

OliveOmics – Understanding the influence of climate change on olive fruit development and olive oil: an omics' approach

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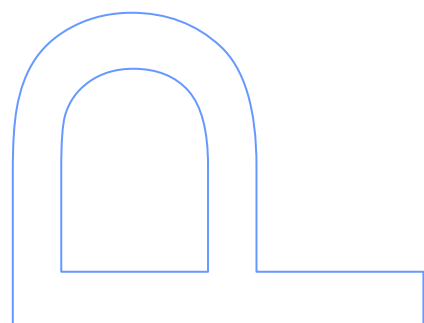
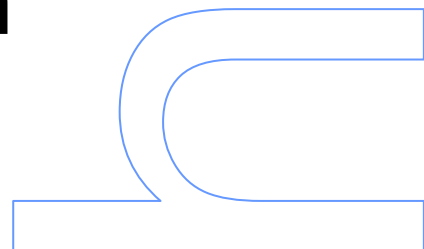
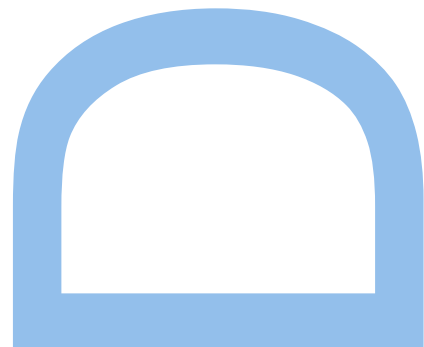
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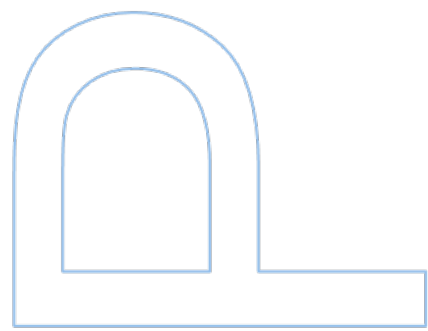
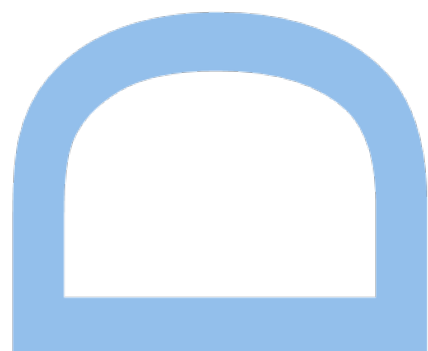
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Foreword

According to the General Regulation of Doctoral Programs of the University of Porto (number 2, 4th Article) and the Decree Law 74/2006 (Article 31, 24 of March) revised under the Decree law 230/2009 (14th of September), this thesis includes manuscripts published or in consideration for publication in peer-reviewed scientific journals. These manuscripts are the result of collaborations with several co-authors. The candidate declares that she actively contributed to the ideas and the development of the research work, including the compilation, analysis, results, discussion and writing as in its current publication form. The candidate was supported by the National Foundation for Science and Technology (FCT), through PhD grants: (SFRH/BD/116801/2016) and (COVID/BD/151706/2021). This thesis' research was developed in the context of the Doctoral Program in Biology (Faculty of Sciences, University of Porto) and the work was conducted at University of Coimbra (CFE, Centre for Functional Ecology), University of Aveiro (Department of Chemistry), University of Porto (Department of Biology, Faculty of Sciences), Polytechnic Institute of Bragança (CIMO, Mountain Research Center) and University of Trás-os-Montes and Alto Douro (CITAB, Centre for the Research and Technology of Agro-Environmental and Biological Sciences).



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Abstract

Climate change is affecting our planet, and the Mediterranean basin, the world's leading region for olive production, is being affected by the ongoing extreme climate episodes. Both olive oil and table olives are agri-food products with enormous socioeconomic impact in this region. Here it is discussed the olive socioeconomic value; olive production models, and the cultivars used in each model; the impact of heat and drought on plants and how plants are able to recover from it; and sustainable alternatives to fertilizers and pesticides aligned with the farm to fork EU strategy. This Ph.D. thesis aimed to i) characterize plant functional-plasticity to single and/or combined abiotic stresses (heat/drought) as well as the olive recovery ability, integrating functional/metabolomics/gene-expression data; ii) understand if an algae-based biostimulant (*Ascomyces nodosum*) may improve the olive tolerance to drought; and iii) understand if heat and/or drought affect in orchards the performance of plants and fruits, regarding the phenolic and lipid profiles of olive leaves, fruits and oil, and if it influences oil quality both at the physicochemical and sensory level. For that, controlled assays (greenhouse) were performed in young cv. 'Arbequina' olive trees with single and/or combined heat/drought stresses, and the recovery ability of the trees was evaluated (Chapter II) using a diverse range of physiological parameters (e.g., P_N , E , F_v/F_m), oxidative stress (e.g., MDA) and molecular transcripts of protective proteins (e.g., AQPs, HSPs). To understand the role of a biostimulant to improving the abiotic stress tolerance, young olive trees cv. 'Arbequina' and 'Galega' were tested (Chapter III). Biostimulant was applied, and drought was implemented; fruit development and their lipophilic profile by GC-MS were evaluated in two different development stages. Finally, field experiments were performed (Chapter IV) using adult cv. 'Cobrançosa' olive trees to test the effects of 2017' heat waves in Portugal. Olive trees grown in dryland farming (traditional olive groves) were exposed to drought due to the heat waves, leaves antioxidants were evaluated; leaves, fruits, and olive oil lipophilic (GC-MS) and phenolic (UHPLC-MS) profiles were assessed; and olive oil quality and organoleptic profile were evaluated. Our results demonstrate that olive trees present high plasticity to the tested abiotic factors (heat and drought) even in early development stages (Chapter II). Biostimulant application is a promising solution to counteract the effects of drought and could be a valuable solution to improve fruit quality under optimal irrigation (Chapter III). In adult olive trees, despite the increased levels of reactive oxygen species in leaves, the antioxidant battery was sufficient to counteract the stress negative effects. In fruits from adult olive trees, the stress conditions reduced some fatty acids (e.g., palmitic and linoleic acids) but increased e.g., carbohydrates, β -sitosterol, α -tocopherol and oleuropein. While in olive oil, despite the yield reduction caused by drought, the levels of β -sitosterol and oleacein increased, showing that

climate events can stimulate the accumulation of bioactive compounds that improve oil quality. This Ph.D. thesis contributes to the knowledge of how olive trees respond to heat and drought, which could be helpful to the producers to promote sustainable water management and open the potential use of biostimulants as a complement to fertilizers.

Keywords

Olea europaea, climate change, drought, heat, photosynthesis, aquaporins, dehydrins, heat shock proteins, biostimulants, *Ascophyllum nodosum*, flavonoids, secoiridoids, fatty acids

Resumo

As alterações climáticas estão a afetar o nosso planeta e a bacia do Mediterrâneo, região líder mundial na produção de azeitona, está a ser afetada pelos atuais episódios climáticos extremos. Tanto o azeite como a azeitona de mesa são produtos agroalimentares com enorme impacto socioeconómico nesta região. Nesta tese, é discutido o valor socioeconómico da oliveira; modelos de produção do olival e as cultivares utilizadas em cada modelo; o impacto do calor e da seca nas plantas e como estas são capazes de recuperar; e, são apresentadas alternativas sustentáveis aos fertilizantes e pesticidas alinhadas com a estratégia da UE - do prado ao prato. Esta tese de doutoramento teve como objetivos i) caracterizar a plasticidade funcional da oliveira a stresses abióticos isolados e/ou combinados (calor/seca), bem como a capacidade de recuperação da oliveira, integrando dados funcionais/metabolómicos/expressão génica; ii) compreender se um bioestimulante à base de algas (*Ascophyllum nodosum*) pode melhorar a tolerância da oliveira à seca; e iii) compreender se o calor e/ou a seca afetam o desempenho das plantas e frutos, no que diz respeito aos perfis fenólico e lipídico das folhas, frutos e azeite, se influencia a qualidade do azeite tanto a nível físico-químico como sensorial. Para isso, foram realizados ensaios controlados (estufa) em oliveiras jovens da variedade 'Arbequina' com stresses de calor/seca isolados e/ou combinados, sendo avaliada a capacidade de recuperação das árvores (Capítulo II) usando uma gama diversificada de parâmetros fisiológicos (por exemplo, P_N , E , F_v/F_m), stress oxidativo (por exemplo, MDA) e transcritos codificantes de proteínas protetoras (por exemplo, AQPs, HSPs). Para entender o papel de um bioestimulante na melhoria da tolerância ao stress abiótico das plantas, foi testada a sua aplicação em oliveiras jovens das variedades 'Arbequina' e 'Galega' (Capítulo III). Aplicou-se bioestimulante e implementou-se a seca; e avaliou-se o desenvolvimento dos frutos em dois estádios de desenvolvimento diferentes, bem como o seu perfil lipofílico por GC-MS. Por fim, foram realizadas experiências de campo (Capítulo IV) em oliveiras adultas da variedade 'Cobrançosa' para testar os efeitos das ondas de calor de 2017 em Portugal. As oliveiras cultivadas em sequeiro (num olival tradicional) foram expostas a seca devido às ondas de calor; foram avaliados os antioxidantes das folhas; e os perfis lipofílicos (GC-MS) e fenólico (UHPLC-MS) nas folhas, azeitonas e azeite; avaliou-se ainda a qualidade do azeite e o seu perfil organoléptico. Os resultados demonstraram que as oliveiras apresentam alta plasticidade aos fatores abióticos testados (calor e seca) mesmo em estágios de desenvolvimento jovens (Capítulo II). A aplicação de bioestimulantes é uma solução promissora para neutralizar os efeitos da seca e pode ser uma solução valiosa para melhorar a qualidade dos frutos sob irrigação ideal (Capítulo III). Em oliveiras adultas, apesar do aumento dos níveis de espécies reativas de oxigénio nas folhas, a bateria antioxidante foi suficiente para neutralizar esses efeitos. Em azeitonas de árvores

adultas, a seca reduziu alguns ácidos gordos (por exemplo, ácidos palmítico e linoleico), mas aumentou, por exemplo, hidratos de carbono, β -sitosterol, α -tocoferol e a oleuropeína. Enquanto no azeite apesar da redução de rendimento devido à seca, os níveis de β -sitosterol e oleaceína aumentaram, mostrando que eventos climáticos podem estimular a acumulação de compostos bioativos que melhoram a qualidade do azeite. Esta tese de doutoramento contribui para o conhecimento de como as oliveiras respondem ao calor e à seca, o que pode ser útil para os produtores promoverem uma gestão sustentável da água da rega e também fomentar o uso de bioestimulantes como complemento aos fertilizantes.

Palavras-chave

Olea europaea, alterações climáticas, seca, calor, fotossíntese, aquaporinas, desidrinas, proteínas de choque térmico, biostimulantes, *Ascophyllum nodosum*, flavonoides, secoiridoides, ácidos gordos

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ANOVA	Analysis of variance	MUFA	Monounsaturated fatty acids
APX	Ascorbate peroxidase	NBT	Nitroblue tetrazolium
AQPs	Aquaporins	NI	Non-irrigated plants at control temperature
CAT	Catalase	NI^H	Non-irrigated plants with a heat shock
CDD	Consecutive dry days	NPQ	Non-photochemical quenching
C_i	Intercellular CO ₂ concentration	OS	Oxidative stability
CMP	Cell membrane permeability	PCA	Principal component analysis
DHNs	Dehydrins	PDO	Protected Designation of Origin
DMSO	Dimethyl sulfoxide	PMSF	Phenylmethylsulfonyl fluoride
DPPH	<i>2,2-diphenyl-1-picrylhydrazyl</i>	P_N	Net CO ₂ assimilation rate
DTT	Dithiothreitol	PUFA	Polyunsaturated fatty acid
DW	Dry weight	PV	Peroxide value
E	Transpiration rate	PVP	Polyvinylpyrrolidone
EA	Elenolic acid	qP	Photochemical quenching
EDTA	Ethylenediaminetetraacetic acid	ROS	Reactive oxygen species
EU	European Union	RWC	Relative water content
F_v/F_m	Efficiency of excitation energy capture by open PSII reaction centres	SFA	Saturated fatty acids
F2F	Farm to Fork	sHSP	small Heat Shock Proteins
FA	Free acidity	SOD	Superoxide dismutase
FC	Field capacity	SP1	Sampling time 1
F_v/F_m	Maximum quantum efficiency of photosystem II	SP2	Sampling time 2
FW	Fresh weight	TBA	Thiobarbituric acid
FWC	Fruit Water Content	TCA	Trichloroacetic acid
GC-MS	Gas chromatography mass spectrometry	TSS	Total soluble sugars
GPADHEA	1-β-D-glucopyranosyl acyclodihydroelenolic acid	TW	Turgid weight
GPOX	Guaiacol peroxidase	UHPLC-MS	Ultra-high-performance liquid chromatography-mass spectrometry
Gr	Glutathione reductase	WI	Well-irrigated plants at control temperature
g_s	Stomatal conductance	WI^H	Well-irrigated plants with a heat shock
HD	High density	Φ_{PSII}	Effective quantum efficiency of PSII
HS	Heat shock		
HSPs	Heat shock proteins		
IOC	International Olive Council		
K₂₃₂	Coefficients of extinction at 232 nm		
K₂₇₀	Coefficients of extinction at 270 nm		
LOD	Limit of detection		
LOQ	Limit of quantification		
LWC	Leaf water content		
MDA	Malondialdehyde		

Chapter I

General introduction

“The olive is a symbol of wealth, fame, and peace.”

H. Schafer-Schuchardt *In* World Olive Encyclopaedia



Author Márcia Araújo

OLIVE: A MEDITERRANEAN CROP

Botanical characterization

The olive tree (*Olea europaea* L.) belongs to the genus *Olea* within the Oleaceae family, which comprises 600 species within 25 genera (Obied et al., 2008). This species is an evergreen tree with a medium size (grow up to 10 meters in height). It is a xerophyte species well adapted to the harsh environment of the Mediterranean able to support high temperatures and drought. This species can also support temperatures a few degrees below 0 °C (Lavee, 1996; Torres et al., 2017).

The olive tree has a multi-leader root system that, depending on the soil texture and soil aeration, can reach a depth up to 6 - 7 m, if the soil is aerated. However, in less aerated soils, the root system depth decreases, developing a superficial and wide root network, generally spreading from 0.9 m to 1.2 m (Chiappetta and Muzzalupo, 2012; Lavee, 1996).

Depending on the irrigation level, the bark and wood of the olive trunk are very different. The bark is thin in irrigated trees and develops a thick corky layer when the trees are under dryland conditions (Lavee, 1996).

The olive leaves, when fully expanded, are usually elongated and spindle-shaped. However, some cultivars can show different leaf forms, ranging from narrow elongate (lancifolia) to wide rounded forms (Lavee, 1996). Typically, the olive leaf is around 5 to 6 cm long and 1 to 1.5 cm wide in its middle. The leaves grow oppositely arranged, and each one of the leaves grows for two years (Chiappetta and Muzzalupo, 2012). The leaf's upper layer is shiny and dark green due to the waxy cuticle, whereas the leaf's lower layer is greyish. The stomata are located in the lower layer, nestled in peltate trichomes and are also present in multicellular hairs (Chiappetta and Muzzalupo, 2012; Koudounas et al., 2015).

Flower buds occur on the axils of the leaves (developed in the previous season) (Chiappetta and Muzzalupo, 2012). The development of the flower buds occurs during a season (can be dormant for over a year), and in the next season, start to develop the inflorescences, which contain about 15 to 30 flowers along with the shoot. This is cultivar dependent (Chiappetta and Muzzalupo, 2012). Olive inflorescences are green, and the flowers are yellow-white, presenting both a small size (Figure 1.1). The floral initiation starts in November. Nevertheless, only in March do the flower parts start to form (Chiappetta and Muzzalupo, 2012; Lavee, 1996). Some cultivars require chilling time to properly induce flower, breaking the flower buds from dormancy. Chilling time is the number of hours that plants need to break the dormancy. The chilling time is cultivar dependent, varying from 50-60 hours at 7.2 °C to more than 1200 hours (Chiappetta and Muzzalupo, 2012; Lavee, 1996; Rallo and

Martin, 1991). Interestingly, olive trees can only tolerate temperatures below -8°C for a week and prolonged temperatures above 30°C limit their yield performance; also, temperatures above 40°C reduce their photosynthetic rate (Araújo et al., 2018; Mancuso et al., 2002).



Figure 1.1 Olive inflorescences. The presented scale is in cm. Author Márcia Araújo.

Similar to almond, peach, or plum, olive fruit is also a drupe. The drupe consists of seed inside of the endocarp (pit), and inside of the mesocarp (pulp) wrapped in the exocarp (skin). During approximately 6 weeks, after full bloom, the pit enlarges to full size and hardens, while the mesocarp also grows. Initially, the olive fruit is green and during their development, to maturity, the color changes from green to black (full maturation). The fruit color and pit size vary according to the cultivar (Chiappetta and Muzzalupo, 2012; Lavee, 1996; Sanz-Cortés et al., 2002).

The Mediterranean olive cultivation: an historical perspective

This species has been cultivated in the Mediterranean basin for centuries. It can be said that olive cultivation has evolved alongside the Mediterranean civilizations (Blázquez, 1996). It is estimated that the cultivated olive appeared in Asia Minor (Blázquez, 1996), with archaeological findings from Ebla showing that oil production in Syria dates back to the 3rd millennium BC (Blázquez, 1996). Over the years, the olive tree plantations increased mainly in Syria, Palestine, and Egypt, where oil was used in the lamps and also for health purposes (used of oil to protect the skin against cracks and sunburns) (Blázquez, 1996). The expansion

of cultivated olive in the Mediterranean basin occurred mostly during the Roman Empire, persisting in the current days (Blázquez, 1996).

The Mediterranean olive cultivation: the socioeconomic value

The olive crop comprises several socioeconomic benefits for the Mediterranean region, for instance, their products (table olives and olive oil) are known to have several health benefits, which lead to a huge demand by the consumers, generating wealth for the producers, and generation of employments. Due to its influence on the Mediterranean socio-economy, cultural heritage and landscape, several research is being dedicated to the olive chain of value. Around 98% of the world's olive trees are growing in this region. Also, most of the producing countries in the Mediterranean are in the European Union, and Figure 1.2 shows the production of olives for olive oil of EU in 2017, 2018, 2019, and 2020.

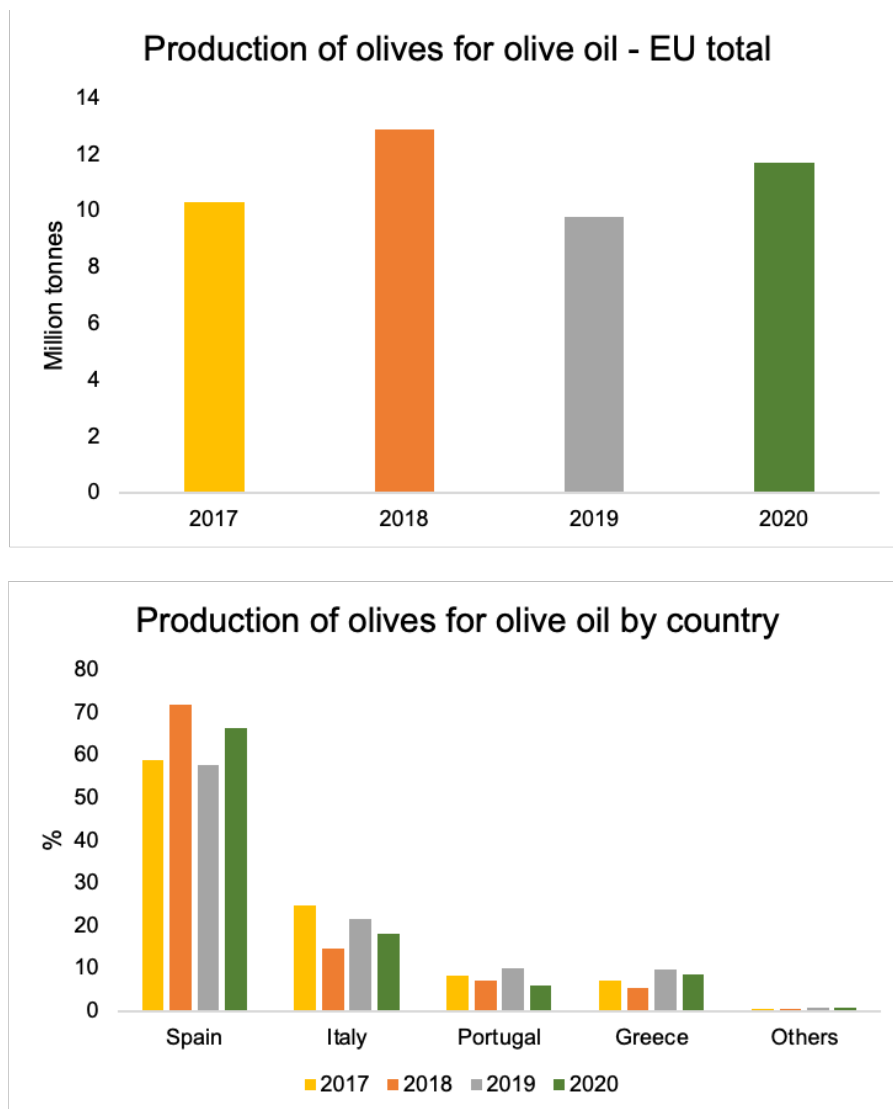


Figure 1.2 Production of olives for olive oil of EU in 2017, 2018, 2019, and 2020. Data are from Eurostat (Eurostat, 2020, 2019b, 2018, n.d.)

About two-thirds of the world's olive oil is produced in the EU. In 2017 the area occupied by olive trees covering about 4.5 million hectares in the European Union (EU). Being Spain, Italy, Greece, and Portugal the countries where most of the olive groves are located. A smaller percentage are located in France, Croatia, Cyprus, and Slovenia (Eurostat, 2019a). From these 4.5 million hectares, 2.5 million hectares have trees at least 50 years old (Eurostat, 2019a).

The production of olives is not constant. In general, the trees follow a production cycle of two years, meaning that in one year, the production is higher than in the other one. Besides that, the role of weather influences the levels of production too (Eurostat, 2020).

The EU provided the last four years about around 10 million tons of olives for olive oil each year (Figure 1.2). In 2020, 11.7 million tons of olives were harvested in the EU (Eurostat, n.d.), in 2019, 9.8 million tons (Eurostat, 2020), in 2018, 12.9 million tons (Eurostat, 2019b), and in 2017, 10.3 million tons (Eurostat, 2018). In the last four years, the major producer country was Spain, followed by Italy, and depending on the year, Greece and Portugal contest for the third position (Figure 1.2).

The socioeconomic value of the olive crop in Portugal

As in the other Mediterranean countries, in Portugal, the population reaches for olive oil not only for its health benefits but also because it is a cultural aspect of the consumption of olive oil and table olives. This increased the demand for this product, which contributed to the modernization and enhancement of the olive crop sector. Farmers' organizations, companies and universities are involved and committed to growing this sector in Portugal. Besides that, olive culture also helps the fixation of people on the rural areas of the country and promotes new ways to share the olive culture knowledge by, for example, promoting the tourism associated with the olive culture and consumption.

In Portugal, there are two major regions of olive cultivation, Trás-os-Montes in the North and Alentejo in the south center. The major producer region of olive oil in Portugal is Alentejo followed by Trás-os-Montes. For instance, in 2020, Trás-os-Montes produced 177 897 hectolitres of olive oil while in Alentejo 807 866 hectolitres of olive oil were produced (INE, 2021a). As a form of protection of Portuguese olive oil from counterfeiting, some regions adopted the Protected Designation of Origin for their olive oils (Council Regulation (EEC) No 509/2006 of 20 March 2006 on agricultural products and foodstuffs as traditional specialties guaranteed). This mechanism certifies the origin and the method of production. In the case of olive oil, it also delimits a geographical area of the production, the edaphoclimatic characteristics, soil, and cultivars used for the oil production (DGADR, n.d.). In Portugal, there are six Protected Designation of Origin regions for olive oil and two for preserved table olives

(Table 1.1). The Protected Designation of Origin regions for olive oil are “Moura”, “Trás-os-Montes”, “Alentejo Interior”, “Beira Interior (Beira Alta e Beira Baixa)”, “Norte Alentejano”, and “Ribatejo” (DGADR, n.d.). “Negrinha de Freixo” and “Elvas e Campo Maior” are the two Protected Designation of Origin regions for preserved table olives and “Azeitona Galega da Beira Baixa” has the status of Protected Geographical Indication (Agricultural products and foodstuffs) (DGADR, n.d.).

Table 1.1 Protected Designation of Origin (PDO) regions of olive oil and table olives in Portugal and cultivars used in each region. Data are from Direção-Geral de Agricultura e Desenvolvimento Rural (DGADR, n.d.).

Protected Designation of Origin region	Cultivars
Olive oil	
Moura	Cordovil
	Galega
	Verdeal
Trás-os-Montes	Cobrançosa
	Cordovil
	Madural
	Verdeal transmontana
Alentejo Interior	Cobrançosa
	Cordovil de Serpa
	Galega
Beira Interior (Beira Alta e Beira Baixa)	Cobrançosa
	Cordovil
	Galega
	Verdeal
Norte Alentejano	Azeiteira
	Blanqueta
	Carrasquenha
	Cobrançosa
	Galega
	Redondil
Ribatejo	Cobrançosa
	Galega
	Lentisca
Table olives	
Negrinha de Freixo	Negrinha
Elvas e Campo Maior	Azeiteira
	Carrasquenha
	Conserva de Elvas
	Redondil

Olive cultivation practices and cultivars distribution in Portugal

In Portugal, there are two major models of olive cultivation. In the traditional model, where olive trees are dryland farming, with 40 to 240 trees/ha, the use of phytopharmaceuticals and fertilizers is low (Reis, 2014), and are usually used based on recommendations for targets and based on analyses (e.g., soils, leaves). As the name indicates, olive trees grow traditionally, with space between trees. In some way, the grove is allowed to self-regenerate, farmers only cut the weeds to facilitate access to the olive trees. Also, biodiversity is higher in this model of production (Calabrese et al., 2012; Guerrero-Casado et al., 2021). However, in some cases, aged farmers with low levels of training overuse fertilizers, pesticides, and herbicides, compromising the existing biodiversity (Reis, 2014).

