments to pomegranates trees on fruit size, crop yield (in terms of early harvest), respiration rate, fruit quality characteristics and sensory traits, as well as on bioactive compound content and antioxidant activity (**Publication 7**). In 2016, three OA concentrations were applied 1, 5 and 10 mM, and the best results were obtained with the 10 mM concentration. Thus, the 10 mM OA concentration was used in 2017 in order to confirm the 2016 results. Faced with the need for studies that evaluate the effect of OA on table grape quality as well as to elucidate the mechanism of action or possible role of this elicitor on the benefits obtained regarding fruit quality, the latest publication of the present PhD Thesis arises (**Publication 8**). Therefore, the main aim of the present study was to assess the potential effect of OA preharvest treatment, applied at three key points of table grape development in 2018-growing season, on quality parameters, mainly berry colour, of 'Magenta' poor-coloured table grape at harvest and during postharvest cold storage. This effect will be elucidated based on a metabolomic (ABA, ABA catabolites and antioxidant enzymes) approach and throughout the study of the relative *Vv*NCED1 gene expression about the action or role of OA during berry development, on-vine ripening and postharvest senescence. OA treatments were also performed by foliar spray application at 5 mM. This concentration was chosen as the best among three concentrations tested (1, 5 and 10 mM) in previous experiments (growing seasons 2016 and 2017) in terms of yield, berry maturity-quality and bioactive compounds (data not shown).

5.1. Effect of elicitation strategies on improving total anthocyanin content and other functional parameters at harvest

The main results obtained in the present PhD Thesis are focused on the overall aim defined, which was improving colour of both fruit species by applying elicitation strategies at preharvest. Anthocyanins quantification is interesting for determining the quality of fruits and vegetables, as previously commented in the introduction section, since their quality traits, mainly colour, are directly related to the content of these pigments and other phenolic compounds presence. The colour measurement can be used as an indirect way of analyzing the coloured components of fruits and vegetables, since it is simpler and faster compared to chemical analyses (Alves et al.,

2008). Some studies demonstrate a correlation between colour measurements of the CIELab system, which determine a three-dimensional colour space and represent the colour stimulus as an achromatic signal (L*) and two chromatic channels representing blue-yellow (b*) and red-green (a*), and the presence of bioactive compounds such as carotenoids, anthocyanins and other phenolics, betalaine and chlorophyll in plants (Sant'Anna et al., 2013). Therefore, the total anthocyanin content is a key study parameter in all the publications included in the present PhD Thesis. Overall, an increase in total anthocyanin content at harvest was found as a consequence of elicitation treatments, the most effective results being dependent on the applied compound, concentration tested, cultivar and fruit species at which treatments were performed (**Table 5**).

Table 5: *Comparative fold increase analysis among the effect of preharvest treatments tested in the present PhD Thesis on total anthocyanin content of table grape (flesh + skin tissues) and pomegranate fruit (arils) at harvest.*

Total anthocyanin content at harvest			
Treatment♦	Table grape		Pomegranate fruit
	'Magenta'	'Crimson'	'Mollar de Elche'
MeJa 0.01 mM	↑ 1.05	↑ 1.46	-
MeJa 0.1 mM	↑ 1.26	↑ 1.58	-
MeJa 1 mM	↑ 1.38	↑ 1.56	↑ 1.17
MeJa 5 mM	-	-	↑ 1.18
MeJa 10 mM	-	-	↑ 1.15
SA 0.01 mM	↑ 1.37	↑ 1.83	-
SA 0.1 mM	↑ 1.09	↑ 1.52	-
SA 1 mM	↑ 1.34	↑ 1.52	↑ 1.01
SA 5 mM	-	-	↑ 1.15
SA 10 mM	-	-	↑ 1.28
ASA 0.01 mM	↑ 1.26	↑ 1.23	-
ASA 0.1 mM	↑ 1.69	↑ 1.83	-
ASA 1 mM	↑ 1.40	↑ 1.91	↑ 1.01
ASA 5 mM	-	-	↑ 1.01
ASA 10 mM	-	-	↑ 1.20
MeSa 0.01 mM	↑ 1.40	↑ 1.31	-
MeSa 0.1 mM	↑ 2.00	↑ 2.11	-
MeSa 1 mM	↑ 1.79	↑ 1.58	↑ 1.01
MeSa 5 mM	-	-	↑ 1.01
MeSa 10 mM	-	-	↑ 1.03
OA 1 mM	-	-	↑ 1.07
OA 5 mM	↑ 1.99	-	↑ 1.24
OA 10 mM	-	-	↑ 1.46

♦ These data of treatments have been calculated with respect to the results of untreated or control fruits for each of the publications used; MeJa - **Publications 1 and 2**, SA, ASA and MeSa - **Publications 4 and 5**, and OA – **Publications 7 and 8**, according to the fruit species under study.

It is worth noting that MeJa treatments increased total and individual anthocyanin concentration at harvest in both table grapes cultivars, the higher effects being found for 1 mM (Magenta) and 0.1 mM (Crimson) MeJa treatments (**Publication 1** and **Table 5**). On the other hand, the arils hue angle at harvest was significantly lower in fruit from 1, 5 and 10 mM MeJa-treated trees than in controls, showing a deeper red colour in the arils of MeJa treated fruits, especially for 5 mM dose in which the lowest hue value was obtained. These differences among MeJa treatments on red colour of the arils were also observed on total anthocyanin content, which was higher in arils from 5 mM MeJa-treated pomegranate fruit at harvest and during 60 days of storage at 10 ºC (**Publication 2** and **Table 5**). Moreover, the concentration of antioxidant compounds (phenolics, individual anthocyanins and ascorbic acid) and total antioxidant activity were higher in arils from pomegranate treated fruit than in controls, at harvest and during storage at 10 °C, a non-chilling temperature, the highest concentrations being also found with 5 mM dose (**Publication 2**).

