ESCUELA TÉCNICA SUPERIOR DE INGENIEROS AGRÓNOMOS Y MONTES
Programa de Doctorado Interuniversitario en Ingeniería Agraria, Alimentaria, Forestal y del Desarrollo Rural Sostenible

The effect of increasing temperature on olive trees (Olea europaea L. subsp. europaea) biology: An integrated morphological, phenological and biomolecular study

Estudio del efecto del aumento de la temperatura asociado a las condiciones del cambio climático sobre el olivo (Olea europaea L.), mediante el uso integrado de técnicas de fisiología y bioquímica y bioquímica clásica, proteómica, metabolómica y transcriptómica

Memoria de tesis doctoral presentada por
Rosa Sánchez Lucas

DIRECTORES

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TITULO: *The effect of increasing temperature on olive trees (Olea europaea L. subsp. europaea) biology: An integrated morphological, phenological and biomolecular study*

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TÍTULO DE LA TESIS:

Estudio del efecto del aumento de la temperatura asociado a las condiciones del cambio climático sobre el olivo (Olea europaea L.*), mediante el uso integrado de técnicas de fisiología y bioquímica clásica, proteómica y metabolómica*".

DOCTORANDO/A:

Rosa Sánchez Lucas Rosa

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

La presente Tesis Doctoral, ha sido realizada por Rosa Sánchez Lucas, durante los años 2015 a 2019, dentro del "Programa de Doctorado de la Universidad de Córdoba "Ingeniería Agraria, Forestal, Alimentaria y Desarrollo Rural Sostenible ". Ha sido dirigida por los Profesores Jesús V. Jorrín Novo (AGR-164; Bioquímica, Proteómica, y Biología de Sistemas Vegetal y Agroforestal) y María Benlloch González (AGR-174; Arboricultura). Parte del trabajo lo ha realizado en la Universidad de Rennes, bajo la dirección del Profesor Francisco Cabello Hurtado, de la Universidad de Rennes (UFR Sciences de la Vie et de l´Environnement UMR-CNRS 6553 Ecosystemes, Biodiversité, Evolution, ECOBIO). Cumple los requisitos para su presentación en la modalidad "Doctorado Europeo", y atendiendo a esta circunstancia, el manuscrito ha sido escrito en inglés, lo que le dará mayor visibilidad al trabajo.

La trayectoria de Rosa Sánchez Lucas durante este periodo de formación ha sido muy brillante, habiendo logrado excelentes resultados. Ha demostrado una gran capacidad de trabajo, superando con creces los objetivos y plan de trabajo originalmente propuestos. Su grado de formación es óptimo, tanto a nivel científico-técnico como académico. Prueba de ello es su gran *curriculum vitae.* Como dato indicativo señalaremos, su índice RG (Research Gate, https://www.researchgate.net/profile/Rosa_Sanchez-Lucas) de 23,79, está muy por encima de investigadores de su edad. Ha estado a la altura de las expectativas generadas durante sus estudios de Ingeniero Agrónomo y su Master de Producción, Protección y Mejora Vegetal, lo que le hizo merecedora de una beca FPU (FPU14/00186).

El trabajo realizado es de un gran interés, ya que ha abordado el estudio del efecto del aumento de temperatura sobre la fenología del olivo, un cultivo estratégico para nuestra región, un trabajo que nos permitirá predecir los efectos del cambio climático, tal y como se justifica en los Capítulos I y II de la Tesis. Es muy novedoso en su abordaje metodológico (Capítulo III de la

Tesis), abarcando ensayos de campo, cámara de cultivo (Capítulo IV) y laboratorio (Capítulos V, VI y VI). Se ha empleado un diseño experimental apropiado para estudios de campo que permite utilizar como única variable experimental la temperatura, el "Open Top Chambers" u OTC. La gran novedad es la integración de ensayos de campo, con la medida de parámetros morfométricos, con los de fisiología y las más modernas técnicas moleculares, las –ómicas (transcriptómica, proteómica y metabolómica). Un abordaje similar no se ha descrito hasta le fecha en la literatura. El trabajo ha contribuido a la formación de Rosa Sánchez Lucas en áreas muy diversas, desde la Agronomía a la Bioquímica Biología Molecular, pasando por la Fisiología Vegetal. Dicha formación transversal no es muy frecuente ente los estudiantes de doctorado.

Los resultados son de enorme transcendencia, tanto en lo que suponen un avance en el conocimiento de la respuesta de olivo a temperaturas elevadas, como de las consecuencias que se derivan en términos de productividad del cultivo.

Los datos de los ensayos de campo y de cámara de cultivo se recogen en los Capítulos III y IV, respectivamente, y han dado lugar a las siguientes publicaciones: "*An approach to global warming effects on flowering and fruit set of olive trees growing under field conditions", "Global warming effects on yield and fruit maturation of olive trees growing under field conditions", "Effect of moderate high temperature on the vegetative growth and potassium allocation in olive plants"* and *"Effects of olive root warming on potassium transport and plant growth"*. Se ha visto que el aumento de la temperatura del ambiente en 4 °C adelanta tanto la floración como la maduración pero prolonga ambos procesos. Favorece el desarrollo de flores incompletas limitando su capacidad potencial para originar frutos. Disminuye por tanto el número de frutos, es decir la producción del árbol, haciendo además que estos sean de menor tamaño con un menor contenido de grasas debido un retraso en la lipogénesis. Esto lleva asociado un menor contenido de antocianinas de los frutos. Por otro lado los arboles sometidos a temperaturas más altas han resultado ser de mayor tamaño.

Los estudios moleculares del efecto del aumento de temperatura sobre la maduración de la aceituna han constituido un gran reto y supuesto un esfuerzo importante en la optimización de protocolos que permitan el análisis del perfil de RNA (tanscriptómica), proteínas (proteómica) y metabolitos (metabolómica) en un material altamente recalcitrante, siendo este trabajo pionero en este sentido. Los resultados se presentan en los Capítulos V, VI y VII. Dichos manuscritos serán enviados para su publicación inmediatamente después de la defensa de la Tesis a revistas de alto impacto como Journal of Proteomics y Frontiers in Plant Sciences. Los trabajos han sido presentados en diferentes congresos, siendo elegidos como comunicación oral algunos.

A modo de resumen, los resultados presentados indican como un aumento de 4 °C originaron un cambio importante en la composición química de la aceituna. Causando un descenso de los principales componentes de la pulpa de aceituna y, por consiguiente, la calidad de aceite. Se corrobora el descenso de contenido total de lípidos, disminución de flavonoides y terpenos. Así mismo, en estos análisis se corrobora la necesidad de nuevos criterios para la selección del momento de colecta. El color magenta que aparece como consecuencia del incremento térmico

aplicado, no corresponde a nivel de pulpa con el estado de envero, estado asignado por su color de piel.

El número de actividades formativas realizadas por Rosa Sánchez Lucas ha sido muy superior al propuesto y exigido por el propio Programa de Doctorado. Ello demuestra sus enormes ganas de aprender y vocación por la actividad investigadora y universitaria. De cada una de las actividades realizadas ha presentado un detallado informe avalando el excelente aprovechamiento de los mismos.

Finalizaremos este informe señalando que el trabajo de Rosa Sánchez Lucas durante su periodo de doctorado ha transcendido el ámbito de la presente tesis. En correspondencia a los requisitos de la beca FPU que ha disfrutado, ha participado en actividades docentes, con una impartición de 20,21 créditos durante los cuatro años. Ha sido un miembro relevante del grupo de investigación Bioquímica, Proteómica, y Biología de Sistemas Vegetal y Agroforestal. Ha participado en otras investigaciones llevadas a cabo en el grupo, siendo coautora de un buen número de publicaciones. Ha dirigido trabajos fin de grado y tutorizado a estudiantes de otros países que realizaron estancias en el ciado grupo.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 21 de septiembre de 2019

Firma de los directores

Fdo.: Jesús V. Jorrín Novo

Fdo.: María Benlloch González

ABSTRACT

The effect of increasing temperature on the olive tree (*Olea europaea* L. subsp. e*uropaea*) phenology: An integrated morphological, phenological and biomolecular study

This Doctoral Thesis is focused on olive tree (*Olea europaea* L. subsp. *europaea*), the dominant tree crop over large areas of the Western Mediterranean Basin, with a production of 20,872,788 tonnes in 2018. Olive oil is appreciated worldwide because of its chemical composition, nutraceutical value, and organoleptic properties. Like most crops, the olive tree is facing important problems and challenges related to adverse environmental conditions, either biotic or abiotic ones, a situation that can be worsened in a climate change scenario. Hence, understanding the physiological and molecular basis/mechanism of olive tree adaptability and resilience to different environmental stresses, especially to high temperature, is crucial for predicting the consequences of climate warming, for implementing agricultural practices, designing classic or biotechnological breeding programs, and, lately, increasing its production in order to cope with the current demand.

In the present work, both the effect and the responses to an increase of 4 °C above ambient temperature have been studied at phenological, physiological and molecular,-omics, levels, in an attempt to integrate all the results in the novel Systems Biology direction. The use of the –omics techniques (transcriptomics, proteomics, and metabolomics), and its integration with physiological and phenological studies constitute the great novelty of this work. It has required an important effort in optimizing different protocols for transcriptome (RNA isolation and qRT-PCR), proteome (shotgun nLC-MS/MS Orbitrap), and metabolome (UHPLC-MS/MS Q-tof) profiling, together with computational tools for the identification and quantification of the different biomolecules, their functional classification, statistical analysis, clustering and networking. Both newly developed tools and knowledge generated will establish the basis for ongoing and future studies to obtain a better understanding of the molecular mechanisms mediating phenotypes of interest (productive, tolerant to environmental cues, nutraceutical value and the selectionof genotypes more resilient to climate warming).

The manuscript has been organized and presented in nine chapters as follows:

Chapter I summarises the state of the art of the topics covered in this thesis: the experimental system, objectives, hypothesis and methodologies. It starts with the description of the olive tree as a plant species and crop, its economic and cultural importance, problems and challenges related to its cultivation. The chapter continues with the description of, and publications on, environmental stresses with an emphasis on warming temperatures and the

denominated “Climate Change”. Responses and mechanisms of tolerance to temperature stress and climate warming will be discussed. This chapter finishes with the description of the methodology employed and the integration of the different approaches.

Chapter II presents the general and specific objectives of the Thesis.

Chapter III corresponds to two published papers on the warming effect on olive tree phenology:

“An approach to global warming effects on flowering and fruit set of olive trees growing under field conditions”

and

“Global warming effects on yield and fruit maturation of olive trees growing under field conditions”

Both were published in *Scientia Horticulturae*. They present alterations in the flowering and ripening processes, caused by the permanent thermal increase of 4ºC above ambient temperature (AT) applied to adult olive trees (cv. `Picual´) growth underfield conditions. In this chapter, three years were surveyed, analysed and data analysed in relation to climate conditions. In general, earlier and prolonged flowering and ripening were observed under warmer (AT+4ºC) conditions. Temperature increase reduced the quantity of perfect (well- developed) flowers and fruits. A decrease in oil quality-related chemical parameters (fat, phenolics, and anthocyanins) was observed under thermal increase conditions.

Chapter IV corresponds to two published papers on the effect of different temperature regimes in roots and shoots of olive tree seedlings (cv. Arbequina), a plant growth chamber experiment:

“Effect of moderate high temperature on the vegetative growth and potassium allocation in olive plants”

and

“Effects of olive root warming on potassium transport and plant growth”

They were published in *Journal of Plant Physiology*.

The effect of differential thermal 37ºC /25ºC, exposurebetween root and aerial parton growth, and K uptake and transport were evaluated. At 37ºC root, 25ºC shoot, potassium uptake increased. 37 °C in both organs inhibited growth, reduced K uptake and transport, and diminished water content.

Chapters V, VI and **VII** are devoted to the different –omics approaches (proteomics, transcriptomics and metabolomics) employed in the molecular study of the effect and responses to

warming temperature (+ 4ºC) in fruit pulp at three ripening stages: green (0), turning red (called veraison) (3) and purple epicarp (4). A discrepancy between the colouring of the skin, traditionally observed as a reference for phenology studies and harvesting, and the olive pulp's internal ripeness stage and chemical composition has been found. The veraison stage was the one most affected by the temperature increase. Overall, a reduction in the abundance of major compounds related to olive oil quality (lipids, phenylpropanoids, flavonoids, and terpenes) was observed under warming conditions.

Chapter VIII contains an integration of all the data, and a general discussion, with focus on those most relevant parameters from an agronomic point of view, those related to crop productivity, either from a quantitative (fruit production) or qualitative (fruit chemical composition) perspective. Based on the present study, a predictive hypothesis on the olive tree situation and production in a future climate change scenario is presented, proposing alternative management practices to deal with the social demand for this crop.

Chapter IX corresponds to the conclusions.

RESUMEN

Estudio del efecto del aumento de la temperatura asociado a las condiciones del cambio climático sobre el olivo (*Olea europaea* L.), mediante el uso integrado de técnicas de fisiología y bioquímica y bioquímica clásica, proteómica, metabolómica y transcriptómica

Esta Tesis Doctoral se centra en el olivo (*Olea europaea* L. subsp. *europaea*), el cultivo arbóreo dominante en grandes áreas de la cuenca del Mediterráneo Occidental, con una producción de 20.872.788 toneladas en 2018. El aceite de oliva es apreciado en todo el mundo por su composición química, su valor nutracéutico y sus propiedades organolépticas. Como la mayoría de los cultivos, el olivo se enfrenta a importantes problemas y desafíos relacionados con las condiciones ambientales adversas, ya sean bióticas o abióticas, situación que puede empeorar en un escenario de cambio climático. Por lo tanto, comprender la base/mecanismo fisiológico y molecular de la adaptabilidad y resiliencia del olivo a las diferentes tensiones ambientales, especialmente a altas temperaturas, es crucial para predecir las consecuencias del calentamiento climático, implementar prácticas agrícolas, diseñar programas de mejoramiento genético clásicos o biotecnológicos y, en los últimos tiempos, aumentar su producción a fin de hacer frente a la demanda actual.

En el presente trabajo se han estudiado tanto el efecto como las respuestas a un aumento de 4 °C por encima de la temperatura ambiente a nivel fenológico, fisiológico y molecular, -ómico, en un intento de integrar todos los resultados en la nueva dirección de la Biología de Sistemas. El uso de las técnicas de la -ómica (transcriptómica, proteómica y metabolómica) y su integración con estudios fisiológicos y fenológicos constituyen la gran novedad de este trabajo. Se ha solicitado un importante esfuerzo en la optimización de diferentes protocolos de perfiles de transcriptoma (aislamiento de ARN y qRT-PCR), proteoma (Orbitrap de escopeta nLC-MS/MS) y metaboloma (UHPLC-MS/MS Q-tof), junto con herramientas computacionales para la identificación y cuantificación de las diferentes biomoléculas, su clasificación funcional, análisis estadístico, clustering y networking. Tanto las herramientas de nuevo desarrollo como los conocimientos generados sentarán las bases de los estudios en curso y futuros para obtener una mejor comprensión de los mecanismos moleculares que median los fenotipos de interés (productivos, tolerantes a las señales ambientales, de valor nutracéutico y a la selección de genotipos más resistentes al calentamiento climático).

El manuscrito ha sido organizado y presentado en nueve capítulos como a continuación se detallan:

El **capítulo I** resume el "estado del arte" de los temas tratados en esta tesis, el sistema experimental, los objetivos, las hipótesis y las metodologías. Comienza con la descripción del olivo,

como especie vegetal y cultivo, su importancia económica y cultural, problemas y retos relacionados con su cultivo. El capítulo continúa con la descripción y publicaciones sobre el estrés ambiental con énfasis en el calentamiento de las temperaturas y el denominado "Cambio Climático". Se discutirán las respuestas y los mecanismos de tolerancia al estrés de la temperatura y al calentamiento climático. Este capítulo finaliza con la descripción de la metodología empleada y la integración de los diferentes enfoques.

En el **capítulo II** se presentan los objetivos generales y específicos de la Tesis.

El **capítulo III** corresponde a dos artículos publicados sobre el efecto de calentamiento en la fenología del olivo:

"Una aproximación a los efectos del calentamiento global sobre la floración y el cuajado de los olivos que crecen en condiciones de campo".

y

"Efectos del calentamiento global sobre el rendimiento y la maduración de los frutos del cultivo del olivo en condiciones de campo".

Ambos fueron publicados en *Scientia Horticulturae*.

Presenta alteraciones en los procesos de floración y maduración, causadas por un aumento térmico permanente de 4 °C por encima de la temperatura ambiente (AT) aplicada a olivos adultos (cv. `Picual´) en condiciones de campo. En este capítulo se han estudiado, analizado y analizado los datos relativos a las condiciones climáticas durante tres años. En general, se observó una floración y maduración más temprana y prolongada en condiciones más cálidas (AT+4 °C). El aumento de la temperatura redujo la cantidad de flores y frutos perfectos (bien desarrollados). Se observó una disminución de los parámetros químicos relacionados con la calidad del aceite (rendimiento graso, fenólicos y antocianos) en condiciones de aumento térmico.

El **capítulo IV** corresponde a dos artículos publicados sobre el efecto de los diferentes regímenes de temperatura en las raíces y brotes de las plántulas de olivo (cv. `Arbequina´), un experimento de cámara de crecimiento vegetal:

"Efecto de las altas temperaturas moderadas sobre el crecimiento vegetativo y la asignación de potasio en las plantas de olivo".

y

"Efectos del calentamiento de la raíz del olivo en el transporte de potasio y el crecimiento de las plantas"

Ambos fueron publicados en *Journal of Plant Physiology.*

Se evaluó el efecto de la diferencia térmica de 25 °C/37 °C, la exposición entre la raíz y la parte aérea sobre el crecimiento, y la absorción y el transporte de K^+. En la raíz a 25 °C, brote a 37 °C, la absorción de potasio aumentó. 37 °C en ambos órganos inhibió el crecimiento, redujo la absorción de K^+ y el transporte, y disminuyó el contenido de agua.

Los **capítulos V, VI** y **VII** están dedicados a los diferentes enfoques -ómicos (proteómica, transcriptómica y metabolómica) empleados en el estudio molecular del efecto y las respuestas al calentamiento de la temperatura (+4 °C) en la pulpa del fruto en tres etapas de maduración: verde (0), rojo (llamado envero) (3) y epicarpio púrpura (4). Se ha encontrado una discrepancia entre la coloración de la piel, tradicionalmente observada como referencia para los estudios fenológicos y la recolección, y la fase de maduración interna de la pulpa de la aceituna y la composición química. La fase de envero fue la más afectada por el aumento de temperatura. En general, se observó una reducción en la abundancia de los principales compuestos relacionados con la calidad del aceite de oliva (lípidos, fenilpropanoides, flavonoides y terpenos) en condiciones de calentamiento.

El **capítulo VIII** contiene una integración de todos los datos y una discusión general, centrada en los parámetros más relevantes desde el punto de vista agronómico, relacionados con la productividad de los cultivos, ya sea desde el punto de vista cuantitativo (producción de fruta) o cualitativo (composición química de la aceituna). A partir del presente estudio se presentan hipótesis predictivas sobre la situación del olivo y la producción en un futuro escenario de cambio climático, proponiendo prácticas de gestión alternativas para hacer frente a la demanda social de este cultivo.

El **capítulo IX** corresponde a las conclusiones.

“Una a una se cogen las aceitunas”

“One by one the olives are harvested”

INDEX

Chapter I. General Introduction

This Doctoral Thesis is focused on olive tree (*Olea europaea* L. subsp. *europaea*): the dominant tree crop over large areas of the Western Mediterranean Basin, with a production of 20,872,788 tonnes for mill oil purpose and table olives (FAO, 2019). Olive oil is worldwide appreciated, because of its chemical composition and derived nutraceutical value on beneficial effect on the human health.

As for most crops, olive tree is facing important problems and challenges relate to adverse environmental conditions, either biotic or abiotic, a situation that can be worsen in a climate change scenario. Hence, understanding the physiological and molecular basis of olive tree adaptability to different environmental stresses, especially to high temperature, is crucial for predicting the effect of climate warming, implementing agricultural practices, designing classic or biotechnological breeding programs and, lately, to increase its production in order to cope with the current demand. In the present work, the effect and the responses to temperature rising have been studied at the phenological, physiological and molecular, -omics, with the results being integrated using a Systems Biology approach. The use of the –omics techniques, transcriptomics, proteomics and metabolomics, and its integration with physiological and phenological studies constitute the great novelty of the present work. It requested an effort in optimizing different protocols for transcriptome (RNA isolation and qRT-PCR), proteome (shotgun nLC-MS/MS Orbitrap), and metabolome (UHPLC-MS/MS Q-tof) profiling, together with computational tools for the identification and quantification of the different biolmolecules, its functional classification, statistical analysis, clustering and networking. The newly developed tools and the knowledge generated will set the basis for ongoing and future studies to obtain a better understanding of the molecular mechanisms, mediating phenotypes of interest (productive, tolerant to environmental cues, nutraceutical value (Güllüce *et al.*, 2004, Karioti *et al.*, 2011)), and the future selection of genotypes more resilient to the climate warming.

In this introductory chapter, we will make reference to the three elements of a research project: the experimental system (olive tree, *Olea europaea* subsp. *Europaea*), `Picual´ cultivar, the objective (to study the effect on the phenology and production of the temperature raising and the responses to it at the molecular level), and the methodology (physiological parameters, classic biochemical and –omics approaches). Special attention will be paid on the fruit. The structure of this `General Introduction´ will start with the olive tree taxonomy and morphology, continuing with the phenology (vegetative growth, flowering and ripening). Stress conditions as the main cause of the crop productivity reduction and the derived economic losses will be the next section, paying special attention to climate warming. Finally, the omics- approaches and its use in plant biology research will be presented, it illustrated with examples of olive tree. All these sections will

be supported with data from the literature, without pretending an exhaustive list of published papers, but the most relevant and close related to this Thesis.

1. OLIVE TREE TAXONOMY AND MORPHOLOGY

The olive tree (*Olea europaea* L. subsp. *europaea* var. *europaea*) is one of the most important fruit trees cultivated in the Mediterranean basin (Besnard *et al.*, 2008), with its close relative, the wild olive (*Olea europaea* L. subsp. *europaea* var. *sylvestris* (Mill.) Lehr) being the key element, together with Holm oak (*Quercus ilex*) of the warm Mediterranean sclerophyllous forest.

Olea europaea is divided into six subspecies: *europaea*, *laperrinei*, *guanchica*, *maroccana*, *cerasiformis*, and *cuspidata* (Besnard *et al.*, 2009; Vargas *et al.*, 2000). The genus belongs to the subtribe Oleinae (40 taxa), tribe Oleeae, family Oleaceae, and order Lamiales (Refulio-Rodríguez and Olmstead, 2014; Chase *et al.*, 2016).

In Spain, the *Olea europaea* var. *europaea* taxon include more than 260 cultivars that are classified, according to its distribution and importance, into the following categories: principals, secondaries, disseminated and locals (Figure 1.1). In particular, `Picual´ cultivar is the most representative due to its largest cultivation area, approximately 860.000 ha, followed by `Cornicabra´ cultivar with 269.000 ha.

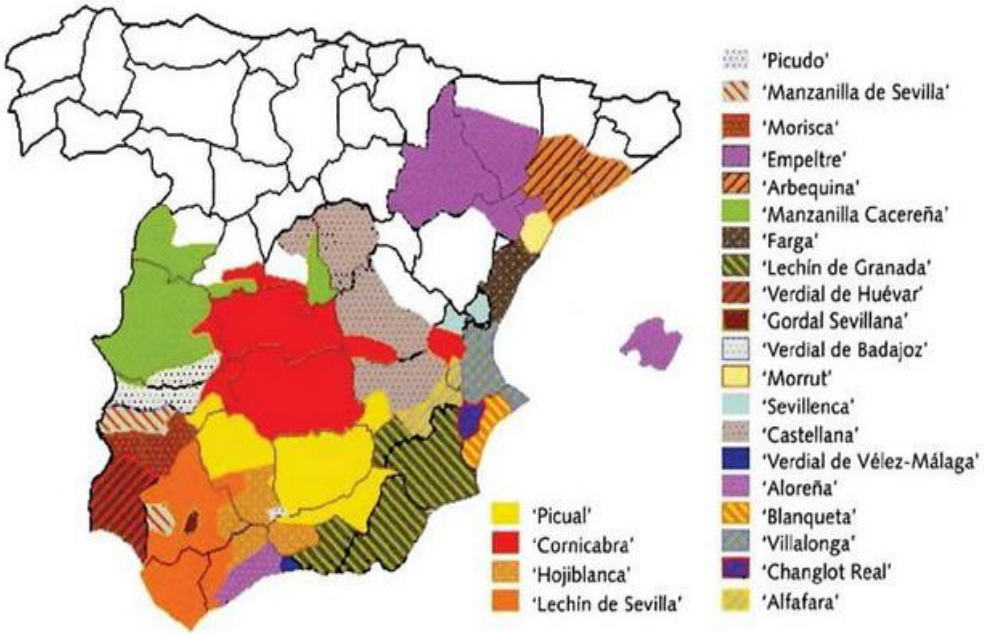


Figure 1.1. Distribution map of the 24 principal olive cultivars in Spain. The coloured area represents the localization not the cultivated area.

`Picual´ cultivar supposes more than 95 % of production in Jaen (Andalusia, Spain), around the 50 % of Spanish production and the 20 % of world production. This cultivar (also called `Marteño´, `Nevadillo´ and `Lopereño´) is used for the olive oil extraction. It is appreciated by its early production, high fat yield and easy cultivation. Its oil, although scarce

appreciated by the common consumer due to its spicy and bitter taste, stands out for its high stability index and for its high oleic acid and polyphenols content (Table 1.1) (Rallo *et al.*, 2005).

Table 1.1. `Picual´agronomic and commercial characteristics

Fruit Size (g)	Pulp/Pit Ratio	Fat Yield (%)	Oleic Acid (%)	Fruit Retention/ Weight (cN/g)	Oil Stability (h at 98.8 °C)	Polyphenols Content (mg/kg)*	Tocopherols Content (mg/kg)**
3.2	5.6	22.1	78.4	<100	119.4	450-600	270-292

*Values referenced to total content equivalents of caffeic acid
**Values referenced to total content equivalent of α-tocopherol

The olive tree is a polymorphous, evergreen and sclerophyllous tree, with a tortuous trunk. It has simple, opposite, short petiole leaves with leathery, elliptical or lanceolate-oblong contour, being the top face of strong cuticle and green-black color while the back is shiny upholstered by lepidot hairs (Figure 1.2 A).

Flowers appear at the end of spring as inflorescences in axillary bunches (Figure 1.2 B) those size and fruit-giving potential are intimately related to the growth and environmental conditions (Figure 1.2 B) (Barranco *et al.*, 2008). Flowers consist of a chalice formed by four welded green sepals and four white petals, also soldiers by the base. It is considered a perfect flower when it is hermaphrodite, that is, with two stamens and gynoecium fully formed and functional, with a large yellow anthers divided into two lobes (Figure 1.2 C). Its pollen (Figure 1.2 D), spherical or subcircular grains, (Valdés *et al.*, 1987; Trigo *et al.*, 2008) contains allergens, becoming the main cause of environmental allergy in some areas of Mediterranean climate countries (D'Amato *et al.*, 2007; Barber *et al.*, 2008).

Figure 1.2. Morphological details of: A) leaves, B) inflorescences, C) perfect and male flowers, D) pollen and E) drupes with tissues defined.

The olives fruits, olives, are, ellipsoidal or globose drupes, measuring between 1-4 cm in length and 0.6-2 cm in diameter, that develop during the summer and ripens during the autumn (Lavee, 1996). These drupes consist of 3 major tissues: epicarp (skin), mesocarp (flesh or pulp) and endocarp (pit) (Figure 1.2 E) (Valdés *et al.*, 1987). The endocarp is a sclerified tissue that protects the single seed inside and covered by cultivar characteristic grooves. The mesocarp contains oil (20-30 %, that suppose the 90 % of total oil content), simple sugars (3-4 %), polysaccharides (4 %), protein fractions (1-3 %) and phenolics compounds (1-3 %) of the total fresh weight among others. The epicarp formed by the epidermis and the cuticle, with some stomata, becoming lenticels as little dots on the surface and has a protective role against biotic or abiotic stress.

2. OLIVE TREE BIOLOGICAL CYCLE

In general, the plant biological cycle includes the following stages: germination, plant growth, flowering, pollination, fruit development and seed dispersion. In olive tree, as in most crops, these stages are grouped into two: vegetative (shoot and growth) and reproductive cycle (flowering and fruiting). Both are cyclic phenomena, repeating annually its pattern; but, while shoot growth is completed within the same, processes leading to fruit bearing require two consecutive seasons (Figure 1.3).

Olive tree has adapted its vegetative growth to the annual climatic cycle of the Mediterranean Basin. In the Northern Hemisphere, the beginning of vegetative growth occurs after the winter latency and continues along all the year when the thermal and rainfall regimes are favorable (10-30 °C; 300-700 mm, respectively).

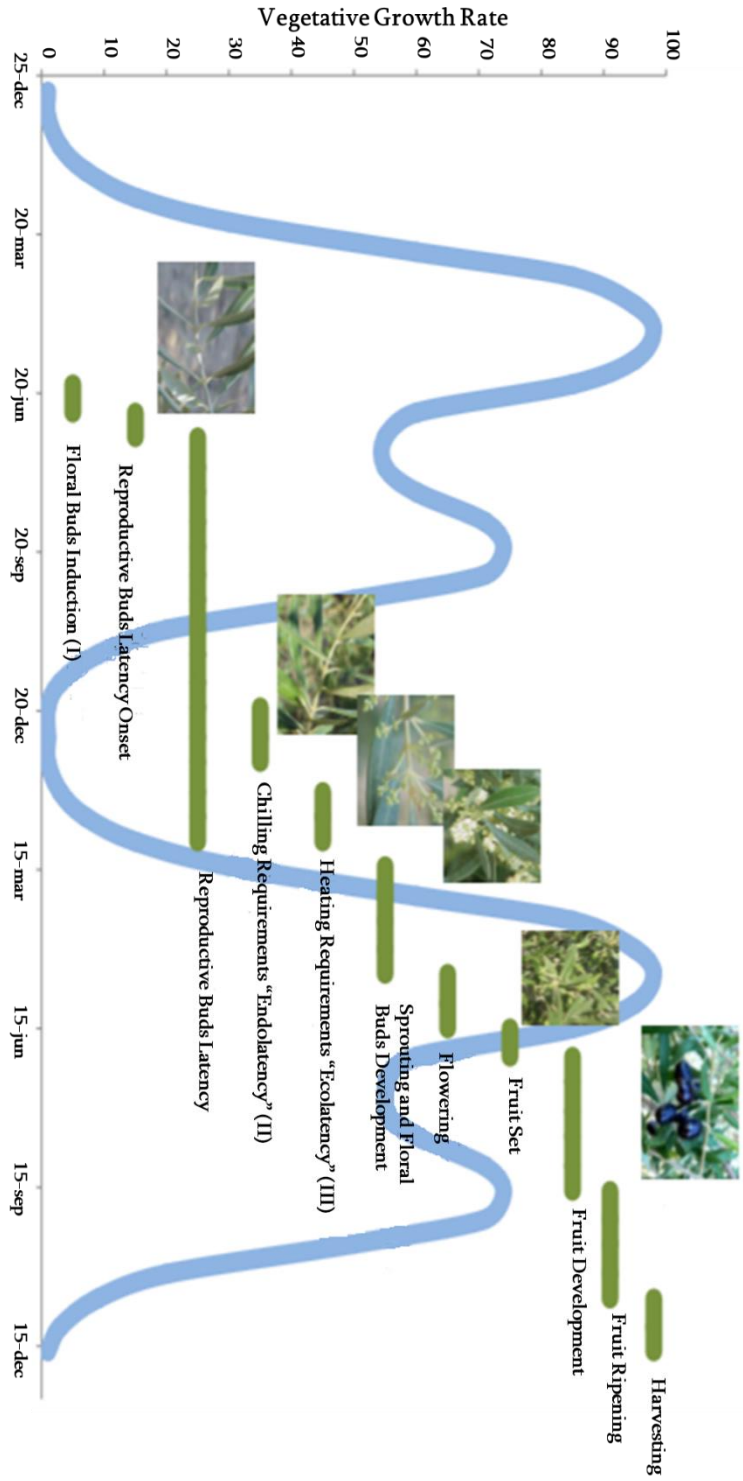


Figure 1.3. Biannual pattern of olive tree. In this chronogram the critical phases of the reproductive cycle are represented: floral induction, buds latency, floral differentiation, flowering and ripening processes.

During spring, the vegetative growth is more accused (limited to ≤ 35 °C) than in the last growing period, during the autumn and before the winter dormancy, when the degree of growth is usually favored by the abundant rainfall. This annual trend of double peak of growth is described in a Mediterranean climate. Extreme temperatures and hydric deficits can produce important damages and stop vegetative growth (Mancuso *et al.*, 2002; Cansev *et al.*, 2011; 2012; Bacelar *et al.*, 2007; 2009; Rapoport *et al.*, 2012). The size of the plant, trunk diameter, leaves and

internode length constitute the most obvious signs of vegetative growth. Hence, monitoring these parameters allow to evaluate this process (Lavee, 1996; Barranco *et al.*, 2008).

The reproductive cycle of olives trees follows a biannual pattern in the organs formation that corresponds to an inverse behavior of the vegetative growth (Díaz-Fernández *et al.*, 2004; Lavee, 2007). Fructification is derived from processes for two consecutive years (Figure 1.3). It is remarkable the fact that within a given population, trees present asynchronous bearing coexisting among trees, and even within its branches of a given tree (Dag *et al.*, 2010). Initiation, differentiation and development of flower buds are a continuous and relatively short process that takes about 4-5 weeks (Barranco *et al.*, 2008) and it is explain bellow following the schedule shown in Figure 1.4.

Figure 1.4. Zofairon parthenocarpic fruits details with an olive to compare size and development

Flowering occurred exclusively in shoots that developed vegetatively during the early spring with the inflorescence buds still dormant for 2-3 months. In this way, the vegetative buds, developed during the autumn, are those not differentiated into inflorescences (Lavee, 1996). The differentiation of the flower buds begins at the end of winter, but the induction occurs much earlier, a few weeks after the fertilization occurs (seeds play a role as an inhibitor of floral induction (Fernández-Escobar *et al.*, 1992; Rallo and Martín, 1991; Andreini *et al.*, 2008).

After a state of rest, called latency (both “endolatency” and "ecolatency”) (Galán *et al.*, 2001; De la Rosa *et al.*, 2000), in late winter or early spring, the active bud development initiates and the morphological differentiation between vegetative and reproductive buds can be distinguished visually (De la Rosa *et al.*, 2000; Mert *et al.*, 2013): differences between reproductive and vegetative buds were not observed during autumn or summer (Andreini *et al.*, 2008; Malik and Bradford, 2006; Pinney and Polito, 1990).

The inflorescences of an olive tree are formed by 10-35 flowers but just 1-3 % of flowers results in fruit set. The major reason of this elevated fruit abscission is the competition between the young fruits with other that are lagging behind in its ripening. An interesting phenomenon to mention at this point, is the “gynosterility”, favoured by winter drought, in which flowers with a sterile gynoecium are produced acting as imperfect male flowers (Figure 1.2 D), that produce viable pollen, and cause a scarce fruit set (Barranco *et al.*, 2008).

During full flowering, occurs an effective pollination period in which the plants produces most of the fertilization (Orlandi *et al.*, 2005; Cuevas *et al.*, 2009). The olive tree is partially self-incompatible being the degree of self-incompatibility dependent on the environmental conditions (Farinelli *et al.*, 2008; Spinardi and Bassi, 2012; Zhu *et al.*, 2013). This fact is responsible for the production of parthenocarpic fruits of low commercial value called zofairons (Fernández-Escobar and Gómez-Valledor, 1985; Koubouris *et al.*, 2010) (Figure 1.4).

Once the ovule is fertilized, a drupe fruit develops going through different phases (Figure 1.5). On Phase I, the fruit can be seen 10-15 days after fertilization. Subsequently, the development of the endocarp takes place at the beginning of the summer (Phase II) (Rapoport *et al.*, 2013) culminating in the development of the embryo and endocarp. At the end of July occur a thickening of mesocarp (Phase III), the pulp formation and the biosynthesis of oil, the fruit reaching green ripening in September. The veraison begins around September-November period and the complete ripening of most cultivars is only reached in winter (Shulman and Lavee, 1979).

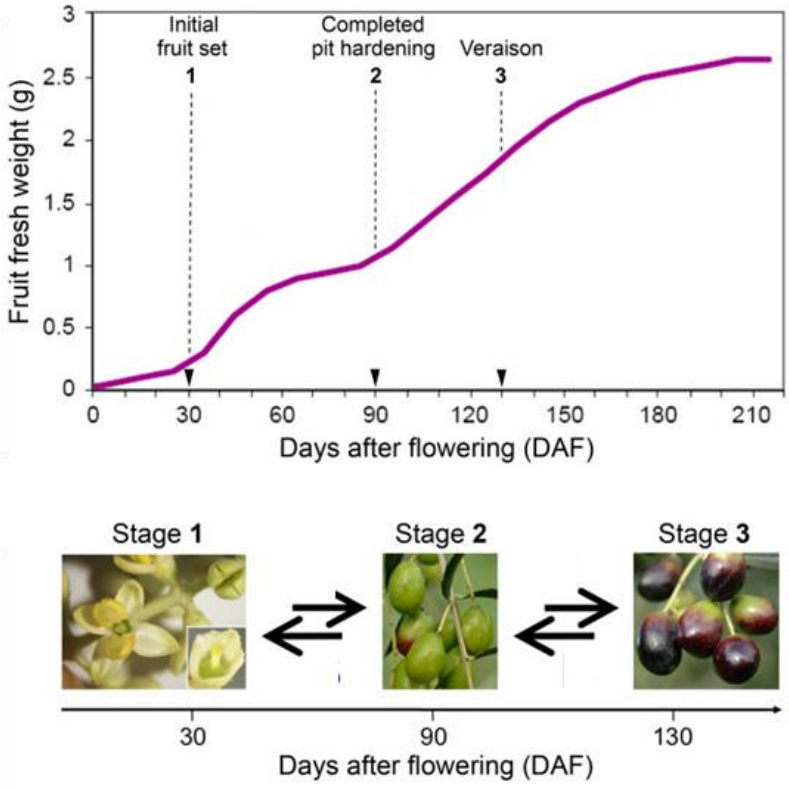


Figure 1.5. Olive fruit development phases. The increase on fresh weight for each development phase is represented indicating with image the fruit stage (full flowering, pit hardening, veraison and final maturity, and ripening).

Olive ripening is accompanied by changes in weight, pulp/pit ratio and epicarp pigmentation, fruit firmness as well as changes in chemical composition, oil contents and lipids profile, enzyme activity, secondary metabolism, among others that are involved on olive oil composition and organoleptic characteristics (Beltrán, 2000). Fruit growth and oil accumulation depend on the cultivar but ripening process also is influenced by agronomical and environmental

conditions as crop load and water availability (Lavee and Wodner, 1991; Barone *et al.,* 1994; Lavee *et al.*, 1990; Inglese *et al.*, 1996; Tombesi, 1994).

The harvesting period is defined by the optimal ripening stage, when olives achieve their maximum oil content and best oil quality. Olive growers use as traditional harvesting dates, changes in fruit skin colour (Figure 1.7) and natural fruit drop as guidelines. However, these parameters are not valid for this purpose because the development and ripening process of olive fruit changes with the cultivar and environmental conditions (Barranco *et al.*, 2000) and thus is different for each growing area and yield.

3. OLIVE TREE PHENOLOGY

Phenology is defined as the science that comprises the study and the observation of the reproductive and vegetative stages of plant development in relation to environmental parameters with applications for many different studies fields (Schwartz, 2003). Although the concept of Phenology was first used in 1853 by the botanist Carles Morren, the term was first defined in 1985 by Font Quer, and the first continuous systematic observations of events are not recorded until 18th century (Menzel, 2002). Nowadays, phenology has becoming of a considerable interest, as several studies show that the phenology events are an excellent bio-indicator of the effects of climate change (García Mozo *et al.*, 2010b; Gordo and Sanz, 2010; Menzel, 2002). In addittion recently, phenological data have taken an added value as calibrators and evaluators of NDVI satellite information or by their importance as an ecological variable (Tucker *et al.*, 2001; Chen *et al.*, 2001; Bradley *et al.*, 2011). Therefore, from the agronomic point of view, phenology can be used to guide the various actions that are carried out on a cultivated plant, such as phytosanitary treatments, pruning, etc.

However, the collection of phenological data requires a methodology set up determined to compare temporal evolutions. During 3 campaigns, in the present thesis the keys for phenophases identification have been followed standardized phenology described by Colbrant and Fabre (1975) for the olive floral phenology (Figure 1.6); and suggested by Ferreira (1979) to determine the ripening index (RI) (Figure 1.7). On the one hand, each stage in the floral phenological scale represents a sequential state in the evolution of the buds which give rise to buds, on the one hand, and to flowers until the formation of fruits on the other. Sequential phenology subdivides the development known with the name of phenophases.

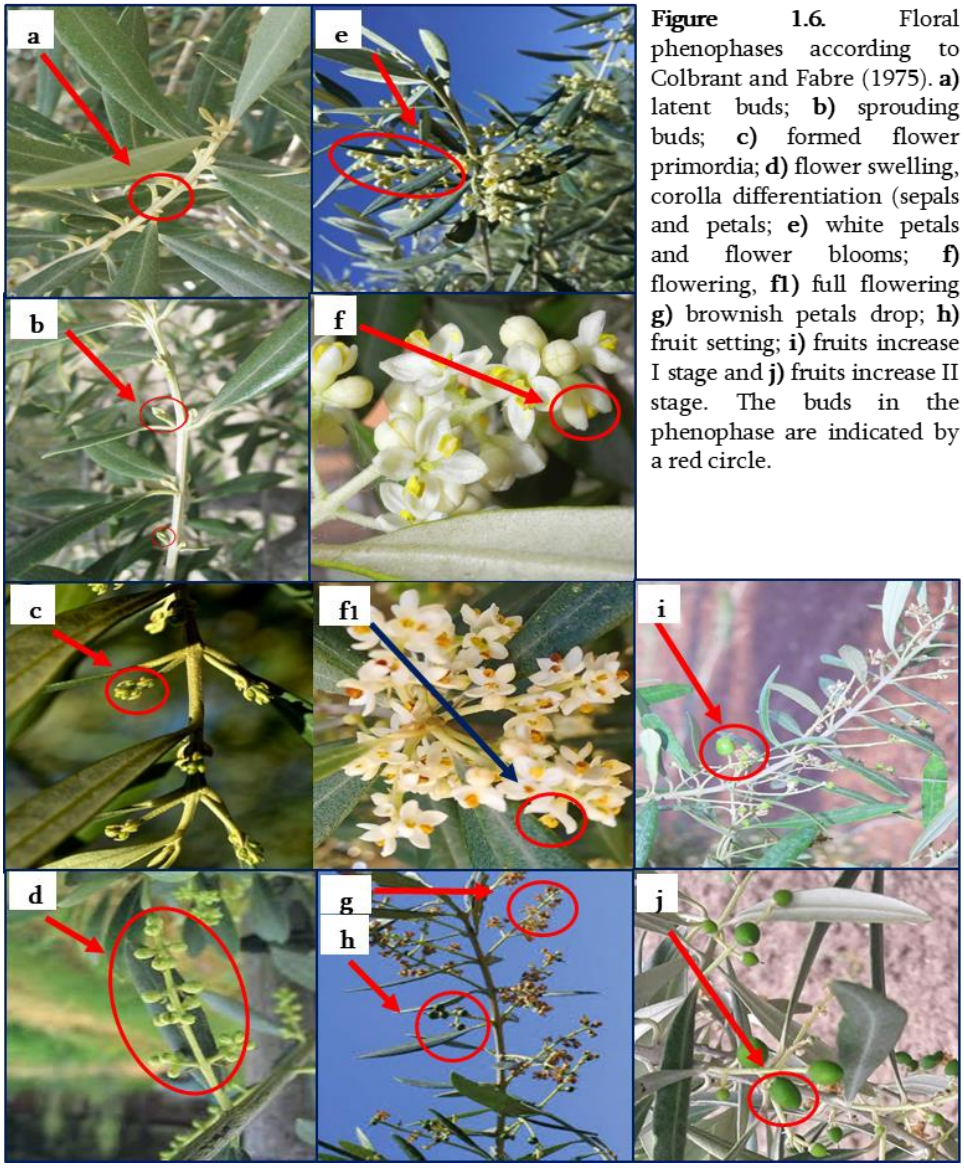


Figure 1.6. Floral phenophases according to Colbrant and Fabre (1975). **a)** latent buds; **b)** sprouding buds; **c)** formed flower primordia; **d)** flower swelling, corolla differentiation (sepals and petals; **e)** white petals and flower blooms; **f)** flowering, **f1)** full flowering **g)** brownish petals drop; **h)** fruit setting; **i)** fruits increase I stage and **j)** fruits increase II stage. The buds in the phenophase are indicated by a red circle.

Figure 1.6. Floral phenophases according to Colbrant and Fabre (1975).

On the other hand, the olives are categorized into 8 classes depending its skin and flesh colour. For this classification, 2kg taken around the tree and 100 olives are sampled to separate in the 8 ripening stages (from 0 to 7) and the RI is calculated according to the followed formula:

$$RI = \frac{A * 0 + B * 1 + C * 2 + D * 3 + E * 4 + F * 5 + G * 6 + H * 7}{100}$$

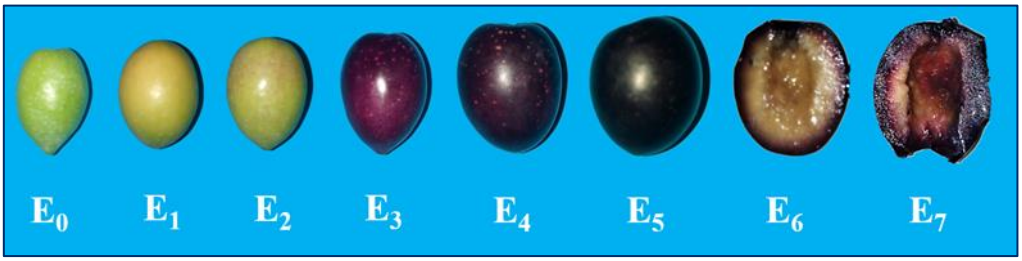


Figure 1.7. Fruit ripening classification. E_0= Deep green skin; E_1= Yellowih-green skin; E_2= green skin with reddish spots <50 % fruit surface skin; E_3= reddish patches >50 % fruit surface skin, veraison phenophase; E_4= Purple skin; E_5= Black skin with whitish flesh; E_6= Black skin with purple flesh covering less than half of the flesh; E_7= Black skin with purple flesh up to pit.

4. OLIVE TREE CULTIVATION AND ECONOMIC ASPECTS

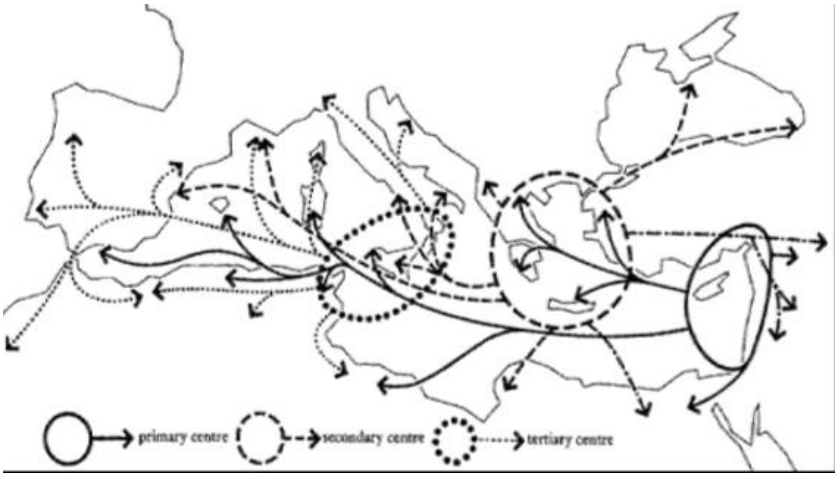
As the poet George Duhamel said, "the Mediterranean ends where the olive tree no longer grows", which can be capped by saying that "There where the sun permits, the olive tree takes root and gains ground".

The olive tree was first cultivated in the Middle East some 6000 years ago (Barranco *et al.*, 2008; Kaniewski *et al.*, 2012). Subsequently, the crop spread to the entire Mediterranean Basin (Simmonds, 1976) by Iberian population or to an expansion of the oleaster during the pre-Roman period (Martin-Puertas *et al.*, 2009).

In the Modern age, with the discovery of America, its cultivation has now spread to countries such as the United States, Canada and The United States, Argentina, Chile or Australia. Its distribution is restricted between 30º and 45º latitude, both in the north and in the south.

The profitability of olive cultivation lies mainly in olive oil, this product representing more than 90 % of the commercial output of the olives, being the second commercial outlet of the fruit (Barranco *et al.*, 2008). The increase in production of olive oil coincides with the growing demand for the product (FAO, 2019). This increase has been achieved thanks to the expansion of the cultivation area, as well as the development of strategies for densification, intensification of production and irrigation, and of technical improvements at growth level. However, in the case of Spain there is no evidence of a parallel relationship and a stagnation in consumption.

A)



B)

Figure 1.8. A) Three main diversification centres during olive tree spread. **B)** Olive tree and oleaster Andalusian distribution

The total production for campaign 2017/2018 was 20,872,788 tonnes. Europe is the main productor with 61.8 % of the total followed by Africa with 17.9 %, values very close to the production of Asia, 16.9 %. The new growth systems in California and Australia place America with 2.8 % and Oceania with a negligible value of 0.6 % (FAO, 2019) (Figure 1.9).

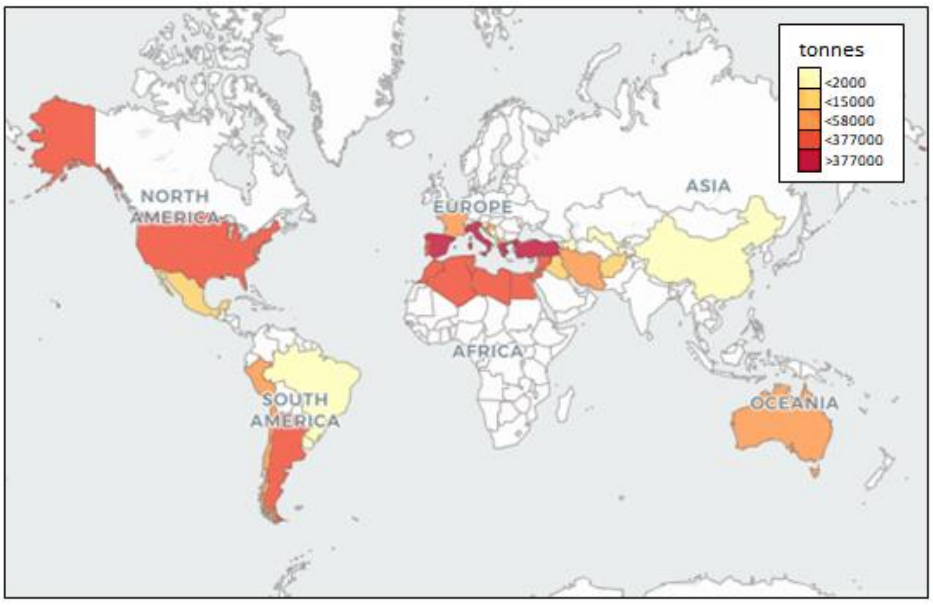


Figure 1.9. Olives world production

According to data from the International Olive Oil Centre (IOC, 2019), Spain is the first olive oil producing country in the world, having obtained in the campaign 2017/2018 close to 50.8 % of european production (31,4 % of world production), followed by Greece (21 %) and Italy (10 %) (Figure 1.10).

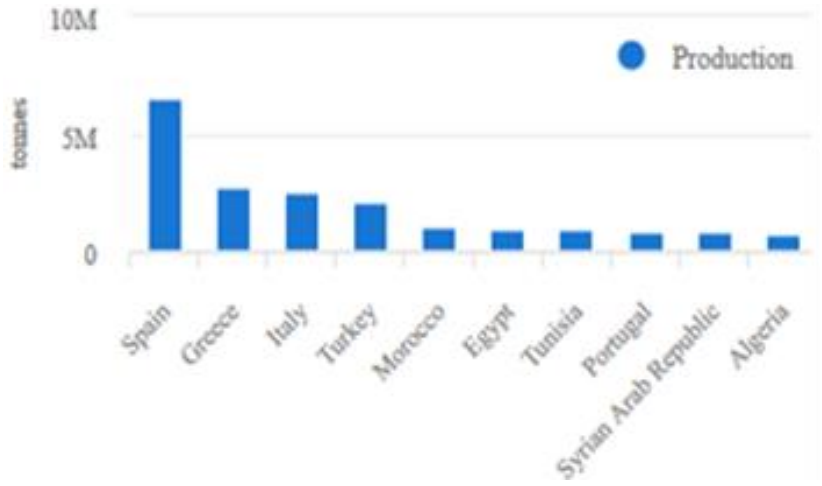


Figure 1.10. Top 10 of country by total olives production.

Andalusia, specifically, produces 80-90 % of the Spanish production and the province of Jaen around 40-60 % of Andalusian olive oil production followed by Cordoba (25-30 %) (MAPAMA 2017, 2018 and 2019). In fact, a fairly homogeneous landscape was characteristic of the nucleus of the Andalusian olive grove. Since traditional agricultural management favors the elimination of any other vegetation not. On the other hand, it is frequent to observe a great homogeneity within the olive groves themselves formed by clones of the same plant (Ipek *et al.*, 2009). When the Jaen and Cordoba production was compared with the principal cultivar distribution (Figure 1.2), `Picual´ cultivar suppose around 70-80 % of the Spanish olive oil production.

5. ENVIROMENTAL STRESS AND CLIMATE WARMING

Plants, as sessile organisms, must cope with a different range of environmental stresses, either biotic or abiotic, that imposes restrictions to its growth and productivity. It is the reason why during the course of the evolution plants have evolved mechanisms of adaptation and resilience to extreme conditions. Among them the environmental conditions, and in the context of the present Thesis, reference to temperature will be discussed. It affects life processes in all organisms being a critical factor that determines plant growth and development (Sung *et al.*, 2003).

The temperature is considered to be the main source of geographical variation in the phenology of the olive tree (Wielgolaski, 2003; Milla *et al.*, 2009; Aguilera, 2012; Jochner *et al.*, 2012). Hence, trees grown in cold climates are usually smaller than those cultivated in warmer growing conditions, provided that water is not a limiting factor. High temperatures (above 35 °C) lead to stoma closure which prevents gas exchange, and decreases the photosynthesis rate up to 70-80 % at 40 °C. In additions, several studies have reported that pollen germination and pollen tube growth that bot are sensitive to elevated temperatures (Fernández-Escobar *et al.*, 1983; Vuletin Selak *et al.*, 2013). Constant temperatures above 33 °C after fertilization may affect fruit setting (Graniti *et al.*, 2011) and subsequent fruit development (De Andrés Cantero, 2001). In fact, the anthocyanins accumulation in many fruits and plant tissues, used as ripening marker, depends on temperature (Steyn *et al.*, 2002) affecting fruit color (Koshita, 2015). Further, temperatures above 25 °C reduced fruit fresh weight, oil concentration, and oil fatty acid composition (García-Inza *et al.*, 2014; García-Inza *et al.* 2018).

Climate change has already led to a wide range of impacts on the environment, the economy, and society (Giorgi, 2006; IPCC, 2014). The intensity and frequency of extreme weather events are predicted to increase in many regions. High-temperature events like hot days, tropical nights, and heat waves have become more frequent, while low-temperature events (e.g., cold spells, frost days) have become less frequent in Europe. Heat waves may have short-term durations of a few days with temperature increases over 2-5 °C above the normal temperatures and the average length of summer heat waves over Western Europe doubled over the period 1880 to 2005, while the frequency of hot days almost tripled.

The Mediterranean Basin has been considered as one of the most vulnerable regions to climatic changes in the near future (Giorgi, 2006; Lionello, 2012), where are expected increases of global average temperature of 2 to 4 °C by the end of this century (Figure 1.11) (Giannakopoulos *et al.*, 2009; Gualdi *et al.*, 2013).

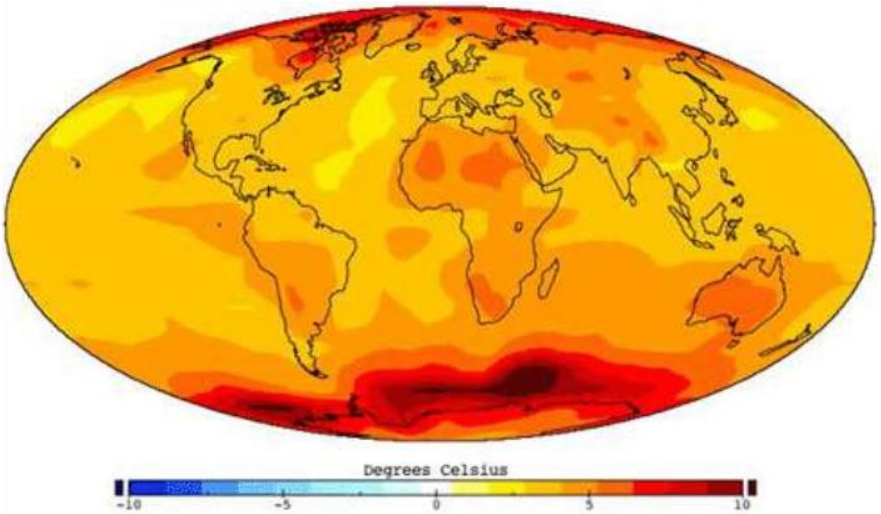


Figure 1.11. Prediction of surface air temperature increase from 1960 to 2060.

Beyond the knowledge on plant responses to the altered climatic conditions in other plant systems (Beckling *et al.*, 2016), this scenario demands the particular study of woody plants, and more specifically olive tree response and adaptation to the changing environmental conditions, in order to manage this culture.

6. OMICS APPROACHES IN PLANT RESEARCH

Omics technologies allow the comprehensive molecular monitoring of all of the changes that take place when the genetics, nutritional state, or environment of an organism is altered. The information obtained from the combination of multiple omics analysis provides functional knowledge on these responses.

Then, below, an overview of each of the omics technologies integrated in this thesis and their use in plant studies are presented according to the flow of the biological information in the Central Dogma of Biology.

6.1. Transcriptomics

A transcriptome is the complete set RNA molecules expressed by an organism, cellular type, or tissue under specific conditions. Usually is used to describe just the array of mRNA transcripts that will be translated into proteins (Mattick and Makunin, 2006; Kukurba and Montgomery, 2015). Unlike the genome, which is roughly fixed for a given cell line, the transcriptome is dynamic as it is essentially a reflection of the genes that are actively expressed at any given time including stage of development and environmental conditions, such as under biotic and abiotic stresses. Thus, the study of the transcriptome in any specific condition will allow identifying the functional elements of the genome involved in this particular response.

From the different methodologies available for its study, in this PhD Thesis, Quantitative PCR (qRT-PCR) was employed to study the response of olive tree and olive ripening process to temperature increase. The qRT-PCR consists in the real time estimation of the levels of the transcript of the selected genes, often calculated as fold changes with respect to a control gene, or housekeeping (Olias *et al.*, 2014).

The number of the studies devoted to the transcriptional profile of heat response in different plant species have increased in the last two years, consistent with the growing concern for climate change/warming (Fernie and Stitt, 2012; Ueda *et al.*, 2012; Yángüez *et al.*, 2013), It is known that approximately 5 % of the plant transcriptome is up-regulated two fold or more in response to heat stress in plants (Larkindale and Vierling, 2008; Saidi *et al.*, 2011). Most of the differential transcripts correspond to genes acting in primary and secondary metabolism, translation, transcription, regulation, and responses to environmental stresses, and in processes

such as calcium and phytohormone signalling, sugar and lipid signalling, or protein phosphorylation.

6.2. Proteomic

Proteome are considered the entire set of proteins that is, or can be, expressed by a genome, cell, tissue, or organism at a given time, under defined conditions (Wilkins, 1995). Proteins are everywhere in plants and the thousands of distinct proteins play key functional roles for the texture, yield, flavour, and nutritional value of virtually all food products. Through protein expression profiling, proteins can be identified at a specific time as a result of expression to a stimulus.

Advances in mass spectrometry have enabled the analysis of cellular proteins in unimaginable scale. Gene translation into the functional proteins that ultimately determine what happens in a living system. These proteins also undergo posttranslational modifications, proteolysis, recycling, multicomplex formation and subcellular translocation that are key events to regulate protein functions in cellular processes (Park, 2004).

With the aim of profiling proteins in biological samples, proteomics has long been associated with the techniques of two-dimensional gel electrophoresis (2DE) and MS (Wilkins *et al.*, 1996; Shevchenko *et al.*, 1996). The classical 2DE allows the direct visualization of full proteomes and it is quantitative and suitable for the study of protein isoforms and posttranslational modifications (PTMs). However, 2DE had limitations in resolution and reproducibility of gels, as well as low abundance proteins generally are masked by the more abundant ones. For this reason, gel-free MS-based approaches are the current gold standard for the analysis and quantification of complex peptides mixtures. Gel-free MS technology was developed for the ionization of proteins and peptides, allowing automated analyses of peptide mixtures that were generated from complex protein samples (Washburn *et al.*, 2001; Wolters *et al.*, 2001). This MS techniques offer high sensitivity and mass accuracy (Aebersold and Mann, 2003). The use of a next-generation spectrometers such as Orbitrap, MS coupled to liquid chromatography separation systems, allowed a peptide characterization without precedent in terms of speed, resolution, dynamic range and accuracy, being possible the analysis of full proteomes (Nagaraj *et al.*, 2012).

Changes in transcript abundances were not always coherent with the changes at protein level (Vogel and Marcotte, 2012). Therefore, proteomic analysis is the most straightforward and powerful tool for the discovery of key molecular mechanisms involved in abiotic stress. In fact, studies of proteome changes under heat stress can significantly contribute to detection and identification of stress tolerance biomarkers (Weckwerth, 2011).

6.3. Metabolomic

Metabolomics is the study of chemical processes providing a linkage between genotypes and phenotypes. Proteomics identifies the gene products produced, whereas metabolomic studies bring us closer to the function. It determine whether the expressed proteins are metabolically active and identify biochemical processes and the roles of the resulting various metabolites. The metabolome is dynamic and subject to environmental and internal conditions, which can aid in the development of improved crop varieties and a basic understanding of changes resulting from biotic or abiotic stress by systems biology.

The major challenge of metabolomics is related to the chemical complexity of the metabolome, the biological variance inherent in most living organisms, and the dynamic range limitations of most instrumental approaches together with the databases improvement requirement (Sumner *et al.*, 2003). The perfect selective extraction must be able to extract simultaneously all different metabolites (e.g., ionic inorganic species, hydrophilic carbohydrates, hydrophobic lipids, complex natural products) from a unique sample using also multiple analytical technologies to provide a comprehensive view of the metabolome. In this context, currently, MS-based platforms and the combination of different techniques are the best option to increase metabolome coverage. The use of ultra-performance liquid chromatography coupled to high resolution Fourier Transformation MS (UPLC-Orbitrap-MS) is an excellent option in order to profiling the complex metabolome of stressed plants. Databases are far to include all the metabolic pathways, being remarkable the underrepresentation of secondary metabolisms, and the interaction between the different plant tissues (Grafahrend-Belau *et al.*, 2013).

Metabolic profiling provides an instantaneous picture of what is occurring in the cell, for example, during fruit ripening, identifying key compounds important for imparting taste and aroma. In plants, it has been reported that high temperature has negative effects in various physiological processes such as photosynthesis, primary and secondary metabolism, water relations, or lipid metabolism (Xu *et al.*, 2006; Escandón *et al.*, 2018). Especifically, high-temperature stress can promotes the production of phenolic compounds such as flavonoids, phenylpropanoids, anthocyanins, and lignins which are related to the suppression of stress-induced oxidation of most cell molecules (Wahid *et. al.*, 2007).

6.4. Systems Biology

The use of available high-throughput methodologies such the different omics and its combination in the direction of the Systems Biology would be an ideal approach to understand the different mechanism of plant response. In addition, considering the large amount of experimental data generated and the need for a clear, integrated and meaningful view of the investigated biological events, we resorted to the construction of interpretable metabolic pathways (Valledor *et al.*, 2018; Hizashi *et al.,* 2018).

Systems biology encompasses a holistic approach to the study of biology and the objective is to simultaneously monitor all biological processes operating as an integrated system, including data set of DNA sequences, RNA and protein measurements, protein-protein and protein-DNA interactions, metabolic biomolecules, signalling and gene regulatory networks, cells, organs, individuals, populations, and ecologies (Figure 1.12) (Valledor *et al.*, 2014). The amount of data generated by high-throughput platforms led to the rapid growth of computational biology and bioinformatics (Guerrero-Sánchez *et al.*, 2018). The interactions between biomolecules and physiology are often missed when each functional level is studied in isolation, leading to an increased number of false positives, loss of information (false negatives) and irreproducible findings (Angione *et al.*, 2016; Bruggeman and Westerhoff, 2007).

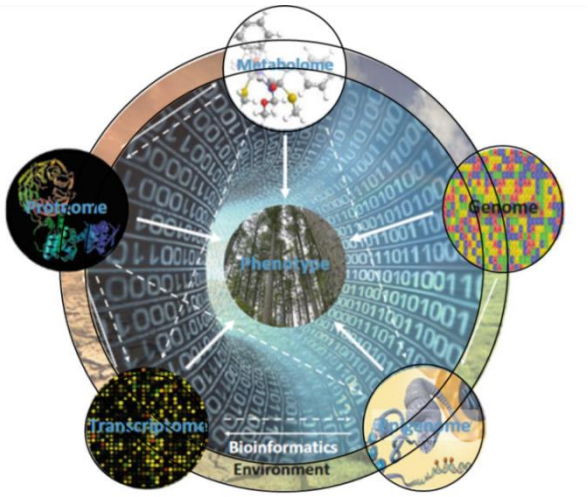


Figure 1.12. Scheme of Systems Biology integration. Systems biology aims to predict phenotype based on the integration of the different omic levels and environmental factors by using specific bioinformatic tools. Adapted to Valledor et al., 2016.