In the intensive and/or high density (HD) olive groves model, the density of the trees per hectare can vary between 300 to 1500/ha (INE, 2021b), where, in the high density, trees can be planted in hedgerows. The trees are irrigated and the use of phytopharmaceuticals and fertilizers are more used. However, here the phytopharmaceuticals and fertilizers are usually used based on recommendations for targets and based on analyses (e.g., soils). The farmer sends samples from soil and/or plant material to analyze and used the recommended doses. This model of crop is made to be super productive and with high income (Reis, 2014). Considering the biodiversity that can be found in this model of production, it is poorer when compared with the traditional model (Calabrese et al., 2012; Guerrero-Casado et al., 2021). The distribution of the olive groves in Portugal can be found in Figure 1.3. The intensive/HD model of olive groves can be found mostly in Alentejo and the traditional model is the most used in the other regions (Figure 1.3).

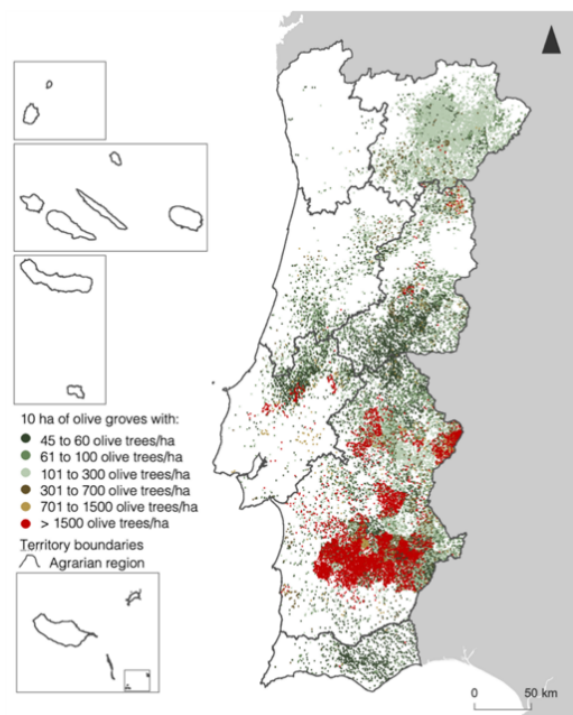


Figure 1.3 Distribution of olive groves in Portugal (adapted from INE (INE, 2021b)).

Besides requiring different cultivation practices, the intensive/HD and the traditional models of olive cultivation also require cultivars with different characteristics. Regarding the olive cultivars, there are registered sixty five different cultivars in the National Catalogue of Fruit Cultivars able to produce fruit (Direção Geral de Alimentação e Veterinária (DGAV), 2016). Among those cultivars, this work focuses on the cultivars 'Galega' and 'Cobrançosa', mostly used in the traditional model, and the cultivar 'Arbequina', mostly used in the intensive/HD model of cultivation.

Galega

'Galega' is the cultivar most used in Portugal in the traditional model of cultivation. This cultivar is also known as 'Galega Vulgar', 'Galega Meuda', 'Molar', 'Negruça', 'Coimbreira' (Direção Geral de Alimentação e Veterinária (DGAV), 2016). It can be found in Beira Interior, Alentejo, Ribatejo and Algarve, being present in variable proportions in olive oils from Portugal PDO regions (Table 1). Fruits from 'Galega' (Figure 1.4) are used to extract oil that present high quality and stability (Peres et al., 2016). The oils extracted from 'Galega' are characterized by higher levels of oleic, palmitoleic and palmitic acids and γ -tocopherol (Peres et al., 2016).



Figure 1.4 Phenological evolution of 'Galega' cultivar olive fruits. A, green olives; B, fruit coloring; C, full maturity fruits with the typical cultivar fruit coloring. Author Márcia Araújo.

Cobrançosa

'Cobrançosa', also known as 'Quebrançosa' or 'Salgueira', is a Portuguese cultivar that can be found in different regions in Portugal, such as Trás-os-Montes, Ribatejo, Beira Alta and Alentejo (Direção Regional de Agricultura da Beira Litoral, n.d.). 'Cobrançosa' is used in the traditional mode of cultivation and also can be found in the intensive model of cultivation. The fruits (Figure 1.5) are mostly used for oil extraction and the oils present good quality, being present in variable proportions in olive oils of some PDO regions of Portugal (Table 1.1). The 'Cobrançosa' extra virgin olive oils are characterized by the higher quantities of oleic and palmitic acid (Peres et al., 2016).

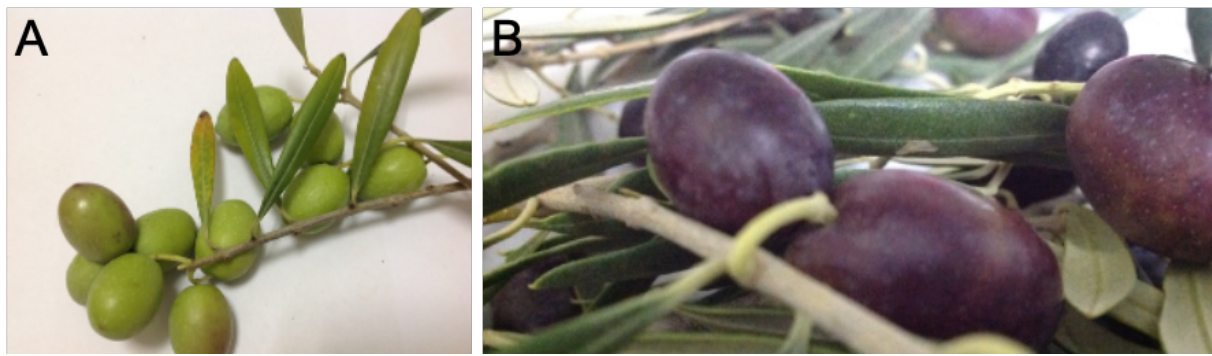


Figure 1.5 Different phenological stages of 'Cobrançosa' olive fruits. A, green fruits and B, fruits coloring. Author Márcia Araújo.

Arbequina

Arbeca (Lleida, Catalonia, Spain) is a municipal district that gave the name to this cultivar and it is where it was first grown (Criado et al., 2004). The cultivar 'Arbequina' is one of the most known Spanish cultivars and is mostly used in the intensive/HD olive groves, in hedgerows. It is also known in the international oil market for its taste and flavor (Criado et al., 2004). It is a high productivity cultivar with small-sized fruits (~1.9 g) with high oil yield (~20.5%) (Criado et al., 2004), mostly used for oil extraction (Figure 1.6). As 'Galega' and 'Cobrançosa', 'Arbequina' extra virgin olive oils are also rich in oleic and palmitic acids (Reboredo-Rodríguez et al., 2016). In Portugal, this cultivar is typically used in the intensive/HD olive farming, being this model of production mostly used in Alentejo and Ribatejo (see Figure 1.3).

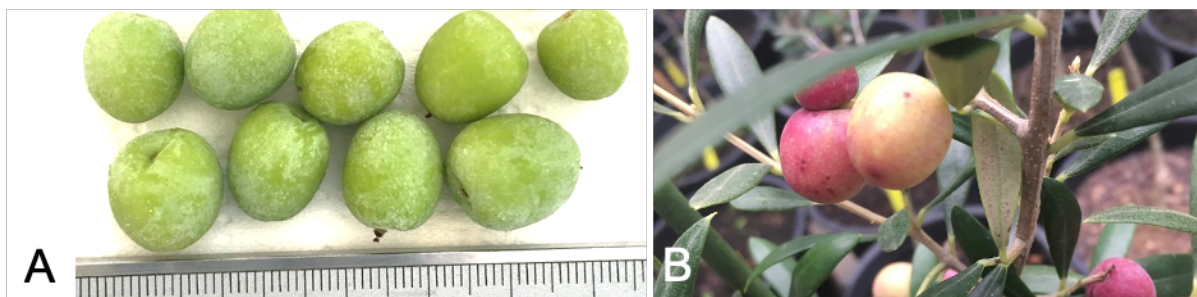


Figure 1.6 Different ripeness stages of 'Arbequina' fruits. A, green fruits and B, fruits coloring. Author Márcia Araújo.

OLIVICULTURE: CHALLENGES UNDER A CLIMATE CHANGE SCENARIO

Climate change in Portugal

Climate change has already deeply impacted biodiversity (Figure 1.7), ecosystems, and agroforestry systems (Kapitza et al., 2021; Kilroy, 2015; Muluneh, 2021). The Mediterranean region is being highlighted as one of the most sensitive regions of the globe to the ongoing climate changes, particularly susceptible to a large reduction of precipitation and an increase in average temperature (Cramer et al., 2018; Lange, 2020).

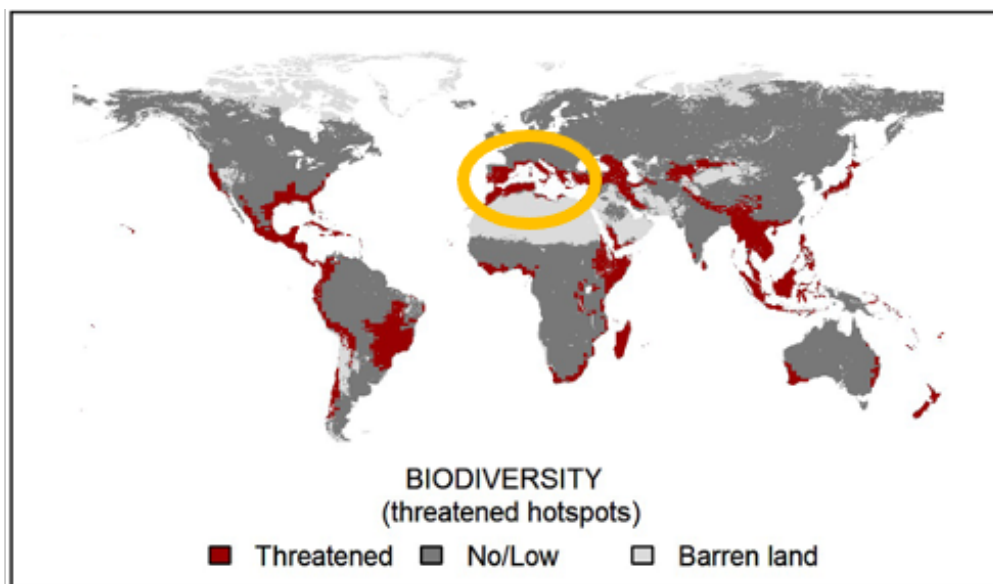


Figure 1.7 The biodiversity threatened hotspots, marketed between an orange circle the Mediterranean basin, adapted from IPCC (2019).

The Earth is suffering from climate change, meaning heatwaves, droughts, heavy precipitation, and tropical cyclones, mostly due to human activities (IPCC, 2019). Changes in temperature and precipitation patterns are key factors triggering shifts in the climate of a region (Andrade et al., 2021; Santos et al., 2019). For instance, in the last 30 years, the temperature in Portugal has increased, and it is clear the trend toward augmenting the maximum and the average temperature of the air (Figure 1.8 A), especially when the anomalies in the temperature are analyzed (Figure 1.8 B). Since 1995 there is a clear tendency to increase the maximum and average temperatures yearly (Figure 1.8 B). Contrarily to temperature, the levels of precipitation are decreasing over the years, especially in the winters (Figure 1.8 C). Since 2003, the yearly rainfall has been diminishing (Figure 1.8 D). The increasing temperature associated with the diminishing precipitation in recent years influenced the

climate in Portugal. A recent study (Andrade et al., 2021) points out that the area with the climate CSa (warm Mediterranean climate with hot summer, Köppen–Geiger climate classification) increased 18.1%. The climate in Portugal used to be 53.9% CSa, 45.8% CSb (warm summer) and 0.03% Cfb (warm temperate/Mediterranean fully humid with hot summer) between 1950-1979 and now (1990-2019) is mostly CSa (72%) and CSb (28%) (Andrade et al., 2021).

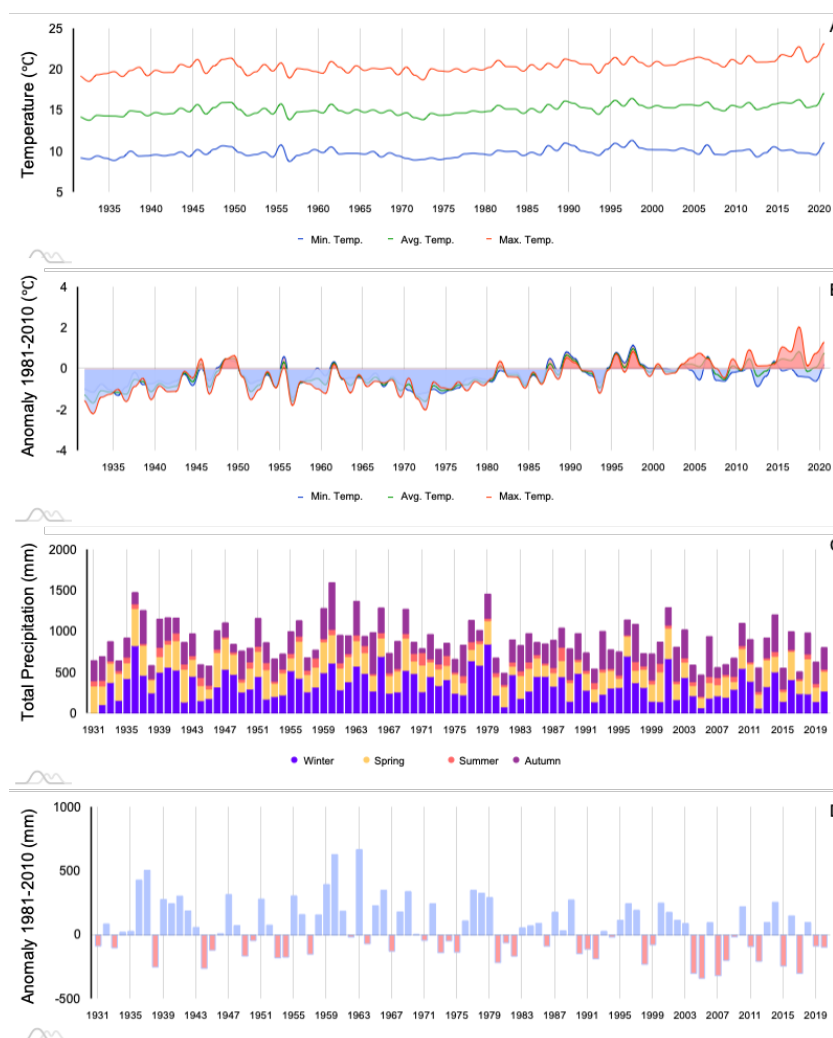


Figure 1.8 Portugal's temperature and precipitation from 1931 to 2019, adapted from IPMA. The minimum, average and maximum temperature (A), the temperature anomalies (B), the total precipitation in the year's four seasons (C) and the precipitation anomalies (D).

Elevated temperatures associated with the low precipitation levels will continue in the next years. It is now projected that the number of consecutive dry days (CDD, Figure 1.9 A and B) will increase in Portugal (Santos et al., 2019). Interestingly, the locations where it is projected that the number of CDD will be higher are the Alentejo region and Algarve. Alentejo (Figure 1.9 C) is where most of the olive groves are located and where the intensive/HD model

of production is used (Figure 1.3). As this model requires a large amount of water for the irrigation of the olive trees, the future of this model of production needs to be discussed at light of sustainability.

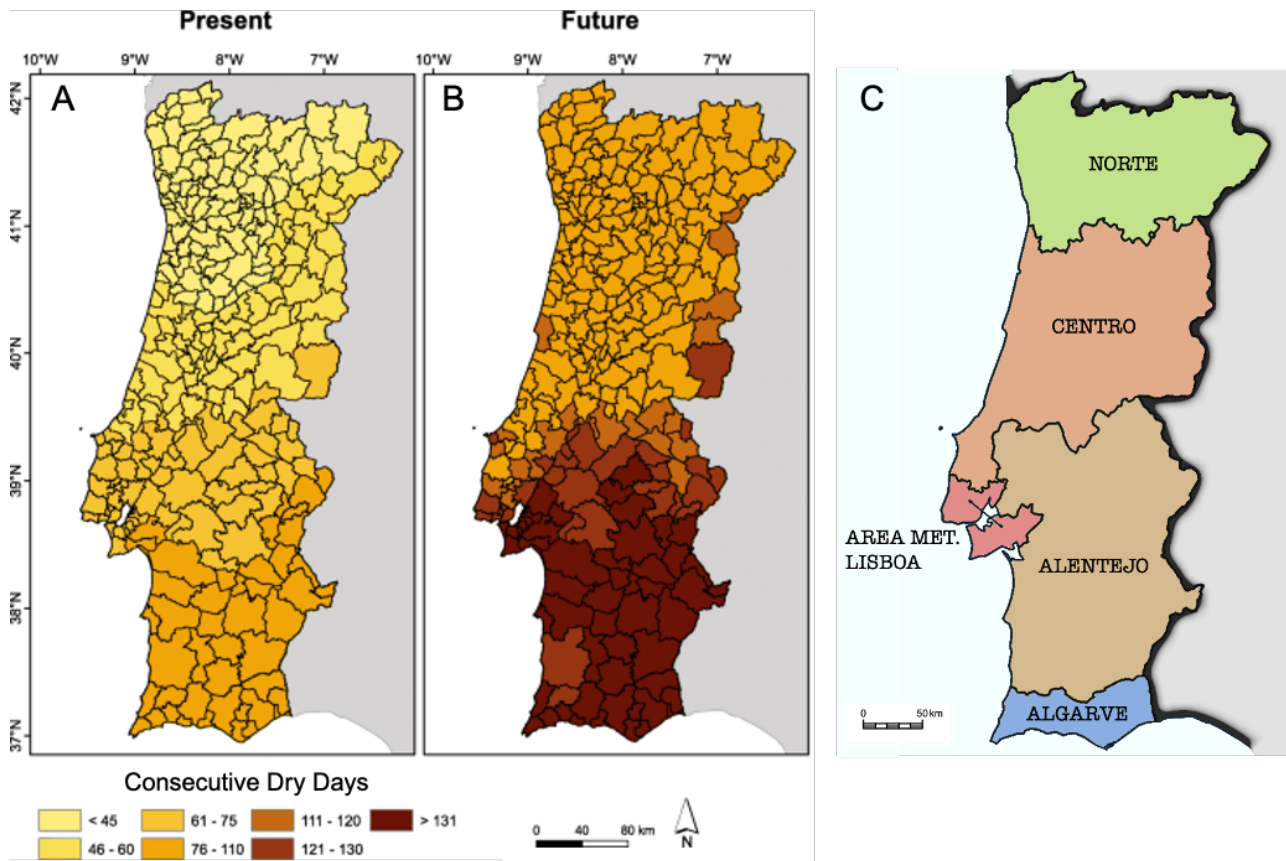


Figure 1.9 Present (1950–2003) (A), future (2046–2065) (B) of the consecutive dry days in mainland Portugal, aggregated by municipality and the nomenclature of territorial units for statistical purposes (NUTS) of Portugal. Adapted from (Santos et al., 2019).

As these natural phenomena are likely to increase in the next years, it is imperative to study these effects on plants, as they are the primary source of food on the planet. These events will reduce crop yield and compromise agricultural production in a world with an increasing population. That may lead to a lack of resources to feed populations and/or more pronounced social differences worldwide. As shown above, olive and its products have crucial importance to the socio-economy and cultural-landscape heritage in the Mediterranean basin. The Mediterranean region is expected to suffer from hot extremes and agricultural and ecological drought (IPCC, 2019). As known, the high temperatures increase the evaporation of water from the soil, leading to water deficit and drought perceived by plants and animals. Here we discuss how these two abiotic stressors (high temperature and drought) impact plants, particularly olive trees.

High temperature: exposure and recovery

One of plants' most deleterious abiotic stresses is excessive heat (Araújo et al., 2018). It affects plant growth and yield, physiology, including photosynthesis and nutrition, transpiration, metabolic pathways and associated metabolome-proteome and transcriptome. Heat stress also influences the ultrastructure and anatomy of plants (Araújo et al., 2018; Mittler et al., 2012). Plants perceive heat as a whole process involving several signaling cascades in different cellular organelles to originate a response (Araújo et al., 2018). Therefore these molecules resulting from the signaling cascades converge to regulate gene expression that encodes proteins/enzymes enrolled in stress metabolism (Zandalinas et al., 2018). For example, at the cell level, this stress may disturb the cell homeostasis at membrane and protein stability levels (Brestic et al., 2013) causing a state of metabolic imbalance (Mittler et al., 2012). That can compromise plant development, growth, productivity, yield, and even reproduction (Araújo et al., 2018; Mittler et al., 2012). Ultimately, heat stress can lead plants to severe stress and cause plant death (Zhao et al., 2021).

Plants/crops can develop mechanisms of heat tolerance up to certain levels. Key mechanisms (besides the anatomic and morphological adaptations, e.g., wax cuticle) help the plant maintain the stability of proteins and membranes by adjusting their redox status. This adjustment can be achieved by scavenging excessive reactive oxygen by increasing enzymatic or non-enzymatic antioxidant pathways, and by increasing the levels of protective proteins like the heat shock proteins (Araújo et al., 2019; Mittler et al., 2012; Raja et al., 2017) (see subchapter 2.2).

Analyzing the gene expression and the transcriptome, proteome, and metabolome of these stressed plants shows how these stressors impact the plants at different levels and may also identify the most sensitive pathways that determine the plant's susceptibility, or tolerance, and the mechanisms of adaptation. These metabolic responses, anchored on molecular signals, are integrated with multiple signal transduction pathways involved in different cell compartments (Brestic et al., 2013). For example, photosynthesis is a sensitive metabolic process in plant cells to heat stress. In general, the photosynthetic apparatus can tolerate moderate temperatures involving reversible changes or may be severely damaged as a result of severe heat (Brestic et al., 2013). Also, the temperature increase may lead to the accumulation of reactive oxygen species (ROS) that induce the plant cells to activate specific antioxidant enzymes in response (see subchapters 2.2 and 4.1). ROS, such as H₂O₂, can also impair photosynthesis, as chloroplasts are a major organelle of the generation of ROS (Zandalinas et al., 2018).

Heat tolerance can also be achieved in orchards by selecting and using more tolerant species/varieties, by developing breeding programs to improve genetically improve heat-

tolerant cultivars, and/or by the exogenous application of elicitors of tolerance, e.g., biostimulants, and finally by in some cases using cultural practices like the use of protective nets.

Some main changes in plants in response to heat stress are summarized in Figure 1.10. Heat stress in young olive plants is discussed in the Chapters II and IV of this thesis.

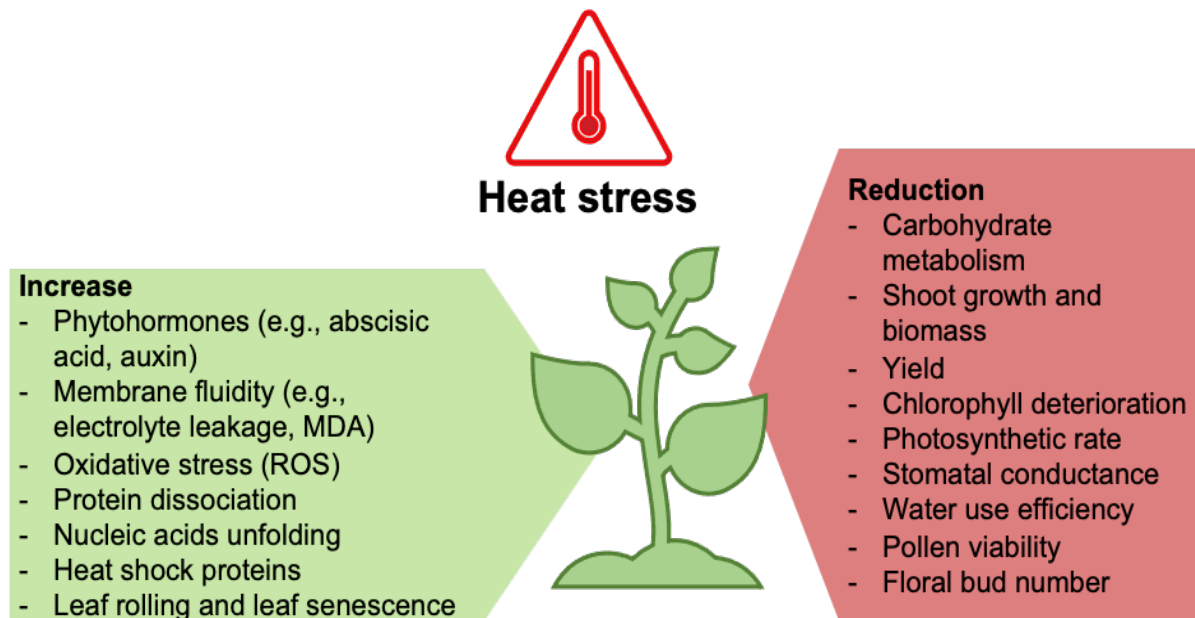


Figure 1.10 Summary of heat stress consequences in plants based on the works of Ali et al. (2020), Araújo et al. (2019), Li et al. (2021), Mittler et al. (2012) and, Zhao et al. (2021). MDA, malondialdehyde; ROS, reactive oxygen species.

The study of how plants recover, and the most limiting factors regulating plant homeostasis after the removal of heat stress, remains less studied than the plants response during the stress period. The plants' recovery may involve, or not, repair mechanisms. For example, physiological processes (e.g., stomatal conductance) that were impaired by the stress, but without structural damages (e.g., tissues, cells), may immediately be reactivated. On the contrary, if stress led to structural damages, there is a need to redirect energy for reconstruction, and recovery may take longer, or require less growth, among other aspects (Ruehr et al., 2019). In a review on the behavior of tree plants after heat or drought removal, Ruehr et al. (2019) highlighted that physiological indicators (e.g., senescence, stomatal conductance, hydraulic conductance in xylem, functioning of PSII) are pivotal to determine the recovery ability of woody species after heat or drought stress. The authors proposed that in general, a "Mild stress" is reversible, allowing that stomata reopen and photosynthesis increases, after the increase of the plants' turgor. The "Moderate stress" causes functional impairment and reversal involves active repair mechanisms. Finally, the "Severe stress"

results in structural damages, which led to a slower recovery, dependent on the regrowth of damaged tissues (Ruehr et al., 2019).