When pomegranate fruits were treated with salicylates in preharvest, the hue angle in the husk and arils was significantly lower in all treated fruits with respect to control ones, showing a deep red colour of both husk and arils because of treatments, although no significant differences were observed among treatments. In this sense, SA, ASA, and MeSa treatments also induced a significant increase on total phenolics and anthocyanin concentrations in pomegranate arils, which was in general dose-dependent. The best results obtained in the 2017 experiment were achieved with 10 mM dose of the three assayed compounds, which was chosen for the storage experiment in 2018, and especially with SA (**Publication 4** and **Table 5**). During the whole storage period (90 days of storage at 10 °C), anthocyanin concentration was also higher in arils from all treated fruits than in controls, the highest increase being found in arils of SA and ASA treated fruits. In conclusion, the effects of salicylate treatments on increasing total and individual anthocyanin concentration in pomegranate arils, mainly with SA at 10 mM, led to arils with a deeper red colour and, in turn, fruit that would be more appreciated in the international market.

Total anthocyanin concentration at harvest was also significantly increased by preharvest salicylate treatments in both table grape cultivars, and the highest concentrations of these pigments were found for 0.01 mM SA, 0.1 mM ASA and 0.1 mM MeSa treatments in 'Magenta' cultivar and for 0.01 mM SA, 1 mM ASA and 0.1 mM MeSa in 'Crimson' table grapes (**Publication 5** and **Table 5**). Thus, total anthocyanin content during 45 days of storage at 2 °C was measured in table grapes from the treatments cited above and results showed increases during storage, with higher concentrations in berries from treated vines than in those from control ones until the last sampling date. Based on these results, MeSa at 0.1 mM was the most

effective salicylate treatment increasing berry colour for both table grape cultivars at harvest and during cold storage.

With respect to OA preharvest treatments of pomegranate fruit, results indicated that the husk red colour increased as a consequence of these treatments, leading to improved external red colouration of 'Mollar de Elche', which is an important quality trait for this cultivar in terms of market acceptance and early harvest. As we well know, the red colour of pomegranate husk and arils are a consequence of anthocyanin biosynthesis and accumulation in the cells and is directly influenced by the amount and composition of anthocyanins presented. Our results show that preharvest treatments with OA, especially applied at 10 mM, increased the total phenolics and total anthocyanins content (**Publication 7** and **Table 5**). Finally, berry colour, expressed as hue angle, was improved at harvest and during postharvest cold storage in 5 mM OA-treated table grapes. This fact is related with the effect on increasing the total and individual anthocyanins content in both whole fruit (flesh + skin tissues) and skin tissue by applying OA treatments at preharvest (**Publication 8** and **Table 5**). In general, this content was mainly improved in the skin tissue of berries.

Accordingly, similar results have been reported by other authors about the effect of these elicitors as pre- or postharvest treatments on increasing the colour or promoting anthocyanin biosynthesis and accumulation in fruit and vegetables, as discussed below. For instance, treatments with MeJa at 0.05 and 0.1 mM 30 days before harvest also promoted anthocyanin biosynthesis in peach by increasing the expression of genes codifying enzymes involved in anthocyanin biosynthesis pathway (Wei et al., 2017). As postharvest treatment, MeJa at 1 and 0.1 mM promoted anthocyanin accumulation by up-regulating related genes while 10 mM inhibited anthocyanin biosynthesis in apple fruit (Feng et al., 2017).

On the other hand, SA, ASA, and MeSa applied as preharvest treatments on sweet cherry and plum trees increased fruit total phenolic and anthocyanin concentrations at harvest with respect to controls, and these differences were maintained during cold storage (Giménez et al., 2014, 2015, 2017; Martínez-Esplá et al., 2018). Accordingly, SA preharvest treatments of vine led to higher levels of these bioactive compounds in table grape 'Flame Seedless' at harvest and during postharvest storage (Champa et al., 2015). On pomegranate fruit, postharvest treatments with SA, ASA or MeSA have been reported to maintain total phenolics, anthocyanins, and antioxidant activity at higher levels than in control fruit during cold storage (Sayyari et al., 2011a, b) as well as SA and ASA on sweet cherry (Valero et al., 2011), and SA on cornelian cherry fruit (Dokhanieh et al., 2013) and apricot (Wang et al., 2015e). These enhancements have been attributed to an increase in the activity of phenylalanine ammonia lyase (PAL), which is the main enzyme involved in the biosynthetic phenolic pathway (Sayyari et al., 2011a, b; Valero et al., 2011; Dokhanieh et al., 2013; Wang et al., 2015a).

Similar results inducing accumulation of phenolic and anthocyanins at harvest have also been found in other climacteric and non-climacteric fruits when OA was applied at pre- or

postharvest stages (Valero et al., 2011; Martínez-Esplá et al., 2014b, 2019; Deng et al., 2015; Razzaq et al., 2015; Razavi and Hajilou, 2016; Zhu et al., 2016; Kok and Bal, 2019), leading to fruits with higher antioxidant capacity. The mechanism by which OA increased the bioactive compounds and antioxidant properties is not well understood (Razzaq et al., 2015) and deserves further research, although it could be also attributed to the activation of PAL activity (Deng et al., 2015; Razavi and Hajilou, 2016; Martínez-Esplá et al., 2019). Contradictorily, no stimulation of this enzyme was observed by the OA treatment in a previous report (Zheng et al., 2012). In this sense, the higher content of total and individual anthocyanins in table grapes treated with OA could be related with endogenous ABA accumulation, as reported by Keller et al. (2015) and Shahab et al. (2020). According to our results, we hypothesize that OA could improve berry colour throughout the on-vine ABA metabolomic stimulation, mediated at the same time by the homeostasis of its catabolites and transcribed by the *Vv*NCED1 gene, as discussed later. Nevertheless, for better understanding of the possible mode of action, more molecular, metabolomic or proteomic studies are needed.