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Chapter II. Objectives

The general objective of this Doctoral Thesis aimed to investigate how an increase of 4 °C in ambient temperature, associated with the predicted climate change thermal conditions, affects the olive tree (*Olea europaea* L.) phenology. Adult trees of the cultivar `Picual´ growing under natural conditions were permanently exposed to 4 °C above ambient temperature, throughout their complete reproductive cycle, using temperature open top chamber (OTC) structures. The temperature effect on olive phenology (particularly ripening processes) was analyzed at eco-physiological and biomolecular level.

This general objective was divided into the following specific ones:

1. To study under field conditions the effect of warmer temperatures on the occurrence and duration of the different olive phenophases: vegetative growth, flowering, fruit set, growth and development and maturation. In addition, total tree production, fruit characteristics and some oil quality parameters were determined.

2. To analyze the effect of warmer temperature in the olive root system on potassium transport and plant growth, a set of experiments in hydroponic medium under controlled temperature conditions were performed using `Arbequina´ cultivar seedlings. The temperature effect on K^+(Rb^+) uptake and translocation to the different plant organs and consequently on plant vegetative growth was analyzed.

3. To evaluate the effect of increasing air temperature on the chemical and biomolecular response of the olive fruit at three different ripening stages (green, veraison and purple skin) utilizing classical biochemistry, and the integration of the modern –omics approaches: transcriptomic, proteomic and metabolomic.

Chapter III. Global warming effects on reproductive and vegetative phases of olive trees growing under field conditions

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Temperature in the Mediterranean Basin, the main area of olive tree culture, has been predicted to rise in the near future threatening olive production. To determine the potential effects of increasing temperature on the olive reproductive cycle and vegetative growth, a study with the `Picual´ cultivar has been developed simulating global warming conditions under field experiment over three years. Temperature-controlled open top chambers (OTC) systems were used to increase 4 °C above the day/night ambient temperature throughout the complete reproductive cycle of olive trees. The study has shown that the applied thermal increase advances the date of flowering and ripening processes in the olive, and lengthens both periods. Pistil abortion and the consequently reduction in fruit set was more pronounced in trees subjected to warmer temperatures. Moreover, those results indicate that 4 °C increase of ambient temperature reduces total fruit production and affects fruit characteristics. Smaller fruits, lower pulp stone ratio, oil yield reduction and significant differences on anthocyanins and total phenols contents were associated to rising temperatures. In addition, the vegetative growth was stimulated by the thermal treatment resulting in higher, more vigorous and branched trees.

1. INTRODUCTION

The olive (*Olea europaea* L.) is one of the most emblematic crops in the Mediterranean Basin having a great economic, social and cultural impact. However, the environmental conditions of this region are expected to change in the near future (Giorgi, 2006). In particular, the mean air temperature has been projected to rise drastically in the range of 2-5 °C (Giorgi, 2006; Giannakopoulos *et al.*, 2009; Gualdi *et al.*, 2013; IPCC, 2014). In addition, more frequent and extreme events such as drought periods and heat-waves are expected to happen in this region in the near future (Giorgi and Lionello, 2008; Tanasijevic *et al.*, 2014). Temperature is a primary environmental factor controlling plant growth and development, hence changes in seasonal temperatures can affect the biological processes of the reproductive cycle of traditional Mediterranean crops, including olive crops where a constant increase in ambient temperature may affect its phenology and, consequently, may reduce yield and total production.

Olive production depends on vegetative and reproductive processes occurring along a biennial cycle. Both processes are repeated annually, but while shoot growth is completed within the same year, processes leading to fruit bearing require two consecutive seasons. In addition, olive trees exhibit a strong alternate bearing phenomenon. Olives flowers appear on one-year-old wood and display two inflorescences per node or, exceptionally, directly at the shoot apex. The inflorescences contain a mixture of perfect (hermaphroditic) and imperfect (staminate) flowers.

The former group contains well-developed stamens and pistil while in the latter a residual atrophied pistil is often visible in the center as a consequence of varying degrees of pistil abortion (Cuevas and Polito, 2004; Reale *et al.*, 2006). Usually, flower bud induction is manifested by July in the northern hemisphere (Fernández-Escobar *et al.*, 1992), and floral differentiation is evident by March (Hartmann, 1951) after floral bud dormancy. Anthesis occurs by May, and shortly after that, massive abscission of flowers and fruits appear (Rallo and Fernández-Escobar, 1985). The remaining fruits usually persist on the tree until harvest, which takes place during the autumn and winter (October-February) depending on the year and the cultivar drupes ripening.

In the Northern Hemisphere two vegetative growth flushes can be observed: the main one from March to mid-July, and the second from September to mid-October, provided that water is not a limiting factor. The optimum temperature for olive shoot growth and development ranges from 10 to 30 °C, but when temperature rises above 35 °C shoot growth could be limited (Rallo and Cuevas, 2008; Therios, 2009). This effect has been recently observed in olive when plants were exposed to moderately high temperature (37 °C) during a period of time (Benlloch-González *et al.*, 2016; 2017). From this information, it is not clear in which sense the change in temperature patterns along the year, especially during winter and autumn, will affect the vegetative growth in this species.

Most of the information available on the potential effects of warmer temperature on olive fruitfulness has been focused on floral phenology, paying little attention on the following reproductive processes as the fruit set is partly influenced by flower quality. It is not clear if warmer temperatures during pre-flowering and flowering phenophases could modify any morphological or developmental characteristics of the flower affecting its ability to set and form a fruit. Although it has been informed that environmental factors and cultural practices have a significant effect on floral quality (Hartmann, 1950; Uriu, 1953; 1960), some studies have reported that poor N_2 nutritional status, low leaf-to-bud ratio and water deficit favor pistil abortion (Fernández-Escobar *et al.*, 2008; Uriu, 1956; 1960; Cuevas and Polito, 2004; Reale *et al.*, 2006; Rapoport, 2012). There is also a cultivar-related tendency for producing imperfect flowers (Campbell, 1911; Morettini, 1950; Lavee *et al.*, 1996; Rosati *et al.*, 2011).

On the other hand, the success of a flower to become a fruit not only depends on its quality but also on the pollination and fertilization processes. Studies that have analyzed the effect of temperature on pollen germination and pollen tube growth have reported that both are sensitive to elevated temperatures (Fernández-Escobar *et al.*, 1983; Vuletin Selak *et al.*, 2013). Constant temperatures above 33 °C after fertilization may affect fruit setting (Graniti *et al.*, 2011) and subsequent fruit development (De Andrés Cantero, 2001). After fruit set, olive drupes growth and development are completed in approximately 4-5 months, following a double sigmoid growth curve pattern (Hartmann, 1949; Lavee, 1996; Rallo and Cuevas, 2008). During this period structural changes and chemical transformations such as cell division, cell expansion and

metabolites storage, take place in the different fruit tissues. For study purpose, these transformations can be divided in different phases. Phase I is characterized by an exponential growth, where cellular divisions of the different fruit tissues are predominant, and the mesocarp and the endocarp increase in size; in addition, the sclerification and hardening of the endocarp begin. In phase II, fruit growth slows down or even stops, both the embryo and the endocarp reach their final size and the endocarp hardening process is completed. Finally, during phase III, there is a fast growth of the fruit due to the enlargement of the mesocarp cells which determines the final fruit size. Besides, lipogenesis in flesh parenchyma cells begins (Manrique *et al.*, 1999). This phase ends in early autumn when fruit undergoes the first pigmentation changes.

All these processes are genetically controlled and influenced by several environmental factors (Connor and Fereres, 2005; Costagli *et al.*, 2003; Gucci *et al.* 2009), being water availability the most studied. Briefly, when water shortage occurs at phase I, smaller endocarps are observed (Lavee, 1986) which can lead to fruits with unusually high pulp/stone ratios, compromising fruit viability. Moreover, water reduction during this stage has been informed to affect cell size rather than cell number in the mesocarp (Rapoport *et al.*, 2004). On the other side, water limitation in phase III results in small fruits with reduced oil content (Beltrán *et al.*, 2004). In contrast to the well described effects of water limitation on fruit development and growth, the information related to higher temperature is scarce. There is some information from studies performed in the Southern Hemisphere, in which the effect of warmer temperatures has been analysed on fruit dry weight, oil concentration, and oil fatty acid composition. Fruiting branches from 'Arauco' olive cultivar were enclosed in transparent plastic chambers with individualized temperature control during the oil accumulation phase (García-Inza *et al.*, 2014; 2018). Under these experimental conditions, temperatures above 25 °C reduced fruit fresh weight. A reduction in fruit oil concentration was also observed when temperature was increased during the period of active oil accumulation.

During the ripening process, the fruit darkens from lime-green to purple-black while oil content increases. The anthocyanins content in a fruit (biosynthesis, accumulation and degradation) determines its color, purple or black in the case of olive (Roca and Mínguez-Mosquera, 2001) and temperature influences these processes. High temperature has been described to reduce anthocyanins accumulation in many fruits and plant tissues (Steyn *et al.*, 2002) affecting fruit color (Koshita, 2015). This has been observed in different fruit species such as grapes (Tomana *et al.*, 1979; Naito *et al.*, 1986), apple (Creasy, 1968; Yamada *et al.*, 1988; Arakawa, 1991), Satsuma mandarin (Utsonomiya *et al.*, 1982), and Japanese persimmons (Taira *et al.*, 2000; Isobe and Kamada, 2001), but it is not clear in olive fruit. The final changes in fruit colouring is an important stage during the olive fruit maturation after which oil accumulation processes are ceased (Lavee and Wodner, 1991).

Regional Climate Models and olive-pollen capture traps studies, revealed that the development of flower structures will be completed faster compared with the historical data leading to earlier flowering dates (García-Mozo *et al.*, 2010; Oteros *et al.*, 2013; Osborne, 2000; Giannakopoulos *et al.*, 2009; Orlandi *et al.*, 2010; Aguilera *et al.*, 2015). There is an actual tendency to compare floral phenology and ripening processes in olive orchards located in geographical regions differing in annual mean temperatures. But considering that floral events not only are governed by mean temperature but also by seasonal temperature fluctuations and the photoperiod along the different annual seasons, the results from these studies may lead to misunderstandings. These variables are difficult to be the same in different geographical regions because they partly depend on latitude and altitude.

However, long-term temperature field experiments are necessary to verify the differences of phenophases occurrence between actual and historical data results. Open top chambers (OTCs) are useful tools for the simulation of global change under field conditions (Allen *et al.*, 1992; Ceulemans and Mousseau, 1994; Norby *et al.*, 1997). These systems are able to simulate real conditions of global warming, including daily and seasonal fluctuations in weather. They allow trees to grow in the ground for several growing seasons avoiding the potential negative effects of container artefacts: root mechanic stress or the increase in soil temperature above ambient temperature due to containers overheating.

The aim of this study was to determine under field conditions, in a Mediterranean climate area in which temperature and photoperiod fluctuations occur along the year, the effect of 4 °C increase in air temperature on olive floral phenology, fruit set, olive maturation and yield as well as vegetative growth. To reach this goal, olive trees `Picual´ cultivar, were subjected to warmer temperature than ambient throughout their complete reproductive cycle using temperature-controlled OTCs. The information resulting from this study, about the impact of high temperature on flower quality, fruit set, growth and development, maturation period, ripening processes, oil production, gives an important overview of the impact of global warming on olive production.

2. MATERIAL AND METHODS

2.1. Plant material and growth conditions

'Picual' olive trees (*Olea europaea* L.) growing in the experimental farm of 'Campus de Rabanales', University of Córdoba, Spain (37°55'N 4°43'W) were used to perform the experiment. The orchard soil is classified as Calcic Luvisols with a clay-loam to clay texture, pH moderately alkaline (7-8), organic matter around 2 %, and moderate to high cation exchange capacity (Del Campillo *et al.*, 1993). The trees were planted in autumn 2009 spaced 8 x 6 m apart, with a drip irrigation system. Depending on the season, water was applied over five to six months during the

dry season (from late May to middle October, approximately). During this period the dose applied was about 55.2 l per tree and day. The experiment was conducted from 2014 to 2017.

2.2. Experimental design and treatments

Sixteen trees, consecutively distributed in two lines, were selected from the experimental orchard to perform this study. Trees were subjected to two temperature treatments, ambient temperature (AT) and 4 °C above ambient temperature (AT+4 °C), along three consecutive years. The experimental plot was divided into four blocks containing each one two trees (replicates) per temperature treatments (AT vs. AT+4 °C) which were randomly distributed. To maintain trees at 4 °C above ambient temperature, eight temperature-controlled OTC, specifically designed for this study, were constructed around the trees (Figure 3.1). Each OTC (hexagonal prism-shape), consisting of a steel frame covered by panels (panel size: 1.80 cm width x 3.60 cm height) of plastic film of high transparency (transmission: 90-85 % of the solar photosynthetically active radiation), was equipped with heating and ventilation devices regulated by an automaton. In this way, a constant day/night temperature gradient between the tree and the surrounding environment of 4 °C was maintained throughout the complete reproductive cycle of this species.

The 4 °C thermal gradient was maintained by two mechanisms that operated independently: 1) A close circuit came into operation at night or under low solar radiation conditions to inject heat to the chamber. This circuit recirculates the air from the chamber through a turbined equipped with electric heating elements (Figure 3.1).

An automaton regulates the warm air flow by controlling the turbine speed and the number of electric heating elements which comes into operation; 2) During the day, under high solar radiation conditions, the excess of heat accumulated in the chamber was partly eliminated by the injection of air from outside. The automaton selects one or another mechanism, acting on the closing or opening of two vents placed in the system (Figure 3.1).

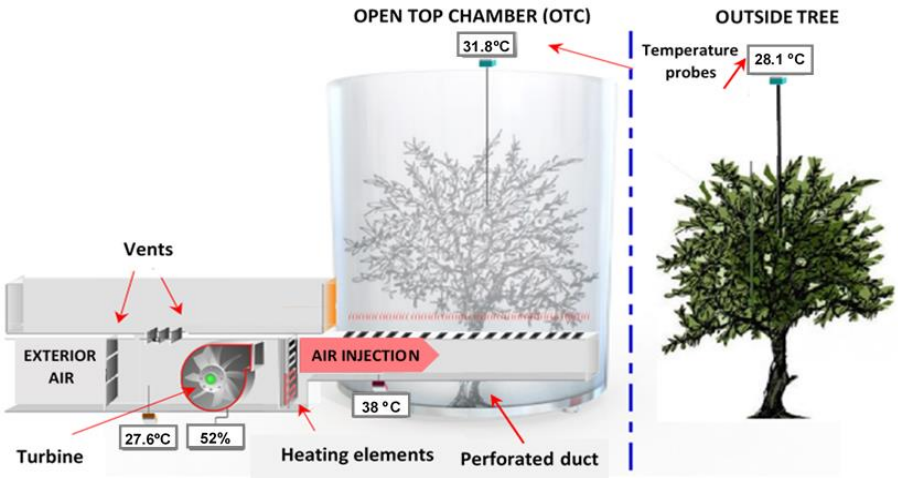


Figure 3.1. Scheme of functioning of the Open Top Chamber.

The information processed by the automaton was provided by a temperature probe placed at the middle position of the tree canopy, both in control trees (TA) and those confined in the chambers (TA+4 ºC). The air injected into the chamber diffuses upwards along the tree from a 35 cm diameter duct, resting on the ground, provided with circular perforations (4 cm in diameter) (Figure 3.1). The system provides a uniform air-flow of low flow once it reaches the branches of the tree.

2.3. Measurements

Vegetative growth (shoot length, trunk diameter and weight of the pruning material) was measured once a year at the end of the vegetative period (late autumn). Shoot length was measured on fifteen uniformly distributed shoots per tree, which were previously selected and tagged each spring. After harvesting, the trees were pruned and the trunk circumference measured at 30 cm above the ground surface.

A fresh sample of four fully expanded leaves per tree was collected at noon at different times along the reproductive cycle of the olive to determine the leaf relative water content (RWC). Leaves were weighed, rehydrated over 24 h, and re-weighed, according to the procedure reported by Stocker (1929).

Fifteen uniformly distributed bearing shoots per tree were selected and tagged each spring. Shoot length was recorded at this time. Flowering was determined visiting periodically the trees from late February until the end of the flowering period, and recording the phenological stages described by Colbrant and Fabre (1975). The time of flowering was determined according to the method described by Fleckinger (1954). Flowering intensity per tree was determined using a visual scale from 0 (no flowering) to 3 (maximum flowering) at full bloom (FB). The number of inflorescences and flowers per inflorescence were counted in each tagged shoot before anthesis. Fertile inflorescences and the number of perfect flowers per inflorescence were determined before petal autumn. Fertile inflorescences were considered those with at least one perfect flower (Rallo and Fernández-Escobar, 1985). The number of fruits per tagged shoot was determined 40 days after full bloom (FB), when June drop is finished and fruit population is maintained until harvest (Rallo and Fernández-Escobar, 1985). To determine the fruit maturation period, the experimental trees were visited every 3-4 days from late August to December, recording the fruit phenological stages of each tree according to the following visual scale: 1 deep green skin; 2 yellowish-green skin; 3 veraison, green skin with reddish patches over more than half of the fruit; 4 purple skin; 5 black skin and white flesh. The maturation period was determined according to Barranco *et al.* (1998).

At harvest, fruits of each tree were collected to determine total weight. Fifty to eighty fruits per tree, depending on the year, were used to determine fruit size (average weight of the

fruits sampled), pulp/stone ratio, expressed as fresh weight, and the maturity index (MI). The MI was determined according to Ferreira (1979). The fruits were classified into 8 categories (1-8) according to the visual scale mentioned above (1-5) extended to 3 more categories: 6 black skin and purple pulp over less than half of the pulp; 7 black skin and purple pulp not reaching the stone but covering more than half of the pulp, and 8 black skin and purple pulp up to the stone. MI is the sum of the multiplication of the number of fruits in each category by the numerical value of each category, divided by total number of fruits.

Fruit oil content was determined by nuclear magnetic resonance (NMR) (Minispec mq 20, Bruker Analytik Gmbh). The results were expressed as percentage of fresh and dry weight (% FW and DW). Anthocyanin fraction was extracted from olive pulp using extraction methods described by Lee *et al.* (2005) with some modifications. Total anthocyanin content of diluted fruit extract was estimated by the pH differential spectroscopic method proposed by Cheng and Breen (1991). Absorbance (A) was measured with a UV-visible spectrophotometer at 510 nm and 700 nm in diluted buffers at pH 1 and pH 4.5, where $A = (A_{510}-A_{700})_{pH\ 1} - (A_{510}- A_{700})_{pH\ 4.5}$. Data was expressed as cyanidin-3-glucoside equivalent per kg of fresh weight. The extraction of the phenolic fraction from the pulp was carried out following the method proposed by Gómez-Rico *et al.* (2008). Total polyphenols content was determined by the colorimetric method described by Vázquez-Roncero *et al.* (1973), using the reagent Folin–Ciocalteu. Absorbance was measured with a UV- visible spectrophotometer (CaryBio50, Varian) at 725 nm. Results were expressed as mg of caffeic acid per kg of pulp.

3. RESULTS

As a previous and necessary step prior to perform the experiments, the temperature inside the chambers was monitored and compared with the outside temperature. The temperature gradient of around 4 °C between the air inside of the OTC and the surrounding environment, was maintained relatively constant from January to July of each year along three consecutive reproductive cycles (2015-2017) (Figure 3.2). This data shows the reliability of the experimental system used to subject trees, growing under field conditions, to warmer temperatures than the ambient one.

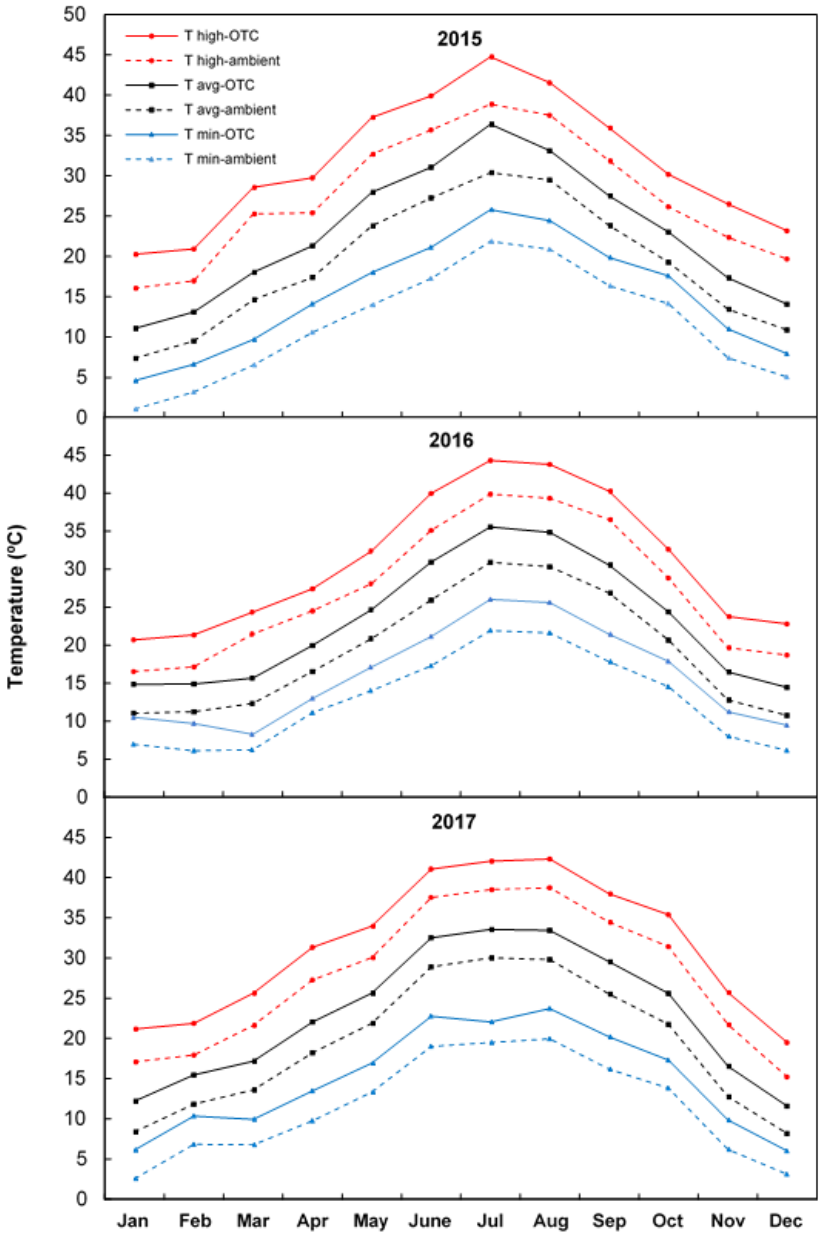
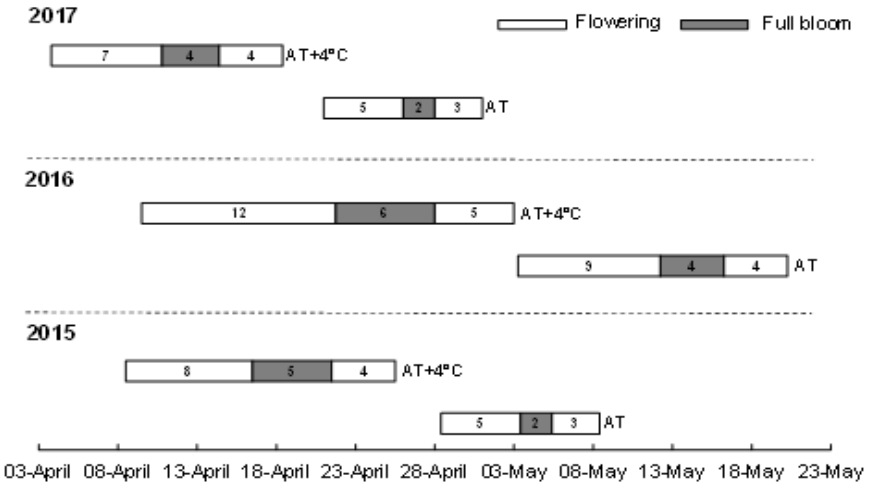


Figure 3.2. Average monthly temperatures recorded from January to December during three consecutive years (T_{avg}). Dotted lines show ambient temperatures and the straight lines show OTC temperatures. T_{high} and T_{low} represents average high and low temperatures by month, respectively.

Under these conditions, trees exposed to 4 °C above the ambient temperature (AT+4 °C) initiated flowering between 18 and 24 days before than control trees, those exposed to the AT treatment, this depending on the year (Figure 3.3 A). The fruit maturation period, i.e., days from the start of veraison to ripeness, was also affected by temperature (Figure 3.3 B). The appearance of reddish spots in the fruits was forwarded about 17-30 days in trees subjected to AT+4 °C treatment when compared with AT trees. This effect was periodically observed in 2015-2017 period. In addition, the warmer temperature prolonged the maturation period in 2015 and 2016, while no effect was observed in 2017 (Figure 3.3 B).

A)



B)

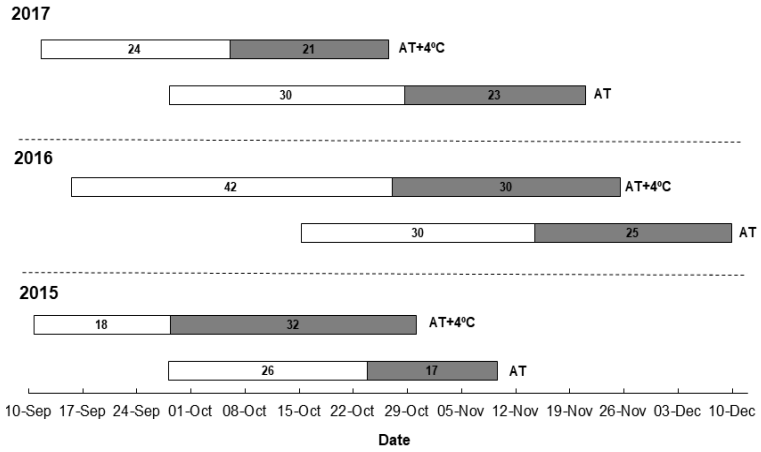


Figure 3.3. Phenological phases occurrences. A) Flowering period under AT and AT+4 °C treatments in three consecutive years. Inside of each bar from left to right it is represented the periods of time (days) from the start of flowering to the start of full bloom, from the start of full bloom to the end of full bloom, and from the end of full bloom to the end of flowering. B) Fruit maturation period under AT and AT+4 °C treatments in three consecutive years. Inside each bar from left to right are represented the periods of time (days) from the start to the end of veraison and from the end of veraison to final ripeness.

In addition, the flowering period and the full bloom period were between 5 and 7 days and 2 to 3 days longer, respectively. Flowering intensity, expressed as the quantity of flowers per tree, estimated on a visual scale of 0 (non-flowering) to 3 (abundant flowering), did not show significant differences between temperature treatments (Figure 3.4).

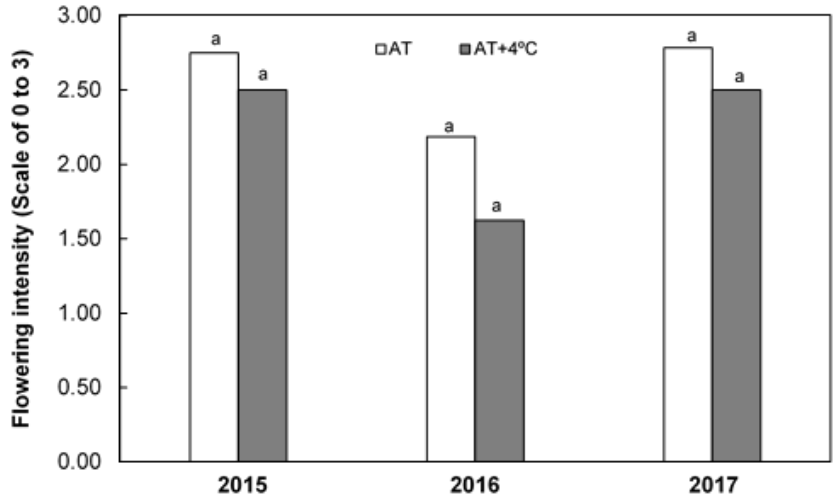


Figure 3.4. Effect of AT treatment vs. AT+4 °C treatment on the quantity of flowers produced by the trees in three consecutive years. Flowering rating: scale from 0-3 (none-very abundant flowering). Mean separation within each year by non-parametric Friedman test.

Flower quality was affected by warm temperatures (Table 3.1). The percentage of perfect flowers and fertile inflorescences significantly decreased in AT+4 °C trees in 2015 and 2016. No effect was observed in 2017. In 2015 pistil abortion was very intense even in control trees, which produced only 23.9 % of fertile inflorescences and 5.9 % of perfect flowers. On the contrary, in 2016 pistil abortion was very low, with 93.9 % of fertile inflorescences and 56.4 % of perfect flowers in AT trees. An intermediate behavior was observed in 2017.

Table 3.1. Effect of a 4 ºC increase in ambient temperature (AT vs. AT+4ºC) on flower quality.

	Flower quality[a,b]											
	2015				2016				2017			
	Flowers		Inflorescences		Flowers		Inflorescences		Flowers		Inflorescences	
Temperature treatment	Total	Perfect (%)	Total	Fertile (%)	Total	Perfect (%)	Total	Fertile (%)	Total	Perfect (%)	Total	Fertile (%)
AT	110.5 a	5.9 a	10.5 a	23.9 a	113.4 a	56.4 a	9.1 a	93.9 a	137.1 a	11.5 a	11.8 a	40.4 a
AT+4ºC	79.2 b	1.4 b	10.3 a	6.4 b	86.4 a	23.4 b	8.2 a	75.6 b	113.6 b	12.6 a	12.1 a	39.5 a
[c]*CV (%)*	*22.3*	*39.7*	*8.2*	*32.9*	*23.7*	*5.9*	*22.7*	*5.5*	*11.1*	*48.1*	*8.4*	*36.8*

[a]Means within each column followed by different letters are significantly different at P≤0.05 by F-test
[b]Each data is the mean of eight trees and was obtained from 15 bearing shoots per tree.
[c]Coefficient of variation

Fruit set was also affected by the treatments. When fruit set was expressed as the percentage of fruits per flower or per inflorescence, and also if it is expressed as fruit per cm of bearing shoot, a significantly decrease in AT+4 °C trees was observed (Table 3.2). It was not occurred in 2016, when fruit set was expressed as percent of fruits per perfect flowers.

Table 3.2. Effect of a 4 ºC increase in ambient temperature (TA vs. TA+4ºC) on fruit set determined 40 days after FB.

	Fruit set[a,b]								
	2015			2016			2017		
Temperature treatment	Fruit/cm	Flowers (%)	Inflorescences (%)	Fruit/cm	Flowers (%)	Inflorescences (%)	Fruit/cm	Flowers (%)	Inflorescences (%)
AT	0.17 a	14.5 a	31.1 a	0.70 a	10.5 a	75.6 a	0.35 a	26.8 a	59.4 a
AT+4ºC	0.01 b	1.1 b	1.9 b	0.23 b	10.7 a	34.0 b	0.16 b	8.1 b	23.8 b
[c]*CV (%)*	*64.9*	*52.6*	*66.8*	*26.5*	*14.8*	*11.2*	*19.8*	*17.3*	*17.5*

[a]Means within each column followed by different letters are significantly different at P≤0.001 by F-test
[b]Expressed as number of fruits per cm of bearing shoot or as the percentage of perfect flowers or fertile inflorescence. Each data is the mean of eight trees, and was obtained from 15 bearing shoots per tree
[c]Coefficient of variation

Table 3.1. Effect of a 4°C increase in ambient temperature (TA vs. TA+4 °C) on fruit set determined 40 days after FB.e (AT vs.

The relative water content (RWC) of leaves was significantly lower in AT+4 ºC trees in winter 2015 and in spring 2016, and unaffected by the treatments at these times in 2017 (Table 3.3). These results indicate that the AT+4 ºC trees was stressed around floral differentiation in 2015 and 2016 compared to control trees. During the summer, there were significant differences between treatments in all the years.

Table 3.3. Effect of a 4 ºC increase in ambient temperature (TA vs. TA+4ºC) on leaf relative water content (RWC).

	Leaf RWC (%)[a]								
	2015			2016			2017		
Temperature treatment	Winter	Spring	Summer	Winter	Spring	Summer	Winter	Spring	Summer
AT	93.2 a	-	89.3 a	89.1 a	92.6 a	87.8 a	88.9 a	90.7 a	90.6 a
AT+4ºC	89.6 b	-	86.9 b	88.4 a	90.5 b	86.1 b	88.2 a	91.0 a	89.4 b
[b]*CV*	*1.3*	-	*1.37*	*1.88*	*0.8*	*1.26*	*1.43*	*1.08*	*0.78*

[a]Means within each column followed by different letters are significantly different at P≤0.05 by F-test
[b]Coefficient of Variation

The increase of ambient temperature in 4 ºC affected the vegetative growth of trees (Table 3.4). There was no effect of this treatment on the growth of tagged shoots in 2015 and 2016, but there were significant differences in 2017. The trunk diameter (cm) of tress at the beginning of the experiment was similar [7.3 (AT) vs. 7.4 (AT+4 ºC)]. However, after exposing trees to 4 ºC above ambient temperature differences were observed. The increment (Δ) of the trunk diameter was significantly greater in AT+4 ºC than in AT trees at the end of each growing season (2015-2017). The same tendency was observed with the pruning material. This is, 4 ºC above ambient temperature applied constantly along the three growing seasons, promoted the vegetative growth of AT+4 ºC trees resulting in trees of significantly bigger size (Table 3.4).

Table 3.4. Effect of a 4 ºC increase in ambient temperature (AT vs. AT+4ºC) on vegetative growth.

	Vegetative growth[a,b]								
	2015			2016			2017		
Temperature treatment	Shoot growth (cm)	Trunk diameter[c] (cm)	Pruning material (kg)	Shoot growth (cm)	Trunk diameter (cm)	Pruning material (kg)	Shoot growth (cm)	Trunk diameter (cm)	Pruning material (kg)
AT	1,6 a	8,1 (0,8) b	4,2 b	9,8 a	9,4 (1,3) b	4,9 b	4,8 b	10,6 (1,2) b	3,9 b
AT+4ºC	1,4 a	8,7 (1,3) a	8,1 a	11,2 a	10,7 (2,0) a	11,0 a	7,6 a	12,5 (1,8) a	12,6 a
[d]*CV (%)*	*31.0*	20.7	*31.9*	*14.0*	*12.5*	*37.5*	*21.3*	*18.6*	*25.9*

[a]Means within each column followed by different letters are significantly different at P≤0.05 by F-test
[b]Each data is the mean of eight trees. In the case of shoot growth, it was obtained from 15 vegetative shoots per tree.
[c]Numbers in parenthesis are the increment of the trunk diameter of trees in two consecutive years.
[d]Coefficient of Variation

Although fruit yield in trees growing under ambient temperature (AT) was not drastically elevated, this was significantly affected in AT+4 ºC trees due to the temperature increase applied during the complete reproductive cycle of the trees (Table 3.5). It was highly impacted in 2015 and in less but similar proportion in 2016 and 2017 (about 60-70 % of reduction, respectively). Fruit oil content values, expressed as percentage of dry and fresh fruit weight (% DW and FW), were also significantly lower in those trees subjected to warmer temperatures (AT+4 ºC vs. AT) (Table 3.5). The reduction in fruit oil content by the high temperature treatment was similar (about 30 % compared to AT trees) along the tree years when expressed over FW. While it was

expressed over DW this effect was more marked in 2015 than in 2016 and 2017 (about 33 and 20 % of reduction, respectively).

Table 3.5. Effect of a 4 ºC increase in ambient temperature (AT vs. AT+4ºC) on fruit yield and oil content.

	2015			2016			2017		
Temperature treatment	Fruit yield (kg per tree)	Oil content (%DW)	Oil content (%FW)	Fruit yield (kg per tree)	Oil content (%DW)	Oil content (%FW)	Fruit yield (kg per tree)	Oil content (%DW)	Oil content (%FW)
AT	5,1 a	53,7 a	18,1 a	16,7 a	52,1 a	19,1 a	22,4 a	51,2 a	20,6 a
AT+4ºC	0,4 b	35,7 b	12,3 b	6,6 b	42,0 b	12,7 b	6,8 b	39,9 b	14,2 b
[a]*CV (%)*	*61.23*	*23.86*	*14.43*	*39.39*	*3.93*	*8.59*	*25.08*	*9.56*	*5.73*

Means within each column followed by different letters are significantly different at P≤0.05 by F-test
Each data is the mean of eight trees
[a]Coefficient of Variation

At harvest, differences in fruit characteristics were observed between temperature treatments (AT vs. AT+4 °C) (Table 3.6). Fruits size was reduced by the AT+4 °C treatment in 2015 and 2016. In 2017 fruits grown under ambient temperature (AT) were smaller in size when compared with those of previous growing seasons and no effect was observed due to the AT+4 °C treatment. Pulp/stone ratio was smaller in AT+4 °C fruits than in AT ones in all seasons. There were no differences in MI between treatments in 2015. However, it was decreased by the AT+4 °C temperature treatment in 2016 and 2017 seasons (Table 3.6).

Table 3.6. Effect of a 4 ºC increase in ambient temperature (AT vs. AT+4ºC) on fruit size, pulp/stone ratio and maturity index (MI).

	Fruit characteristics[a,b]								
	2015			2016			2017		
Temperature treatment	Fruit size (g/fruit)	Pulp/stone	MI	Fruit size (g/fruit)	Pulp/stone	MI	Fruit size (g/fruit)	Pulp/stone	MI
AT	5,7 a	9,0 a	3,9 a	5,5 a	8,3 a	4,3 a	4,5 a	9,2 a	4,7 a
AT+4ºC	3,3 b	4,9 b	3,8 a	5,1 b	7,1 b	4,0 b	4,8 a	6,9 b	4,0 b
[c]*CV (%)*	*15.15*	*15.84*	*9.42*	*3,35*	*8,20*	*4,71*	*14,56*	*12,53*	*7,93*

[a]Means within each column followed by different letters are significantly different at P≤0.05 by F-test
[b]Each data is the mean of eight trees and was obtained from 50 fruits per tree
[c]Coefficient of Variation

The accumulation of anthocyanins in fruits during the maturity period was significantly different in AT and AT+4 °C trees (Table 3.7). Greater values were observed in fruits of AT trees in all seasons, so warmer temperatures during this period could decreased the accumulation of anthocyanins in fruits of AT+4 °C trees. No significant differences were observed in fruit polyphenols contents between treatments in 2016. The high temperature treatment decreased fruit polyphenols content in 2017 (Table 3.7).

Table 3.7. Effect of a 4 ºC increase in ambient temperature (AT vs. AT+4ºC) on fruit anthocyanins and polyphenols content.

	2015		2016		2017	
Temperature treatment	Anthocyanins (cyanidin-3-glucoside equivalent kg^{-1} FW)	Polyphenols (mg caffeic acid kg^{-1} pulp)	Anthocyanins (cyanidin-3-glucoside equivalent kg^{-1} FW)	Polyphenols (mg caffeic acid kg^{-1} pulp)	Anthocyanins (cyanidin-3-glucoside equivalent kg^{-1} FW)	Polyphenols (mg caffeic acid kg^{-1} pulp)
AT	192,6 a	--	993,9 a	8726 a	293,4 a	9511,2 a
AT+4ºC	106,4 b	--	490,5 b	11482 a	127,2 b	6347,6 b
[a]*CV (%)*	*21.75*		*12.91*	*12.21*	*33.33*	*11,05*

Means within each column followed by different letters are significantly different at P≤0.05 by F-test
Each data is the mean of eight trees
[a]Coefficient of Variation

4. DISCUSSION

The evaluation of the effect of global warming on the behaviour of agricultural crops results of interest since an increase of temperature is expected in the near future (IPCC, 2014). For that reason, many studies have been developed to analyse the responses of different crops to temperature. Usually, these studies have been conducted contrasting the behaviour of these crops in different environments (Yaacoubi *et al.*, 2014), that is, in different climatic regions, or developing models to predict the behaviour of a particular crop (De Melo-Abreu *et al.*, 2004). In the present work, we evaluated the effect of global warming on olive floral phenology and fruit set installing, under field conditions, temperature controlled by OTC systems, which maintain inside, 4 °C above the ambient temperature. The reliability of the system for this experiment was first evaluated and shown in Figure 3.2. The advantage of this system is that allows observing just the effect of an increase of temperature in each particular region, excluding the effect of other climatic particularities associated with this region.

Under the conditions of the experiment, it has been observed that an increase of 4 °C in air temperature induced an earlier date of flowering. This effect was predicted to occur in southern Spain by Aerobiological studies and Regional Climatic Circulation Models (Galán *et al.*, 2005; Osborne, 2000; Pérez-Lopéz *et al.*, 2008; Giannakopoulus *et al.*, 2009; Orlandi *et al.*, 2014; Gabaldón-Leal *et al.*, 2017). In addition to that, the data presented here showed that the increase in air temperature extent the flowering period in about six days, when comparing with control trees growing at ambient temperatures. The flowering period is usually shortened when temperature increase during flowering and it is longer with lower temperatures (Rallo, 1997). A possible explanation to the extent of flowering in AT+4 °C trees, could be that despite an increase of temperature of 4 °C, the advancement of the date of flowering to the beginning of April can make that flowering occurs at lower temperatures than flowering in control trees. However, according to the data recorded in Figure 3.2, the average temperatures in April inside the OTCs were similar to external May temperatures, when control trees bloomed. We have no explanation for this apparent paradox, other than other factors may affect the extension of the flowering period, since this effect was observed in all the years analyzed.

Percentage of perfect flowers is important for olive yield. This characteristic has an important varietal component, but it is influenced by environmental conditions, particularly by water deficit during flower differentiation (Uriu, 1960; Hartmann and Panetsos, 1961; Rapoport *et al.*, 2012). In our experiment, pistil abortion occurs in 2015 and 2017 in both AT+4 °C trees and AT trees, probably because the low rainfall at the end of the winter in both years. A low abortion rate was observed in AT tree in 2016, related to the rainfall was higher than in the other years studies. But in 2015 and 2016, pistil abortion was significantly higher in AT+4 °C trees and no significant differences were observed in 2017. The relative water content (RWC) of leaves was

significantly different in AT and AT+4 °C trees in winter 2015 and spring 2016, suggesting a different water status of these trees in dates close to floral differentiation, which may explain the differences observed in pistil abortion in these years. No significant differences in water status were observed in winter or spring of 2017 between AT and AT+4 °C trees, which may explain the lack of differences in pistil abortion in this year.

In addition to the reduction of perfect flowers and fertile inflorescences in AT+4 °C trees, characteristics which may reduce yield, fruit set was also significantly reduced in these trees. The temperature increase treatment may influence flower fertilization, resulting in a poor fruit setting. In other species, temperature affects stigma receptivity (Burgos *et al.*, 1991), ovule longevity (Postweiler *et al.*, 1985) and pollen tube growth (Lewis, 1942; Williams, 1970). In the olive, the optimum temperature for pollen germination and pollen tube growth has been observed to be 25º C. When temperature is above 30 °C, these processes fail affecting fruit setting (Fernández-Escobar *et al.*, 1983; Cuevas *et al.*, 1994). Temperatures above 30 °C have been reached during the pollination period under the experimental conditions of this study, and may have limited flower fertilization and fruit set. Cross-pollination, the pollination with pollen of a different cultivar, allows a greater pollen germination and pollen tube growth at higher temperatures than the own pollen (Fernández-Escobar *et al.*, 1983), suggesting a higher fruit set under these conditions. In fact, the need for cross-pollination in the olive has been reported in areas of Israel, Argentina or Mexico (Rallo and Cuevas, 2017), where temperatures during olive flowering are usually higher than in southern Spain. Results suggest that, under an increase of temperature during flowering, cross-pollination may be a technique to increase fruit set and, consequently, yield.

It was emphasized that in other studies the effect of global warming was analyzed contrasting the behavior of crops located in regions with different mean temperatures or developing models to predict the performance of a particular crop (De Melo-Abreu *et al.*, 2004; Giannakopoulos *et al.*, 2009; Orlandi *et al.*, 2014; El Yaacoubi *et al.*, 2014; Gabaldón-Leal *et al.*, 2017). In our study, we evaluated the effect of global warming on the olive installing temperature-OTC systems under field conditions in an area vulnerable to climatic changes in the near future (Giorgi, 2006; Lionello, 2012; IPCC, 2014). The accuracy of the system is shown in Figure 3.1.

We concluded that when temperature increases 4 °C above the ambient temperature in this region, olive flowering would be earlier and prolongated, significantly increase pistil abortion leading to a reduction in fruit set. Results presented in the present work were obtained from the same experimental trees along the same years. Consequently, they show the end of the fruiting cycle of these trees. The reduction in fruit set, led to a significant reduction in fruit yield, but also in oil content. A reduction in fruit yield usually led to an increase in fruit size. This phenomenon is common in fruit tree species, including the olive and, in fact, fruit thinning is done to increase fruit size at harvest in apple, peaches, and other fruit tree species (Dennis, 2000; Looney, 1993; Wertheim, 2000; Costa and Vizzoto, 2000). However, in our study fruit size was smaller in AT+4

°C than in AT trees, producing a fruit production significantly lower. Yield, fruit size and oil content depend on both genetic and environmental conditions (Lavee and Wodner, 1991). Despite that olive is well adapted to adverse environmental conditions, water stress along the different stages of fruit growth and maturity have been reported to highly affect those parameters (Lavee, 1996). Fruit size has been described to be reduced when water deficit is applied during early fruit growth (Rapoport *et al.*, 2004; Gucci *et al.*, 2009). This effect was mainly attributed to a failure in mesocarp cells extension rather than division. If water limitation is constant along the dry season, the metabolic activity is slowed down and consequently fruit growth and oil accumulation reduced (Lavee, 1996). When occurring at the end of the dry season, when fruit growth rate and oil production are intense, fruits of smaller size and lower oil content are produced. The results obtained under the experimental conditions of the present study could be explained by a water stress effect associated to a higher evapotranspiration demand of trees due to elevated temperature inside the open-top-chamber during the dry season. From the results of this study we cannot elucidate what stage of fruit growth and maturity was more sensitive to the high temperature treatment, but it is clear that it affects any of the developmental processes that determine final fruit size and oil accumulation. Although studies performed in the southern hemisphere with the cultivar 'Arauco' have shown a reduction in fruit size and oil content when high temperature was applied during the oil accumulation phase (García-Inza *et al.*, 2014; 2018). Nevertheless, there is not much information on how global warming will affect these parameters increasing the need of further research on this respect.

During fruit maturation changes in fruit color occur at the same time that oil content increases. In our study the fruit maturity period was forwarded and extended in AT+4 °C trees. Therefore, the reduction in oil content could be also due to a delay in lipogenesis because of the delay in fruit maturation. In fact, the maturity index indicates that the trees subjected to higher temperatures at the time of harvest showed a lower MI than those growing at ambient temperatures. Also, anthocyanins found in AT+4 °C trees, a flavonoid responsible of the fruit color in the olive fruit, was significantly lower than in AT trees, supporting the delay in fruit maturation. This reduction in fruit anthocyanins content due to high temperature has been observed for long in other species (Utsonomiya *et al.*, 1982; Naito *et al.*, 1986; Yamada *et al.*, 1988, Arakawa, 1991; Taira *et al.*, 2000; Isobe and Kamada, 2001). The same effects on fruit maturation were observed in trees subjected to high doses of nitrogen (Fernández-Escobar *et al.*, 2014), indicating the probable relationships between those effects mechanism involved.

Olive trees grow, as most woody species, by forming new shoots and extending and thickening the former ones. The measure of each shoot defines the annual growth of the tree and the tree size. In this experiment, vegetative growth was exuberant in AT+4 °C trees. The annual growth of the trees, estimated measuring shoot growth at the end of the vegetative period and by the weight of the pruning material, was higher in AT+4 °C than in AT trees, probably because of

the stimulating effect of temperature (Way and Oren, 2010). Trunk girth has been related to tree weight (Westwood, 1993) and, consequently, is an estimation of tree size (Fernández-Escobar, 2014). In our work, trunk diameter was higher in AT+4 °C trees, suggesting that the effect of higher temperatures have a direct effect on olive tree growth. The lower temperature threshold for olive vegetative and trunk growth has been estimated to be around 15 and 7 °C, respectively (Pérez-López *et al.,* 2008). Thus, it seems that warmer temperatures during winter and autumn prolonged the growing season in AT+4 °C trees resulting in an increase in tree size.

5. CONCLUSIONS

In conclusion, increasing temperature 4 °C above the actual ambient temperature may lead to an advance of the date of flowering in the olive, an extent of the flowering period, an increase of pistil abortion, and a reduction in fruit set, conditions which may reduce yield. These results suggest that it could be necessary to reduce trees stress during the winter, probably providing irrigation water during floral differentiation or by prolong the irrigation period during the autumn and winter. Also, cross-pollination could be necessary to guarantee a normal fruit set and productivity. Furthermore, increasing temperature 4 °C above the actual ambient temperature could lead to: 1) a delay in fruit maturation, reducing oil content because a delay in lipogenesis; 2) fruit of smaller size; 3) a reduction in yield, due to a reduction in fruit set and an increase in pistil abortion; and 4) an increase in the size of trees. These results suggest that under future warmer conditions, plantation density must be lower to prevent interferences between nearby trees. In addition, actual irrigation management practices should be revised to reduce pistil abortion and increase fruit size and, consequently, yield.

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Chapter IV. Radicular responses to thermal stress on seedlings of *Olea europaea*

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Several studies carried out in olive tree (*Olea europaea* L.) reported an inhibition in the shoot growth when the air temperature rises above 35 °C. However, there is no reference on how growth is affected when the soil warms. In order to examine the temperature effect in the olive aerial and root part, three independent experiments were carried out. The experiments evaluated the effect of high temperatures (37 °C) only in the aerial part in mist-cuttings plants (*experiment 1*) compared to 25 °C temperature control, as well as in combination (aerial and root medium at 37 °C) in seed plants (*experiments 2* and *3*), and even evaluated recovery 48 h and 96 h after heat treatment in root medium (*experiment 3*). The role of K^+ accumulation was evaluated, as well as the determination of K^+ uptake and transport capacity using rubidium (Rb^+) as tracer. The recovery this capacity after being exposed to high temperature was determined as well. In our results, plant dry matter accumulation was reduced when the temperature of both the air and the root medium was moderately high. However, when the medium root temperature was 25 °C, the inhibitory effect of air high temperature on plant growth was not observed. The exposure of both the aerial part and the root to moderate high temperature also reduced the accumulation of K^+ in the stem and the root, the water use efficiency and leaf relative water content. Furthermore, the exposition of the root system at 37 °C for 24 h inhibited K^+(Rb^+) transport from root to shoot having no effect on its uptake. Even though when the root system was exposed permanently to 37 °C, the K^+(Rb^+) uptake and translocation to the aerial part were inhibited as well as the growth in all plants organs. When only the aerial part was exposed to moderate high temperature, the accumulation of K^+ in the stem, the water use efficiency and leaf relative water content were not modified, while the recovery of K^+(Rb^+) root transport capacity after high root temperature was slow. Any signal of recovery was observed after 48 h without stress: both K^+ root uptake and subsequent transport to above organs were inhibited. Whereas 96 h without stress led to restoration K^+ upward transport capacity, the uptake was partially inhibited. These results shown the root system of olive seedling is very sensitive to high temperature related to root K^+ transport and growth of the plant. Taking into account the two processes involved in root K^+ transport, the discharge of K^+ to the xylem vessels was more affected than the uptake at the initial phase of high root temperature stress. However, it was the first process to be re-established during recovery. All this suggest that olive is very efficient in regulating the water and K^+ transport through the plant when only the atmosphere surrounding the aerial part is warmed up. While an increase in the soil temperature decrease root K+ uptake and its transport to the aerial parts resulting in a reduction in water status, shoot growth and leaves with low levels of K^+ contents and dehydration symptoms.

1. INTRODUCTION

The Mediterranean basin is the largest area in the world with an optimal climate for olive (*Olea europaea* L.) cultivation; however, if the current trends in greenhouse gas emissions continue, the environmental conditions of this region will be expected to change in the near future. In particular, air temperature has been projected to rise drastically in the range of 2-5 ºC coupled with more frequent and extreme droughts and heatwaves (Giorgi, 2006; Giorgi and Lionello, 2008; Gualdi *et al.*, 2013; Tanasijevic *et al.*, 2014). Soil temperature is generally lower than that of the air, although seasonal fluctuations occur depending on aboveground factors. The increase in atmospheric temperature is, therefore, expected to be accompanied by a gradual rise in soil temperature, especially in the upper soil layers (IPPC, 2014). Temperature is a primary environmental factor for plant growth and development. Each species has an optimal thermal range, hence when temperature lies outside this range, the physiological and biochemical processes involved in plant growth are impaired, resulting in growth decline (Mahan *et al.*, 1995; Wahid *et al.*, 2007).

The olive is the dominant tree crop in the Mediterranean Basin, having an enormous ecological an economic importance in this region. It is well adapted to the Mediterranean climate characterized by hot and dry summers and mild winters with little rainfall. The optimum temperature for olive vegetative development ranges from 10 to 30 ºC, provided that nutrient and water availability is not a limiting factor. It has been suggested that temperature above 35 ºC could limit olive vegetative growth (Rallo and Cuevas, 2008; Therios, 2009). Considering that temperature values in the Mediterranean region are predicted to exceed the optimal levels for olive performance, this crop is likely to experience frequent periods of temperature stress of both long and short duration, which may affect growth, development and productivity.

Most of the studies addressing plant adaptation to warmer temperatures have been focused on plant responses to increasing air temperature (Paulsen, 1994; Wahid *et al.*, 2007) and pay scarce attention to the underlying processes that occur when the soil warms up. Given that the microbial activity, mineralization processes and movement of ions in the soil are temperature-dependent (Zak *et al.*, 1999; Hussain and Maqsood, 2011), higher temperatures may contribute to loss of soil fertility and diminish the availability of nutrients for plants (St Clair and Lynch, 2010). The scarcity of soil water and mineral nutrients under warmer conditions could negatively affect crops, as they are primary soil resources for plant growth. Among the essential mineral nutrients, potassium (K^+) is directly involved in plant growth processes: its accumulation in the cell contributes to the creation of the osmotic component of the water potential needed to absorb water (Kramer, 1983) and generate the cell turgor required for cell elongation (Wyn Jones *et al.*, 1979; Mengel and Arneke, 1982; Hsiao and Läuchli, 1986; Shabala and Lew, 2002; Chen *et al.*, 2007).

Despite the olive tree adaptation to arid climates, a syndrome of dehydration is often observed, which, in many cases, has been associated with a poor K^+nutritional status of the plant (Fernández-Escobar *et al.*, 1994). Plants need to accumulate large amounts of K^+ in their cells, reaching concentrations in the cytoplasm of between 100 and 200 mM. Therefore, it seems that plant water economy depends on plant K^+ status. In this regard, in olive trees and other crops, it has been observed that K^+ starvation inhibits the effect of water stress on stomatal closure (Arquero *et al.*, 2006; Benlloch-González *et al.*, 2008) and enhances water loss through transpiration (Sudama *et al.*, 1998; Cabañero and Carvajal, 2007). As a result, the plant displays lower water use efficiency and its growth is diminished (Fournier *et al.*, 2005; Arquero *et al.*, 2006). It is, therefore, not surprising to observe that when a plant is supplied adequately with K^+ its tissues are better hydrated (Mengel and Kirkby, 2001) and is more resistant to drought and other unfavourable environmental conditions such as salinity, waterlogged soils or toxicity caused by other nutrients (Cakmak, 2005; Zörb *et al.*, 2014).

Changes in the morphology and distribution of the root system through the soil profile have been observed in response to certain abiotic stresses, most likely to enhance nutrient uptake (Smucker and Aiken, 1992; Feddes and Raats, 2004; Benlloch-González *et al.*, 2014). In different olive cultivars, the hydraulic properties of the root system were adjusted differently according to the grade of salt tolerance of the cultivars (Rewald *et al.*, 2011). Therefore, it might seem reasonable to think that in a near future the nutrient status of crops will be determined in part by the root function under warmer conditions. In relation to this, it has been reported that crops survival under adverse environmental conditions is linked to the nutritional status of plants (Marschner, 1995; Wang *et al.*, 2013). Among the essential mineral nutrients, K^+, as mentioned above, has a key role in this respect (Zörb *et al.*, 2014). Most plants are able to regulate K^+ homeostasis in response to different environmental challenges (Anschütz *et al.*, 2014). This is because plant tissues have very efficient mechanisms for the uptake and redistribution of K^+, in which different families of membrane proteins are involved (Ashley *et al.*, 2006; Nieves-Cordones *et al.*, 2014). Plants are able to adjust to variations in the availability of K^+ through changes in root architecture and the activation or inhibition of K^+ transporter systems (Nieves-Cordones *et al.*, 2014). Nevertheless, the potential effects of high temperature on these mechanisms and the consequences on plant K^+ nutrition are not clear. The movement of ions through the cell membranes seems to be very sensitive to changes in soil or root temperature (Chapin, 1974; BassiriRad *et al.*, 1993). As temperatures rise, membrane protein-transporters change their configuration (Epstein and Bloom, 2004). A reduction in the absorption of K^+ (Rubidium ion, Rb^+) has been observed in whole sunflower plants, isolated corn roots, and tomato plants when the temperature of the root medium was above 33, 30 and 35 °C, respectively (Bravo-F and Uribe, 1981; Benlloch *et al.*, 1989; Falah *et al.*, 2010). In a recent study using tomato plants, the reduction in root K^+-uptake rate under high root medium temperature was correlated to a decrease in the concentration/activity of the potassium transporter KT1 (Giri *et al.*, 2017). In creeping bentgrass,

exposure of the root system to high soil temperature (35 °C), while maintaining the shoot at normal temperature significantly reduced K^+ uptake by the root (Huang and Xu, 2000).

The influence of high soil temperatures on root growth and physiology and the impact on root-shoot relationships has not been researched in depth (BassiriRad, 2000; Huang *et al.*, 2012). This is of great importance in olive orchards, where the irrigation systems commonly used mean that the most active roots are located in the upper soil layers (Fernandez and Moreno, 1999), which are the most exposed to high temperatures, because these soils tend to have poor plant cover. Despite growing recognition of this fact, there is little information on how warmer soil temperatures will affect olive root growth and the uptake of K^+, and its subsequent allocation to the different plant organs, which results in plant growth. The accumulation of a high level of K^+ in plant tissues under warmer conditions would support both the development of organs and the transpiration of leaf area, contributing in this way towards alleviating the possible detrimental effects of high temperatures.

The olive root system is expected to be exposed to short and long periods of high temperature stress in the future, however, how this organ will cope under these circumstances is unknown. It is not clear how high temperature in the root will affect K^+ accumulation and distribution through the plant, alteration on K^+ transporter systems involved in K^+ nutrition, and consequently on growth and water relations. To reach this goal, firstly, different olive plant material, mist-rooted cuttings and young plants generated from seeds, were grown under moderate high temperature (37 °C) for 64 and 42 days, respectively. In order to clarify the effect on K^+ transporter, olive seedlings generated from seeds is twofold. The immediate and prolonged effect of moderately high temperature applied in the root medium on $K^+(Rb^+)$ uptake and its subsequent allocation to the different plant organs and, consequently, plant growth, was examined. Secondly, it evaluates if the $K^+(Rb^+)$ uptake and transport capacity of the root system is recovered after being exposed to a period of high temperature. Interpreting root responses to temperature is often a complicated task due to interactions with experimental conditions and surrounding environmental factors. Therefore, in this study, root medium and shoot air temperatures were controlled independently and the nutritional status of the root surface was maintained relatively constant. The plants were grown in a liquid hydroponic system, to ensure that potential changes in root functions were not due to any alterations in the nutrient supply. Considering all these, the aim of this study was to examine in olive adaptability to global warming.

2. MATERIAL AND METHODS

2.1. Plant material and growth conditions

Experiment 1. Rooting cuttings of olive 'Picual' were used. Previously, 20 cm length semi-hardwood cuttings recollected on March, with two pairs of leaves, were propagated. The basal end

of cuttings was treated with IBA (indole-3-butyric acid) (3000 ppm) applied for 10 s, and placed in a mist bend with basal temperature control (22 °C).

After 40 days, 20 rooting cuttings were individually transferred to 3.5 l cylindrical plastic pots (11 cm diameter, 37 cm deep) filled up with a sand/peat mixture (2:1v/v). Below the soil substrate a 6-7 cm layer of gravel was placed to facilitate drainage of excess water. To this end, a drainage tube was also connected to the bottom of every container. After transplantation, each plant was watered in excess causing the drainage of water. Olive plants were placed in a controlled growth chamber with a relative humidity between 60 and 80 %, a constant temperature of 25 °C (day/night), a photoperiod of 14h of light and a photosynthetic photon flux density of 350µmolm^{-2}s^{-1} (fluorescent tubes, Sylvania cool-white VHO). Every week, the plants were irrigated and fertilized using 2gL^{-1} of Hakaphos® Verde fertilizer 15-10-15 (Compo) containing 15 % N, 4.4 % P, 12.4 % K, 1.2 % Mg, 12 % S, 0.01 % B, 0.05 % Fe, 0.05 % Mn, 0.02 % Zn, 0.02 % Cu and 0.001 % Mo.

After three weeks of acclimation, half of the plants were transferred to another controlled growth chamber with the same characteristics described above except for the temperature set at 37 °C. In this way, the plants were exposed to two air temperature regimes: 25 °C (control) and 37 °C (high temperature) for 64 days. While the former was irrigated every week, plants exposed to 37 °C were irrigated every 3-4 days. The volume of water drained was individually collected after each irrigation. Weekly, both types of plants were fertilized as described above.

Experiment 2. Young plants generated from seeds of the olive 'Arbequina' were used. Seeds were soaked in a Ziram fungicide solution (2gL^{-1}) for 5 min and stratified on filter paper moistened with water, in covered petri-dishes, at 14 °C for 21 days. After stratification, the seeds were sown in recipients with perlite moistened with water and placed in a germination chamber at 25 °C. After 10 days, the seedlings were individually transferred into 680mL flasks containing a Hoagland's type nutrient solution (NS) and placed in a controlled growth chamber with a relative humidity between 60 and 80 %, a temperature of 25/22 °C (day/night), a photoperiod of 12 h of light and a photosynthetic photon flux density of 350 µmol m^{-2} s^{-1} (fluorescent tubes, Sylvania cool-white VHO). The NS had the following composition: 2.5 mM $Ca(NO_3)_2$; 2.5 mM KCl; 0.25 mM $Ca(H_2PO_4)_2$; 1.0 mM $MgSO_4$; 12.5 µM H_3BO_3; 1.0 µM $MnSO_4$; 1.0 µM $ZnSO_4$; 0.25 µM $CuSO_4$; 0.2µM $(NH_4)_6Mo_7O_{24}$ and 10µM Fe-ethylenediamine-di-o-hydroxy-phenylacetic acid. The NS was aerated continuously and renewed every week during the whole experiment. Plants were grown under these conditions for 14 days before different temperature treatments were imposed.

After this growth period, the aerial part and the root system of the plants were exposed to different air/root-medium temperature regimes: 25/25 °C, 37/37 °C and 37/25 °C for 42 days. To apply these three temperature regimes, some plants were transferred to another controlled growth chamber with the same characteristics as described above except that the temperature was set at 37 °C. In this way, the air of the atmosphere and the root-medium were maintained at the ambient

temperature of each growth chamber, 25 and 37 °C, so the aerial part and the root system were exposed to 25/25 °C and 37/37 °C, respectively. To maintain the root medium at 25 °C, when the ambient temperature was 37 °C (37/25 °C), a cooling system was designed. The flasks were introduced in a water bath maintaining the desire temperature (25 °C) using a thermostat connected to an electric valve, which regulated the flow of cold water passing throw a glass coil. Water level was maintained at the top edge of each bath during the experimental period. Plants were under these temperature regimes for 42 days.

Experiment 3. Olive 'Arbequina' seeds were germinated and grown as described in *Experiment 2* until temperature treatments were applied. After growth and acclimation, the root system of a group of plants was maintained at the ambient temperature of the growth chamber (25 °C), while in others the temperature of the root medium was increased to 37 °C. In order to reach this temperature in the root medium, the flasks were placed in a water bath at 37 °C positioned in the growth chamber. An immersion-heater regulated by a thermostat kept the roots at the desired temperature. To maintain a constant temperature of 37 °C in the entire root zone of the plants, the water level was kept at the top edge of the bath throughout the experimental period. In all the plants, the aerial part was exposed to the ambient temperature of the growth chamber (25 °C). The plants were kept under these conditions for 33 days. In order to determine the immediate effect of high temperature in the root system, a set of plants grown in the root medium at 25 °C, 24 h prior to the end of the experiment, was introduced in a water bath at 37 °C. In this way, three root temperature treatments were applied: 25 °C, 37 °C and 25/37 °C (24 h) with the aerial part kept at 25 °C in all cases. To study the recovery capacity of the root system after being exposed to a high temperature period, another experiment was performed. Firstly, the root system of the plants was exposed to either 25 or 37 °C, with the aerial part kept at ambient temperature (25 °C), for 31 days. The same procedure described above was followed. After that, those plants whose root systems were exposed to 37 °C were taken out of the water bath and kept at the ambient temperature, 25 °C, for recovery for either 48 h [37to25 °C] or 96 h [37to25 °C].

In all cases, the nutrient solution of all the plants was changed for another with the same composition as the basic one, except that KCl was replaced by RbCl (2.5mM). The plants were kept under this condition for 24 h prior to the end of the experiment. This assay was performed as follows: 1) in plants whose root system had been exposed to 25 °C and 37 °C permanently and to 37 °C for a short period (24 h) [25; 37; 25/37 (24 h) root temperature treatment, respectively]; and 2) in plants which were subjected to different recovery periods from high temperature (37 °C) in the root medium [25; 37/25 (48 h), 37/25 (96 h) root temperature treatments]. In both groups of experiments, at the end of this assay, the plants were harvested as described below.