Also, Liu et al. (2012) found that some genes that were up/down-regulated by heat stress and during recovery shared a common group, while other genes were regulated in opposite directions. Finally, some studies also showed that pretreatments could alleviate the impact of the stress and help the recovery. For example, pretreatment with salicylic acid in grapevines alleviated the negative effects of heat stress on photosynthesis (P_N) and accelerated the recovery of this parameter (Wang et al., 2010). Also, biostimulants may have beneficial effects in the recovery, despite this is scarcely studied in olives (Hasanuzzaman et al., 2021; Van Oosten et al., 2017).

Drought: exposure and recovery

The impacts of drought stress are increasing (in severity and/or duration) with climate change. Also, drought stress is intimately linked to the dynamics of other environmental factors, including temperature, light intensity, and low rainfall. Therefore, other factors like the plant species, ecotypes, must be considered when evaluating the impact of drought. In glycophytes (representing most relevant crops), drought generally influences plant morphological, physiological, biochemical and molecular characteristics (Hura et al., 2022). Photosynthesis and redox status are among the most sensitive (Chaves et al., 2003; Hura et al., 2022). For example, the overproduction of ROS is critical, and if not counteracted by the protective antioxidant (enzymatic and non-enzymatic) battery, may lead to biomolecules peroxidation and ultimately to organelle/membrane damages and cell death (Araújo et al., 2018) (see subchapters 2.2 and 4.1). Being chloroplasts a major source of ROS, the well-functioning of the photosynthetic apparatus depends on the plant's capacity to balance the cell/chloroplast redox status under heat and/or drought (Gollan et al., 2015; Tamburino et al., 2017). Besides, photosynthesis is also directly linked to other drought and/or heat protective strategies such as the stomatal control, which is important to regulate water content, but may compromise gas exchange (reducing intercellular CO₂ availability) and related photosynthetic reactions (Araújo et al., 2016; Dias et al., 2014). Also, enhancing energy dissipation and reducing light absorption are protective mechanisms preventing photoinhibition-related damages that often occur as a result of drought and/or heat stress (Mittler et al., 2012; Pinheiro and Chaves, 2011). Thus, with a compromised photosynthesis, the mobilization of carbohydrates for fruiting and ripening is affected.

To cope with drought, plants developed different adaptation/resistance mechanisms. Besides the anatomical characteristics (e.g., xerophytics, of which olive trees have some

traits) physiological and biochemical responses, are also modulated as immediate response, and as longer-term adaptation to the stress to improve the water stress tolerance and adaptation (Brito et al., 2019). For example, some changes include changes in the growth pattern and structural dynamics, increased efficiency of transpiration rates and of water use, and increased stomatal resistance, leaf rolling, higher plasticity in the root to shoot ratio (e.g., root length increase), accumulation of compatible solutes, osmotic and hormonal regulation (Seleiman et al., 2021). Using omics to understand the most sensitive genes and pathways to the conditions of stress is crucial to elucidate how to improve plants' resilience and develop targeted mechanisms of breeding (Hura et al., 2022) (see subchapter 2.2). Also, more recently, the addition of biostimulants has shown to improve, in a sustainable way, the efficiency of the plants, at e.g., increasing water use efficiency, or increasing metabolic pathways for resilience, and or higher homeostasis (Del Buono, 2021).

Some main changes in plants in response to drought stress are summarized in Figure 1.11. Drought is discussed in the Chapters II, III and IV of this PhD thesis.

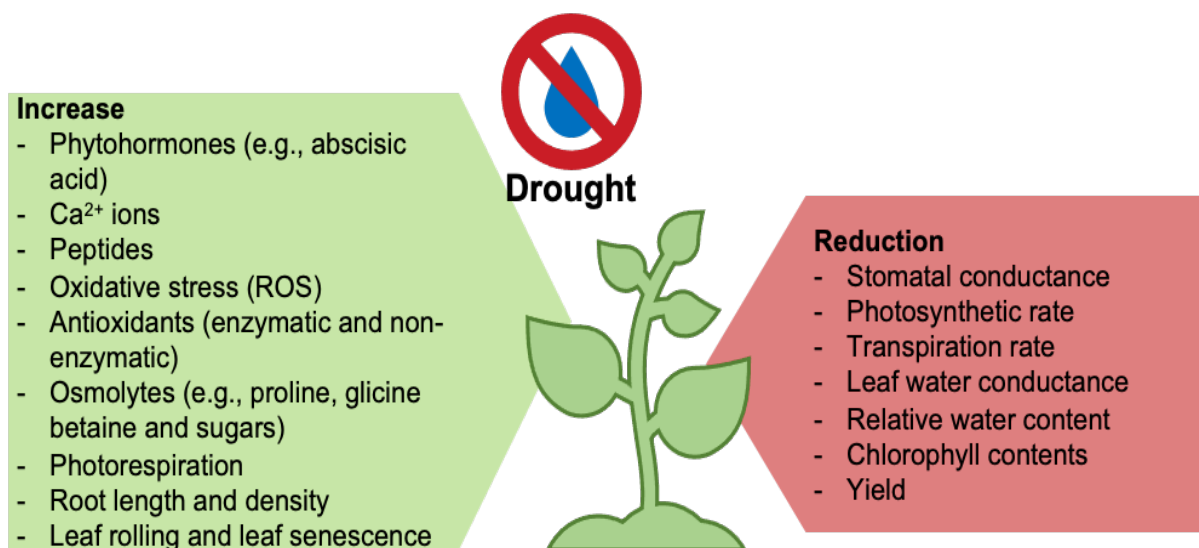


Figure 1.11 Summary of the main effects of drought in plants according to Chaves et al. (2003) and Seleiman et al. (2021).

Like in the recovery of plants after heat stress removal, the recovery of plants after rewatering remains less studied, depending on the duration and intensity of the stress, besides the species/genotype tolerance (Ruehr et al., 2019). In addition to the review of Ruehr et al. (2019) on physiological aspects of woody plants during the recovery to heat and drought, already mentioned above, in the recovery process mostly for mild drought stress, it is often assumed a recovery of the parameters to values close to those of unstressed plants. For example, after rewatering, plant growth and photosynthesis may recover short after through

growing new plant parts, re-opening the stomata, and decreasing peroxidation. However, these responses depend on the drought intensity, duration, and species (Ruehr et al., 2019).

The plasticity of the plant to shift its metabolites pathways, including in the production of protective osmolytes and to manage the antioxidative battery (both enzymatic and non-enzymatic) may also be pivotal to the plant not only during the stress but also during the recovery period (Araújo et al., 2019; Brito et al., 2019).

Finally, although the effects of drought or heat stress on plant performance are well characterized individually, few studies have investigated the molecular and physiological responses to a heat episode combined with drought conditions (see subchapter 2.2).

COMBINED DROUGHT AND HEAT ON OLIVE TREES: FROM NURSERIES TO GROVES

Climate change, particularly drought and heat, threatens food security and production worldwide (IPCC, 2019; Muluneh, 2021). Water availability will reduce, leading the regions to face drought, the hot and dry days will increase throughout the year and the rain when it comes will be more intensive (Andrade et al., 2021; Santos et al., 2019). These phenomena are already occurring, leading to a vast socioeconomic and biodiversity losses (IPCC, 2019; Muluneh, 2021).

Olive trees are known to tolerate a broad range of environmental stresses, including heat and drought. For this tolerance, olive trees developed several morphological and physiological adaptations (e.g., Brito et al., 2019; Carr, 2013; Tripepi et al., 2011), including some xerophytic morpho-anatomic characteristics (e.g., Rapoport et al., 2016).

However, the physiological adaptations regulating the olive adaptation to heat episodes are less studied (see chapter II). Understanding these physiological/metabolic adaptations and the physiological differences associated with more tolerant varieties, would be essential to better select cultivars more suitable for extreme heat and/or drought episodes. This research is particularly important as an increase in temperatures is ongoing in the Mediterranean region, including Portugal (Andrade et al., 2021; IPCC, 2019; IPMA, n.d.; Santos et al., 2019), naturally affecting the olive trees physiology, yield and the olive/oil quality (see chapter IV). The quality of the olive fruit and its oil is conditioned by the cultivar and the edaphoclimatic conditions like the soil and climatic conditions (Criado et al., 2007; Mafra et al., 2021). Therefore, producers need to select the fittest cultivars coping with heat/drought conditions.

Several reports on the effects of water deficit in olive plants' metabolism and physiology have been produced (Araújo et al., 2021, 2019; Bacelar et al., 2007; Correia et al., 2005; Dias et al., 2021, 2018a; Ennajeh et al., 2008, 2006; Gonçalves et al., 2020; Silva et al., 2018). The studies show that the response to water deficit is cultivar sensitive, time of exposure to stress, age of trees and if the experiment is controlled or in the field. For instance, after drought and heat shock exposure, young olive trees of 'Cobrançosa', 'Cordovil de Serpa' and 'Cordovil de Castelo Branco', the 'Cobrançosa' plants coped better with water deficit (Dias et al., 2018). 'Cobrançosa' also showed higher tolerance when compared with 'Madural' and 'Verdeal Transmontana' cultivars (Bacelar et al., 2007a).

As previously mentioned, several reports focus only on one stress and rarely focus on the effects of the combination of abiotic stresses. Also, there is a lack of studies on different plant parts, the majority of those focus on the physiology of the olive tree (Araújo et al., 2019;

Bacelar et al., 2007b, 2006, 2004; Mancuso and Azzarello, 2002; Sofo, 2011; Sofo et al., 2008). It is important not only to study plant physiology but also to study the influence of these abiotic stresses on plant metabolites from different plant organs, e.g., leaves, fruits, roots and even its products, as in the olive case, the olive oil. There are several reports about the constitution of fruits and oil (Machado et al., 2015; Malheiro et al., 2011, 2015; Marx et al., 2021; Peres et al., 2013; Rodrigues et al., 2020; Špika et al., 2021). From the constitution of fruit, the composition on fatty acids (e.g., oleic acid, palmitic acid, linoleic acid), antioxidants compounds (e.g., phenolic compounds such as oleuropein or luteolin) to the organoleptic profiles [olfactory sensations (e.g., dry fruits, fresh grass) and gustatory-retronasal sensations (e.g., sweetness, bitterness, pungent)] of olive oil. However, still, a lack of studies on the effects of drought and/or heat on the fruits and oil and how these abiotic stresses interfere with the plant physiology in the different cultivars this species has. There are studies around the irrigation theme in order to see how irrigation can improve the oil yield without interfering with the oil quality (e.g., Caruso et al., 2017; Fraga et al., 2020) or if the lack of irrigation has effects on the olive yield, productivity and quality (e.g., Ahumada-Orellana et al., 2018).

Water management is a challenge nowadays and is also one of the most important resources on our planet. Without it, no life exists. In the case of olive trees and their products, an increased number is observed in the use of the intensive/HD model of production in Portugal, as this model offers a better cost/benefit for the producers. However, the cost to the environment is often neglected. As this model of olive production uses more fertilizers and pesticides, increases soil erosion and the rates of soil fertility loss, degradation of habitats and landscapes, and over-exploitation of vulnerable and scarce water resources (Fernández-Lobato et al., 2021). A commitment must be done in terms of socioeconomic benefits without degradation of the ecosystem that will be important for the generations to come.

Sustainable olive crop practices must address the challenges posed by climate change. The intensive/HD model of cultivation has to be replaced by less water-demanding practices like dry farming or the emerging partial root-zone drying (e.g., Fraga et al., 2021; Wang et al., 2017) or even integrate the olive cultivation in agroforestry models. Or it turns unsustainable olive production as it demands irrigation systems in areas already suffering from drought or severe drought (Andrade et al., 2021; IPMA, 2022, 2020). Among the cultivars more intensively used worldwide, 'Arbequina', considered tolerant to drought (Bacelar et al., 2004; Benlloch-González et al., 2008), has been widely used in the intensive/HD model of cultivation (Larbi et al., 2015) with unsustainable water demands. In comparison, 'Galega' and 'Cobrançosa' cultivars, mostly used in the traditional model of cultivation, are present in areas less affected by drought in Portugal (Andrade et al., 2021; IPMA, 2022) and are mainly used in dryland farming, as previously mention.

The Mediterranean countries are the most producers of olive products and are also one of the climate change most affected parts of the world. Nowadays, we are facing an extreme challenge that implies the increase of food production associated with the increase of population in the world. We must make changes in our practices to manage better ways to produce safe food for all without depleting our resources. And to achieve those goals we, as society, must invest in research. For olive, it is still lacking studies on the effects of different abiotic stresses isolated and combined, e.g., drought, heat, flooding, freezing in the different cultivars that *O. europaea* has. And in different scenarios, such as greenhouse experiments and field ones. For instance, in this thesis, we only studied 'Cobrançosa', 'Galega' and 'Arbequina', several other cultivars need to be studied too.

SUSTAINABLE SOLUTIONS TO IMPROVE OLIVE GROVES RESILIENCE TO CLIMATE CHANGE

Sustainable solutions aligned with the Farm to Fork Strategy

Mediterranean and European olive producers' countries most implement strategies to be more sustainable in relation to the food. Aligned with the Farm to Fork (F2F) strategy, the EU aimed that the food systems are fair, healthy and environmentally friendly. To be able to achieve these goals, those of F2F, agriculture must be neutral and have a positive impact on the environment, promote climate change mitigation and adapt to its impacts, promote biodiversity, ensure food security, nutrition and public health, make sufficient, safe, nutritious and sustainable food access to everyone promoting fair trade and a fairer economic return. When implementing those measures, EU countries will also need to ensure that the agrochemicals and pollutants are reduced or even eliminated and replaced by natural alternatives. Considering that those alternatives are able to maintain or even improve the nutritional value of soil (and consequently the plants' nutritional status and the food nutritional balance) and promote and/or improve water management. EU countries need to promote organic farming and agroforestry systems to improve biodiversity and food security. Also, more surveillance of each EU country needs to be implemented to achieve those goals.

Another important aspect to achieve the F2F EU goals is valorizing the native biodiversity, promoting strategies to reduce or eliminate alien species, and educating people to achieve those goals and improve our food security. Several strategies involve e.g., nanotechnologies, biotechnological approaches, including the selection of resilient genotypes. One strategy emerging among producers is the use of biostimulants, alone or combined with other strategies (reviewed by Sani and Yong, 2022).

Plant biostimulants as sustainable complements to fertilizers in olive groves

As defined by the Regulation (EU) 2019/1009 a *plant biostimulant* shall be a product “to stimulate plant nutrition processes ... with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: i) nutrient use efficiency, ii) tolerance to abiotic stress, iii) quality traits, or iv) availability of confined nutrients in the soil or rhizosphere” (European Parliament and the Council, 2019). This definition focused more on the function rather than on the composition, characteristics, or origin of the biostimulants. Thus, research has been settled towards developing plant biostimulants to improve (directly or indirectly) plants/crop physiology, yield, and quality, by improving the nutritional conditions

(e.g., availability in soil and/or the plant's use efficiency of nutrients), soils, or even improving water usage by the plant (Rouphael and Colla, 2020).

The beneficial impacts of these biostimulants led researchers to hypothesize that they may help the plant to resist better environmental stresses (du Jardin, 2015; Rouphael and Colla, 2020), namely heat and drought. The advantage of using these substances is due to their effectiveness in improving crop productivity and quality (Rouphael and Colla, 2020). In line with the F2F strategy and to face the challenges of climate change, biostimulants thus emerge as promising alternatives to the overuse of hazardous agrochemicals, putatively suitable for a more sustainable agriculture. Additionally, these biostimulants are often considered to be of low cost (Xu and Geelen, 2018), and may have multiple natural origins, including algae (Rouphael and Colla, 2020).

There are several sources of biostimulants, animal and vegetal protein hydrolysates, humic and fulvic acids, macroalgae seaweed extracts, silicon and beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) and N-fixing bacteria strains (e.g., genera *Rhizobium*, *Azotobacter*, and *Azospirillum*) (Drobek et al., 2019; du Jardin, 2015; Rouphael and Colla, 2020). Each one has different beneficial properties but also enables plants to be able to have more protection against biotic and abiotic stressors (Drobek et al., 2019). However, here it is presented the *Ascophyllum nodosum* L. macroalgae based biostimulant.

The application of biostimulants derived from brown seaweeds (e.g., extracts of *Ascophyllum nodosum*) are environmentally sustainable, and the positive results lead an increasing number of growers to apply biostimulants in their fields (Carmody et al., 2020). They may increase biomass, water use efficiency, plant vigor, promote root development and earlier flowering, enhance chlorophyll synthesis, fruit set and uniformity of fruit, delay senescence, improve photosynthesis and nutrient uptake, and promote tolerance to abiotic stress, such as drought or heat (Carmody et al., 2020; Drobek et al., 2019; Shukla et al., 2018).

The characterization of these extracts, and the identification of the mechanisms by which these biostimulants act remains however still uncovered.

In olive, the combination of the seaweed *A. nodosum* with boron and nitrogen increased the productivity and oil content, accelerated the fruit maturation (color and firmness), and in leaves increased the content of potassium, iron and copper and reduced the amounts of manganese (Chouliaras et al., 2009). Also, the total phenol was not affected in oil, the linolenic and oleic acids increased and the linoleic, stearic, and palmitoleic acids decreased in olive oil from the Greek olive cultivar 'Koroneiki' (Chouliaras et al., 2009) when applied boron, nitrogen and *A. nodosum*. Another study (Graziani et al., 2022) where a mixture of seaweed biostimulant (*A. nodosum* + *Laminaria digitate*) increased the leaf relative water content in 33%, however this result does not decipher the impact of the application of isolated

A. nodosum. The concept of biostimulant is evolving with time, with a tendency to board their roles, and include compounds that traditionally are not considered as a biostimulant like kaolin or free amino acids as reviewed by Rouphael and Colla (2020). To support this evolution of the concept Brito and colleagues (Brito et al., 2021) proposed that kaolin improves the olive tree performance and yield under sustained deficit irrigation by reducing the stomatal and the non-stomatal limitations to photosynthesis at high light intensity, improving plant water status, and decreasing the oxidative damages. Another study demonstrated that the use of fulvic acid in soil polluted with trace elements under semi-arid conditions increased the N content and the chlorophyll content without increase of Cd, Cu, Pb, Tl or Zn (Murillo et al., 2005). Yet, still remains unknown all the mechanism of action of plant biostimulants applied to olive trees and more studies are needed to decipher it. How *A. nodosum* act in olive physiology and fruit development is still an unanswered question (see chapter III).

RESEARCH OBJECTIVES

Climate change is already affecting our planet. The increasing temperature associated with the reduction of rain affects the ecosystems and the existing biodiversity in the agroforestry systems. Worldwide, the Mediterranean basin is considered one of the most sensitive regions to the climate change challenges, being a case study for combined stressors as heat and drought act together. Thus, traditional crops (like olive trees) and production modes may be challenged in the next decade.

Despite the fact that drought or heat stress's effects on olive tree performance are well characterized, few studies integrate the combination of these two conditions, which will be dominant in the Mediterranean under climate change. Even less studied is the performance of the plants to recover from the stress after their removal. Innovative solutions to help olive producers face these challenges must be sustainable (aligned with the Farm to Fork Strategy) and provide high-quality food products to address the consumer's perception and demand. So, this thesis aims to:

- Characterize plant/fruit functional-plasticity of Portuguese autochthonous olive cultivars (adapted to dryland farming - traditional model of production) and a foreign cultivar (adapted to intensive production) to the
 - o single or combined stresses (integrating functional/metabolomics/gene-expression data)
 - o recovery ability and profile after the removal of the stressor(es)
- Evaluate the effects of biostimulants on increasing the resistance of olives to extreme conditions of drought and heat under a climate change perspective and towards providing sustainable solutions to improve orchards resilience;
- Understand if heat and drought affect orchards' performance of plants and fruits, regarding the phenolic (e.g., oleuropein) and lipid profiles of olive leaves and fruits/oil, and if it influences oil quality at physicochemical and sensorial level.

THESIS STRUCTURE

The thesis is organized to address the objectives mentioned above.

In **Chapter I**, an integrated general Introduction presents the problem that olive groves face in Portugal, aligned with the current state of the art on climate change and the general morphophysiological responses of plants to heat and drought. Also, key aspects of oliviculture, namely the olive cultivars studied here, are presented.

Chapter II addresses mostly Objective I, and is dedicated to unveiling the response of young olive plants (under controlled conditions of greenhouse) exposed to drought and heat conditions and their recovery. Different stresses (drought and heat) applied separately or simultaneously were implemented for these experiments. In addition, a detailed evaluation of physiological and molecular mechanisms (namely photosynthetic, redox status, and protective proteins) was evaluated immediately after stress exposure and ten days after stress relief (recovery). These studies provided the information for one book chapter published (Araújo et al., 2018) and one manuscript published in the *Planta* journal (Araújo et al., 2019).

Chapter III addresses mostly Objective II. It is dedicated to understanding if a biostimulant may improve the olive tolerance to drought. Experiments were conducted with one Portuguese and one Spanish olive cultivars. For these experiments, young potted olive plants were previously treated with a biostimulant and then maintained at 100% and 50% of the field water capacity during 69 days. Studies focused on the response to drought of olive fruits at two different stages of development (focusing on fruit development and the GC-MS analysis of the lipophilic profile), and if the biostimulant improves the metabolomic profile of the fruits (from this work, one manuscript Araújo et al. 2022 was submitted to an ISI journal).

Chapter IV addresses mostly Objective III. It is dedicated to study the impact of drought and heat under extreme conditions in the field. The experiment was in Trás-os-Montes olive groves and aimed to unravel the state of olive leaves, fruit and oil under extreme weather conditions. For that a work was done with leaves where the antioxidant battery (enzymatic and non-enzymatic) and lipophilic and phenolic metabolites profile (using GC-MS, and UHPLC-MS) were studied that resulting in one published paper in the journal *Plants* (Araújo et al., 2021) and another work involving the study of metabolites of fruit and oil was produced (a second paper Araújo et al 2022, was submitted to an ISI journal).

Finally in **Chapter V**, an integrated discussion, main conclusions and future perspectives, provides information on the main advances regarding the objectives, and the remaining key research questions.

CHAPTER I

General Introduction

From controled conditions to

field conditions

CHAPTER II

Unveiling olive response
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CHAPTER III

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CHAPTER V

General discussion, main conclusions and future perspectives

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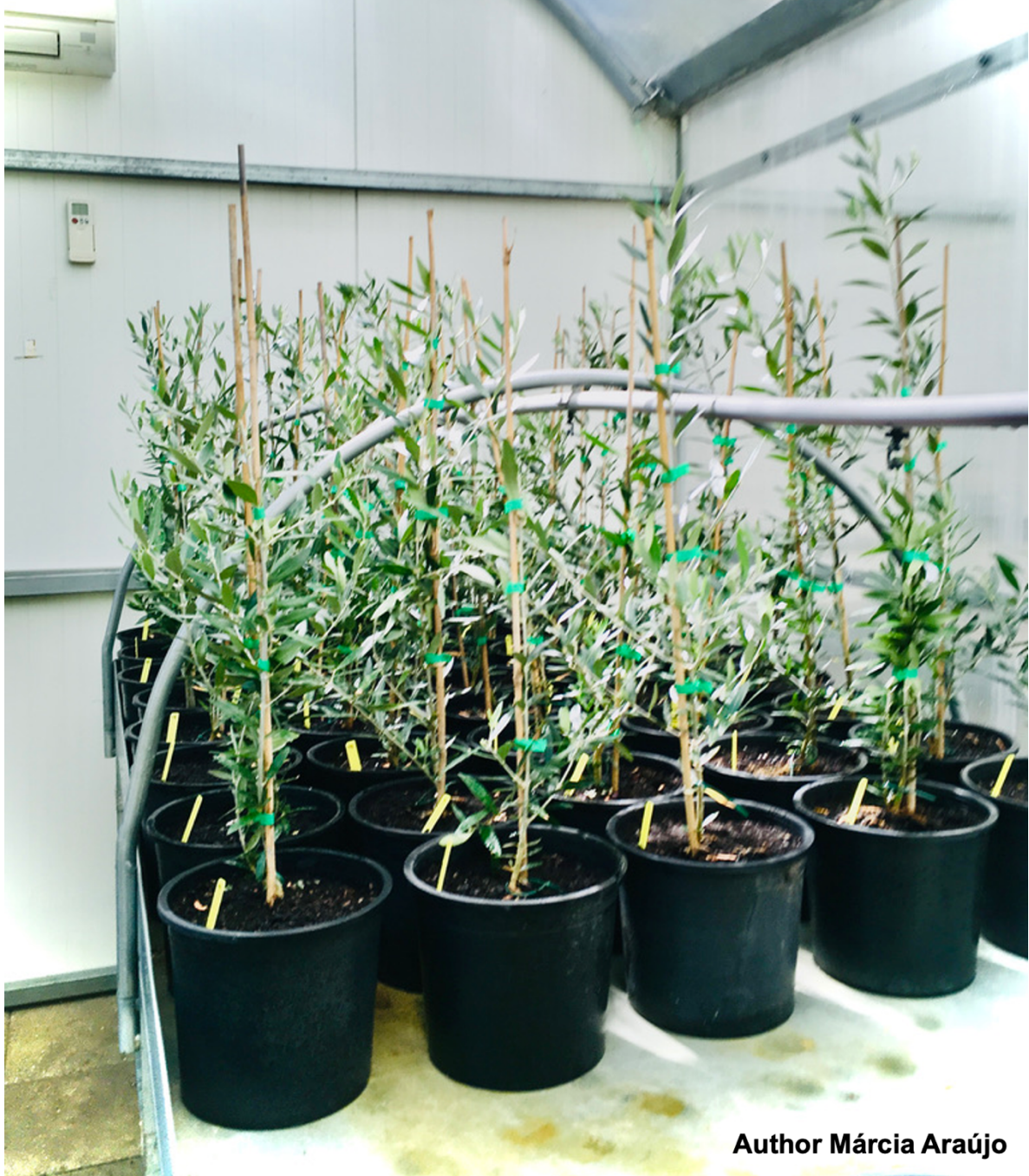
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Chapter II

Unveiling olive responses under greenhouse conditions



Author Márcia Araújo

Chapter II is constituted by two sub-chapters.

Here are presented works under controlled conditions (green house).

Both subchapters are already published. The subchapter 2.1. is a book chapter published in *Theory and Practice of Climate Adaptation* and the subchapter 2.2. is an original article published in *Planta*.