A comparative fold increase analysis among the preharvest treatments tested in the present PhD Thesis on total anthocyanin content of table grape and pomegranate fruit at harvest has been carried out as a tool to decide which is the most effective in terms of fruit colour improvement, since this is the overall aim (**Table 5**). Based on this comparative study, OA preharvest treatment at 10 mM showed a significant 1.46-fold increase on total anthocyanin content of arils at harvest in 'Mollar de Elche' pomegranate fruit, followed by SA at 10 mM which increased this content 1.28-fold than control fruits. Accordingly, OA treatment applied at 5 mM was one of the most effective treatments enhancing total anthocyanin concentration in 'Magenta' table grape with a 1.99-fold increase with respect to control berries. Nevertheless, 0.1 mM MeSa-treated berries also showed a significant 2.00 and 2.11-fold increase on this content in 'Magenta' and 'Crimson' cultivars, respectively, compared to control grapes. Thus, 5 mM OA and 0.1 mM MeSa could be useful preharvest tools to improve colour in these poorly coloured table grape cultivars, leading to an equal increase on colour of the whole berry (flesh + skin tissues).

Although in terms of improving total anthocyanins content in the skin tissue of table grapes, where these coloured pigments are mainly synthesized and concentrated, MeSa preharvest treatment showed a significantly lower effectiveness increasing this content than OA (1.5-fold increase *vs.* 2.4-fold increase in MeSa and OA-treated berries skin, respectively, than control berries skin) (**Publication 6** and **Publication 8**). Therefore, OA preharvest treatment could be the most effective elicitation strategy in preharvest compared to other plant growth regulators tested in the present PhD Thesis (MeJa, SA, ASA or MeSa) on improving quality traits, mainly in terms of colour, on table grape and pomegranate fruit.

Furthermore, the most effective preharvest treatments increasing colour of pomegranate arils; 5 mM MeJa, 10 mM SA and 10 mM OA, were also the most effective ones improving

pomegranate crop yield (≈1.40-fold increase of kg harvested per vine than control pomegranate trees) (**Publication 2**, **Publication 4** and **Publication 7**). On the other hand, MeJa 0.1 mM, MeSa 0.1 mM and OA 5 mM were the most effective in terms of improving berry colour on both table grape cultivars and, in addition, all these treatments increased crop vine compared to control vines in a cultivar-dependent manner: 1.3 and 1.1-fold increase in 0.1 mM MeJa-treated vines for 'Magenta' and 'Crimson', respectively; 1.2 and 1.1-fold increase in 0.1 mM MeSa-treated vines for 'Magenta' and 'Crimson', respectively; 1.5-fold increase in 5 mM OA-treated vines for 'Magenta' cultivar (**Publication 1**, **Publication 5** and **Publication 8**). Therefore, OA preharvest treatment applied at its proper concentration according to the fruit species could be an effective tool to improve crop yield in addition to fruit colour.

5.2. Effect of elicitation strategies on crop yield

MeJa treatments affected grape ripening process and vine yield differently depending on applied concentration (**Publication 1**). In the experiment performed in 2016, MeJa 1 mM treatment accelerated the berry ripening process in both cultivars, since vine yield (kg harvested per vine) was higher than in control vines at the first and second harvest dates. On the contrary, a delay in the ripening process was observed for grapes treated with MeJa 5 and 10 mM, this effect being dose-dependent. In addition, MeJa treatments at 1, 5 and 10 mM decreased total yield in both cultivars, the effect being dose-dependent and higher in 'Crimson' than in 'Magenta' cultivar. In the view of these results, treatments with MeJa at 0.01, 0.1 and 1 mM were applied in 2017 and all of them accelerated grape ripening since at the first harvest date more kg of grapes were harvested from all MeJa-treated vines with respect to controls, the effect being significantly higher as increased MeJa concentration from 0.01 to 1 mM. Nevertheless, as in 2016 experiment, MeJa 1 mM decreased vine total yield. However, concentrations of 0.01 and 0.1 mM increased total yield with respect to control vines, without significant differences between both doses. Not only MeJa treatments affected vine yield but also berry size and weight since both were reduced by 1, 5 and 10 mM MeJa treatments, in both cultivars, and in a dose-dependent way while increases in size and weight were obtained with 0.1 and 0.01 mM treatments.

Contradictorily to the effect of MeJa-tested concentrations on table grape crop yield in 2016 and 2017 seasons, similar results were obtained about the effect of MeJa treatments on pomegranate crop yield in both seasons (**Publication 2**). Preharvest MeJa treatments at 1, 5 and 10 mM increased pomegranate crop yield compared to control trees. This increase was due to the number of fruit that were harvested per tree which was significantly increased by 1, 5 and 10 mM MeJa, while no significant effects were observed in fruit mass attributed to MeJa treatments. MeJa applied at these concentrations could have reduced the abscission process during fruit growth and ripening on tree together with an increment in the plant photo-assimilates available to support fruit growth. Nevertheless, similar effects of preharvest MeJa treatments to those obtained in on-vine ripening have been found in pomegranate crop, although with different MeJa concentrations. Thus, the on-tree growth and ripening processes were accelerated by 1 and

5 mM MeJa treatments since more pomegranate fruits had reached their commercial harvesting attributes at the first harvest date, while the contrary occurred with 10 mM MeJa treatment. The effect of 1 and 5 mM MeJa treatments on advancing pomegranate fruit ripening has important economic implications since early harvested fruit usually reach higher prices in the market.

With respect to salicylates treatments, in the 2017 experiment, pomegranate trees were treated with SA, ASA, and MeSa at 1, 5, and 10 mM, the application method and key moments being similar to those explained in MeJa treatments, and a higher crop yield (kg tree^{-1} and number of harvested fruit tree^{-1}) at harvest was obtained (**Publication 4**). On table grape, these treatments were applied in 2016 and at 1, 0.1 and 0.01 mM concentration in 2017, since in general, salicylates at 5 and 10 mM decreased vine yield and delayed berry ripening process, according to the effect observed with MeJa preharvest treatments on crop yield and on vine-ripening process, which could be hastened by lower doses (**Publication 5**). Results demonstrated that treatments with salicylates affected vine yield and grape ripening depending on the applied compound, concentration and cultivar. Thus, grapevine treatment with SA or ASA at 5 and 10 mM led to a significant decrease on total yield of 'Magenta' while these effects only were significant for 10 mM dose on 'Crimson' in 2016 experiment. The effects of salicylate treatments at high doses on reducing total yield were attributed to a delay or inhibition on the ripening process since many berries failed to ripen properly and some clusters did not reach the requested commercial quality and were discarded. However, when lower doses of SA and ASA were applied, the ripening process was accelerated, as was observed for SA and ASA at 0.1 and 0.01 mM in 'Magenta' and at 1 mM in 'Crimson'. On the contrary, all the applied MeSa concentration, except 0.01 mM, accelerated the ripening process in 'Magenta' and except 10 mM in 'Crimson', although for both cultivars the highest effect was found for 0.1 mM MeSa. Thus, to accelerate the ripening process and achieve higher prices at market the most appropriate concentration of each salicylate should be established for each particular table grape cultivar.