2.2. Data collection

Once the experiments were over, after 64 days (*Experiment 1*), 42 days (*Experiment 2*) or 33 days (*Experiment 3*), the same set of data were collected. In all the experiments described, the same procedure was followed. The plants were harvested and separated into leaves, stems and roots. Each organ was individually stored in an oven at 70 °C to determine dry matter. Before harvesting, the length of the primary shoot and the secondary shoots of each plant was determined (only *experiment 2*) as well as the primary root length. A fresh sample of four fully expanded leaves per plant was also collected to determine the leaf relative water content (RWC) according to the procedure reported by Stocker (1929): leaves were first weighed, then rehydrated over 24 h and re-weighed.

Along the *Experiments 1 and 3*, the length of new shoots was determined every week as well as water consumption. Water consumption was estimated as the difference between applied and drained volume (only *experiment 1*). The water use efficiency (WUE) was determined as the grams of biomass produced (dry matter) per L of water consumed during the whole growth cycle. Stem and root relative growth rate (RGR) were calculated at different moments during the experiment using the following equation:

$$RGR = (\ln(\text{length at time 2}) - \ln(\text{length at time 1}))/(\text{time 2-time 1}).$$

In the three experiments, both K^+ and Rb^+ accumulation in each plant organ was determined by atomic absorption spectrophotometry (Perkin Elmer 1100 B) after extraction from the leaves, stems and roots by ashing each tissue at 600 °C; ashes were dissolved with 1N HCl. The rates of Rb^+ uptake (V_{Rb}) and Rb^+ translocated to the shoot by the root were calculated from the Rb^+ accumulated in all plant organs and in the aerial organs (stem plus leaves), respectively, and the values were referred to the dry weight of the root and the time of uptake ($\mu molg^{-1}$ root DWd^{-1}).

2.3. Statistical analysis

The experimental design was a randomised complete block design, with two temperature treatments and ten replicates per each (n=2 x 10) in the *Experiment 1* and three temperature treatments and five replicates per each (n=3 x5) in *Experiment 2* and in *Experiment 3*. The values shown are means ± standard error (SE). Statistical analyses were made using Statistix 8.0 software package (Analytical Software, Tallahassee, FL, USA). The significance of the differences between the means values was determined by analyses of variance using Tukey's test and a 5 % rejection level. In all analyses, residual plots were generated to identify outliers and to confirm that variance was common and normally distributed.

3. RESULTS

To estimate the effect of global warming on the growth of olive plants, K^+ allocation in the different plant organs and water relations, mist-olive cuttings were grown in a sandy soil substrate in a growth chamber at 37 °C for 64 days. A moderately high temperature treatment had a negative effect on the growth of plants.

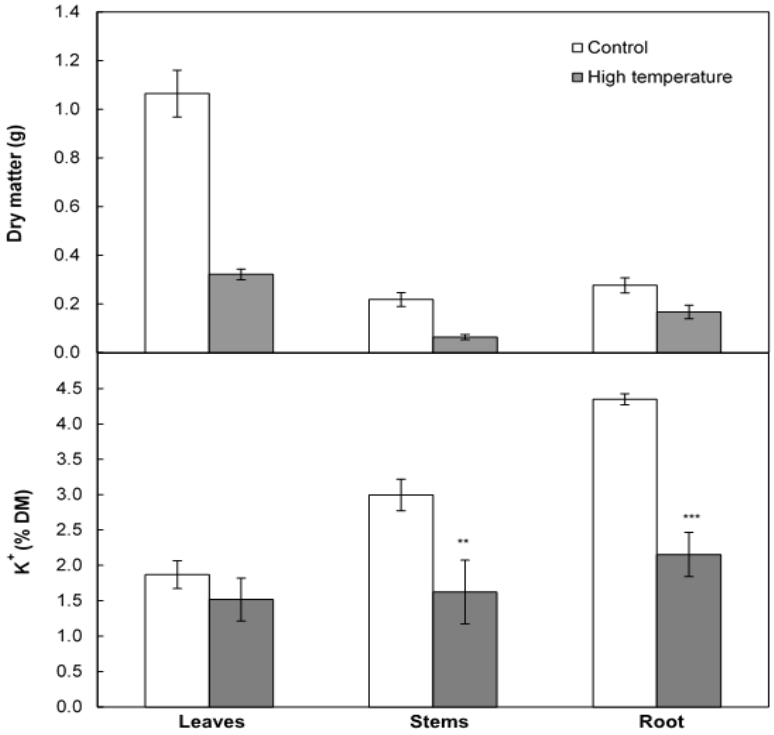

Figure 4.1. Effect of aerial high temperature (Control vs. High temperature) on dry matter accumulation (g) and K^+ concentration (% DM) in leaves, stems and root. Mist-rooted olive cuttings grown in 3.5L pots containing a sand/peat mixture were subjected to high temperature (37 °C) for 64 days. For all treatments, values are the mean of 10 replicates ± SE. The level of significance by Tukey´s test is shown at the top of each bar * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

Plants exposure to high temperature (37 °C) led to a reduction in plant biomass when comparing with plants grown at 25 °C (control) (Figure 4.1). The accumulation of dry matter was inhibited in all plant organs - root, stem and leaves - although this effect was more accused in the aerial organs than in the root. The high temperature also reduced the elongation of the stem. This effect was short notice; three weeks after the application of the temperature treatment, significant differences in stem elongation were observed between high temperature and control plants. At the end of the experiment, the reduction in total shoot length was of 74 % (Figure 4.2). In the same direction, plants grown under high temperature showed a reduction in the length of the taproot of 65 % (Figure 4.3).

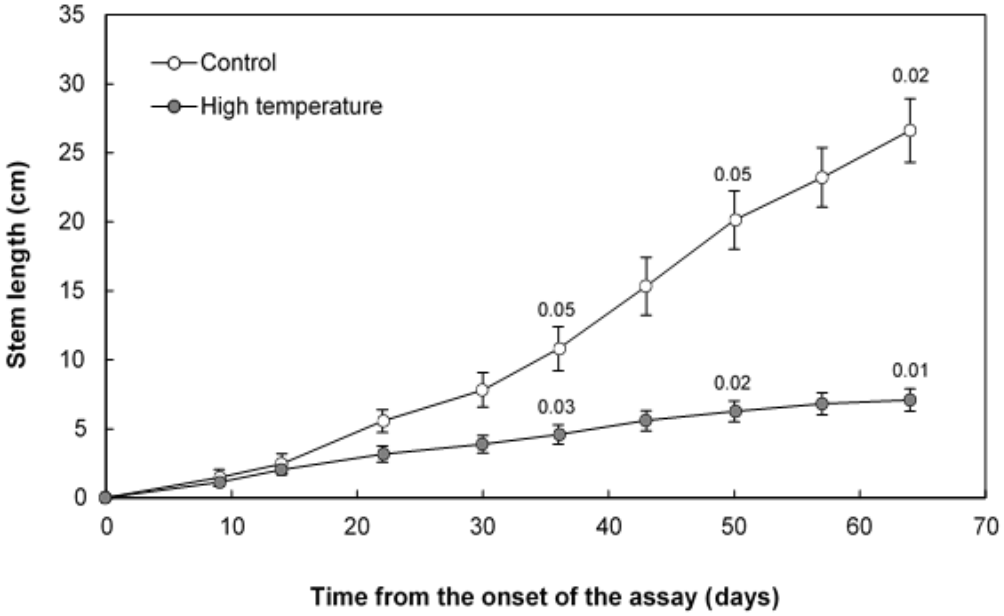

Figure 4.2. Effect of aerial high temperature (Control vs. High temperature) on stem growth (cm). Mist-rooted olive cuttings grown in 3.5L pots containing a sand/peat mixture were subjected to high temperature (37 °C) for 64 days. Stem length was measured only on that part of the plant growing during the assay (64 days). Values are the mean of 10 replicates ± SE. Numbers above symbols indicate values (cm cm^{-1} day^{-1}) of the stem relative growth rate (RGR) at 14 days intervals from day 22 to day 64 after the onset of the assay (stem growth during 22-36 days interval, 36-50 days interval and 50-64 days interval).

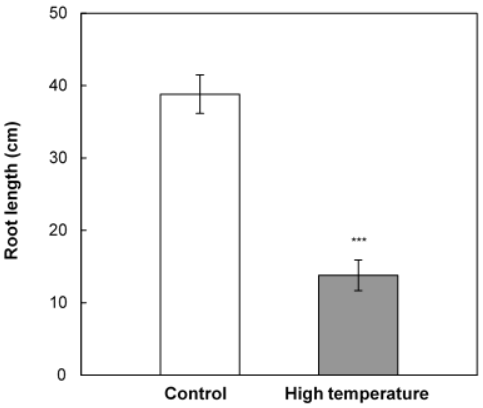

Figure 4.3. Effect of aerial high temperature (Control vs. High temperature) on root length (cm). Root length refers to primary root growth. Values are the mean of 10 replicates ±SE. The level of significance by Tukey´s test is shown at the top of the bar *** ($P < 0.001$).

The high temperature did not affect the K^+ nutritional status of the leaves. The leaf K^+ concentration in both control and high temperature treated plants was above 1.5 % DM (Figure 4.1). In control plants, the K^+ distribution in the different organs was not uniform. The root showed the highest K^+ concentration, followed by the stem and leaves. The high temperature treatment significantly inhibited the accumulation of K^+ in both stem and root, although this effect was more pronounced in root (Figure 4.1).

The high temperature treatment altered the water status of the plant: not only RWC significantly reduced, but also WUE (Figure 4.4). WUE was drastically reduced in plants under high temperature showing values approximately 75 % lower than control plants.

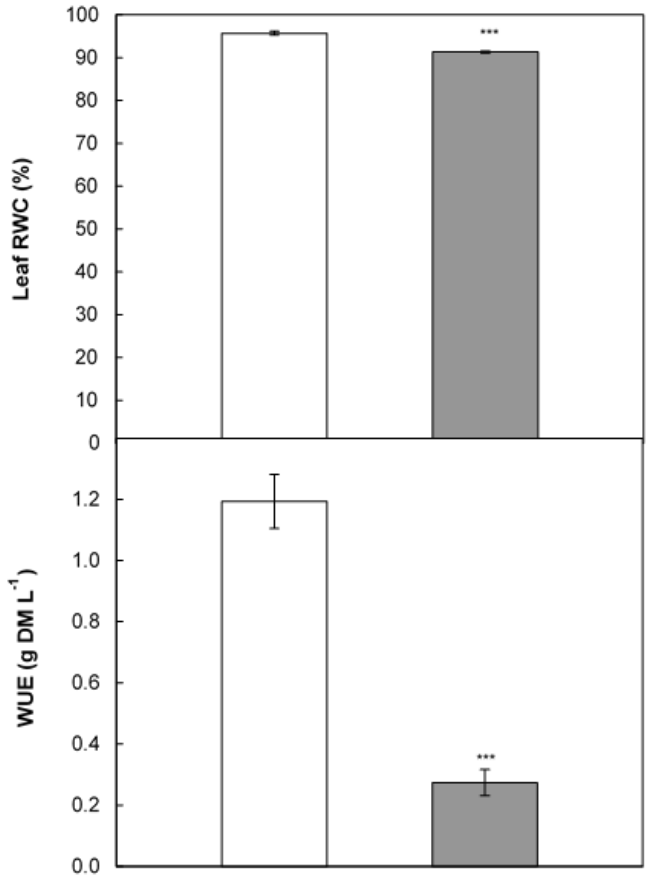

Figure 4.4. Effect of aerial high temperature (Control vs. High temperature) on leaf relative water content (RWC) and water-use efficiency (WUE). Values are the mean of 10 replicates ± SE. The level of significance by Tukey´s test is shown at the top of each bar *** (P <0.001).

Although the atmospheric temperature of the growth chamber at 37 °C was maintained constant along the experiment, a rise in the temperature of the superficial soil layers was observed. It ranged from 41 to 37 °C at 10 and 20 cm-depth from the soil surface, respectively. In order to check the specific effect of the high temperature treatment on the aerial organs, regardless of the temperature of the root system, another experiment was performed with young olive seedlings in a hydroponic medium to regulate more accurately the temperature of each part of the plant. To reach this goal, the aerial organs and the root were exposed to three air/root-medium temperature regimes: 25/25 °C, 37/37 °C and 37/25 °C.

The high air temperature (37 °C) did not affect the growth of the aerial organs of the plant (leaves and stems) when the temperature in the hydroponic root-medium was 25 °C (37/25), but it did significantly favour root growth (Figure 4.5). Although this treatment (37/25) did not affect the accumulation of dry matter in the aerial part, it had an important effect on its morphology.

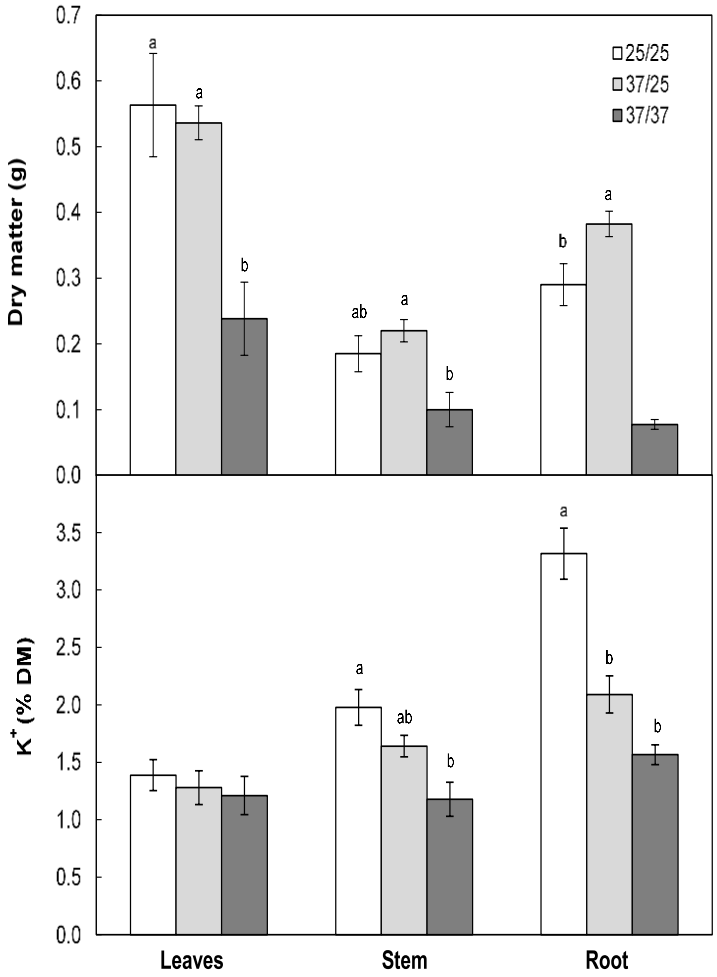

Figure 4.5. Dry matter accumulation (g) and K^+ concentration (% DM) in leaves, stems and root of young olive plants after exposing the aerial part and the root to different air/root-medium temperature regimens (25/25 °C, 37/25 °C and 37/37 °C) for 42 days. The plants were grown in nutrient solution. Values are the mean of 5 replicates ± SE. Within each organ, different letters at the top of each bar indicate significant differences (Leaves dry matter P< 0.01; Stem P< 0.05; Root P< 0.001). Means separation by Tukey´s test.

The growth of secondary shoots was enhanced (Figure 4.8), their total length (Figure 4.6 A) and the length of the main root (Figure 4.6 B, Figure 4.8). However, under high temperature conditions in both areas of the plant (37/37), the growth of all organs was significantly inhibited (Figures 4.5 and 4.6 B).

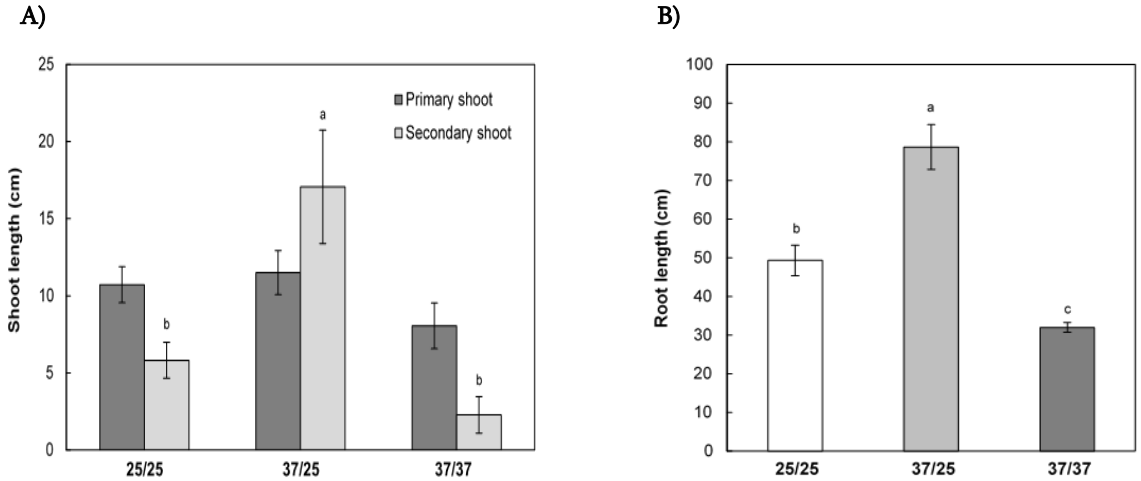

Figure 4.6. **A)** Primary shoot length and secondary shoots length (cm) of young olive plants after exposing the aerial part and the root to different air/root-medium temperature regimens (25/25 °C, 37/25 °C, 37/37 °C) for 42 days. **B)** Root length of young olive plants after exposing the aerial part and the root to different air/root-medium temperature regimens (25/25 °C, 37/25 °C, 37/37 °C) for 42 days. The plants were grown in nutrient solution. Root length refers to primary root growth. All values are the mean of 5 replicates ± SE. Different letters at the top of the bars indicate significant differences (P< 0.001). Means separation by Tukey´s test.

The high temperature conditions in the growth chamber did not affect the potassium accumulation in the aerial part, but it did inhibit its accumulation in the root, when the temperature of the hydroponic root medium was 25 °C (37/25). When the temperature of the aerial surrounding and the root-medium were both high (37/37), the K^+ concentration in the stem and root system was significantly lower than the control, although it did not affect K^+ accumulation in the leaves (Figure 4.5).

The high temperature treatment did not significantly affect the water status of the plant when the temperature was only applied to the aerial part (37/25). However, high temperatures in both areas of the plant (37/37) led to a significant fall in RWC (Figure 4.7).

To evaluate the high temperature effect on the K^+(Rb^+) uptake and transport capacity of the root system were monitored. Young olive plants generated from seeds were cultivated for 33 days in a liquid hydroponic medium. During the cultivation, a group of plants was subjected to a high temperature treatment (37 °C), aimed exclusively at the root, while the aerial part remained at 25 °C, the same temperature as the root and aerial part of the control plants.

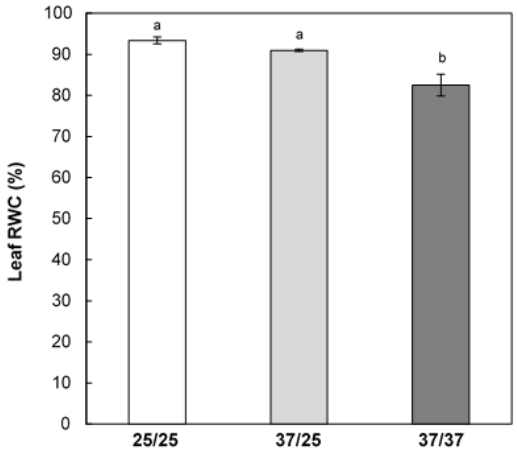

Figure 4.7. Leaf RWC of young olive plants after exposing the aerial part and the root to different air/root-medium temperature regimens (25/25 °C, 37/25 °C and 37/37 °C) for 42 days. The plants were grown in nutrient solution. Different letters at the top of each bar indicate significant differences ($P < 0.001$). Means separation by Tukey´s test.

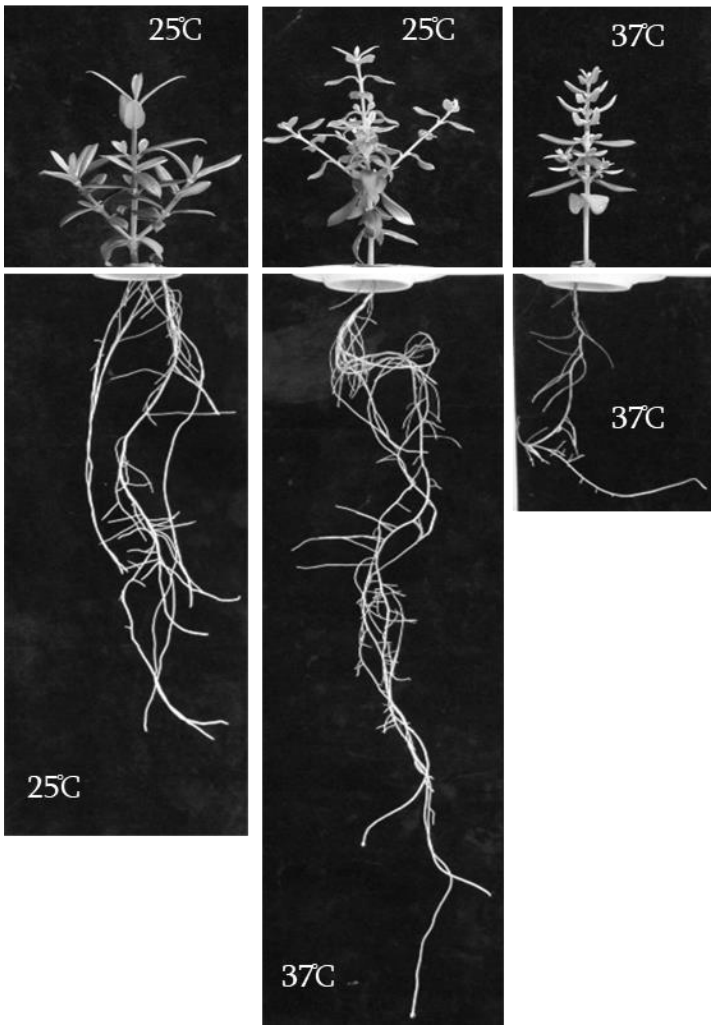

Figure 4.8. View of the aerial part and the root of young olive plants after exposure to different air/root-medium temperature regimens for 42 days.

The high temperature located in the root inhibited the growth of the plant, although the effect was more marked in the root than in the aerial part (Table 4.1). The accumulation of dry matter in the root of plants under high root temperature was less than 50 % of that of the control plants at the end of the experiment (Table 4.1). The high temperature in the root also significantly affected the accumulation of dry matter in the aerial part: the leaves were more sensitive than the stem to high root temperature with 39 % and 33 %, respectively, less dry matter compared with the control plants (Table 4.1).

Table 4.1. Effect of high temperature in the root medium (37 ºC vs. 25 ºC) on dry matter (g) and K^+ concentration (% DM) in leaves, stems and root. Young olive plants were subjected to high root temperature (37 ºC) remaining the aerial part at 25 ºC for 33 days. Values are the mean of 5 replicates ± standard error. Means separation by Tukey´s test.

	Dry matter (g)			K^+ concentration (% DM)		
Temperature (ºC)	**Leaves**	**Stem**	**Root**	**Leaves**	**Stem**	**Root**
25	0.44 ± 0.06	0.15 ± 0.02	0.21 ± 0.02	1.17 ± 0.07	1.41 ± 0.05	1.36 ± 0.16
37	0.27 ± 0.02	0.10 ± 0.02	0.10 ± 0.01	0.43 ± 0.01	1.04 ± 0.05	0.74 ± 0.08
Significance	*P=0.025*	*ns*	*P=0.001*	*P=0.0004*	*P=0.002*	*P=0.014*

The effect of the high temperature treatment applied in the root on the length of the primary root and the shoot was more marked than that observed on the accumulation of dry matter in these organs. At the end of the experiment, the reduction in the length of the primary root and stem was 80 % and 50 %, respectively, compared to control plants (Figure 4.9).

In addition to that, both organs responded differently. The effect in the root was extremely rapid: 7 days after the onset of the treatments, RGR fell by 40 % compared with the control, and continued dropping sharply until growth was stopped at day 28 (Figure 4.9 A). In the case of the stem, the differences in length were less marked: they became apparent 14 days after the onset of the treatments, although, actually, they never stopped their growth (Figure 4.9 B).

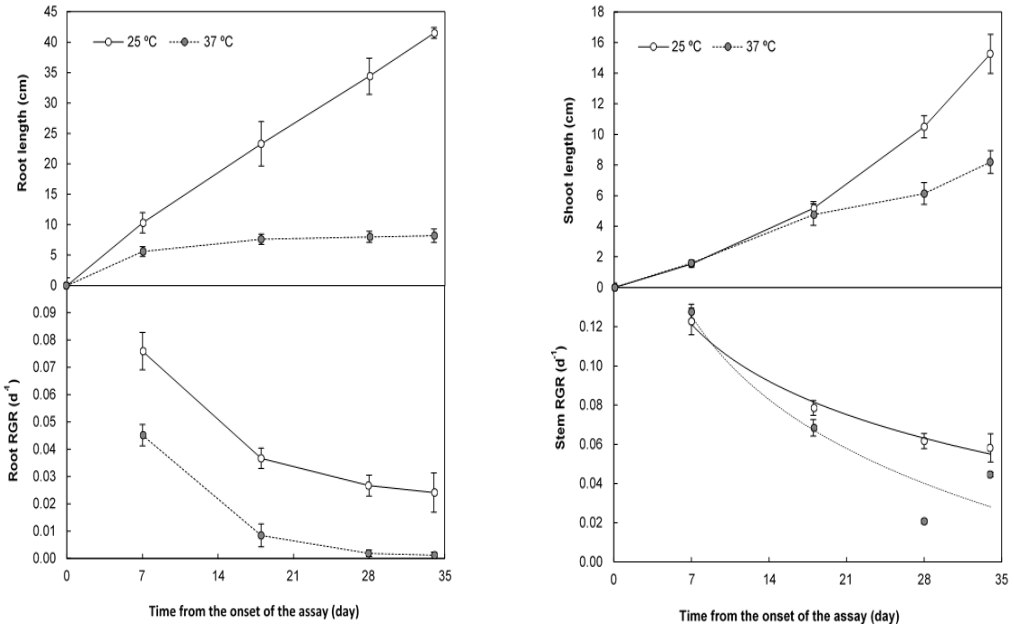

Figure 4.9. Effect of high temperature in the root medium (37 °C vs. 25 °C) **A)** On root length and root relative growth rate (RGR) in young olive plants; **B)** On shoot length and stem RGR in young olive plants. Length (cm) refers to new growth generates (stem or primary root) during the assay measured every 7-10 days. All plants were grown in hydroponic medium exposing the root system either to 25 or to 37 °C while the aerial part to 25 °C for 33 days. RGR, expressed as cm cm^{-1} day^{-1}, was calculated at 7-10 days intervals from day 7 to day 33. Full or empty symbols represent 25 or 37 °C root temperature medium, respectively. Values are the mean of 5 replicates ± SE.

At the end of the experiment, the concentration of K^+ in the different plant organs was measured (Table 4.1). The high temperature in the root reduced the accumulation of K^+ in all the organs, albeit/although unequally. The greatest reduction was observed in the leaves, followed by the root and finally the stem. 24 hours prior to the end of the experiment, the uptake of Rb^+ by the root and its translocation to the aerial organs was determined. The root systems were kept at the same temperature as during the growth period (25 and 37 °C), except for a group of control plants, whose root medium temperature was changed from 25 to 37 °C (Figure 4.10).

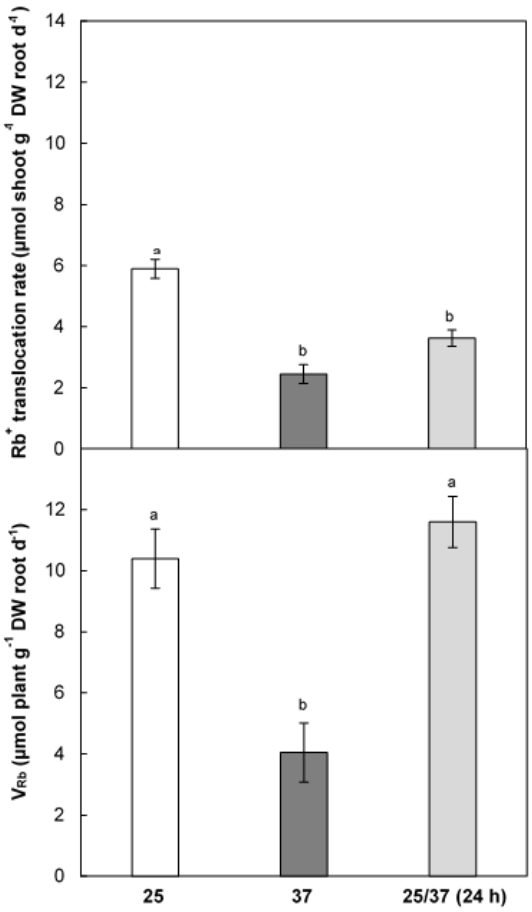

Figure 4.10. Rb^+ uptake rate (V_{Rb}) and Rb^+ translocation rate in young olive plants subjected to different root temperature regimens. Plants were grown in hydroponic medium exposing the root system to 25 or to 37 °C for 33 days and to 37 °C for a short period (24 h) (root medium temperature (°C): 25; 37; 25/37 (24 h)). The aerial part was maintained in all cases at 25 º C. 24 h prior the end of the assay K^+ was replaced by Rb^+ in the nutrient solution. Values are the mean of 5 replicates ± SE. Different letters at the top of the bars indicate significant differences among root temperature treatments ($P< 0.001$). Means separation by Tukey´s test.

The plants were kept during this period in the same hydroponic medium used during growth, except that, in this case, K^+ was replaced by Rb^+. The effect of the high temperature in the root on the transport of Rb^+ depended on the length of the treatment. In plants grown under a 37 °C root medium for 33 days, the high temperature treatment strongly inhibited both the uptake and translocation of Rb^+ to the shoot. However, when the high temperature acted for 24 hours, only the translocation to the aerial part was inhibited, and no effect was observed on the absorption (Figure 4.10). In the first case, the accumulation of Rb^+ was reduced in all the organs, whereas in the second, it was reduced in the stem, but favored in the root (Figure 4.11).

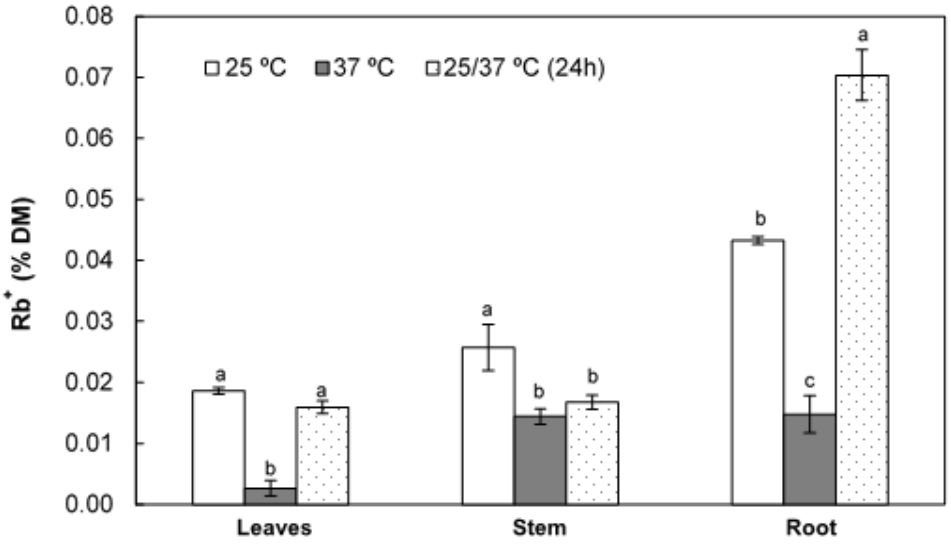

Figure 4.11. Effect of prolonged and immediate high temperature in the root medium on Rb^+ accumulation in different plant organs. Plants were grown in hydroponic medium exposing the root system to 25 or to 37 °C for 33 days and to 37 °C for a short period (24 h) (root medium temperature (°C): 25; 37; 25/37 (24 h)). The aerial part was maintained in all cases at 25 °C. 24 h prior the end of the assay K^+ was replaced by Rb^+ in the nutrient solution. Values are the mean of 5 replicates ± SE. Different letters at the top of the bars indicate significant differences among root temperature treatments (Leaves and Root $P< 0.001$; Stem $P<0.05$). Means separation by Tukey´s test.

In previous experiments, the recovery capacity of K^+ transport after root temperature stress was determined. The plants were grown with a constant root temperature of 25 °C or 37 °C for 31 days. Thereafter, in the treatments at 37 °C, the temperature of the root medium was changed from 37 °C to 25 °C for the last 48 or 96 hours. In all cases, the K^+ in the root medium was replaced by Rb^+ 24 hours prior to the end of the assay. After 48 hours of recovery (48 h with the root kept at 25 °C), the uptake and translocation of Rb^+ was significantly lower than in the control (where the root was kept at a constant 25 °C). However, after 96 hours of recovery (96 h with the root at 25 °C), Rb^+ translocation was similar to that of control, and inhibition of absorption was less pronounced. The uptake of Rb^+ after 96 hours of recovery was significantly lower than that of the control and greater than after 48 hours of recovery (Figure 4.12).

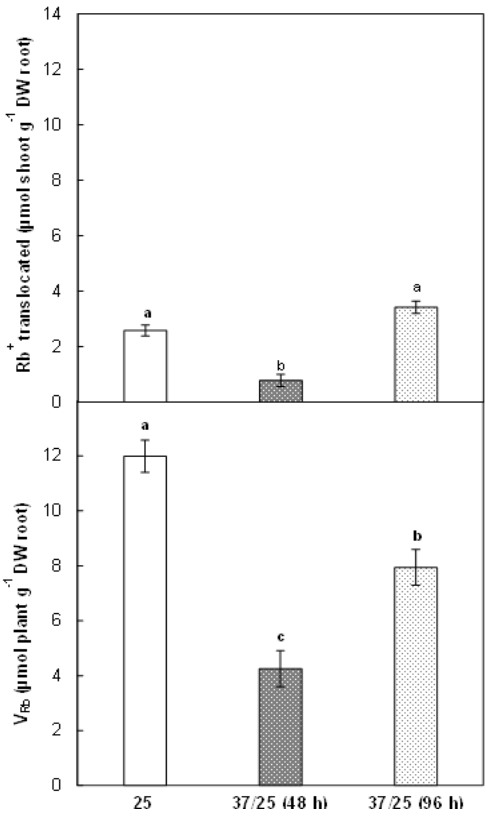

Figure 4.12. Rb^+ uptake rate (V_{Rb}) and Rb^+ translocation rate in young olive plants after being subjected to recovery from high root temperature stress for 48 and 96 h. Plants were grown in hydroponic medium exposing the root system either to 25 or to 37 °C while the aerial part to 25 °C for 31 days. Thereafter, in 37 °C root temperature plants, the temperature of the root medium was changed from 37 °C to 25 °C for 48 or 96 hours (root medium temperature (°C): 25; 37to25 (48 h); 37to25 (96 h)). 24 h prior the end of the assay K^+ was replaced by Rb^+ in the nutrient solution. Values are the mean of 5 replicates ± SE. Different letters at the top of the bars indicate significant differences among root temperature treatments (V_{Rb} $P< 0.001$; Rb translocation rate $P<0.01$). Means separation by Tukey´s test.

Concerning the K^+ content in the different organs analyzed at the end of the experiment, showed that in the root temperature stress treatment it was significantly lower than in the control, except in the leaves of the plants given the 96-hour recovery treatment. In this case, no significant differences were observed compared with control plants (Figure 4.13).

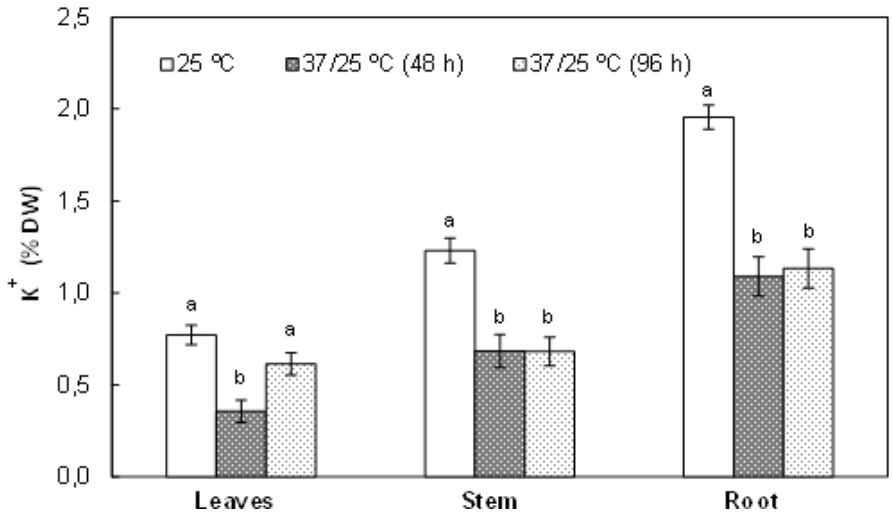

Figure 4.13. K^+ accumulation in different organs of young olive plants subjected to high root temperature stress and thereafter to recovery for 48 and 96 h. Plants were grown in hydroponic medium exposing the root system either to 25 or to 37 °C while the aerial part to 25 º C for 31 days. Thereafter, in the treatments at 37 °C, the temperature of the root medium was changed from 37 °C to 25 °C for 48 or 96 hours (root medium temperatures (°C): 25; 37to25 (48 h); 37to25 (96 h)). Values are the mean of 5 replicates ± standard error. Different letters at the top of the bars indicate significant differences among root temperature treatments (Leaves $P< 0.01$; Stem and Root $P<0.001$). Means separation by Tukey´s test.

4. DISCUSSION

Evidence of global warming has aroused a lot of interest in the possible effects of the projected increase in air temperature on crop growth and development. The effect of heat stress caused by extreme high temperatures applied for short periods of time has been studied at the molecular level (Sachs and Ho, 1986; Queitsch *et al.*, 2000). However, on the plant level, few studies have analysed the prolonged effect of a moderately high temperature treatment. While most of these studies have focused on the effect of high temperature on the aerial part of the plant (Paulsen, 1994; Wahid *et al.*, 2007), there is some information in the same species on the effect of high temperature when applied in both atmosphere and soil (Graves *et al.*, 1991; Huang *et al.*, 2012). In the case of olive, there are no studies on this respect, even though the issue is of great importance considering that under future climate scenarios, agriculture will become increasingly dependent on irrigation. In this respect, the prevailing dripping irrigation systems in olive groves prompt the location of the most active roots in the upper soil layers, which at the same time are the most exposed to high temperature.

It has been suggested that the vegetative growth of olive is inhibited when the temperature of the air rises above 35 °C (Rallo and Cuevas, 2008) - a temperature which is regularly reached, these days, for long periods in the areas where this crop is cultivated. There is no reference on how the increase in the temperature of the soil affects growth in olive trees. In our study, the high air temperature (37 °C) reduced plant dry matter accumulation only when the temperature of the root medium was also high (37/37). When the temperature of the root medium was 25 °C (37/25), the inhibitory effect of high air temperature on plant growth was not observed (Figures 4.1 and 4.5). However, although these conditions are apparently not harmful to the plant, the 37/25 treatment led to morphological changes in the aerial part, and to a greater root growth (Figures 4.6 A and 4.6 B, Figure 4.8). This suggests that the aerial part can sense the increase in the temperature of the air and send signals to the root to enhance growth in this organ. This response might be related with a mechanism to escape from stress. In this regard, Xu and Huang (2000) observed, in Creeping Bentgrass, that high temperatures in the aerial part encouraged the transport of carbohydrate to the root, as long as the root temperature was not high.

It is known that the growth of the stem is highly dependent on the turgor of the cells of the growing zones of this organ (Hsiao, 1973), and that the transport of water and K^+ into these zones contributes to the maintenance of stem growth (Benlloch and Benlloch-González, 2016). Small losses in cell turgor can lead to the inhibition of growth. It is also widely known that the increase in air temperature generally results in an increase of the evaporative demand, which may cause the dehydration of the plant (Kramer, 1983). Under these conditions, the regulation of stomatal opening may be involved in avoiding the dehydration of the aerial part of the plant. Potassium is known to play a direct role in this mechanism (Fischer and Hsiao, 1968), in fact when plants are starved in K^+ the effect of water stress on stomatal closure is inhibited (Benlloch-

González *et al.*, 2008). This work has shown that the dehydration of the aerial part of the plant and the reduction in growth are only induced when the temperature of both the air and the root medium is high (Figures 4.1, 4.4, 4.5 and 4.7). These results suggest that the olive is very efficient in regulating the movement of water through the plant when only the atmosphere surrounding the aerial part is warmed up. However, an increase in soil temperature leads to a loss in water use efficiency (Figure 4.4). Both the inhibition of photosynthesis and the promotion of water flow through the plant could be the cause of this loss of water use efficiency. It has been reported that high soil temperature (35 °C) inhibits photosynthesis in *Agrostis stolonifera*, which is a heat-sensitive species (Huang *et al.*, 2001); however, no evidence has been shown that soil warming favours the hydraulic conductivity of the plant - the opposite effect is, in fact, more likely (Graves *et al.*, 1991). However, it is definitely known that a moderate deficiency of K^+ in the root can enhance the hydraulic conductivity in this organ (Quintero *et al.*, 1998).

One of the functions of the root system is to provide mineral nutrients to the shoot, this function can be modified if the temperature of the root medium rises. It is well known that the maintenance of the cell turgor requires the accumulation of K^+ in the cells of the expanding zones of the plants (De La Guardia and Benlloch, 1980; Leigh and Wyn Jones, 1984; Hsiao and Läuchli, 1986). Considering this, the reduction in plant growth due to high temperature in the root medium, could be attributed to a lower rate of K^+ transport from the root to the shoot. It has been observed in different species that temperatures over 33 °C inhibit the absorption of K^+ by the root (Bravo-F and Uribe, 1981; Benlloch *et al.*, 1989; Falah *et al.*, 2010). In this work, the high temperature applied simultaneously to both parts of the plant did not alter the leaf's nutritional status in K^+, although it did inhibit its accumulation in the stem, and especially in the root (Figures 4.1 and 4.5). Similar results have been reported in two Creeping Bentgrass cultivars, with differing degrees of heat tolerance (Huang and Xu, 2000). In both cultivars, the high temperature in the plant inhibited the accumulation of K^+ in all plant organs. The lower K^+ content in the stem of the olive tree, observed when the temperature of the root medium was high, could account for a lower growth in this part of the plant. Conversely, when the high temperature was only applied in the shoot, there was no inhibition of any organ, as already mentioned, and the K^+ concentration in leaves and stems was not affected, although its content in the root was drastically reduced. This effect was not observed in the case of Creeping Bentgrass (Huang and Xu, 2000) for which the accumulation of K^+ in the root was not affected, although the K^+ accumulation was inhibited in the aerial part in the cultivar most sensitive to higher temperatures. Our results have shown that the olive tree has a great capability for accumulating K^+ in the root. When the temperature was adequate, the concentration of K^+ in this organ was found to be two and half times higher than in the aerial part. With high temperature, either in the whole plant or only in the aerial part, the K^+ concentration in the root was smaller, but the concentration was never lower than that in the aerial part (Figures 4.1 and 4.5).

The growing concern about climate change has drawn increased attention to the effect of high temperature on plant production. However, despite the numerous studies on plant molecular responses to high temperature (Sachs and Ho, 1986; Queitsch *et al.*, 2000), few have analyzed the effect of moderate high temperature in the root on growth and ionic transport. This effect is much more noticeable in the case of the olive tree. This crop has been suggested to impair its vegetative growth when the atmospheric temperature exceeds 35 °C (Rallo and Cuevas, 2008; Therios, 2009). Surprisingly, the species is extremely well adapted to areas where this temperature is regularly exceeded during the summer season. In a recent study using different olive plant material, it has been observed that the exposition of the aerial part to moderate high temperature (37 °C) did not affect the accumulation of dry matter in the plant and favored primary root growth (Figures 4.1 and 4.5). However, only when both the root and the aerial part were exposed to 37 °C, the high temperature treatment was harmful. It can be hypothesized that moderate high temperature in the root could inhibit the transport of K^+ towards the aerial part.

There is no information on how olive root warming affects the transport of K^+ in this organ, or whether it negatively affects the growth of the plant. Given the importance of K^+ for plant growth (Marschner, 1995), and considering that the cultivation techniques used in new olive plantations favor root warming - bare soil at the base of the tree, drip irrigation with drippers and pipes close to the surface, water stored in open-air reservoirs (Alcántara *et al.*, 2017; Orgaz *et al.*, 2017). It is crucial to bring to light the specific effects of olive root warming on K^+ transport and plant growth. Further information is also necessary to elucidate on the one hand, whether the potential effects of root temperature stress are immediate or take time to trigger out and on the other hand, the recovery capacity of root K^+transport after the stress. In the present study, the exposition of the root system of young olive plants to a moderate high temperature (37 °C), with the aerial part remaining at an optimal temperature for growth (25 °C), inhibited both root and shoot growth (Table 4.1). Another work dealing with the same species has focused on the effect of temperature stress localized exclusively in the aerial part of the plant, and in this case, no effect was observed on the accumulated dry matter - on the contrary, it favored the elongation of the root (Benlloch-González *et al.*, 2016). In our current work, the most significant effect of temperature stress located exclusively at the root on plant growth was the inhibition of the stem and primary root elongation (Figure 4.9 A and 4.9 B). These results suggest that the root system of young olive plants is very susceptible to soil warming and that the perception of the temperature stress in the root triggers out signals that induce a negative regulation of growth in the aerial part. We do not know what kind of signals are generated in olive roots in response to temperature stress; however, taking into account the role of K^+ in maintaining the cellular turgor needed for cell elongation (De La Guardia and Benlloch, 1980), as well as the role played by the transport of water and K^+ in the root in the recovery of growth after different kinds of stress (Benlloch and Benlloch-González, 2016), it is logical to believe that the inhibition of root K^+transport under high

temperature is one of the primary effects of stress. The severe inhibition of the stem and primary root elongation due to high root temperature supports this hypothesis (Figure 4.9 A and 4.9 B).

Two independent transport processes acting in series are involved in the transport of ion in the root from the external medium to the xylem vessels. The first one is located in the plasma membrane of epidermal and cortical cells while the second in the cells of the stele. The former allows the uptake and incorporation of ions in the symplast of the root and the latter the discharge or release of ions to the apoplast of the stele which is in contact with the xylem vessels. The joint action of both processes coupled with the effect of the Caspari Band on the endodermis cells, avoiding the apoplastic flow of water and ions, allows the selection of ions by the root and subsequent accumulation in the xylem. The upward movement of ions from the root to the aerial part is produced by mass flow created by root pressure or by transpiration (Marschner, 1995).

In the case of K^+, the mechanisms involved in uptake process are better identified than those involved in xylem loading. In *Arabidopsis*, there are five types of transporters involved in K^+ absorption: the Shaker and two-pore K^+ channels, K^+ uptake permeases (KUP / HAK / KT), HKT and K^+/H^+ antiporters (Very and Sentenac, 2003; Gomez-Porras *et al.*, 2012; Sharma *et al.*, 2013). Only one Shaker family channel type, the Stelar K^+ Outward Rectifier (SKOR), has been identified, related with K^+ discharge into the xylem, and its expression has been localized in the tissues of the root stele (Gaymard, 1998; Liu *et al.*, 2006).

It is not known how high temperature in the olive root affect the transport of K^+ in this organ. However, there is some information on this respect in other species, which suggest that a moderate high temperature in the root can cause a negative effect on the transport of K^+ in this organ. The exposition of the root system of different Creeping Bentgrass cultivars to a supraoptimal temperature (35 °C) throughout the growing period inhibited the accumulation of K^+ in the aerial part and the root. This effect was more marked with K^+ than with other mineral nutrients such as phosphorus and nitrogen (Huang and Xu, 2000). In tomato, when plants were grown in nutrient solution at high temperature (35 °C), the K^+ concentration in the xylem sap was reduced, suggesting that root warming inhibited the K^+ translocation towards the aerial part of the plant (Falah *et al.*, 2010). In sunflowers, the effect of root temperature on the rate of Rb^+ uptake has been studied for short periods of 20-minutes. Under these conditions, it was only possible to determine the absorption of Rb^+, but not the discharge into the xylem. The effect of temperature was dependent on the K^+ nutritional status. In plants with adequate K^+ levels, the temperature did not affect the uptake rate of Rb^+. However, in plants starved in K^+, temperatures of over 33 °C sharply inhibited the uptake of Rb^+, acting in both cases transporters kinetically different (Benlloch *et al.*, 1989).

The results obtained in this work have shown that the transport of K^+ in the root system of young olive plants is very sensitive to root warming. The warming of the root (37 °C) uninterruptedly for 33 days caused the inhibition of the Rb^+ uptake rate as well as the

translocation to the aerial part (Figure 4.10). These results suggest that prolonged warming of the root adversely affects the transport systems involved in uptake and loading processes. It is impossible to say whether gene expression and/or activity of transporters are responsible for that, or neither what type of transporters are expressed in both processes. However, these results also suggest that the nature of the transporters involved in both uptake and loading is different, since they show different sensitivity to temperature changes. The occasional heating of the root (24 h), from 25 ºC to 37 ºC, inhibited the translocation of Rb^+ to the aerial part, but not its uptake. The absence of a rapid response in the inhibition of K^+ uptake by high temperature was similar to that described in sunflower plants with adequate K^+-levels (Benlloch *et al.*, 1989). However, the effect of high temperature on the discharge of K^+ was rapid, which leads us to believe that the signaling routes trigger out by high temperature are different in both processes. In *Arabidopsis*, it has been reported that abscisic acid rapidly inhibits the SKOR expression in root stellar tissue and that this transporter is involved in the release of potassium in the xylem (Gaymard, *et al.*, 1998). Since the role of abscisic acid in different stress situations is well known (Lee and Luan, 2012), it is reasonable to think that the warming of the olive root may play a role in the inhibition of K^+ translocation to the aerial part, possibly inhibiting the expression of transporters of the Shaker family. On the other hand, the fact that, after heat stress, the translocation of K^+ to the aerial part recovers before its absorption supports the idea that different mechanisms are involved in the regulation of both processes, the uptake and the loading.

5. CONCLUSIONS

The obtained results suggest that the root behaves as a powerful source of K^+ that can be used by the plant to maintain the growth of the aerial part in situations of heat stress in that organ. This hypothesis is supported by the adequate water and K^+ status in the aerial part and the maintenance of growth under high temperature conditions only in the aerial part, together with the promotion of root growth and the reduction in K^+ concentration in that organ. When high temperature affects the whole plant, the root loses that capability, and water use efficiency, leaf water status, stem K^+ content and plant growth are all inhibited. These results suggest that high temperatures in the root of the olive tree can inhibit K^+ transport in that organ.

Also, olive tree is known as a species which is well adapted to arid climates with hot and dry summers; however, there is also a widespread idea that it declines growth when the ambient temperature is above 35 ºC. In this work using young olive plants, it has been shown that this observation is true whenever root warming occurs. In natural environments, with spontaneous vegetation, this situation is very uncommon, however, some cultivation practices can favor soil warming, causing harmful effects to the plant by inhibiting K^+ absorption and transport to the aerial part. Along with all this, the water deficit, created by the high vapor pressure gradient

between the plant and the atmosphere, together with the loss of ability to regulate stomatal closure induced by K^+ starvation (Benlloch-González *et al.*, 2008) could explain the symptoms frequently observed in olive orchards when dry and high temperature spells occur: a reduction in stems growth and leaves with low levels of K^+ contents and dehydration symptoms (Fernández-Escobar *et al.*, 1994).

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Chapter V. A "shotgun" proteomic approach to study changes in the protein profile along the olive fruits ripening process under field warming conditions

Presented in:

Sánchez-Lucas, R., Benlloch-González, M., Valledor, L., Jorrín-Novo J. (2018). A proteomic approach to study the effect of global warming on the ripening processes of olive fruits. INPPO2018. Internacional Plant Proteomics Organization. Padova, Italy. From 09-09-2018 to 12-09-2018

Olea europaea L. subsp. *europaea* is one of the most emblematic crops of the Mediterranean Basin, where temperature regimes were expected to rise drastically. This fact could affect any of the phenological stages of this crop and consequently its productivity. Ripening of fleshy fruits is a complex process that involves dramatic changes in chemical composition resulting in colour, texture, flavour, and aroma. To evaluate the impact of warmer temperatures on the mesocarp development, the protein profiles in olive fruits at different ripening stages were analyzed. Fruit samples were collected from trees (cv. `Picual´) growing under field conditions at 4 °C above ambient temperature. This temperature gradient between the trees and the surrounded environment was obtained by temperature-controlled Open-Top-Chamber, equipped with heating and ventilation devices. Fruits were sampled at three ripening stages following the Ferreira (1979) modified scale: green (0), turning red or veraison (3) and purple (4). Proteins were extracted (TCA-acetone/Phenol method) and subjected to LC-LTQ-Orbitrap-MS analysis. A total of 3,522 proteins were identified by using a specie-specific database with FDR<0.01. Of the total identified proteins, 665 proteins showed qualitative or quantitative significant differences among stages and treatments of which 186 proteins varied among ripening states and present sensibility to warming treatments. The greatest ratios between treatments corresponded to proteins of the carbohydrate, lipids and secondary metabolism. These results agree with those obtained in previous analysis of fatty acid, anthocyanin and polyphenol contents. The results from this study suggest that global warming will affect the ripening processes modifying the fruit characteristics and the final oil quality. This should be considered under future climate change scenarios. Further experiments will be carried out by screening the transcripts of the most relevant identified proteins from this experiment.

1. INTRODUCTION

Olive tree is considered the major and emblematic tree crops of the Mediterranean Basin, where temperature regime is expected to rise drastically in the near future in the range 2-5 °C (Gualdi *et al.*, 2013; Giorgi *et al.*, 2006). In addition, more frequent and extreme events such as drought periods and heat-waves are expected to happen in this region. However, long-term temperature field experiments are necessary to study the plant response and adaptability to current conditions. Open top chambers (OTCs) are useful tools for the simulation of global warming under field conditions (Allen *et al.*, 1992; Ceulemans and Mousseau, 1994; Norby *et al.*, 1997).

The economic impact of olive tree resides on the olive oil production, mechanically extracted to olives fruits (olives), that is worldwide appreciated because of its chemical

composition and derived nutraceutical value on beneficial effects on human health. The harvesting period is defined by the optimal ripening stage, when olives achieve their maximum oil content and best oil quality. Olive breeders use as traditional harvesting dates, changes in fruit skin colour (Barranco *et al.*, 2008; Ferreira, 1979) and natural fruit drop as guidelines. However, these parameters are not valid for this purpose because the development and ripening process of olive fruit changes with the cultivar and environmental conditions (Barranco *et al.*, 2000). Particularly, the olives skin colour darkens from lime-green (chlorophylls and carotenes) to purple-black (anthocyanins). The anthocyanins content in a fruit (biosynthesis, accumulation and degradation) determines its color, purple or black in the case of olive (Roca and Mínguez-Mosquera, 2001) and temperature influences these processes. High temperature has been described to reduce anthocyanins accumulation in many fruits and plant tissues (Steyn *et al.*, 2002) affecting fruit color (Koshita, 2015). This fact alters the naturally ripening processes as previous result was concluded when adult trees under field warming conditions was evaluated (Benlloch-González *et al.*, 2018).

Fruit ripening is a highly coordinated, genetically programmed, and an irreversible phenomenon involving a series of physiological, biochemical, and organoleptic changes that finally leads to the development of a soft edible ripe fruit with desirable quality attributes (Brady, 1987). Olive fruit ripening begins around August-September and the complete ripening of the most cultivars is only reached in in winter (Shulman and Lavee, 1979). Olives, considered as non-climateric fruits, ripening is accompanied by changes in weight, pulp/pit ratio and epicarp pigmentation, fruit firmness as well as changes in chemical composition, oil contents and lipids profile, enzyme activity, secondary metabolism, among others that are involved on olive oil composition and organoleptic characteristics (Beltrán, 2000). All of them are influenced by agronomical and environmental conditions as crop load and water availability (Lavee and Wodner, 1991; Barone *et al.,* 1994; Lavee *et al.*, 1990; Inglese *et al.*, 1996; Tombesi, 1994).

It is well established that the expression of specific genes and the subsequent synthesis of proteins and enzymes, both affected by internal and external factors, are required for the onset and the progression of ripening (Giovannoni, 2004). In the recent years, the proteomic approach became widely recognized as a powerful tool in elucidating complex pictures, such as those characterizing fruit development. Some of these studies addressed their attention to the effects induced by heat treatment (Prinsi *et al.*, 2011; Lara *et al.*, 2009; Cheng *et al.*, 2016) or chilling damages (Dagar *et al.*, 2010). However, most of these studies deal with the effect of high temperature in post-harvest trials. In addition, comparative proteomics, based on two-dimensional electrophoresis (2-DE) coupled to tandem mass spectrometry was carried out on olive fruits during drupes development in order to elucidate the protein responsible for quality traits (Bianco *et al.*, 2013). However, gel-based techniques have some limitations such as low proteome coverage and reproducibility (Canovas *et al.*, 2004). As an alternative, third-generation strategies based on liquid

chromatography coupled to mass spectrometry have emerged; this is known as gel-free strategy or shotgun approach. It provides simpler experimental designs, without the need to employ protein separation techniques, and that involve less sample manipulation; however, both techniques are complementary and can be combined in a single experiment (Canovas *et al.*, 2004). In addition, the development of high-resolution and high-precision mass spectrometers allow it has become an important approach in the quantitative analysis of biological samples. These techniques allow a greater coverage of the proteome, as well as an estimation of the amount of proteins in absolute terms (Gonzalez-Fernandez *et al.*, 2013). This new technique is a powerful tool to carry out the study of gene expression through the corresponding translation products and the characterization of complex biological systems (Davalieva *et al.*, 2016). Nowadays the "shotgun" strategy is employed to several descriptive and comparative studies focused on plant stress response

The aim of this study was to determine under field conditions, in a Mediterranean climate, the effect of 4 °C increase in air temperature on protein profile of olive fruits during ripening, selecting 3 critical ripening stage: green (0), veraison (3) and purple (4) (Barranco *et al.*, 2008; Benlloch *et al.*, 2019). To reach this goal, olive trees `Picual´ cultivar, were subjected to warming conditions, increasing 4 °C above the ambient temperature than ambient using temperature-controlled OTCs systems (Benlloch-González *et al.,* 2018; 2019). Proteomic data were integrated with some phenotypic (skin colour, fatty yield, total contents of polyphenols and anthocyanins) ones, and results revealed differences are proposed in warming conditions tolerance between ripening stages and underlying molecular mechanisms involved. The information resulting from this study, about the impact of warmer temperatures on ripening processes gives an important overview of the impact of global warming on olive oil quality and production.

2. Material and Methods

2.1. Plant material and experimental design

Adult 'Picual' olive trees (*Olea europaea* L.) growing in the experimental farm of 'Campus de Rabanales', University of Córdoba, Spain (37°55'N 4°43'W) were used to perform the experiment such as described by Benlloch-González *et al.* (2018). Trees were subjected to two temperature treatments, ambient temperature (AT) and 4 °C above ambient temperature (AT+4 °C) though the OTC structures such as is described on Chapter III.

To determine the fruit maturation period, the experimental trees were visited every 3-4 days from late August to December, recording the fruit phenological stages of each tree according to the following visual scale: 1 deep green skin; 2 yellowish-green skin; 3 veraison, green skin with reddish patches over more than half of the fruit; 4 purple skin; 5 black skin and white flesh. The maturation period was determined according to Barranco *et al.* (1998) (Chapter I. Phenological data recompilation). During ripening, 20 fruits per each ripening stage are collected at random

from each tree (taken as biological replicate). The 20 fruit were pooled and divided into pericarp (skin and flesh) and endocarp (pit) when the samples were homogenized in liquid nitrogen and then stored at -80 °C until biomolecular analysis. For such purpose three critical stages were selected to the biomolecular approaches: the initial phase, green (0); the phase that present more alterations under AT+4 °C treatment respect AT, veraison (3) and the previous phase to harvesting, purple (4) (Figure 5.1).

At harvest, fruits of each tree were collected to determine total weight. Fifty to eighty fruits per tree, depending on the year, were used to determine fruit size (average weight of the fruits sampled), pulp/stone ratio, expressed as fresh weight, and the maturity index (MI). The MI was determined according to Ferreira (1979).

Figure 5.1. Ripening stages selected and visualized during the field experiment on olive trees under warming conditions. **A)** Green skin (0); **B)** Advanced Veraison (3) at AT conditions; **C)** Advanced veraison (3) at AT+4 °C conditions. **D)** Purple skin (4) that suppose change of ripening stage.

2.2. Chemical determinations

Olives on veraison stage (3) and at harvesting ripening stage (4.5-5) were collected to the chemical determinations. Fruit oil content was determined by nuclear magnetic resonance (NMR) (Minispec mq 20, Bruker Analytik Gmbh). The results were expressed as percentage of fresh and dry weight (% FW and DW). Anthocyanin fraction was extracted from olive pulp using extraction methods described by Lee *et al.* (2005) with some modifications. Total anthocyanin content, expressed as cyanidin-3-glucoside equivalent per kg of fresh weight, was estimated by the pH differential spectroscopic method proposed by Cheng and Breen (1991). Absorbance (A) was measured with a UV-visible spectrophotometer at 510 nm and 700 nm in diluted buffers at pH 1 and pH 4.5, where $A = (A510\text{-}A700)_{pH\ 1} - (A510\text{-}A700)_{pH\ 4.5}$. The extraction of the phenolic fraction from the pulp was carried out following the method proposed by Gómez-Rico *et al.* (2008). Total polyphenols content, expressed as mg of caffeic acid per kg of pulp, was determined by the colorimetric method described by Vázquez-Roncero *et al.* (1973), using the reagent Folin–Ciocalteu. Absorbance was measured with a UV- visible spectrophotometer (CaryBio50, Varian) at 725 nm.

2.3. Protein extraction and sample preparation

Three independent protein extractions (200 mg fresh weight tissue) were performed per experimental condition (one per biological replicate and developmental stage previously described) using trichloroacetic acid (TCA)/ acetone-phenol methods with modifications (Wang *et al.,* 2008; Romero-Rodríguez *et al.*, 2019). The protein concentration in the extract was determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard. Protein extracts were cleaned-up in SDS-PAGE at 12 % polyacrilamyde (Laemmlii, 1970). Samples, 80 µg of BSA protein equivalents, were loaded to the gel and 80V was applied until the electrophoresis front entered 1 cm into the resolving gel, then the gel was stained with Commasie Blue (Mathesius *et al.*, 2001). Protein bands were cut off, diced and kept in water until digestion. Gel pieces were distained in 200 mM ammonium bicarbonate (AB)/50 % acetonitrile for 15 min followed by 5 min in 100 % Acetonitrile. Protein was reduced by addition of 20 mM dithiothreitol in 25 mM AB and incubated for 20 min at 55 °C. The mixture was cooled down to room temperature, followed by alkylation of free thiols by addition of 40 mM iodoacetamide in 25 mM AB, in the dark for 20 min and the gel pieces were then washed twice in 25 mM AB. Proteolytic digestion was performed by addition of Trypsin (Promega, Madison, WI) at 12.5 ng/µl of enzyme in 25 mM AB and incubated at 37 °C overnight. Protein digestion was stopped by addition of trifluoroacetic acid at 1 % final concentration and the digested samples were finally Speedvac dried (Valledor and Weckberth *et al.*, 2014; Castillejo *et al.*, 2016; Romero-Rodríguez *et al.*, 2019).

2.4. nLC-MS/MS analyses

nLC was performed in a Dionex Ultimate 3000 nano UPLC (Thermo Scientific) with a C18 75 µm x 50 Acclaim Pepmam column (Thermo Scientific). The peptide mix was previously loaded on a 300 µm x 5 µm Acclaim Pepmap precolumn (Thermo Scientific) in 2 % acetonitrile/0.05 % TFA for 5 min at 5 µl/min. Peptide separation was performed at 40 °C for all runs. Mobile phase buffer A (A) was composed of water, 0.1 % formic acid. Mobile phase B (B) was composed of 20 % acetonitrile, 0.1 % formic acid. Samples were separated at 300 nl/min. Elution conditions were: 4-35 % B for 60 min; 35-55 % B for 3 min; 55-90 % B for 3 min followed by 8 min wash at 90 % B and 12 min re-equilibration at 4 % B. Total time of chromatography was 85 min.

Eluting peptide cations were converted to gas-phase ions by nano electrospray ionization and analysed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo Scientific) mass spectrometer operated in positive mode. Survey scans of peptide precursors from 400 to 1500 m/z were performed at 120 K resolution (at 200 m/z) with a 4 × 105 ion count target. Tandem MS was performed by isolation at 1.2 Da with the quadrupole, CID fragmentation with normalized collision energy of 35, and rapid scan MS analysis in the ion trap. The AGC ion count target was set to 2 x 103 and the max injection time was 300ms. Only those precursors with charge state 2–5

were sampled for MS2. The dynamic exclusion duration was set to 15 s with a 10ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top 30 mode with 3s cycles, meaning the instrument would continuously perform MS2 events until a maximum of top 30 non-excluded precursors or 3s, whichever is shorter.