2.1. Can Young Olive Plants Overcome Heat Shock?

Araújo, M., Santos, C., & Dias, M. C. (2018). Can Young Olive Plants Overcome Heat Shock? In U. Alves, F., Leal Filho, W. Azeiteiro (Ed.), *Theory and Practice of Climate Adaptation* (pp. 193–203). https://doi.org/10.1007/978-3-319-72874-2_11

2.2. Responses of olive plants exposed to different irrigation treatments in combination with heat shock: physiological and molecular mechanisms during exposure and recovery.

Araújo, M., Ferreira de Oliveira, J. M. P., Santos, C., Moutinho-Pereira, J., Correia, C., & Dias, M. C. (2019). Responses of olive plants exposed to different irrigation treatments in combination with heat shock: physiological and molecular mechanisms during exposure and recovery. *Planta*, 249(0123456789), 1583–1598. <https://doi.org/10.1007/s00425-019-03109-2>

2.1. Can young olive plants overcome heat shock?¹

ABSTRACT

Climate change is bringing more frequent and intense heat waves over the last years. Under this circumstance, it is important to understand whether species can tolerate stress and which mechanisms are involved in this adaptation process. Olive tree (*Olea europaea* L.) have been known for centuries to be drought tolerant, but less is known about the impact on the physiological response of this species to heat. To understand how young olive plants deal with heat shock, one-year-old plants (cv. 'Arbequina'), grown at 23±2°C, were exposed to heat, 40°C, for 2 hours. Relative water content, gas exchange, carbohydrates content, cell membrane permeability and lipid peroxidation were assessed immediately after heat exposure. The heat shock treatment compromised plant water status, photosynthesis and induced stomatal closure. However, neither membrane damage nor carbohydrates content (total soluble sugars and starch) were affected. The results indicate that young olive plants can overcome short heat shock episodes.

KEYWORDS

climate change, *Olea europaea* L., photosynthesis, abiotic stress

¹ Araújo, M., Santos, C., & Dias, M. C. (2018). Can Young Olive Plants Overcome Heat Shock? In U. Alves, F., Leal Filho, W. Azeiteiro (Ed.), *Theory and Practice of Climate Adaptation* (pp. 193–203). https://doi.org/10.1007/978-3-319-72874-2_11

INTRODUCTION

By the end of this century, the global temperature is expected to increase by 4°C compared to the average 20th century values (IPCC, 2014). This phenomenon will increase the frequency and intensity of high temperature episodes, which will impact on crops' yield and compromise agricultural production (Bita and Gerats, 2013). Heat is considered one of the most deleterious abiotic stresses and severely affects plants (Bita and Gerats, 2013; Zhao et al., 2014). Heat deleterious effects are observed at morphological, physiological, biochemical and molecular levels, which ultimately compromise plant growth, productivity and yield (Galán et al. 2001; Koubouris et al. 2009, Zhao et al. 2014; Bita & Gerats 2013). Whilst fluctuations in temperature occur naturally throughout the plant life cycle, during hot summers the elevated temperatures can induce severe stress in non- adapted plants.

Olive trees (*Olea europaea* L.) are known for centuries in the Mediterranean region for their economic value. About 98% of the world olive and 78% of world olive oil are produced in this region (Rigueiro-Rodríguez et al., 2009). This species is also known to tolerate a broad range of environmental stresses, including heat, mostly due to a variety of morphological and physiological adaptations (e.g., Bacelar, Santos, et al. 2007; Carr 2013; Tripepi et al. 2011), but less is known about the mechanisms regulating their adaptation to heat episodes. Due to climate change, the increase in temperatures is expected in the Mediterranean region (IPCC, 2014). The quality of olive products (olive fruit and oil) is influenced by cultivar, soil and climatic conditions (Criado et al., 2007). Climate change may induce changes in the quality and production/yield of olive plants. Therefore, it is important for producers to select the fittest cultivars coping heat conditions and also to explore the way highly productive cultivars like 'Arbequina' interact with heat.

Heat is perceived to plants as a whole process, meaning that there is no specific molecule playing a thermosensor role (Brestic et al., 2013). In fact, diverse intracellular signalling molecules are expressed in response to heat exposure. These molecular signals are integrated with multiple signal transduction pathways involved in different cell compartments (Brestic et al., 2013). The changes in the regulation of gene expression followed by changes in the transcriptome, proteome and metabolome enable plants to acclimate and survive to heat stress (Brestic et al., 2013). A sensitive metabolic process in plant cells to heat stress is photosynthesis. The photosynthetic apparatus can tolerate moderate temperatures involving reversible changes or may be severely damaged as result of severe heat (Brestic et al., 2013).

In this study, our aim is to understand how young olive plants deal with heat shock. To achieve this objective, we exposed *O. europaea* cv 'Arbequina' to heat shock. Water status, gas exchange, carbohydrates content, cell membrane permeability and lipid peroxidation were

measured. By investigating these physiological parameters, we expect to decipher which mechanisms are involved in the adaptation process of young olive plants to heat stress.

MATERIAL AND METHODS

Plant material and experimental conditions

O. europaea L. cv 'Arbequina' plants (1-year-old) were obtained at *Viveiros Vitor Santos* (Mealhada, Portugal). Plants were maintained for one month under controlled conditions in a phytotron: $23\pm 2^{\circ}\text{C}$, with a photoperiod of 16h/8h (day/night), $30\pm 6\%$ of relative humidity, a light intensity of $\approx 600 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by lamps (Osram cool white fluorescent). All plants were watered to field capacity before the experiment. Plants were randomly divided into two groups as follows: i) control, where plants were maintained in the same conditions mentioned above, and ii) heat shock treatment, where plants were exposed for 2 hours at $40\pm 2^{\circ}\text{C}$. After the heat shock treatment, gas exchange, plant water status and cell membrane permeability were evaluated. Also, leaf samples were collected, frozen in liquid nitrogen and stored at -80°C for further analysis of carbohydrates and lipid peroxidation.

Plant water status

As described by Araújo et al. (2016), leaf fresh weight (FW), turgid weight (TW) and dry weight (DW) were determined to calculate the relative water content (RWC). RWC is calculated as $100 \times (\text{FW}-\text{DW})/(\text{TW}-\text{DW})$ and expressed as a percentage (%).

Gas exchange

A portable infrared gas analyser (Infra-Red Gas Analyzer: IRGA, LCpro+, ADC, Hoddesdon, UK) was used to measure net CO_2 assimilation rate (P_N) and transpiration rate (E) in leaves. Also, stomatal conductance (g_s) was calculated according to (von Caemmerer and Farquhar, 1981).

Evaluation of carbohydrates content

To evaluate the total soluble sugars (TSS) (Irigoyen et al., 1992), 80% (v/v) ethanol was added to frozen leaves powder and incubated for one hour at 80°C . After centrifuged ($5000\times g$, 10 min, 4°C), the supernatant was incubated with an anthrone solution (40 mg anthrone: 1 mL dH_2O : 20 mL H_2SO_4) for 10 min at 100°C . When cooled, samples were centrifuged ($5000\times g$, 10 min, 4°C) and the absorbance of the supernatant was read at 625 nm

using a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S). The concentration of TSS was obtained against a D-glucose standard curve.

Starch content was quantified according to Osaki et al. (1991). To the resultant pellet of TSS extraction was added 30% (v/v) perchloric acid and incubated for one hour at 60°C. After centrifuged (10000×g, 10 min, 4°C), the supernatant was incubated with an anthrone solution for 10 min at 100°C. Then, the supernatant was cooled, centrifuged (5000×g, 10 min, 4°C), and the absorbance was read at 625 nm using a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S). The starch concentration was obtained against a D-glucose.

Determination of cell membrane permeability and lipid peroxidation

Electrolyte leakage was used to evaluate cell membrane permeability as described by Dias et al., (2014). Leaves were enclosed in vials with de-ionized water and incubated overnight at room temperature on a rotary shaker. Electrical conductivity was measured (L_t) after 24h. Samples were autoclaved at 120°C for 20 min and after reached room temperature, electrical conductivity (L_0) was read. Electrical leakage was calculated as L_t/L_0 and expressed as a percentage (%).

The production of malondialdehyde (MDA) was used to evaluate lipid peroxidation (Hodges et al., 1999). Samples were homogenised with 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged (10000×g, 10 min, 4°C). An aliquot of supernatant was homogenized with 20% TCA (w/v) (-TBA) and another with 20% TCA (w/v) and 0.5% thiobarbituric acid (TBA) (w/v) (+TBA). Both samples (-TBA and +TBA) were incubated for 30 min at 95°C, cooled on ice and centrifuged as previously. Absorbance was read at 440, 532 and 600 nm using a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S). MDA were calculated as $(A - B/157\,000) \times 10^6$, where A is $(\text{Abs } 532_{+TBA}) - (\text{Abs } 600_{+TBA}) - [(\text{Abs } 532_{-TBA}) - (\text{Abs } 600_{-TBA})]$ and B is $[(\text{Abs } 440_{+TBA}) - (\text{Abs } 600_{+TBA})] \times 0.0571$.

Data analysis

The experiments were conducted with 3 plants per treatment and were analysed using a SigmaPlot (version 3.1) program for performing statistical analysis. Data were analysed by one-way analysis of variance (ANOVA) follow by the Holm-Sidak comparison test.

RESULTS AND DISCUSSION

The emerging global climate change is already having a considerable impact on agricultural production, particularly in the Mediterranean region where *O. europaea* plays a

crucial economic role. Several studies have shown that olive plants are tolerant to drought (Bacelar et al., 2007b, 2007a, 2006), nonetheless the physiological response of juvenile olive plants to heat episodes remains less studied. In this experiment all juvenile plants survived to the heat treatment, and showed no significant morphological changes (necrosis, chlorosis, leaf decay, etc.), supporting that even at an early stage they present tolerance mechanisms. Similarly to other stresses, such as water deficit (e.g., Dias et al., 2014a) or high UV-B radiation (e.g., Araújo et al., 2016), the photosynthetic apparatus is one of the main targets of heat stress (Brestic et al., 2013). In this experiment, whilst no chlorosis was observed, our data support that the juvenile plants exposed to 2 hours' heat shock (HS) episode suffered an impairment of the net CO₂ assimilation rate (decrease of 70.6%, $p \leq 0.05$, Figure 2.1.1 A), which supports some damage in the photosynthetic apparatus. Also, transpiration rate was reduced ($p \leq 0.05$) by around 71.6% and stomatal conductance by 79.6%, relative to control plants (Figures 2.1.1 B and C). These changes indicate that olive plants respond to HS by closing the stomata. Stomatal closure is a common defence mechanism in heat-stressed plants and can prevent the plant from water loss. At the same time, it also restricts CO₂ uptake, which conditions the availability of this gas in the intercellular spaces of mesophyll cells, which is the main cause of net CO₂ assimilation rate declination under stress (Araújo et al., 2016). In these young olive plants, the HS episode decreased ($p \leq 0.05$) only 7.5% leaf RWC (Table 2.1.1), indicating that stomatal closure prevented from severe water loss and putatively from cell dehydration. Our data are in line with Koubouris et al. (2015), who also reported a strong reduction in olive net CO₂ assimilation rate, stomatal closure and a decrease in the intercellular CO₂ concentration (C_i) after a longer (28h at $38 \pm 4^\circ\text{C}$) HS treatment.

Plant growth and carbon metabolism are closely associated since carbohydrate (particularly sucrose), the final product of photosynthesis, is the main source of the production and maintenance of biomass. Abiotic stress leads, in some species, to significant reductions in photosynthesis in source tissues, leading to a reduction of the supply of soluble sugars to sink tissues (Wahid et al., 2007). In the present work, we analysed total soluble sugars and starch, a reserve carbohydrate (Table 2.1.1). Despite the strong decrease of net CO₂ assimilation rate observed in olive plants after the HS, the pools of starch and total soluble sugars were not significantly affected indicating that carbon partitioning/translocation was not affected. Also, Bacelar, Santos, et al. (2007) verified a similar amount of total soluble sugars after different water regimes applied despite a low P_N , indicating that olive plants accumulate osmotic compounds to maintain the maximum amount of water as possible. Similar strategies of maintaining the carbohydrates high are used by other species such as sugarcane (Wahid et al., 2007) under heat stress.

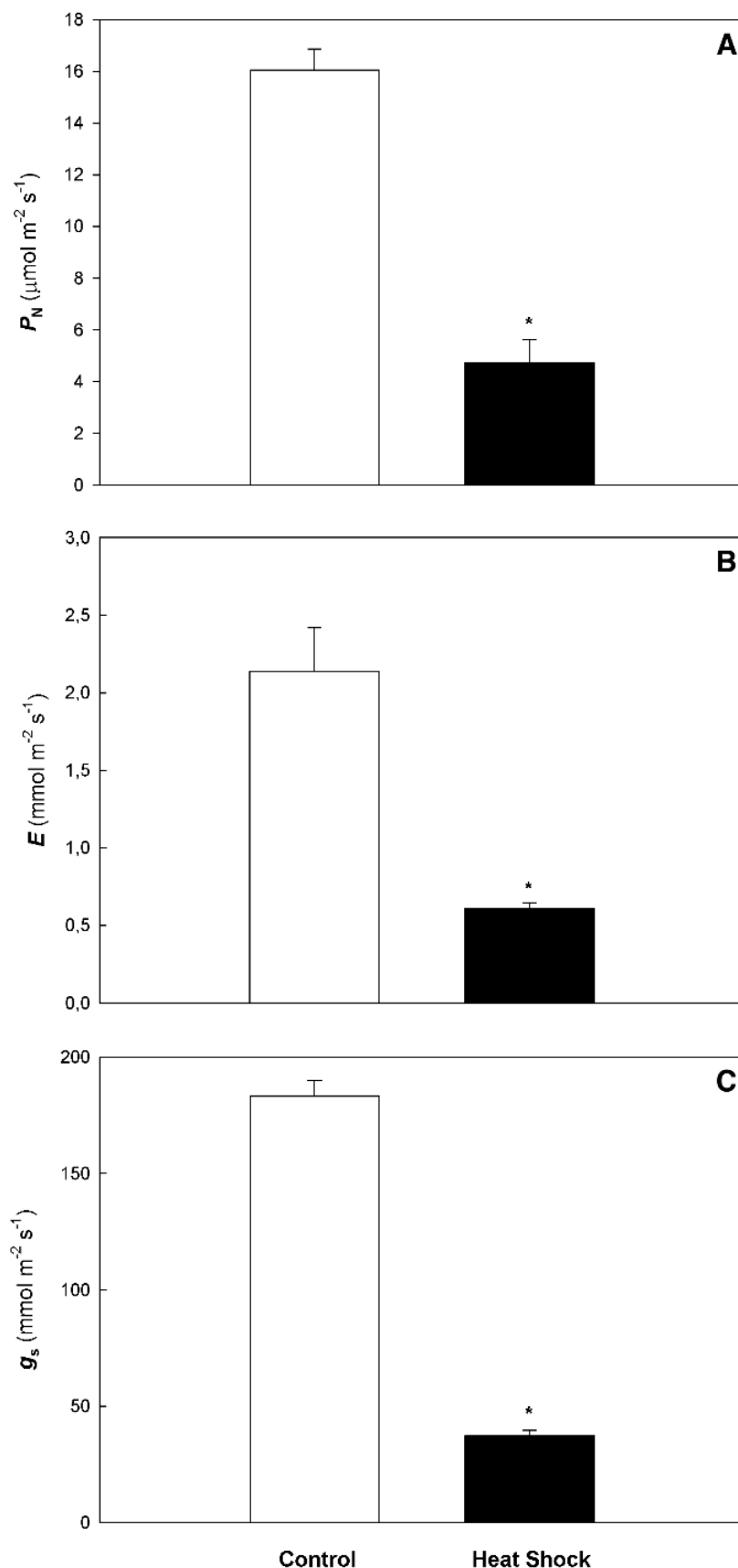


Figure 2.1.1 Net CO₂ assimilation rate, P_N (A), transpiration rate, E (B) and stomatal conductance, g_s (C) in leaves of young *O. europaea* plants under control conditions and heat shock treatment. Data shown are mean $\pm$ SEM (n=3). *, Significant differences between control and heat shock treatment (p ≤ 0.05).

Table 2.1.1 Relative Water Content (RWC), total soluble sugars (TSS) and starch in leaves of young *O. europaea* plants under control conditions and heat shock treatment. Data shown are mean $\pm$ SEM ($n = 3$). *, Significant differences between control and heat shock treatment ($p \leq 0.05$).

Parameter	Control			Heat Shock		
RWC (%)	85.3	$\pm$	0.14	78.9	$\pm$	0.49*
TSS ($\mu\text{g mg}^{-1}$ DW)	105.6	$\pm$	2.80	98.1	$\pm$	2.63
Starch ($\mu\text{g mg}^{-1}$ DW)	62.1	$\pm$	4.75	70.9	$\pm$	7.01

A common response of plants to heat stress is the increased production of ROS. ROS attack essential cellular components and structural elements, and accumulation of ROS are associated with lipid peroxidation, making cellular membranes particularly susceptible to oxidative damage (Feki et al., 2015; Mittler et al., 2011). Thylakoid membranes are the site where the light-dependent reaction of photosynthesis occurs and are particularly sensitive to ROS. Therefore, damages on these membranes can result in reduced photosynthetic activity. We analysed one parameter widely used as ROS induced oxidation in macromolecules, namely lipids (the MDA). The leaves of olive plants exposed to the heat episodes tested here showed to cope with the stress with no increase of MDA. Increases in cellular MDA levels may, indirectly, be correlated with parameters of cell membrane integrity, as is the CMP (Figures 2.1.2 A and B). Therefore, the absence of significant changes in the CMP ($p > 0.05$) of plants exposed to heat shock, is in line with the MDA results, again suggesting that these juvenile plants tolerate the imposed heat stress. Mechanisms underlying this tolerance may involve the increase of heat shock proteins, which are documented to have a crucial role in the heat shock response (McLoughlin et al., 2016). Heat shock proteins family act as first line of defence against heat stress (Assab et al., 2011; McLoughlin et al., 2016), and some preliminary data also support an increase of these proteins in these olive plants (data not shown), and may justify at least partially the absence of membrane damages and lipid peroxidation.

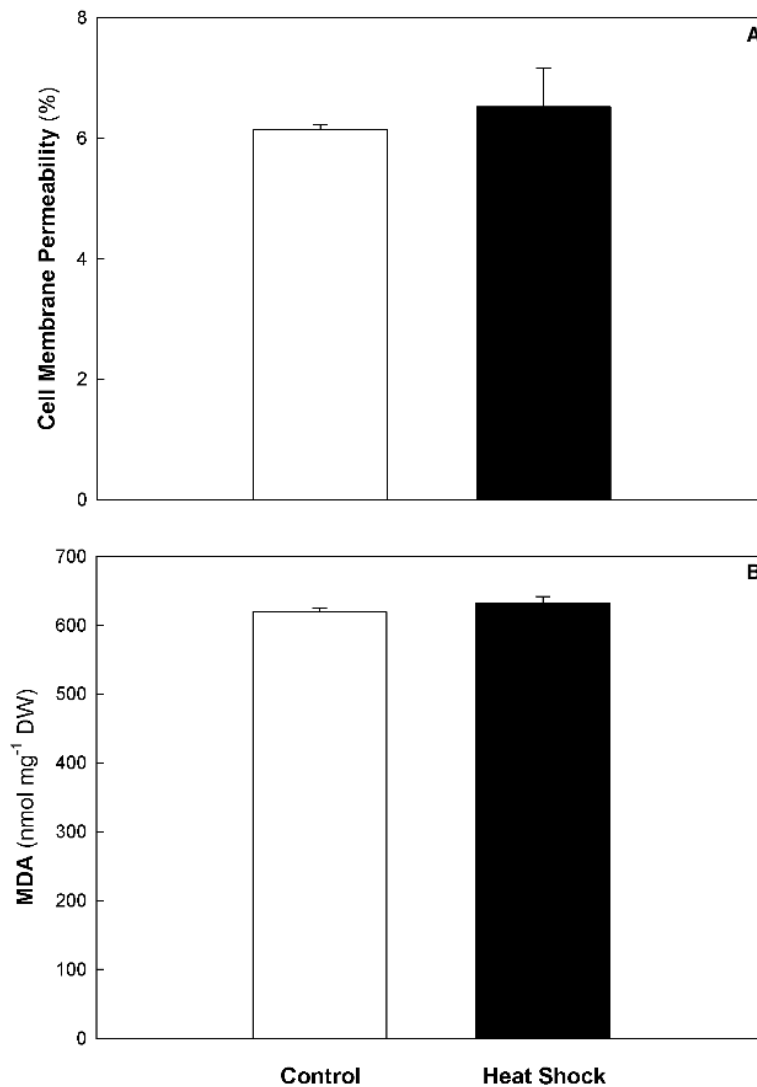


Figure 2.1.2 Cell membrane permeability (A) and MDA content (B) in leaves of young *O. europaea* plants under control conditions and heat shock treatment. Data shown are mean $\pm$ SEM (n =3). *, Significant differences between control and heat shock treatment ($p \leq 0.05$).

CONCLUSION

In conclusion, our data showed that after a short period of HS, young olive plants survived, but showed functional adjustments at the photosynthetic level. Plants closed their stomata to decrease the transpiration levels, but at the same time, this may reduce the amount of CO₂ availability, resulting in an impairment of net CO₂ assimilation rate. Despite the observed stomatal closure, plant water status decreased. However, changes seem not be deleterious to these plants as no increased lipid peroxidation nor cell membrane damage were observed. These mechanisms performed by young olive plants shows that this species is capable to overcoming a short heat shock episode.

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2.2. Responses of olive plants exposed to different irrigation treatments in combination with heat shock: physiological and molecular mechanisms during exposure and recovery²

ABSTRACT

Olive is traditionally cultivated in dry farming or in high water demanding irrigated orchards. The impact of climate change on these orchards remains to unveil, as heat and drought episodes are increasing in the Mediterranean region. To understand how young plants face such stress episodes, olive plants growing in pots were exposed to well-irrigated and non-irrigated treatments. Subsequently, plants from each treatment were either exposed to 40 °C for 2 h or remained under control temperature. After treatments, all plants were allowed to grow under well-irrigated conditions (recovery). Leaves were compared for photosynthesis, relative water content, mineral status, pigments, carbohydrates, cell membrane permeability, lipid peroxidation and expression of the protective proteins' dehydrin (OeDHN1), heat-shock proteins (OeHSP18.3), and aquaporins (OePIP1.1 and OePIP2.1). Non-irrigation, whilst increasing carbohydrates, reduced some photosynthetic parameters to values below the ones of the well-irrigated plants. However, when both groups of plants were exposed to heat, well-irrigated plants suffered more drastic decreases of net CO₂ assimilation rate and chlorophyll *b* than non-irrigated plants. Overall, OeDHN1 and OeHSP18.3 expression, which was increased in non-irrigated treatment, was potentiated by heat, possibly to counteract the increase of lipid peroxidation and loss of membrane integrity. Plants recovered similarly from both irrigation and temperature treatments, and recovery was associated with increased aquaporin expression in plants exposed to one type of stress (drought or heat). These data represent an important contribution for further understanding how dry-farming olive will cope with drought and heat episodes.

KEYWORDS

Aquaporins, Climate change, Dehydrins, Drought, Heat-shock proteins, Photosynthesis

² Araújo, M., Ferreira de Oliveira, J. M. P., Santos, C., Moutinho-Pereira, J., Correia, C., & Dias, M. C. (2019). Responses of olive plants exposed to different irrigation treatments in combination with heat shock: physiological and molecular mechanisms during exposure and recovery. *Planta*, 249(0123456789), 1583–1598. <https://doi.org/10.1007/s00425-019-03109-2>

INTRODUCTION

Olive (*Olea europaea* L.) is a major crop adapted to Mediterranean climate (Brito et al., 2018; Ramos and Santos, 2010). Considering that sustainable olive culture practices must address the challenges posed by climate change in this region, the most used practice of (super)intensive olive production has to be replaced by less water demanding practices like dry farming or the emerging partial root-zone drying (e.g. Wang et al. 2017). Also, within a climate change scenario, olive orchards are exposed to other stress episodes, namely heat, which may differently impact the orchard's productivity according to its agricultural system and cultivar. Among the cultivars more intensively used worldwide, 'Arbequina', whilst considered tolerant to drought (Bacelar et al., 2004; Benlloch-González et al., 2008), has been widely used in super-intensive productive orchards, with unsustainable water demands (Larbi et al., 2015).

Although the effects of drought or heat stress on plant performance are well characterised, few studies have investigated the molecular and physiological responses to a heat short-term episode of plants under different irrigation treatments. A first sign of these stressful conditions is the overproduction of reactive oxygen species (ROS), which if not counteracted by the protective antioxidant (enzymatic and non-enzymatic) battery, may lead to biomolecules peroxidation and ultimately to organelle/membrane damages and cell death (Araújo et al., 2018). Being chloroplasts a major source of ROS, the well-functioning of the photosynthetic apparatus thus depends on the plants' capacity to balance the cell/chloroplast redox status under heat and/or drought (Gollan et al., 2015; Tamburino et al., 2017). Besides, photosynthesis is also directly linked to other drought and/or heat protective strategies such as the stomatal control, which is important to regulate water content, but may compromise gas exchange (reducing intercellular CO₂ availability) and related photosynthetic reactions (Araújo et al., 2016; Dias et al., 2014a). Also, enhancing energy dissipation and reducing light absorption are protective mechanisms preventing photoinhibition-related damages that often occur as result of drought and/or heat stress (Mittler et al., 2012; Pinheiro and Chaves, 2011).

Other mechanisms that include specific proteins, like aquaporins (AQPs) and dehydrins (DHNs) also contribute to the protective responses to environmental challenges (Hara et al., 2013; Kumar et al., 2014; Liu et al., 2013). AQPs plasma membrane-intrinsic proteins PIPs (PIP1 and PIP2) are the main transmembrane transporters of water and CO₂ through plant cell membranes (Chaumont and Tyerman, 2014; Yaneff et al., 2015). In particular, AQPs determine water flux and CO₂ assimilation rates via stomatal control, affecting photosynthesis through their effect on mesophyll conductance (Moshelion et al., 2015). Although several reports demonstrated that PIPs are responsive to abiotic stresses, gene expression studies showed inconsistent patterns of these proteins (Kapilan et al., 2018;

Reddy et al., 2017). Under water deficit, the upregulation of PIP1 and PIP2 expression seems to promote water movement inside leaves via symplast enhancing membrane permeability to water (Perez-Martin et al., 2014; Pou et al., 2013). In turn, PIP2 downregulation can be seen as a strategy for cellular water conservation, reducing membrane water permeability and preventing water loss (Secchi et al., 2007). The small proteins DHNs are located in the cytosol, nuclei, chloroplasts, mitochondria and vacuoles, and during drought stress promote a protective response to cellular dehydration (Chiappetta et al., 2015), acting as chaperones, buffering water, sequestering ions and stabilising membranes (Kumar et al., 2014; Tripepi et al., 2011). Specific heat shock proteins (HSPs) such as the HSP100, HSP70 and small heat shock proteins (sHSPs, with 12-42 kDa) are also in the first line of defence against heat and/or drought stress since they interact with denaturing proteins (Assab et al., 2011; McLoughlin et al., 2016). How these combined protective responses behave in olives under a dry farming system (where plants are frequently exposed to multiple stress conditions), and their contribution to the plants recovery after the stress removal is not yet understood.