On the other hand, our results show different effects of salicylate treatments on crop yield depending not only on applied doses but also on cultivar. Thus, in 'Magenta' cultivar, SA, and ASA at 0.1 and 0.01 mM and MeSa at 0.1 mM increased total yield significantly while it decreased by 5 and 10 mM SA and ASA doses. However, in 'Crimson' total yield was significantly reduced by SA and ASA at 10 mM but not significantly enhanced by any of the salicylate treatments. The increase in yield in 'Magenta' was due to an increase on berry volume of 5 %, 7.6 % and 13 % as a consequence of 0.01 mM SA, 0.1 ASA and 0.1 MeSa treatments, respectively. It is worth noting that as clusters with higher berry size are more appreciated by consumers and reach higher prices at markets than small ones. In this sense, these treatments could increase economic benefit of this crop, apart from their effects on increasing total yield. Finally, results of the present research show that a lower SA concentration could be even more effective to increase yield in the 'Magenta' cultivar, although it is not applicable to all table grape cultivars because no significant increases on yield were observed in 'Crimson' for the wide range of SA doses assayed.

Harvesting of 'Mollar de Elche' pomegranate fruit is based on fruit husk colouration and increase in red husk colour will allow early harvest. All OA concentrations used in the 2016 and 2017 experiments promoted the on-tree ripening process and improved early ripening and early harvest of this cultivar, 10 mM OA being the most effective (**Publication 7**). In the **Publication 8**, OA preharvest treatment was applied at 5 mM in 'Magenta' table grapes, as the best concentration in terms of yield, berry maturity-quality and bioactive compounds (data not shown). This concentration was chosen as the optimum among three concentrations tested (1, 5 and 10 mM) in previous experiments (growing seasons 2016 and 2017).

In 2016 and 2017, OA was applied at 1, 5 and 10 mM concentrations and cluster were harvested when berries reached colour, size and TSS content characteristic of this cultivar, according to commercial criteria. For both growing cycles, 5 mM OA accelerated the on-vine berry ripening process since higher amount of cluster were harvested from these OA-treated vines at the first harvest date, while no significant effects were observed for 1 or 10 mM concentrations. In addition, it is worth noting that accumulated yield at the last harvesting date was significantly higher in 5 mM OA treated vines with respect to controls, while yield was not affected by 1 mM OA treatment and was reduced by 10 mM OA treatment. Thus, OA 5 mM was chosen as the best dose for the 2018 experiment, and similar results were obtained in term of accelerating the on-vine ripening and increasing vine yield (**Publication 8**). The effects of 5 mM OA treatment on increasing vine-yield were due to an enhanced berry volume and the reduction in yield observed in 10 mM OA treatment to a lower berry size. In addition, in 'Magenta' cultivar, the ripening process of the berries within a cluster is also heterogeneous, and then, berries not fully coloured are cut and discarded after harvesting by operators in the field. These poorly coloured berries were reduced in clusters from 5 mM treated vines, which also contributed to increase vine yield, apart from the effect on increasing berry size. In this sense, our results showed that 5 mM OA preharvest treatment significantly increased the berry volume (mm^3) by 11 %, 16 % and 13.5 % in 2016, 2017 and 2018 seasons, respectively, than control berries.

Generally, the effects of elicitation treatments on vine yield were due to their effect on berry size which were different depending on applied compound and concentration, without affecting the number of berries per cluster. Similar results increasing pomegranate crop yield with MeJa at 0.5 mM have been obtained in 'Rabab' cultivar by Asghari et al. (2020). The effect of MeJa on fruit size has been published in a limited number of papers and contradictory results have been observed depending on fruit species, applied doses and fruit development stage (Rudell et al., 2005; Ziosi et al., 2008; Ruiz et al., 2013; Martínez-Esplá et al., 2014a). It is interesting to note that 10 mM MeJa concentration applied before veraison reduced the grape ripening process while when applied at veraison this effect was not observed in previous experiments with wine grapes cultivars (Portu et al., 2015, 2018a, 2018b; Gómez-Plaza et al., 2017). Moreover, this process can be accelerated by applying lower concentration. However, the molecular mechanism involved in these effects deserves further research.

On the other hand, the increase in fruit number by salicylate treatments could be due to: (i) an increased flowering rate, (ii) an increased rate of set fruits, or (iii) a decrease in fruit abscission. However, in our experiments, treatments were performed when fruit had reached *ca.* 30 % of their final size so that flowering or fruit set were not affected and the increase in fruit number was due to the reduction of fruit abscission that naturally occurs during the fruit developmental process. The results about crop yield prove that treatment with salicylates increases net photo-assimilate production in plants and/or the sink strength of developing fruits. In addition, given the pivotal role of SA on increasing tree tolerance to environmental stresses (Tiwari et al., 2017; Koo et al., 2020), its effect allowing pomegranate tress to overcome drought and high temperature stresses during summer seasons in the Spain Southeast cannot be discarded. In fact, maximum mean temperatures in summer, from June to September, were very high in the field crop, ca. 31.5 °C in both years. On the other hand, it is worth noting that the percentage of fruits harvested in the first picking date was higher in 5 and 10 mM salicylates-treated pomegranate trees and in all ASA-treated ones than in controls. This effect could be attributed to an increase of net photosynthesis and/or sink strength induced by treatment with salicylates previously commented (Fariduddin et al., 2003; Elgamaal and Maswada, 2013; Ghasemzadeh and Jaafar, 2013).