The raw data were processed using Proteome Discoverer (version 2.1.0.81, Thermo Scientific). MS2 spectra were searched with SEQUEST engine against a combined database derived from the olive and oleaster genomes draft (Cruz *et al.,* 2016; Unver *et al.*, 2017). Peptides were generated by theoretical tryptic digestion allowing up to one missed cleavage, carbamidomethylation of cysteine as fixed modification and oxidation of methionine as variable modification. Precursor mass tolerance was 10 ppm and product ions were searched at 0.1Da tolerance. Peptide identifications were grouped into proteins according to the law of parsimony and filtered to 1 % FDR and peptide quantification was based on the peak area intensity.

2.5. Statistical analysis and functional classification of proteins

Protein values were normalized by sum, and log2-transformed before statistical analyses. All statistics procedures were conducted with the R programming language running under the open-source computer software RStudio v1.2.1335: Integrated development environment for R (RStudio Boston, MA) available from http://www.rstudio.org/. Three biological replicates for all statistical procedures were used. The "agricolae" package (function aov()) was used for univariate one-way analysis of variance (ANOVA) followed by post-hoc multiple comparisons using Tukey's test (function HSD.test) to estimate the significance of the results. For cluster analysis, the Genesis v1.7.7 software (http://www.mybiosoftware.com/genesis-1-7-6-cluster-analysis-microarray-data.html) was utilised. Proteins were functionally classified according to Mapman (Thimm *et al.*, 2004) functional bins through the MERCATOR server (Lohse *et al.*, 2014). In addition, some of the statistics and enrichment analysis were performed through pRocessomic v.1 (a filtered and processing tool for biologic data) available on web direction: http://github.com/Valledor/pRocessomics.

3. RESULTS

To study olive fruits ripening processes, a gel-free (shotgun) strategy was used in order to obtain the largest possible proteome coverage and hence, maximal information on the changes in the protein profile throughout this complex process. After obtain this information, the comparison between AT and AT+4 °C treatments was performed determining the effect of warming on ripening processes. Parallelly, the phenotypic data of fatty yield, total polyphenols and

anthocyanins contents was determined. Results obtained by both phenotypic and proteomics approaches was combined to elucidate the mechanisms involved in ripening processes and its alteration under warming conditions.

Contrary to what would be expected to have a low number of fruits, they are smaller with less ratio pulp-pit (due to the low competition for photoassimilates between fruits) and also with a fat yield of 37 % lower under warming conditions compared to AT conditions. In addition, a decrease in the levels of priority compounds in the pulp as total polyphenols or total anthocyanin contents at harvesting period was observed (Figure 5.2). It is curious the pattern of veraison stage because there seems to be a gap between veraison at AT+4 °C treatment respect the control AT.

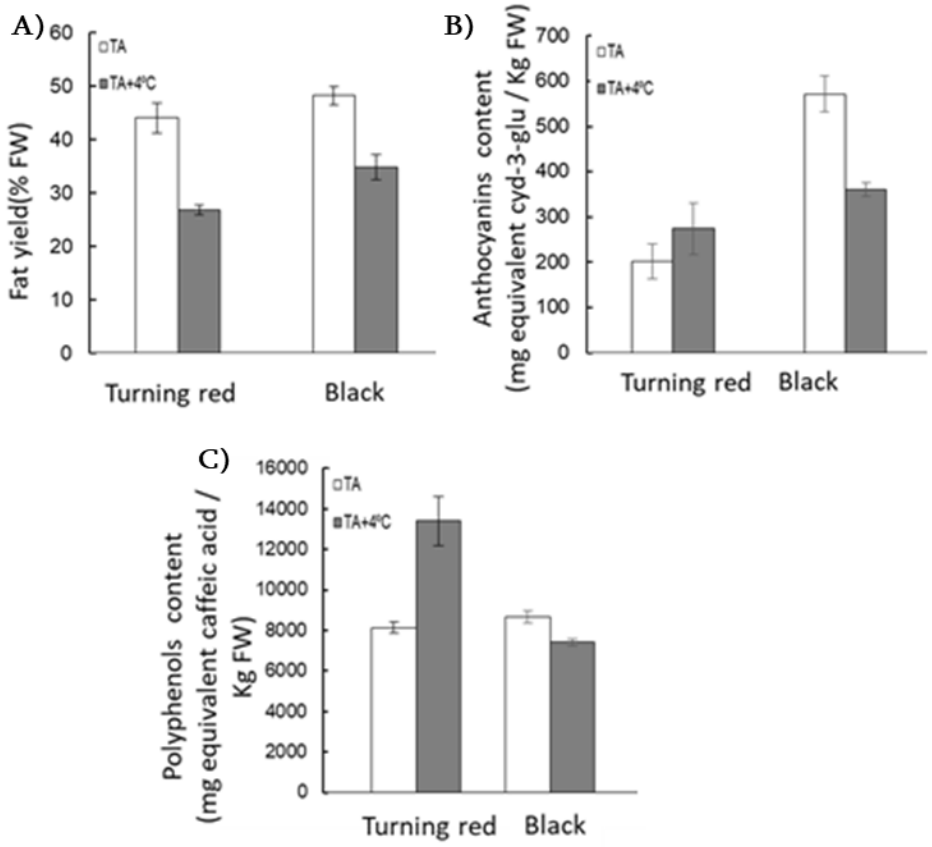

Figure 5.2. Chemical analyses of olives fruit at veraison (3) and black skin (5) (harvesting) ripening stages. **A)** Fat yield referred to percentage of oleic acid per DW. **B)** Total anthocyanins contents values referred to mg equivalents of cyanidine-3-glucoside per kg of FW. **C)** Total polyphenols contents values referred to mg equivalents of caffeic acid per kg of FW.

Proteins were extracted from flesh and skin of three ripening stages by using the TCA/acetone and phenol precipitation method. Statistically significant variations were not observed in protein yield between ripening stage and treatment (but a slight increase on treated samples was observed), ranging from 3.92 ± 1.38 mg/g FW to 5.21 ± 1.64 mg/g FW.

A specific *Olea europaea* database was used revealing a total of 3522 identified proteins (FDR<0.01), of which 3048 conformed to the confidence parameters: score > 2, % coverage > 10 % and at least 2 peptides) those 2599 proteins had unique peptide>1were. A low number of proteins

(21) appear like uncharacterized or non-named or putative due to the employ a specific database. These proteins were categorized using MERCATOR software (http://www.plabipd.de/portal/mercator-sequence-annotation), visualizing 34 functional categories (Figure 5.3) according to MapMan software (Thimm *et al.*, 2004). The greatest percentages correspond to: processing, folding and degradation of protein (23.15 %); amino acid metabolism (4.48 %); signalling (4.35 %); lipid metabolism (4.04 %); stress response (3.81 %); and secondary metabolism (3.16 %), among others.

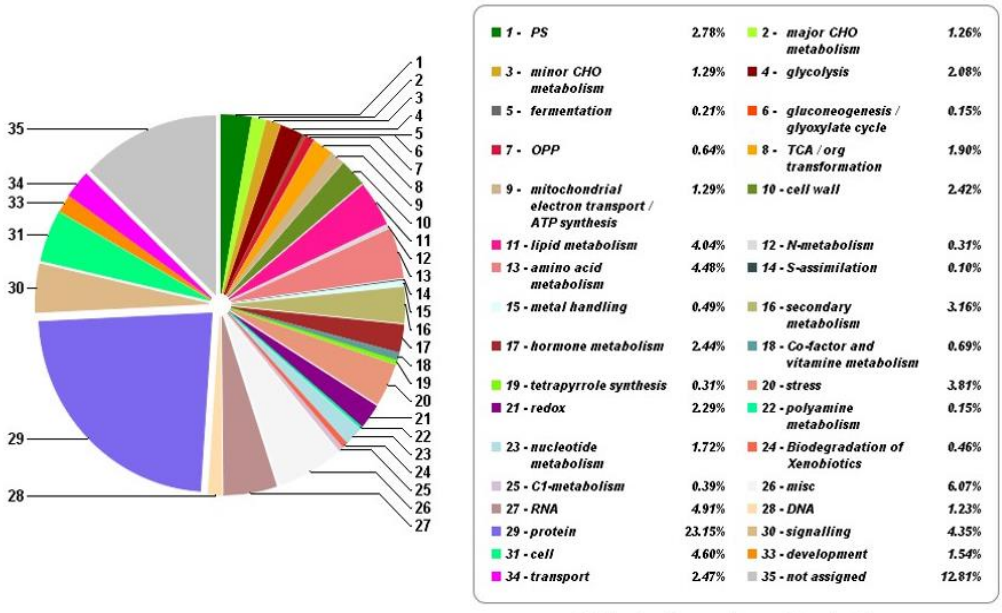

Figure 5.3. Mercator functional categorization of 3522 total identified protein in 34 Mapman categories.

Proteins were subjected to an analysis of variance (ANOVA) to identify those that presented significant differences ($p<0.05$) between the different conditions studied, finding a total of 1085 proteins. Due to the elevated number of differences, the data set was divided as follow: first an ANOVA were performed with normal ripening processes and then we applied a t-student test to evaluate the sensitivity to temperature on each ripening stage, obtained 186 proteins meeting both requirement (Supplemental Material Figure 5.1). Figure 5.4 shown the intersection of significant differences per each statistical analysis: ANOVA with ripening at AT conditions, ANOVA with ripening at AT+4 °C, and t-student per each ripening stage (green, veraison and purple) comparing AT+4 °C vs AT conditions.

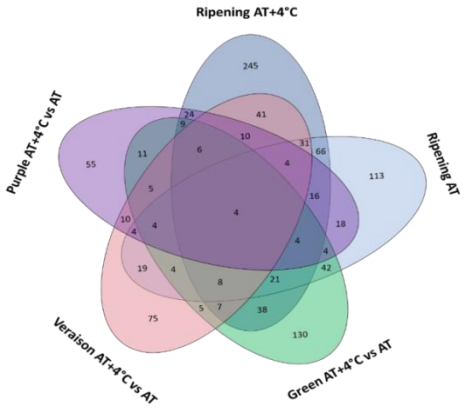

Figure 5.4. Venn diagram with significant differences corresponded to 1085 proteins. The graphic showns the common differences and the unique differences because of both treatment and ripening stages.

The PCA analysis for the complete data set (Figure 5.5 A) revealed that the first component (PC1) clearly separates ripening stages (Green, Veraison and Purple) and PC2 separates the temperature conditions, explaining both components 68.23 % of the variability of the experiment. The hierarchical grouping analysis (Figure 5.5 B) similarly grouped the ripening stages following the logical criteria of colour skin, and it separated the samples belonged to the temperature treatment. It is also worth mentioning the grouping of the biological replicas for each of the treatments, which confirms the reproducibility of the experiment. To obtain a visual representation of functional categories involved on PCA separation, an analysis with pRocessomics was performed, however, not significant contribution of a specific categories was observed (Figure 5.5 C).

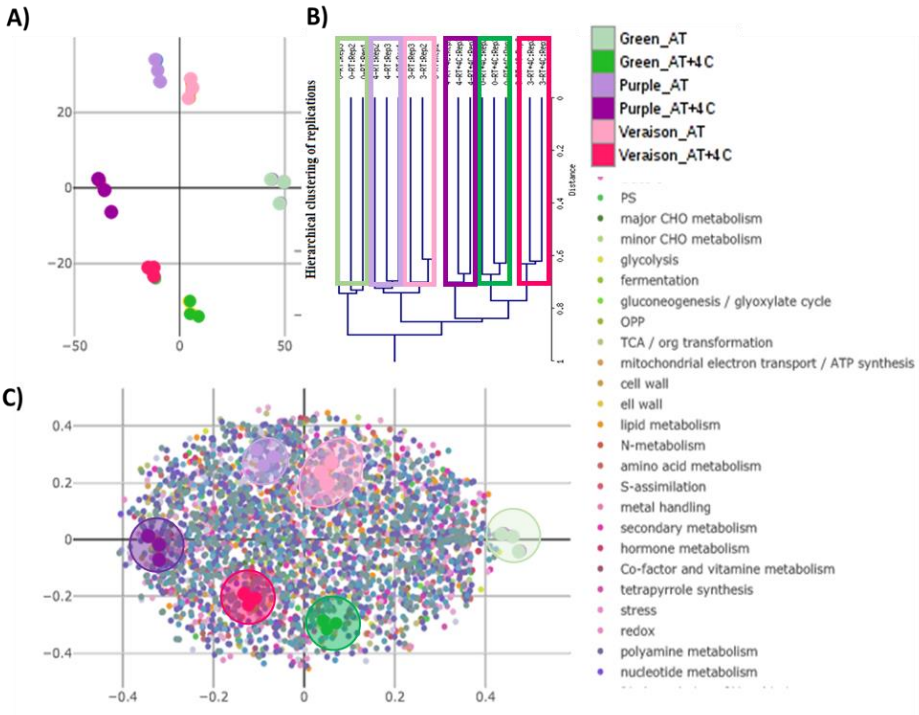

Figure 5.5. A) PCA analysis with total identified protein obtained by nLC-MS/MS. B) Hierarchical clustering analysis of the total proteins. C) Representation of the Mapman functional categories involved into PCA separation.

A quantitative analysis of the 186 differential proteins was carried out comparing between treatments. For this purpose, the proteins that changed ±2 times (Fold Change) in warming conditions with respect to the control were selected for the two temperature conditions, finding a mayor differences on the veraison AT+4 ºC than the other conditions (Figure 5.6), generally there are a lower abundant of the protein related to lipids metabolism, carbohydrate metabolism and secondary metabolism (phenylpropanoids pathway). These ratios are grouped into the 36 main biological functions, with transcription/translation, protein degradation and carbohydrate metabolism predominant by Mapman software (Figure 5.6). In addition, the green stage presented an inverse pattern between normal and warming conditions. Generally, protein related to gluconeogenesis, signalling and hormone metabolism, transport and secondary metabolism are more abundant on warming conditions; while proteins related to lipids metabolism, development, cell wall or degradation showed lower abundance.

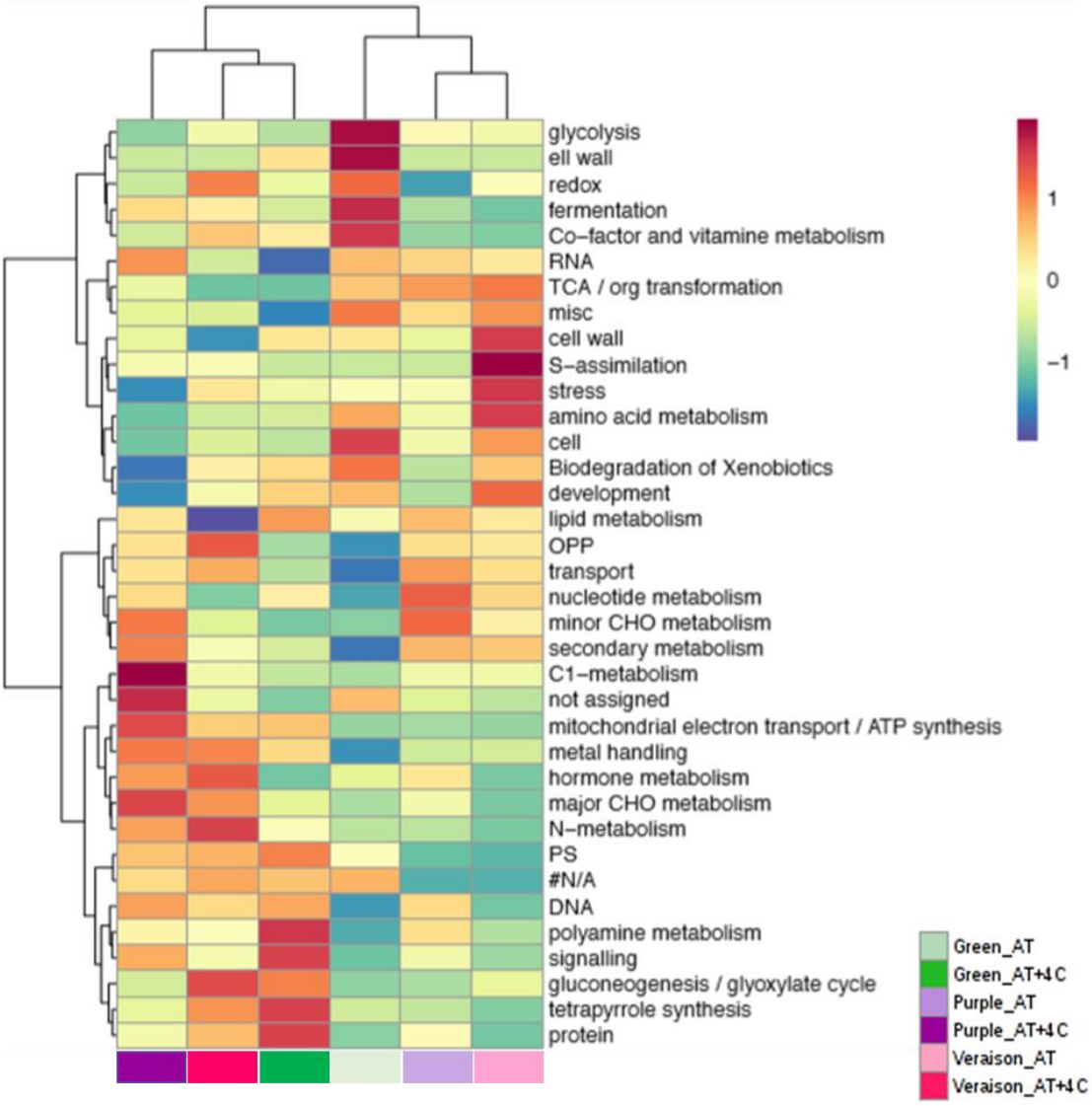

Figure 5.6. Enrichment analysis heatmap with the protein abundances per categories in each condition, combining ripening stages and temperatures treatments. This graphic was obtained through pRocessomics tool.

Additionally, the graphic representation of the altered pathway was performed to identify concretely which proteins are indicatives to warming conditions and recognize the trends among ripening processes at normal condition and warming treatment with Fold Change ±2 values (Figure 5.7).

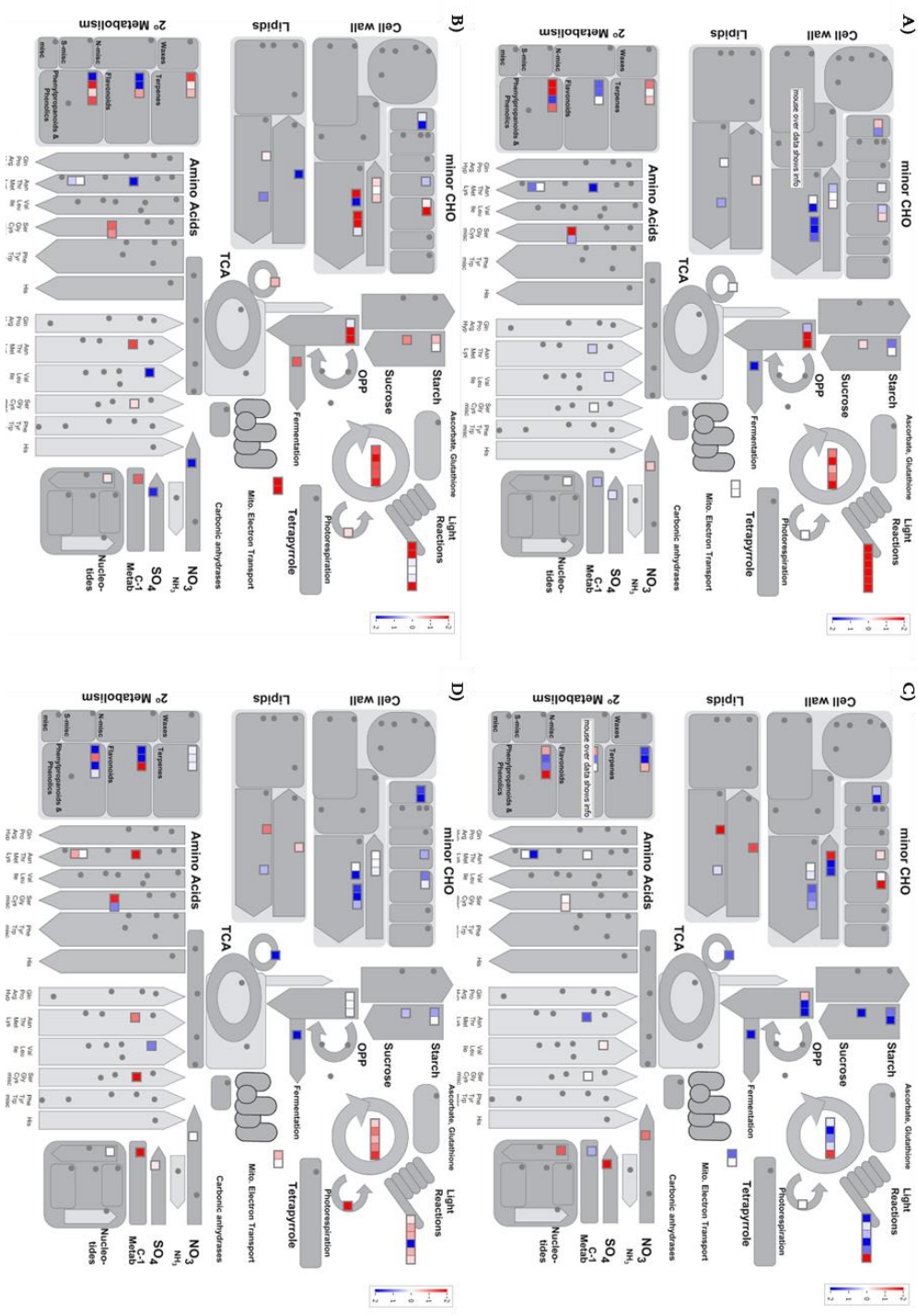

Figure 5.7. Visualization of the differential proteins in response to drought in the context of general metabolism using MapMan software. The functional distribution and relative abundance of the protein values (log2 of the normalized values) is represented. **A)** Ratio veraison/green skin at AT treatment; **B)** Ratio purple skin/veraison at AT treatment; **C)** Ratio veraison/green skin at AT+4 °C treatment.

The significant differences per each ripening stage at warming vs normal conditions also was represented categorically by Mapman software (Figure 5.8 and Supplementary Figure 5.2). This comparation was performed in order to visualize only the warming condition on the specific

ripening stage. In this analysis the proteins related to abiotic stress appear while they are absent when comparison firstly by normal ripening processes was performed (Figure 5.6).

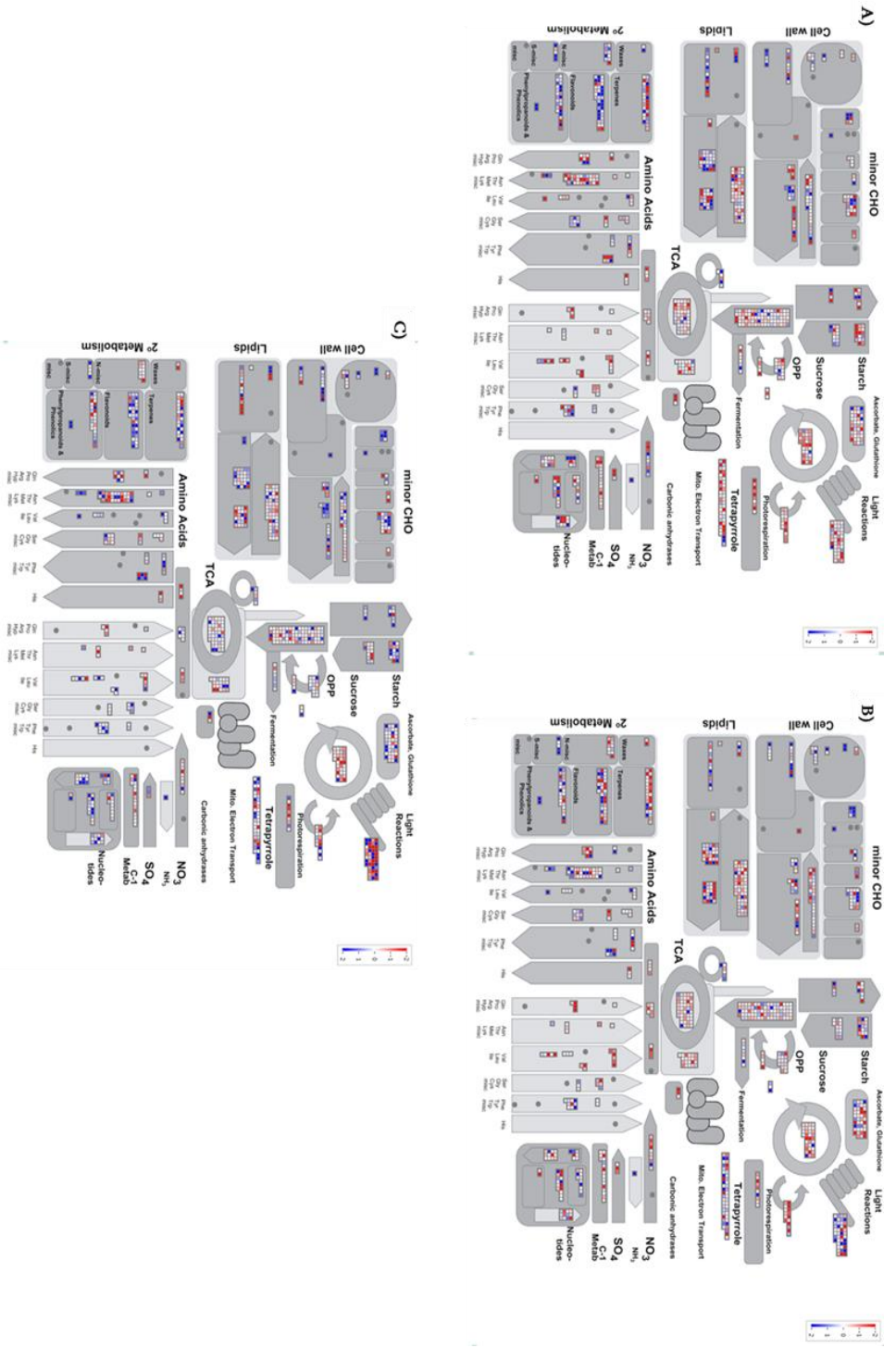

Figure 5.8. Visualization of the differential proteins in response to drought in the context of general metabolism overview using MapMan software. The functional distribution and relative abundance of the protein values (log2 of the normalized values) is represented. **A)** Ratio green skin at AT+4 °C treatment/AT treatment; **B)** Ratio veraison at AT+4 °C treatment/AT treatment; **C)** Ratio purple skin at AT+4 °C treatment/AT treatment.

4. DISCUSSION

High temperatures have negatively effects in various physiological processes such as photosynthesis, primary and secondary metabolism, water relations, lipid and hormonal signalling. Specifically heat stress generates damage to cell membrane, senescence, inhibition of photosynthesis and cell death (Xu *et al.*, 2006). Another mechanism of injury under high

temperature involves the overproduction of reactive oxygen species (ROS). This is represented on the great number of identified proteins in this experiment related to mentioned functions as raffinose synthase, glucosyltransferases, MAP-kinases, catalases or Acyl-CoA-transferases among others. Therefore, in this experiment, proteins related to redox processes are more abundant in warming conditions than in ambient temperature such as glutathione peroxidase, glutathione reductase, ascorbate peroxidases, peroxiredoxins and thioredoxins.

Heat stress also induces relevant changes in cytoskeleton composition indicating its reorganization (Ferreira *et al.*, 2006). Plants response to heat shock leads to changes in the cellular membrane structure, protein metabolism, level of enzymes and photosynthetic activity. In our study, proteasomes, peptidases and proteases were identified in major abundance on warming conditions and advanced ripening processes. Proteases are involved in all aspects of the plant life cycle ranging from the mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs (Schaller, 2004). In addition, some proteins have been described to have both general molecular chaperone and proteolytic activities. The chaperone function dominates at low temperature, while the proteolytic activity is present at elevated temperatures (Spiess *et al.*, 1999). In this sense, we also have identified more than 70 proteins annotated as HSP, such as 70HSP, 90HSP and 16.9HSP present in AT+4 °C treatment and absent in ambient conditions. However, when we compared firstly the ripening processes and subsequently the sensitivity to warming treatments, these proteins were not well represented, ones only appear in veraison stage at AT conditions due to a warm autumn. HSPs reveal a specific role in protein folding (chaperone function) and protection of proteins during their transport into specific organelles such as plastids and mitochondria (Ge *et al.*, 2012). In addition, HSPs plays important roles in different kinds of environmental stress conditions, such as heat and drought, low, and high temperatures and salinity (Zhang *et al.*, 2015). Several studies demonstrated an increase of plant HSPs under different stress conditions, indicating an enhanced need for protein protection under stress (Bian *et al.*, 2009; Hassan *et al.*, 2015; Castillejo *et al.*, 2016).

High temperature modifies the activities of carbon metabolism enzymes, starch accumulation, and sucrose synthesis by down-regulating specific genes in carbohydrate metabolism (Ruan *et al.*, 2010). It contrasted with our results, possibly due to the greater influence of the ripening process, which is characterised by the hydrolysis of complex sugars. In addition, the green stage is active photosynthetically. Proline, glycine betaine, and soluble sugars are some of first metabolites which are accumulated in response to heat stress (Wahid *et al.*, 2007). This response is accompanied by a decrease in the synthesis of primary metabolism proteins and the accelerated transcription and translation of heat shock proteins (HSPs) (Bray *et al.*, 2000), the production of phytohormones of the stress signalling such as abscisic acid (ABA) and antioxidants and other protective molecules (Maestri *et al.*, 2002).

Anthocyanin biosynthesis is increased at cold temperatures, and this increase has been suggested to enhance the survival of plants under cold stress (Catala *et al.*, 2011). By contrast, the benefit of suppressing anthocyanin biosynthesis at high temperatures is unknown. Given that both morphological changes and anthocyanin biosynthesis are energy-demanding processes, the energy balance between these two processes might be optimized in response to the environmental conditions. In this respect, the suppression of anthocyanin biosynthesis might be more beneficial for plants grown at high temperature because thermomorphogenic growth at a cost of reduced anthocyanin biosynthesis might enhance survival under high temperature stress. Enzymes related to anthocyanins biosynthesis showed differences due to the temperature and ripening stage, such as leucoanthocyanidin deoxygenase or naringenin 3 dioxigenase among others. These proteins were more represented on normal conditions than warming treatment at green and purple skin stages, with veraison presented a relevance pattern alteration and more susceptibility to warmer conditions.

In plants, increased synthesis of phenols is a common response to stress (Dixon and Paiva, 1995) and the same environmental conditions that cause oxidative stress are associated with the induction of phenylpropanoid metabolism. Phenylpropanoids and flavonoids are involved in the protection against oxidative stress (Grace, 2005). The level of lipid peroxidation and how the total antioxidant capacity and the phenolic compound content evolve can both be used as indicators of stress and the response to it. One response to stressors during ripening is an increase in the phenolic contents. These include flavonoids and phenylpropanoid glycosides (Dixon and Paiva, 1995; Fortes *et al.*, 2011), where flavonoids participate in protecting against oxidative stress by eliminating ROS (Grace and Logan, 2000; Grace, 2005). We observed an accumulation of proteins related to flavonoids compounds in warming conditions, especially patent on veraison stage. In addition, proteins related to non-mevalonate pathway are more abundant in the mentioned conditions, the relevant pathway of major polyphenol compounds on olive oil (Alagna *et al.*, 2012).

The results as a whole show a great complexity of the ripening processes Therefore, in response to warming conditions there is an activation of the defence system (stress proteins, redox and secondary metabolites), as well as a metabolic adjustment to re-establish homeostasis in the face of unfavourable conditions similar to the response to drought (Echevarría-Zomeño *et al.*, 2009; Kosová *et al.*, 2011; Simova-Stoilova *et al.*, 2015). Thus, the mechanisms involved in both stresses (drought and high temperatures) are related, probably due to the evapotranspiration ratio that is more elevated in warmer conditions. In our study dehydrin, LEA and DELLA proteins were identified with significant differences between AT and AT+4 °C, being more abundant on warming conditions. All of them play an important role in protecting cells from abiotic stress, and in plant normal growth and development. More importantly, LEA expression is often induced by abiotic stresses such as cold, drought, or high salinity (Huang *et al.*, 2016).

5. CONCLUSIONS

A total of 3,522 proteins were identified by nLC-LTQ-Orbitrap MS strategy using a specific database based on translated CDS sequences with high values of scores and coverages. The elevated complexity of ripening processes makes it necessary to separate each of the stages in order to evaluate the effect of the applied warming conditions. The major functional categories affected by AT+4 ºC, independently of ripening processes were the following: Photosynthesis, fatty acids synthesis, plant cell wall degradation, and terpene, phenylpropanoids and flavonoids biosynthesis. Data were corroborated by fatty yield, total polyphenols and anthocyanins contents obtained by classical biochemistry analysis. The veraison stage presented more sensitivity to warming conditions, showing drastic changes on the natural colour, lipids content and polyphenols amounts among others. Veraison is considered a critical point where the harvesting can be start due to all the desirable compounds are present on the olive fruits. The obtained results suggest that global warming will affect the ripening processes modifying the fruit characteristics and the final oil quantity and quality, as well as the harvesting optimal period

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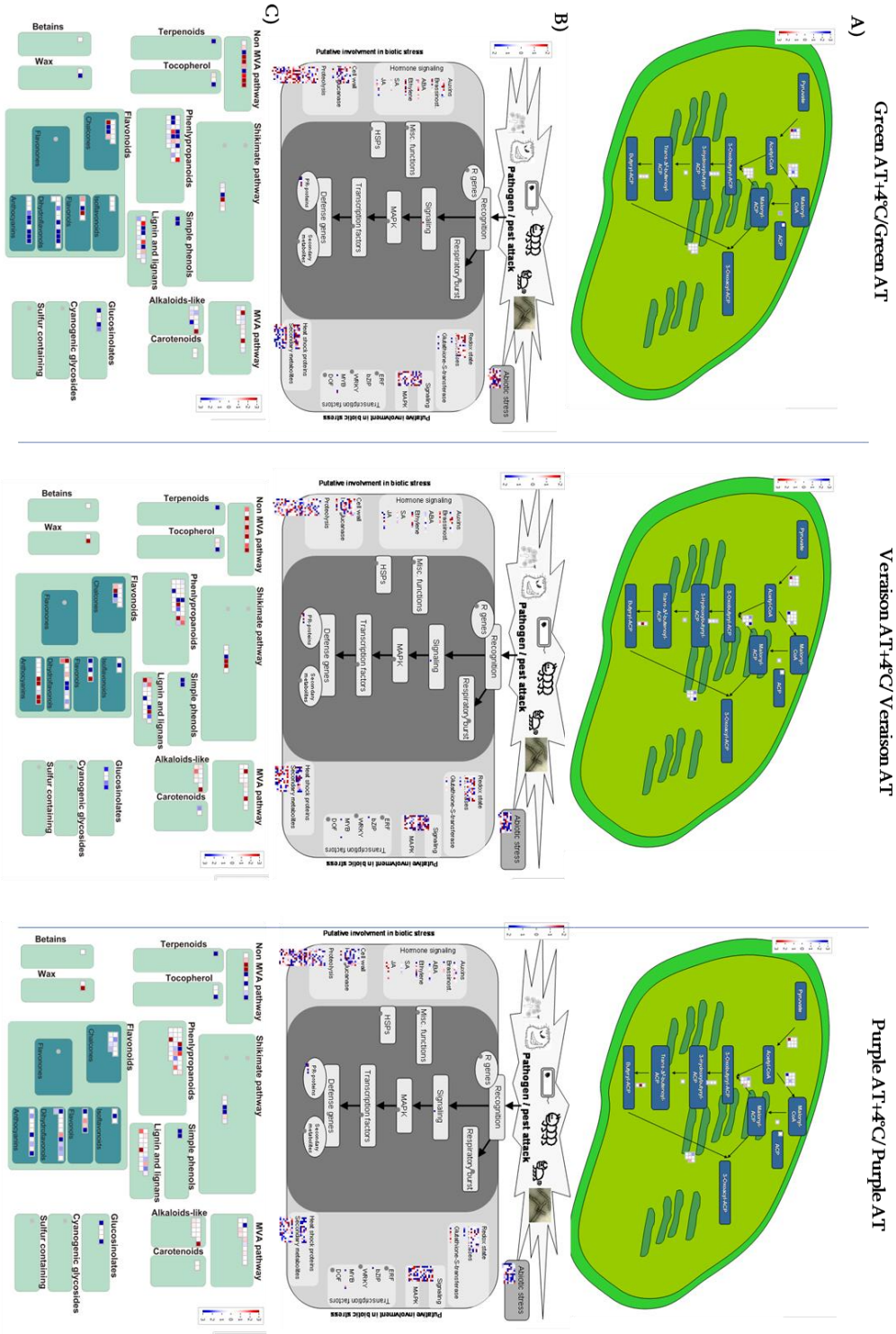
7. SUPPLEMENTARY MATERIAL

Supplementary Figure 5.1. HeatMap with the log 2 ratios corresponding first to each stage (AT+4°C vs AT): secondly ripening (Veraison vs green: Purple vs Green) at AT conditions and finally ripening under warming conditions (Veraison vs green: Purple vs Green).



Supplementary Figure 5.2. Details of metabolic pathways affected by warming conditions. A) Lipid metabolism: B) Abiotic or biotic stress proteins related: and C) Secondary metabolism.

Chapter VI. Targeted transcriptomic analysis of the effect of environmental temperature increase along olive fruit ripening

Crop yield improvement is essential for feeding the growing world population without concomitant increases in the land area allocated to agriculture. Particularly, oil crops are proposed as a strategy to increment the caloric ingest using a scarce increase on feedstock. Olive tree growing for mill oil purposes would therefore be an incentive to exploit. This crop, of great economic interest in the Mediterranean Basin, especially for Spain, faces adverse environmental conditions typical of the Mediterranean climate that could aggravate in the current context of climate change. For this reason, knowledge about olive tree response against warmer conditions is required to maintain or improve the oil production. To provide information in this regard, a targeted transcriptomics analysis has been developed to study the effect of warming conditions on the olive ripening process. Fruit samples were collected from trees (cv. `Picual´) growing under field conditions at a permanent 4 °C above ambient temperature, obtained by temperature-controlled Open-Top-Chamber (OTC). Fruits were sampled at three ripening stages: green skin (0), turning red or veraison (3) and purple skin (4). Total mRNA from those samples was extracted (Macherey-Nagel Plant NucleoSpin Kit) and subjected to qRT-PCR analysis using a Wafergen Biosystem to monitored gene expression. A total of 112 genes were selected in basis of the data of the previous proteomic analysis (Chapter V) and transcriptomic information available in the literature along ripening in *Olea* and other related species. All of them belong to the functional categories of signalling, lipid metabolism, secondary metabolites and transcription factors, mainly. The gene transcript profiles for 112 genes were analysed in biological triplicates and analytical duplicates, resulting in 85 suitable for further expression analysis due to the presence of unique melting curve, primers efficiency ranges 1.4-2.2 and C_T values <30 on 4/6 of replicates. A clustering analysis allowed the analysed transcripts to be grouped according to the behaviour. The greatest ratios of transcripts profiles between treatments corresponded were obtained on veraison. Some results agree with those obtained in previous analysis (phenology and proteomic approach, Chapter III and V, respectively). All together this study suggests that global warming affects the visual perception of ripening stages, especially veraison. A discrepancy between the colouring of the skin, traditionally taken as a reference for harvesting, and the internal flesh ripeness stage was observed. This should be considered under future climate change scenarios to change the harvesting period in order to keep the fruit characteristics and the final oil quality. Nevertheless, further experiments are required to validate at the metabolic level the proposed mechanisms involved on the warming response.

1. INTRODUCTION

Nowadays, the world's population faces one of its main challenges: ensuring the sustainability of resources to feed the growing population (up to about 9 billion people by 2050). One of the possible solutions is to provide a higher caloric content through the addition of products from oil crops (FAO, 2018). For this reason, demand for vegetable oils is expected to reach above the average across agriculture. However, the required yield improvements and timeframe cannot longer achieve using traditional means because of the changing weather patterns into the current climate change scenario. A much greater understanding of the underlying mechanisms of plant response to stress and environmental factors is required in order to achieve an increment in crop yields into the near future. In this direction, omics research constituted a useful tool to go beyond looking at oil synthesis (improve fat yield) and fruit development (Hadjuch *et al.*, 2011; Landridge and Fleury, 2011; Troncoso-Ponce *et al.*, 2011; Ferni and Schauer, 2009; Chiapetta *et al.*, 2015).

Olive oil is the second most economically important crop that accumulate high levels of triacylglycerols in its mesocarp (also called flesh), due mainly to its role on human health and nutritional properties (Pérez *et al.*, 2007). This specie is the major tree crop located in Mediterranean Region which drought and high temperatures periods that will be aggravate in climate change predictions. So, a deepen understanding of biomolecular mechanisms of response to warming conditions are needed. Particularly, the knowledge of the effect on the production, development and ripening of olives is a critical step in designing an appropriate crop management to face the changing situation.

Genetically, olive tree (*Olea europaea* subsp. *europaea*) belongs to Lamiales Order where there are five other Lamiales species with available genome sequences: *Fraxinus excelsior, Mimulus guttatus*, *Sesamum indicum*, *Utricularia gibba*, and *Salvia miltiorrhiza* (Julca *et al.*, 2018) added to the drafts (not well depurated) of the genome of olive tree `Farga´ cultivar (Cruz *et al.*, 2016) and oleaster (Unver *et al.*, 2018) coupled to the improvement of the quantity and quality of identified protein identification. This information is mandatory to identify and study the genes involved in processes of interest; and for primers design through qRT-PCR technique. Transcriptomic and proteomic research in olive mostly focused on flowering (Alagna *et al.*, 2016; González-Plaza *et al.*, 2016; Yanik *et al.*, 2013; Bianco *et al.*, 2013). While most metabolomic studies have focusing on lipids, sugars, phenolics and other secondary metabolites during fruit development and ripening (Conde *et al.*, 2008; Ghanbari *et al.*, 2012; Martinelli *et al.*, 2013).

The production of fleshy fruit involves three distinct stages, namely, fruit set, fruit development, and fruit ripening. The ripening process activates a whole set of biochemical pathways that make the fruit attractive, desirable, and edible for consumers, in which the biochemistry and physiology of the organ are developmentally altered to influence appearance, texture, flavour, and aroma (Giovanonni 2001; 2004). Since the majority of the quality attributes

are elaborated during the ripening process, it has always been considered essential to better understand the mechanisms underlying this ultimate fruit developmental stage. In olives, the main compounds are lipid, sugars, polyphenols and anthocyanins as determinant of oil quality and optimal harvesting period (Beltrán *et al.*, 2008).

During fruit ripening, changes in carbohydrate content in olive included the increase of soluble sugars (glucose, fructose and mannitol) contents up to 90 days after fruit set followed by a decreased during fruit ripening up to 120 days. A marked transient increase was evident at the beginning of fruit colour changes. The content of reducing sugars in fruit was considerably higher than that of non-reducing ones. The changes in glucose and fructose content followed a reverse trend up to 135 days after fruit set, thereafter concomitant to decrease of glucose and fructose, mannitol content increased. Insoluble sugars of fruit and leaf in off-year were higher than that of on-year. The main constituents of this fraction after hydrolysis were glucose, rhamnose, arabinose, fructose, galactose, ribose, xylose and mannitol, in different amounts (Ebrahizadeh *et al.*, 2000).

Several studies concerning phenolic compounds in the olive have already been carried out in relation with the technical problems posed in processing the fruit (Fernandez Diez, 1971; Vázquez Roncero and Janer del Valle, 1977). In fact, these molecules are relevant for the organoleptic qualities of the fruit (Cohen *et al.*, 1967), and o-diphenols in particular can play a role in browning (Ben Shalom *et al.*, 1977) or act as antioxidants in the conservation of oil (Vazquez Roncero, 1978). Oleuropein is the main bitter component in the olive, (Shasha and Leibowitz, 1961) as a heterosidic ester of elenolic acid and dihydroxyphenyl ethanol (Panizzi *et al.*, 1960). Demethyloleuropein, a demethylated derivative, has also been reported in ripe olives (Ragazzi *et al.*, 1973). Besides these compounds, there are also flavonol glycosides, in particular rutin and luteolin 7-glucoside (Vázquez-Roncero *et al.*, 1976) and derivatives of hydroxycinnamic acids, the main one of which was identified recently as being verbascoside (Fleuriet *et al.*, 1984) and the heterosidic ester of caffeic acid and dihydroxyphenyl ethanol (Andary *et al.*, 1982).

The biochemical machinery of oil biosynthesis has been well characterized in both oil seeds and oil-bearing fruits (Hardwood, 1993). Omics investigations of oil crops have mostly focused on oil biosynthesis within the seeds (Gupta *et al.*, 2016; Rawsthorne, 2002). For example, proteomics approaches to study near-isogenic sunflower varieties differing in seed oil traits revealed that fructokinase, plastid phosphoglycerate kinase, enolase proteins were up-regulated in the high-oil line while phosphofructokinase, cytosolic phosphoglucomutase, and cytosolic phosphoglycerate kinase were up-regulated in the low-oil variety (Hajduch *et al.*, 2007). In the fatty acid biosynthetic pathway, the transcripts of acyl-carrier protein (*ACP1*, *ACP3*), pyruvate dehydrogenase (*PDHE12*), dihydrolipoamide dehydrogenase (*LPD2*), biotin carboxyl carrier protein (*BCCP1*), hydroxyacyl-[ACP] dehydratase (*HAD*), acyl-ACP thioesterase A (FatA) and long-chain acyl-CoA synthetases (*LACS*) and stearoyl-ACP desaturase (*SAD*) were overexpressed in the seeds of canola (annual) compared to the seeds of castor (perennial). While, transcript levels

of *PHD-E12*, dihydrolipoamide acetyltransferase (*LTA1*), *LPD1*, *BCCP2*, *LACS9*, *ACP2* and *DES6* were more abundant in castor compared to canola seeds (Li *et al.*, 2006).

The accumulation of anthocyanins in the ripening of grapevine or apple has been reported to be reduced at high temperatures (Mori *et al.*, 2007; Lin-Wang *et al.*, 2011). Anthocyanin biosynthesis is increased at cold temperatures, and this increase has been suggested to enhance the survival of plants under cold stress as osmoprotector molecule (Catala *et al.*, 2011). By contrast, the benefit of suppressing anthocyanin biosynthesis at high temperatures is unknown. The anthocyanin biosynthesis pathway has been studied in numerous plant species and most of the genes involved in this process have been identified. In the model plant *Arabidopsis thaliana*, the genes encoding anthocyanin biosynthesis enzymes are grouped into two classes: early biosynthetic genes [chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), and flavonoid 3O-hydroxylase (*F30H*)], all of which are common to other flavonoids; and late biosynthetic genes [dihydroflavonol 4-reductase (*DFR*), leucoanthocyanidin oxygenase (*LDOX*), UDP-glucose flavonoid 3-O-glucosyltransferase (*UF3GT*), and anthocyanin acyltransferase (*AAT*)] that are specific to the anthocyanin pathway (Tanaka *et al.*, 2008). Several transcription factors that regulate the expression of anthocyanin biosynthetic genes have been identified. The R2R3-type MYB transcription factors PAP1/MYB75, PAP2/MYB90, MYB113, and MYB114 positively regulate anthocyanin biosynthesis in vegetative tissues (Borevitz *et al.*, 2000; Teng *et al.*, 2005; Gonzalez *et al.*, 2008). In contrast, the small MYB protein MYBL2 negatively regulates anthocyanin biosynthesis by repressing the expression of the late biosynthetic genes *DFR* and *LDOX* (Dubos *et al.*, 2008; Matsui *et al.*, 2008).

The aim of this study was to evaluate the mechanisms involved in the response to warming conditions under field conditions, in a Mediterranean climate, of olive fruits ripening employing a targeted transcriptomic analysis. For this purpose, olive trees `Picual´ cultivar, were subjected to warming conditions, 4 °C above ambient using temperature-controlled OTCs (Benlloch-González *et al.*, 2018; 2019) and 3 critical ripening stage: green (0), veraison (3) and purple skin (4), were selected for the study. The candidate genes were selected based on the results obtained in proteomic and phenological data and evidences found in the literature. This strategy was performed to integrate the transcriptomic data with some phenotypic (skin colour, fatty yield, total contents of polyphenols and anthocyanins) data. The information resulting from this study, gives an important overview of the impact of global warming on olives ripening (and optimal harvesting time), olive oil quantity and quality; and could be useful for the selection of putative biomarkers for future screening.

2. Material and Methods

2.1 Plant material and experimental design

'Picual' olive trees (*Olea europaea* L.) growing in the experimental farm of 'Campus de Rabanales', University of Córdoba, Spain (37°55'N 4°43'W) were used to perform the experiment as previously described by Benlloch-González *et al.* (2018). Trees were subjected at two temperature treatments, ambient temperature (AT) and 4 °C above ambient temperature (AT+4 °C) though the OTC structures as described on Chapter III.

To determine the fruit maturation period, the experimental trees were monitored every 3-4 days from late August to December, recording the fruit phenological stages of each tree according to the following visual scale: 1 deep green skin; 2 yellowish-green skin; 3 veraison, green skin with reddish patches over more than half of the fruit; 4 purple skin; 5 black skin and white flesh. The maturation period was determined according to Barranco *et al.* (1998). During ripening, 20 fruits per each ripening stage were collected randomly from each tree. The 20 fruits were pooled and divided into pericarp (skin and flesh) and endocarp (pit) when the samples were homogenized in liquid nitrogen and then stored at -80 °C until biomolecular analysis. For the molecular analysis, Green (0), Veraison (3) and Purple (4) skin were selected as critical stages of the initial phase, the phase that present alterations under AT+4 °C treatment respect AT (Figure 6.1), and the previous phase to harvesting, respectively.

Figure 6.1. Ripening stages selected and visualized during the field experiment on olive trees under warming conditions. A) Green skin (0); B) Advanced Veraison (3) at AT conditions; C) Advanced veraison (3) at AT+4 °C conditions; D) Purple skin (4).

At harvest, fruits of each tree were collected to determine total weight. Fifty to eighty fruits per tree, depending on the year, were used to determine fruit size (average weight of the fruits sampled), pulp/stone ratio, expressed as fresh weight, and the maturity index (MI). The MI was determined according to Ferreira (1979).

2.2. RNA extraction and sample preparations

RNA was extracted from 75 mg of lyophilised (frozen-dry) olive fruit flesh powder per triplicate using the NucleoSpin Plant Mini Kit from Macherey-Nagel following the manufacturer

recommendations with modifications. For cell lysis, a mix 1:1 (v:v) of RA and RAP buffers (supported by Macherey-Nagel commercial kit) was performed. Desalted step with MDB buffer (also supported Macherey-Nagel commercial kit) was conducted twice. Finally, RNA was eluted twice with the provided Elution Buffer. Potential DNA contamination was removed by a second DNAse treatment using the DNAse Ambion Kit (Life Technologies, United States) and following the manufacturer recommendations. Quantity and quality of RNA samples were tested with a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, United States) and an Agilent BioAnalyzer (Agilent Technologies, United States), respectively. Synthesis of cDNA was conducted using iScriptIV™ (Invitrogen, Carlsbad, CA) in 20 µL reactions containing 1500 ng of RNA. cDNA synthesis was performed following the manufacturer instructions and tested by gel electrophoresis.

2.3. Selection of candidate genes and genes expressions analysis by qPCR

The 110 genes were selected following two major criteria. On the one hand, 55 CDS obtained thought the differential protein sequences obtained by previous proteomic data coming from to the same field experiment and; on the other hand, 55 genes whose expression has been reported that could be altered by ripening and/or stress (mainly high temperature treatments). Out of 185 differential proteins obtained in the shot-gun proteomics, 55 were selected for further studies based on a |Fold Change|>3, being sensitives to ripening and to warming conditions or proteins always present under warming conditions, and belonging to the major categories (MERCATOR classification of local BLASTp results) relevant on oil quantity or quality.

Primers were designed for 110 genes by using 3 complementary tools: first using Primer3 v4.1.0 (http://bioinfo.ut.ee/primer3/, Untergasser *et al.*, 2012) to obtain the primers sequences; subsequently on-line server OligoCalculator v3.27. (http://biotools.nubic.northwestern.edu/OligoCalc.html, Kibbe *et al.*, 2015) was utilised to check self-complementary and hairping; and finally PrimerBlast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye *et al.*, 2012) was employed to specificity verification. Two housekeeping genes, *αTUB* and *EF1*, were also used. Primer sequences and characteristics are described in the Supplementary Table S1. Previous to the qPCR, the primer amplifications were checked on gDNA at different annealing temperatures (Tm) ranges 52-58 °C, selecting 56 °C as common Tm.

The high throughput qPCR was performed using a WaferGen™ SmartChip MyDesign Chips (WaferGen Biosystems, Fremont, CA) (216x24) with 100 nL reaction volume from Roche (Switzerland) and the accompanying SmartChip Dispenser Software (version 2.8.6.1) for results visualization and analysis. Each qPCR contained 1µM of cDNA, 0.5µM of each forward and reverse primers and 5µL of Master Mix PowerUp™ SYBR™ Green (ThermoFischer Scientific, USA). The qPCR program was set as follows: an initial activation step at 50 °C for 2 min, a first

denaturation step at 95 °C for 2 min following by 45 cycles of denaturation at 95 °C for 15 s, primer annealing at 56 °C for 15 s, extension at 72 °C for 1 min before a dissociation step composed by 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For relative expression quantification, the comparative cycle threshold (Ct) and the calculated primer efficiencies (Supplementary Table 1) was used as described by Livak and Schmittgen (2001).

2.4. Statistical analysis

A data quality screen on amplification presence, primers and PCR efficiency, Tm curves, and Ct and Tm variability was performed to remove any outlier data before ΔCt computations (Livak and Schmittgen, 2001). ΔCt computations were calculated for each temperature treatment. The two-tailed unpaired Student's *t* test or one-way ANOVA with Tuckey multiple means comparison was used to determine the significance of difference, $P \leq 0.05$. For analysis of gene-expression data, fold change (log2) values are subjected to hierarchical clustering was performed with the Genesis software (avaible on http://genome.tugraz.at) using Pearson squared distance for the similarity distance calculation and the complete linkage clustering for the linkage rule. To stablish correlations between protein abundance and transcripts relative expression a Pearson correlation test was performed by using Statgraphics Centurion v 16.1.15 Software.

3. RESULTS

This work aims to monitor the expression profile of a group of selected genes related to the ripening process in response to warming conditions trough a targeted transcriptomic analysis. This analysis could shed light towards the identification of biomarkers for ripeness and temperature response. The results of amplification and the curves for primers efficiency allowed the study of 85 out of 110 candidate genes. In general, the expression levels of the genes monitored were more sensitive to the ripening processes than to warming conditions, just as previous proteomic analysis suggested. For this reason, a t-student two tails test was employed comparing AT with AT+4 °C per each ripening stage.

The relative expression of the 85 genes was represented in the Supplementary Figure 6.1, in which data corresponding to means ± SE per each ripening stage and temperature treatment was shown. Those protein coding genes are involved in different processes/pathways related to the ripening process under warming conditions: i) Lipid metabolism (*ACAA1*, *SAD1*, *fabI*, *accA*, *fabG*, *KASI*, *KASIII*, *LACS8*, *FAD7.2*, *FAD2.2*, *CXE6*, *fadD*, *ACCA* and *CYP2 member*); ii) Hormone regulation or signalling (*DRM2*, *PNAE*, *ASR1*, *ARR1*, *DELLA GAI1*, *GID1*, *TIP120*, *BISCPL1*, *ACCO*, *OPR*, *LOX2*, *MAP3K9/MEKK1*, *AP2/ERF-AP2-1*, *PAP1 MYB75/PAP1*, *EIN3*, *ERF1B*, *MYC2* and *NDL1*); iii) Carbohydrate metabolism (*PK1*, *MIPS*, *M6PR*, *RS4*); iv) Amino-acid or protein metabolism (*AK-HSDH*, *CYS-C1*, *CTSF*, *M41FTSH*, *SKP1*, *pakA*); v) Secondary

metabolism, phenylpropanoids pathway (*CO*, *PAL*, *CYP98A3*, *4CL*, *CHI3*, *DFR*, *3GGT*, *ANS*, *CISZOG*, *F3H*, *UGT73C*, *SDH*, *UGT72E*, *COM*) and terpenoids pathways (*HAD*, *AK1D1*, *MDV2, FDPS, ispD*, *RGB*, *DXS*, *TPS3*, *SQS*, *GE10H*, *CYP72A219*, *ispG/HBMPP*, *CDP-MEK*, *FDPS*, *MVD2*); vi) Senescence processes related (*β-GAL*, *PL1*, *GMD*, *PPIASE CYP18-3*, *PHB1*, *ANAXA13*); and vii) Stress response (*LEA2*, *ERD4*, *HSP70*, *ChiB*, *KRP4*).

The clustering analysis revealed 6 different patterns concerning their relative expression, as shown the Figures 6.2 and 6.3. The cluster named 1A, 1B, 2, 3, 4A and 4B group a total of 3, 5, 20, 25, 4 and 25 genes, respectively. However, the genes compilated on each determined cluster by relative expression pattern did not represented a especific functional category (Figure 6.2). Cluster 1A compiled genes underexpressed under warming condition respect environmental temperature at unripe olives, but with overexpression at veraison AT+4 ºC respect green stage AT+4 ºC. Cluster 1B included genes overexpressed at green stage AT+4 ºC and underexpressed at veraison AT+4 ºC that cannot be detected along ripening processes. Cluster 2 represented genes absent at green stage but overexpressed under warming conditions in veraison without a clear pattern at purple stage apparently without differences. Genes grouped on Cluster 3 were overexpressed at green stage, underexpressed at veraison stage and without significant differences at purple stage under warming condition. Cluster 4A corresponded to genes underexpressed along ripening progress and under warming conditions. Finally, Cluster 4B comprised genes absent at green stage, underexpressed at veraison and without differences at purple stage under warming conditions respect ambient temperature. Most of genes (cluster 1A, 2, 3 and 4B) showed an inverse tendency along ripening progress between AT and AT+4 ºC.

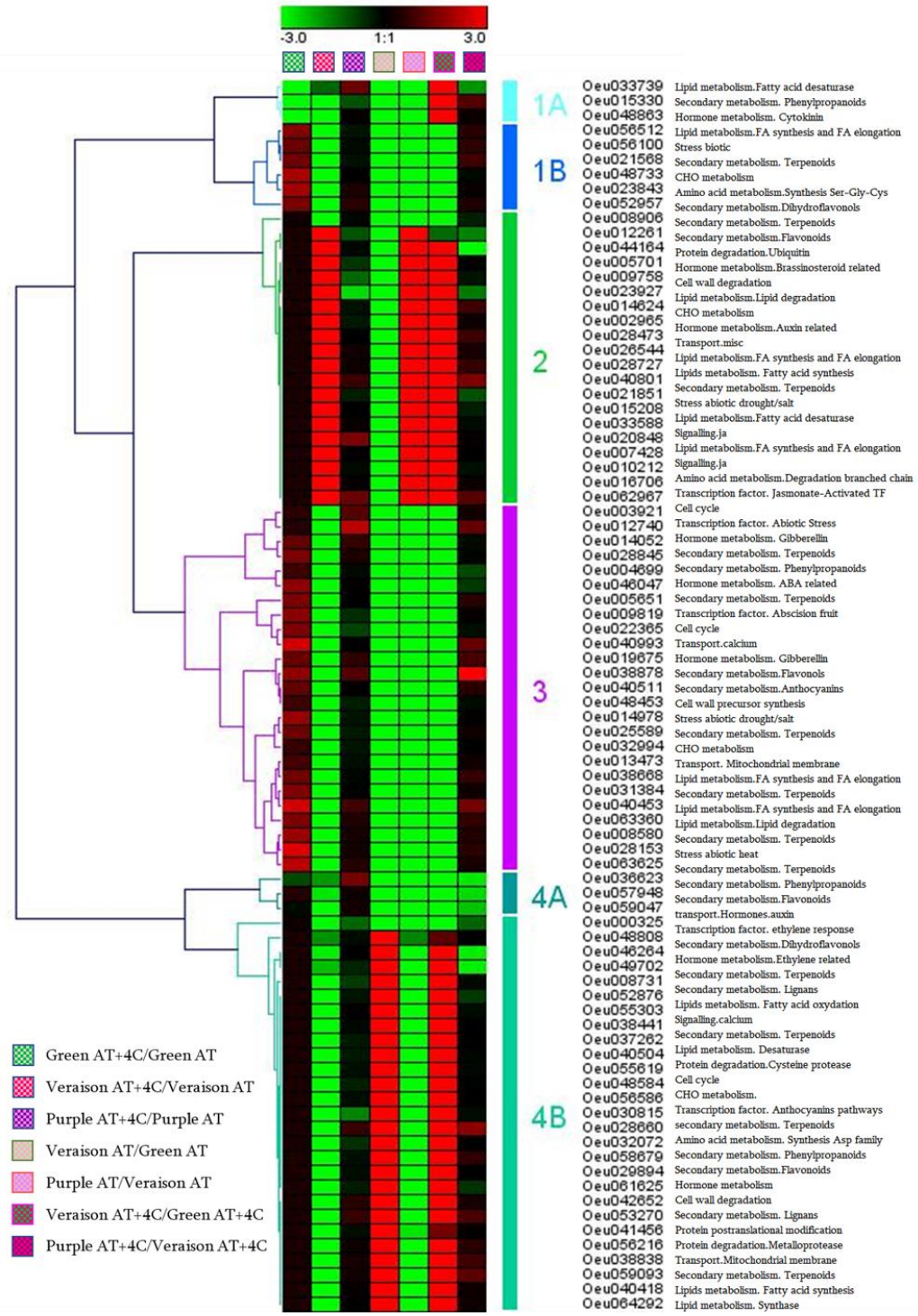


Figure 6.2. Schematic representation of the expression pattern of the 85 highly-responsive genes in the comparisons of the warming treatment vs control treatment. Average linkage hierarchical clustering, where each horizontal line displays the expression data for one gene. The colours red or green indicate respectively up- and downregulation.

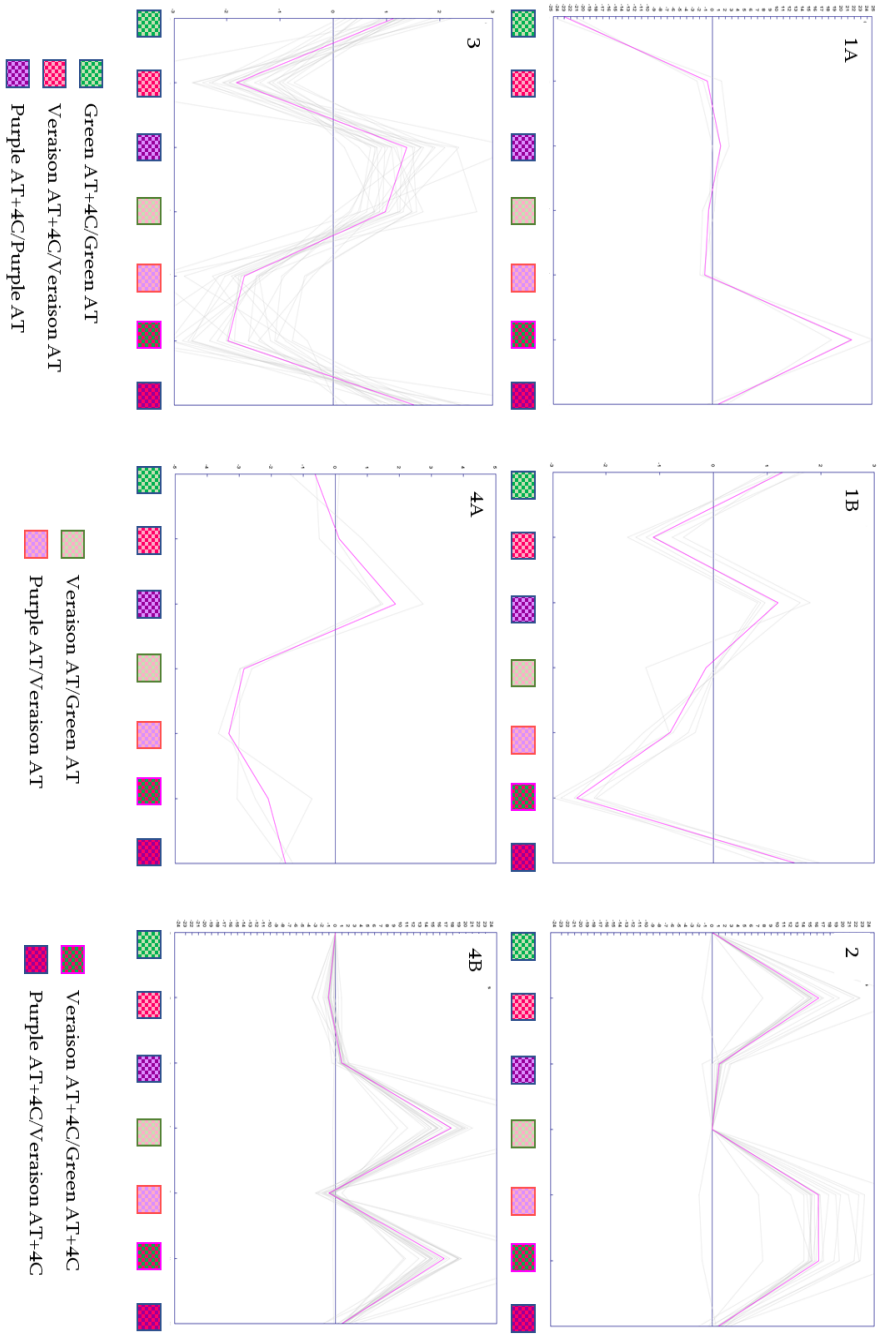


Figure 6.3. Representation of the 6 clusters associated to the hierarchical clustering employed. Firstly, ratios comparing temperatures treatment are showed, secondly the ripening at AT conditions are represented and finally, riepning procees at AT+4 ºC are displayed.