Aligned with the environmental benefits evidenced by dry farming approaches, we hypothesise that olive plants exposed to a non-irrigation treatment may develop a functional plasticity that will increase the plant's ability to cope with an additional heat shock episode, preventing cumulative damages during, and after, the heat-stress episode. This protective plasticity may involve photosynthetic adjustments and protective proteins (AQPs, DHNs and sHSPs). In the present study, well-irrigated and non-irrigated young plants were exposed to a heat-shock episode. A functional approach covering photosynthetic performance, water status, nutritional balances and membrane damages was performed. In parallel, the expression level of key genes related to the protective proteins AQPs (*OePIP1.1* and *OePIP2.1*), DHNs (*OeDHN1*) and HSPs (*OeHSP18.3*) were analysed to better understand if, and how, prolonged non-irrigation influences the capacity of these plants to cope with (and recover from) heat episodes.

MATERIAL AND METHODS

Plant material and experimental conditions

Seven-month-old *Olea europaea* L. cv 'Arbequina' potted plants (2L black plastic pots with a loam soil) provided by the nursery *Viveiros Vitor Costa* (Mealhada, Coimbra, Portugal) were acclimated for one month in a climate room with photoperiod of 16h/8h (day/night), temperature of 23 ± 2 °C, and relative humidity of $30 \pm 6\%$. Light was provided by lamps (Osram cool white fluorescent) that gave a light intensity of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were weekly fertilised (NPK 12-4-6, Complesal, Bayer). Two irrigation treatments were performed, one well-

irrigated and another non-irrigated. The day before the beginning of the treatments, all plants ($n = 40$) were watered to 100% field capacity. While one group of plants were maintained well-irrigated ($n=20$), another group of plants were non-irrigated ($n=20$) for one month. After this period, half of the plants of each group was subjected to a heat episode: for that, plants were transferred to a climate chamber (with the same light intensity and relative humidity, where the temperature was $40 \pm 2^\circ\text{C}$ and maintained inside for 2 hours at the same temperature (WI^{H} , well-irrigated plants with a heat shock, $n=10$; NI^{H} , non-irrigated plants with a heat shock, $n=10$). The other half of plants was maintained at control temperature conditions (WI , well-irrigated plants at control temperature, $n=10$; NI , non-irrigated plants at control temperature, $n=10$). Gas exchange, chlorophyll *a* fluorescence, relative water content and cell membrane permeability were analysed in fresh leaves. Additionally, leaf samples were collected, snap frozen in liquid nitrogen and stored at -80°C until further determination of pigments, carbohydrates, malondialdehyde (MDA) and gene expression. After collecting samples, the plants were maintained under optimal growth condition (WI conditions) and used to evaluate the plant recovery capacity 10 days after stresses relief. The same parameters described above were evaluated on the same plants.

*Gas exchange and chlorophyll *a* fluorescence*

Gas exchange was measured using a portable infrared gas analyser (ADC, Hoddesdon, England, UK). Net CO_2 assimilation rate (P_{N}), and transpiration rate (E) were measured in leaves under environmental growth conditions. Stomatal conductance (g_{s}) was calculated according to von Caemmerer and Farquhar (1981).

Chlorophyll *a* fluorescence was measured using a pulse amplitude modulation system (Hansatech Instruments, Norfolk, England, UK). After dark adaptation, the minimum fluorescence was measured by applying a weak-intensity modulated light. The maximum fluorescence was measured after applying, during 0.7 s, a saturating pulse of white light ($> 1500 \mu\text{mol m}^{-2} \text{s}^{-1}$). Then, leaves were adapted to light and the steady-state fluorescence was averaged over 2.5 s, followed by exposure to saturating light ($5000 \mu\text{mol m}^{-2} \text{s}^{-1}$, during 0.7 s) to establish the maximal fluorescence in the light. Then, the actinic light was turned off and the minimal fluorescence was determined. The maximum quantum efficiency of photosystem II ($F_{\text{v}}/F_{\text{m}}$), the efficiency of excitation energy capture by open PSII reaction centres ($F'_{\text{v}}/F'_{\text{m}}$), the effective quantum efficiency of PSII (Φ_{PSII}), the photochemical quenching ($q\text{P}$) and the non-photochemical quenching (NPQ) were calculated according to Dias et al. (2016).

Relative water content and mineral status

Leaf relative water content (RWC, %) was calculated as $100 \times (\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight})$. For mineral analysis, leaves were washed in Milli-Q water, weighed and dried at 80°C, till constant weight. Samples were incinerated at 530 °C during 8h. After cooling, ashes were weighed. Then some drops of Milli-Q water and 1 mL of concentrated HCl were added to the ashes. This mixture was heated to boiling and then another 1 mL of HCl was added and heated to boiling again. Finally, 10 mL of HCl 10% (v/v) was added and filtered (2 µm filter). Samples were analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES Jobin Yvon Activa M) to determine Mg, Ca, K, Fe, Zn and Cu contents.

Pigment content

Frozen leaves powder was mixed with cold acetone/50 mM Tris pH 7.8 (80:20, v/v) and centrifuged at 5 000g for 5 min at 4 °C (Sims and Gamon, 2002). Absorbance was read at 470, 537, 647 and 663 nm with a Thermo Fisher Scientific spectrophotometer (Genesys 10-uvS). The contents of chlorophyll *a*, chlorophyll *b* and carotenoids were calculated (Sims and Gamon, 2002).

Carbohydrates content

Total soluble sugars (TSS) were quantified according to Irigoyen et al. (1992). Briefly, 80% (v/v) ethanol was added to frozen leaves powder and placed in an 80 °C bath for one hour. Samples were centrifuged at 5 000g for 10 min (4 °C). The supernatant was incubated with anthrone solution (40 mg anthrone: 1 mL dH₂O: 20 mL H₂SO₄) for 10 min at 100 °C. After cooling, samples were centrifuged as previously, and the absorbance was read at 625 nm using a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S). TSS concentration was calculated against a D-glucose standard curve (, $R^2 = 0.952$).

Starch was extracted from the TSS remaining pellet according to Osaki et al. (1991). Perchloric acid (30%, v/v) was added to pellet and the mixture was incubated at 60 °C for 1 h and then centrifuged at 10 000g for 10 min (4 °C). The supernatant was incubated with anthrone solution at 100 °C for 10 min. After centrifugation (5 000g, 10 min, 4 °C), the absorbance was read at 625 nm using a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S). Starch content was determined against a D-glucose standard curve (, $R^2 = 0.989$).

Protective proteins (AQPs, DHNs and HSPs)

To assess the impact of heat episodes in protective proteins, the transcripts of selected AQPs, DHNs and HSPs were quantified. RNA was extracted using the method described by Le Provost et al. (2007) with modifications. Leaf RNA was extracted with 900 µL of extraction buffer (20 mM Na₂EDTA pH 8.0; 2% cetyltrimethylammonium bromide (m/v); 0.1 M Tris pH 8.0; 2% polyvinylpyrrolidone (w/v) and 1.4 M NaCl) for 15 min at 65 °C with vortex mixing every 5 min, and then cooled on ice for 5 min. Then, 900 µL of chloroform: isoamyl alcohol (24:1, v/v) was added to samples, followed by vigorous mixing for 5 s. Samples were placed 5 min at room temperature and then centrifuged at 16 000g for 10 min at 4 °C. The upper phase was transferred to another tube, 900 µL of chloroform: isoamyl alcohol (24:1, v/v) was added, and the mixing and centrifugation process was repeated twice. After the third extraction with chloroform: isoamyl alcohol, 0.1 volumes of 3 M sodium acetate and 2 volumes of absolute ethanol (-20 °C) were added and the samples were gently mixed. Samples were incubated at -20 °C for 1 hour. The mixture was centrifuged at 16 000g for 30 min at 4 °C and the supernatant was discarded. To wash the pellet, 400 µL of 70% ethanol (-20 °C) was added and centrifuged at 16 000g for 3 min at 4 °C. The supernatant was discarded and 250 µL of absolute ethanol was added. Samples were centrifuged at 16 000g for 3 min at 4 °C, supernatant discarded, and pellet dried out. To re-suspend the pellet, 30 µL of DEPC-treated Milli-Q water was added, and samples were stored at -80 °C. Before cDNA synthesis, 2 µg of total RNA were incubated with 1U DNase I Amplification grade (Life Technologies, Carlsbad, CA-USA) to remove any contaminating genomic DNA. Prior and after this treatment, total RNA was quantified on a Thermo Fisher Scientific spectrophotometer (NanoDrop 1000). After DNase I treatment, 2 µg of total RNA was reverse-transcribed with 1 µM Oligo(dT)₁₈, using the Omniscript RT Kit (Qiagen, Hilden, Germany). cDNA samples were diluted in ultrapure Milli-Q water (1:20). All qPCR reactions were performed in 12 µL volumes containing iTaq SYBR Green Supermix (BioRad, USA), 150 nM each gene-specific primer (Table 2.2.1) and 3 µL pre-diluted cDNA (1:20). The reactions were subjected to an initial denaturation of 30 s at 95 °C, followed by 2 cycles of 1 min at 95 °C, 1 cycle of 6 s at 95 °C, 60 cycles at 94 °C for 5 s, 58 °C for 15 s, 72 °C for 15 s and 68 °C for 6 s. A melting program was performed at the end of qPCR. Average PCR efficiency values and cycle thresholds (CTs) were estimated from the fluorescence data using the algorithm Real-Time PCR Miner (Zhao and Fernald, 2005). At least 3 qPCR technical replicates were performed per sample from each of 2 independent pools of three plants. Gene-specific primers (Table 2.2.1) were designed from published *O. europaea* coding sequences using the Primer3 design tool according to Rozen and Skaletsky (1999). Gene expression levels of target genes were calculated relative to control (WI) and

normalised with *OeACT1* and *OeGAPB* reference genes according to $\Delta\Delta CT$ method (Applied Biosystems, 2008), after CT transformation with efficiency values.

Table 2.2.1 Primers used for qPCR

Gene Name	GenBank Accession No.	Primer Sequence (5'-3')
<i>OeACT1</i>	AF545569.1	F: GAGAGGGTATATGTTACACCTCA R: GGACTTCTGGACAACGGAATCT
<i>OeGAPB</i>	EF506494.1	F: GCTCAAGGGCAAGCTCAA R: ATCGCATACGGCCAATACAC
<i>OeDHN1</i>	KR349290.1	F: CCCACCACCACCATATCAG R: CCTTTTCCTTCTCTTCCTCAGTC
<i>OeHSP18.3</i>	FN554869.1	F: CAGCCGTCTCTGCTCCAA R: CTCCCTGCTCCTCTCTCCTC
<i>OePIP1.1</i>	DQ202708.1	F: GGCTGTTTTTGGGGAGGA R: CCATCGCCCTTGGTGTA
<i>OePIP2.1</i>	DQ202709.2	F: GGTTGGTTTGGGTGCTGA R: GCAGGGTTGATGCCAGTT

Oxidative status

Lipid peroxidation was evaluated by measuring the production of MDA as described by Hodges et al. (1999). Frozen leaves powder was homogenised with 0.1% (w/v) trichloroacetic acid and centrifuged (10 000g, 10 min at 4 °C). An aliquot of supernatant was mixed with 20% trichloroacetic acid (w/v) as a negative control and another with 20% trichloroacetic acid (w/v) and 0.5% thiobarbituric acid (w/v) as a positive control. Both positive and negative control groups were incubated at 95 °C for 30 min, cooled on ice and centrifuged (10 000g, 10 min, 4 °C). Absorbance was read at 440, 532 and 600 nm using a Thermo Fisher Scientific spectrophotometer (Genesys 10-uvS). MDA equivalents were calculated according to Hodges et al. (1999).

Cell membrane permeability (CMP) was accessed by electrolyte leakage (Dias et al., 2014b). Leaf was enclosed in vials with de-ionised water and incubated overnight at room temperature on a rotary shaker. After 24h, electrical conductivity was measured (L_t) and then samples were autoclaved at 120 °C for 20 min. Conductivity was read (L_0) when samples reached room temperature. Electrolyte leakage (%) was calculated as $L_t/L_0 \times 100$.

Statistical analysis

The program SigmaPlot for Windows version 3.1 was used. Two-way ANOVA was used to assess the effects of irrigation ($WI+WI^H$ vs $NI+NI^H$) and temperature ($WI+NI$ vs WI^H+NI^H), as well as their interactions, on olive physiology and on the expression of genes related to stress protection. Pairwise differences among treatments were compared by Holm-Šidák Comparison Test at the 5% significance level. Pearson's Correlation was analysed also using the same statistical program. The heat map analysis was performed using the Microsoft® Excel for Mac (version 16.15).

RESULTS

Gas exchange and chlorophyll a fluorescence

Figure 2.2.1 presents the gas-exchange in olive plants immediately after stress and recovery. After stress, only the irrigation factor influenced g_s and E , which presented lower values under the non-irrigated treatment. Meanwhile, P_N was reduced in the non-irrigated and in the heat shock treatments, with larger differences at the control temperature and at the well-irrigated condition, respectively. After the recovery period, g_s was particularly high under the non-irrigated treatment.

Immediately after stress exposure, the F_v'/F_m' was reduced in the non-irrigated treatment, being more pronounced under the heat shock condition (Table 2.2.2). The Φ_{PSII} and qP were reduced under the non-irrigated treatment (Table 2.2.2). Only temperature factor influenced NPQ, which presented higher values under heat shock (Supplementary material 1). After the recovery period, the qP presented lower values under the non-irrigated treatment whereas the F_v/F_m was reduced under the heat shock treatment (Table 2.2.2 and Supplementary material 2.2.1). In addition, the F_v'/F_m' and Φ_{PSII} were not only reduced under the non-irrigated treatment, but also under the heat shock treatment (Table 2.2.2).

Relative water content and nutritional status

Immediately after stresses, the RWC was lower under the heat shock treatment (Figure 2.2.2 a). Concerning nutrients (Table 2.2.3), immediately after stress the Fe content was reduced under the well-irrigated with heat shock condition as well as under the non-irrigated under control temperature condition. In addition, the Cu, Ca and K contents were affected by the temperature factor with the lowest values obtained under control temperature and with major differences found in the non-irrigated condition. Both the irrigation and the temperature factors influenced Zn and Mg contents. In particular, the content of Zn was reduced under the non-irrigation and the heat shock treatments, whereas the opposite was

observed for Mg. After the recovery period, Ca content presented lower values in the non-irrigated and in the heat shock treatments, with larger differences observed under control temperature and under the well-irrigated conditions, respectively. The contents of Fe and K were lower in the non-irrigated and in the control temperature treatments. Cu content was reduced in the non-irrigated treatment and Mg content was reduced in the heat shock treatment.

Pigment content and carbohydrates

After stress application, chlorophyll *a* content was lower under the well-irrigated treatment, with major differences observed under control temperature condition (Figure 2.2.3 a). The content of chlorophyll *b* presented lower values in the non-irrigated and the heat shock treatments, with larger differences found under control temperature and under the well-irrigated conditions, respectively (Figure 2.2.3 b). For the carotenoids, their content was reduced in the non-irrigated and in the heat shock treatments, in particular, larger differences were found under heat shock and under the non-irrigated conditions, respectively (Figure 2.2.3 c). After recovery, the level of chlorophyll *a* was lower in the control temperature and in the non-irrigated treatments, with major differences in the latter observed under heat shock temperature condition (Figure 2.2.3 d). In addition, the level of chlorophyll *b* was only influenced by the temperature factor, being higher in the heat shock treatment (Figure 2.2.3 e). Carotenoids were lower in the non-irrigated and in the heat shock treatments (Figure 2.2.3 f).

Concerning carbohydrates (Figure 2.2.4) after stress exposure, only irrigation factor influenced TSS and starch levels, which presented higher values in the non-irrigated treatment. After the recovery period, TSS levels were higher in the non-irrigated and in heat shock treatments.

Protective proteins: relative expression of OeDHN1, OePIP1.1, OePIP2.1 and OeHSP18.3

Figure 2.2.5 presents the relative expression of *OeDHN1*, *OePIP1.1*, *OePIP2.1* and *OeHSP18.3* in olive plants after stress application and recovery. *OeHSP18.3* relative expression after stress was higher in the non-irrigated and in the heat shock treatments. Concerning the relative expression of *OeDHN1*, higher values were obtained under the non-irrigated treatment. After recovery, the expression of *OeHSP18.3* was higher in the heat shock treatment. *OePIP1.1* relative expression was particularly high under the well-irrigated with

heat shock condition. Regarding *OePIP2.1*, the expression levels were higher in the non-irrigated treatment, with larger differences found in control temperature condition.

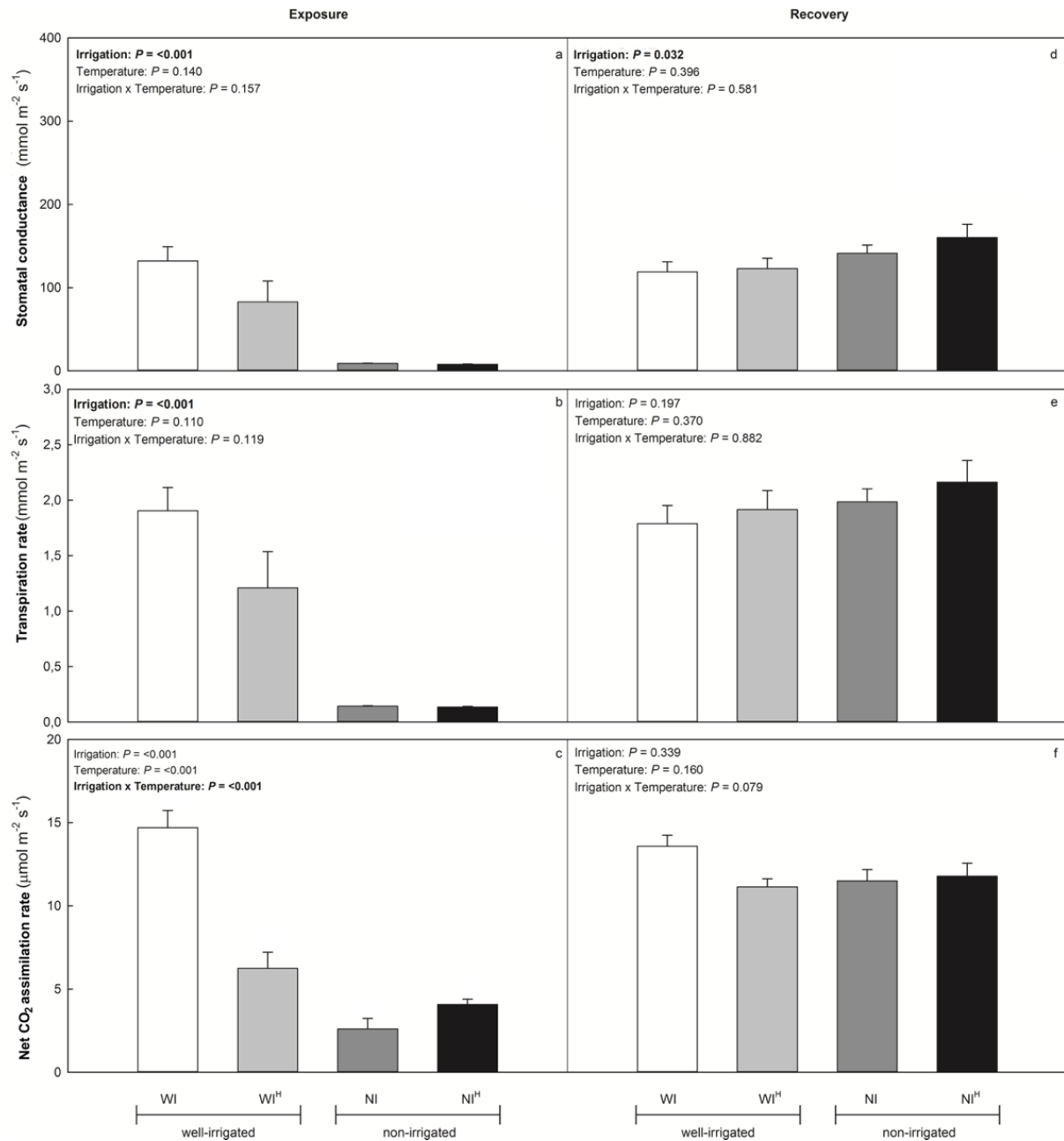


Figure 2.2.1 Stomatal conductance (a and d), transpiration rate (b and e) and net CO₂ assimilation rate (c and f) of exposed and recovered *O. europaea* plants under well-irrigated (WI) and WI with heat shock (WI^H), non-irrigated (NI) and NI with heat shock (NI^H). Values are means ± SE ($n = 6 - 14$)

Table 2.2.2 Efficiency of excitation energy capture by open photosystem II (PSII) reaction centres (F_v/F_m), effective quantum efficiency of PSII (Φ_{PSII}) and photochemical quenching (qP) measured in leaves of *O. europaea* after stress exposure and recovery from well-irrigated (WI) and WI with heat shock (WI^H), non-irrigated (NI) and NI with heat shock (NI^H).

Source of variation	Exposure			Recovery		
	F_v/F_m	Φ_{PSII}	qP	F_v/F_m	Φ_{PSII}	qP
Irrigation						
well-irrigated	0.750 ± 0.009	0.398 ± 0.015	0.519 ± 0.018	0.777 ± 0.008	0.434 ± 0.013	0.558 ± 0.012
non-irrigated	0.673 ± 0.012	0.218 ± 0.011	0.348 ± 0.021	0.718 ± 0.009	0.281 ± 0.010	0.394 ± 0.014
Temperature						
no heat	0.723 ± 0.009	0.305 ± 0.022	0.423 ± 0.026	0.768 ± 0.009	0.373 ± 0.019	0.486 ± 0.021
heat	0.698 ± 0.017	0.316 ± 0.029	0.444 ± 0.030	0.729 ± 0.011	0.350 ± 0.025	0.462 ± 0.026
Irrigation x Temperature						
well-irrigated						
WI	0.741 ± 0.008	0.381 ± 0.018	0.513 ± 0.020	0.795 ± 0.007	0.441 ± 0.018	0.561 ± 0.016
WI ^H	0.759 ± 0.016	0.416 ± 0.026	0.525 ± 0.032	0.762 ± 0.012	0.427 ± 0.019	0.554 ± 0.019
non-irrigated						
NI	0.704 ± 0.013	0.221 ± 0.014	0.333 ± 0.024	0.745 ± 0.011	0.306 ± 0.011	0.411 ± 0.011
NI ^H	0.642 ± 0.014	0.216 ± 0.019	0.362 ± 0.036	0.689 ± 0.007	0.249 ± 0.010	0.379 ± 0.025
Two-way ANOVA (<i>P</i> values)						
Irrigation	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Temperature	0.095	0.453	0.482	<0.001	0.033	0.328
Irrigation x Temperature	0.004	0.301	0.760	0.252	0.172	0.507

Values are means ± SE (*n* = 7 – 10)

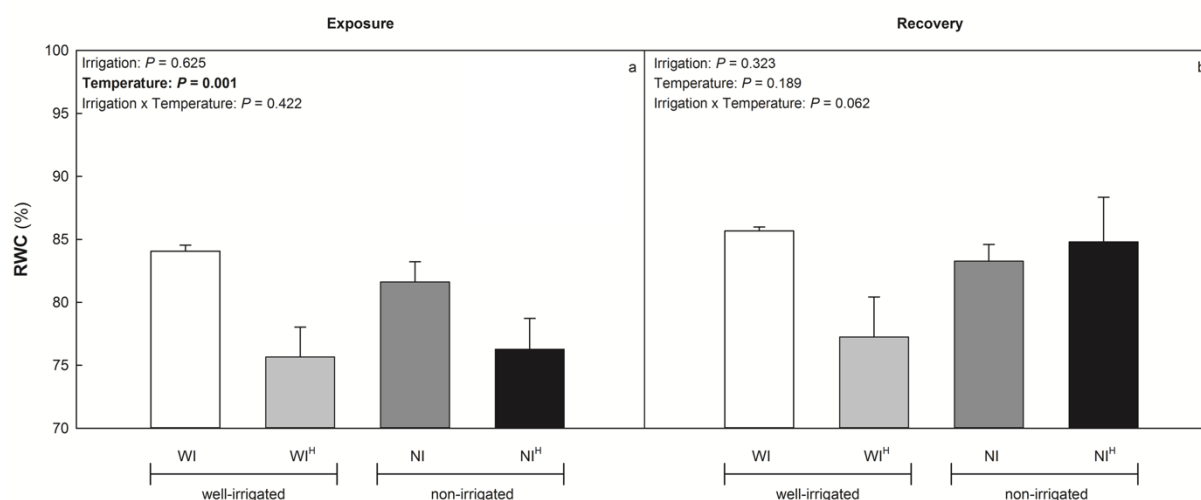


Figure 2.2.2 Variation of relative water content (a and b) in leaves of *O. europaea* plants after stress exposure and recovery from well-irrigated (WI) and WI with heat shock (WI^H), non-irrigated (NI) and NI with heat shock (NI^H). Values are means ± SE (*n* = 7)

Table 2.2.3 Mineral content in leaves of *O. europaea* plants after stress exposure and recovery from well-irrigated (WI) and WI with heat shock (WI^H), non-irrigated (NI) and NI with heat shock (NI^H).