Results showed that OA treatments increased total crop yield due to an increase in the number of fruits that were harvested from each tree, which had a similar mass, independently of the applied concentration. However, in both experiments, treatments were performed when fruit had reached *ca.* 30 % of their final size so that flowering or fruit set were not affected and the increase in fruit number was due to the reduction of fruit abscission that naturally occurs during the fruit developmental cycle due to wind or other environmental factors, as previously discussed. In addition, results of the yield on the first harvest date showed that OA treatment hastened the time of harvest in a concentration-dependent manner since the largest number of fruits were harvested in the first harvest, leading also to a greater yield in kg tree^{-1} than non-treated trees. This effect of OA on increasing crop yield and promoting early harvest could be related with an increase in the abundance of RuBisco activase enzyme which could lead to an increase in photosynthesis rate as a consequence of OA treatment (Wang et al., 2009b). However, metabolomic studies are needed to fill this knowledge gap. Finally, the effect of OA preharvest treatment on increasing berry size has also been reported in sweet cherry cultivars, 'Sweet Heart' and 'Sweet Late', by Martínez-Esplá et al. (2014b), manifested by higher fruit volume and weight in cherries from treated trees than from controls, the higher effect being found with 2 mM OA.

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

Skin colour, fruit firmness, TSS, and TA are indicators of fruit quality (Nuncio-Jáuregui et al., 2014; Pareek et al., 2015). Respect to fruit quality traits, all preharvest treatments included in

the present PhD Thesis increased these parameters at harvest and maintain them during postharvest storage experiments for both fruit species. In addition, all the treatments tested enhanced fruit bioactive compounds from harvest until consumption. Specifically, preharvest MeJa treatments at 0.1 mM led to increase table grape organoleptic quality parameters, such as size, weight, firmness and TSS, as well as the bioactive compound content, leading to berries with increased health properties, highly appreciated by consumers (**Publication 1**). For the first time, our results have reported that MeJa treatments (at 1, 5 and 10 mM) applied during on-tree 'Mollar de Elche' pomegranate fruit growth delayed the postharvest ripening during storage at 10 °C, manifested by lower losses in fruit weight, firmness and organic acids, leading to fruit quality maintenance (**Publication 2**).

The best results in terms of fruit quality parameters and the concentration of phenolics, anthocyanins, and ascorbic acid among salicylate treatments applied on pomegranate fruit were achieved with 10 mM dose of the three assayed compounds (SA, ASA and MeSa), since these quality and functional traits were maintained at higher levels in pomegranate fruit from treated trees than in controls during prolonged storage at 10 °C (**Publication 4**). Similarly, results of hue angle show that salicylate treatments improved colour appearance in 'Magenta' and 'Crimson' table grapes, since the lower values of hue angle were obtained in salicylates-treated berries, either at harvest or during storage, show deeper red and purple colours which were due to increases in anthocyanin biosynthesis. In addition, these treatments significantly enhanced the bioactive compound content in the treated berries. These effects would be even higher after prolonged cold storage since, in general, the highest differences among control and treated berries in total phenolic and total anthocyanin concentrations were found at the last sampling date. With respect to other quality parameters, the evolution of weight loss, TSS and TA during storage was delayed and berry firmness was maintained at higher levels in salicylate-treated grapes of both cultivars during storage (**Publication 5**).

Respect to berry maturity-quality parameters, preharvest treatment significantly affected the content of TSS and TA at harvest (**Publication 5**). For both cultivars, MeSa at 0.1mM significantly increased the content of both TSS and TA with respect to control grapes, while a reduction in TSS was observed for 'Crimson' and 'Magenta' treated with ASA 1 and 0.1 mM, respectively. However, preharvest treatment with SA did not show significant differences comparing with control berries. Similarly, the ripening index or ratio TSS/TA revealed that ASA treatment induced a delay of ripening process. Similarly to TSS, glucose and fructose were enhanced in grapes treated with MeSa and reduced in those treated with ASA. On the other hand, the concentration of organic acids was different depending on treatment and cultivar. All treated grapes with salicylates showed the maximum concentration of tartaric acid. However, with respect to ascorbic, succinic and fumaric acids, only grapes treated with MeSa at 0.1 mM has significant higher concentrations compared with controls and other treated grapes. Interestingly, from these results it can be highlight the increase of ascorbic acid or vitamin C.

OA preharvest treatment reduced the respiration rate at harvest in comparison to untreated fruit. This effect on decreasing the respiration rate at harvest would indicate an effect of OA on reducing the cell metabolism rate on tree, which, in turn, could be attributed to a lower metabolic activity induced by the treatment. On the other hand, OA treatment increased TSS content and TA without delaying or promoting the ripening index (RI) of pomegranate fruit at harvest for both seasons (**Publication 7**). In this work, physico-chemical data and sensory evaluation proved that pomegranate fruit treated with OA at 10 mM had appropriate sensory characteristics and higher overall quality than the control fruit. Finally, berry firmness at harvest was significantly higher in 5 mM OA-treated table grapes than in controls. Significant decreases occurred in firmness values during storage at 2 °C, although the effect of OA delaying firmness losses was maintained during postharvest storage, retarding softening and postharvest ripening (**Publication 8**).