Finally, a correlation analysis was performed to determined the relationships between transcript and its protein translated to elucidate probable post-transcriptional modifications. Only 24 out of 55 genes was a good correlation, of which 13 were positively correlated and the rest negatively correlated

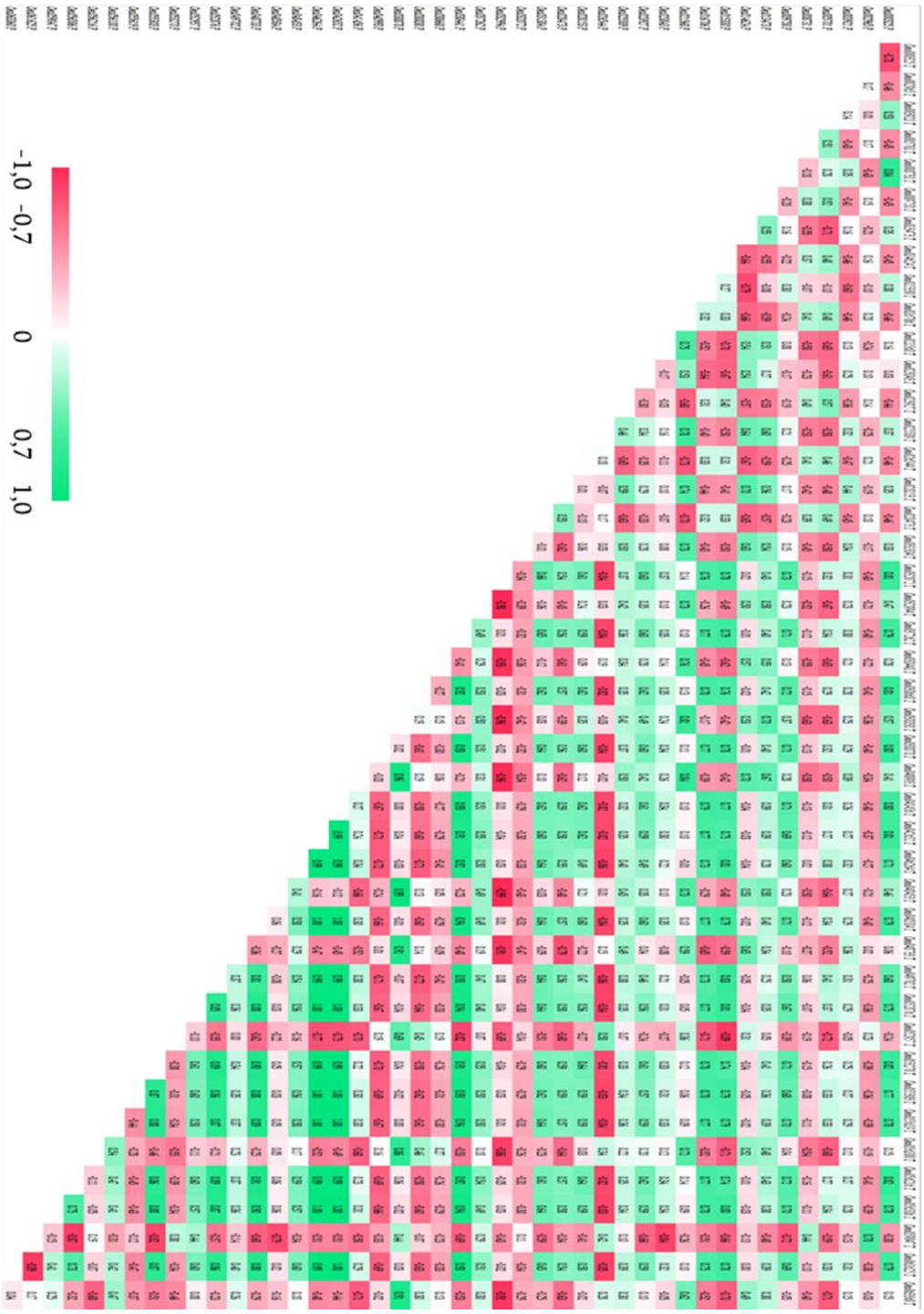


Figure 6.4. Correlation matrix between differential proteins (horizontal axis) and genes (vertical axis). Pearson Correlations values were represented. The colours red or green indicate respectively positive and negative correlation, |r|=>0.7 was the threshold selected.

4. DISCUSSION

Plants have adapted to potential air temperature changes in their growing environment and can usually adjust to conditions slightly above and below optimum air temperature ranges. Physiological adaptations to high temperatures include increased leaf wax, leaf rolling, changes in leaf orientation and in leaf size and the production of heat shock proteins (Taiz and Zeiger, 1998) among others. In general, temperature changes can either delay or hasten the ripening as previous under field experiments demonstrated in olive tree (Benlloch-González *et al.*, 2019).

Under warmer air temperature, the percentage of unsaturated fatty acid chains in plant membranes decreases, specifically thylakoid membranes, to counter the greater membrane fluidity (Jun *et al.*, 2001; Taiz and Zeiger, 1998). Acyl carrier proteins (ACPs) are involved in a diverse array of metabolic pathways including the biosynthesis of fatty acids (Rock and Cronan, 1981; Rock and Jackowski, 2002), polyketides (Cane and Walsh, 1999), membrane-derived oligosaccharides (Tang *et al.*, 2007), lipopolysaccharides (Brozek and Raetz, 1990; Sweet *et al.*, 2004) and phospholipids (Cronan, 2003). Modeling studies, based on the information provided by the interaction of ACP with fabH and fabG (Zhang *et al.*, 2001; Zhang *et al.*, 2003), suggest that ACP should interact with a cluster of basic residues adjacent to the fabI substrate binding loop. In our experiment under warming conditions genes involved on lipid metabolism are absent or overexpressed at green stage, underexpressed at veraison and newly overexpressed at purple stage; while at environmental conditions, the peak of expression (lowest values than the observed at AT+4 °C) occurred at veraison but decreasing level of transcripts at purple stages. Interestingly, *fabI* and *fabG* followed this tendency. The decrease of lipids content observed on field experiment (Chapter III) had not a direct correlation at the transcript or proteomic level; long chain fatty acids synthetases and desaturases were present/increase in harvested fruits in warming conditions (*KASII*, *KASIII*, *CAA1*, *accA*, *SAD1, FAD7.2* or *FAD2.2*). A plausible explanation could be the delay of the lipogenesis (at veraison) or functional regulation by post-transcriptional mechanisms (Chapter V). Furthermore a probable lipid oxidation to obtain secondary metabolites, or derived towards hormone synthesis pathway could be occurring.

Lipids can be oxidized by enzymes like lipoxygenases, cyclooxygenases, and cytochrome P450. Plant cytochrome P450s are involved in a wide range of biosynthetic reactions and target a diverse range of biomolecules. These reactions lead to various fatty acid conjugates, plant hormones, secondary metabolites, lignins, and a variety of defensive compounds (Schuler and Reichnard, 2003). Plant genome annotations reported that cytochrome P450 genes make up as much as 1 % of the plant genes. The number and diversity of P450 genes is responsible, in part, for the multitude of bioactive compounds (Mizutani and Sato, 2011). CYP2 subfamily plays a role in metabolizing polyunsaturated fatty acids (PUFAs) to biologically active, intercellular cell signalling molecules (eicosanoids) and/or metabolize biologically active metabolites of the PUFA to less active or inactive products. These CYPs possess cytochrome P450 omega hydroxylase

and/or epoxygenase enzyme activity. Here were analysed 4 CYP members genes, *PPIASE CYP18-3*, *CYP98A3*, *CYP72A219* and a *CYP2 member like,* of which *PPIASE CYP18-3* are related to lipids synthesis through amino-acid degradation, *CYP72A219* are involved on terpenoids synthesis and *CYP2-member like* are annotated as lipid oxidant enzymes. All of them follow the same behaviour described above for the….

The plant ability to detect and respond to the level of carbohydrates may act as a controlling mechanism, influenced by environmental conditions (e.g. light, nutrients, biotic and abiotic stress factors) with internal developmental programs, controlled directly by hormones (Koch, 2004; Rolland *et al.,* 2006; Hammond and White, 2008; Loreti *et al.,* 2008; Ramon *et al.,* 2008; Agulló-Antón *et al.,* 2011). The progress in research on molecular mechanisms of sugar sensing and signaling in plants shows that signal molecules include glucose, fructose, sucrose, and trehalose (Rolland *et al.,* 2002; Koch, 2004; Ramon *et al.,* 2008; Rosa *et al.,* 2009; Cho and Yoo, 2011). Additionally, biochemical studies provide evidence for the involvement of a variety of protein kinases, i.e. Snf1-related kinases (SnRKs) (Smeekens *et al.,* 2010), calcium-dependent protein kinases (CDPKs), mitogen-activated protein kinases (MAPKs), and protein phosphatases, 14-3-3 proteins, Ca^{2+} ions as a second messenger, and G-proteins (Rolland *et al.,* 2006) in sugar signal transduction (Rolland *et al.,* 2002; Sinha *et al.,* 2002). Recently, several studies revealed that genes encoding mitogen-activated protein kinases (MK1 and MK2), protein phosphatase 2C (PP2C1), homeodomain and ERF transcriptional factors (HD1 and ERF1-4), phosphoinositide-specific phospholipase C (PI- PLC1), F-box protein (DRF1), diacylglycerol kinase (DK1), zinc finger protein (RF1), DEAD-box RNA helicase (RH1), and serine carboxypeptidase-like protein (SCPL1) are involved in disease resistance and other abiotic stress responses (Song and Goodman, 2002a; 2002b; Luo *et al.*, 2005a; 2005b; Hu *et al.*, 2006; Cao *et al.*, 2006a; 2006b; 2008; Song *et al.*, 2006; Zhang *et al.*, 2008; Liu *et al.*, 2008a; 2008b; Li *et al.*, 2008). A range of biological processes that occur in different tissue types, under a multitude of environmental conditions, might be regulated by temporal and spatial coordination between ROS and other signals. In response to stress stimuli, early signalling events in plants include increased flux of Ca^{2+} into the cytosol, activation of mitogen-activated protein kinases (MAPKs), and protein phosphorylation (Benschop *et al.,* 2007).

Plant hormones in general and ethylene in particular, have a major role in the ripening process (Vendrell, 1984). More than increasing respiration rates, they are even marking the transition between the fruit growth phase and its ageing is the increase in ethylene biosynthesis up to stimulating concentrations (Barceló *et al.*, 1980). From that moment on, there is a constant increase until a peak is reached at ripeness (Maxie *et al.*, 1960). Conforming ripening processes advances, fruit become more sensitive to small doses of ethylene; without effect of external application or its precursor (Bouzayen *et al.*, 2010). Ethylene releasing compounds have been used in an attempt to reduce fruit retention force and to facilitate mechanical harvesting (Sun and

Martin, 1982). Nevertheless, this has led to undesirable effects, including leaf drop (Lang and Martin, 1980) and lower flowering rates the following year (Hartmann *et al.*, 1970). Although there is not information on abscisic acid action mechanism, it is believed that it may have a role at the star of fruit ripening. An increase in its concentration has been observed at green stage, also stimulate the ripening when is artificially applied (Vendrell, 1984). Giberellic acid action delays ripening and reduces fruit respiration despite its scarce level in ripe fruit. When this hormone was applied externally, a delay in the onset of purple colouring was observed (Lavee, 1986). Cytokinins have an anti-ageing action, so they usually counteract some of the ripening processes. Cytokinin levels are found in low concentrations in developing olives and increase with ripening (Shulemna and Lavee, 1976). Other proteins have relevance in the senescence process as prohibitin. The prohibitin-1 and prohibitin-2, are polypeptide complex appearing as a chaperone, stabilizing components of the respiratory chain (Artal-Sanz *et al.*, 2003; McClung *et al.*, 1995; Nijtmans *et al.*, 2002; Steglich *et al.*, 1999). There is a body of evidence suggesting that prohibitins play a role in the regulation of aging, senescence and/or stress response. Our results revealed that *PHB1* levels were higher under warming conditions and the earlier apparition acted as indicative the hasten senescence process.

The cyclin-dependent kinase inhibitory kinase and the anaphase promoting complex activator CCS52A both participate in the control of cell size and the endoreduplication process driving cell expansion during early fruit development in tomato. Moreover, the fruit-specific functional analysis of the tomato CDK inhibitor KRP1 reveals that cell size and fruit size determination can be uncoupled from DNA ploidy levels, indicating that endoreduplication acts rather as a limiting factor for cell growth (Chevalier *et al.*, 2011). In our experiment, KRP4 transcript levels showed a maximum peak at veraison under control (environmental conditions); however, this peak appears at green stage under warming condition. This could explain the lowest pulp/pit ratio observed under warming conditions.

The ripening period varies according to climate conditions and the varietal characteristics. Furthermore, the number of fruits per tree may delay ripening. In addition, when there are many fruits in each bearing brunch, anthocyanins biosynthesis may be partially inhibited and fruit colour may only reach reddish tones, probably due to the phytohormones activity as ABA. ABA-mediated development-dependent anthocyanin biosynthesis and fruit coloration during *Lycium* fruit maturation was proposed. The activated NCED1 promotes accumulation of the phytohormone ABA, that in turn stimulates transcription of the MYB-bHLH-WD40 transcription factor complex to upregulate the expression of structural genes in the flavonoid biosynthetic pathway, and thereby promoting anthocyanin production and fruit coloration (Li *et al.*, 2019). Our results revealed that MYB2 and NDL1 transcripts had a reduction along ripening progress at environmental conditions. However, the levels of these transcripts showed different expression peaks along ripening under warming conditions. Other possible reason for reduction of

anthocyanin contents could be the higher oxidation rates. Under warming conditions, transcripts for polyphenol oxidases (*PO* and *CO*) were overexpressed. The transcriptional profiles of phenylpropanoids related genes followed different patterns depending on the ripening stage: underexpression at veraison stage, but overexpressed at green and purple stages were observed for *ANS*, *DFR*, *C3H*, *4CL*, *MYC2* and *MYB113like*-transcriptional factors; while their corresponding proteins involved on anthocyanidins biosynthesis directly correlated with a decrease of flavonoids/phenylpropanoids content (Chapter V and VII). Accumulation of trans-cinnamic acid due to C4H knockdown was accompanied by the reduction of p-coumaric acid, total phenolics, anthocyanin, cinnamate-4-hydroxylase (C4H) and phenylalanine ammonia lyase (PAL) activities but an increase in salicylic acid (SA) and artemisinin (Kumar *et al.*, 2016). In *Arabidopsis thaliana*, anthocyanin synthesis through the phenylpropanoid pathway was associated to increased transcript levels for flavonoid biosynthetic genes, including *PAL* and *CHS* (Leyva *et al.*, 1995). On the contrary, high temperature (35 °C) reduced the total anthocyanin content in *Vitis vinifera* berries compared to control temperature (25 °C), as a result of anthocyanin degradation and transcriptional downregulation of anthocyanin biosynthetic genes (Mori *et al.*, 2007). The intended use of the olive will determine the most suitable ripeness stage for harvesting. For oil extraction, in the cultivars that develop colour (Picual, Hojiblanca, Arbequina, etc) olive oil is fully formed when the ripeness index reaches values close to 3-3.5 (closely to veraison stage) according to Ferreira scale (Ferreira, 1979). This fact reveals the relevance of a correct relationship between olives skin colour and their internal ripening stage. Moreover, UDP-glucosyltransferase (UGT) family are key enzymes for anthocyanin biosynthesis, which by catalysing glycosylation of anthocyanidins, increases their solubility and accumulation in plants. UGT activity is inhibited by anthocyanidin, the sugar acceptor substrate, while calcium/calmodulin binding to some of these UGT proteins enhances anthocyanin accumulation competing with this substrate. In our study, *UGT72E* and *UGT73C* relative expression drop abruptly at veraison under warming conditions and then increase the levels slightly at purple stage.

Terpenoids plays an important role as oil antioxidant and stabilising. Moreover, they are responsible to olive oil organoleptic and nutritional value. Oleuropein is the main secoiridoid, representing up to 82 % of total phenolic compounds, and it is known as the bitter principle of olives (Alagna *et al.*, 2009). The first step in the oleuropein biosynthesis pathway is initiated from geranyl diphosphate and catalyzed by geraniol synthase (GES), following which the product geraniol is converted into 8-hydroxygeraniol by geraniol 8-hydroxylase (G8H). Secoiridoids are derived from iridoids by opening of the cyclopentane ring, and in the Oleaceae family, the resulting carbonyl group is oxidized and conjugated with a phenolic moiety. Recent studies have elucidated the pathway from geranyl diphosphate to secologanin in *Catharanthus roseus* (Miettinen *et al.*, 2014) and *O. europaea* (Mougiou *et al.*, 2018). The pathway involves seven enzymes catalyzing successive reactions from 8-hydroxygeraniol to secologanin: 8-hydroxygeraniol oxidoreductase (8-HGO), iridoid synthase (IS), iridoid oxidase (IO), 7-

deoxyloganetic acid-O-glucosyl transferase (7-DLGT), 7-deoxyloganic acid hydroxylase (7-DLH), loganic acid methyltransferase (LAMT), and secologanin synthase (SLS). In addition, *HAD*, reported as negative regulator of isopentenyl diphosphate biosynthetic process (isoprenoids), present high transcript level at veraison stage underwarming condition. Our results showed lower transcripts levels for genes involved in the synthesis of the major terpenoids under warming conditions. However, genes involved on these pathways as *SQS*, *LS*, *DXPS*, are overexpressed. Terpenoids metabolism is the less represented at proteomic level. The general pattern for terpenoids related transcripts was the overexpression at green stage and purple but and underexpression at veraison stage under warming condition. This could negatively affect the olive oil quality but could add a sweeter taste increasing the acceptance of consumer accustomed to seed oils.

5. CONCLUSIONS

Targeted transcriptomics allowed monitoring gene expression of 85 out of 110 selected candidate genes involved in: i) Lipids metabolism ii) Hormone regulation or signalling iii) Carbohydrates metabolism; iv) Amino-acid or protein metabolism; v) Secondary metabolism, phenylpropanoids and terpenoids pathways; vi) Senescence processes related; and vii) Stress response. Clustering analysis grouped the differential transcripts in six clusters as follow: 1A (3 genes), 1B (6 genes), 2 (20 genes), 3 (25 genes), 4A (3 genes) and 4B (27 genes). Clusters determined by relative expression pattern did not compilated a determined functional category. However, most of genes involved in Phenylpropanoid Pathway were absent at green stage, with overexpression at veraison and underexpressed at purple stage under warming conditions. Genes involved in Mevalonate Pathway (terpenoids) followed the same pattern at advance ripening stages but these were present at green stage with the higher relative expression observed. Many genes involved in Lipid metabolism showed a quite similar expression that genes involved in Mevalonate Pathway. In general, relative expression at veraison rise closely related values to purple stage. This should be considered under future climate change scenarios to change the harvesting period in order to keep the fruit characteristics and the final oil quality. Further experiments are required to validate a metabolic level the mechanisms involved on warming response.

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7. SUPPLEMENTARY MATERIAL

Supplementary Table 6.1 Characteristics of primers disegned to the targeted transcriptomic analysis.

Gene Description	Primer Code	Sequence	Amplicon Size
	Oeu000325.1_F	CCTCTGTGCAAAGCTCCATC	
N-MYC DOWNREGULATED-LIKE 3 PROTEIN	Oeu000325.1_R	GCTCTGCCTCTCATCTAGCA	201
	Oeu002662.1_F	ACCACCTCGTGTCTAGCCA	
ASPARTYL PROTEASE	Oeu002662.1_R	CGTGCCACACCAAGCTTG	130
	Oeu002965.1_F	TGCCCACAATTCACACAGGA	
Mannose-6-phosphate 6-reductase	Oeu002965.1_R	CCATACCGCGCCATGTTG	105
	Oeu003921.1_F	CGTCGTGCACTTAACCAGC	
annexin A13	Oeu003921.1_R	TAGTCGCCCGAAGTGTCCT	162
	Oeu004699.1_F	CGGCATGCATTTACCGGCT	
polyphenol oxidase	Oeu004699.1_R	GAATCGCTGCCGCTGAAGT	98
	Oeu004908.1_F	GGATGTTGGAAAAGAGGGCA	
DESICCATION-RELATED PROTEIN	Oeu004908.1_R	CACTCACCTTCGTCGCCTT	162
	Oeu005651.1_F	ACGAGTGAGACATGCCTTGG	
(R)-Limonene synthase	Oeu005651.1_R	GCCCACATCCTTCCACCAA	197
	Oeu005701.2_F	TCTGCTCGACCTCACATGTC	
SKP1	Oeu005701.2_R	TTTCCTCTTCCTCCTCCGGG	114
	Oeu005716.1_F	AGACTGGCCCAACAACGATG	
Catechol oxidase / Tyrosinase	Oeu005716.1_R	ACCTGCGGCATTGACTGT	161
	Oeu007428.1_F	GGCAATACGAACAGGCTGG	
Beta-ketoacyl-[acyl-carrier-protein] synthase I	Oeu007428.1_R	TTGGACAATGCCACCTTGAT	122
	Oeu008580.1_F	GCGCTTGACACTGTTGAGG	
Squalene synthase	Oeu008580.1_R	AGCAGTGGAAACGTGATGGA	161
	Oeu008731.1_F	AAGAGCGTGGGAATGCAGG	
HALOACID DEHALOGENASE-LIKE HYDROLASE	Oeu008731.1_R	CAGCCGCCAAGTCAGGTAA	103

	Oeu008906.1_F	AGTTCCCAACACAGCTGCA	
cis-zeatin O-glucosyltransferase	Oeu008906.1_R	ATCGCCTCCTGAACAACCG	147
	Oeu009758.1_F	TGGCGTCCTTGGTTGGATG	
SERINE PROTEASE S10	Oeu009758.1_R	AGTGAAAGCTCTCTGGGGC	120
	Oeu009819.2_F	GTGGAAAGGGTGAACGGGT	
Apetalla 2/Ethylene Response Factor 1	Oeu009819.2_R	GCTCACTCCCCAACGAACA	183
	Oeu010212.1_F	AGCCGAATGACCAAGCTCC	
12-oxophytodienoic acid reductase	Oeu010212.1_R	CGTTACGAGCAGCCAACCT	144
	Oeu012051.1_F	GCTCGACTGAAGCAACCGA	
Iridoid synthase	Oeu012051.1_R	AATTAGTGCAGGCCGGTGG	105
	Oeu012261.1_F	AAGACAATGCCAGCGCCA	
1-deoxy-D-xylulose-5-phosphate synthase	Oeu012261.1_R	CGCCTTCGTCTGCTTTCCT	141
	Oeu012740.1_F	TCACCTTCCTCCTCGTCGA	
Mitogen-activated protein kinase kinase kinase 1	Oeu012740.1_R	CCGCCACAGGAATCGAGAA	180
	Oeu012990.1_F	CACAACGGCACCACTCTTC	
7-deoxyloganetic acid-O-glucosyl transferase	Oeu012990.1_R	CCTGAACTCATTGAAGCCGG	341
	Oeu013473.1_F	TGCAGGACCAGAAAACGCA	
solute carrier family 25 (mitochondrial phosphate transporter), member 3 (SLC25A3, PHC, PIC) (PAC:37701501)	Oeu013473.1_R	AGCTTCAAAGGGGCAGAGC	102
	Oeu013924.1_F	CTGCAGCACACACACCCTT	
FAD2.1	Oeu013924.1_R	AGTCGCCTCCATTGCATGA	185
	Oeu014052.2_F	TGGGCCGGTTTTCTTGGAC	
DELLA GAI1	Oeu014052.2_R	CCTCACACGCCACCACATT	174
	Oeu014624.1_F	TGCTGTTTACACCCGCCT	
MEMBER OF 'GDXG' FAMILY OF LIPOLYTIC ENZYMES	Oeu014624.1_R	GCCAGAGGAGTGCATCGAA	121
	Oeu014978.1_F	TCCCATCACCTTTAAGCCC	
Late embryogenesis abundant protein	Oeu014978.1_R	TGTACCACCCTCCTTGCTG	147
	Oeu015208.1_F	GTTCCTGGTGACCTGCTGA	
EARLY-RESPONSIVE TO DEHYDRATION STRESS PROTEIN	Oeu015208.1_R	ACAAGCCATCCCAAGCCA	103
	Oeu015330.2_F	ATTGGTGGTGGCCGTCTTG	
SPERMIDINE HYDROXYCINNAMOYL TRANSFERASE	Oeu015330.2_R	AACCAATGGCTGCTCGTGAA	179

	Oeu016706.1_F	GCTGCTTATCGAACCGCAC	
acetyl-CoA acyltransferase 1	Oeu016706.1_R	TGGAGCCAAAACTGTGCCA	158
	Oeu019675.1_F	TTCCTCAGCCAACAGTGCC	
Gibberellin receptor GID1	Oeu019675.1_R	CCCACTCCTTAACCACGACC	167
	Oeu020848.1_F	TGTTGGCAGGAGTGAACCC	
Linoleate 13S-lipoxygenase	Oeu020848.1_R	CGTCCTGATTGCCTCGTCA	160
	Oeu021568.1_F	ATGACACCACAAGCGCCAA	
CHITINASE-RELATED	Oeu021568.1_R	ACACCATAACCAGGCACCC	103
	Oeu021851.1_F	TGGACCTATTCGTTGCGGG	
GE10H	Oeu021851.1_R	AGGGCCTCTTTCACCATGC	120
	Oeu023843.2_F	GTGAGGCCCGTGAGTTTCT	
PYRUVATE KINASE	Oeu023843.2_R	ATGCCAAGGTTCCCACGT	160
	Oeu023927.1_F	CCCCTGCCAATCCTTTTGC	
PECTATE LYASE 1-RELATED	Oeu023927.1_R	GCCCCGTTAAGCATCAGGT	124
	Oeu025589.3_F	CAGGGATGCGTGAGACTGT	
Diphosphomevalonate decarboxylase	Oeu025589.3_R	GGCGAAGTATCCAGGCAGA	138
	Oeu026544.2_F	CACCCTGAATCCTGATGGC	
apolipoprotein D	Oeu026544.2_R	CGCCTCGTCACTATTGGGA	181
	Oeu027591.1_F	AGTGGTTGGTGGGTGGAAC	
4-hydroxy-3-methylbut-2-enyl diphosphate reductase	Oeu027591.1_R	TGCATGGGGAGTTGGTAGG	190
	Oeu027984.2_F	TGTTGTGCAGGTGTTGAGC	
tocopherol O-methyltransferase	Oeu027984.2_R	GAAGGATGCGGACGACTGT	145
	Oeu028153.2_F	GATGGCTTTGCAGGACCGT	
HEAT SHOCK PROTEIN 70KDA	Oeu028153.2_R	TTGGTTTCATCCTCGCCGT	120
	Oeu028473.1_F	TTCTGCTGCGGTGTTCCA	
TIP120	Oeu028473.1_R	TCGTCATCCTCCTCCACCA	101
	Oeu028660.1_F	TGGGGCGATGCTGTAAATGA	
Secogolanin synthase	Oeu028660.1_R	GCGAAACTCTGTCCAATGCA	142
	Oeu028727.1_F	ACCGACGCTGACAAACTGA	
acetyl-CoA carboxylase carboxyl transferase subunit alpha (accA)	Oeu028727.1_R	CGGACACCCCTGATTTTGC	136

	Oeu028845.1_F	GCGATGATCCAACAAAGCCG	
beta_glucosidase	Oeu028845.1_R	CCAATCCGAACCAGCCTGT	125
	Oeu029894.1_F	TGCTCCCCAAAGACCCCAA	
4-coumarate--CoA ligase (4CL)	Oeu029894.1_R	TTGGCCGGTGTGAAAATCAA	142
	Oeu030756.1_F	CAAGAGCCAAACCGACCCA	
allene oxide cyclase (AOC)	Oeu030756.1_R	CGGAGACCTGCAGAAACGA	123
	Oeu030815.1_F	TGGTCGCTAATTTCCGGTAGA	
MYB113- like//PAP1 MYB75	Oeu030815.1_R	TGGGCTTTTCTCTTCTCCTCT	157
	Oeu031384.1_F	TGGCAAATCTGAATGGGACG	
farnesyl diphosphate synthase (FDPS)	Oeu031384.1_R	TCCAGCATCCGTTCAACCC	146
	Oeu032072.1_F	AGTCAACTGTCGGTGCTGG	
ASPARTATE KINASE // HOMOSERINE DEHYDROGENASE	Oeu032072.1_R	TTTCCGCCTTCTACCTCGC	182
	Oeu032994.1_F	TGGAGCACTACGACACAGC	
raffinose synthase (E2.4.1.82) (PAC:37720293)	Oeu032994.1_R	ACCCAAGCCATGAACAGCA	102
	Oeu033588.2_F	TGGCTGGTTGGGTGTTGT	
FAD7.2	Oeu033588.2_R	GCCTTGGAATTGACCCTGG	134
	Oeu033739.1_F	CTGATGCTGGCGTTCTTGC	
FAD2.2	Oeu033739.1_R	AGGCAGGGAGGGATGTGTA	167
	Oeu033769.1_F	AGTGGGAGGACTGGGAGTT	
ESSS subunit of NADH:ubiquinone oxidoreductase (complex I)	Oeu033769.1_R	TAGTGCTTTCTGGTGGGCC	158
	Oeu036623.1_F	CGCCACAATTCCAAGGTCG	
phenylalanine ammonia-lyase	Oeu036623.1_R	CCATTGACAAGTGCAAGGCC	164
	Oeu037262.1_F	TGACCCTCCTTTCCACGAG	
3-oxo-5-beta-steroid 4-dehydrogenase	Oeu037262.1_R	TGGCCTATGAACCGACCAC	123
	Oeu037647.1_F	CCCAAACCCATATCGCCGA	
beta-fructofuranosidase	Oeu037647.1_R	TGCATTTTCACCGCTGGC	148
	Oeu038441.1_F	AGAATTGGGGCAGGCACA	
calcium-binding EF hand family protein	Oeu038441.1_R	CGCCATCCAGTTGCTTTTCA	167
	Oeu038668.1_F	AGCATCAGTTGTCGGCCT	
3-oxoacyl- (fabG)	Oeu038668.1_R	TGCAATGAACCCAGGAGCA	198

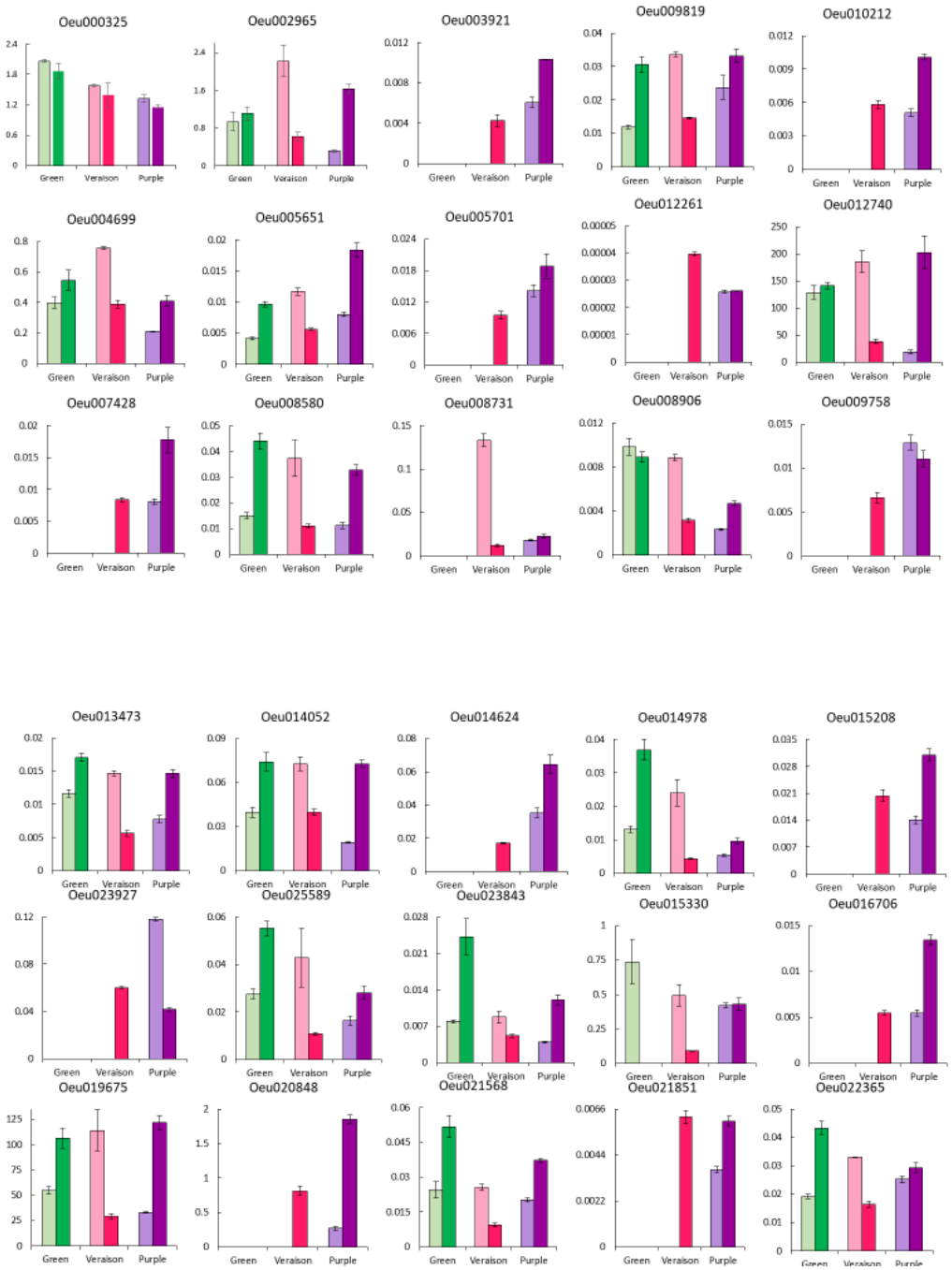
solute carrier family 25 (mitochondrial oxoglutarate transporter), member 11 (SLC25A11, OGC) (PAC:37739068)	Oeu038838.2_F	AGCCGTGAAGCCCTTTGT	
	Oeu038838.2_R	ACCAGCCGACAAACCCTT	124
UDP-glucosyl transferase 73C	Oeu038878.1_F	AGCCAGCTCCAAGTTGCA	
	Oeu038878.1_R	CGTCTCGGGCACACTTTCA	122
long-chain acyl-CoA synthetase (ACSL, fadD) (PAC:37742565)	Oeu040418.1_F	GCGCTCCTCTTTCTGCTGA	
	Oeu040418.1_R	GGCCAACTCGACCAACAGA	136
FAMILY NOT NAMED // LONG CHAIN ACYL-COA SYNTHETASE 8 (PAC:37729763)	Oeu040453.2_F	TTACAGCCAGCGACGGAA	
	Oeu040453.2_R	TCTGCCCGAGTTTCAGCA	127
ACYL-[ACYL-CARRIER-PROTEIN] DESATURASE 1	Oeu040504.1_F	TCTTTCTGGAGAGGGGCGT	
	Oeu040504.1_R	TGCCTGCTTGACCTTCGA	165
Anthocyanidin 3-O-glucoside 2''-O-glucosyltransferase (PAC:37697620)	Oeu040511.1_F	TGCAACCTTTTCTCCGGCA	
	Oeu040511.1_R	TGCAGGGCTTACAACGGA	123
acetyl-CoA carboxylase carboxyl transferase subunit alpha (accA) (PAC:37703866)	Oeu040801.1_F	TTGACACTCCAGGGGCAT	
	Oeu040801.1_R	TCCACCAGAGCCACCTTCT	158
CALCIUM-TRANSPORTING ATPASE 1 ENDOPLASMIC RETICULUM-TYPE-RELATED (PAC:37737722)	Oeu040993.2_F	GTCTCATTCGGCCTGCACT	
	Oeu040993.2_R	TGCCACTTGTACACCGACC	159
SERINE-THREONINE PROTEIN KINASE // PROTEIN KINASE FAMILY PROTEIN (PAC:37723856)	Oeu041456.1_F	ACGGGAAGTGGAAGGGTTC	
	Oeu041456.1_R	TCGCGCCAAAACTCCACA	147
N-HYDROXYCINNAMOYL/BENZOYLTRANSFERASE, PUTATIVE-RELATED (PAC:37703617)	Oeu043317.1_F	AACGACAACCACCCTGCA	
	Oeu043317.1_R	CGAATGACCGGCACTCCTT	124
CHALCONE--FLAVONONE ISOMERASE 3-RELATED	Oeu044164.1_F	TGCAGCAGAGGGAAAAGAAG	
	Oeu044164.1_R	CTCGGCAGATAGAGCAGTGG	126
abscisic stress-ripening protein 2-like	Oeu046047.1_F	AGAGAAGAAGCACCACGGC	
	Oeu046047.1_R	TACTCGCCTCCAACACCAG	157
naringenin 3-dioxygenase	Oeu046264.1_F	GGGGCTGGAAAAGGATGCT	
	Oeu046264.1_R	CCAAGTGTGATGTCGGGCT	158
GDPmannose 4,6-dehydratase	Oeu048453.1_F	TCTTCTCCGTTCCACCCCA	
	Oeu048453.1_R	AAGTTTTCGCCCCTCCTGG	126
prohibitin 1	Oeu048584.1_F	TCACTTGTCCTACGGTGCG	
	Oeu048584.1_R	GATCAGGCCCATTCCAGCA	24

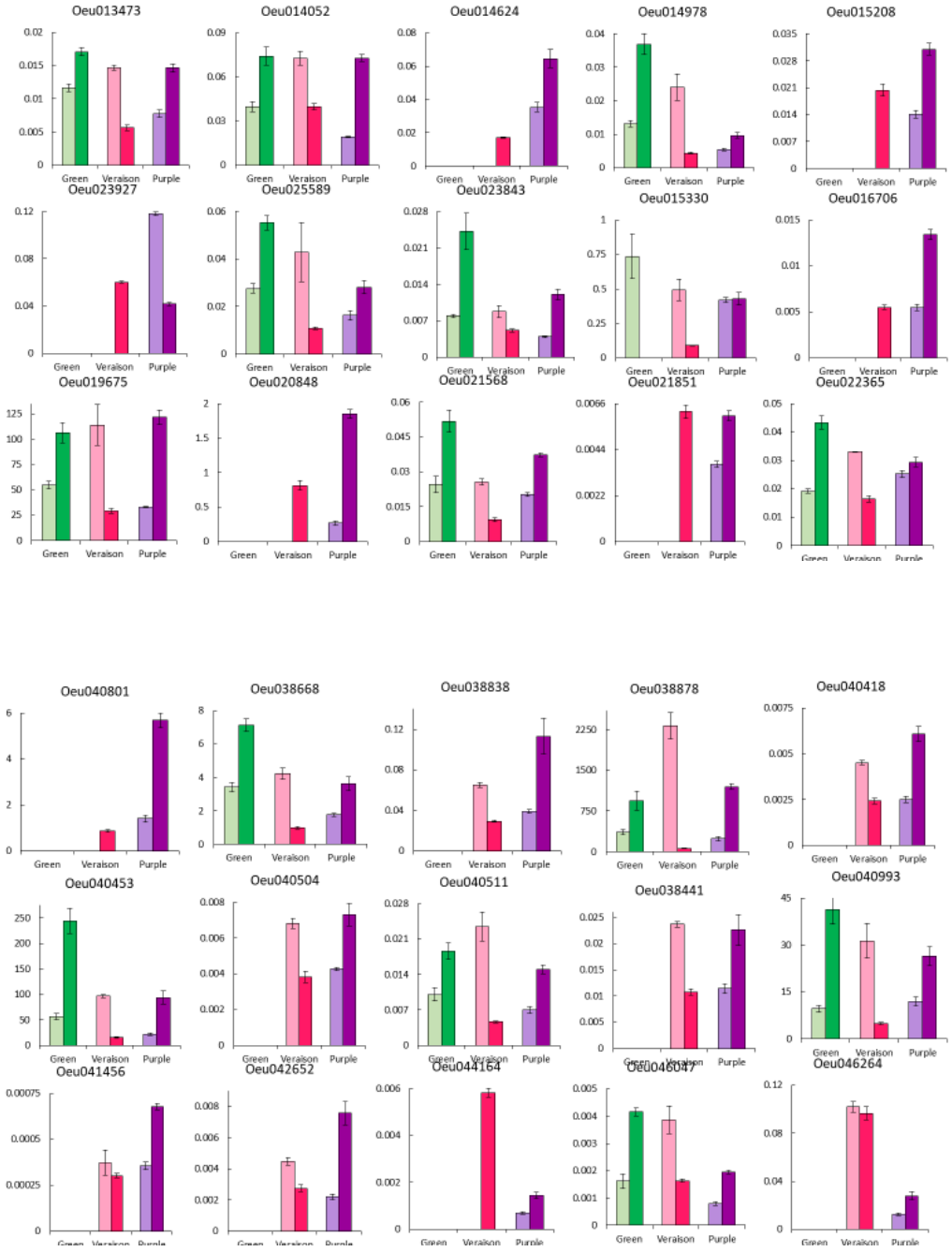
	Oeu048733.1_F	GGCCATTGTTTGTACCGCG	
Isopiperitenol dehydrogenase	Oeu048733.1_R	GCCAGCCCAATTTTCGCA	159
	Oeu048808.1_F	AGCGAGGAATGGTGGCAT	
EIN3	Oeu048808.1_R	CGAGCTTGCGAATCTTGGC	135
	Oeu048863.2_F	ACCGATTCGCATTGAGGCA	
ARR1	Oeu048863.2_R	TCGACTGGGTTTTGCTGCT	102
	Oeu049702.1_F	TGGGGTTTCTTTGAGCTGGT	
aminocyclopropanecarboxylate oxidase	Oeu049702.1_R	GATGGCGGAGGAAGAAGGT	101
	Oeu051855.1_F	ATGCTGCGTCCATTGCTG	
(NADPH Trans-2-enoyl-CoA reductase	Oeu051855.1_R	TGATCCTGCCCTGTCCCT	124
	Oeu052876.1_F	GCAAAGACTCAACGCCACG	
coniferyl-alcohol glucosyltransferase (UGT72E) (PAC:37738614)	Oeu052876.1_R	CGCCTTCGTCGTCCATCA	102
	Oeu052957.1_F	ACTGAAGGCTGTGGTGCT	
Cysteine synthase / OAS sulfhydrylase // L-3-cyanoalanine synthase / Beta-cyanoalanine synthase (PAC:37718456)	Oeu052957.1_R	TGCCATGCTGATCCCCAT	139
	Oeu053270.1_F	GACGCTGCCCTTTGTGTTG	
Caffeate O-methyltransferase (PAC:37700642)	Oeu053270.1_R	ATGGGACGCAATCTCGCC	164
	Oeu055004.1_F	TGCAGCCAAAGTAGACTCGT	
loganic acid O-methyltransferase	Oeu055004.1_R	TGATGCTTGCTCGGGATGT	158
	Oeu055203.1_F	CGTTTTCAATGCGTGGCCA	
ERF1-B	Oeu055203.1_R	CCCTGCTTTTCTTGTTCCCG	158
	Oeu055303.1_F	GGACCGTGGCAGAGCTTT	
Cytochrome P450	Oeu055303.1_R	GGAGCGGGAGTATGAAGTCG	148
	Oeu055619.1_F	GTTCGTGCGACTCTGGCT	
Cathepsin F	Oeu055619.1_R	ACCTTGGCAGCAACCTTGT	146
	Oeu056100.2_F	GCGGCCTCAAACACCTCA	
Dormancy/auxin associated protein (Auxin_repressed) (PAC:37735995)	Oeu056100.2_R	CGGAGTCAAAGGCGTCGA	159
	Oeu056216.1_F	ACTGTGCCCAATGACCCG	
METALLOPROTEASE M41 FTSH // ATP-DEPENDENT ZINC METALLOPROTEASE FTSH 1 CHLOROPLASTIC-RELATED (PAC:37717079)	Oeu056216.1_R	TGTGCCCGCCTGAACAAA	197
	Oeu056512.1_F	GAGGTCCCATCAGAACGCA	
Beta-ketoacyl-[acyl-carrier-protein] synthase III / KASIII (PAC:37702786)	Oeu056512.1_R	GCTCCAAATCCCGCAGCT	193

	Oeu056586.1_F	CCACTCATTCCACCCCGT	
Inositol-3-phosphate synthase / Myo-inositol-1-phosphate synthase (PAC:37734105)	Oeu056586.1_R	CCGCTGCTTCGAAAGTGC	154
	Oeu057948.1_F	TGGTCCATTCATCACGCCA	
bifunctional dihydroflavonol 4-reductase/flavanone 4-reductase (DFR) (PAC:37722891)	Oeu057948.1_R	GCACATACTGACCTTGCGC	164
	Oeu058679.1_F	ACGAGTGGGCACAAACCT	
coumaroylquinate(coumaroylshikimate) 3'-monooxygenase (CYP98A3, C3'H) (PAC:37729353)	Oeu058679.1_R	ATGCCTGTCCGCCAACTG	157
	Oeu059047.1_F	CGGCGGAGGAGAAGGAAA	
leucoanthocyanidin dioxygenase (E1.14.11.19) (PAC:37732629)	Oeu059047.1_R	TCACTTGTTGCTGGGCTGT	149
	Oeu059093.1_F	AGAGCAGTTGACCAAGCCA	
1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase	Oeu059093.1_R	GCCACGCACAGAAACCAC	136
	Oeu060418.1_F	GCTGGGCGTATTGTCATGG	
PEPTIDYL-PROLYL CIS-TRANS ISOMERASE CYP18-3-RELATED (PAC:37705459)	Oeu060418.1_R	AGCTGTGAAGTCTCCGCC	153
	Oeu061625.1_F	AGCTTCGCACATTTCCCGA	
Polyneuridine-aldehyde esterase / Polyneuridine aldehyde esterase (PAC:37696431)	Oeu061625.1_R	GCAAAAGCCAAGTTCAAGCC	187
	Oeu062547.1_F	AGTTTGCTGCCACTCGGA	
17.6 KDA CLASS I HEAT SHOCK PROTEIN 1-RELATED	Oeu062547.1_R	TCCTCTCCATACGGTGCCA	169
	Oeu062967.2_F	TTGCCCGAGAATGACCCG	
MYC2	Oeu062967.2_R	ACCACCCGAACACGAAGG	164
	Oeu063360.1_F	GTCCTCGTGCTGGGCTTT	
3-hydroxyacyl-CoA dehydrogenase	Oeu063360.1_R	AGATCGCTGACCCCTGGA	147
	Oeu063625.1_F	CGACGGCTTCTGATTCGA	
4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase	Oeu063625.1_R	CCATCTTCCCTTTTGCCCG	178
	Oeu064292.1_F	GCCTTTGCCTGGATTGCC	
enoyl- (fabI)	Oeu064292.1_R	GTGCAGGAACCCATGTACCA	191
	Oeu016530.1_F	AGGTTCCCAAGACTCCCCA	
SPERMIDINE HYDROXYCINNAMOYL TRANSFERASE	Oeu016530.1_R	ACTCCGTCGTTAGGTCCCA	185
	Oeu024513.3_F	TCGAGTCATGCCTCCTCCT	
PECTATE LYASE 1-RELATED	Oeu024513.3_R	ACTGAAGTTTCCGCTGCCA	113

	Oeu022365.1_F	TGCCATCCAAACTCCGAGT	
GE10H	Oeu022365.1_R	GCTGCTTTTCTTCACGGGC	124
	Oeu042652.1_F	ACTGTCATGTCTGCTGGCC	
BETA-GALACTOSIDASE RELATED // BETA-GALACTOSIDASE 1	Oeu042652.1_R	GCACGCCAACATTCGCAA	115

Supplementary Figure 6.1 Transcript profiles of the total candidate genes employed.

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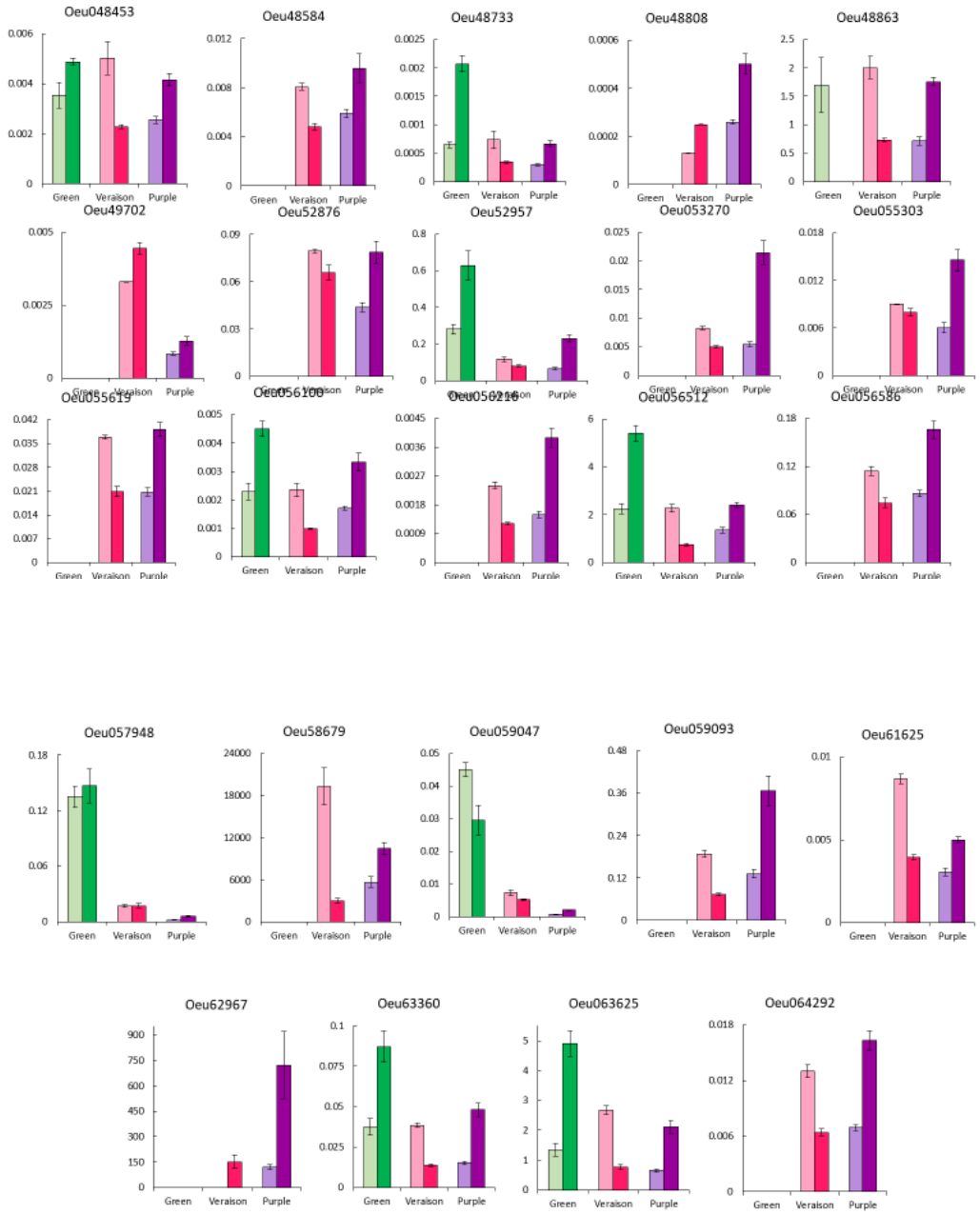
Green
Veraison
Purple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Oeu057948
Oeu58679
Oeu059047
Oeu059093
Oeu61625
Oeu62967
Oeu63360
Oeu063625
Oeu064292
Green
Veraison
Purple

Chapter VII. Metabolome profiling in olive fruits ripening and changes associated to environmental warming

Olive oil is an important constituent of the Mediterranean diet with proven health benefits due to its chemical composition, which is rich in unsaturated fatty acids and phenolic compounds that contribute to its organoleptic and nutraceutical properties. According to previous studies, climate change affected the olive tree phenology, increasing growth, anticipating flowering and ripening processes, accompanied by a reduction in yield and fruit quality. The present experiment aimed studying the effect of warming conditions on the metabolite profile of olive fruits. Changes in the metabolite profile at 4 °C above ambient temperature was performed under field experiment with adult trees (cv. `Picual´) by using a UPLC-MS/MS approach as well as colorimetric analysis. For the warming treatment, a temperature-controlled Open-Top-Chamber was employed. Twenty fruits per tree (3 trees per treatment) were sampled at 3 ripening stages; green skin, veraison and purple skin. Metabolites were extracted from olive pulp using aqueous and organic solvents, being the extracts subjected to UPLC-qTOF analysis. In parallel, colorimetric determination was carried out to quantify pigments together flavonoids, reducing sugar, phenolics and free amino-acids total contents. Metabolite identification was carried out by MS/MS spectra with different public plant/food specifics databases using Progenesis QI software. A total of 1162 and 9877 compounds were found in negative and positive mode, respectively. Total prefiltered and grouped metabolites (6977) were detected. A total of 159 metabolites were confidently identified (Score>40 and fragmentation spectra). Qualitative and quantitative differences have been found between treatments (AT and AT+4 °C). Changes on pigments contents were observed along ripening process, chlorophylls and carotenes (being more abundant in warming conditions) decrease with ripening, contrary to anthocyanin accumulation starting from veraison. On veraison, higher anthocyanin quantity was observed in AT+4 °C although in purple stage, anthocyanins were more abundant in AT treatment. Reducing sugar, phenolics and free amino-acid follows the same pattern in both treatment during ripening but the amount present differences. In addition, 110 unique and differential identified metabolites corresponded mainly to the phenylpropanoids and terpenes classes followed by alkaloids, carbohydrates and derivatives chemical families. Terpenes (mainly iridoids) are more abundant in AT than AT+4 °C, trend that share with organic acid, alkaloids and flavonoids. Phenylpropanoids and derivates follows the contrary pattern together chromenes subclass, this could explain the total phenylpropanoids contents. Lipids are not well represented due to the extraction and mobile phase employed during LC-MS/MS. The obtained results suggest firstly, veraison is the most susceptible stage to warming conditions and secondly, warming conditions alter the major secondary metabolites classes responsible of the organoleptic and nutritional properties of the olive oil.

1. INTRODUCTION

The interest for natural products is growing because of the high cost and potentially side effects of commonly available synthetic drugs, therefore, there has been an increase in the consumption of foods with nutraceutical properties. *Olea europaea* extracts have been employed widely in folk medicine in European Mediterranean area, Arabia peninsula, India and other tropical and subtropical regions, as diuretic, hypotensive, emollient and for urinary and bladder infections (Samova *et al.*, 2003; Visioli *et al.*, 1998). Concretely, the olive oil obtained by mechanical extraction from the olive tree drupes (denominated as Virgin olive oil, VOO) is worldwide appreciated for its nutraceutical properties, especially against cardiovascular disorders (Pérez-Jiménez, 2007; Colomer and Menendez, 2006). These healthy benefits are due to the presence of high levels of monounsaturated fatty acids and other valuable minor components such as phenolics, phytosterols, tocopherols, carotenoids, chlorophyll and squalene (Colomer and Menendez, 2006; Ghanbari *et al.*, 2012; Ribarova *et al.*, 2003) biosynthetised during ripening processes.

The production of olive oil is considered to be one of the largest agricultural business sectors in the Mediterranean area. For this reason, olive tree constituted a strategic crop for Spain due to the cultivated area, 2584564ha approximately (MAPA, 2019). However, the Mediterranean Basin has been considered as one of the most vulnerable regions to climatic changes in the near future with an increase in air temperature in the range of 2-5 °C, (Giannakopoulos *et al.*, 2009; Giorgi, 2006; Lionello, 2012) coupled with more frequent and extreme events such as drought periods and heat-waves episodes (Giorgi and Lionello, 2008; Tanasijevic *et al.*, 2014). Temperature is one of the main environmental determinants of plant physiology and phenology. Consequently, high temperature adversely affects quality and yield of this fruit. Recent studies about olive tree phenology reveal an altered pattern of flowering and ripening processes under warmer conditions (Benlloch-González *et al.*, 2018; 2019). This can be assumed to be an alteration in the underlying biomolecular machinery that produces an alteration of the chemical compounds involved in olive oil quality parameters.

The chemical composition of harvested olives fruits varies depending upon different factors including agronomical factors (e.g., olive cultivar) (Romani *et al.*, 1999), the ripening stage of the fruit (Amiot *et al.*, 1986), agroclimatic conditions (Vigna *et al.*, 2005) and/or irrigation management (Martinelli *et al.*, 2012; Sánchez-Rodríguez *et al.*, 2019). One of the most appreciated cultivar for mill oil is `Picual´ cultivar that produce a high quality of oil due to its spicy and bitter taste, stands out for its high stability index and for its high oleic acid and polyphenols content compared with other cultivars such as `Arbequina´(Figure 7.1) (Rallo *et al.*, 2005; García *et al.*, 2003).

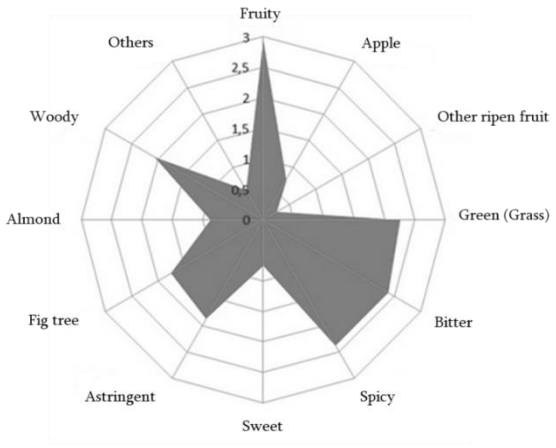

Figure 7.1 Diagram of `Picual´ sensory profile adapted to Uceda *et al.*, 2010.

Generally, the ripening processes in fleshy fruits represents the final development stage in which the biochemistry, physiology and structure are altered to determine appearance, texture, flavour and aroma. These changes include: modification of skin colour (content and composition of chlorophylls, carotenoids and/or flavonoids); textural modification via alteration of cell turgor and cell wall structure; modification of reducing sugars, organic acids and volatiles (Giovanni *et al.*, 2004).

The oil content can reach up to 28–30 % of the total pulp fresh weight, with the accumulation peak after the onset of ripening. Olive oil is particularly enriched in the monounsaturated fatty acid oleate (C18:1) (75–80 % of total fatty acids), followed by linoleate (C18:2), palmitate (C16:0), stearate (C18:0) and linolenate (C18:3) (Conde *et al.*, 2008). However, the final acyl composition enormously varies throughout olive fruit development, according to genotype and environmental conditions (Connor and Fereres, 2005).

Olive drupe mesocarp can accumulate other important secondary metabolites (SMs), including polyphenols, carotenoids, chlorophylls, sterols, terpenoids and a wide range of volatile compounds, all directly or indirectly affecting the olive oil quality and aroma (Conde *et al.*, 2008). Secondary metabolites play a crucial role in the plant stress response, so this presence/ absence or changes in the quantity on the tissue could be considered as biomarker of these stresses. Several groups of these compounds have been implicated in activation and reinforcement of defense mechanisms in plants (Harbourne, 1999; Bourgaud *et al.*, 2001). Generally, raising temperature might almost enhance all of SMs in plant species. For instance, the positive correlations between temperature and the composition of phenolic compounds have been demonstrated (Zheng *et al.*, 2012). Therefore, alkaloids accumulation after heat shock was reported (Jansen *et al.*, 2009; Guo *et al.*, 2007). On the contrary, high temperature (<35 °C) reduced the total anthocyanin content (Mori *et al.*, 2007; Dela *et al.*, 2003; Benlloch-González *et al.*, 2019).

Olive fruits phenolics compounds include phenolic acids, phenolic alcohols (e.g., hydroxytyrosol (3,4-DHPEA) and tyrosol (p-HPEA), flavonoids and lignans (Oliveras *et al.*, 2008;

Owen *et al.*, 2003)). High amounts of verbascoside (Servili *et al.*, 2004) and other phenolics present on ripen olives, such as homovanillic alcohol, 3,4-dihydroxyphenylacetic acid (DHPAC), caffeic acid, p-coumaric acid, phloretic acid, vanillic acid (Boskou *et al.*, 2006) and low amounts of comselogoside (Jerman *et al.*, 2010) were previously described. Other compounds include triterpenic acids, maslinic and oleanolic acids (Goulas and Manganaris, 2012), and tocopherols (Dabou *et al.*, 2009). Also, the terpenoids group represented a highlighted category, especially the secoiridoids, as oleuropein, demethyloleuropein, oleuroside, ligstroside, nüzhenide and their aglycon forms (Obied *et al.*, 2008). Sterols, such as β-sitosterol, cycloartenol and 24-methylenecycloartanol, progressively accumulate when the olive fruit reaches its final size and veraison begins (Stiti *et al.*, 2007). Squalene, a precursor of α- and β-amyrins and the triterpenic diols erythrodiol and uvaol, is another important compound with recognised effects on human health that is present in consistent amount only in olive and other vegetable oils (Waterman and Lockwood, 2007).

Secondary metabolites are the major molecules group in plants, whose different nature and specificity, even at the level of plant species, supposes a challenge when it comes to studying and identifying them. In this sense, modern-day mass spectrometers (MS) interfaced with liquid chromatography (LC) have allowed the unprecedented detection of several hundreds of peaks from a single biological sample (Patti *et al.,* 2013; Humston *et al.,* 2011). In addition, while chromatography provides highly efficient separations, MS is an invaluable tool serving both as an additional dimension for separation and/or for structural elucidation of analytes. Liquid chromatography coupled to tandem mass spectrometry (LC–MSn) (Patti *et al.,* 2013) is a bioanalytical method for quantitative analysis of metabolites which has several applications on biomedicine, plant stress response, bioactive compounds research, among others. Particularly, ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) was employed for the separation, detection, and identification of the secondary metabolites to identify potential biomarkers.

The aim of this work was to evaluate under field conditions the changes on the olive fruits metabolites profile during ripening processes due to warming conditions. In order to characterize the effects of predicted increase in air temperature on global warming scenario, 3 critical ripening stages (green (0), veraison (3) and purple (4)) were selected (Barranco *et al.*, 2008; Benlloch *et al.*, 2019). Adult olive trees `Picual´ cultivar, were subjected to warming conditions, increasing 4 °C above the ambient temperature than ambient using temperature-controlled OTCs systems (Benlloch-González *et al.*, 2018; 2019). Metabolomic data were integrated with some phenotypic ones such as skin colour, fatty yield, total contents of pigments (chlorophylls, carotenoids and anthocyanins), flavonoids, phenolic compounds, reducing sugars and amino acids. This study, about the impact of warmer temperatures on ripening processes gives an important overview of

the impact of global warming on olive oil quality and its nutraceutical properties, proposing putative biomarkers of warming conditions tolerance between ripening stages.

2. MATERIAL AND METHODS

2.1. Plant material and experimental design

Adult 'Picual' olive trees (*Olea europaea* L.) growing in the experimental farm of 'Campus de Rabanales', University of Córdoba, Spain (37°55'N 4°43'W) were used to perform the experiment such as described by Benlloch-González *et al.* (2018). Trees were subjected to two temperature treatments, ambient temperature (AT) and 4 °C above ambient temperature (AT+4 °C) though the OTC structures such as is described on Chapter III.

To determine the fruit maturation period, the experimental trees were visited every 3-4 days from late August to December, recording the fruit phenological stages of each tree according to the following visual scale: (1) deep green skin; (2) yellowish-green skin; (3) veraison, green skin with reddish patches over more than half of the fruit; (4) purple skin; (5) black skin and white flesh. The maturation period was determined according to Barranco *et al.* (1998) (Chapter I. Phenological data recompilation). During ripening, 20 fruits per each ripening stage are collected at random from each tree (taken as biological replicate). The 20 fruit were pooled and divided into pericarp (skin and flesh) and endocarp (pit) when the samples were homogenized in liquid nitrogen and then stored at -80 °C until biomolecular analysis. For such purpose three critical stages were selected to the biomolecular approaches: the initial phase, green (0); the phase that present more alterations under AT+4 °C treatment respect AT, veraison (3) and the previous phase to harvesting, purple (4) (Figure 7.2).

At harvest, fruits of each tree were collected to determine total weight. Fifty to eighty fruits per tree, depending on the year, were used to determine fruit size (average weight of the fruits sampled), pulp/stone ratio, expressed as fresh weight, and the maturity index (MI). The MI was determined according to Ferreira (1979).

Figure 7.2. Ripening stages selected and visualized during the field experiment on olive trees under warming conditions. **A)** Green skin (0); **B)** Advanced Veraison (3) at AT conditions; **C)** Advanced veraison (3) at AT+4 °C conditions; **D)** Purple skin (4).

2.2. Metabolites extraction

Metabolites were extracted from plant tissue as described by Valledor *et al.* (2014), with minor modifications, in order to extract the largest number of metabolites from olive fruits pulp. Buffer containing 600 µL of cold methanol: chloroform: water (5:2:2) was added to 50 mg of frozen tissue and grinded in a mortar, vortexed (10 s), and sonicated (ultrasonic bath, 40 kHZ, 10 min). After centrifugation (4 °C, 4 min, 20000 × g) the supernatant was transferred to new tubes. Then, 200 µL of cold methanol: chloroform: water (5:2:2) was added to the pellets and the process was repeated once. After combining the both supernatant, they were vacuum dried at 30 °C (SpeedVac, Eppendorf Vacuum Concentrator Plus/5301) and reconstituted in 1000 mL of pure methanol. After performing the samples extraction, the extracts were centrifuged at 10000 x g for 10 min and were filtered using 0.22 µm PTPE membrane filter (Thermo Scientific, MA, USA) and collected in 1.5 mL LC/MS certified sample vials for their posterior analysis by UPLC-QTof-MS. In parallel an equivalent extraction was employed to classical biochemistry determination of polyphenols, reducing sugars, free amino-acids and flavonoids total contents. For these determinations, metabolites were reconstituted in 1000mL of 70 % methanol.

2.2.1. Determination of total phenolic compound, reducing sugars and free amino acids content

For the determination of the content of different families of compounds, such as phenolic compounds, reducing sugars, free amino acids and flavonoids, different colorimetric methods were used as described below.

Phenolics total contents was determined by the Folin-Ciocalteu method as described by Ainsworth *et al.* (2007). A standard curve using gallic acid dissolved on 70 % methanol was performed. The equation obtained was A765 nm = 1.2134x (R2 = 0.9946); where x represents mM gallic acid equivalents and R2, the regression coefficient.