Source of variation	Fe ($\mu\text{g g}^{-1}\text{DW}$)	Zn ($\mu\text{g g}^{-1}\text{DW}$)	Cu ($\mu\text{g g}^{-1}\text{DW}$)	Mg ($\mu\text{g g}^{-1}\text{DW}$)	Ca ($\mu\text{g g}^{-1}\text{DW}$)	K ($\mu\text{g g}^{-1}\text{DW}$)
Exposure						
Irrigation						
well-irrigated	42.4 $\pm$ 1.1	39.3 $\pm$ 1.3	352.5 $\pm$ 14.7	586.9 $\pm$ 7.9	7221.9 $\pm$ 64.2	11502 $\pm$ 154.3
non-irrigated	43.2 $\pm$ 1.8	35.0 $\pm$ 1.5	357.7 $\pm$ 28.5	650.7 $\pm$ 24.6	7109.0 $\pm$ 127.2	10951.2 $\pm$ 465.1
Temperature						
no heat	41.9 $\pm$ 1.4	39.4 $\pm$ 1.6	331.7 $\pm$ 19.0	582.5 $\pm$ 17.1	7056.3 $\pm$ 106.8	10704.2 $\pm$ 282.7
heat	43.8 $\pm$ 1.6	34.5 $\pm$ 0.9	380.9 $\pm$ 25.1	660.9 $\pm$ 19.5	7289.6 $\pm$ 72.5	11749 $\pm$ 352.6
Irrigation x Temperature						
well-irrigated						
WI	45.3 $\pm$ 0.5	42.5 $\pm$ 1.0	391.4 $\pm$ 9.3	566.6 $\pm$ 3.1	7323.9 $\pm$ 99.1	11563.8 $\pm$ 155.2
WI ^H	39.0 $\pm$ 0.6	35.4 $\pm$ 0.6	305.9 $\pm$ 7.6	607.1 $\pm$ 8.2	7119.8 $\pm$ 64.5	11440.1 $\pm$ 281.2
non-irrigated						
NI	38.5 $\pm$ 1.9	36.3 $\pm$ 2.6	272.0 $\pm$ 8.8	595.7 $\pm$ 31.4	6788.7 $\pm$ 108.5	9844.6 $\pm$ 179.1
NI ^H	47.9 $\pm$ 1.2	33.7 $\pm$ 1.5	443.3 $\pm$ 23.4	705.8 $\pm$ 21.6	7493.3 $\pm$ 60.6	12057.8 $\pm$ 655.5
Two-way ANOVA (<i>P</i> values)						
Irrigation	0.414	0.029	0.998	0.008	0.371	0.158
Temperature	0.239	0.010	0.004	0.002	0.011	0.012
Irrigation x Temperature	<0.001	0.202	<0.001	0.122	<0.001	0.006
Recovery						
Irrigation						
well-irrigated	45.6 $\pm$ 1.3	34.0 $\pm$ 1.2	299.9 $\pm$ 13.9	596.4 $\pm$ 32.0	6447.2 $\pm$ 159.6	11137.1 $\pm$ 263.8
non-irrigated	40.8 $\pm$ 2.2	31.9 $\pm$ 1.2	241.0 $\pm$ 6.8	597.2 $\pm$ 37.5	6048.8 $\pm$ 42.8	9757.8 $\pm$ 290.4
Temperature						
no heat	38.1 $\pm$ 1.4	31.5 $\pm$ 0.8	284.1 $\pm$ 8.6	694.6 $\pm$ 17.6	6494.5 $\pm$ 138.7	10020.0 $\pm$ 364.2
heat	47.7 $\pm$ 1.4	34.2 $\pm$ 1.4	260.0 $\pm$ 19.9	507.1 $\pm$ 21.8	5997.3 $\pm$ 68.6	10976.5 $\pm$ 245.4
Irrigation x Temperature						
well-irrigated						
WI	42.1 $\pm$ 0.3	32.7 $\pm$ 0.3	311.2 $\pm$ 4.7	690.1 $\pm$ 11.8	6856.3 $\pm$ 170.1	10841.8 $\pm$ 387.0
WI ^H	48.6 $\pm$ 1.6	35.2 $\pm$ 2.1	288.5 $\pm$ 27.8	518.2 $\pm$ 31.4	6038.2 $\pm$ 127.3	11432.4 $\pm$ 348.7
non-irrigated						
NI	34.7 $\pm$ 1.4	30.5 $\pm$ 1.3	256.9 $\pm$ 3.4	698.3 $\pm$ 32.2	6132.7 $\pm$ 57.5	9198.1 $\pm$ 404.5
NI ^H	46.8 $\pm$ 2.4	33.2 $\pm$ 1.9	217.1 $\pm$ 2.7	496.1 $\pm$ 32.6	5948.1 $\pm$ 21.4	10429.4 $\pm$ 107.8
Two-way ANOVA (<i>P</i> values)						
Irrigation	0.013	0.227	<0.001	0.817	0.002	0.001
Temperature	<0.001	0.128	0.065	<0.001	<0.001	0.016
Irrigation x Temperature	0.113	0.946	0.598	0.616	0.013	0.322

Values are means $\pm$ SE (*n* = 6).

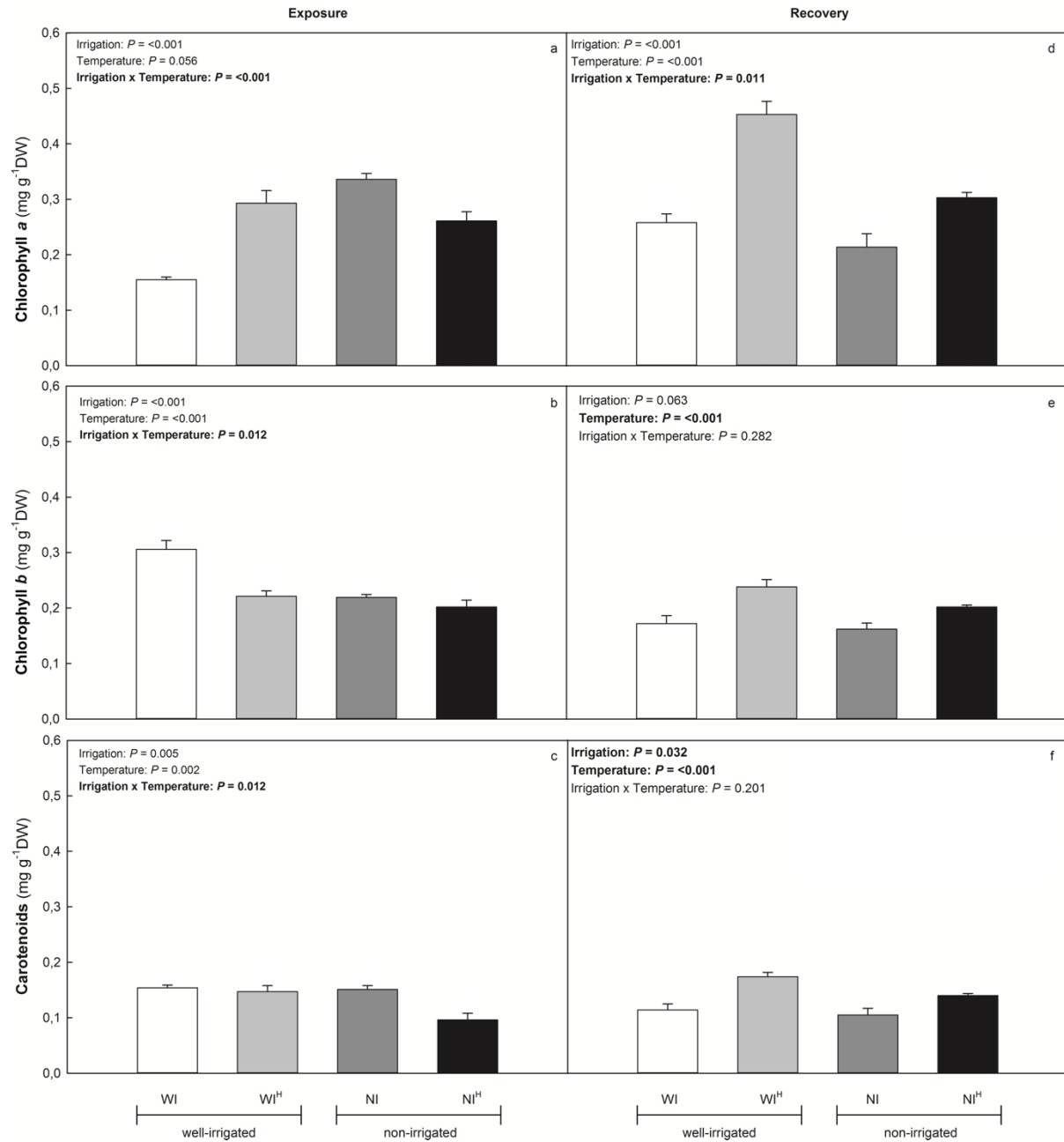


Figure 2.2.3 Chlorophyll a (a and d), chlorophyll b (b and e) and carotenoids (c and f) contents on leaves of *O. europaea* after stress exposure and recovery from well-irrigated (WI) and WI with heat shock (WI^H), non-irrigated (NI) and NI with heat shock (NI^H). Values are means ± SE (n = 4 – 8)

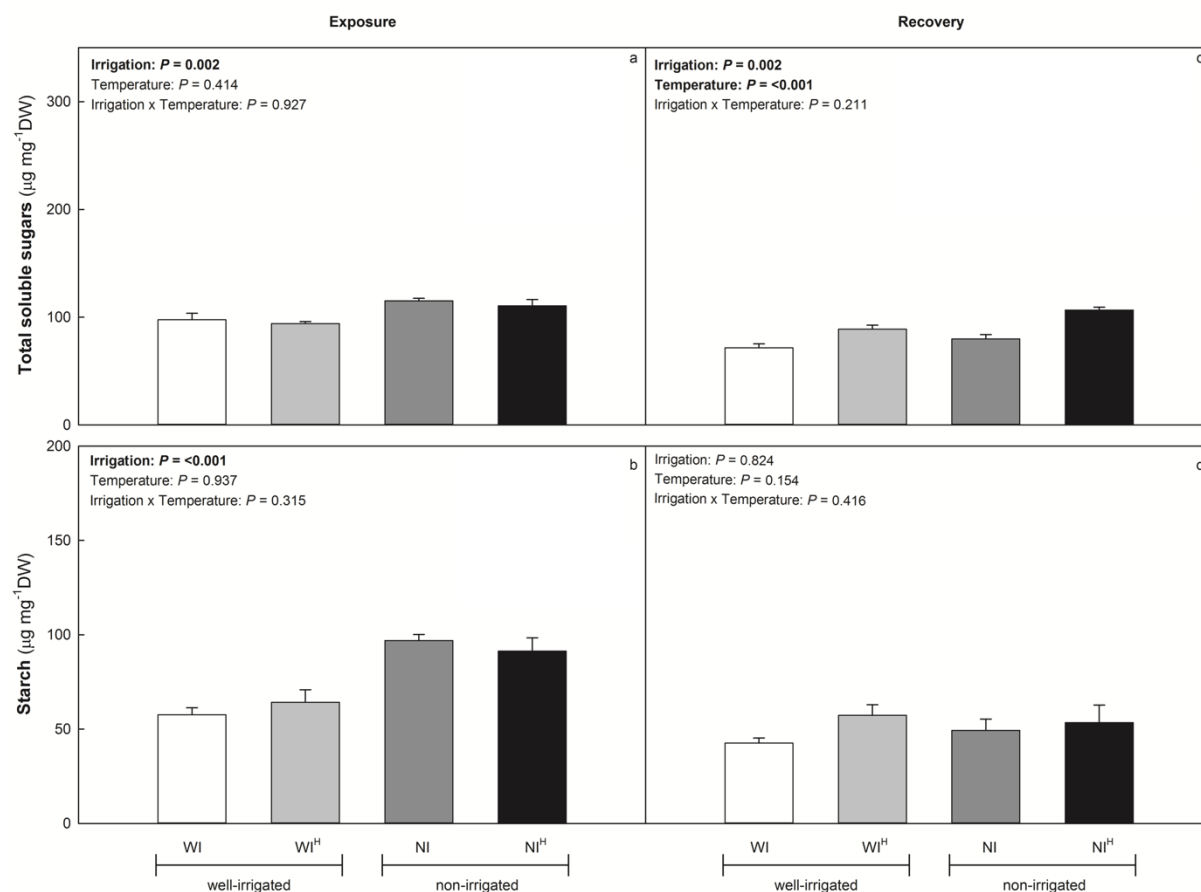


Figure 2.2.4 Total soluble sugars (a and c) and starch (b and d) in leaves of *O. europaea* after stress exposure and recovery from well-irrigated (WI) and WI with heat shock (WI^H), non-irrigated (NI) and NI with heat shock (NI^H). Values are means $\pm$ SE ($n = 5 - 8$)

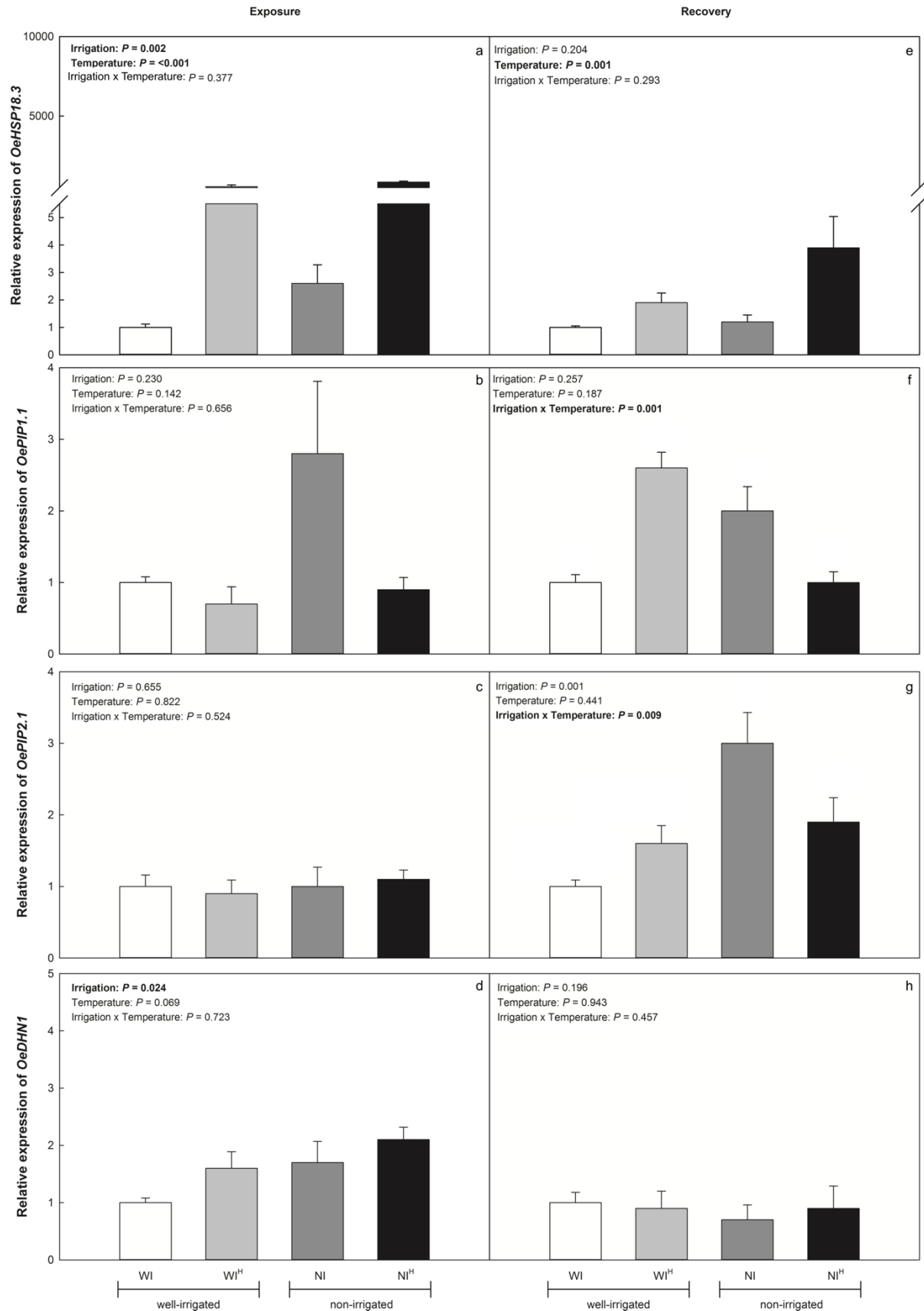


Figure 2.2.5 Relative expression of *OeHSP18.3* (a and e), *OePIP1.1* (b and f), *OePIP2.1* (c and g) and *OeDHN1* (d and h) of exposed and recovered *O. europaea* plants under well-irrigated (WI) and WI with heat shock (WI^H), non-irrigated (NI) and NI with heat shock (NI^H) treatments. Values are means $\pm$ SE ($n = 6$)

Oxidative status

Figure 2.2.6 presents the contents of CMP and MDA in olive plants immediately after stress application and recovery. After stress exposure, the CMP was higher in the non-irrigated treatment, with major differences found at control temperature condition. Concerning MDA, only the non-irrigated treatment showed the highest levels. Regarding the recovery period, MDA levels were higher in the non-irrigated treatment and lower in the heat shock treatment.

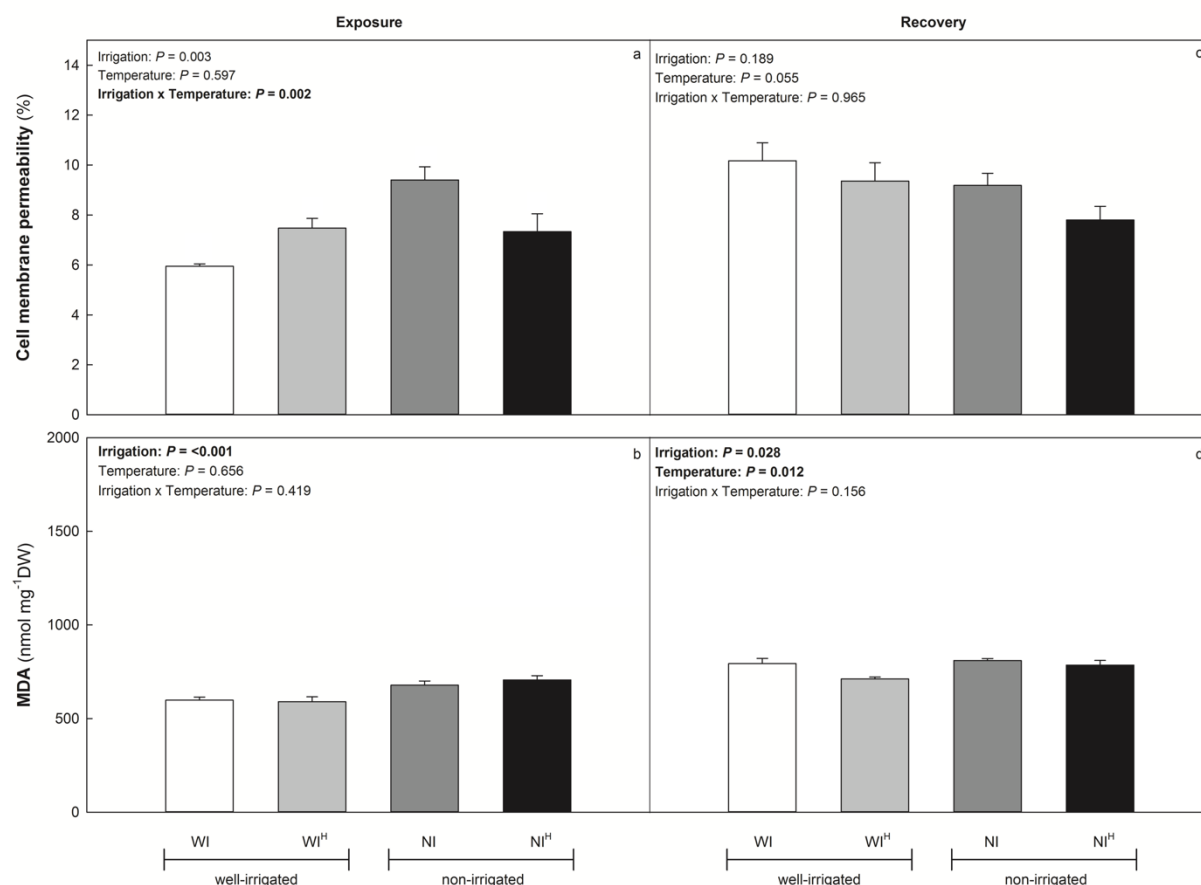


Figure 2.2.6 Cell membrane permeability (a and c) and MDA (b and d) of exposed and recovered *O. europaea* plants well-irrigated (WI) and WI with heat shock (WI^H), non-irrigated (NI) and NI with heat shock (NI^H). Values are means $\pm$ SE ($n = 6 - 8$)

DISCUSSION

Plant response immediately after stress exposure

Irrigation and temperature induced changes in the physiological and gene expression profiles of olive plants, as it can be seen in the heat map analysis (Figure 2.2.7). The expected reductions of leaf gas exchange by non-irrigated treatment regarding their well-irrigated counterparts (e.g. NI vs WI; Figure 2.2.7) suggest that the former plants adjusted their g_s to

protect from water loss, a typical response of olive to this stress (Brito et al., 2018). Contrarily to the non-irrigated, in the well-irrigated treatment, heat dramatically compromised P_N (WI^H vs WI and NI^H vs NI ; Figure 2.2.7), despite the negligible effect on g_s and E , which remained high, probably because they need to control leaf temperature (Martin-StPaul et al., 2017). These data demonstrated that plants from the non-irrigated treatment can operate similarly, achieving comparable P_N to those under the well-irrigated with a heat shock (WI^H), but with a lower g_s and E . This advantage may be a consequence of the drought pre-exposure, that could promoted some tolerance mechanisms allowing plants to cope better with further stress episodes, as suggested in other studies with olive (Abdallah et al., 2017; Dias et al., 2018a).

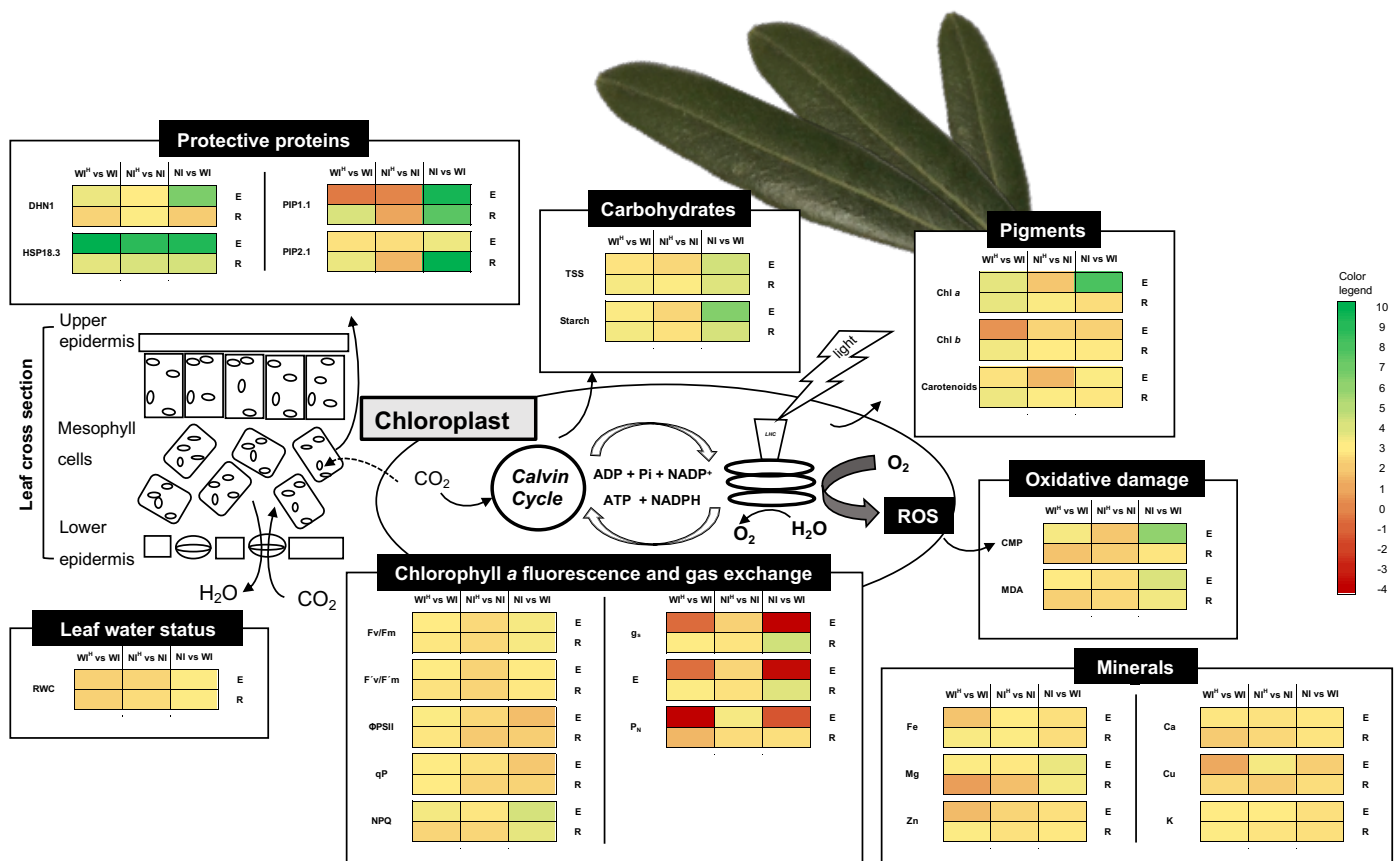


Figure 2.2.7 General overview of the physiological and gene expression changes in olive leaves immediately and 10 days after a heat shock episode. Relative levels are expressed as $\log_2$ (WI^H/WI) or (NI^H/NI) or (NI/WI), and given besides each parameter as a heat-map. WI, well-irrigated, WI^H , well-irrigated with heat shock, NI, non-irrigated, NI^H , non-irrigated with heat shock, E, exposure to stress treatments and R, recovery from the stress treatments. Abbreviations: CMP (cell membrane permeability), DHN1 (dehydrin 1), HSP18.3 (heat shock protein 18.3), MDA (malondialdehyde), PIP1.1 (aquaporin 1.1), PIP2.1 (aquaporin 2.1), RWC (relative water content), TSS (total soluble sugars)

Light dependent reactions of photosynthesis were less affected than gas exchange (Figure 2.2.7). Φ_{PSII} and qP decreased in non-irrigated plants, but contrary to leaf gas exchange (e.g. P_N), these parameters were less sensitive to heat. The reduction of Φ_{PSII} in the

non-irrigated treatment represents less ATP and NADPH availability for the Calvin Cycle (Nouri et al., 2015) and may contributed to the general impairment of P_N on this treatment (NI vs WI: $r = 0.73$, $P < 0.01$). In turn, Φ_{PSII} loss was a consequence of a decrease in the proportion of open PSII reaction centres - qP (NI vs WI: $r = 0.98$, $P < 0.001$) and in the efficiency of excitation energy capture by open PSII reaction centres - F_v/F_m , particularly for NI^H plants. Also, the decrease of some PSII-essential micronutrients (Fe and Cu) in non-irrigated plants interfered negatively with Φ_{PSII} (NI vs WI: $r \geq 0.61$, $P < 0.05$), suggesting that their deficiency contributes to light-dependent photosynthetic reactions efficiency. Φ_{PSII} also depends on chlorophylls content and olive plants under abiotic stress have shown decreases in these two parameters (e.g. Dias et al. 2018a, b, c). Unexpectedly, the lower Φ_{PSII} found in olive plants from the non-irrigated treatment was accompanied by higher levels of chlorophyll *a* and lower chlorophyll *b* contents. Chlorophyll *a* increase can be attributed to the degradation of chlorophyll *b* (WI vs NI: $r = -0.79$, $P < 0.001$), which involves its conversion in chlorophyll *a* (Sato et al. 2015). In *Arabidopsis*, chlorophyll *b* degradation (particularly in the LHCII) promotes nutrient remobilisation, suppression of photodamage and regulates light-harvesting capacity (Sato et al., 2015; Zhu et al., 2017).