The effect of elicitation treatments on enhancing TSS and sugar content on table grapes could be due to an increase of both the net photosynthetic rate of vine and the sink strength of berry cells which would lead to increase sugar accumulation, leading to enhance berry volume and weight. In turn, these treatments would increase available photo-assimilates to support fruit growth. The reduction on respiration rate by the exogenous applications may be associated with the corresponding reduction in ethylene production through inhibition of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity, according to Razzaq et al. (2015). On the other hand, the effect of these elicitors, mainly OA, on delaying fruit softening process during storage has been attributed to a reduction in the activity of cell wall hydrolytic enzymes, such as exo-polygalacturonase (exo-PG) and pectin methyl-esterase (PE) in mango and plum fruits treated in postharvest (Wu et al., 2011; Razzaq et al., 2015). In this sense, we hypothesize that this possible reduction on these two-cell wall hydrolytic enzymes, exo-PG and PE, could be in turn related with the higher activity of antioxidant enzymes mediated by these treatments, which could delay the senescence process in the fruit. The enhance of the antioxidant systems could lead to efficient scavenging of ROS which are generated during fruit ripening, a process considered as a functionally modified prolonged form of senescence (Hodges et al., 2004). Furthermore, the formation of oxalate-pectin as a result of OA treatment (Razzaq et al., 2015), leading to reinforcing wall structure of mesocarp cells and slowing down the softening process, could be associated to the effect of this treatment.

The effectiveness on reducing chilling injury incidence, as a fruit physiological disorder, by MeJa treatments in pomegranate fruit has been studied (**Publication 3**). Thus, MeJa was applied in preharvest (Pre) and in a combined treatment of pre- and postharvest (Pre + Post) at 5 mM concentration, which showed the best results in terms of improving fruit quality, mainly colour, and crop yield. Based on the results obtained, both Pre and Pre + Post MeJa treatments would have an effect on maintaining membrane structure since lower CI symptoms and ion leakage (IL) values were observed in treated fruit in comparison to the control. It was found that both, external and internal CI indexes were correlated with IL ($r^2 = 0.823$ and 0.733, respectively)

by considering data of control and treated fruit for all sampling dates. Thus, IL could be used as an indicator of CI and cell membrane integrity, according to previous reports in a wide range of fruit species including pomegranate (Sayyari et al., 2011a; Ehteshami et al., 2019). However, significant differences between Pre and Pre + Post MeJa treatments were observed only in internal CI while external CI and ion leakage were similar in both treatments. Thus, for practical purposes in order to reduce CI, MeJa preharvest treatments could be enough, with similar effects on reducing CI than the postharvest treatments previously reported (Sayyari el al., 2011a).

It is well known that damage of the membrane structure and subsequent changes in lipid constituents is correlated with the occurrence of CI. These changes in lipid composition show mainly decrease in the UFA/SFA ratio. This could be affecting the phase transition of membrane lipids from a liquid-crystalline to a solid-gel state, and in turn leading to membrane peroxidation and damage, accelerating the occurrence of CI (Wongsheree et al., 2009). Cao et al. (2009) showed that postharvest treatment with MeJa of loquat fruit significantly reduced CI and maintained higher UFA/SFA ratio during storage, suggesting that MeJa induced CI tolerance in fruit tissues by reducing losses in UFA and maintaining a higher UFA/SFA ratio. Our results are in agreement with this previous report and show that MeJa reduced CI throughout maintenance of cell membrane stability. These effects could be described as a mechanism of acclimation to low temperature and would account for maintenance of membrane semi-permeability, leading to lower losses of intracellular water, ions and metabolites and, in turn, being responsible for the lower weight loss, IL and CI index scores found in MeJa-treated fruit. For the first time, we identified twelve new fatty acids in the pomegranate husk.

Secondly, and based on the chosen concentrations for salicylates treatments, in which the best results for these treatments in terms of yield, berry maturity-quality and bioactive compounds were obtained, preharvest treatments with salicylates were also performed in 2018 in order to find out their effects on the incidence and severity of decay on table grapes inoculated with *Botrytis cinerea*, as well as the possible mechanism of action involved in the alleviation of this fungal disease (**Publication 6**). Thus, MeSa and SA were applied at 0.1 and 0.01 mM for both cultivars, while ASA was applied at 1 mM in 'Crimson' and 0.1 mM in 'Magenta'. In this experiment, grapes were artificially injured and inoculated by spraying them with 7500 UFC mL^{-1} of *Botrytis cinerea* suspension, and the berries were ranked for visual decay incidence after 5 days of inoculation. For both cultivars, similar results were obtained. The lowest decay incidence was obtained in those berries treated with MeSa. The ASA treatment also showed good control of *Botrytis cinerea* disease, but with lower performance than in MeSa-treated grapes. Our results clearly demonstrated that preharvest treatment with salicylates, and specially MeSa, were able to induce resistance of table grape to be colonized by *B. cinerea*.

Furthermore, all salicylates preharvest treatments enhanced the concentration of total phenolics, total anthocyanins and total antioxidant activity (TAA) due to hydrophilic (H-TAA) and lipophilic compounds (L-TAA) in the grape skin for both cultivars. It is worth noting that

no significant differences were observed among SA, ASA and MeSa-treated berries on phenolic content and H-TAA. According to the results obtained about total anthocyanins, as expected, all treated grapes showed significant higher concentrations in the major individual anthocyanins than controls. On the other hand, the activity of the antioxidant enzymes APX, CAT and POD in the skin of the two grape cultivars at harvest was significantly enhanced due to all salicylate treatments. This effect was especially important for MeSa-treated berries which had the highest levels of APX, CAT and POD while control grapes showed the lowest activities. Therefore, two possible mechanisms of action involved in the induced resistance effect against *Botrytis cinerea* are supported by data in this publication, that is the increase of phenolic compounds, including anthocyanins, and the greater activity of the antioxidant enzymes. The increase in total phenolic compounds has been regarded as an indicator for plant defence response since they are able to scavenge free radicals (Beckman, 2000). ROS are accumulated in plants under pathological and senescence conditions, leading to oxidative injury. It is well-known that APX, CAT and POD are the key enzymes involved in ROS elimination, and the decrease in them may lead to high levels of ROS, as has been shown in control grapes. Therefore, the higher levels of antioxidant enzymes in treated berries could be involved in fruit resistance against *Botrytis cinerea*.