Reducing sugar contents were quantified with 3,5-dinitrosalicic acid (DNS) protocol as described by Miller (1959). A D-glucose (in 70 % methanol solution) standard curve was employed. The equation obtained was A570 nm = 0.0236x (R2 = 0.9939); where x represents mM D-glucose equivalents and R2, the regression coefficient.

Free amino-acid contents were calculated by Ninhydrin method as described by Smith and Agiza (1951). A standard curve with L-glycine methanolic solution as reference was performed. The equation obtained was A575nm=3.5191x (R2=0.9866); where x represents mM L-glycine equivalents and R2, the regression coefficient.

Flavonoids contents were determined using aluminium chloride method as described by Lamaison and Carnat (1990). Rutin was utilized as reference on the standard curve. The equation

obtained was A430nm=0.0124x (R2= 0.9969); where x represents mM rutin equivalents and R2, the regression coefficient.

2.2.2. UPLC-ESI-qTof-MS analyses and metabolites identification

The chromatography analysis was performed in an UPLC Acquity H-Class (Waters, Milford, USA) using a C18 column 2.1×100mm, 1.7µm (Waters, Milford, USA). The flow rate was 0.450mL·min−1 and the injection volume was 5µL. The column temperature was set at 45 °C. Mobile phases consisted of 0.1 % formic acid in Milli-Q water (A) and methanol (B). The compounds separation was carried out under the following conditions (time, % B): 0 min, 2 % B; 0.25 min, 2 % B; 12.25 min, 99 % B; 13.0 min, 99 % B; 13.01 min; 2 % B; 17.00 min; 2 % B and then the column was equilibrated for 5 min prior to each analysis.

The UPLC system was couple to quadrupole time of flight (QTof) G2-XS mass spectrometer (Waters, Milford, USA). The MS acquisition was performed in negative and positive ionization modes in a scan range from m/z 100 to 1200 and time acquisition of 0 to 17 min. The analysis type performed was accurate mass screening on MSE data with a low collision energy of 4.00 eV and a high energy ramp of 10.00 to 45.00 eV. The capillary and cone voltage were set at 2.50 kV and 40 V, respectively. The desolvation gas was set to 600 L h−1, the cone gas set to 50 L h−1 and the source and desolvation temperature was set to 100 °C and 250 °C, respectively in negative mode and to 120 °C and 550 °C in positive mode.

For automated accurate mass measurement, a solution of leucine-enkephalin (200 ng mL−1) in methanol: water (50:50) with 0.1 % formic acid was used as lock mass and pumped at a flow rate of 5 µL min−1. The molecule of leucine-enkephalin (m/z 556.2766 in ESI+ and m/z 554.262 in ESI−) was used for recalibrating the mass axis and ensuring a robust accurate mass measurement at any time. In addition, for continuous quality assurance and to provide confidence in our data, quality control (QC) samples were prepared by mixing all the sample extracts and were injected between every five samples in the batch along with methanol as a blank run to correct a drift of the raw signal intensity during the analysis. In the set, a phenolic standard mixture was injected to validate results.

All data acquisitions were operated by Waters UNIFI software and spectra data treatment and metabolite identification was performed by Progenesis QI software v.2.1. For the alignment of corresponding features in multiple samples chromatograms was employed a QC sample chromatogram. The peak picking was applied with default parameters sensitivity, absolute ion intensity and retention time limits. Identification was achieved using a combination of MS/MS fragmentation spectra, accurate mass (under 10ppm), isotope analysis, and database matches (PlantCyc, AraCyc, Carotenoids Database, FooDB, Plant Metabolic Network, KEGG, MassBank

and FDA) and finally manually putative annotation by METLIN database. All detected compounds were manually checked for false positive and negative assignments. Ambiguous compounds were manually assigned in accordance with previous reported plant compound. Moreover, the total intensity peak area values were subjected to statistical analysis. Finally, the differential metabolites were manually classified following KEGG annotation in to different classes and subclasses.

2.3. Pigments quantifications

Chlorophyll a, chlorophyll b, carotenoids and anthocyanins contents were determinated by spectrophotometry as described by Sims and Gamon (2002). Chlorophyll a, chlorophyll b and carotenoids were extracted using 100 % Acetone: 1M Tris pH 8: Water (80:5:15) (v:v:v); while anthocyanins were extracted using Methanol / 1 % HCl/ Water (98:1:1) (v:v:v). The concentration (μmol mL-1) was calculated as follow:

$$Chla = 0.01373\ A663nm - 0.000897\ A537nm - 0.003046\ A647nm$$

$$Chlb = 0.02405\ A647nm - 0.004305\ A537nm - 0.002228\ A663nm$$

$$Anthocyanin^{*} = 0.08173\ A537nm - 0.00697\ A647nm - 0.002228\ A663n$$

*This equation was not employed for analyzing anthocyanins contents but it is necessary to carotenoids calculation.

$$Carotenoids = \frac{A_{470}nm - 17.1(Chl_a + Chl_b) - 9.479\ Anthocyanin^{*}}{119.26}$$

$$Anthocyanins = A529nm - 0.288\ A650nm$$

The selected molecular weight for chlorophyll a, chlorophyll b, carotenoids and anthocyanins contents values were 893.51, 907.47, 537 and 449.2 g/mol, respectively.

2.4. Statistical analysis

The total detected metabolites were pre-filtered to assure the confident detection (compounds present on 2/3 replicates but not in blank, and present on Quality Control or Standard phenolics mixture). The resultant compounds were processed for grouping by m/z and RT through pRocessomic v.1 (a filtered and processing tool for biologic data on R programming language running under the open-source computer software RStudio v1.2.1335:) available on web direction: http://github.com/Valledor/pRocessomics. The relative quantification of the features was performed after filtering data employing the total intensity peak area values. Intensity values was normalized with the total sum of the peak area values per each sample transformed by a log2 transformation. The statistical analyses, PCA, sPLS-DA, hierarchical clustering, ANOVA t-student

test and Tukey test, were performed trough R code underlying MetaboAnalyst v.4.0 web server (https://www.metaboanalyst.ca). In addition, an enrichment analysis was employed with differential metabolites classification with the same web server using the total normalized peak area values of all differential identified metabolites per each category.

All the data of colorimetric determination was subjected to Analyses of variance performed using Statistix 9.0 software package (Analytical Software, Tallahassee, FL, USA). In all analyses, residual plots were generated to identify outliers and to confirm that variance was common and normally distributed. All values were transformed using log2 transformation. T-student test and Tukey test was used to means comparison.

3. RESULTS

In this study aims to elucidate changes on the metabolite profile due to the warming conditions on the olive fruit ripening trough colorimetric determination and UPLC-MS/MS. The colorimetric determinations are used as a relatively fast and inexpensive method (compared to -omics techniques) for screening samples. And then, UPLC-MS/MS is employed to obtain massive data. Generally, in this experiment, the effects of ripening processes are sharpest than warming conditions effects. For this reason, a t-student two tails test was performed comparing AT with AT+4 °C per each ripening stage. At exception of reducing sugar pattern, with a remarkable peak at veraison AT+4 °C condition but without significant differences between treatment (Figure 7.3 B), the ripening progression had the same effect on free amino-acid, phenolics and flavonoids total contents in both temperature treatments (Figure 7.3).

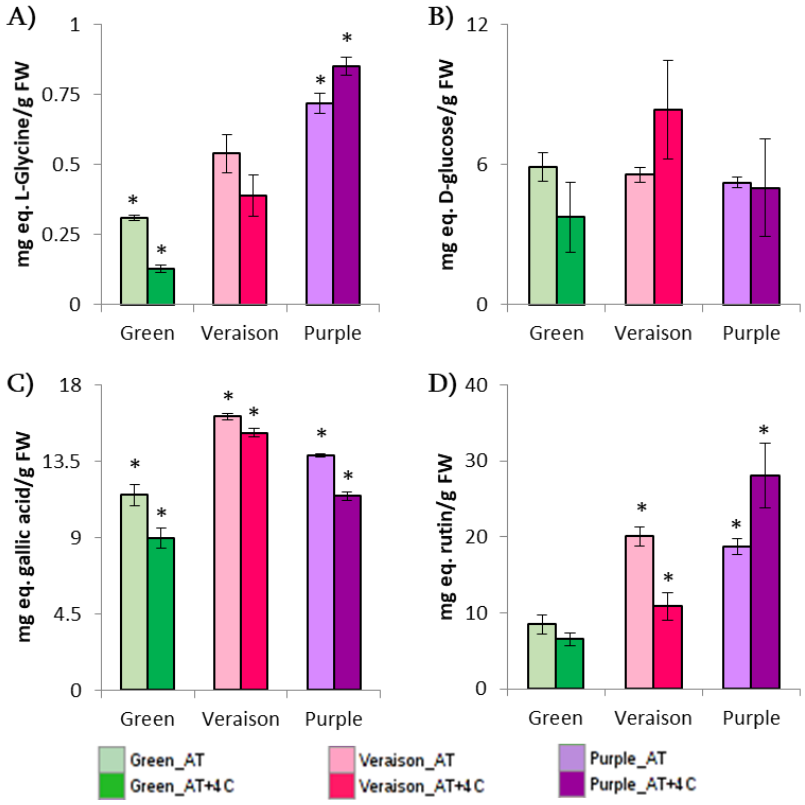

Figure 7.3. Colorimetric determinations on olive fruit pulp among ripening stages. A) Free amino-acids total contents resulting to Ninhydrin method. B) Total phenolic compounds contents calculated with Folin-Ciocalteu. C) Total reducing sugar contents obtained by DNS method. D) Total flavonoid contents determined by aluminum chloride method. * Represent significant differences $p<0.05$ between temperature conditions. Light colours indicate AT treatment and dark colours AT+4 °C.

A gradual accumulation of free amino acids was observed along ripening, to a lesser extent for the green and veraison stages for AT+4 °C and to a greater extent in purple AT+4 °C in comparison with AT. The values range 0.13±0.01 to 0.85±0.03 mg equivalents of L-Glycine/g of FW in warming conditions and 0.31±0.01 to 0.72±0.04 mg equivalents of L-Glycine/g of FW in ambient conditions (Figure 7.3 A).

Reducing sugar had not presents significant differences between ripening stages or temperature conditions. However, a scarce decrease was observed in AT (5.89±0.6 to 5.23±0.24 mg equivalents of D-glucose/g of FW). Warming conditions presents an elevated heterogenicity but a light increase peak on veraison stage was observed (8.34±2.12 mg equivalents of D-glucose/g of FW) (Figure 7.3 B).

The phenolic total contents present a maximum on veraison stage in both AT and AT+4 °C conditions (16.13±0.18 and 15.17±0.26 mg equivalents of gallic acid/g of FW, respectively). In warming conditions low total phenolic quantities was observed for all ripening stages (Figure 7.3 C).

Flavonoids total contents increase during the ripening processes. The values range 8.51±1.23 to 20.1126 mg equivalents of gallic acid/g of FW 1.26 mg equivalents of gallic acid/g of FW (green and veraison, respectively) in control (AT) treatment. While values range 6.56±0.81 to 28.10±4.30 mg equivalents of gallic acid/g of FW (green and purple, respectively) in AT+4 °C treatment. There were significant differences between temperature treatments on veraison and purple stages, with lower flavonoids levels in AT+4 °C on veraison than AT, and higher levels in AT+4 °C on purple respect AT (Figura 7.3 D).

Changes on pigments contents were observed along ripening process (Figure 7.4). Chlorophylls a and b (Figures 7.4 A and 7.4 B, respectively) decrease strongly along ripening with maximum values on green stage in control conditions (223.68±5.6 mg equivalents of chlorophyll a/kg of FW and 57.07±2.1 mg equivalents of chlorophyll b/kg of FW) and in warming conditions (239.1±4.48 mg equivalents of chlorophyll a/kg of FW and 59.71±1.23 mg equivalents of chlorophyll b/kg of FW). Major significant differences were observed in veraison and purple stages. Also, chlorophylls reaching almost null values in purple AT+4 °C but not in AT, especially chlorophyll a (55.2±0.8 vs 0.91±0.013 mg equivalents of chlorophyll a/kg of FW) (Figure 7.4 A).

Carotenes total contents presented a similar pattern that chlorophylls decreasing during ripening process but significant differences only appeared on green stage between temperature treatments (being more abundant in warming conditions, 18.07±1.62 vs 12.29±0.92 mg equivalent of β-carotenes/kg FW) (Figure 7.4 C). On veraison and purple stages there were still amounts of carotenes, around 4 mg equivalent of β-carotenes/kg FW in both temperature conditions.

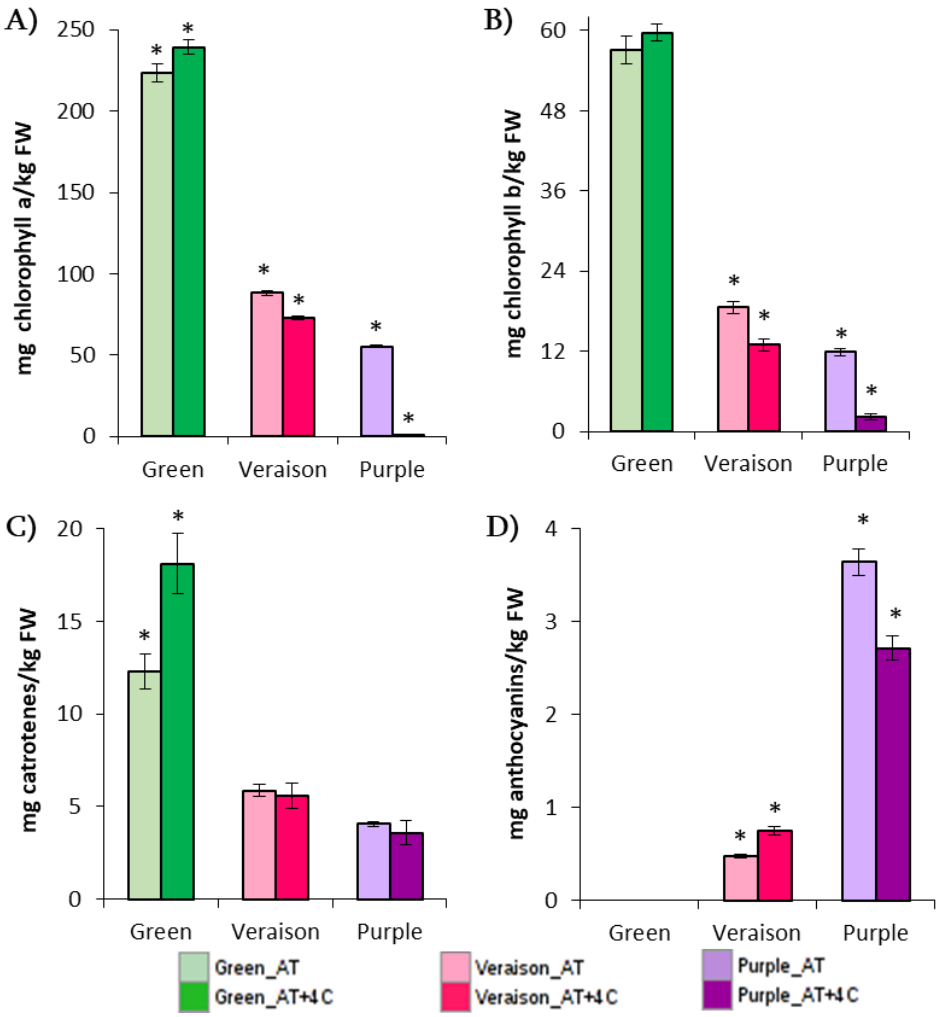

Figure 7.4. Pigments quantification on olive fruit pulp among ripening stages of A) Chlorophyll a content; B) Chlorophyll b content; C) Carotenoids total content; and D) Total anthocyanin contents * Represent significant differences $p<0.05$ between temperature conditions. Light colours indicate AT treatment and dark colours AT+4 °C.

Anthocyanins accumulation started from veraison in both treatments. On veraison, higher anthocyanin quantity was observed in AT+4 °C (0.79±0.04 mg equivalent of cyanidin-3-glucoside/kg FW) respect AT (0.48±0.02 mg equivalent of cyanidin-3-glucoside/kg FW); although in purple stage, anthocyanins were more abundant in AT treatment that AT+4 °C treatment (3.64±0.14 vs 2.71±0.13 mg equivalent of cyanidin-3-glucoside/kg FW).

Lipids peroxidation, measured as membrane damage parameter, revealed significant differences due to the temperature conditions and ripening process. In general, an increase along ripening was observed. This increase was far more accused in control treatment (0.29±0.05 to 4.86±0.61mM MDA) comparing to warming treatment (0.05±0.01 to 0.82±0.12mM MDA).

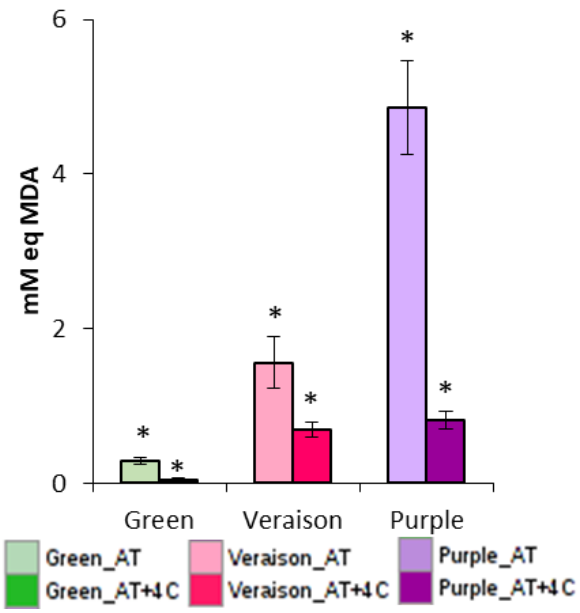

Figure 7.5. Lipids peroxidation of olive fruit pulp among ripening stages * Represent significant differences $p<0.05$ between temperature conditions. Light colours represent AT treatment and dark colours AT+4 °C.

The non-targeted metabolomic analysis displayed a total of 6977 found metabolites thought a comparison with the public plant/food specific libraries and mass spectra interpretation.

All of them were used in the PCA to determine the percentage of variation represented. More than 5000 compounds are involved in the separation of the all PC. PC1 explained 39.8 % of the variability separating the temperature treatment clearly. PC2 explained 17.6 % of variability grouping the samples according to its ripening stage. All of replicates were well grouped as demonstrated by the correspondent hierarchical clustering (Figure 7.7).

These compounds were grouped, filtered and identified with fragmentation spectra resulting of 179 tentative identified metabolites. A complete list of the confidently identified compounds with their respective RT and the mass-to-charge ratios (m/z) has been included in Supplementary Tables 7.S1.

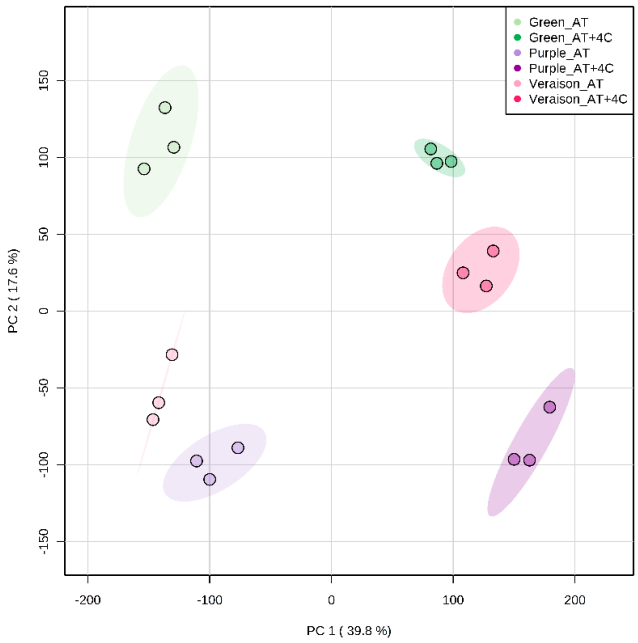

Figure 7.6. Principal Component Analysis (PCA) representation with the total detected metabolites through the ProgenesisIQ software employing the Metaboanalyst server.

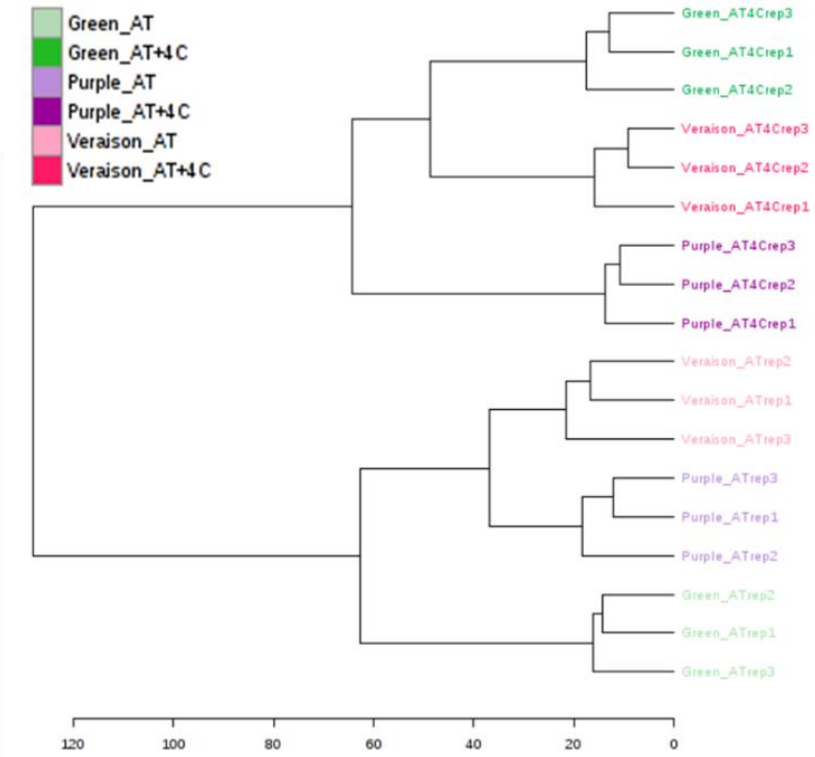

Figure 7.7. Hierarchical clustering analysis using Pearson correlation and average linkage model employing the Metaboanalyst server.

The above mentioned 179 tentative identified metabolites were analyzed statistically obtaining 79 differential metabolites (FDR<0.01) for green stage, 78 differential metabolites for veraison and 80 for purple stage. A total of 110 common differential metabolites were found (Figure 7.8. and Supplementary Table 7.1).

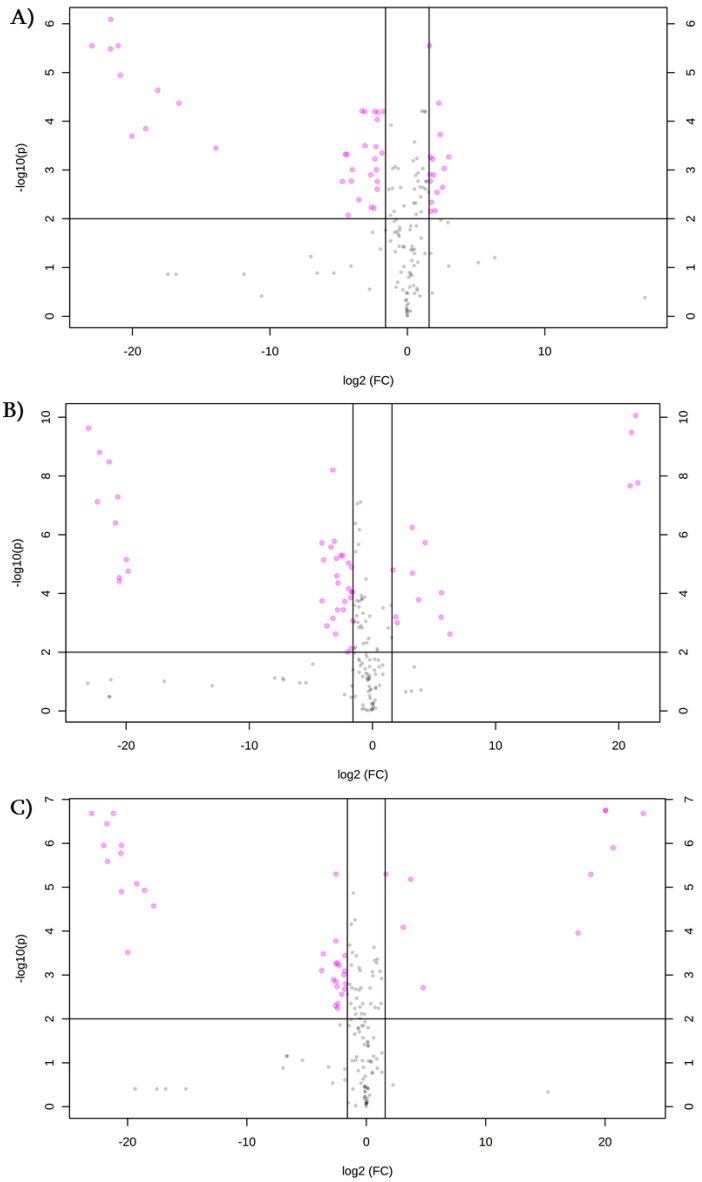

Figure 7.8. Volcano plots resulting of the t-student test using Fold Change ≥|3| and FDR<0.01 for A) green AT+4 °C vs green AT; B) veraison AT+4 °C vs veraison AT; and C) purple AT+4 °C vs purple AT. Reddish colour represents the metabolites with significant differences.

The common differential metabolites were classified following KEGG categorization, so metabolites not defined in this database and with unclear chemical category, appear into the "Others" classification together metabolites with just one representation/category (Table 7.1). The major represented classes were Phenylpropanoids and Derivates/Conjugated and/or Precursors (39) followed by Terpenes/Terpenoids and Derivates (29). The principal subclasses were Phenylpropanoid ester derivated, Flavonoids, Chromenes and Monoterpenes Conjugated (Iridoids). Alkaloids (10), Carbohydrates and Derivates (10) were also well represented. In minor order metabolites related to Organic acids (5), Amino-acid (4) and Poliketides class were also identified as well as Methanopterins (3) and Fatty acid related (2) (Supplementary Table 7.1).**

This classification allowed the representation of enrichment analysis to elucidate the major changes caused by warming conditions and to detect probable biomarker (Figure 7.9).

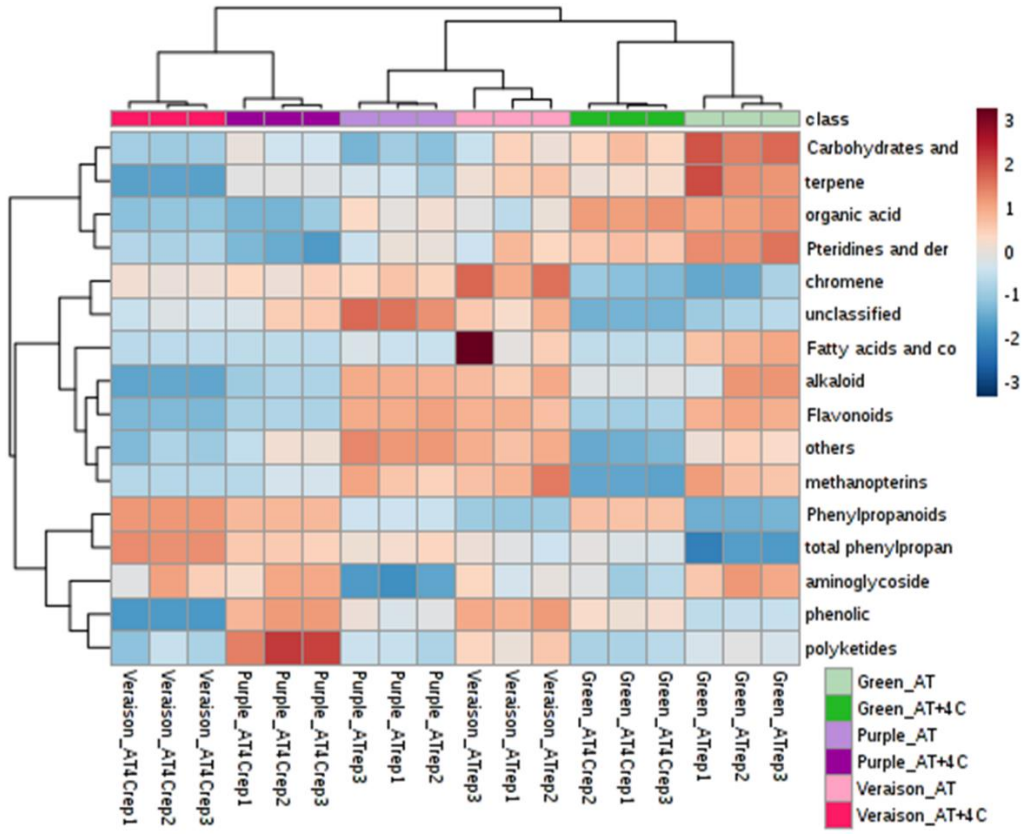

Figure 7.9. Heat map of the differential metabolite categorization. In this figure, the total intensity of all metabolites was employed to calculate the relative representation of each category depending on ripening stage and temperature treatment. Fold Change was used as colour scale (red-blue).

Terpenes (mainly iridoids) are more abundant in AT than AT+4 °C except at purple stage, trend that share with organic acid, methanopterins, alkaloids and flavonoids related compounds. Phenylpropanoids and derivates follows the contrary pattern together chromenes subclass, this could explain the total phenylpropanoids contents. Lipids are not well represented due to the extraction and mobile phase employed during LC-MS/MS.

4. Discussion

As one of the major weather variables, temperatures can significantly influence the composition of secondary metabolites (SMs) present in plants (Yang *et al.*, 2018). For this reason, the knowledge about warming conditions effects on ripening processes acquires high importance in the climate change scenario coupled to requirements to feed a growing population with value-added products.

Ripening processes are visually followed by colour variations on fruit (skin, pulp or both of them. The initial phases are characterized by a light photosynthesis on unripen fruits that disappear as ripening progresses, when other pigmentations as carotenes, licopenes and anthocyanins are accumulated (Mlodzinska, 2009). This natural pattern was observed in this study

(Figure 7.4, light colours) but in warming conditions there are changes on pigments contents, generally (Figure 7.4, dark colours). On the one hand the chlorophylls loss was sharpest on AT+4 ºC conditions, founding more similarities between veraison AT+4 ºC and purple AT than between purple AT+4 ºC and AT treatments. Studies reported an increase chlorophylls contents at moderately elevated temperature but decreased markedly at high temperature compared with the control condition (Song *et al.*, 2016). In the other hand, only in green stage carotenoids amount presents significant differences between treatments (Figure 7.4 C) with higher amounts in warming conditions. In plants, carotenoids function as light harvesting antennae pigments, as important free radical scavengers, and have photoprotective roles (Demmig-Adam *et al.*, 1996; Miki, 1991; Tracewell *et al.*, 2001), so is expected that stress increase the levels of carotenoids. In the olive fruits, although the content of carotenoids is low, their presence is important because together with the chlorophylls, supply colour to virgin olive oil and `Picual´ cultivar has been reported by their high pigment content (Roca and Mínguez-Mosquera, 2001). Several results match reports of unusual carotenogenesis during green-yellowish turning (Roca and Mínguez-Mosquera, 2002). Therefore, anthocyanins accumulations at advanced ripening stages (veraison and purple) differed depending on the temperature conditions. In veraison, higher anthocyanin quantity was observed in AT+4 ºC although in purple stage, anthocyanins were more abundant in AT treatment (Figure 7.4 D). Anthocyanin content in leaf sheaths of Zea mays seedlings increased with intensity and exposure time to cold, due to the induction of anthocyanin biosynthetic pathway genes (Christie *et al.*, 1994). Similarly, low temperature induced anthocyanin accumulation in leaves and stems of Arabidopsis thaliana, and facilitated anthocyanin synthesis through the phenylpropanoid pathway associated with increased transcripts of flavonoid biosynthetic genes including phenyl alanine ammonia lyase and chalcone synthase (Leyva *et al.*, 1995). On the contrary, high temperature (35 ºC) reduced the total anthocyanin content of *Vitis vinifera* berries respect a control temperature (25 ºC), as a result of anthocyanin degradation and the inhibition of anthocyanin biosynthetic genes transcription (Mori *et al.*, 2007). Moreover, several studies in different plant species, Petunia hybrid, Citrus sinensis, or Rosa sp., (Shvarts *et al.*, 1997; Lo Piero, 2007; Dela *et al.*, 2003) corroborated the anthocyanin accumulation inducing by low temperature and inhibited by high temperature. All this studies were agree with the results obtained in this experiment because of warming conditions was coupled to lower flavonoids quantities (anthocyanins among others) identified by UPLC-MS/MS and the decrease of total anthocyanins content measured spectrophotometrically at AT+4 ºC treatment on overripe olives. The flavonoids changes along ripening shows quantitative and qualitative differences among olive tree cultivars, being related to the differences in anthocyanin biosynthesis patterns, concretely, the presence of different anthocyanins molecules, cyanidin-3-rutoside together cyanidin-3-glucoside, has been associated to lower content on flavonol and flavones glycosides as quercetin-3-rutoside and luteolin-7-glucoside (Roncero *et al.*, 1970; Vlahov, 1992). In addition, levels of delphinidin-3-O-glucoside, delphinidin-3-O-rutinoside, and myricetin-3-O-glucoside showed

positive correlations with temperature in Ribes nigrum (Zheng *et al.*, 2012). Elevating temperature resulted in a decrease in the amounts of total flavonoids in bark as well as total catechins and total acetophenones in needles (Virjamo *et al.*, 2014).

The relevance of flavonoids antioxidant defenses (against ROS) has been identified in several studies, and had been widely reported to involve in environmental stress (Farrant *et al.,* 2004; Ogweno *et al.,* 2008; Xu *et al.,* 2011; Cakmak and Horst, 1991). Under prolonged stress, the declining scavenging ability within plant cells occurs (Liu and Huang, 2000; Sofo *et al.,* 2004; Reddy *et al.,* 2004; Yang *et al.,* 2014; Talbi *et al.,* 2015).

The modulation of temperature to alkaloids accumulation was reported, and high temperature preferable to induce the biosynthesis of alkaloids. The total accumulation of alkaloids (morphinane, phthalisoquinoline and benzylisoquinoline) in dry Papaver somniferum was restricted at low temperature (Bernáth and Tétényi, 1979). In contrast, the total level of phenolic acids and isoflavonoid (such as genistein, daidzein and genistin) increase were reported after the exposure at low temperature in comparison to the control (Janas *et al.*, 2002). High temperature incubation led to an instinct rise of 10-hydroxycamptothecin (HCPT) described as indicating HCPT was involved in the defence against heat shock from the environment. Moreover, higher alkaloid content was observed with higher temperatures (Jansen *et al.*, 2009). C. roseus showed under short-term heat shock, the contents of vindoline, catharanthine and vinblastine in the seedling leaves were higher at elevated temperatures, and in a long-term experiment at warming conditions, the concentrations of monomeric alkaloids catharanthine and vindoline showed a sharp increase (Guo *et al.*, 2007). Besides, the concentrations of total piperidine alkaloids in needles of Picea abies exposed to high temperature were significantly higher than those in the needles under ambient temperature (Virjamo *et al.*, 2014).

In recent years, the correlation between terpenoid yield and temperature were investigated, for example the capacity for isoprene emission of *Quercus rubra* and *Q. alba* in warm conditions was twice that in cold conditions (Hanson and Sharkey, 2001). Most terpenes in Daucus carota were increased under warmer conditions temperature. causing a strong bitterness (Rosenfeld *et al.*, 2002). The sesquiterpene compounds (SQTs) emission of from Pinus sp. was induced by higher temperature (β-caryophyllene, α-bergamotene, α-farnesene, and β-farnesene) (Helmig *et al.*, 2007; Ibrahim *et al.*, 2010). However, there was no temperature effect on the total terpenoid emissions in Picea abies (Filella *et al.*, 2007; Kivimaempaa *et al.*, 2013).

Regarding to the effect of warming conditions on olive oil organoleptical and nutraceutical properties, we detected some of bioactive compounds present on the olive as differential metabolites with different accumulation in warming temperature,generally less abundants than ambient temperature, most of them phenylpropanoids and terpenes (iridoids primarily) as verbascoside, oleuropein and loganic acid devivatives (both of them responsible to bitter, spicy

and astringent characteristics) as well as gentisin, sweroside are the best known in olive tree. It is remarkable the ascorbic acid 2-Glucoside as unique, stabilized form of vitamin C that we identified in our samples. 3,4-DHPEA-EA is the major form of the oleuropein-aglycone found naturally in unripe olive fruit and decreasing along ripening progresses rise a minimum amount at warming conditions. Also, sweroside a bioactive herbal ingredient used for the osteoporosis treatment and anti-inflammatory and analgesic activities was more abundant at environmental conditions

Generally, temperature increase has been confirmed to reduce the concentrations of SMs in plants, data that we observed in warming conditions as has been presented (Figure 7.9). Obtained results showed concordance to described previously in olive fruits by Pérez *et al.* (2018) respect a decrease of phenolic compound contents observed with warmer/higher temperatures.

5. Conclusions

Our results confirm metabolite profiling is a valid method to study the effects of stress in the composition of olives fruits during ripening processes. In fact, we discriminate and evaluate the effect of warming temperatures in olive fruit composition and highlighted phenylpropanoids and derivatives together terpenes such us major metabolic pathways that are highly affected on warming conditions. Veraison stage has been demonstrated as the most susceptible ripening stage presenting the major differences at metabolic level. In addition, a ripening advance at the pulp internal level indicates that the veraison stage in warming conditions is displaced towards the purple one. The obtained results suggest the negative impact of warming conditions on major secondary metabolites classes responsible of the organoleptic and nutritional properties of the olive oil, decreasing the terpenes, flavonoids and carotenes (flavour, aroma, colour and stability, among others).

6. References

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7. SUPPLEMENTARY MATERIAL

Supplementary Table 7.1

ID Compound	Neutral Mass (Da)	RT (min)	Accepted ID	Accepted Description	Total Score	Frag. Score	Formula	MassError (ppm)	Isotope Similarity	Subclass/ specifications
Phenylpropanoids/ Phenylpropanoids Derivatives and Conjugated/ Precursors Phenylpropanoids										
Simple Phenolic Compounds										
neg_2.47_313.0927m/z	314.1004877	2.4721	CSID4954574	Avenein	56.5	97	C14H18O8	-0.7161	86.52	Phenolic (volatile)
neg_3.15_477.1605m/z	478.1683755	3.1475	CSID59696396	2-Hydroxy-4-(2-hydroxyethyl)phenyl 6-O-beta-D-glucopyranosyl-beta-D-glucopyranoside	55.5	92.4	C20H30O13	-1.7109	87.23	Phenolic glucoside
neg_3.82_223.0613m/z	224.069138	3.8245	CSID23245203	5-(3.4.5-Trihydroxybenzyl)dihydro-2(3H)-furanone	41.5	20.4	C11H12O5	0.5286	87.65	Furanone
Phenylpropanoids and Derivatives/Conjugated										
neg_4.74_621.1830m/z	622.1907966	4.7379	CSID23327073	Crenatoside	52.4	85.3	C29H34O15	0.7796	77.78	Phenylpropanoid and derivatives
neg_5.27_623.1978m/z	624.2056793	5.2650	CSID4445086	OUH5BQ893P	44	23.7	C29H36O15	-0.5098	97.04	Phenylpropanoid and derivatives
neg_2.33_329.0875m/z	330.0953466	2.3311	CSID28533627	1-O-vanilloyl-beta-D-glucose	55	89.2	C14H18O9	-0.9087	86.81	Benzaldehide
neg_4.07_163.0403m/z	164.0481418	4.0697	CSID11012	3-coumaric acid	40.2	9.29	C9H8O3	1.4643	93.25	Probable (p-coumaric)
neg_4.65_639.1925m/z	640.2003564	4.6497	CSID4445101	Plantamajoside	55.4	99	C29H36O16	-0.8031	78.78	Phenylpropanoid and derivatives
neg_6.17_609.1229m/z	610.1307524	6.1695	CSID30900775	prodelphinidin B1	41.6	23.5	C30H26O14	-3.3832	88.61	Proanthocyanidins/precursor tannins
neg_4.00_385.1137m/z	386.1214972	4.0007	CSID24785464	1-O-[(2E)-3-(4-Hydroxy-3.5-dimethoxyphenyl)-2-propenoyl]hexopyranose	57.4	98.3	C17H22O10	-0.9410	90.11	Phenylpropanoid and derivatives
neg_6.13_623.1972m/z	624.2049943	6.1343	CSID4445112	Verbascoside	47.3	49.9	C29H36O15	-1.6073	88.47	Phenylpropanoid and derivatives
neg_4.92_785.2513m/z	786.2591389	4.9229	CSID10128243	Purpureaside C	45.9	56.3	C35H46O20	0.4392	73.76	Phenylpropanoid and derivatives
neg_3.10_353.0873m/z	354.095141	3.1034	CSID1405788	Chlorogenic acid	56.1	98.1	C16H18O9	-1.4278	84.31	Phenylpropanoid and derivatives
neg_3.32_163.0405m/z	164.0483303	3.3238	CSID4445164	3.4-Dihydroxycinnamaldehyde	56.6	96.2	C9H8O3	2.6135	90.09	Phenylpropanoid and derivatives
Flavonoids and Derivatives										
neg_4.07_637.1778m/z	638.1856374	4.0697	CSID24785427	dalpatein 7-O-β-D-apiofuranosyl-(1->;6)-β-D-glucopyranoside	47.5	58.8	C29H34O16	0.6140	79.24	Isoflavonoid
neg_6.83_593.1299m/z	594.137746	6.8289	CSID9702000	(+)-gallocatechin-(4alpha->8)-(-)-epicatechin	43.5	32.6	C30H26O13	-0.2299	85.38	Biflavonoid
neg_4.61_609.1462m/z	610.1540311	4.6057	CSID8161778	kaempferol 3-O-beta-D-glucosylgalactoside	53.6	89	C27H30O16	0.1583	79.10	Flavonoids

pos_6.40_409.0 326m/z	408.0247799	6.4035	CSID64953	flavodic acid	40.2	12	C19H14O8	1.5504	90.79	Flavonoids
neg_5.55_285.0 403m/z	286.0481453	5.5454	CSID4444102	Luteolin	42.4	25.4	C15H10O6	-0.4799	87.26	Flavonoids
neg_4.14_609.1 467m/z	610.1545634	4.1402	CSID8546981	Quercetin-3-O-alpha-L-rhamnopyranosyl-(1-->6)-beta-D-galactopyranoside	53.2	89.6	C27H30O16	1.0306	77.65	Flavonoid-3-o-glycosides
neg_4.64_785.2 510m/z	786.2588278	4.6409	CSID4445084	Echinacoside	55.8	99.2	C35H46O20	0.0435	80.13	Flavonoids
pos_2.11_455.0 939m/z	454.0860853	2.1139	CSID26367429	79YJI9GIF1	41.8	33.7	C21H20O10	-2.2157	77.74	Flavonoids
neg_6.13_621.1 816m/z	622.1894558	6.1343	CSID147700	Pectolinarin	52.4	79.3	C29H34O15	-1.3754	84.49	Flavonoids
neg_5.13_593.1 503m/z	594.1581233	5.1328	CSID17283650	Isoorientin 2''-O-rhamnoside	40.2	10.7	C27H30O15	-1.4765	91.86	Flavonoids
neg_4.98_609.1 458m/z	610.1535891	4.9846	CSID4444362	Rutin	43.2	19.2	C27H30O16	-0.5663	97.39	Flavonoids
pos_3.46_581.1 826m/z	580.1747244	3.4592	CSID138307	Mulberrofuran C	40.8	26.6	C34H28O9	3.3404	81.30	Flavonoids
neg_5.92_447.0 940m/z	448.1017888	5.9155	CSID102753	Isoorientin	53.8	85.1	C21H20O11	1.5180	85.75	Flavonoids
neg_4.94_447.0 926m/z	448.1004387	4.9405	CSID4678039	Quercetin-7-O-rhamnoside	55.9	86.7	C21H20O11	-1.4948	94.65	Flavonol
neg_4.94_284.0 322m/z	285.0400449	4.9405	CSID24784679	3-(2.4-Dihydroxyphenyl)-5-hydroxy-4-oxo-4H-chromen-7-olate	54.5	92.9	C15H9O6-	-3.4196	83.80	Isoflavone
neg_5.04_300.0 271m/z	301.0349486	5.0359	CSID24808102	7-Hydroxy-2-(3.4.5-trihydroxyphenyl)-3.5-chromeniumdiolate	42.1	29.4	C15H9O7-	-3.2387	84.83	Isoflavone
Chromenes										
neg_5.75_551.1 399m/z	552.1477072	5.7481	CSID58829950	2-Hydroxy-3-(5-hydroxy-3.6.7-trimethoxy-4-oxo-4H-chromen-2-yl)-6-methoxyphenyl beta-D-glucopyranoside	43.7	24.6	C25H28O14	-1.3301	95.26	
neg_6.21_535.1 451m/z	536.1529718	6.2136	CSID58829946	6-(5-Hydroxy-3.7-dimethoxy-4-oxo-4H-chromen-2-yl)-2.3-dimethoxyphenyl beta-D-glucopyranoside	51.6	61.5	C25H28O13	-1.1130	97.83	
neg_5.76_581.1 521m/z	582.1598844	5.7569	CSID59696280	(2R)-Naringenin 8-C-α-L-rhamnopyranosyl-(1->2)-β-D-glucopyranoside	48.1	65.4	C26H30O15	1.5182	76.88	
neg_7.01_361.1 291m/z	362.1369172	7.0140	CSID35013674	2-(2-Hydroxy-3.4.5-trimethoxyphenyl)-7-methoxy-8-chromanol	40	20.7	C19H22O7	-0.5588	79.97	
neg_4.74_651.1 944m/z	652.2022753	4.7379	CSID58837445	dalnigrein 7-O-&;β;-D-apiofuranosyl-(1-6)-&β;-D-glucopyranoside	50.3	73.9	C30H36O16	2.1538	80.31	
Coumarins and Derivatives										
neg_5.48_553.1 924m/z	554.2002226	5.4765	CSID30791654	Osthenol-7-O-beta-D-gentiobioside	56.5	95.8	C26H34O13	-0.5088	87.41	Coumarins
neg_6.40_569.1 869m/z	570.1946843	6.3987	CSID390647	Decuroside III	49.3	51	C26H34O14	-1.3228	97.04	Coumarins
neg_3.37_445.1 347m/z	446.1425066	3.3678	CSID4474618	Gaultherin	52.6	85.5	C19H26O12	-1.0600	78.99	Lignin precursor
Chalcones										
neg_4.75_595.1 667m/z	596.1745735	4.7467	CSID4444668	Isobutrin	55.8	97.3	C27H32O15	-0.1897	81.82	Chalcone
Terpenes/Terpenoids and Derivatives										

neg_4.35_197.0821m/z	198.0898892	4.3517	CSID35013302	2-Oxo-5-[(3E)-3-penten-1-yl]tetrahydro-3-furancarboxylic acid	57	97	C10H14O4	0.6693	88.79	Lactone
neg_6.40_291.0872m/z	292.0950345	6.3987	CSID16498838	Picrotoxinin	50.5	63.8	C15H16O6	-0.7365	89.55	
neg_1.77_361.1134m/z	362.1212456	1.7687	CSID390858	Anthirrinoside	49.2	57	C15H22O10	-1.6982	91.28	Iridiod
neg_5.28_389.1448m/z	390.1526263	5.2826	CSID79111	Loganoside	52.7	82.1	C17H26O10	-1.3206	82.89	Iridoid
neg_6.71_358.1291m/z	359.1369501	6.7056	CSID24784733	1-(Hexopyranosyloxy)-7-methyl-1.4a.5.6.7.7a-hexahydrocyclopenta[c]pyran-4-carboxylate	55	98.2	C16H23O9-	4.5402	82.18	Secologanin and strictosidine biosynthesis
neg_5.72_345.0977m/z	346.105571	5.7217	CSID10128508	(6R)-2-Acetyl-6-(3-acetyl-2.4.6-trihydroxy-5-methylphenyl)-3-hydroxy-6-methyl-2.4-cyclohexadien-1-one	45.3	43.3	C18H18O7	-0.6864	83.98	
neg_10.39_487.3428m/z	488.350628	10.3870	CSID414049	Euscaphic acid	55.5	98.5	C30H48O5	-0.2345	79.27	Triterpenoids
neg_10.39_515.3376m/z	516.3453848	10.3870	CSID2271900	Fusidic acid	54.1	95.7	C31H48O6	-0.5070	75.28	steroid
neg_3.61_345.1187m/z	346.1264933	3.6130	CSID82585	Aucubin	44.2	34.3	C15H22O9	-1.2518	88.29	monoterpenoid
neg_4.20_357.1191m/z	358.1269217	4.2019	CSID391589	10-Deoxygeniposidic acid	56.3	98.2	C16H22O9	-0.0135	83.44	monoterpene
neg_2.12_395.1552m/z	396.1630668	2.1196	CSID4946020	Tiaprost	54.1	95.5	C20H28O6S	4.7158	80.39	arachidonic derivative
neg_2.84_359.1336m/z	360.1414775	2.8406	CSID391568	8-Epideoxyloganic acid	47.5	58.7	C16H24O9	-3.0684	82.17	
neg_5.10_525.1612m/z	526.168987	5.0976	CSID390593	Inumakilactone A glycoside	45.8	46.3	C24H30O13	-0.3926	83.04	
neg_3.86_413.1085m/z	414.1163621	3.8598	CSID76046	Asperuloside	54.2	92.2	C18H22O11	-0.9718	80.07	terpenoid
neg_3.43_413.1087m/z	414.116571	3.4295	CSID8539900	Futalosine	54.5	96	C19H18N4O7	-3.7105	81.00	terpenoid
neg_2.84_389.1446m/z	390.1524521	2.8406	CSID522957	Loganin	57.1	95.4	C17H26O10	-1.7671	92.38	
neg_2.46_361.1133m/z	362.1211488	2.4633	CSID30791176	(1aR.1bR.2R.5aS.6R.6aR)-6-Hydroxy-1a-(hydroxymethyl)-1a.1b.2.5a.6.6a-hexahydrooxireno[4.5]cyclopenta[1.2-c]pyran-2-yl beta-L-glucopyranoside	58	96.7	C15H22O10	-1.9654	95.41	
neg_2.30_421.1343m/z	422.1421341	2.3046	CSID391577	10-Hydroxymorroniside	53.6	76.7	C17H26O12	-2.0027	93.90	monoterpenoid
neg_2.62_375.1292m/z	376.1369928	2.6203	CSID80905	loganate	56.9	98.3	C16H24O10	-1.3101	87.98	monoterpenoid
neg_5.89_555.2073m/z	556.2151568	5.8891	CSID391583	11-Hydroxyiridodial glucoside pentaacetate	46.6	62.1	C26H36O13	-1.7490	72.96	monoterpenoid
neg_0.57_267.0721m/z	268.0799111	0.5733	CSID26332738	2-O-(alpha-D-glucopyranosyl)-D-glyceric acid	51.9	71.2	C9H16O9	-0.2963	88.66	monoterpenoid
neg_4.41_405.1395m/z	406.1473415	4.4134	CSID390865	Ipolamiide	56.5	97.8	C17H26O11	-1.8073	87.02	monoterpenoid
neg_4.00_481.1349m/z	482.1427707	4.0007	CSID30791175	Catalposide	54.4	91.5	C22H26O12	-0.4333	80.77	

neg_4.67_555.1 717m/z	556.179532	4.6674	CSID391585	7-Dehydrologanin tetraacetate	55.9	98.8	C25H32O14	-0.3965	81.39	Terpenoid
neg_3.37_403.1 247m/z	404.1325304	3.3678	CSID390873	6beta-hydroxygeniposide	52.9	80.3	C17H24O11	0.2743	84.30	monoterpenoid
neg_2.90_345.1 185m/z	346.1262855	2.9023	CSID391588	Deutzioside	56.9	92.3	C15H22O9	-1.8521	94.30	
neg_6.87_377.1 235m/z	378.1313359	6.8730	CSID30776771	3.4-DHPEA-EA	53.7	80.7	C19H22O8	-1.8185	90.03	monoterpenoid
neg_2.84_357.1 186m/z	358.1264471	2.8406	CSID141471	Sweroside	55.1	90.4	C16H22O9	-1.3387	86.73	monoterpene
neg_2.30_375.1 289m/z	376.1367535	2.2958	CSID391578	7-Epiloganic acid	55.6	88	C16H24O10	-1.9462	92.10	monoterpene
Alkaloids and Derivatives										
pos_2.05_635.1 776m/z	634.1697245	2.0467	CSID30790812	(1S.2R)-1-(3.4-Dihydroxyphenyl)-6.7-dihydroxy-N.N'-bis[2-(4-hydroxyphenyl)ethyl]-1.2-dihydro-2.3-naphthalenedicarboxamide	41.8	32.2	C34H32N2O8	-2.4900	79.89	
neg_5.12_352.1 034m/z	353.111218	5.1152	CSID24785713	1-O-[(2-Oxo-2.3-dihydro-1H-indol-3-yl)acetyl]hexopyranose	45.9	48.8	C16H19NO8	-1.1004	82.11	Monocarboxylic acid (auxin);
pos_6.45_118.0 651m/z	117.057294	6.4474	CSID776	Indole	56.6	91.6	C8H7N	0.0130	91.35	Indol
neg_4.98_655.1 502m/z	656.1580695	4.9846	CSID31930	Teniposide	50.8	80.6	C32H32O13S	1.7434	75.56	Topoisomerase
neg_2.47_359.0 978m/z	360.1055942	2.4721	CSID17215958	3-Methoxy-4-hydroxyphenylglycol glucuronide	54.7	88.9	C15H20O10	-1.6977	86.55	
neg_4.07_291.0 984m/z	292.1062184	4.0697	CSID29272789	(4S.5R)-N-(2-Methoxy-2-oxoethyl)-4-methyl-2-oxo-5-phenyl-1.3-oxazolidine-3-carboximidic acid	49.6	58	C14H16N2O5	-0.8976	91.16	
pos_4.10_194.0 802m/z	193.072386	4.0961	CSID19250427	4-Hydroxy-5-phenyl-1.3-oxazinan-2-one	50	67	C10H11NO3	-4.9640	88.79	
neg_6.78_460.1 605m/z	461.1683016	6.7760	CSID4588593	Morphine-3-glucuronide	43	31.4	C23H27NO9	-1.8018	85.54	Morphinane alkaloid
pos_5.79_130.0 978m/z	129.0899911	5.7949	CSID5373018	(2S)-2-Piperazinecarboxamide	47.1	45.2	C5H11N3O	2.5924	93.40	
neg_7.21_474.1 761m/z	475.1839463	7.2063	CSID4590054	Codeine-6-glucuronide	50	69.7	C24H29NO9	-1.7725	82.35	Morphinans
Carbohydrates and Derivatives										
neg_0.58_665.2 121m/z	666.219914	0.5821	CSID388624	Stachyose	45.7	48.4	C24H42O21	-3.7563	84.54	Carbohydrate
neg_0.58_337.0 771m/z	338.0849588	0.5821	CSID18678797	L-Ascorbic acid-2-glucoside	47.2	48.9	C12H18O11	-1.4635	88.98	AA2G
neg_0.53_193.0 353m/z	194.0431197	0.5293	CSID73320	Mannuronic acid	45.2	32.3	C6H10O7	-0.4221	94.36	Carbohydrates derivatives
pos_1.45_489.0 778m/z	488.0699466	1.4475	CSID26333142	O-α-D-glucosyl poly(glycerol phosphate)	41.8	15.1	C12H26O16P2	1.8219	95.94	Carbohydrates and conjugates
neg_0.57_383.1 188m/z	384.1266428	0.5733	CSID9524553	4-O-(6-O-Acetyl-alpha-D-glucopyranosyl)-D-glucopyranose	47.6	42.5	C14H24O12	-1.7723	97.52	Carbohydrates and conjugates
neg_0.58_341.1 087m/z	342.1164899	0.5821	CSID144601	Kojibiose	42.8	28.1	C12H22O11	-0.8030	86.91	Carbohydrates and conjugates
neg_0.57_503.1	504.1695974	0.5733	CSID388379	Raffinose	42.2	32	C18H32O16	0.0136	78.97	Carbohydrates and

618m/z										conjugates
neg_0.57_505.1768m/z	506.1845806	0.5733	CSID24534254	alpha-L-Glucopyranosyl-(1->4)-alpha-L-glycero-hexopyranosyl-(1->3)-(3xi)-D-xylo-hexitol	42	26.1	C18H34O16	-1.3136	85.58	Carbohydrates and conjugates
neg_6.36_439.1599m/z	440.1677334	6.3634	CSID59696256	Cichorioside B	51.8	77.6	C21H28O10	-2.4153	84.30	Carbohydrates and conjugates
neg_0.53_195.0509m/z	196.0587059	0.5293	CSID10240	D-Gluconic acid	44.2	26.7	C6H12O7	-0.7733	95.45	Carbohydrates and conjugates
Organic Acids										
neg_0.90_203.0198m/z	204.0275842	0.8994	CSID389591	1-Oxo-1.2.4-butanetricarboxylic acid	50.9	63.4	C7H8O7	0.1108	91.23	Organic acid
pos_0.52_102.0551m/z	101.0473001	0.5186	CSID4911379	2-Oximino-3-butanone	45.4	33	C4H7NO2	1.7582	95.93	Organic acid
neg_0.91_111.0093m/z	112.0171179	0.9082	CSID15367214	2.3-Dihydroxy-2.4-cyclopentadien-1-one	51.5	69.1	C5H4O3	4.6364	94.06	Organic acid
neg_0.59_133.0144m/z	134.0221863	0.5910	CSID193317	L-(ÔêÆ)-Malic acid	57.5	92	C4H6O5	0.7667	96.53	Organic acid
neg_0.91_191.0196m/z	192.027459	0.9082	CSID305	Citric acid	40.5	5.72	C6H8O7	-0.5340	97.54	Tricarboxylic acids and derivatives
Amino-acids and Conjugated										
neg_0.50_131.0464m/z	132.0541844	0.5044	CSID231	DL-asparagine	57.1	92.6	C4H8N2O3	1.0135	94.35	AA
neg_3.27_309.1089m/z	310.1167747	3.2709	CSID449883	glu-tyr	46.6	50.1	C14H18N2O6	-0.8541	84.00	Dipeptide
pos_1.73_507.2045m/z	506.1967137	1.7298	CSID388681	Gentamycin A	50.8	67.3	C18H36N4O10	-3.7339	91.25	Aminoglycoside
pos_7.33_230.0584m/z	229.0505996	7.3302	CSID30777022	{5-[(Z)-5.6-Dihydro-3(4H)-pyridinylidenemethyl]-2-furyl}methanol	44.5	38.4	C11H13NO2	3.3308	88.02	Aminoglycoside
Polyketides										
neg_6.88_275.0923m/z	276.1001629	6.8818	CSID79667	(+)-dihydromethysticin	42	26.7	C15H16O5	-0.5528	83.93	
pos_2.50_598.0971m/z	597.0893075	2.5035	CSID30791120	dTDP-L-megosamine	40.5	15.2	C18H31N3O13P2	1.3224	88.97	
neg_5.56_633.1249m/z	634.1327107	5.5631	CSID16735642	Actinorhodin	40.4	24.8	C32H26O14	-0.1669	77.30	
Methanopterins										
neg_6.74_472.1576m/z	473.165443	6.7408	CSID140	folinic acid	41.6	33.5	C20H23N7O7	-2.1230	77.12	
neg_6.73_440.1334m/z	441.1412396	6.7320	CSID5815	folic acid	40.6	17.9	C19H19N7O6	2.2829	87.67	
neg_3.33_773.2362m/z	774.2440475	3.3326	CSID389685	7.8-Dihydromethanopterin	51.4	87.3	C30H43N6O16P	-4.9339	75.31	
Fatty Acids and Related										
pos_6.68_277.2165m/z	276.2086816	6.6774	CSID4471933	Stearidonic acid	41.2	25.7	C18H28O2	1.1302	81.74	lipid
neg_7.02_195.0664m/z	196.0742299	7.0228	CSID35014005	5-(3-Furyl)-2-methyl-5-oxopentanoic acid	56.7	92.4	C10H12O4	0.5781	91.62	Fatty acids and conjugates
Others										

pos_5.48_453.0353m/z	452.0274986	5.4793	CSID389386	(7R)-7-(5-carboxy-5-oxopentanamido) cephalosporanic acid	44.7	35.5	C16H18N2O9S	-2.7349	91.07	Pteridines and derivatives
neg_4.94_285.0400m/z	286.0478357	4.9405	CSID58830044	1.3.8-Trihydroxy-2-methoxy-9.10-anthraquinone	52.1	75.5	C15H10O6	-1.5621	86.71	Anthraquinone
pos_4.99_753.2540m/z	752.2461466	4.9949	CSID170815	Urdamycin G	40.6	21.9	C37H46O14	2.8861	84.71	Polyketide Biosynthesis
neg_5.98_853.2769m/z	854.2847356	5.9772	CSID767	Hydroxymethylbilane	53.1	99.3	C40H46N4O17	-1.8553	68.66	Porphyrin
pos_4.16_776.2727n	776.272672	4.1600	CSID19985521	N-(4.5.7-Tricarboxyheptanoyl)-L-gamma-glutamyl-N-[2-(4-{[5-(formamidomethyl)-3-furyl]methoxy}phenyl)ethyl]-L-glutamine	46.9	48.4	C35H44N4O16	-3.2668	90.02	Unclassified

Chapter VIII. General Discussion

The research project behind the present Doctoral Thesis aimed at studying the effect and the response to warming (4°C above the environment temperature) in olive tree (*Olea europaea* subsp. *europaea*),the dominant tree crop in the Mediterranean Basin, using for that purpose the cv. Picual as an experimental system. Related investigations and data have been previously published on this topic using different tree localizations with different thermal regimes (Miserere et al., 2018). The particularities of this work included, among others, its experimental design. Field experiments and adult productive trees were employed, which is quite unusual, as it is difficult to impose gradient temperatures in neighbouring trees. For that reason, Open Top Chambers (OTC) structures were employed (Benlloch-González et al., 2018; 2019). This system has only been occasionally used for olive tree and other crops in order to study, as an example, the effect of high CO_2 concentrations (Yang et al., 2018; Elmendorf et al., 2015).

The olive tree, a long-lived species, has a high level of adaptability to changing environmental conditions. This plasticity allows it to grow in the Mediterranean climate that is characterised by mild winters and warm dry summers (Dell'Amico et al., 2012; Martinelli et al., 2012). However, its maintenance and sustainability face severe problems and challenges, mainly related to extreme and adverse environmental conditions (Fisher and Shärk, 2010; Lobell et al., 2011). Of them, high and extreme temperatures are one of the main limiting factors in crop productivity. Thus, in olive trees and other crops reports have been received of reduction yields of 30% decrease with a decline in global crop production of 2-6% in the last decades associated with rising temperatures (Beck et al., 2007; IPCC, 2019; Hattfield et al., 2011). This topic is of great relevance considering the predicted climate change in which an increase of 2-6°C in the environment temperature is expected to occur by 2050 in the Mediterranean Basin (Giorgi et al., 2006). Temperature is a clear limiting factor and it is considered as being the main source of geographical variation in the phenology of the olive tree (Aguilera, 2012; Jochner et al., 2012; Miserere et al., 2018). With this in mind, the general objective of the Thesis was planned as an attempt to evaluate how a 4°C increase affected the phenology of the tree, concretely the reproductive stage, by determining parameters such as flower development, fruit set, growth, and ripening.

It is assumed that the high quality of olive oil is related to its chemical composition, and derived nutraceutical and organoleptic properties differentiating it from other vegetable oils. It is because the chemical composition of the fruit pulp all through the ripening process was evaluated in order to find out how warmer temperatures affected its chemical composition (The et al., 2017; Pérez-Jiménez, 2007; Colomer and Menendez, 2006). For this purpose, a double classic and holistic biochemical approach has been made, including transcriptomics, proteomics, and metabolomics.

This multi-disciplinary proposal is one of the important novelties of this Thesis. It let us determine chemical parameters related to fruit and oil quality (oil, pigments, volatiles), but also to get a better understanding of the molecular mechanisms involved in the ripening process.

In the experiments performed, two temperature regimes throughout three consecutive years (2015-2018) were imposed: the environment temperature and a four degree C increase, corresponding to the, respectively, control and warming treatments. As a field experiment, a high variability was expected, considering differences in daily and average temperatures during the years monitored (Chapter III, Fig. 2). Also, and for a better interpretation and understanding of the results presented, it was important to keep in mind the typical cause-effect curve and the concept of the optimum or threshold temperature. Whether or not an increase in 4°C takes the effect beyond the optimal temperature depends on the environment temperature and this has been proven for the four major herbaceous crops (Zhao et al., 2017.).

In this general chapter, and considering that detailed discussion of the data has been made in the previous ones, any integration and interpretation of the results will be limited only to those of great relevance from an agronomic point of view, or those closely related to crop productivity, either from a qualitative or quantitative perspective. The economic value of the olive tree is related to its fruit, the olive, with a yearly production of $20x10^6$tonnes, 31.4% in Spain, and 28.9% in Andalusia (FAO, 2019; MAPAMA, 2019). The fruit production is destined for table olives and, more importantly, olive oil (10 and 90% of total production, respectively) (IOC, 2019). Olive oil has been part of the Mediterranean culture since the Phoenicians, through the Greek and Roman Empires, and is a key element in the Mediterranean diet (Kaniewski et al., 2012; Altomare et al., 2013). It is preferred to other plant oils because of its nutritional value, beneficial effect on health (Pérez-Jiménez, 2007; Gouvinhas et al., 2017), and organoleptic properties. These properties are the result of its chemical composition, enriched in polyphenols content, unsaturated fatty acids (C18:1, predominantly), and terpenoids (Ganbari et al., 2012; Kalua et al., 2007). All of them are determined by genetic variability between cultivars, as well as environmental factors and stress that affect the metabolite profile of the fruit, thus acting on its composition and, subsequently, the oil's quality (García et al., 2013; Steyn et al., 2002; García-Inza et al., 2018).