Water deficit modifies carbon assimilate partitioning between sucrose and starch resulting in an accumulation of sucrose (Chaves, 1991). Here, non-irrigation treatment also increased the pool of both TSS and starch (e.g., NI vs WI; Figure 2.2.7). Carbohydrates accumulation in response to drought may contribute to tissue osmoregulation, protecting the subcellular structures against dehydration (Azoulay-Shemer et al., 2016). Also, starch increase may represent a way to maintain a low stomatal aperture (starch degradation provides the carbon skeletons for anion synthesis in guard cells promoting stomata aperture (Lawson et al., 2014), as sustained by the negative correlation between g_s and E with starch (WI vs NI: $r \geq -0.64$, $P < 0.05$). Increases of starch levels were reported to represent a blockage of carbon translocation out of the leaves for other olive cultivars exposed to water deficit (Bacelar et al. 2006).

The contribution of the stabilising proteins, DHN and HSPs, to plant protection was evident in the non-irrigated treatment, but heat particularly potentiates the expression of *OeHSP18.3* (Figure 2.2.7). HSPs and DHNs display several homeostatic functions related to the cell protection from environmental stress, such as osmoprotection, ROS scavenging activities, stabilisation of enzymes and cell membranes (Park and Seo 2015; Liu et al. 2017). This protection is evident in the non-irrigated treatment (and heat condition), where plants were more exposed to oxidative stress (higher CMP and MDA, particularly in NI vs WI; Fig. 2.2.7). It can be hypothesised that upregulation of these genes is a strategy of olive to counteract the observed lipid peroxidation (WI vs NI: $r = 0.62$, $P < 0.05$) and loss of membrane

integrity (WI vs NI and WI vs WI^H: $r \geq 0.64$, $P < 0.05$) as proposed by Ré et al. (2016) and Halder et al. (2018) for other species. Similarly, olive DHNs participated in defence mechanisms to water deficit (Tripepi et al. 2011; Chiappetta et al. 2015). While the role of HSPs, as the first line of defence under heat stress, has also been suggested in olive (Assab et al. 2011), the contribution of PIPs seemed similar in the irrigation treatments, not being influenced by temperature. The role of PIPs according to the cultivar and stress conditions deserves further clarification as, for example in 'Manzanilla' plants (Perez-Martin et al., 2014) exposed to 13 days of drought, *OePIPs* were differently up/downregulated, suggesting that in this cultivar and for a lower period of drought they may act to maintain cell homeostasis.

Plant response after a stress recovery period

Ten days after stress relief, plants from both irrigation and heat treatments recovered similarly, showing a great plasticity to these environmental challenges. The heat map (Figure 2.2.7) and data reveal that, in general, gas exchange, pigments and membrane damages achieved, at least, control levels (WI). Starch pools were re-established in the non-irrigated treatment, but the maintenance of high levels of TSS, particularly in the non-irrigated and heat treatments, may be necessary to restore cellular processes and growth (Abid et al., 2018). Some nutrient pools, such as the Ca, and not so markedly the Mg, reduced during the recovery from WI^H, NI and NI^H, suggesting their contribution to plant recovery of photosynthesis and membrane damages, since Mg plays a crucial role in chlorophyll biosynthesis and carbon fixation, as well as a cofactor of enzymes involved in carbon metabolism, and Ca is a key ion to maintain membrane structure and rigidity of the cell walls (Guo et al., 2015; Hepler, 2005). The incomplete reestablishment of Φ_{PSII} in plants of the non-irrigated treatment still correlated to the unrecovered of qP (NI vs NI^H, $r = 0.95$, $P \leq 0.001$), and in some part also to F_v/F_m (NI vs NI^H, $r = 0.84$, $P \leq 0.001$). However, these low values of Φ_{PSII} do not compromise P_N reposition, since plants from the non-irrigated treatment achieved values similar to WI. Overall, these data corroborate previous studies in olive, which reported a good recovery capacity of the photosynthetic apparatus from water deficit (Dias et al., 2018a; Sofo, 2011; Torres-Ruiz et al., 2013), and support that olive tree exhibits a high photosynthetic plasticity to tolerate water deficit and heat episodes, being able to recover their physiological performance.

OeDHN1 and *OeHSP18.3* which were highly involved during the stress period showed overall to be less responsive ten days after stress relief (Figure 2.2.7). Interestingly, the PIPs seem to be more active during olive recovery. This role was particularly evident for *OePIP2.1*, which was, in general, more abundant in plants recovering from the NI condition (e.g., NI vs WI, Figure 2.2.7). Contrarily, been only overexpressed in the WI after heat shock, *OePIP1.1* mechanism involving this protein-channel are more solicited to participate in the recovery to

heat in a yet unknown way. Our different profiles of both PIPs are consistent with the data of Perez-Martin et al. (2014) in olive under drought where during recovery a peak of *OePIP1.1* expression was reported. This more active role of PIPs during recovery (regarding DHN and sHSP), support that they play a later role on cell homeostasis, putatively by regulating CO₂ and/or water transport (Perez-Martin et al. 2014). This may also explain the fast recovery of leaf RWC, g_s and P_N .

CONCLUSIONS

We demonstrate that, whilst plants from the non-irrigated treatment showed some photosynthetic parameters below the ones of the well-irrigated treatment, the imposition of a heat episode induced lower effects in the non-irrigated treatment (e.g. P_N , chlorophyll *b*, Zn and Fe). This suggests that the water deficit period modulated some tolerance mechanisms, putatively the overexpression of protective proteins, such as sHSP and DHN, and stomatal control, allowing plants to cope better with further stress episodes and to recover similarly from both irrigation treatments. AQPs also play an important role in olive recovery, and their expression seems to be influenced by the irrigation and temperature. The impact of these adaptive plasticity on field orchards and putative contribution to a better irrigation management of intensive orchards deserves to be further studied.

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SUPPLEMENTARY MATERIAL

Supplementary material 2.2.1 Maximum quantum efficiency of photosystem II (F_v/F_m) and non-photochemical quenching (NPQ) measured in leaves of *O. europaea* after stress exposure and recovery from well-irrigated (WI) and WI with heat shock (WI^H), non-irrigated (NI) and NI with heat shock (NI^H).

Source of variation	Exposure		Recovery	
	F_v/F_m	NPQ	F_v/F_m	NPQ
Irrigation				
well-irrigated	0.852 ± 0.004	0.644 ± 0.044	0.857 ± 0.004	0.658 ± 0.042
non-irrigated	0.848 ± 0.005	0.710 ± 0.035	0.860 ± 0.004	0.722 ± 0.048
Temperature				
no heat	0.854 ± 0.003	0.615 ± 0.038	0.868 ± 0.003	0.714 ± 0.040
heat	0.846 ± 0.005	0.759 ± 0.032	0.849 ± 0.003	0.666 ± 0.051
Irrigation x Temperature				
well-irrigated				
WI	0.852 ± 0.004	0.569 ± 0.060	0.868 ± 0.004	0.129 ± 0.041
WI ^H	0.852 ± 0.008	0.751 ± 0.039	0.846 ± 0.004	0.240 ± 0.076
non-irrigated				
NI	0.856 ± 0.005	0.661 ± 0.045	0.869 ± 0.005	0.224 ± 0.071
NI ^H	0.840 ± 0.007	0.764 ± 0.051	0.852 ± 0.005	0.219 ± 0.069
Two-way ANOVA (<i>P</i> values)				
Irrigation	0.517	0.315	0.506	0.337
Temperature	0.199	0.010	<0.001	0.472
Irrigation x Temperature	0.199	0.447	0.542	0.877

Values are means ± SE (*n* = 7 – 10).

Chapter III

Influence of water stress and biostimulant on fruit development in young plants of two cultivars



Author Márcia Araújo

Chapter III is constituted by one subchapter.

The work present here is between controlled conditions and field conditions as the trees were outside at the Botanical Garden of the University of Coimbra and only the water irrigation was controlled.

The subchapter present here was submitted to the *Food Research International*.

3.1. Biostimulants based on *Ascophyllum nodosum* are beneficial to olive's lipophilic profile during water deficit conditions.

Araújo, M., Santos, C., Pinto, D.C.G.A., Silva, A.M.S., Pereira, J.A., & Dias, M. C. (2022). Biostimulants based on *Ascophyllum nodosum* are beneficial to olive's lipophilic profile during water deficit conditions. *Research Food International* (Submitted, Ref No. FOODRES-D-22-05694)

3.1. *Ascophyllum nodosum* affects differentially the fruit growth and lipophilic profile during development under different irrigation levels³

ABSTRACT

Addressing the Farm to Fork strategies, sustainable oliviculture needs to find new strategies to prevent the demand for more water. We evaluated the effect of a biostimulant (*Ascophyllum nodosum*) pretreatment on olive tolerance to drought and fruit quality. 'Arbequina' and 'Galega' plants were pre-treated with *A. nodosum* and then exposed to two irrigation conditions: 100% of field capacity (FC), 100%FC + Biostimulant, 50%FC, and 50%FC + Biostimulant. *A. nodosum* seems effective to improve the leaf water status of 'Arbequina' under low irrigation, but also increase fruit growth and induce metabolites readjustment in a way dependent on the cultivar, irrigation level and sampling time. Under low irrigation the biostimulant increased β -D-glucopyranose and quinic acid in both cultivars and promoted the fruit water content and α -D-galactopyranose only in 'Galega', contributing to the improvement of the nutritional quality of olives. Under optimal irrigation conditions biostimulant could also be valuable to increase fruit quality.

KEYWORDS

Drought, biostimulant, olive compounds, 'Arbequina', 'Galega'

³ Araújo, M., Santos, C., Pinto, D.C.G.A., Silva, A.M.S., Pereira, J.A., & Dias, M. C. (2022). Biostimulants based on *Ascophyllum nodosum* are beneficial to olive's lipophilic profile during water deficit conditions. *Research Food International* (Submitted, Ref No. FOODRES-D-22-05694)

INTRODUCTION

The olive tree (*Olea europaea* L.) is a pivotal crop in the Mediterranean basin, with its importance covering socio-economic, environmental, landscape, and cultural heritage dimensions. The Mediterranean Basin is the world's producer of olive products, including table olives and/or olive oil (FAOSTAT, 2017). Being highly recognized as having healthy nutritional properties, the consumers' demand for olives and olive oil has been increasing (Brito et al., 2019).

Olive trees are well adapted to the prolonged drought conditions typical of the Mediterranean region (Brito et al., 2019). However, the increasing frequency of extreme climate events, especially the increase of extreme heat and/or prolonged drought episodes represents a threat to oliviculture considering both olive fruit yield and quality (Fraga et al., 2021). Olive fruits are rich in lipids, carbohydrates, proteins, minerals and secondary metabolites (Rodrigues et al., 2019). The nutritional quality and health benefits of olive fruits are mainly attributed to the high content of unsaturated fatty acids as well as to minor compounds such as polyphenols, squalene, tocopherol, pigments and sterols (Talhaoui et al., 2015). The levels of these compounds and the ratio of key compounds that change during the ripening process vary in response to the abiotic stress (Gonçalves et al., 2020; Martinelli et al., 2013; Valente et al., 2020). For instance, the monounsaturated oleic acid, the major fatty acid, and the saturated palmitic acid seem to increase after drought + heat episodes (Valente et al., 2020). However, the contents and ratio of key compounds vary along with ripening and are influenced by environmental conditions. For example, drought conditions alone increased palmitic acid and decreased oleic acid (Martinelli et al., 2013). Also sugars and polyols tend to accumulate in response to drought + heat conditions (Gonçalves et al., 2020; Valente et al., 2020), but reductions of these metabolites were also reported when olive plants were exposed only to drought (Martinelli et al., 2013). Besides varying along the maturation stage, the levels of other metabolites, such as terpenes (e.g., squalene) and sterols, showed a tendency to decrease in response to drought + heat (Valente et al., 2020).

To counteract the adverse effects of climate change stress and to increase intensive olive orchards production, irrigation system strategies were implemented in several regions of the Mediterranean basin. However, these water necessities for irrigation are acknowledged as not sustainable, as the water shortage is already a problem. Climate change and weather accentuate the water deficit in several Mediterranean regions (Santos et al., 2019). Thus, the water availability for agriculture needs to be used in a more efficiently way. New approaches to improving water use efficiency and agriculture sustainability, like the application of biostimulants, are emerging. Biostimulants are natural compounds, or microorganisms that enhance plant nutrition efficiency and crop quality, as well as (a)biotic stress tolerance (Shukla

et al., 2019). Biostimulants include various products, including protein hydrolysates or amino acids, seaweed, humic substances, and/or beneficial microbes, like bacteria and fungi (Sible et al., 2021). The seaweed biostimulants are among the most commercialized compounds, and those based on the brown algae *Ascophyllum nodosum* L. are already used in some agricultural practices (González-Morales et al., 2021). This biostimulant is described to promote plant growth, trigger early flowering and increase fruit set in several species, like tomato, pepper, and bean (Ali et al., 2021; Shukla et al., 2019). These benefits are mainly associated with this algae's composition, which includes several polysaccharides, lipids, proteins, hormones, vitamins, alginate and minerals (Pereira et al., 2020). The effects of biostimulants remain underexplored in olive trees, but one study with *A. nodosum* suggested an increase in productivity and oil quality in olives from rainfed orchards (Chouliaras et al., 2009). Contrarily, protein hydrolysates did not affect the yield of fruits and oil of potted olive trees growing under well-watered conditions (Almadi et al., 2020).

Stress tolerance is one of the major beneficial effects of biostimulants, but the detailed mechanisms of the protective action triggered by biostimulants are yet to unveil. It is known that biostimulants can prime plants to withstand abiotic stresses, by modulating several metabolic and physiological processes (Kerchev et al., 2020). These adjustments activated by biostimulants provide some benefits, improving plant performance and stress resilience. However, the impact of these metabolic and physiological changes at the fruit level (e.g., growth, development and quality) is not well studied.

In the present study, we aim to evaluate the effect of a pretreatment of olive trees with a biostimulant on the fruit quality along with the development under two irrigation levels (well-watered and water deficit). We hypothesize that biostimulant treatment will affect differentially the fruit growth and metabolite richness (lipophilic composition) of the two cultivars during development, under two different irrigation levels. To test this, potted olive plants with fruits from both cultivars were used, the Spanish 'Arbequina', usually used in the high-intensive irrigated orchards, and the Portuguese 'Galega', often used in the traditional dryland farming. Plants were treated with *A. nodosum* and then exposed to water deficit conditions. Leaf and fruit water content, fruit biometric parameters, and the metabolites present in the lipophilic phase of the fruits were evaluated during the water deficit treatment.

MATERIAL AND METHODS

Plant material and experimental conditions

Olea europaea L. cultivars 'Arbequina' and 'Galega' plants with one-year-old were kindly offered by Viveiros Miguel Vaz, Sociedade Agricola Lda (Gaite, 3220-414 Semide, Coimbra, Portugal) in August 2018. Plants were potted in a 3 L black plastic pots with universal

substrate (EcoGrow, balanced mixture of vegetable mulch and peat, pH (CaCl₂): 5.5-6.5, humidity: 50 – 60%, conductivity: 0.6 – 1.2 μscm^{-1} , nitrogen: 80 – 150 mg L⁻¹, phosphorus: 80 – 150 mg L⁻¹, potassium: 300 – 500 mg L⁻¹ and organic matter: > 70 %). Plants were monthly fertilized with Complestal 12-4-6 NPK or Complestal 5-8-10 NPK (according to the manufacture instructions and the olive stage of development) and sprayed with 0.3% (m/v) of sodium borate (Borax, Sociedade Portuguesa de Drogas S.A., Portugal) at the beginning of inflorescence buds. Plants were grown in the open-air at the Botanic Garden of the University of Coimbra. To minimize the effects of solar irradiation, every week all plants were rotated and, every month, plants were relocated. A datalogger (LOG 32 TH, Dostmann electronic GmbH, Germany) was installed to record hourly temperature and humidity every day. Data from April 2019 to November 2020 was compiled in Figure 3.1.1, which represents the monthly maximum, minimum and average temperature and humidity. Olive plants were grown in a large range of temperatures, being July the hotter month in both 2019 and 2020, and the dryer months between May and September of 2019 (Figure 3.1.1). Between September 2019 and April 2020, olive plants had 646 cold hours (where the temperature ranged between 4°C and 10°C). When olive plants were about 3 years-old (in 2020), the majority of the olive plants started to develop inflorescence buds at leaf axils, corresponding to the 50 phenological stage (BBCH scale, Sanz-Cortés et al., 2002). Before the start of the experiments, all the olive plants were height and divided in random groups. Both 'Arbequina' and 'Galega' olive plants were divided into two groups of 20 plants each. The first group was sprayed four times with the biostimulant Fitoalgas Green® (*Ascophyllum nodosum*, Tradecorp, Portugal) at a concentration of 3 mL L⁻¹, while the other group was sprayed with water. The application of the biostimulant occurred once on March 17th before flowering [between 53 and 55 phenological stage (BBCH scale, Sanz-Cortés et al., 2002)], and during fruit formation and development (June 30th, July 7th and 14th). All plants were well watered [100% field capacity (FC)] during the application of water or biostimulant. Then, each group was subdivided into two groups. One group was assigned as Control [plants treated with water (treatment 100%FC 0) or with biostimulant (treatment 100%FC B)] and the plants were watered at 100% FC twice a week. The second group was assigned as Stress [plants treated with water (50%FC 0) or with biostimulant (50%FC B)] and the plants were watered at 50% FC twice a week. A final application of the biostimulant was done on September 7th.

To characterize different olive fruit development stages, two sampling times were performed. One on September 21st (69 days after the beginning of the water deficit) – *Sampling Time 1 (SP1)* –, where olive fruits were at 79 phenological stage (BBCH scale, Sanz-Cortés et al., 2002) corresponding to 128 days after flowering; and another on November 9th (118 days after the beginning of the water deficit) – *Sampling Time 2 (SP2)* – when olive fruits

were at 89 phenological stage (BBCH scale, Sanz-Cortés et al., 2002) corresponding to 176 days after flowering. Olive leaves were collected in both sampling times to access the plant water status. At the first sampling time, between 1 and 10 fruits per tree were collected and at the second sampling time all the remaining fruits were collected. Fruits were used to evaluate the fruit water content, biometric parameters, and lipophilic profile.

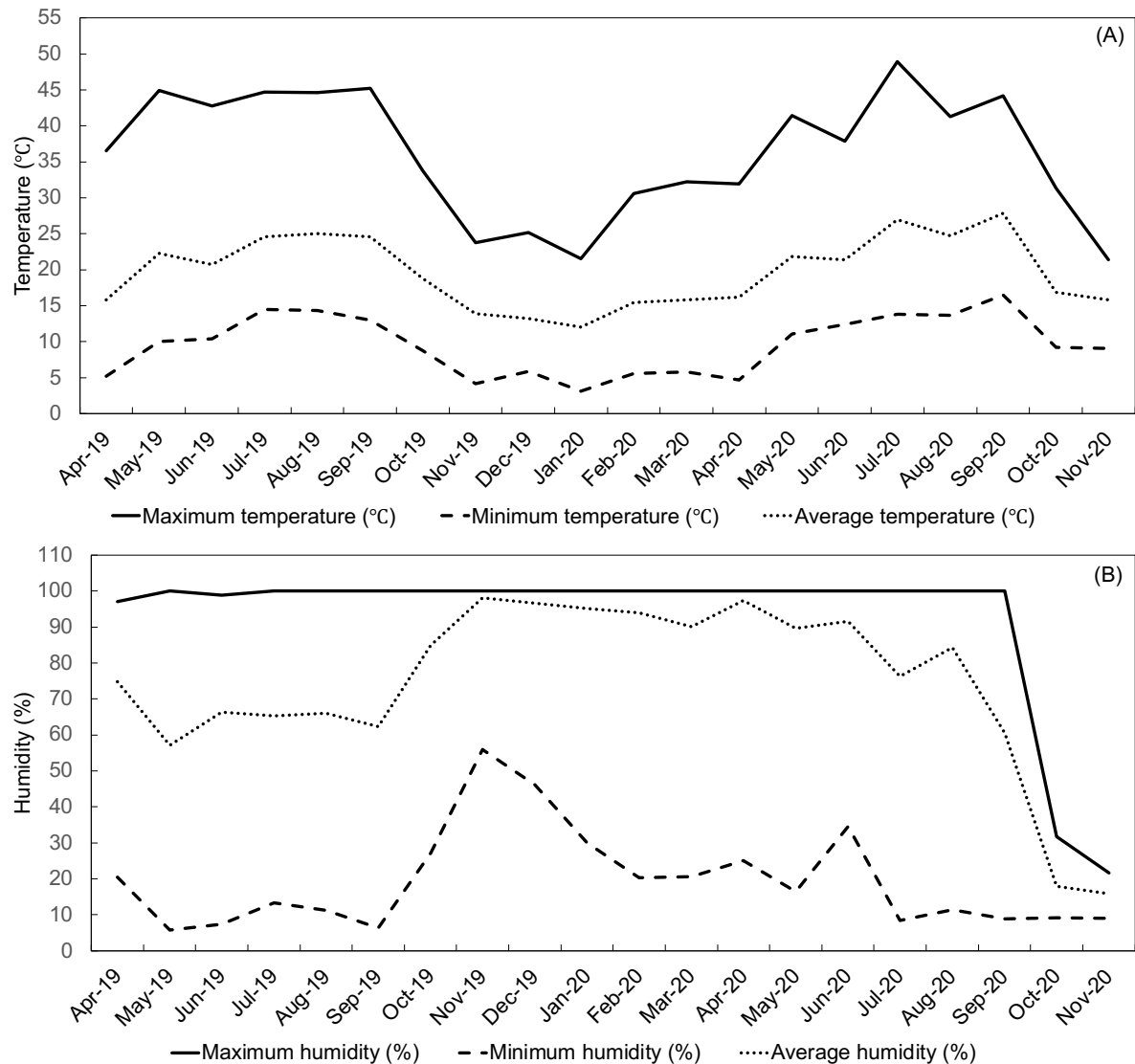


Figure 3.1.1 Monthly maximum, minimum and average temperatures (A) and humidity (B) between April 2019 and November 2020.

Water status and biometric parameters

From each olive plant, at least two fully expanded leaves were collected, the fresh weight (FW) was immediately measured, and each leaf placed in a tube filled with distilled water for 48h at 4°C. Leaves were weighed (turgid weight, TW) and then dried at 40°C until

the weight was stable (dry weight, DW). The leaf relative water content (RWC) was calculated according to the formula: $RWC (\%) = (FW - DW) / (TW - DW) \times 100$.

Fruits were collected and immediately weighted (fresh weight, FW) and then were dried at 40°C until constant weight (dry weight, DW). The fruit water content (FWC) was calculated according to the formula: $FWC (\%) = (FW - DW) / FW \times 100$.

Regarding biometric parameters, only healthy fruits were evaluated. Each fruit was evaluated for weight, length, and width. The length, or the maximum diameter (the distance from the apex to the olive's base), and the width or minimum diameter, were measured using a digital calliper.

Lipophilic extracts preparation and gas chromatography mass spectrometry (GC-MS)

Healthy olive fruits were pitted, weighed, and dried at 40°C until the weight was stable. Using a mortar and pestle the dried fruits were macerated. The macerated fruits were weighed, and *n*-hexane (10 mL per gram of sample) was added to extract the metabolites. The mixture of fruit and *n*-hexane was left in the dark with orbital agitation at room temperature for 72 h. Then the solvent was renewed and left in agitation for another 24 h. After that, the solvent was evaporated using a rotatory evaporator until the extract was completely dry. The dry extract was used for the GC-MS analysis, but before the analysis a silylation procedure was performed. To the extract solution (250 µL, 30 mg mL⁻¹ in dichloromethane) was added 200 µL of internal standard (tetracosane, 0.46 mg mL⁻¹), 250 µL of pyridine, 250 µL of *N,O*-bis(trimethylsilyl) trifluoroacetamide and 50 µL of trimethylsilyl chloride. This solution was incubated in closed glass tubes in a water bath at 70°C for 30 min (Araújo et al., 2021). Each extract was injected in a GC-MS QP2010 Ultra Shimadzu equipped with a FactorFour Capillary Column VF-5ms 30 m × 0.25 mm ID DF = 0.25. The chromatographic conditions were similar to those previously reported in Araújo et al. (2021). Briefly, the carrier gas was set at a linear velocity of 40.0 cms⁻¹, the column oven temperature was at 70°C, the injection temperature was 320°C, the split mode was used for the injection, at 76.1 kPa, the total flow was 63.7 mL min⁻¹, the column flow was 1.19 mL min⁻¹, the purge flow was 3.0 mL min⁻¹ and the split ratio as 1:50. The initial temperature was 70.0°C for the initial 5 min, followed by an increase of 4°C up to 250 °C and then 2°C up to 300°C for 5 min. The full program time was 80 min. Regarding mass spectrometry conditions, the ion source temperature was 200°C, the interface temperature was 300°C, the solvent cut time was 6.5 min, the micro scan width time was 0 µs, threshold was 0, the detector voltage (relatively to the tuning result) was 0.1 kV. The initial time was 6.5 min until the finish time at 80.0 min. The scan mode was used for

acquisition with event time at 0.10 s, speed time of 20000 and the start/end m/z at 50.0/1000.0, respectively. The compounds were identified based on a direct comparison with the mass spectra database's libraries (NIST14 Mass spectral and Wiley Registry of Mass Spectra Data), comparing with previous studies using the same chromatographic conditions and comparing the retention times and mass spectra of the standard compounds injected using the same chromatographic conditions of the samples. At least four independent analyses per condition were performed. A semi-quantitative analysis was used, where each compound area was divided by the internal standard area and the sum of those areas was considered the total (100%) compounds present in the sample. Then, each compound area was compared with the sum of all compound areas and the result was expressed in percentage.