In the last publication included in the present PhD Thesis, the role of OA preharvest treatment improving colour and quality of 'Magenta' table grape has been studied through a metabolomic approach, including ABA metabolism and antioxidant system, studying the relative *Vv*NCED1 gene expression (**Publication 8**). The OA treatment application significantly increased the ABA content at 3 days after treatment 1 (3DAT1) stage compared to control grapes, whilst its effect was maintained at 3 days after treatment 2 (3DAT2) or veraison stage, but to a lesser extent. These biochemical changes may be supported by the up-regulation of *Vv*NCED1 gene by OA treatment at 3DAT1 and 3DAT2 stages. However, the 2.7-fold increase of ABA content in OA-treated berries at 3DAT1 showed a lack of correlation with the 2-fold up-regulation of *Vv*NCED1 expression and the ABA-GE or other catabolites content at this developmental stage. In contrast to the results obtained at 3DAT1, the relative *Vv*NCED1 gene expression showed a concomitant increment (≈ 7-fold increase) in OA-treated berries at the onset of ripening or 3DAT2 stage. Thus, we suggest that the greatest ABA content in the OA-treated grapes at 3DAT1 began prior to the highest up-regulation of endogenous ABA biosynthesis observed at 3DAT2 and mediated by the relative expression of the rate-limiting *Vv*NCED1 gene. In this sense, this increment on ABA levels could be supported by an ABA-translocation from leaves to berries, accordingly with others authors (Shiozaki et al., 1999; Keller et al., 2015), as a physiological plant response to OA preharvest treatment.

On the other hand, the 7-fold increase on relative *Vv*NCED1 gene expression of OA-treated berries at 3DAT2 stage was not proportionally correlated with the 10 % increase on ABA content at this developmental stage. Thus, values of free ABA at 3DAT2 developmental stage could be regulated by different metabolic pathways: 1) higher conjugation of ABA with glucose in treated berries, increasing ABA-GE content which acts as a reservoir of ABA; and 2) higher

ABA catabolism at the position 8′, leading to a higher PA content, which is biologically active and serves as a bona fide ABA-like phytohormone. However, very similar levels of ABA-GE and ABA catabolites were observed at 3DAT2 between both treatments. Thus, although there is no available information reporting ABA translocation from table grape berries to other parts of the vine, including the leaves, it is highly probable according to the reported metabolomic and molecular data that OA preharvest treatment leads to a dynamic balance or redistribution of berry-derived ABA through the phloem into the aerial parts of the vine. Increasing evidence indicates that various plant membrane transport systems play a significant role in adaptation to stress conditions, such as drought. The role of various transport systems in ABA translocation, stomatal, cuticular, and root responses, as well as osmotic adjustment has been summarized by Jarzyniak and Jasiński (2014). In contrast, other reports have shown that *Vv*NCED1 gene was up-regulated by ABA and ethephon treatments (Pilati et al., 2017; Suehiro et al., 2019), explaining the intracellular ABA level measured by Pilati et al. (2017). In relation to colour development, previous studies (Castellarin et al., 2016) showed that a disruption of phloem transport of ABA to berry clusters occurred (induced through girdling), prior to the onset of ripening, leading to completely inhibition of colour development. Therefore, we concluded that OA preharvest treatment improves colour and quality of 'Magenta' table grape upregulating on-vine ABA metabolism and relative *Vv*NCED1 gene expression.

During the stress response, plant cells exhibit defence mechanisms to detoxify the synthesized ROS including enzymes such as superoxide dismutase (SOD), CAT, POD and APX. SOD converts $O2^{\bullet -}$ to H_2O_2, which could be finally eliminated by CAT, POD and APX activities (Hodges et al., 2004). In general, these antioxidant enzymes showed higher activity at three studied stages in table grapes from OA-treated vines than in controls. Our results suggest that preharvest application of OA 5 mM could delay the senescence of treated table grapes in postharvest throughout the upregulation of these antioxidant systems during the developmental cycle and their maintenance during storage. Finally, a significantly lower incidence of *Botrytis cinerea* was observed in berries from OA-treated vines than non-treated vines. OA contributes to induce systemic resistance in plants, which may be due to an increase in both, POD activity and synthesis of new POD isoforms, upregulating defence-related enzymes (Tian et al., 2006). In addition, the effect of OA on decreasing fruit decay incidence might also be attributed to the delay of berry senescence, as well as was reported by Zheng et al. (2007b). In fact, a significantly lower ABA and catabolite content in OA 5 mM-treated berries than in controls were found during 15, 30 and 45 days of postharvest storage at 2 °C. OA-treated berries showed a less advanced stage of senescence than non-treated table grapes. Furthermore, the effectiveness of 5 mM OA and 0.1 mM MeSa reducing the percentage (%) of decayed berries at the most advanced decay stage (S5) was similar (≈ 4-fold decrease) in 'Magenta' table grapes (**Publication 6** and **Publication 8**).

Globally, all the results published within the present PhD Thesis offer us useful tools to solve the problems presented by the studied cultivars of pomegranate fruit and table grape. Mainly, all preharvest treatments are effective for improving the fruit colour and quality at harvest as well as to maintain them during their postharvest storage, reducing the quality losses which usually occurs in these fruit species. Therefore, these preharvest treatments could be considered as a safe, natural and effective solution to increase economic profit of pomegranate and table grape growers and, in turn, those treated fruit would be more appreciated in the markets by the consumers due to their higher sensory quality traits and antioxidant properties.

6

Conclusions - Conclusiones

6. CONCLUSIONS / CONCLUSIONES

CONCLUSIONS

In the present PhD Thesis it has been developed different elicitation strategies in preharvest to increase the quality, mainly fruit colour, and shelf-life on table grape and pomegranate fruit. These eco-friendly strategies could be considered as safe tools with the potential to improve fruit quality attributes at harvest and maintain them during postharvest storage in both crops. Therefore, the **overall conclusions** are:

1. Berry skin colour of 'Crimson' and 'Magenta' table grapes was improved at harvest throught the preharvest application of 1 mM (Magenta) and 0.1 mM (Crimson) methyl jasmonate (MeJa), 0.01 mM salicylic acid (SA), 1 mM (Crimson) and 0.1 mM (Magenta) acetylsalicylic acid (ASA), 0.1 mM methyl salicylate (MeSa) and 5 mM oxalic acid (OA), which accelerated colour evolution during on-tree ripening due to increase anthocyanin biosynthesis in both poor-coloured cultivars. According to overall results obtained, the most effective one increasing berry skin colour was oxalic acid applied at 5 mM concentration.