Under our experimental conditions, an increase of 4°C above ambient temperature induced an earlier date of flowering, fruit development, and ripening, in the three seasons investigated (Chapter III, Fig. 3). This could be due to the minimal chilling requirement of olive tree (900-1000 chilling units) to get over endolatency (Orlandi et al., 2002), and the heat accumulation (800-700 degree-days) to go through ecolatency (Pérez-López et al., 2008). Although the reproductive phase was advanced, in two out of the three experimental seasons (16-17, and 17-18), the complete cycle from the beginning of flowering to the final ripening (harvesting period) did not show any significant differences between AT and AT+4°C (Chapter III, Fig. 3). In the 2015/2016 season an

elongationof 20 days in the development of the fruit under warming conditions occurred. This may be observed under typical Mediterranean climate conditions, with yearly variable temperatures between seasons. This cycle prolongation was predicted to take place in southern Spain by aerobiological studies and regional climate circulation models (Galán et al., 2005; Osborne, 2000; Pérez-Lopéz et al., 2008; Giannakopouluset al., 2009; Orlandi et al., 2014; Gabaldón-Leal et al., 2017).

High temperatures during the flowering period are detrimental to crop production since they can lead to a high degree of ovarian abortion (Rallo, 1994; Rapoport et al., 2012; Çamoglu, 2013; Benlloch-González et al., 2018), cause vegetative damage (Mancuso et al., 2002; Cansev et al., 2012), and a poor fecundation coupled with a poor fecundation and pollen quality (Koubouris et al., 2009). On the other hand, moderate temperatures favour photosynthetic activity and mineral nutrition (Zufferey, 2000; Vuletin-Selak et al., 2013). Throughout the 3 years of the present trial, we have observed a 30% reduction in fruit production under warming conditions, largely due to ovarian abortion and a more pronounced bearing, which is unexpected in the young olive trees employed with periodic irrigation (Chapter III, Table 1). A + 4°C treatment induced an earlier flowering from May to April, in which normal environment temperatures are lower (from an average of 28-30°C in May, optimal ones for fertilization and fruit set, to 25-28°C in April), this being the cause of the higher ovarian and pistil abortion observed under warming conditions (Chapter III, Fig. 2).

The phenology and fruit production characteristics of the species are the main factors responsible for determining bearing behaviour (Fernández-Escobar et al., 1992; Ryan et al., 2003; Cuevas et al., 1994; Lavee, 2007; Troncoso de Arce et al., 2012), that is of great importance in terms of crop productivity. The different temperature regimes for the Control, and warmed trees, affected bearing, which is a consequence of the photoassimilate competition between vegetative and reproductive buds. The prevalence of a specific phase is dependent on the accumulated chilling temperature. Thus, an increase in the environment temperature reduced the accumulated (below 12°C) low temperatures, favouring vegetative growth. The bearing phenomenon could be considered as being a mechanism of resilience and adaptability to transient extreme temperatures (as it favours vegetative growth, increasing the number of reproductive buds in the next period) which affects crop productivity negatively (Galán et al., 2008). Cultivation techniques, such as irrigation, early harvesting or "artificial fruit thinning" (Dag et al., 2010) may be a valid management practices to prevent bearing. Bearing could explain the increase in vegetative growth observed in our field experiment (Chapter III, Table 4)

While being mostly focused on the reproductive phase of the tree cycle, we observed some changes in the vegetative one and in the plant growth. In our experiment, we noticed an exuberant vegetative growth under warming conditions, approximately 125% on shoot growth, 154% trunk diameter growth and 244% pruning material weight with respect to ambient

temperature. Rather than the temperature increase treatment, it is the environment temperature that determines the tree behavior, the optimal temperature being dependent on the plant species. Norton et al., (1999) found that an increase of 3°C in air temperature significantly promoted the growth of *Agrostis curtisii*. However, a higher temperature (+4°C) significantly decreased biomass and seed weight in *Chenopodium album*. Song et al., (2016) also found that moderate temperature increase (1.5°C to 2°C) is beneficial to the biomass accumulation of *Stipagrandis* and *Leymuschinensis*, but a higher temperature increase (4°C to 6°C increase) has negative effects. These effects are more pronounced when adding the hydric stress, corroborating the theory that watering requirements and management should be revised (Issarakraisila et al., 2007; Wu et al., 2011).This result reporting the effects of high temperatures applied on root and/or shoot conditions on seedlings growth was observed in our culture chamber experiment, since the soil (in this case also well irrigated) was subjected to a lower temperature, a condition equivalent of 25/37°C (root/shoot) treatment (Chapter III, Fig. 8).

Fruit retention force determines its natural drop and therefore affects harvesting. In general, as the ripening advances, there is a sharp decrease in this force (Tous and Romero, 1993; Humanes and Civantos, 1992). It was related to fruit natural abscission (Vioque and Albi, 1975). Consequently, a drop in iron concentration rates in the peduncle has been linked to increased indolaceticoxydase activity that reduced the auxin activity to suitable levels to cause the abscission. Moreover, this activity is associated with the last phase of ethylene biosynthesis (Fernandez et al., 1985). This coincides with phase III of fruit development, during which all the necessary metabolic pathways and physiological processes are aimed at changes in fruit colour, chemical composition and texture. These changes are more significant in climateric fruits, in contrast to non-climateric ones (Prasanna et al., 2010) such as olives. However, at the molecular level, and under our experimental conditions, ethylene responsive genes were induced. Such is the case of transcriptional factors associated with ethylene response (*AP2/ERF1, EIN3* and *ERF1B*) under warming conditions, especially at the purple skin stage. The maximum is observed earlier under +4°C conditions. Taking into account all the data obtained in our study, it can be concluded that it is caused by advanced ripening, which gives overripe, and therefore senescent, fruits, which occurred later at ambient temperatures. The level of other transcripts associated with senescence processes was observed under warming conditions. At the proteomics levels, higher abundances of putrescines and enzymes related to advance cell wall degradation were noted earlier under warming conditions than in ambient temperature (Chapter V).

In the present work, we have analysed the chemical composition of the olive fruit throughout its developmental process and in two temperature regimes, environment temperature and 4°C increase. Three stages were analysed: green, veraison, and purple (Chapter V. Fig 1). This objective has been approached by classic colorimetric methods and metabolomics, and nLC-MS/MS. Previous publications have addressed the chemical analysis using independent,

complementary approaches, either classic (Tonutti and Martinelly, 2012; Beltrán et al., 2004; Martinelli et al., 2013; Conde eta al., 2008) or holistic ones (Alagna et al., 2012).

The data on the different chemical families and variable compounds identified have been discussed in the corresponding Chapter VII, and this general discussion focuses on those more related to the quality of the fruit and derived oil, namely olive oil, phenolics, terpenoids, chlorophylls, and carotenoids. These compounds showed different tendencies throughout the fruit ripening phases. Thus, fatty acids, indirectly estimated with the peroxidation test, increased in control plants from 0.29 mM equivalent MDA (green), to 4.86mMequivalent MDA(purple) (Chapter VII, Fig. 9). Warming treatment kept the fatty acid constant during the three stages, with much lower values than that corresponding to the control (0.05 to 0.82 mM equivalent MDAat green and purple stages, respectively). The amount of total phenolics was maximal at the veraison stage under ambient and warming conditions (16.13and 15.17mg equivalents of gallicacid/g of FW, respectively), the values of the warming treatment being slightly lower than the control ones. A reduction in phenolic compound content has been reported previously by Pérez et al. (2018) when comparing two geographical plantations with contrasting temperatures. Flavonoid content increased from green to purple stages (8.51to 20.1126mg equivalent of rutin/g of FW), with a larger amount under the warming condition at the final maturity stage. Although a similar tendency was observed for anthocyanins, lower values in warming treatment were detected at the purple stage. Finally, the content in pigments, chlorophylls and carotenoids, decreased from green to purple stages, the values being much lower in the + 4°C treatment. In short, temperature increase caused a decrease in the content of most of the compounds analysed, and is assumed to significantly affect the quality of the oil derived. The change in fruits colour as a consequence of the temperature regime may also confuse the farmers with respect to the harvesting time as this is deduced from the olive skin colour (Koshita, 2015). The appearance of a new colour phenotype under warming conditions is worth noting. Olive fruits acquire in warmer conditions a magenta coloration covering the whole surface of the fruit, while this colour did not appear under ambient conditions (See Material and Methods, Chapter V, VI and VII). The reason for that is not clear, although it is possible to speculate on the appearance or disappearance of a chemical compound with specific spectral characteristics, i.e. the bathochromic effect associated with pH changes (Khoo et al., 2017), or other unknown ones. A reduction in fruit anthocyanin content due to high temperature has been observed for a long time in other species (Utsonomiya*et al.*, 1982; Naito *et al.*, 1986; Yamada *et al.*, 1988, Arakawa 1991; Taira*et al.*, 2000; Isobe and Kamada, 2001). Changes in fruit colour occur at the same time as the oil content increases throughout ripening. In our study, the fruit-ripening period was forwarded and extended in AT+4°C trees. In fact, the ripening index, RI,(Chapter I, Fig. 8) indicates that the trees subjected to higher temperatures at harvest time showed a lower RI than those growing at ambient temperatures. Olives are initially green and turn yellowish as a result of a sharp reduction in chlorophyll (Fernández Díaz, 1971). Then, anthocyanin starts to accumulate in the cells determining colour intensity (Maestro and Vázquez,

1976), which may range from reddish to intense purple and black. In most cultivars, skin colouring begins at the apex (start of the onset of ripening) and continues towards the other end next to the stem (end of the onset of ripening). Under warming conditions, the pigmentation evolution was more or less affected depending on the ripening stage analysed (Chapter VII): chlorophyll loss was sharpest, carotenoid level was higher only at green skin, and anthocyanin contents were higher at veraison but lowest at the purple stage. Differently from carotenogenic fruits, in which the chlorophyll concentration drops while carotenoid values may remain stable or even increase through the synthesis of more complex compounds, olives are characterized by a significant drop in chlorophylls and fewer carotenoids, and by an increase in anthocyanins (Chapter VII). The changes in these compounds in ripening has been described by several authors (Vázquez-Roncero et al., 1970; Amio et al., 1986; Vlahov, 1992), who have concluded that anthocyanin content increases during the onset of ripening, reaches its peak, and later remains constant but drops slightly in tree remainder or overripe fruits. The harvesting period is established based on the optimal ripening stage by using the fruit skin colour and natural drop as guidelines (Barranco et al., 2000). The optimal RI corresponded to 3-3.5 values (veraison-purple skin stage) in `Picual´cultivar.

A more precise picture of the chemical composition of the fruit was provided by the metabolomics analysis. Through LC-MS/MS, we were able to confidently identify150 compounds out of the 6000 detected. This is a common characteristic of the metabolomics approach; the low number of identified compounds, it due, among other reasons, to the absence of spectral data in databases (Escandón et al., 2017). The identified compounds belonged to 14 chemical families, which are discussed in detail in Chapter VII. Most of them were variable in developmental stages and/or treatments. Different evolution tendencies were observed (Chapter VI, Fig. 9), with some of them unclear from a biological point of view. In general, similar data have been previously reported (Conde et al., 2008). In this general discussion, as stated above, we will focus on those compounds more related to the quality of the oil, and the differences between treatments. As shown in Figure 9, a general decrease in most of the identified compounds as a consequence of the warming treatment was discerned, including phenylpropanoids, terpenoids, alkaloids, organic acid and carbohydrates among others. This observation is in good agreement with the previous colorimetric analysis data. It is not possible to contrast the metabolomics data with the effect of temperature as there are no similar works published. The closest paper found in the literature is an analysis of olive oils from trees at different geographical locations with different temperatures and climate conditions (Sarolic et al., 2014).

The metabolomics analysis revealed that the monosaccharides glucose and fructose are the most abundant ones in olive fruits (Chapter VII, Supl. Table 1). Their concentration dropped as the fruit developed and ripened. At the two final stages the values remained constant (Chapter VII, Fig. 3). Other sugar monosaccharides identified were sucrose, xylose and ramnose, found in

smaller concentrations without significant differences between temperature treatments. This was also described previously by Beaulieu et al. (2017) on palm and Fonseca et al. (2019) on banana.

Fatty acids, derivatives, and other apolar, hydrophobic compounds, are elusive to the metabolomics nLC-MS analysis, so they were indirectly estimated from the proteomics data on the enzymes implicated in their biosynthesis. The only fatty acid identified in our analysis was arachidonic (20:4) and stearidonic(18:4) acids, both of them absent under warming conditions. This diminution of lipid agreed with the values of fatty yield observed (Chapter III, Table 5), and the values of lipid peroxidation obtained (Chapter III, Fig. 5).

Olive flesh has a high polyphenol content of up to 5% of its dry weight, mainly phenolic acids, flavonoids and secoiridioids. In this work, quite a number of such phenolic compounds have been identified, either in an aglycon-free or sugar-derivative forms. The family included phenylpropanoid precursors, flavonoids, and anthocyanins, which are related to oil quality because of the colour of olives being a clue for harvesting period; moreover, phenolics present in olive oil act as an antioxidant, increasing stability and flavour. The major compounds were flavonoids and chromenes. Most of them were present in a small amount under warming conditions, the only exception was chromenes throughout the fruit's development. Anthocyanins and flavonoids responsible for the olive colour, were in a significantly smaller amount in AT+4°C than in AT trees, supporting the idea of a delay between onset and end in the ripening period (Chapter III, V and VII). This reduction in fruit anthocyanin content due to high temperature has been observed for a long time in other species (Utsonomiya *et al.*, 1982; Naito *et al.*, 1986; Yamada *et al.*, 1988, Arakawa 1991; Taira *et al.*, 2000; Isobe and Kamada, 2001). The colour and stability of these pigments are influenced by pH, light, temperature, and structure (Khoo et al., 2017). The red-coloured pigments of anthocyanins are predominantly in the form of flavylium cations that are most stable at a lower pH solution (Bakowska, 2005). In our field experiment, a new colour stage appeared under the warming condition as mentioned above. The latest possibility related to the greater H^+-ATPase activity found under the warming condition (Chapter V). Apart from the pH, anthocyanin-tannin polymerization could also increase colour stability at a lower pH, helped by the co-pigmentation of anthocyanin aglycones. In addition, glycosylation and acylation increase the colour strength of anthocyanin (Castaneda et al., 2009); however, anthocyanins are less stable at higher temperatures. A previous study reports that heat treatment at a maximum of 35°C reduced the total anthocyanin content in the common grape to less than half the amount in control berries at 25°C (Mori et al., 2007). At up to 40°C, the colour of anthocyanin changes from red to orange although the pH of the solution was low (West and Mauer, 2013).

Verbascoside is one of the most complex phenolics present in olive. From a chemical point of view it is a prenylated and glucosylated derivative. It has been reported as being an antioxidant and an oxygen radical scavenger. Also, antitumour, antimicrobial, anti-inflammatory, anti-thrombotic and wound healing properties have been previously described (Funes et al., 2010). This

compound and similar ones found in olive oil may contribute to its medicinal value. It was not detected in young green fruit, showed its maximum at veraison, and declined at purple stages, with the content being lower at + 4°C, which has been previously reported (Vázquez-Roncero et al., 1974).

Terpenoids are among the major secondary metabolites detected in olives, with oleuropein being the most abundant one. Because of its secoiridioid phenolic structure, it has a browning or over-oxidation ability, and is responsible for the intensely bitter taste in green fruits. It has anti-oxidant properties, being one of the chemical parameters related to olive oil quality (Macheix et al., 1990; Visioli and Galli, 1998). Oleuropein content decreased with the start of the pigmentation change from green to purple (Amiot et al., 1986). Moreover, an increase in dimethyloleuropein and oleoside was observed during fruit ripening. Like oleuropeine, these compounds affect the oil's organoleptic properties, protecting it from oxidation processes (Beltran, 2000; Beltran et al., 2004; Andrewes et al., 2003). Other iridoids related to flavour and oil taste were identified as loganic acid and derivatives (Chapter VII, Supplementary Table 1).A plausible biosynthetic route from deoxyloganic acid, 7-epiloganic acid, 7-ketologanic acid, 8-epikingisidic acid, oleoside 11-methyl ester, 7-β-1-D-glucopyranosyl 11-methyl oleoside and ligustroside to oleuropein was proposed by Damtoft et al. (1995).

In conclusion, a reduction in the concentration of the major terpenoid compounds was found, in good agreement with previous published data Pérez et al. (2018). Last, but not least, phenolics are potent antioxidant compounds and any reduction in their concentration under warming conditions may lead topoor-quality oil in which oxidation reactions take place, diminishing its stability and changing its nutritional value and organoleptic properties.

As metabolites are the products of chemical reactions integrated into metabolic pathways in which specific enzymes play a pivotal role, the proteomics analysis helps to understand the mechanisms mediating the accumulation of specific compounds. In addition, the olive oil making process, based on cold-pressing, instead of its extraction with organic solvents in other plant oils, justifies the presence of some proteins in the final olive oil, which may affect the product's quality and stability. Previous published work on protein content in fruit and oil also used proteomics but with other platforms, such as 2-DE, with a lower potential than the shotgun one employed here (Bianco et al., 2013). In total, we identified more than 3000 proteins, with 1500 variable ones (qualitative or quantitative differences) among developmental stages and /or temperature regimes. They belonged to 34 functional categories, with Photosynthesis, FattyAcids, Terpene, Phenylpropanoids and Flavonoid Synthesis, and Plant Cell Wall Degradation being those most affected by warming conditions. The evolution of the different protein families and the diversities between samples have been discussed in detail in Chapter V.

Proteogenomics is an area of research at the interface of proteomics and genomics. In this approach, customized protein sequence databases generated using genomic and transcriptomic information are used to help identify novel peptides (not present in reference protein sequence databases) from mass spectrometry–based proteomic data. In turn, the proteomic data can be used to provide protein-level evidence of gene expression and to help refine gene models (Guillot et al., 2019). This was the approach employed to perform our targeted transcriptomic analysis of the differential genes expression profile of 85 protein coding genes involved in different processes/pathways related to the ripening process under warming conditions. Proteomics data is discussed in parallel with those of targeted transcriptomics, as the list of selected genes was generated on the basis of the proteomics results.

Changes in carbohydrate and lipid metabolism have been reported as mechanisms to maintain the homeostasis and equilibrium between energy and reserve requirements, where lipids have an additional relevance due to mill purpose olives and unsaturated fatty acid composition. Olive fruit is a deep green colour and has a high photosynthetic ability up to 20 days after full bloom. From that time on, photosynthesis drops for some 60 days and remains at steady-stationary levels until almost all the chlorophyll has disappeared from the fruit (black stage) (Proietti and Tombesi, 1991). In supporting this hypothesis, we observed that olives with green skin had high levels of photosynthesis-related enzymes, which was accompanied by a peak in glycolytic and mitochondrial respiratory ones, more notable in environment than in warmer temperatures. In both treatments, all these metabolic proteins declined with very low levels at veraison and purple stages, this effect being more marked at +4°C than AT. Genes over-expressed in ripening stages, were mainly implicated in the switch transition from the photoautotrophic to heterotrophic metabolism, that occurred earlier under warming conditions.

At the veraison and purple stages, an increase in the levels of reducing sugar was observed at environment temperature and, to a lesser extent, in the +4°C treatment. This could be the result of an induction in polysaccharide-degrading enzymes, acting on starch or cell wall components. Thus, an increase in β-amylases, pectin-methyl esterases, pectate lyases, and polygalacturonases, occurred throughout fruit ripening and senescence (veraison and purple stages). An earlier peak was observed [first] (veraison) at +4°C than at the environment temperature (purple).The increase in protein abundance was accompanied by an induction of the corresponding mRNAs.

Pectic substances, including anhydrogalacturonic acid, are closely related to fruit texture (Alba, 1982). All along the ripening process, there was a continuous decrease in the anhydrogalacturonic acid content, that correlated with a softer texture, and the esterification rate dropped to near undetected levels in the final ripening stages (Minguez Mosquera, 1982). In parallel with the drop in the esterification rate, pectin esterase activity begans, increasing during the first stage (green), reaching its peak (veraison) and then falling to very low levels when polygalacturonase activity appeared and, in accordance, polygalacturonase appeared in ripened

and overripe olives. A high metabolic activity in the ripening processes was observed by identification of polygalacturonase in proteomic and metabolomic analyses. Its levels generally started to increase earlier under warming conditions (veraison) than under ambient ones (purple stage). This was corroborated by the levels of transcripts involved in cell wall degradation *as β-GAL*, *PL1*, *GMD,* that presented over-expression under warming conditions, and ripe olive fruits, especially PL1 appeared first at veraison AT+4°C treatment. The level of other transcripts associated with senescence processes were observed under warming conditions. At the proteomics levels, larger abundances of putrescines and enzymes related to advanced cell wall degradation were observed earlier under warming conditions than at the ambient temperature (Chapter V). The up-accumulation of a β-Galactosidase could be related to the higher transcript levels of *DELLA-GA1* and *GID1* as being indicative of starting senescence under warming conditions earlier than at ambient temperatures (Chapter VI, Supplementary Figure 1). In a previous study it was reported that GAs induce genes related to cell wall modification (Andriotis et al., 2016).This change might be associated with the mobilization of storage polysaccharides (starch) due to an increased catabolism of the glucose towards leaves because of fruit senescence and drop.

Oil accumulation in olives begins immediately after the pit has hardened, while a widening of the parenchymal cells in the flesh is observed. The sources of carbon for lipid biosynthesis are the leaf or the fruit skin (Sánchez, 1995), employed as substrateacetyl-CoA. The oil accumulates as TAG and fatty acids are incorporated into the oil through the Kennedy pathway. In this pathway, the enzymes KAS involved in the last phase of TAG from DAG are sensitive to high temperatures; their activities decrease significantly above 40°C (Sánchez et al., 1990). This could explain our results when the warming temperature is compared with ambient temperature, a similar situation being observed in the low oil content obtained from fruit during very warm summer years (Chapter III). The levels of *KASIII* transcripts (Chapter VI) reinforce this hypothesis, as well as the protein abundances of fatty acid elongases (Chapter V). `Picual´ cultivar has the highest lipid accumulation rate compared to other mill oil cultivars (García Martos and Mancha, 1992; Beltran et al., 2010), with the accumulation rate peak coinciding with the colour change from green to yellowish-green. Water stress conditions during lipid biosynthesis cause a low flesh/pit ratio producing a decrease in oil accumulation ability (Lavee, 1991). This correlation between fruit size and oil content was also described by Barone et al. (1994). Palmitic and other saturated fatty acids decreases during ripening, while oleic acid may remain constant or increase slightly together with a linoleic acid increase (Gutierrez et al., 1999; Beltran et al., 2000). The ratio monounsaturated: polyunsaturated is related to oxidative stability (oil resistance to rancidity) with its values decreasing as ripening advances. Olive oil is particularly rich in the monounsaturated fatty acid oleate (18:1), reaching percentages of up to 75–80% of total fatty acids, followed by linoleate (C18:2), palmitate (C16:0), stearate (C18:0) and linolenate (C18:3). The final acyl composition varies enormously throughout olive fruit development, according to genotype and environmental conditions. Drought and heat stress increase lipid oxidation and, consequently,

trigger more severe oxidative damage to the leaf membrane integrity (Jiang and Huang 2001; Xu and Zhou 2005; Sekmen et al. 2014). Therefore, the reduction in oil content observed in our results could also be due to a delay in lipogenesis because of the delay in fruit ripening.

The transcriptional analyses were extended with a prospecting of differentially expressed genes between warming and control conditions. It was found that in warming treatment the genes most over-expressed were related to the secondary metabolism at the green and purple stages, while all of them were under-expressed at veraison. This expression pattern might be caused by the double peaks of expression showed in order to modulate several responses, as previously demonstrated for the JA and LOX activity regulation, as well as ROS response (Xu et al., 2016).These data were in good agreement with the results obtained for genes relative expression. A high transcript level of *LEA2* was found together with the *ERD4* and *HSP70*, as protective molecules under warming conditions (Chapter VI). The accumulation of oxidative stress-related enzymes (Chapter V) was interpreted as being a defence strategy against the accumulation of ROS under warming conditions; CAT, APX, SOD and GST, among others, were more abundant under warming conditions.

Anti-oxidant activity was also exerted by polyphenol compounds such as flavonoid or terpenoid compounds, all of them identified at –omic levels in this study. The anti-oxidative defence represented by phenolic synthesis and accumulation (PAL, 4CL, CHI3, DFR, UGT73C, UGT72E, COM over-expression) appeared as being altered under warming conditions, showing as smaller amounts of these compounds, a lesser abundance of enzymes involved in its synthesis and under-expressed transcripts, especially at advanced ripening stages. Anthocyanins can be degraded by polyphenol oxidases (PPO and CO) as was described by Patras et al. (2010). Under warming conditions, we identified several genes of the phenylpropanoid pathway, under-expressed ones (*ANS*, *DFR*, *C3H*, *4CL*, *MYC2* and *MYB*-75/PAP1 transcriptional factors) (Chapter VI) and their transcript products involved in anthocyanidins biosynthesis (Chapter V) directly correlated with a decrease in flavonoids identified at the metabolomic level at the purple stage (Chapter VII), while an over-expression of these genes was observed at veraison under warming conditions. Metabolite profiling showed smaller amounts of major terpenes present in olive oil under warming conditions; while genes involved in these pathways such as *HMBPPS*, *GE10H*, *SQS*, *LS*, *DXPS*, were over-expressed, as well as polyphenol oxidase transcripts *PPO* and *CO*. Terpenes (responsible for the organoleptic and nutraceutical properties of olive oil) showed the opposite pattern observed with total compounds of phenylpropanoid derivatives.

Finally, the results presented in this PhD Thesis deepened the knowledge of the physiological and molecular changes taking place during *Olea europarea L. subsp. europaea* fruit ripening and abiotic stress responses through a multidisciplinary analysis. We found elevated similarities between veraison under warming conditions and the purple stage under environmental ones. All the molecular levels (transcripts, proteins and metabolites), which

suggested a discrepancy between the colouring of the skin, taken as harvesting criteria, and the traditional pulp ripeness stage. However, the rapid but not immediately molecular response together with post-transcriptional and posttranslational modifications may be the reason for the scant correlation between transcripts, proteins and metabolites when the same stage was analysed. Thus, an extension of this study is required to analyse all ripening stages to confirm our hypothesis.

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Chapter IX. General Conclusions

The conclusions obtained on this Doctoral Thesis were as follow:

1. Warming conditions (4 °C above environment temperature) altered the phenology of the olive tree. Earlier bud shooting, flowering and fruit ripening period were observed under a warmer temperature. Moreover, a prolongation of flowering and ripening occurred.

2. The pistil abortion that occurred under higher temperatures decreased the fruit set. It was accompanied by a reduction in fruit size, with both contributing to a significant crop yield reduction estimated at 30 %.

3. Different temperature 25 °C/37 °C (root/shoot) regimes altered seedling growth, potassium intake and transport, and leaf water status. Concretely, high temperature (37 °C /37 °C) decreased K^+ uptake and its transport to the aerial parts, reduction in water content, and inhibition of shoot and leaf growth.

4. Warming temperature altered the transcriptomic, proteomic, and metabolomic profiles of olive fruit at the three ripening stages analysed: green, veraison, and purple. The veraison stage was the one most affected.

5. At the ripe, harvesting stage, 4 °C above environment temperature reduced the oil, anthocyanin, and polyphenol content in olive. This reduction was also observed for other pigments, including chlorophyll and carotenoids, both responsible for the oil colour. These diminutions are assumed to alter the quality and organoleptic properties of the olive oil.

6. Through UHPLC-MS/MS metabolomics we were able to confidently identify 150 compounds belonging to 14 chemical families out of the 6000 detected. Those most represented were secondary metabolites, including phenolics, terpenoids, and alkaloids.

7. As a general tendency, the content of most of the compounds identified was lower under warming than under the environment temperature. This occurred for the major sugars detected (glucose and fructose), for the polyunsaturated fatty acids (arachidonic, 20:4, and stearidonic, 18:4), for the phenylpropanoid precursors and derived flavonoids, anthocyanins, and for the most abundant terpenoids (verbascoside and oleuropeine). This decrease would affect the organoleptic, stability, antioxidant and nutritional properties of the olive oil.

8. By using a shotgun proteomics approach and an Olea specific database, around 3000 proteins were identified. Out of them, 150 out were variable among stages and temperature treatments. The differential abundance of these proteins was analysed at the transcriptomic level

and a good correlation was found between protein abundance and number of mRNA copies in 85 out of 110 selected candidate genes.

9. The proteomics and transcriptomics analyses showed the typical gene expression pattern corresponding to the ripening and senescence process, in which auxin, ABA, gibberellin and jasmonic-related genes (ARR1, DELLA-GAI1; GID1, ASR1, TIP120, among others) play a pivotal role.

10. Despite being a non-climateric fruit, the induction of ethylene-responsive genes (ACCO, EIN3, ERD4, ERF1, AP2/ERF1) also occurred. The induction peak of those genes happened earlier in +4 ºC than AT fruits.

11. The different developmental stages had their own metabolic signature and characteristics, as revealed by proteomics and transcriptomics analysis. In green fruits we observed active photosynthesis, and catabolism (glycolysis, TCA cycle, oxidative phosphorylation) metabolism. At veraison, a general overexpression of genes involved in phenylpropanoid and terpenoids synthesis was noted which was accompanied by reduced expression of genes involved in lipid metabolism. At the purple stage, a general decrease in transcript levels was perceived with the exception of genes involved in onphenylpropanoids synthesis. Warming conditions shifted the tendencies and peaks observed towards previous stages of ripening and/or caused a reduction in the abundance of proteins and related genes.

12. An increase in the level of starch and cell wall-degrading protein enzymes (amylases, pectin-methyl esterases, pectate lyases, polygalacturonases) and corresponding transcripts, took place throughout the fruit's ripening and senescence (veraison and purple stages). It was observed first at +4 ºC than in the environment temperature treatments.

13. Other variable proteins and transcripts whose abundance changed were HSP´s (HSP70 and 16.7HSP), and antioxidant enzymes (catalase, peroxidases, and ascorbate peroxidases). Both groups were induced under warming conditions.

14. As a main and general conclusion, under warming (+4 ºC), we noticed a shift in the timing of the fruit maturation stages, with this occurring earlier. The fruit biochemical profile pointed to a discordance or asynchrony between the external colour of the fruit and the internal ripeness state, the latter being more advanced than its colour indicated.

15. From the observations and data presented it is possible to predict the consequences of the climate change on the olive crop and speculate on new recommendations for its management: (i) a lower plantation density to prevent interferences between trees close together; (ii) increasing watering doses during critical phases to reduce pistil abortion and increase fruit size;

and (iii) new phenological and harvesting period evaluation criteria. All the above together will help to maintain a high crop productivity both from a quantitative and qualitative point of view.

Chapter X. Appendix

FIGURES INDEX

TABLES INDEX

List of Abbreviations

2-DE Two dimensional electrophoresis
ABA Abscisic acid
ABA Abscisic acid
ABA-GE Abscisic acid glucosyl ester
ACO 1-aminocyclopropane-1-caboxylic acid oxidase
ACT Actin
AGP Glucose-1-phosphate adenylyltransferase
ANOVA Analysis of variance
APX Ascorbate peroxidase
ATP Adenosine triphosphate
BCA Bicinchoninic acid
BK Castasterone
BL Brassinolide
BLAST Basic local alignment search tool
BRs Brassinosteroids
BSA Bovin serum albumin
CBB Coomassie Brillant blue
CBB R-250 Coomassie Brilliant Blue R-250
CDS Coding sequence
CE Capillary electrophoresis
CHAPS 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonate
Chla Chlorophyll a
Chlb Chlorophyll b
CID Collision induced dissociation
Ck Cytokinins CLPB Casein lytic proteinase B
CKs Cytokinins
CS Castasterone DHAPS Phospho-2-dehydro-3-deoxyheptonate aldolase 1
DAPI 4',6-diamidino-2-phenylindole
dATP Deoxyadenosine triphosphate
DB Database
dCTP Deoxycytidine triphosphte
dGTP Deoxyguanidine triphosphate
DHAR Dehydroascorbate reductase
DHN Dehydrin
DHZR Dihydrozeatin riboside
DIABLO Data Integration Analysis for Biomarker discovery using a Latent component
DTT Dithiothreitol
DTT Ditiotreithol
dTTP Deoxythymidine triphosphate
DW Dry weight
EDTA Ethylenediaminetetraacetic acid
EF Elongation factor
EL Electrolyte leakage

ENO Enolase
ESI Electrospray ionization
EST Expressed sequences tags
FDH Formate dehydrogenase
FDR False discovery rate
FTMS Fourier transform mass spectrometer
FW Fresh weight
GA Gibberellin
GA Gibberellins
GABA γ-4-aminobutyric acid
GADPH Glyceraldehyde 3-phosphate dehydrogenase
GAPDH Glyceraldehyde 3-phosphato dehydrogenase
GASA Giberellin-stimulated arabidopsis
GC Gas chromatography
GC-MS/MS Gas Chromatography coupled to Mass Spectrometry
GeLC Gel electrophoresis liquid chromatography
GST Glutathione S-transferases
HMDB Human metabolome database
HPLC High performance liquid chromatography
HSFs Heat shock factors
HSP Heat shock protein
IAA Indol-3-acetic acid
iP Isopentenyl adenine
iPA isopentenyl adenosine
IPG Immobilised pH gradients
JA Jasmonic acid
KEGG Kyoto Encyclopedia of Genes and Genomes
LC Liquid chromatography
LEA Late embryogenesis abundant
LTQ Linear trap quadrupole
LTQ-Orbirap Linear-quadrupole ion trap-Orbitrap MBROLE2 Metabolites Biological Role 2
MALDI-TOF Matrix-assisted laser desorption/ionization – time of flight
MDA Malondialdehyde
MDHAR Monodehydroascorbate reductase
method for Omics studies
MIP Major intrinsic proteins
MS Mass spectrometry
MTBE Tert-methyl-Butyl-Ether
NDH6 NADH dehydrogenase
NGS Next-Generation Sequencing
NIRs Near-infrared spectroscopy
NRQ Normalized relative quantity
OEE Oxygen evolving enhancer protein
ORFs Open reading frames
PAL Phenylalanine ammonia-lyase
PBS Phosphate buffered saline
PC Principal component
PCA Principal components analysis

PCR Polymerase chain reaction
PLS-DA Partial Least Square Discriminant Analysis
PMSF Phenylmethylsulfonyl fluoride
PMSF Phenylmethylsulphonylfluoride
PP2C Protein phosphatase 2 C
PSII Photosystem II
PSM Peptide spectrum match
PTM Posttranslational modification
PVDF Polyvinylidene difluoride PVPP Polyvinylpolypyrrolidone
PVP Polyvinylpyrrolidone
qRT-PCR Quantitative reverse transcription-PCR
RBCL Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit
RBCS Ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit RIN RNA integrity number
RF Random forest
RH Relative humidity
RIN RNA integrity number
RNA-Seq RNA sequencing
ROS Reactive oxygen species
ROS Reactive oxygen species
RQ Relative quantity
RT-qPCR Reverse transcription quantitative PCR
RWC Relative water content
SA Salicylic acid
SE Standard error
SD Standard deviation
SDS Sodium dodecyl sulphate
SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser-CPs Serine carboxypeptidase-like
sHSPs Small heat sock proteins
SKP S-phase kinase-associated protein
SOD Superoxide dismutase
sPLS Sparse partial least square analysis
sRNA Small RNA
SVM Support vector machine
TBA Thiobarbituric acid
TCA Trichloroacetic acid
TEMED Tetramethylethylenediamine
TMSH Trimethylsulfoniumhydroxide
TPM Transcripts per million
TSS Total soluble sugars
TW Turgid weight
UHPLC Ultra High Performance Liquid Chromatography
UHPLC-MS/MS Ultra-high performance liquid chromatography coupled to Mass Spectrometry Tandem
WC Water content

PUBLICATIONS FOR THESIS BY COMPENDIUM

Global warming effects on yield and fruit maturation of olive trees growing under field conditions.

Benlloch-González, M.; Sanchez-Lucas, R.; Mohamed, A.; Benlloch, M.; Fernández-Escobar, R.

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Horticulture

JCR; Q1; IF 1,961; 5 posición

An approach to global warming effects on flowering and fruit set of olive trees growing under field conditions

Benlloch-González, M., Sánchez-Lucas, R., Benlloch, M., Fernández-Escobar, R. Scientia Horticulturae (2018), 240:405-410.

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Global warming effects on yield and fruit maturation of olive trees growing under field conditions

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ABSTRACT

Temperature in the Mediterranean Basin, the main area of olive (*Olea europaea* L.) cultivation, has been projected to rise drastically in the near future threatening olive production. To determine the potential effects of higher temperature on the olive fruiting cycle and vegetative growth, a study with the cultivar 'Picual' has been carried out simulating global warming conditions under field conditions. Temperature-controlled open top chamber (OTC) systems were used to increase the day/night ambient temperature 4 °C throughout the complete reproductive cycle of this species. Three years of study have shown that 4 °C increase of ambient temperature reduces fruit yield and affects fruit characteristics and maturation processes. Smaller fruits, lower pulp/stone ratio, oil yield and anthocyanin contents were observed. The maturation period was forwarded and extended in trees subjected to warmer temperatures. In addition, the vegetative growth was stimulated by the temperature treatment resulting in trees of bigger size.

1. Introduction

The Mediterranean Basin is the largest area in the world with a specific climate for olive (*Olea europaea* L.) cultivation. However, the environmental conditions of this region are expected to change in the near future (Giorgi, 2006). In particular, the mean air temperature has been projected to rise drastically in the range of 2–5 °C (Giorgi, 2006; Giannakopoulos et al., 2009; Gualdi et al., 2013; IPCC, 2014). In addition, more heatwave days and tropical nights and an extension of the dry season will be observed (Giannakopoulos et al., 2009).

Olive production depends on vegetative and reproductive processes occurring along a biennial cycle. Both processes are repeated annually, but while shoot growth is completed within the same year, processes leading to fruit bearing require two consecutive seasons. Briefly, in the first one, buds are formed in the leaf axils of growing shoots and flowers are induced. After floral bud dormancy, during the second season, inflorescences and flowers develop until flowering, and then fertilization and fruit set occur. In the next stage, fruits develop and grow until ripening (Rallo and Cuevas, 2008). All these processes are regulated, among other factors, by climate conditions and seasonal changes. Thus, higher temperatures in the Mediterranean region associated to global warming may affect any of these processes and, consequently, olive production in the near future. Despite the importance of this issue, there is not much information on this respect.

In the northern hemisphere two vegetative growth flushes can be observed: the main one from March to mid-July and the second from September to mid-October provided that water is not a limiting factor. The optimum temperature for olive shoot growth and development ranges from 10 to 30 °C, but when temperature rises above 35 °C shoot growth could be limited (Rallo and Cuevas, 2008; Therios, 2009). This effect has been recently observed in different olive plant material when plants were exposed to moderately high temperature (37 °C) during a period of time (Benlloch-González et al., 2016, 2017). From this information, it is not clear in which sense the change in temperature patterns along the year, especially during winter and autumn, will affect the vegetative growth in this species.

Most of the available information on the potential effects of warmer temperature on olive fruitfulness has been focused on floral phenology, paying little attention on the following reproductive processes. Regional Climate Models and olive-pollen capture traps studies, have informed that the development of flower structures will be completed faster leading to earlier flowering dates (García-Mozo et al., 2010;

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Oteros et al., 2013; Osborne et al., 2000; Giannakopoulos et al., 2009; Orlandi et al., 2010; Aguilera et al., 2015). An advance in the flowering date has been also observed in a recent study in which olive trees growing under field conditions were exposed during three consecutive years to 4 °C above the ambient temperature using temperature controlled open-top-chambers (Benlloch-González et al., 2018). In addition, the higher temperature affected floral differentiation, favoring pistil abortion, and fertilization processes leading to a reduction in fruit set (Benlloch-González et al., 2018).

After fruit set, olive growth and development are completed in approximately 4–5 months, following a double sigmoid growth curve pattern (Hartmann, 1949; Lavee, 1996; Rallo and Cuevas, 2008). During this period structural changes and chemical transformations such as cell division, cell expansion and storage of metabolites, take place in the different fruit tissues. Phase I is characterized by an exponential growth. During this phase, cellular divisions of the different fruit tissues are predominant, the mesocarp and the endocarp increase in size. The sclerification and hardening of the endocarp begin. In phase II, fruit growth slows down or stops, the embryo and the endocarp reach their final size and the endocarp hardening process is completed. During phase III, there is a fast growth of the fruit due to the enlargement of the mesocarp cells which determines the final fruit size. Lipogenesis in flesh parenchyma cells begins (Manrique et al., 1999). This phase ends in early autumn when fruit undergoes the first pigmentation changes.

All these processes are genetically controlled and influenced by several environmental factors (Connor and Fereres, 2005; Costagli et al., 2003; Gucci et al., 2009), being water availability the most studied. When water shortage occurs at phase I, smaller endocarps are observed (Lavee, 1986) which can lead to fruits with unusually high pulp/stone ratios compromising fruit viability. Moreover, water reduction during this stage has been informed to affect cell size rather than cell number in the mesocarp (Rapoport et al., 2004). Water availability in phase III determines the final size of the fruit and its oil content. Limitation of water during this period results in small fruits with reduced oil content (Beltrán et al., 2004). Although water limitation effects on fruit development and growth have been well described, the information related to higher temperature is scarce. There is some information from studies performed in the southern hemisphere, in which the effect of warmer temperatures has been analysed on fruit dry weight, oil concentration, and oil fatty acid composition. Fruiting branches from 'Arauco' olive cultivar were enclosed in transparent plastic chambers with individualized temperature control during the oil accumulation phase (García-Inza et al., 2014, 2018). Under these experimental conditions, temperatures above 25 °C reduced fruit fresh weight. A reduction in fruit oil concentration was also observed when temperature was increased during the period of active oil accumulation.

During the ripening process, the fruit darkens from lime-green to purple-black at the same time that oil content increases. The purple or black colour of the fruit is due to the formation of anthocyanins (Roca and Minguez-Mosquera, 2001). The amount of anthocyanins in a fruit determines its color and depends on its biosynthesis, accumulation and degradation. Temperature influences these processes. High temperature has been described to reduces anthocyanins accumulation in many fruits and plant tissues (Steyn et al., 2002) affecting fruit color (Koshita, 2015). This has been observed in different fruit species such as grapes (Tomana et al., 1979; Naito et al., 1986), apple (Creasy, 1968; Yamada et al., 1988; Arakawa, 1991), Satsuma mandarin (Utsonomiya et al., 1982), and Japanese persimmons (Taira et al., 2000; Isobe and Kamada, 2001), but it is not clear in olive fruit. The final changes in fruit colouring is an important stage during the olive fruit maturation after which oil accumulation processes are ceased (Lavee and Wodner, 1991).

In order to elucidate how global warming will affect olive maturation and yield as well as vegetative growth, a field study with the cultivar 'Picual' has been carried out in a Mediterranean climate type area. Trees were subjected to warmer temperature than ambient throughout their complete reproductive cycle using temperature-controlled open-top-chambers (OTCs). The impact of high temperature on fruit growth and development, maturation period, ripening processes, oil production and yield has been analyzed. The information from this study along with a previous one (Benlloch-González et al., 2018), gives an important overview of olive production associated to global warming.

2. Material and methods

2.1. Plant material and growth conditions

'Picual' olive trees (*Olea europaea* L.) growing in the experimental farm of 'Campus de Rabanales', University of Córdoba, Spain (37°55′N 4°43′W) were used to perform the experiment. The orchard soil is classified as Calcic Luvisols with a clay-loam to clay texture, pH moderately alkaline (7–8), organic matter around 2%, and moderate to high cation exchange capacity (Del Campillo et al., 1993). The trees were planted in autumn 2009 spaced 8 x 6 m apart, with a drip irrigation system. Depending on the season, water was applied over five to six months during the dry season (from late May to middle October, approximately). During this period the dose applied was about 55.2 l per tree and day. The experiment was conducted from 2014 to 2017.

2.2. Temperature treatments

Sixteen trees, consecutively distributed in two lines, were selected from the experimental orchard to perform this study. Trees were subjected permanently to two temperature treatments, ambient temperature (AT) and 4 °C above ambient temperature (AT + 4°C), during three consecutive years (2014–2017). To increase the ambient temperature in 4 °C, temperature-controlled open-top-chamber (OTC) systems were used. Each OTC, containing a single tree, was equipped with heating and ventilation devices regulated by an automaton to maintain a constant day/night temperature gradient between the tree and the surrounding environment of 4 °C throughout the complete reproductive cycle of this species. Further details about the functioning of this system have been described in Benlloch-González et al. (2018). The experimental design consisted of four blocks, each one with the two temperature treatments (TA; TA + 4°C) randomly distributed.

2.3. Measurements

Vegetative growth (shoot length, trunk diameter and weight of the pruning material) was measured once a year at the end of the vegetative period (late Autumn). Shoot length was measured on fifteen uniformly distributed shoots per tree, which were previously selected and tagged each spring. After harvesting, the trees were pruned and the trunk circumference measured at 30 cm above the ground surface.

To determine the fruit maturation period, the experimental trees were visited every 3–4 days from late August to December, recording the fruit phenological stages of each tree according to the following visual scale: 1 deep green skin; 2 yellowish-green skin; 3 veraison, green skin with reddish patches over more than half of the fruit; 4 purple skin; 5 black skin and white flesh. The maturation period was determined according to Barranco et al. (1998).

At harvest, fruits of each tree were collected to determine total weight. Fifty to eighty fruits per tree, depending on the year, were used to determine fruit size (average weight of the fruits sampled), pulp/stone ratio, expressed as fresh weight, and the maturity index (MI). The MI was determined according to Ferreira (1979). The fruits were classified into 8 categories (1–8) according to the visual scale mentioned above (1–5) extended to 3 more categories: 6 black skin and purple pulp over less than half of the pulp; 7 black skin and purple pulp not reaching the stone but covering more than half of the pulp, and 8 black

skin and purple pulp up to the stone. MI is the sum of the multiplication of the number of fruits in each category by the numerical value of each category, divided by total number of fruits.

Fruit oil content was determined by nuclear magnetic resonance (NMR) (Minispec mq 20, Bruker Analytik Gmbh). The results were expressed as percentage of fresh and dry weight (% FW and DW). Anthocyanin fraction was extracted from olive pulp using extraction methods described by Lee et al. (2005) with some modifications. Total anthocyanin content of diluted fruit extract was estimated by the pH differential spectroscopic method proposed by Cheng and Breen (1991). Absorbance (A) was measured with a UV–vis spectrophotometer at 510 nm and 700 nm in diluted buffers at pH 1 and pH 4.5, where $A = (A_{510}\text{-}A_{700})_{pH\ 1}$ - $(A_{510}\text{-}A_{700})_{pH\ 4.5}$. Data was expressed as cyanidin-3-glucoside equivalent per kg of fresh weight. The extraction of the phenolic fraction from the pulp was carried out following the method proposed by Gómez-Rico et al. (2008). Total polyphenols content was determined by the colorimetric method described by Vázquez-Roncero et al. (1973), using the reagent Folin–Ciocalteu. Absorbance was measured with a UV–vis spectrophotometer (CaryBio50, Varian) at 725 nm. Results were expressed as mg of caffeic acid per kg of pulp.

2.4. Statistical analysis

Analyses of variance were performed on the data using Statistix 9.0 software package (Analytical Software, Tallahassee, FL, USA). In all analyses, residual plots were generated to identify outliers and to confirm that variance was common and normally distributed. All percentages values were transformed using the arcsin of the square root before analysis.

3. Results

Under the experimental conditions of this study, a relatively constant temperature gradient of 4 °C between ambient trees (AT) and trees inside the temperature-controlled open-top-chambers (AT + 4°C), was maintained along three consecutive reproductive cycles (2015–2017) (Fig. 1). This data shows that the experimental system used to subject trees, growing under field conditions, to warmer temperatures than ambient is a reliable method.

The increase of ambient temperature in 4 °C (AT + 4°C) affected the vegetative growth of trees (Table 1). There was no effect of this treatment on the growth of tagged shoots in 2015 and 2016, but there were significant differences in 2017. The trunk diameter (cm) of tress at the beginning of the experiment was similar [7.3 (AT) vs. 7.4 (AT + 4°C)]. However, after exposing trees to 4 °C above ambient temperature differences were observed. The increment (Δ) of the trunk diameter was significantly greater in AT + 4°C than in AT trees at the end of each growing season (2015–2017). The same tendency was observed with the pruning material. This is, 4 °C above ambient temperature applied constantly along the three growing seasons, promoted the vegetative growth of AT + 4°C trees resulting in trees of significantly bigger size (Table 1).

The fruit maturation period, i.e., days from the start of veraison to ripeness, was also affected by temperature (Fig. 2). The appearance of reddish spots in the fruits was forwarded about 17–30 days in trees subjected to high temperature (AT + 4°C) when compared with AT trees. This effect was periodically observed in 2015–2017 interval. In addition, the warmer temperature prolonged the maturation period in 2015 and 2016 while no effect was observed in 2017 (Fig. 2).

Although fruit yield in trees growing under ambient temperature (AT) was not very high, it was significantly affected in AT + 4°C trees due to the temperature treatment applied during the complete reproductive cycle of the trees (Table 2). It was highly impacted in 2015 and in less but similar proportion in 2016 and 2017 (about 60–70% of reduction, respectively). Fruit oil content values, expressed as percentage of dry and fresh fruit weight (% DW and FW), were also

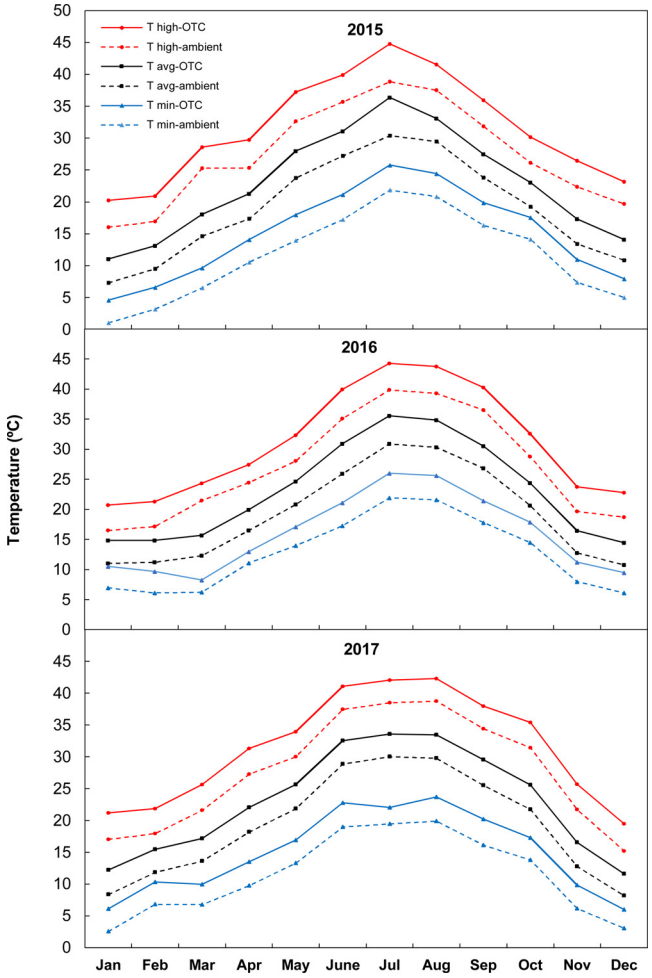

Fig. 1. Average monthly temperatures recorded from January to December during three consecutive years (T_{avg}). Dotted lines show ambient temperatures and the straight lines show OTC temperatures. T_{high} and T_{low} represents average of daily high and low temperatures by month respectively.

significantly lower in those trees subjected to warmer temperatures (AT + 4 °C vs. AT) (Table 2). The reduction in fruit oil content by the high temperature treatment was similar (about 30% compared to AT trees) along the tree years when expressed over FW. While it was expressed over DW this effect was more marked in 2015 than in 2016 and 2017 (about 33 and 20% of reduction, respectively).

At harvest, differences in fruit characteristics were observed between temperature treatments (AT vs. AT + 4°C) (Table 3). Fruits size was reduced by the AT + 4°C treatment in 2015 and 2016. In 2017 fruits grown under ambient temperature (AT) were smaller in size when compared with those of previous growing seasons and no effect was observed due to the AT + 4°C treatment. Pulp/stone ratio was smaller in AT + 4°C fruits than in AT ones in all seasons. There were no differences in maturity index (MI) between treatments in 2015. However, it was lowered by the AT + 4°C temperature treatment in 2016 and 2017 seasons (Table 3).

The accumulation of anthocyanins in fruits during the maturity period was significantly different in AT and AT + 4°C trees (Table 4). Greater values were observed in fruits of AT trees in all seasons, this is, warmer temperatures during this period decreased the accumulation of anthocyanins in fruits of AT + 4°C trees. There were no differences in fruit polyphenols contents between treatments in 2016. The high temperature treatment decreased fruit polyphenols content in 2017 (Table 4).

Table 1
Effect of a 4 ºC increase in ambient temperature (AT vs. AT + 4ºC) on vegetative growth.

Temperature treatment	Vegetative growth[1,2]								
	2015			2016			2017		
	Shoot growth (cm)	Trunk diameter[3] (cm)	Pruning material (kg)	Shoot growth (cm)	Trunk diameter (cm)	Pruning material (kg)	Shoot growth (cm)	Trunk diameter (cm)	Pruning material (kg)
AT	1,6 a	8,1 (0,8) b	4,2 b	9,8 a	9,4 (1,3) b	4,9 b	4,8 b	10,6 (1,2) b	3,9 b
AT + 4°C	1,4 a	8,7 (1,3) a	8,1 a	11,2 a	10,7 (2,0) a	11,0 a	7,6 a	12,5 (1,8) a	12,6 a
[4]*CV (%)*	*31.0*	20.7	*31.9*	*14.0*	*12.5*	*37.5*	*21.3*	*18.6*	*25.9*

[1] Means within each column followed by different letters are significantly different at P ≤ 0.05 by F-test.
[2] Each data is the mean of eight trees. In the case of shoot growth, it was obtained from 15 vegetative shoots per tree.
[3] Numbers in parenthesis are the increment of the trunk diameter of trees in two consecutive years.
[4] Coefficient of Variation.

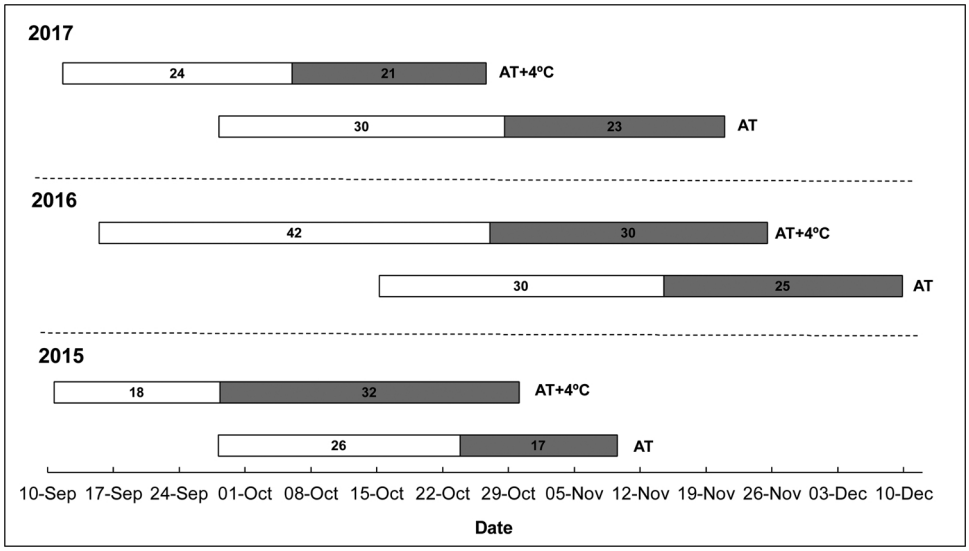

Fig. 2. Fruit maturation period under ambient temperature (AT) and 4 °C above ambient temperature (AT + 4°C) in three consecutive years. Inside each bar from left to right are represented the periods of time (days) from the start to the end of veraison and from the end of veraison to ripeness.

Table 2
Effect of a 4 °C increase in ambient temperature (AT vs. AT + 4°C) on fruit yield and oil content.

Temperature treatment	2015			2016			2017		
	Fruit yield (kg per tree)	Oil content		Fruit yield (kg per tree)	Oil content		Fruit yield (kg per tree)	Oil content	
		(%DW)	(%FW)		(%DW)	(%FW)		(%DW)	(%FW)
AT	5,1 a	53,7 a	18,1 a	16,7 a	52,1 a	19,1 a	22,4 a	51,2 a	20,6 a
AT + 4°C	0,4 b	35,7 b	12,3 b	6,6 b	42,0 b	12,7 b	6,8 b	39,9 b	14,2 b
[1]*CV (%)*	*61.23*	*23.86*	*14.43*	*39.39*	*3.93*	*8.59*	*25.08*	*9.56*	*5.73*

Means within each column followed by different letters are significantly different at P ≤ 0.05 by F-test.
Each data is the mean of eight trees.
[1] Coefficient of Variation.

4. Discussion

In a previous work (Benlloch-González et al., 2018) we presented the results of this experiment relative to the effect of high temperature on flowering and fruit set processes. It was emphasized that in other studies the effect of global warming was analyzed contrasting the behavior of crops located in regions with different mean temperatures or developing models to predict the performance of a particular crop (De Melo-Abreu et al., 2004; Giannakopoulos et al., 2009; Orlandi et al., 2014; El Yaacoubi et al., 2014; Gabaldón-Leal et al., 2017). In our study we evaluated the effect of global warming on the olive installing temperature-OTC systems under field conditions in an area vulnerable to climatic changes in the near future (Giorgi, 2006; Lionello, 2012; IPCC, 2014). The accuracy of the system is shown in Fig. 1.

In the above-mentioned study, we concluded that if temperature increases 4 °C above the ambient temperature in this region, olive flowering would be advanced and last longer, significantly increase pistil abortion leading to a reduction in fruit set. Results presented in the present work were obtained from the same experimental trees along the same years. Consequently, they show the end of the fruiting cycle of these trees. The reduction in fruit set, led to a significant reduction in fruit yield, but also in oil content. A reduction in fruit yield usually led

Table 3
Effect of a 4 ºC increase in ambient temperature (AT vs. AT + 4ºC) on fruit size, pulp/stone ratio and maturity index (MI).

Temperature treatment	Fruit characteristics[1,2]								
	2015			2016			2017		
	Fruit size (g/fruit)	Pulp/stone	MI	Fruit size (g/fruit)	Pulp/stone	MI	Fruit size (g/fruit)	Pulp/stone	MI
AT	5,7 a	9,0 a	3,9 a	5,5 a	8,3 a	4,3 a	4,5 a	9,2 a	4,7 a
AT + 4°C	3,3 b	4,9 b	3,8 a	5,1 b	7,1 b	4,0 b	4,8 a	6,9 b	4,0 b
[3]*CV (%)*	*15.15*	*15.84*	*9.42*	*3,35*	*8,20*	*4,71*	*14,56*	*12,53*	*7,93*

[1] Means within each column followed by different letters are significantly different at P ≤ 0.05 by F-test.
[2] Each data is the mean of eight trees and was obtained from 50 fruits per tree.
[3] Coefficient of Variation.

Table 4
Effect of a 4 ºC increase in ambient temperature (AT vs. AT + 4ºC) on fruit anthocyanins and polyphenols content.

Temperature treatment	2015		2016		2017	
	Anthocyanins (cyanidin-3-glucoside equivalent kg^{-1} FW)	Polyphenols (mg caffeic acid kg^{-1} pulp)	Anthocyanins (cyanidin-3-glucoside equivalent kg^{-1} FW)	Polyphenols (mg caffeic acid kg^{-1} pulp)	Anthocyanins (cyanidin-3-glucoside equivalent kg^{-1} FW)	Polyphenols (mg caffeic acid kg^{-1} pulp)
AT	192,6 a	–	993,9 a	8726 a	293,4 a	9511,2 a
AT + 4°C	106,4 b	–	490,5 b	11482 a	127,2 b	6347,6 b
[1]*CV (%)*	*21.75*		*12.91*	*12.21*	*33.33*	*11,05*

Means within each column followed by different letters are significantly different at P ≤ 0.05 by F-test.
Each data is the mean of eight trees.
[1] Coefficient of Variation.

to an increase in fruit size. This phenomenon is common in fruit tree species, including the olive and, in fact, fruit thinning is done to increase fruit size at harvest in apple, peaches, and other fruit tree species (Dennis, 2000; Looney, 1993; Wertheim, 2000; Costa and Vizzotto, 2000). However, in our study fruit size was smaller in AT + 4°C than in AT trees, even when fruit yield was significantly lower. Yield, fruit size and oil content depend on both genetic and environmental conditions (Lavee and Wodner, 1991). Despite that olive is well adapted to adverse environmental conditions, water stress along the different stages of fruit growth and maturity have been reported to highly affect those parameters (Lavee, 1996). Fruit size has been described to be reduced when water deficit is applied during early fruit growth (Rapoport et al., 2004; Gucci et al., 2009). This effect was mainly attributed to a failure in mesocarp cells extension rather than division. If water limitation is constant along the dry season, the metabolic activity is slowed down and consequently fruit growth and oil accumulation reduced (Lavee, 1996). When occurring at the end of the dry season, when fruit growth rate and oil production are intense, fruits of smaller size and lower oil content are produced. The results obtained under the experimental conditions of the present study could be explained by a water stress effect associated to a higher evotranspiration demand of trees due to elevated temperature inside the open-top-chamber during the dry season. From the results of this study we cannot elucidate what stage of fruit growth and maturity was more sensitive to the high temperature treatment, but it is clear that it affects any of the developmental processes that determine final fruit size and oil accumulation. Although studies performed in the southern hemisphere with the cultivar 'Arauco' have observed a reduction in fruit size and oil content when high temperature was applied during the oil accumulation phase (García-Inza et al., 2014, 2018), there is not much information on how global warming will affect these parameters increasing the need of further research on this respect.

During fruit maturation changes in fruit color occur at the same time that oil content increases. In our study the fruit maturity period was forwarded and extended in AT + 4°C trees. Therefore, the reduction in oil content could be also due to a delay in lipogenesis because of the delay in fruit maturation. In fact, the maturity index indicates that the trees subjected to higher temperatures at the time of harvest showed a lower MI than those growing at ambient temperatures. Also, anthocyanins found in AT + 4°C trees, a flavonoid responsible of the fruit color in the olive fruit, was significantly lower than in AT trees, supporting the delay in fruit maturation. This reduction in fruit anthocyanins content due to high temperature has been observed for long in other species (Utsonomiya et al., 1982; Naito et al., 1986; Yamada et al., 1988; Arakawa, 1991; Taira et al., 2000; Isobe and Kamada, 2001). The same effects on fruit maturation were observed in trees subjected to high doses of nitrogen (Fernández-Escobar et al., 2014), indicating the relationships between those effects.

Olive trees grow, as most woody species, by forming new shoots and extending the old ones, and by thickening those formed. A measure of each defines the annual growth of the tree and tree size. In this experiment, vegetative growth was exuberant in AT + 4°C trees. The annual growth of the trees, estimated measuring shoot growth at the end of the vegetative period and by the weight of the pruning material, was higher in AT + 4°C than in AT trees, probably because of the stimulative effect of temperature (Way and Oren, 2010). Trunk girth has been related to tree weight (Westwood, 1993) and, consequently, is an estimation of tree size (Fernández-Escobar, 2014). In our work, trunk diameter was higher in AT + 4°C trees, suggesting that the effect of higher temperatures have a direct effect on olive tree growth. The lower temperature threshold for olive vegetative and trunk growth has been estimated to be around 15 and 7 °C respectively (Pérez-López et al., 2008). So it seems that warmer temperatures during winter and autumn prolonged the growing season in AT + 4°C trees resulting in an increase in tree size.

In conclusion, increasing temperature 4 °C above the actual ambient temperature could lead to 1) a delay in fruit maturation, reducing oil content because a delay in lipogenesis, 2) fruit of smaller size, 3) a reduction in yield, due to a reduction in fruit set and an increase in pistil abortion, and 4) an increase in the size of trees. These results suggest that under future warmer conditions, plantation density must be lower to prevent interferences between nearby trees. In addition, actual irrigation management practices should be revised to reduce pistil abortion and increase fruit size and, consequently, yield.

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An approach to global warming effects on flowering and fruit set of olive trees growing under field conditions

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ABSTRACT

The increase in air temperature associated to climate change is expected to affect olive (*Olea europaea* L.) tree fructification. To determine the potential effects of global warming on floral phenology and fruit set under field conditions, olive trees growing in a Mediterranean climate type area were subjected to warmer temperature than ambient by the use of temperature controlled Open-top-Chambers (OTCs). Each OTC, equipped with heating and ventilation devices, was able to maintain a day/night temperature gradient between the tree and the surrounding environment of 4 °C throughout the complete reproductive cycle of this species. After three years of study, the results obtained have shown that increasing temperature 4 °C above the actual ambient temperature may led to an advance of the date of flowering in the olive, an extent of the flowering period, an increase of pistil abortion, and a reduction in fruit set, conditions which may reduce yield.

1. Introduction

The Mediterranean Basin has been considered as one of the most vulnerable regions to climatic changes in the near future (Giorgi, 2006; Lionello, 2012). Climate experts have anticipated an increase in air temperature in the range of 2–5 °C (Giorgi, 2006; Giannakopoulos et al., 2009; Gualdi et al., 2013; IPCC, 2014). In addition, more frequent and extreme events such as drought periods and heat-waves will be observed in this region (Giorgi and Lionello, 2008; Tanasijevic et al., 2014). Temperature is a primary environmental factor controlling plant growth and development, so changes in seasonal temperatures can affect the biological processes of the reproductive cycle of Mediterranean crops, affecting crop production. The olive (*Olea europaea* L.) is one of the most emblematic crops in the Mediterranean Basin having a great economic, social and cultural impact. This species is widely spread and well adapted to the rustic conditions of the Mediterranean region, but a constant increase in ambient temperature may affect the phenology of this crop and, consequently, may reduce yield.

Floral phenology is a critical process in determining final yield. Olives flower on one-year-old wood and display two inflorescences per node or, exceptionally, directly at the shoot apex. The inflorescences contain a mixture of perfect (hermaphroditic) and imperfect (staminate) flowers. The former group contains well-developed stamens and pistil while in the latter a residual atrophied pistil is often visible in the center as a consequence of varying degrees of pistil abortion (Cuevas and Polito, 2004; Reale et al., 2006). Flower bud induction is manifested by July in the northern hemisphere (Fernández-Escobar et al., 1992), and floral differentiation is evident by March (Hartmann, 1951). Anthesis occurs by May. Shortly after anthesis, massive abscission of flowers and fruits occurs (Rallo and Fernández-Escobar, 1985). The remaining fruits usually persist on the tree until harvest, which takes place during the fall and winter (October-February) depending on the year and the cultivar. The olive tree exhibits a strong alternate bearing phenomenon.

Olive produces an abundant number of flowers but only a small percent (1–2%) of them set normal fruits that reach maturity (Martin, 1990; Lavee et al., 1996). This phenomenon is partly influenced by flower quality. It is not clear if warmer temperatures during pre-flowering and flowering phenophases could modify any morphological or developmental characteristics of the flower affecting its ability to set and form a fruit. Although it has been informed that environmental factors and cultural practices have a significant effect on floral quality (Hartmann, 1950; Uriu, 1953, 1960), there are not references related to temperature effect. Some studies have reported that poor N nutritional

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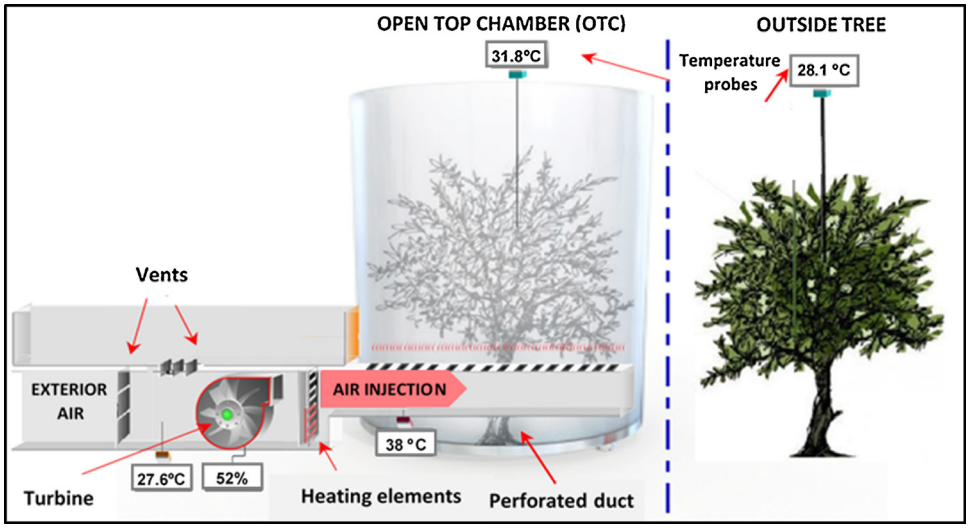

Fig. 1. Scheme of functioning of the Open Top Chamber.

status, low leaf-to-bud ratio and water deficit (Fernández-Escobar et al., 2008; Uriu, 1956, 1960; Cuevas and Polito, 2004; Reale et al., 2006; Rapoport et al., 2012) favor pistil abortion. There is also a cultivar-related tendency for producing imperfect flowers (Campbell, 1911; Morettini, 1950; Lavee et al., 1996; Rosati et al., 2011). On the other hand, the success of a flower to become a fruit not only depends on its quality but also on the pollination and fertilization processes. Studies that have analyzed the effect of temperature on pollen germination and pollen tube growth have reported that both are sensitive to elevated temperatures (Fernández-Escobar et al., 1983; Vuletin Selak et al., 2013). Constant temperatures above 33 °C after fertilization may affect fruit setting (Graniti et al., 2011) and subsequent fruit development (De Andrés Cantero, 2001).

Regional Climate Models and olive-pollen capture tramps have been used for long to predict olive floral timing in the Mediterranean region in the future. It seems that olive flowering date will be modified by the increase in air temperature (García-Mozo et al., 2010; Oteros et al., 2013): the development of floral structures will be completed faster leading to earlier flowering dates (Osborne et al., 2000; Giannakopoulos et al., 2009; Orlandi et al., 2010; Aguilera et al., 2015). However, long-term temperature field experiments are necessary to verify these results. Open top chambers (OTCs) are very useful tools for the simulation of global change under field conditions (Allen et al., 1992; Ceulemans and Mousseau, 1994; Norby et al., 1997). These systems are able to simulate real conditions of global warming including daily and seasonal fluctuations in weather. They allow trees to grow in the ground for several growing seasons avoiding the potential negative effects of container artefacts: root mechanic stress or the increase in soil temperature above ambient temperature due to containers overheating.

There is an actual tendency to compare floral phenology in olive orchards located in geographical regions differing in annual mean temperatures. But considering that floral events not only are governed by mean temperature but also by seasonal temperature fluctuations and the photoperiod along the different annual seasons, the results from these studies may lead to misunderstandings. These variables are difficult to be the same in different geographical regions because they partly depend on latitude and altitude.

The aim of this study was to determine under field conditions, in a Mediterranean climate area in which temperature and photoperiod fluctuations occur along the year, the effect of 4 °C increase in air temperature on olive floral phenology and fruit set. To reach this goal temperature controlled Open-Top-Chambers were used. This study is an approach to global warming effects on olive inflorescences characteristics, timing of the different events during flowering and fruit setting in the near future.

2. Material and methods

2.1. Plant material and growth conditions

'Picual' olive trees (*Olea europaea* L.) growing in a experimental farm of Campus de Rabanales, University of Córdoba, Spain (37°55′N 4°43′W) were used for the experiment. The orchard soil is classified as Calcic Luvisols with a clay-loam to clay texture, pH moderately alkaline (7–8), organic matter around 2%, and moderate to high cation exchange capacity (Del Campillo et al., 1993). The trees were planted in autumn 2009 spaced 8 x 6 m apart, with a drip irrigation system. Depending on the season, water was applied over five to six months during the dry season. The experiment was conducted from 2014 to 2017.

2.2. Experimental design and treatments

To perform this study sixteen trees were selected from the experimental farm. Trees were subjected to two temperature treatments, ambient temperature (AT) and 4 °C above ambient temperature (AT + 4 °C), along three consecutive years. The experimental plot was divided into four blocks containing each one two trees (replicates) per temperature treatments (AT vs. AT + 4 °C) which were randomly distributed. To maintain trees at 4 °C above ambient temperature, eight temperature-controlled Open Top Chambers (OTC), specifically designed for this study, were constructed around the trees (Fig. 1). Each OTC (hexagonal prism-shape), consisting of a steel frame covered by panels (panel size: 1.80 cm width x 3.60 cm height) of plastic film of high transparency (transmission: 90–85% of the solar photosynthetically active radiation), was equipped with heating and ventilation devices regulated by an automaton. In this way, a constant day/night temperature gradient between the tree and the surrounding environment of 4 °C was maintained throughout the complete reproductive cycle of this species.

The 4 °C thermal gradient was maintained by two mechanisms that operated independently. 1) A close circuit came into operation at night or under low solar radiation conditions to inject heat to the chamber. This circuit recirculates the air from the chamber through a turbined equipped with electric heating elements (Fig. 1). An automaton regulates the warm air flow by controlling the turbine speed and the number of electric heating elements which comes into operation. 2) During the day, under high solar radiation conditions, the excess of heat

accumulated in the chamber was partly eliminated by the injection of air from outside. The automaton selects one or another mechanism, acting on the closing or opening of two vents placed in the system (Fig. 1). The information processed by the automaton was provided by a temperature probe placed at the middle position of the tree canopy, both in control trees (TA) and those confined in the chambers (TA + 4 °C). The air injected into the chamber diffuses upwards along the tree from a 35 cm diameter duct, resting on the ground, provided with circular perforations (4 cm in diameter) (Fig. 1). The system provides a uniform air-flow of low flow once it reaches the branches of the tree.

2.3. Measurements

Fifteen uniformly distributed bearing shoots per tree were selected and tagged each spring. Shoot length was recorded at this time. Flowering was determined visiting periodically the trees from late February until the end of the flowering period, and recording the phenological stages described by Colbrant and Fabre (1975). The time of flowering was determined according to the method described by Fleckinger (1954). Flowering intensity per tree was determined using a visual scale from 0 (no flowering) to 3 (maximum flowering) at full bloom (FB).

The number of inflorescences and flowers per inflorescence were counted in each tagged shoot before anthesis. Fertile inflorescences and the number of perfect flowers per inflorescence were determined before petal fall. Fertile inflorescences were considered those with at least one perfect flower (Rallo and Fernández-Escobar, 1985). The number of fruits per tagged shoot was determined 40 days after full bloom (FB), when June drop is finished and fruit population is maintained until harvest (Rallo and Fernández-Escobar, 1985).

A fresh sample of four fully expanded leaves per tree was collected at noon at different times along the reproductive cycle of the olive to determine the leaf relative water content (RWC). Leaves were weighed, rehydrated over 24 h, and re-weighed, according to the procedure reported by Stocker (1929).

2.4. Statistical analysis

Analyses of variance were performed on the data using Statistix 9.0 software package (Analytical Software, Tallahassee, FL, USA). In all analyses, residual plots were generated to identify outliers and to confirm that variance was common and normally distributed. All percentage values were transformed using the arcsin of the square root before analysis. Non-parametric Friedman test was used to compare flowering intensity.

3. Results

The temperature gradient of around 4 °C between the air inside of the Open Top Chambers (OTC) and the surrounding environment, were maintained relatively constant from January to July of each year (Fig. 2). These results validate the treatments, indicating that the experimental trees were effectively subjected to different temperatures regimes.

Under these conditions, trees exposed to 4 °C above the ambient temperature (AT + 4 °C) initiated flowering between 18 and 24 days before than control trees, those exposed to the ambient temperature (AT), depending on the year (Fig. 3). Also, the flowering period and the full bloom period were between 5 and 7 days and 2–3 days longer, respectively. Flowering intensity, expressed as the amount of flowers per tree, estimated on a visual scale of 0 (non-flowering) to 3 (abundant flowering), did not show significant differences between temperature treatments (Fig. 4).

Flower quality was affected by warm temperatures (Table 1). The percentage of perfect flowers and fertile inflorescences significantly

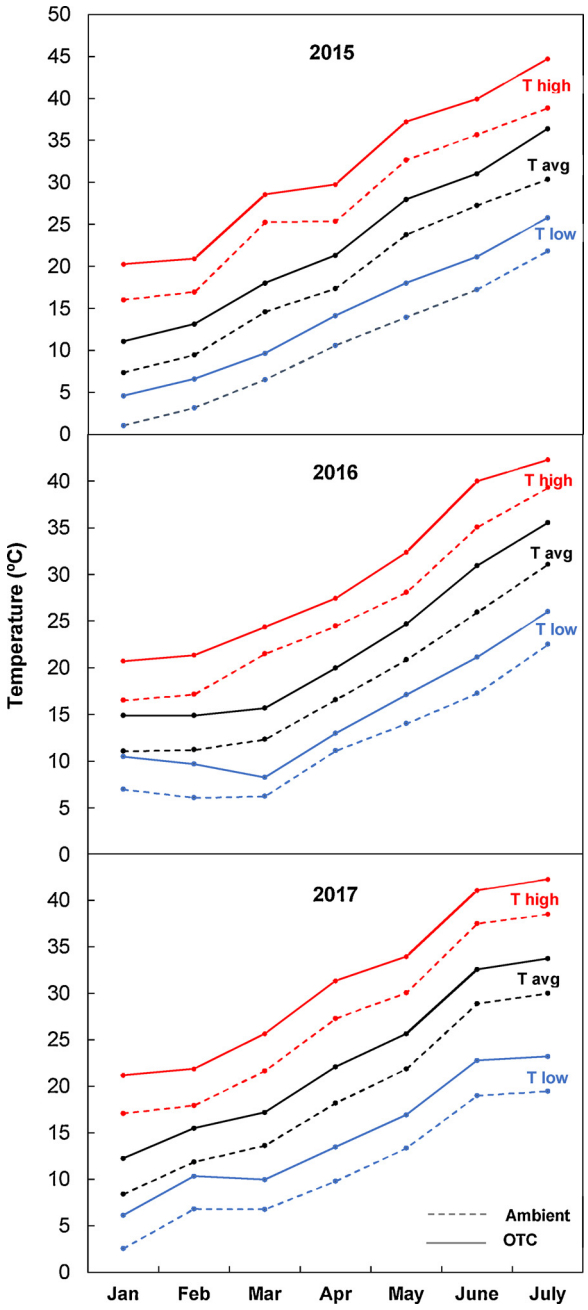

Fig. 2. Average monthly temperatures recorded from January to July during three consecutive years (T_{avg}). Dotted lines show ambient temperatures and the straight lines show OTC temperatures. T_{high} and T_{low} represents average high and low temperatures by month respectively.

decreased in AT + 4 °C trees in 2015 and 2016. No effect was observed in 2017. In 2015 pistil abortion was very intense even in control trees, which produced only 23.9% of fertile inflorescences and 5.9% of perfect flowers. On the contrary, in 2016 pistil abortion was very low, with 93.9% of fertile inflorescences and 56.4% of perfect flowers in AT trees. An intermediate behavior was observed in 2017.

Fruit set was also affected by the treatments. Expressing fruit set as the percentage of fruits per flower or per inflorescence, and also if it is expressed as fruit per cm of bearing shoot, fruit set significantly decrease in AT + 4 °C trees (Table 2). The only exception was in 2016, when fruit set was expressed as percent of fruits per perfect flowers.

The relative water content (RWC) of leaves was significantly lower in AT + 4 °C trees in winter 2015 and in spring 2016, and unaffected by

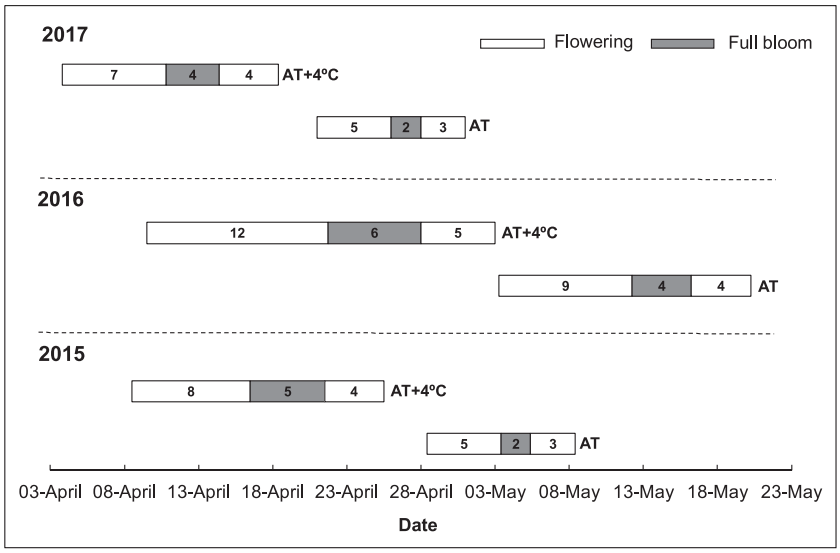

Fig. 3. Flowering period under ambient temperature (AT) and 4 °C above ambient temperature (AT + 4 °C) in three consecutive years. Inside of each bar from left to right it is represented the periods of time (days) from the start of flowering to the start of full bloom, from the start of full bloom to the end of full bloom, and from the end of full bloom to the end of flowering.

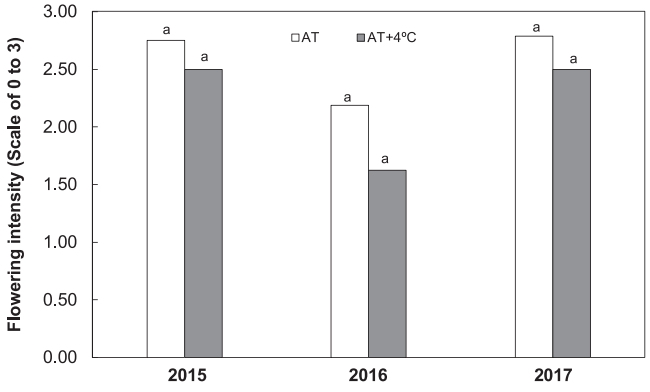

Fig. 4. Effect of a 4 °C increase in ambient temperature (AT vs. AT + 4 °C) on the amount of flowers produced by the trees in three consecutive years. Flowering rating: scale from 0 to 3 (none-very abundant flowering). Mean separation within each year by non-parametric Friedman test.

the treatments at these times in 2017 (Table 3). These results indicate that the AT + 4 °C trees was stressed around floral differentiation in 2015 and 2016 compared to control trees. During the summer, there were significant differences between treatments in all the years.

4. Discussion

The evaluation of the effect of global warming on the behavior of agricultural crops results of interest since an increase of temperature is expected in the near future (IPCC, 2014). For that reason, many works have been developed to study the responses of different crops to temperature. Usually, these studies have been realized contrasting the behavior of these crops in different environments (El Yaacoubi et al., 2014), that is, in different climatic regions, or developing models to predict the behavior of a particular crop (De Melo-Abreu et al., 2004). In the present work, we evaluated the effect of global warming on olive floral phenology and fruit set installing, under field conditions, temperature controlled open top chamber (OTC) systems, which maintain inside, almost constantly, 4 °C above the ambient temperature. That the system works is shown in Fig. 2. The advantage of this system is that allows observing the effect of an increase of temperature in each particular region. Data from the behavior of a particular crop in another region combines both the effect of an increase of temperature and also the other climatic particularities, which probably will not happen in another region.

Under the conditions of the experiment, it has been observed that an increase of 4 °C in air temperature induced an advance of the date of flowering. This effect was predicted to occur in southern Spain by Aerobiological studies and Regional Climatic Circulation Models (Galán et al., 2005; Osborne et al., 2000; Pérez-López et al., 2008; Giannakopoulos et al., 2009; Orlandi et al., 2014; Gabaldón-Leal et al., 2017). But our data also showed that these increase in air temperature extent the flowering period in about six days, when comparing with control trees growing at ambient temperatures. The flowering period is usually shortened when temperature increase during flowering and it is longer with lower temperatures (Rallo, 1997). A possible explanation to the extent of flowering in AT + 4 °C trees, could be that despite an

Table 1
Effect of a 4 ºC increase in ambient temperature (AT vs. AT + 4 ºC) on flower quality.

Temperature treatment	Flower quality[a,b]											
	2015				2016				2017			
	Flowers		Inflorescences		Flowers		Inflorescences		Flowers		Inflorescences	
	Total	Perfect (%)	Total	Fertile (%)	Total	Perfect (%)	Total	Fertile (%)	Total	Perfect (%)	Total	Fertile (%)
AT	110.5 a	5.9 a	10.5 a	23.9 a	113.4 a	56.4 a	9.1 a	93.9 a	137.1 a	11.5 a	11.8 a	40.4 a
AT + 4ºC	79.2 b	1.4 b	10.3 a	6.4 b	86.4 a	23.4 b	8.2 a	75.6 b	113.6 b	12.6 a	12.1 a	39.5 a
[c]*CV (%)*	22.3	39.7	8.2	32.9	23.7	5.9	22.7	5.5	11.1	48.1	8.4	36.8

[a] Means within each column followed by different letters are significantly different at $P \leq 0.05$ by F-test.
[b] Each data is the mean of eight trees and was obtained from 15 bearing shoots per tree.
[c] Coefficient of variation.

Table 2
Effect of a 4 ºC increase in ambient temperature (TA vs. TA + 4ºC) on fruit set determined 40 days after FB.

Temperature treatment	Fruit set[a,b]								
	2015			2016			2017		
	Fruit/cm	Flowers (%)	Inflorescences (%)	Fruit/cm	Flowers (%)	Inflorescences (%)	Fruit/cm	Flowers (%)	Inflorescences (%)
AT	0.17 a	14.5 a	31.1 a	0.70 a	10.5 a	75.6 a	0.35 a	26.8 a	59.4 a
AT + 4°C	0.01 b	1.1 b	1.9 b	0.23 b	10.7 a	34.0 b	0.16 b	8.1 b	23.8 b
[c]*CV (%)*	*64.9*	*52.6*	*66.8*	26.5	14.8	11.2	*19.8*	*17.3*	*17.5*

[a] Means within each column followed by different letters are significantly different at $P \leq 0.001$ by F-test.
[b] Expressed as number of fruits per cm of bearing shoot or as the percentage of perfect flowers or fertile inflorescence. Each data is the mean of eight trees, and was obtained from 15 bearing shoots per tree.
[c] Coefficient of variation.

Table 3
Effect of a 4 °C increase in ambient temperature (TA vs. TA + 4 °C) on leaf relative water content (RWC).

Temperature treatment	Leaf RWC (%)[a]								
	2015			2016			2017		
	Winter	Spring	Summer	Winter	Spring	Summer	Winter	Spring	Summer
AT	93.2 a	–	89.3 a	89.1 a	92.6 a	87.8 a	88.9 a	90.7 a	90.6 a
AT + 4°C	89.6 b	–	86.9 b	88.4 a	90.5 b	86.1 b	88.2 a	91.0 a	89.4 b
[b]*CV*	*1.3*	–	*1.37*	*1.88*	*0.8*	*1.26*	*1.43*	*1.08*	*0.78*

[a] Means within each column followed by different letters are significantly different at $P \leq 0.05$ by F-test.
[b] Coefficient of Variation.

increase of temperature of 4 °C, the advancement of the date of flowering to the beginning of April can make that flowering occurs with lower temperatures than flowering in control trees. However, according to the data recorded in Fig. 2, the average temperatures in April inside the OTCs were similar to external May temperatures, when control trees bloomed. We have no explanation for this disagreement, unless than other factors may affect the extension of the flowering period, since this effect was observed in all the years.

Percentage of perfect flowers is important for olive yield. This characteristic has an important varietal component, but it is influenced by environmental conditions, particularly by water deficit during flower differentiation (Uriu, 1960; Hartmann and Panetssos, 1961; Rapoport et al., 2012). In our experiment, pistil abortion occurs in 2015 and 2017 in both AT + 4 °C trees and AT trees, probably because the low rainfall at the end of the winter in both years. Very low abortion was observed in AT tree in 2016, when rainfall was higher than in the other years. But in both 2015 and 2016, pistil abortion was significantly higher in AT + 4 °C trees, and no significant differences were observed in 2017. The relative water content (RWC) of leaves was significantly different in AT and AT + 4 °C trees in winter 2015 and spring 2016, suggesting a different water status of these trees in dates close to floral differentiation, which may explain the differences observed in pistil abortion in these years. No differences in water status were observed in winter or spring of 2017 between AT and AT + 4 °C trees, which may explain the lack of differences in pistil abortion in this year.

In addition to the reduction of perfect flowers and fertile inflorescences in AT + 4 °C trees, characteristics which may reduce yield, fruit set was also significantly reduced in these trees. The high temperature treatment may influence flower fertilization, resulting in a poor fruit setting. In other species, temperature affects stigma receptivity (Burgos et al., 1991), ovule longevity (Postweiler et al., 1985) and pollen tube growth (Lewis, 1942; Williams, 1970). In the olive, the optimum temperature for pollen germination and pollen tube growth has been observed to be 25 °C. When temperature is above 30 °C, these processes fail affecting fruit setting (Fernández-Escobar et al., 1983; Cuevas et al., 1994). Temperatures above 30 °C have been reached during the pollination period under the experimental conditions of this study, and may have limited flower fertilization and fruit set. Cross-pollination, that is, the pollination with pollen of a different cultivar, allow a greater pollen germination and pollen tube growth at higher temperatures than the own pollen (Fernández-Escobar et al., 1983), suggesting a higher fruit set under these conditions. In fact, the need for cross-pollination in the olive has been reported in areas of Israel, Argentina or Mexico (Rallo and Cuevas, 2017), where temperatures during olive flowering are usually higher than in southern Spain. Results suggest that, under an increase of temperature during flowering, cross-pollination may be a technique to increase fruit set and, consequently, yield.

In conclusion, increasing temperature 4 °C above the actual ambient temperature may led to an advance of the date of flowering in the olive, an extent of the flowering period, an increase of pistil abortion, and a reduction in fruit set, conditions which may reduce yield. These results suggest that it could be necessary to reduce trees stress during the winter, probably providing irrigation water during floral differentiation or by prolong the irrigation period during the autumn and winter. Also, cross-pollination could be necessary to guarantee a normal fruit set and productivity.

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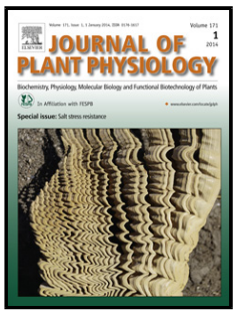

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Effects of olive root warming on potassium transport and plant growth

Running title: Influence of high root medium on K^+ transport

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Abstracts

Young olive (*Olea europaea* L.) plants generated from seed were grown in liquid hydroponic medium exposing the roots system for 33 days or 24 h to high temperature (37 ºC) while the aerial part to 25 ºC aiming to determine the prolonged and immediate effects of root warming on K^+(Rb^+) transport in the root and consequently on plant growth. The exposition of the root system to 37 ºC for 24 h inhibited K^+ (Rb^+) transport from root to shoot having no effect on its uptake. However, when the root system was exposed permanently to 37 ºC both the K^+ (Rb^+) uptake and translocation to the aerial part were inhibited as well as the growth in all plants organs. The ability of the root system to recover K^+ (Rb^+) uptake and transport capacity after being exposed to high temperature was also evaluated. Plants grown in a root medium at 37 ºC for 31 days were transferred to another at 25 ºC for 48 or 96 h. The recovery of K^+ (Rb^+) root transport capacity after high root temperature was slow. Any signal of recovery was

observed after 48 h without stress: both potassium root uptake and subsequent transport to above organs were inhibited yet. Whereas 96 h without stress led to restore potassium upward transport capacity although the uptake was partially inhibited yet. The results obtained in this study have shown that the root system of young olive plants is very sensitive to high temperature related to root potassium transport and growth of the plant. Taking into account the two processes involved in root potassium transport, the discharge of K^+ to the xylem vessels was more affected than the uptake at the initial phase of high root temperature stress. However, it was the first process to be re-established during recovery. All this could explain the symptoms frequently observed in olive orchards when dry and high temperature spells occur: a reduction in shoots growth and leaves with low levels of potassium contents and dehydration symptoms.

Keywords: Climate change; *Olea europaea* L; Potassium transport; Root warming; Rubidium uptake; Vegetative growth

Introduction

The Mediterranean basin is the largest area in the world with a specific climate for olive (*Olea europaea* L.) cultivation; however, if current trends in greenhouse gas emissions continue, the environmental conditions of this region are expected to change in the near future. In particular, air temperature has been projected to rise drastically (Giorgi, 2006; Giorgi and Lionello, 2008; Gualdi et al., 2013). Soil temperature is generally lower than that of the air, although seasonal fluctuations occur depending on aboveground factors. The increase in atmospheric temperature is therefore expected to be accompanied by a gradual rise in soil temperature, especially in the upper soil layers (IPPC, 2014).

Temperature is a primary environmental factor for plant growth and development. Each species has an optimal thermal range, so when temperature lies outside this range, the physiological and biochemical processes involved in plant growth are impaired, resulting in growth decline (Mahan et al., 1995; Wahid et al., 2007). The optimum temperature for olive vegetative development ranges from 10 to 30 ºC, provided that nutrient and water availability is not a limiting factor. It has been

suggested that temperatures above 35 ºC could limit olive vegetative growth (Rallo and Cuevas, 2008; Therios, 2009). Recently, a reduction in plant dry matter accumulation has been observed in olive mist-cuttings and young plants generated from seeds, when the whole plants were exposed to moderately high temperatures (37º C) (Benlloch-Gonzalez et al., 2016). Considering that temperature values in the Mediterranean region are predicted to exceed the optimal levels for olive performance, this crop is likely to experience frequent periods of temperature stress of both long and short duration, which may affect growth, development and productivity.

Most of the studies addressing plant adaptation to warmer temperatures have been focused on plant responses to increasing air temperature (Paulsen, 1994; Wahid et al., 2007) and pay little attention to the underlying processes which occur when the soil warms up. Given that the microbial activity, mineralization processes and movement of ions in the soil are temperature-dependent (Zak et al., 1999; Hussain and Maqsood, 2011), higher temperatures may contribute to loss of soil fertility and diminish the availability of nutrients for plants (St Clair and Lynch, 2010). The scarcity of soil water and mineral nutrients under warmer conditions could negatively affect crops, as they are primary soil resources for plant growth. Among the essential mineral nutrients, K^+ is directly involved in plant growth processes: its accumulation in the cell contributes to the creation of the osmotic component of the water potential needed to absorb water (Kramer, 1983) and generates the cell turgor required for cell elongation (Wyn Jones et al., 1979; Mengel and Arneke, 1982; Hsiao and Läuchli, 1986; Shabala and Lew, 2002; Chen et al., 2007). At the whole plant level, K^+ plays a key role in the regulation of water movement through the plant: it is involved in the osmotic absorption of water by the root (Läuchli, 1984) and in the control of transpiration (Hsiao and Läuchli, 1986). So when a plant is supplied adequately with K^+, its tissues are better hydrated (Mengel and Kirkby, 2001), which favors plant growth and resistance to unfavorable environmental conditions, including heat stress (Gur and Shulman, 1971; Gur et al., 1976; Cakmak, 2005; Zörb et al., 2014). From this information, it can be deduced that the root system may play a critical role in the plant's adaptation to warmer conditions by absorbing enough K^+ to keep the cytosolic K^+ at its optimal level.

It seems that high soil temperature has a greater detrimental effect than high air temperature on the decline in plant growth and mineral nutrient accumulation in various species (Ruter and Ingram, 1990; Udomprasert et al., 1995; Huang and Xu, 2000; Xu

and Huang, 2000a,b). However, the influence of high soil temperatures on root growth and functioning and the impact on root-shoot relationships has not been researched in depth (BassiriRad, 2000; Huang et al., 2012). This is of great importance in olive orchards, where the irrigation systems commonly used mean that the most active roots are located in the upper soil layers (Fernandez and Moreno, 1999) which are the most exposed to high temperatures, because these soils tend to have poor plant cover. Despite growing recognition of this fact, there is little information on how warmer soil temperatures will affect olive root growth and the uptake of K^+ and its subsequent allocation to the different plant organs, which results in plant growth. The accumulation of a high level of K^+ in plant tissues under warmer conditions would support both the growth of developing organs and the transpiring leaf area, contributing in this way towards alleviating the possible detrimental effects of high temperatures.

Plants are able to adjust to variations in the availability of K^+ through changes in root architecture and the activation or inhibition of K^+ transporter systems (Nieves-Cordones et al., 2014). Nevertheless, the potential effects of high temperature on these mechanisms and the consequences on plant K^+ nutrition are not clear. The movement of ions through the cell membranes seems to be very sensitive to changes in soil or root temperature (Chapin, 1974; BassiriRad et al., 1993). As temperatures rise, membrane protein-transporters change their configuration (Epstein and Bloom, 2004). A reduction in the absorption of K^+ (Rb^+) has been observed in whole sunflower plants, isolated corn roots, and tomato plants when the temperature of the root medium was above 33, 30 and 35 °C respectively (Benlloch et al., 1989; Bravo-F and Uribe, 1981; Falah et al., 2010). In a recent study using tomato plants, the reduction in root K^+-uptake rate under high root medium temperature was correlated to a decrease in the concentration/activity of the potassium transporter KT1 (Giri, et al., 2017). In creeping bentgrass, exposure of the root system to high soil temperature (35 ºC), while maintaining the shoot at normal temperature significantly reduced potassium uptake by the root (Huang and Xu, 2000).

In summary, the olive root system will be exposed to short and long periods of high temperature stress in the future, however, how this organ will cope under these circumstances is unknown. It is not clear how high temperature in the root will affect K^+ transporter systems involved in K^+ nutrition and the possible effects on plant growth. In order to clarify these questions, the aim of the present study using young olive plants generated from seeds is twofold. Firstly, it examines the immediate and prolonged

effect of moderately high temperature applied in the root medium on K^+ (Rb^+) uptake and its subsequent allocation to the different plant organs and, consequently, plant growth. Secondly, it evaluates if the K^+ (Rb^+) uptake and transport capacity of the root system is recovered after being exposed to a period of high temperature. Interpreting root responses to temperature is often a complicated task, due to interactions with experimental conditions and surrounding environmental factors. Therefore, in this study, root medium and shoot air temperatures were controlled independently and the nutritional status of the root surface was maintained relatively constant. The plants were grown in a liquid hydroponic system, to ensure that potential changes in root functions were not due to any alterations in the nutrient supply.

Material and methods

Plant material and growth conditions

Olive (*Olea europaea* L.) 'Arbequina' seeds were soaked in a Ziram fungicide solution (2 g L^{-1}) for 5 min and stratified on filter paper moistened with water, in covered petri-dishes, at 14 ºC for 21 days. After stratification, the seeds were sown in recipients with perlite, moistened with water and placed in a germination chamber at 25 ºC. After 10 days, the seedlings were individually transferred into 680-mL flasks containing a Hoagland's type nutrient solution (NS) and placed in a controlled growth chamber with a relative humidity between 60 and 80%, a temperature of 25/22 ºC (day/night), a photosynthetic photon flux density of 350 µmol m^{-2} s^{-1} (fluorescent tubes, Sylvania cool-white VHO) and a photoperiod of 14 h of light. The NS had the following composition: 2.5 mM $Ca(NO_3)_2$; 2.5 mM KCl; 0.25 mM $Ca(H_2PO_4)_2$; 1.0 mM $MgSO_4$; 12.5 µM H_3BO_3; 1.0 µM $MnSO_4$; 1.0 µM $ZnSO_4$; 0.25 µM $CuSO_4$; 0.2 µM $(NH_4)_6Mo_7O_{24}$ and 10 µM Fe-ethylenediamine-di-o-hydroxy-phenylacetic acid. The NS was continuously aerated and renewed every week during the whole experiment. The plants were grown under these conditions for acclimation between 14-17 days, depending on the experiment, before the root temperature treatments were started.

Root temperature treatments

After acclimation, the root system of a group of plants was maintained at the ambient temperature of the growth chamber (25 ºC), while in other the temperature of the root medium was increased to 37 ºC. To reach this temperature in the root medium, the flasks were placed in a water bath at 37 ºC positioned in the growth chamber. An

immersion-heater regulated by a thermostat kept the roots at the desired temperature. To maintain a constant temperature of 37 ºC in the entire root zone of the plants, the water level was kept at the top edge of the bath throughout the experimental period. In all the plants, the aerial part was exposed to the ambient temperature of the growth chamber (25 ºC). The plants were kept under these conditions for 33 days. In order to determine the immediate effect of high temperature in the root system, a set of plants grown in the root medium at 25 ºC, 24 h prior to the end of the experiment, was introduced in a water bath at 37 ºC. In this way, three root temperature treatments were applied: 25 ºC, 37 ºC and 25/37 ºC (24 h) with the aerial part kept at 25 ºC in all cases. To study the recovery capacity of the root system after being exposed to a high temperature period, another experiment was performed. Firstly, the root system of the plants was exposed to either 25 or 37 ºC, with the aerial part kept at ambient temperature (25 ºC), for 31 days. The same procedure described above was followed. After that, those plants whose root systems were exposed to 37 ºC were taken out of the water bath and kept at the ambient temperature, 25 ºC, for recovery for either 48 h [37/25 ºC (48 h)] or 96 h [37/25 ºC (96 h)].

Rb^+ root absorption assays

Rb^+ was used as an analogue of K^+ in order to distinguish between the uptake of K^+ by the root and the K^+ content of the plants. Rb^+ has been widely used as a tracer for K^+ in mineral nutrition studies (Erlandsson et al., 1978; De La Guardia et al., 1985; Benlloch et al., 1989).

In all cases, the nutrient solution of all the plants was changed for another with the same composition as the basic one, except that KCl was replaced by RbCl (2.5 mM). The plants were kept under this condition for 24 h prior to the end of the experiment. This assay was performed: 1) in plants whose root system had been exposed to 25 ºC and 37 ºC permanently and to 37 ºC for a short period (24 h) [25; 37; 25/37 (24 h) root temperature treatment, respectively] and 2) in plants which were subjected to different recovery periods from high temperature (37 ºC) in the root medium [25; 37/25 (48 h), 37/25 (96 h) root temperature treatments]. In both groups of experiments, at the end of this assay, the plants were harvested as described below.

Data collection

Once the root temperature treatments were initiated, the length of the new shoots and the primary root was measured in each plant every 7-10 day. Stem and root relative growth rates (RGR) were calculated, from day 7 after application of root temperature treatments, at different moments during the experiment using the following equation:

RGR = (ln (length at time 2) - ln (length at time 1)) / (time 2 -time 1)

After 33-35 days, the plants were harvested. Each plant was separated into leaves, stems and roots. Each organ was individually stored in an oven at 70 ºC to measure the dry matter. K^+ and Rb^+ accumulation in each plant organ was determined by atomic absorption spectrophotometry (Perkin Elmer 1100 B) after extraction from the leaves, stems and roots by ashing each tissue at 600 ºC; the ashes were then dissolved with 0.1 N HCl. The rates of Rb^+ uptake (V_{Rb}) and Rb^+ translocated to the shoot by the root were calculated from the Rb^+ accumulated in all plant organs and in the aerial organs (stem plus leaves) respectively, and the values were referred to the dry weight of the root and the time of uptake (µmol g^{-1} root DWd^{-1}).

Statistical analysis

The experimental design was a completely randomized design, with three root temperature treatments and five replicates each (n=3 x5) in both groups of experiments. The values shown are means ± SE. The data was analyzed using the Statistical 8.0 software package. The significance of the differences between the mean values was determined by analyses of variance using Tukey's test and a 5 % rejection level. In all the analyses, residual plots were generated to identify outliers and to confirm that the variance was common and normally distributed.

Results

Olive plants generated from seeds were cultivated for 33 days in a liquid hydroponic medium. During the cultivation, a group of plants was subjected to a high temperature treatment (37 ºC), aimed exclusively at the root, while the aerial part remained at 25 ºC, the same temperature as the root and aerial part of the control plants.

The high temperature located in the root inhibited the growth of the plant, although the effect was more marked in the root than in the aerial part (Table 1). The accumulation of dry matter in the root of plants under high root temperature was less than 50% of that of the control plants at the end of the experiment (Table 1). The high temperature in the root also significantly affected the accumulation of dry matter in the aerial part: the leaves were more sensitive than the stem to high root temperature with 39% and 33%, respectively, less dry matter compared with the control plants (Table 1).

The effect of the high temperature treatment applied in the root on the length of the primary root and the shoot was more marked than that observed on the accumulation of dry matter in these organs. At the end of the experiment, the reduction in the length of the primary root and stems was 80% and 50% respectively, compared to control plants (Figures 1 and. 2). In addition, the way both organs responded in time to this treatment was also different. The effect in the root was extremely rapid: 7 days after the onset of the treatments, the root relative growth rate (RGR) fell by 40% compared with the control, and continued dropping sharply until growth was stopped at day 28 (Fig.1). In the case of the stem, the differences in length were less marked: they became apparent 14 days after the onset of the treatments never actually stopping growth (Fig. 2).

At the end of the experiment, the concentration of K^+ in the different plant organs was measured (Table 1). The high temperature in the root reduced the accumulation of K^+ in all the organs, albeit unequally. The greatest reduction was observed in the leaves, followed by the root and finally the stem. 24 hours prior to the end of the experiment, the uptake of Rb^+ by the root and its translocation to the aerial organs was determined. The root systems were kept at the same temperature as during the growth period (25 and 37 °C), except for a group of control plants, whose root medium temperature was changed from 25 to 37 °C (Fig. 3). The plants were kept during this period in the same hydroponic medium used during growth, except that, in this case, K^+ was replaced by Rb^+. The effect of the high temperature in the root on the transport of Rb^+ depended on the length of the treatment. In plants grown under a 37 °C root medium for 33 days, the high temperature treatment strongly inhibited both the uptake and translocation of Rb^+ to the shoot. However, when the high temperature acted for only 24 hours, only the translocation to the aerial part was inhibited, and no effect was observed on the absorption (Fig. 3). In the first case, the accumulation of Rb^+ was

reduced in all the organs, whereas in the second, it was reduced in the stem, but favored in the root (Fig. 4).

In other experiments, the recovery capacity of potassium transport after root temperature stress was determined. The plants were grown with a constant root temperature of 25 °C or 37 °C for 31 days. Thereafter, in the treatments at 37 °C, the temperature of the root medium was changed from 37 °C to 25 °C for the last 48 or 96 hours. In all cases, the K^+ in the root medium was replaced by Rb^+ 24 hours prior to the end of the assay. After 48 hours of recovery (48 hours with the root kept at 25 °C), the uptake and translocation of rubidium was significantly lower than in the control (where the root was kept at a constant 25 °C). However, after 96 hours of recovery (96 hours with the root at 25 °C), rubidium translocation was similar to that of control, and inhibition of absorption was less pronounced. The uptake of rubidium after 96 hours of recovery was significantly lower than that of the control and greater than after 48 hours of recovery (Fig. 5).

The potassium content in the different organs analyzed at the end of the experiment showed that in the root temperature stress treatments it was significantly lower than in the control, except in the leaves of the plants given the 96-hour recovery treatment. In this case, no significant differences were observed compared with control plants (Fig. 6).

Discussion

The growing concern about climate change has drawn increased attention to the effect of high temperature on plant production. However, despite the numerous studies on plant molecular responses to high temperatures (Sachs and Ho, 1986; Queitsch et al., 2000), few have analyzed the effect of moderate high temperature in the root on growth and ionic transport. This effect is much more noticeable in the case of the olive tree. This crop has been suggested to impair its vegetative growth when the atmospheric temperature exceeds 35 °C (Rallo and Cuevas, 2008; Therios, 2009). Surprisingly, the species is extremely well adapted to areas where this temperature is regularly exceeded during the summer season. In a recent study using different olive plant material, it has been observed that the exposition of the aerial part to moderate high temperature (37 °C) did not affect the accumulation of dry matter in the plant and favored primary root growth. However, only when both the root and the aerial part were exposed to

37 ºC, the high temperature treatment was harmful (Benlloch-González et al., 2016). In that work, it was formulated the hypothesis that moderate high temperature in the root could inhibit the transport of potassium towards the aerial part.

There is no information on how olive root warming affects the transport of potassium in this organ, or whether it negatively affects the growth of the plant. Given the importance of potassium for plant growth (Marschner, 1995), and considering that the cultivation techniques used in new olive plantations favor root warming - bare soil at the base of the tree, drip irrigation with drippers and pipes close to the surface, water stored in open-air reservoirs (Alcántara et al., 2017; Orgaz et al., 2017) - it is crucial to bring to light the specific effects of olive root warming on potassium transport and plant growth. Further information is also necessary to elucidate on the one hand, whether the potential effects of root temperature stress are immediate or take time to trigger out and on the other, the recovery capacity of root potassium transport after the stress. In the present study, the exposition of the root system of young olive plants to a moderate high temperature (37 °C), with the aerial part remaining at an optimal temperature for growth (25 °C), inhibited both root and shoot growth (Table 1). Another work dealing with the same species has focused on the effect of temperature stress localized exclusively in the aerial part of the plant, and in this case, no effect was observed on the accumulated dry matter - on the contrary, it favored the elongation of the root (Benlloch-González et al., 2016). In our current work, the most significant effect of temperature stress located exclusively at the root on plant growth was the inhibition of the stem and primary root elongation (Figures 1 and 2). These results suggest that the root system of young olive plants is very susceptible to soil warming and that the perception of the temperature stress in the root triggers out signals that induce a negative regulation of growth in the aerial part. We do not know what kind of signals are generated in olive roots in response to temperature stress; however, taking into account the role of potassium in maintaining the cellular turgor needed for cell elongation (De La Guardia and Benlloch, 1980), as well as the role played by the transport of water and potassium in the root in the recovery of growth after different kinds of stress (Benlloch and Benlloch-González, 2016), it is logical to believe that the inhibition of root potassium transport under high temperature is one of the primary effects of stress. The severe inhibition of the stem and primary root elongation due to high root temperature supports this hypothesis (Fig. 1 and 2).

Two independent transport processes acting in series are involved in the transport of ion in the root from the external medium to the xylem vessels. The first one is located in the plasma membrane of epidermal and cortical cells while the second in the cells of the stele. The former allows the uptake and incorporation of ions in the symplast of the root and the latter the discharge or release of ions to the apoplast of the stele which is in contact with the xylem vessels. The joint action of both processes coupled with the effect of the Caspari Band on the endodermis cells, avoiding the apoplastic flow of water and ions, allows the selection of ions by the root and subsequent accumulation in the xylem. The upward movement of ions from the root to the aerial part is produced by mass flow created by root pressure or by transpiration (Marschner, 1995).

In the case of potassium, the mechanisms involved in uptake process are better identified than those involved in xylem loading. In *Arabidosis*, there are five types of transporters involved in absorption: the Shaker and two-pore potassium channels, potassium uptake permeases (KUP / HAK / KT), HKT and K^+/H^+ antiporters (Very and Sentenac, 2003; Gomez-Porras et al., 2012; Sharma et al., 2013). In the discharge into the xylem, only one Shaker family channel type, the Stelar K^+ Outward Rectifier (SKOR), has been identified, and its expression has been localized in the tissues of the root stele (Gaymard, 1998; Liu et al., 2006).

It is not known how high temperature in the olive root affect the transport of potassium in this organ. However, there is some information on this respect in other species, which suggest that a moderate high temperature in the root can cause a negative effect on the transport of potassium in this organ. The exposition of the root system of different creeping bentgrass cultivars to a supraoptimal temperature (35°C) throughout the growing period inhibited the accumulation of potassium in the aerial part and the root. This effect was more marked with potassium than with other mineral nutrients such as phosphorus and nitrogen (Huang and Xu, 2000). In tomato, when plants were grown in nutrient solution at high temperature (35 °C) the potassium concentration in the xylem sap was reduced, suggesting that root warming inhibited the potassium translocation towards the aerial part of the plant (Falah et al., 2010). In sunflowers, the effect of root temperature on the rate of rubidium uptake has been studied for short periods of 20-minutes. Under these conditions, it was only possible to determine the absorption of rubidium, but not the discharge into the xylem. The effect of temperature

was dependent on the potassium nutritional status. In plants with adequate potassium levels, the temperature did not affect the uptake rate of rubidium. However, in plants starved in potassium, temperatures of over 33°C sharply inhibited the uptake of rubidium, acting in both cases transporters kinetically different (Benlloch et al., 1989).

The results obtained in this work have shown that the transport of potassium in the root system of young olive plants is very sensitive to root warming. The warming of the root (37°C) uninterruptedly for 33 days caused the inhibition of the rubidium uptake rate as well as the translocation to the aerial part (Fig. 3). These results suggest that prolonged warming of the root adversely affects the transport systems involved in uptake and loading processes. It is impossible to say whether gene expression and/or activity of transporters is responsible, and even less what type of transporters are expressed in both processes. However, these results also suggest that the nature of the transporters involved in both uptake and loading is different, since they show different sensitivity to temperature changes. The occasional heating of the root (24 h), from 25 ºC to 37 ºC, inhibited the translocation of rubidium to the aerial part, but not its uptake. The absence of a rapid response in the inhibition of potassium uptake by high temperature was similar to that described in sunflower plants with adequate K-levels (Benlloch et al., 1989). However, the effect of high temperature on the discharge of potassium was rapid, which leads us to believe that the signaling routes trigger out by high temperature are different in both processes. In *Arabidosis* it has been reported that abscisic acid rapidly inhibits the SKOR expression in root stellar tissue and that this transporter is involved in the release of potassium in the xylem (Gaymard, et al., 1998). Since the presence of abscisic acid in different stress situations is well known (Lee and Luan, 2012), it is reasonable to think that in the warming of the olive root may play a role in the inhibition of potassium translocation to the aerial part, possibly inhibiting the expression of transporters of the Shaker family. On the other hand, the fact that, after heat stress, the translocation of potassium to the aerial part recovers before its absorption, supports the idea that different mechanisms are involved in the regulation of both processes, the uptake and the loading.

In conclusion, the olive is known as a species which is well adapted to arid climates with hot and dry summers; however, there is also a widespread idea that it declines growth when the ambient temperature is above 35 °C. In this work using young olive plants, it has been shown that this observation is true whenever root warming

occurs. In natural environments, with spontaneous vegetation, this situation is very uncommon, however, some cultivation practices can favor soil warming, causing harmful effects to the plant by inhibiting potassium absorption and transport to the aerial part. Along with all this, the water deficit, created by the high vapor pressure gradient between the plant and the atmosphere, together with the loss of ability to regulate stomatal closure induced by potassium starvation (Benlloch-González et al., 2008) could explain the symptoms frequently observed in olive orchards when dry and high temperature spells occur: a reduction in stems growth and leaves with low levels of potassium contents and dehydration symptoms (Fernández-Escobar et al., 1994).

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Figure legends

Fig. 1. Effect of high temperature in the root medium (37 ºC vs. 25ºC) on root length and root relative growth rate (RGR) in young olive plants. Plants were grown in hydroponic medium exposing the root system either to 25 or to 37 ºC while the aerial part to 25 º C for 33 days. Root length (cm) refers to primary root growth measured every 7-10 days from the onset of the assay. The root RGR, expressed as cm cm^{-1} day^{-1}, was calculated at 7-10 days intervals from day 7 to day 33. Full or empty symbols represent 25 or 37 ºC root temperature medium, respectively. Values are the mean of 5 replicates ± standard error.

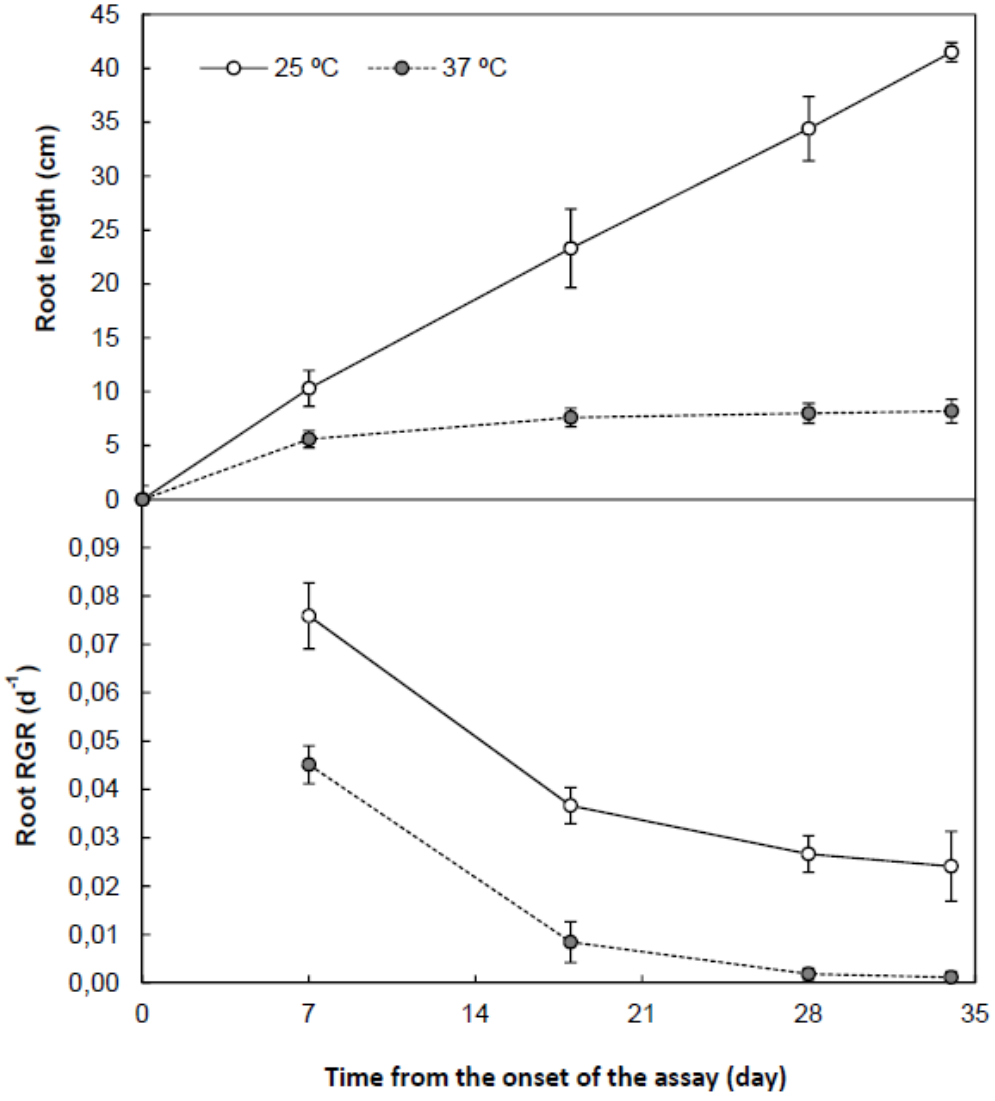

Fig. 2. Effect of high temperature in the root medium (37 ºC vs. 25ºC) on shoot length and stem RGR in young olive plants. Plants were grown in hydroponic medium exposing the root system either to 25 or to 37 ºC while the aerial part to 25 º C for 33 days. Shoot length (cm) refers to new growth generates during the assay measured every 7-10 days. The stem RGR, expressed as cm cm^{-1} day^{-1}, was calculated at 7-10 days intervals from day 7 to day 33. Full or empty symbols represent, 25 or 37 ºC, root temperature medium, respectively. Values are the mean of 5 replicates ± standard error.

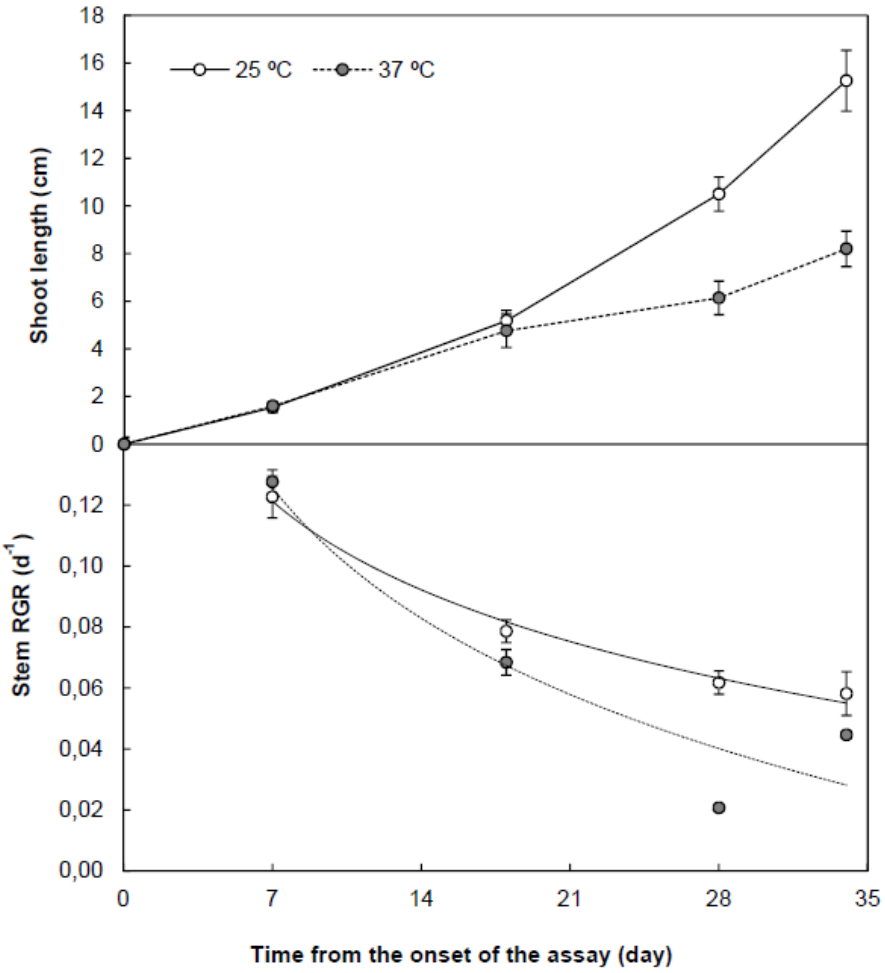

Fig. 3. Rb^+ uptake rate (V_{Rb}) and Rb^+ translocation rate in young olive plants subjected to different root temperature regimens. Plants were grown in hydroponic medium exposing the root system to 25 or to 37 ºC for 33 days and to 37 ºC for a short period (24 h) (root medium temperature (ºC): 25; 37; 25/37 (24 h)). The aerial part was maintained in all cases at 25 º C. 24 h prior the end of the assay K^+ was replaced by Rb^+ in the nutrient solution. Values are the mean of 5 replicates ± standard error. Different letters at the top of the bars indicate significant differences among root temperature treatments (P< 0.001). Means separation by Tukey´s test.

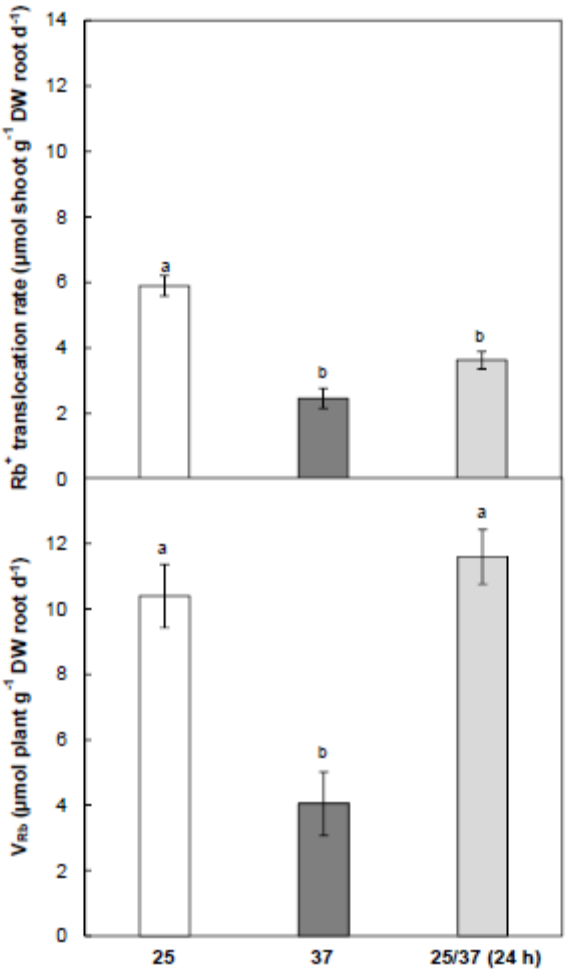

Fig. 4. Effect of prolonged and immediate high temperature in the root medium on Rb^+ accumulation in different plant organs. Plants were grown in hydroponic medium exposing the root system to 25 or to 37 ºC for 33 days and to 37 ºC for a short period (24 h) (root medium temperature (ºC): 25; 37; 25/37 (24 h)). The aerial part was maintained in all cases at 25 º C. 24 h prior the end of the assay K^+ was replaced by Rb^+ in the nutrient solution. Values are the mean of 5 replicates ± standard error. Different letters at the top of the bars indicate significant differences among root temperature treatments (Leaves and Root $P< 0.001$; Stem $P<0.05$). Means separation by Tukey´s test.

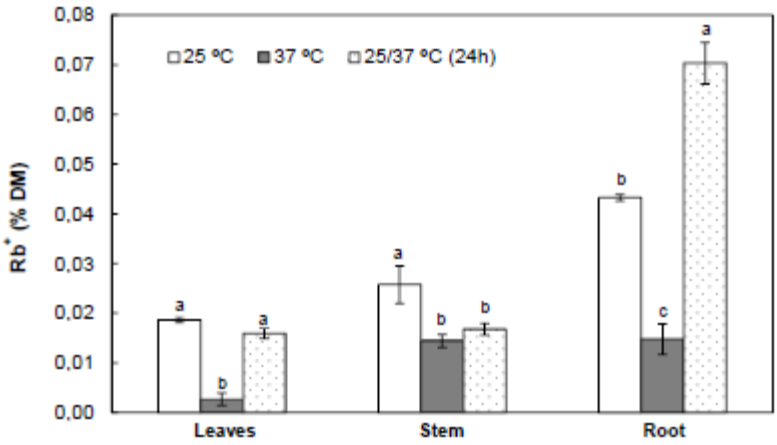

Fig 5. Rb^+ uptake rate (V_{Rb}) and Rb^+ translocation rate in young olive plants after being subjected to recovery from high root temperature stress for 48 and 96 h. Plants were grown in hydroponic medium exposing the root system either to 25 or to 37 ºC while the aerial part to 25 º C for 31 days. Thereafter, in 37 °C root temperature plants , the temperature of the root medium was changed from 37 °C to 25 °C for 48 or 96 hours (root medium temperature (ºC): 25; 37/25 (48 h); 37/25 (96 h)). 24 h prior the end of the assay K^+ was replaced by Rb^+ in the nutrient solution. Values are the mean of 5 replicates ± standard error. Different letters at the top of the bars indicate significant differences among root temperature treatments (V_{Rb} $P< 0.001$; Rb translocation rate $P<0.01$). Means separation by Tukey´s test.

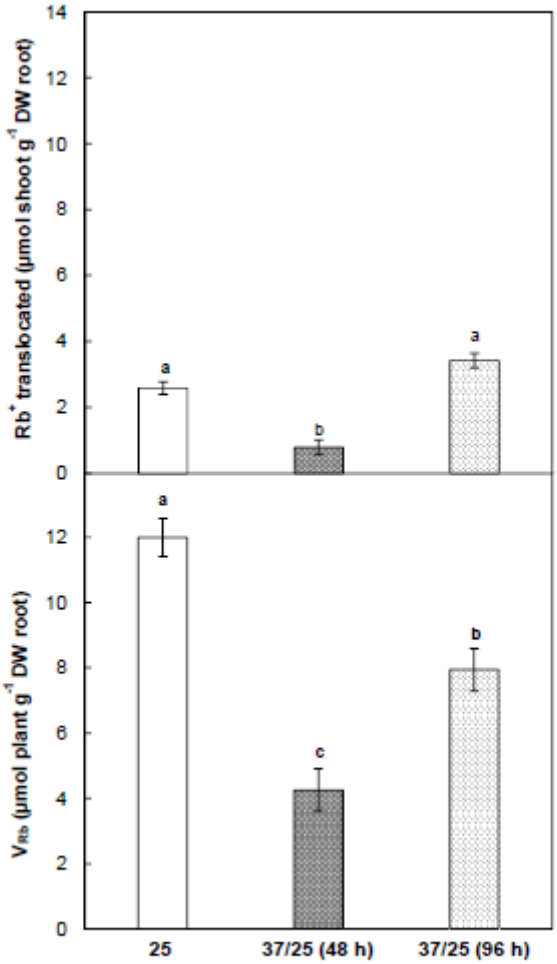

Fig. 6. K^+ accumulation in different organs of young olive plants subjected to high root temperature stress and thereafter to recovery for 48 and 96 h. Plants were grown in hydroponic medium exposing the root system either to 25 or to 37 ºC while the aerial part to 25 º C for 31 days. Thereafter, in the treatments at 37 °C, the temperature of the root medium was changed from 37 °C to 25 °C for 48 or 96 hours (root medium temperatures (ºC): 25; 37/25 (48 h); 37/25 (96 h)). Values are the mean of 5 replicates ± standard error. Different letters at the top of the bars indicate significant differences among root temperature treatments (Leaves P< 0.01; Stem and Root P<0.001). Means separation by Tukey´s test.

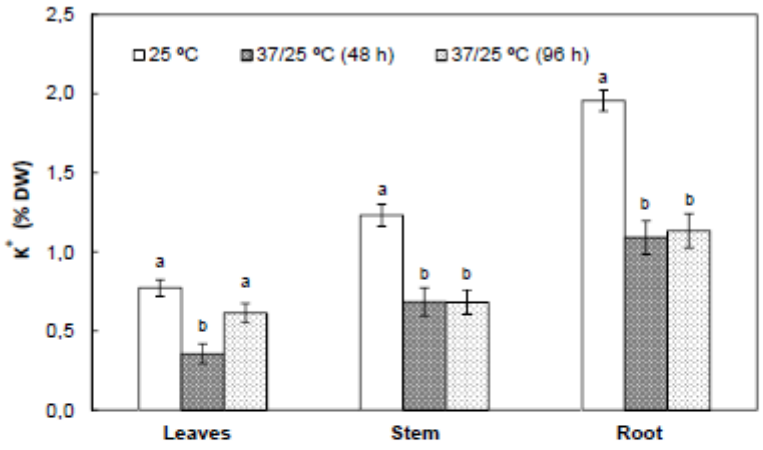

Table 1. Effect of high temperature in the root medium (37 ºC vs. 25 ºC) on dry matter (g) and K^+ concentration (% DM) in leaves, stems and root. Young olive plants were subjected to high root temperature (37 ºC) remaining the aerial part at 25 ºC for 33 days. Values are the mean of 5 replicates ± standard error. Means separation by Tukey´s test.

		Dry matter (g)				**K^+ concentration (% DM)**		
Temperature (ºC)		**Leaves**	**Stem**	**Root**		**Leaves**	**Stem**	**Root**
25		0.44 ± 0.06	0.15 ± 0.02	0.21 ± 0.02		1.17 ± 0.07	1.41 ± 0.05	1.36 ± 0.16
37		0.27 ± 0.02	0.10 ± 0.02	0.10 ± 0.01		0.43 ± 0.01	1.04 ± 0.05	0.74 ± 0.08
Significance		*P=0.025*	*ns*	*P=0.001*		*P=0.0004*	*P=0.002*	*P=0.014*