2. Arils colour of 'Mollar de Elche' pomegranate fruit was improved at harvest throught the preharvest application of 5 mM methyl jasmonate (MeJa) and salicylic acid (SA), acetylsalicylic acid (ASA), methyl salicylate (MeSa) and oxalic acid (OA) at 10 mM concentration, mediated by an increased concentration of total and individual anthocyanins. Based on the final results, the most effective one increasing pomegranate arils colour was oxalic acid applied at 10 mM concentration.

In addition, some **specific conclusions** have been obtained, as follow:

1. Preharvest treatments with elicitors applied at the best tested concentrations increased yield parameters in both crops.

2. Preharvest treatments with elicitors improved fruit quality traits during postharvest storage, delaying losses of weight, firmness, colour and total acidity.

3. Preharvest treatments with elicitors stimulated the accumulation of bioactive compounds and maintained the functional quality during postharvest storage.

4. Preharvest treatments with elicitors induced chilling injury (CI) tolerance in pomegranate fruit under cold storage conditions, increasing its shelf-life in postharvest.

5. Preharvest treatments with elicitors reduced postharvest fungal decay incidence in table grape and, therefore, the effect increased its shelf-life period throughout the commercialization.

CONCLUSIONES

En la presente Tesis Doctoral se han desarrollado diferentes estrategias de elicitación en precosecha para incrementar la calidad, principalmente el color del fruto, y la vida útil en uva de mesa y granada. Estas estrategias respetuosas con el medio ambiente podrían ser consideradas como herramientas seguras con el potencial de mejorar los atributos de calidad del fruto en el momento de la recolección y mantenerlos duante el almacenamiento en postcosecha en ambos cultivos. Por lo tanto, las **conclusiones generales** son:

1. El color de la piel de la baya de las uvas de mesa 'Crimson' y 'Magenta' fue mejorado en el momento de la recoleccion a través de la aplicación precosecha de jasmonato de metilo (JaMe) 1 mM (Magenta) y 0,1 mM (Crimson), ácido salicílico (AS) 0,01 mM, ácido acetilsalicílico (AAS) 1 mM (Crimson) y 0,1 mM (Magenta), salicilato de metilo (SaMe) 0,1 mM y ácido oxálico (AO) 5 mM, los cuales aceleraron la evolución del color durante la maduración en el árbol debido a un incremento en la biosíntesis de antocianinas en ambas variedades con escasez de color. De acuerdo con los resultados generales obtenidos, el más eficaz incrementando el color de la piel de la baya fue el ácido oxálico aplicado a la concentración de 5 mM.

2. El color de los arilos de la granada 'Mollar de Elche' fue mejorado en el momento de la recolección a través de la aplicación precosecha de jasmonato de metilo (JaMe) 5 mM y ácido salicílico (AS), ácido acetilsalicílico (AAS), salicilato de metilo (SaMe) y ácido oxálico (AO) a la concentración 10 mM, mediado por una mayor concentración de antocianinas totales e individuales. Según los resultados finales, el más eficaz para aumentar el color de los arilos de granada fue el ácido oxálico aplicado a una concentración de 10 mM.

Además, algunas **conclusiones específicas** han sido obtenidas, como se muestra a continuación:

1. Los tratamientos precosecha con elicitores aplicados a las mejores concentraciones ensayadas incrementaron los parámetros de rendimiento en ambos cultivos.

2. Los tratamientos precosecha con elicitores mejoraron las características de calidad del fruto durante el almacenamiento en postcosecha, retrasando las pérdidas de peso, firmeza, color y acidez total.

3. Los tratamientos precosecha con elicitores estimularon la acumulación de compuestos bioactivos y mantuvieron la calidad funcional durante el almacenamiento en postcosecha.

4. Los tratamientos precosecha con elicitores inducieron tolerancia a los daños por frío (CI) en granadas almacenadas en condiciones de frío, incrementando su vida útil en postcosecha.

5. Los tratamientos precosecha con elicitores redujeron la incidencia de podredumbres fúngicas en postcosecha en uva de mesa y, por lo tanto, el efecto incrementó su periodo de vida útil a lo largo de la comercialización.

7

Future Research Lines

7. FUTURE RESEARCH LINES

Based on the results obtained during the development of the present PhD Thesis, new scenarios emerge that could be studied in order to: ***1)*** To transfer the scientific knowledge to agri-food industry and ***2)*** Elucidate the elicitation strategies's role on improving table grape and pomegranate fruit colour from a transcriptomic approach, as follow:

1. To transfer scientific knowledge to agri-food industry. This early future research line is focused on society public funds or CDTI (Centro para el Desarrollo Tecnológico Industrial) projects transferring part of the scientific knowledge obtained in the present PhD Thesis as well as in other parallel research lines of our postharvest group, which also study the use of preharvest treatments with elicitors in other fruits and vegetables, such as lemon, sweet cherry, pepper fruit and artichoke, among others, to industry. The aim is the transference of scientific knowledge; making available tools to solve real problems, knowing the most appropriate concentration of each elicitor or the key moments and type of application, among other issues, which is transferable to the agri-food industry to obtain an economic profit.

2. Further research is needed to fully elucidate the elicitation strategies's role on improving table grape and pomegranate fruit colour from a transcriptomic approach. To achieve this long-distance future research line, the genes involved on the anthocyanins biosynthesis pathways, such as phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), UDP-glucose: flavonoid 3-*O*-glucosyl transferase (UFGT), leucoanthocyanidin reductase (LAR) or anthocyanidin reductase (ANR) enzymes, should be analyzed. The aim is the elucidation of the elicitation effect on improving fruit colour at transcriptomic level, since it has not been possible to address it as an objective of the present PhD Thesis.

8

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