Data analysis

Data statistical analysis was performed using SigmaPlot version 3.1 for Windows. A two-way ANOVA was used to access the differences between the irrigation factor and the biostimulant factor used for each cultivar tested. When normality failed, a simple transformation was performed. Pairwise comparisons were made using the Holm-Sidak test. Principal component analysis (PCA) was performed using the software Canoco version 4.5.

RESULTS

Water status and biometric parameters

The relative water content (RWC) in 'Arbequina' was affected by the irrigation factor at the sampling time one (SP1), presenting the stress groups (50%FC) lower values. At the SP2, an interaction between both factors (irrigation and biostimulant supply) occurred, and RWC was lower in the group 50%FC without biostimulant. In 'Galega' at the SP1, it was observed an effect of irrigation and biostimulant factors, both reduced the RWC. At the SP2, the irrigation factor affected the response of olive plants, the stress groups presented lower RWC values (Figure 3.1.2 A).

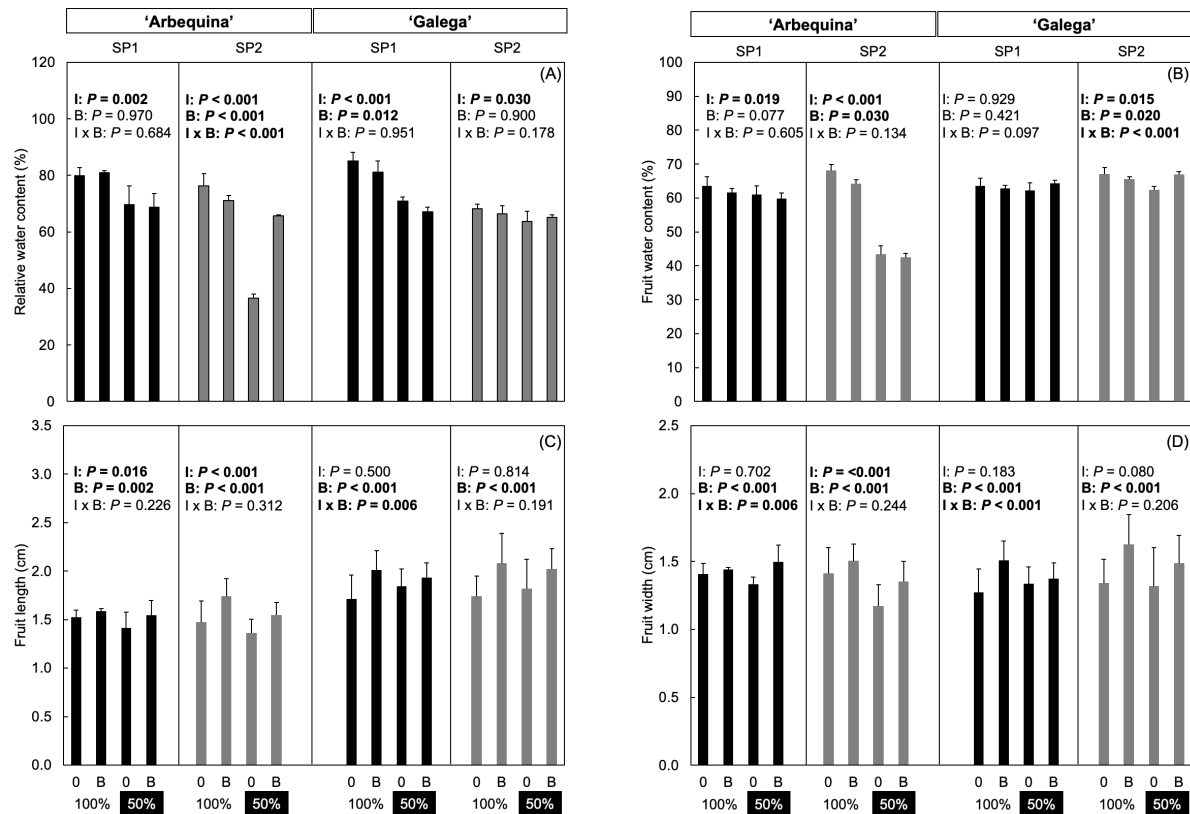


Figure 3.1.2 Relative water content [(A), $n = 3 - 5$], fruit water content [(B), $n = 3 - 9$], fruit length [(C), $n = 10 - 53$] and width [(D), $n = 5 - 53$] of 'Arbequina' and 'Galega' on two different sampling times (SP1 and SP2). 0, zero represents the absence of biostimulant and B represents the application of biostimulant. 100% and 50% indicate the field capacity. I – irrigation factor, B – biostimulant factor, I x B interaction between factors irrigation and biostimulant. Values are mean $\pm$ standard deviation.

Fruit water content (FWC) in 'Arbequina' was affected by the irrigation factor at the SP1, presenting the 50%FC groups lower values. At the SP2 both irrigation and biostimulant factors reduced the FWC. In 'Galega' at the SP1, none of the factors influenced the FWC, however at SP2 an interaction between the factors was observed, the group 50%FC without biostimulant presented the lower values (Figure 3.1.2 B).

Regarding fruit length, in 'Arbequina' it was observed an effect of both irrigation and biostimulant factors in both sampling times, the groups with biostimulant presented a higher length, however the 50%FC reduced those values. In 'Galega', it was observed an interaction between the two factors at SP1, where the control group (100%FC 0) presented the lower value. At SP2 only the biostimulant factor produced an effect, the groups with biostimulant presented the higher values of fruit length (Figure 3.1.2 C).

Fruit width in 'Arbequina' at SP1 showed an interaction between the two factors tested, the group 50%FC without biostimulant presented the lowest fruit width. At SP2 it was observed an effect of irrigation and biostimulant, where the stress groups presented the lower values. In 'Galega', at SP1 an interaction of both factors was observed, the control group with

biostimulant (100%FC B) showed the higher values while the control group (100%FC 0) showed the lower values. At SP2 only the biostimulant factor produced an effect, the groups with biostimulant showed higher values (Figure 3.1.2 D).

Sugars and polyols

The contents of sugars and polyols in treated plants are presented in Figure 3.1.3. The α -D-galactopyranose in 'Arbequina' was affected by the irrigation factor at SP1, where the stress groups (50%FC) presented the lower values. This sugar was not detected at SP2. In 'Galega' at SP1, it was observed no differences between the treatments. At SP2 occurred an interaction between the irrigation and biostimulant factors, where the application of biostimulant at 100%FC reduced the amounts of α -D-galactopyranose while at 50%FC improved the amounts of this sugar (Figure 3.1.3 A). For the D-mannitol in 'Arbequina', it was observed an effect of both irrigation and biostimulant factors at both sampling times, where the 50%FC groups presented higher values however the treatments with biostimulant presented lower values in control and stress groups. In 'Galega', it was not detected any differences between the treatments at the SP1. However, at the SP2, it was observed an interaction between the two factors, where the group 100%FC B presented the lower values (Figure 3.1.3 B). The β -D-glucopyranose was influenced by the irrigation factor at SP1 in 'Arbequina', where the stress groups presented lower values. At SP2, both irrigation and biostimulant factors produced an effect, the 50%FC groups presented lower values, and the groups where the biostimulant was applied produced higher values than the groups without biostimulant. In 'Galega' at SP1, it was observed an interaction between the two tested factors where the group 50%FC without biostimulant presented the lower values. At SP2 it was also verified an interaction between the two factors, the biostimulant application increased the levels of the β -D-glucopyranose in the stress group (50%FC B) and decreased in the control (100%FC B) – Figure 3.1.3 C. Regarding the *myo*-inositol, in 'Arbequina' at SP1 it was observed an interaction between the irrigation and biostimulant factors, where the group 50%FC without biostimulant presented the lower values and no differences were found between the treatments with biostimulant. At SP2, both irrigation and biostimulant factors produced changes in *myo*-inositol, the groups with 50%FC presented the lower values and the application of biostimulant decreased the amounts of this polyalcohol. In 'Galega' at SP1 the biostimulant factor induced changes, the groups with biostimulant presented the higher values. At SP2 it was verified an interaction between the factors tested, where while in the 100%FC the application of biostimulant increased the values of *myo*-inositol, in the 50%FC the groups presented similar values (Figure 3.1.3 D).

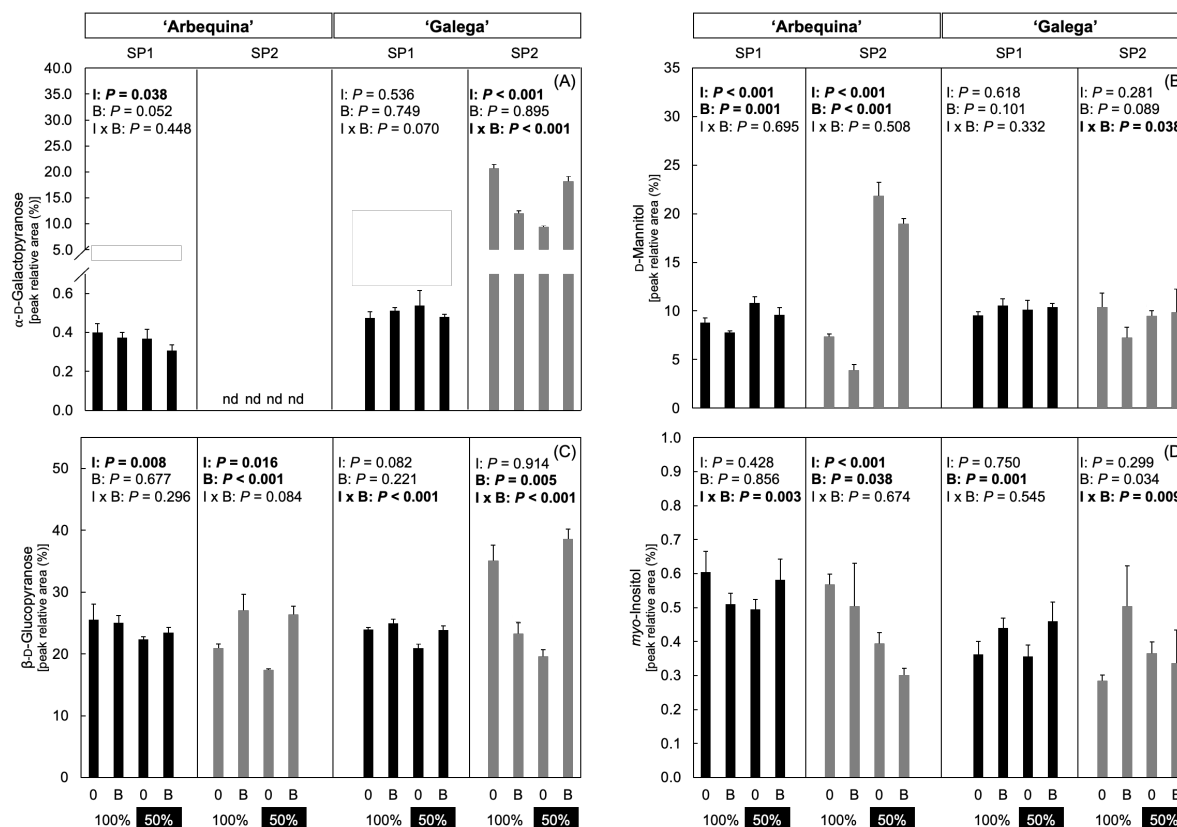


Figure 3.1.3 Sugars and polyols of 'Arbequina' and 'Galega' olive fruits on two different sampling times (SP1 and SP2). 0, zero represents the absence of biostimulant and B represents the application of biostimulant. 100% and 50% indicate the field capacity. I – irrigation factor, B – biostimulant factor, I x B interaction between factors irrigation and biostimulant. Values are mean $\pm$ standard deviation ($n = 4$).

Carboxylic acids and derivatives

Figure 3.1.4 presents the carboxylic acids and derivatives found in fruits from 'Arbequina' and 'Galega' at both sampling times. Similar responses were observed for citric and quinic acids in 'Galega' at SP1, in both was observed an interaction between the factors where the stress group without biostimulant presented the lower value (Figure 3.1.4 B and C). Linoleic acid, stearic acid and squalene at SP1 in 'Galega' also presented an interaction between the tested factors, in both cases the treatment 50%FC 0 presented the highest value (Figure 3.1.4 E, G and I). Also, in 'Galega' at SP1, both the irrigation and biostimulant factors affected the values of 1-monopalmitin and β -sitosterol, the stress (50%FC) induced an increase of the values of these compounds however the biostimulant in both control and stress reduced their amounts (Figure 3.1.4 H and J). Another similarity occurred in palmitic acid, 1-monopalmitin and β -sitosterol in 'Galega' at the SP2, where the interaction effect increased the values of the group biostimulant in the control (100%FC B), but in the stress groups the group without biostimulant (50%FC 0) presented higher values (Figure 3.1.4 D, H and J).

Linoleic, oleic and stearic acids in 'Galega' at SP2 also produced an interaction between the factors, the stress group without biostimulant (50%FC 0) presented the highest value followed by the control with biostimulant (100%FC B) and the stress with biostimulant (50%FC B) (Figure 3.1.4 E, F and G). No differences were found in 'Arbequina' at SP1 for citric, quinic, linoleic, and stearic acids and 1-monopalmitin. In 'Galega', oleic acid does not present differences at SP1 and citric acid at SP2. Regarding butanedioic acid in 'Arbequina', at SP1 an interaction occurred between the factors where the stress groups presented the higher values, however, in these groups, the biostimulant showed lower values than without it. At SP2 both factors (irrigation and biostimulant) influenced the butanedioic acid, the 50%FC groups presented lower values than the 100%FC ones and within those groups, the biostimulant also promotes a reduction of the values. In 'Galega' at SP1, an interaction between irrigation and biostimulant occurred, and biostimulant groups presented the lower values. At SP2 an interaction was also verified, being the higher values in the group 100%FC with biostimulant (Figure 3.1.4 A). Citric acid showed an interaction at SP2 where the 100%FC 0 group presented the highest value in 'Arbequina' (Figure 3.1.4 B). Quinic acid was affected by the irrigation and the biostimulant factors at SP2 in 'Arbequina', the groups with biostimulant presented higher values than those without, despite the 50%FC reduction verified (Figure 3.1.4 C). In 'Galega' at SP2 it occurred an interaction where the 50%FC without biostimulant presented the lower values while the 50%FC with biostimulant presented the highest values for the quinic acid (Figure 3.1.4 C). Palmitic acid in 'Arbequina' at SP1 was influenced by the biostimulant application, biostimulant groups (100%FC B and 50%FC B) presented the higher values. At SP2, both irrigation and biostimulant factors influenced the palmitic acid levels, the application of biostimulant reduced the levels of this fatty acid in both irrigation systems (Figure 3.1.4 D). In 'Galega' at SP1 the palmitic acid produced an interaction between the factors where the treatments without biostimulant presented higher values (Figure 3.1.4 D). An interaction between the factors occurred in 'Arbequina' at SP2 for linoleic acid, the highest values were found in the groups without biostimulant (50%FC 0 and 100%FC 0) (Figure 3.1.4 E). In 'Arbequina' at SP1 it was observed that the biostimulant factor influenced the levels of oleic acid, the groups with biostimulant presented higher values. At SP2, the levels of this fatty acid were influenced by the irrigation factor, the stress groups (50%FC) presented lower values (Figure 3.1.4 F). In 'Arbequina' at SP2, an interaction between irrigation and biostimulant influenced the levels of stearic acid, the control group (100%FC 0) presented the highest value (Figure 3.1.4 G). In 'Arbequina' at SP2, the irrigation and biostimulant factors influenced the levels of the 1-monopalmitin, the stress (50%FC) and the presence of biostimulant reduced the levels of this compound (Figure 3.1.4 H). The levels of squalene in 'Arbequina' at SP1 were influenced by an interaction between the factors tested, the 100%FC

B group presented the highest values and the stress (50%FC) showed similar values between the two groups. At SP2, the levels of squalene were influenced by irrigation and biostimulant, where the stress groups (50%FC 0 and 50%FC B) presented the lower values. In 'Galega' at SP2, the interaction between the factors induced the highest values in the 100%FC B followed by the 50%FC 0 of squalene (Figure 3.1.4 I). The levels of β -sitosterol in 'Arbequina' at SP1 were influenced by the interaction between the factors where the stress groups (50%FC 0 and 50%FC B) presented the highest values. At SP2, the interaction between the factors induced an increased on the groups without biostimulant (100%FC 0 and 50%FC 0) (Figure 3.1.4 J).

Multivariate analysis

Figure 3.1.5 presents the principal component analysis (PCA) of leaf and fruit water status, and biometric parameters, sugars, polyols, carboxylic acids and its derivatives of 'Arbequina' and 'Galega' olive fruits at two sampling point in response to biostimulant pretreatment and irrigation levels. The PCA shows a clear separation between the two cultivars and in 'Galega' it is more notorious a separation between the sampling point. Regarding SP1, in Galega all groups are positioned in the lower half, with 100%FC 0, 100%FC B and 50%FC B close to each other, and the group 50%FC 0 being distant from the previous ones, and correlating with β -sitosterol. In the SP2, the groups 100%FC 0 and 50%FC B are near each other but located in the upper right quadrant (correlating with D-glucopyranose, D-galactopyranose, and quinic acid), and the groups 50%FC 0 and 100%FC are more away from the previously but closer to each other in the downright quadrant. 'Arbequina' clustered differently from Galega, and its groups at the SP1 are all very close to each other, distributed between the upper and down left quadrant. At the SP2, the stress groups 50%FC B and 50%FC 0 are closer and positioned mostly in the upper left quadrant, far away from the control groups (100%FC 0 and 100%FC B).

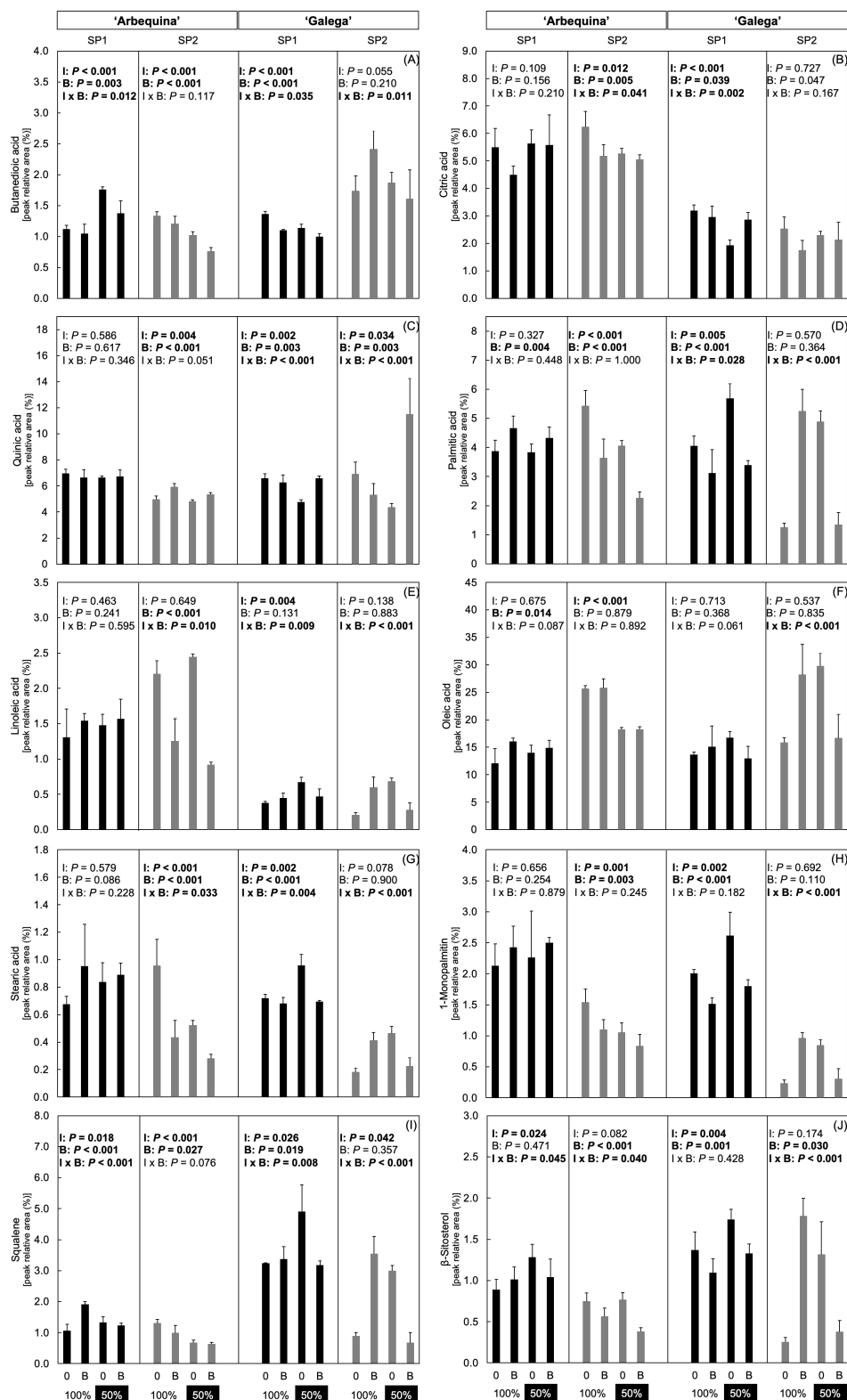


Figure 3.1.4 Carboxylic acids and derivatives, squalene and b-sitosterol of 'Arbequina' and 'Galega' olive fruits on two different sampling times (SP1 and SP2). 0, zero represents the absence of biostimulant and B represents the application of biostimulant. 100% and 50% indicate the field capacity. I – irrigation factor, B – biostimulant factor, I x B interaction between factors irrigation and biostimulant. Values are mean $\pm$ standard deviation ($n = 4$).

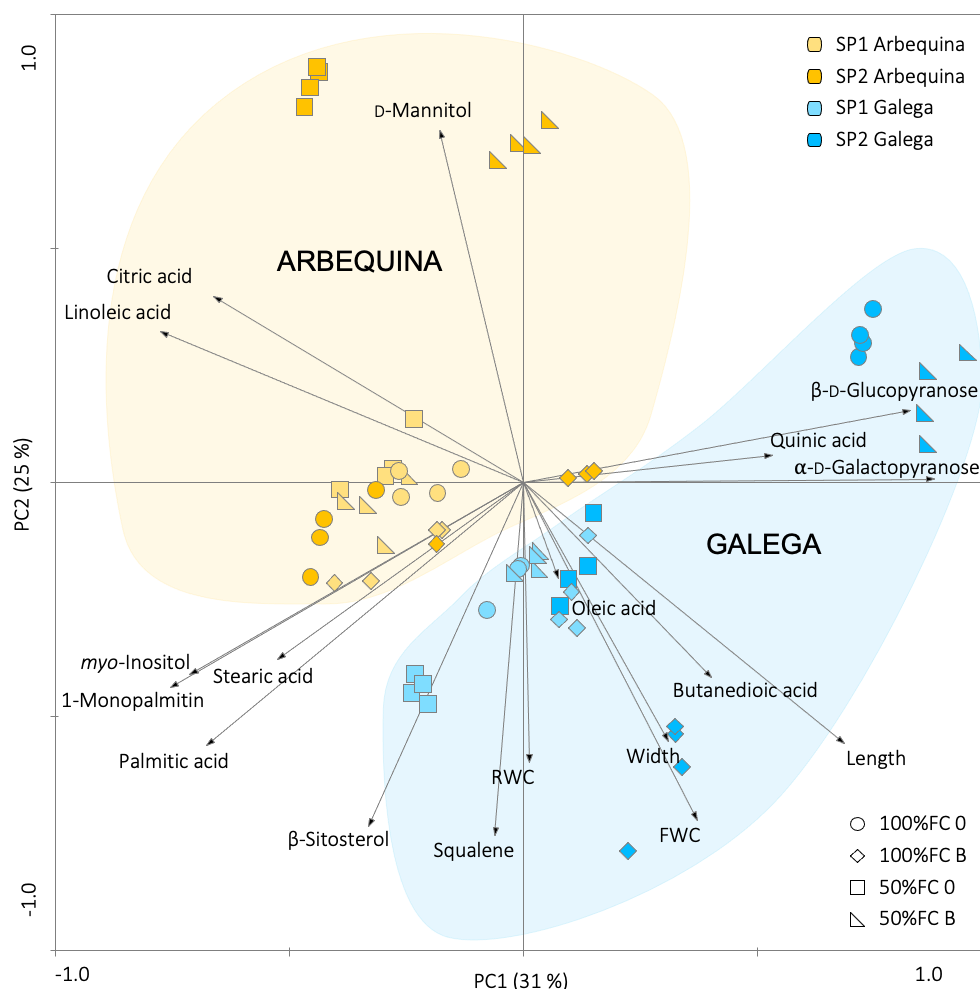


Figure 3.1.5 Principal component analysis (PCA) biplot of the 'Arbequina' and 'Galega' olive leaves and fruits on two different sampling times (SP1 and SP2). PC1 explains 31% of the variance, while PC2 explain 25%.

DISCUSSION

Extreme climate events are increasing in frequency, severity and extension in the Mediterranean region (Fraga et al., 2021) affecting the olive crop. *Olea europaea* is well adapted to the Mediterranean harsh environmental conditions, but may be threatened by the increasing periods of prolonged lack of precipitation and to high temperature (Silva, Santos, Serodio, Silva, & Dias, 2018). For instance, it was demonstrated that extreme climate events reduce the olive production, alter olive fruit and oil quality, change the phenological traits and increase the susceptibility to pests and diseases (Brito et al., 2019; Fraga et al., 2021). Therefore, it is urgent to adopt more sustainable agricultural practices, like the use of biostimulants, to improve plant performance and fruit quality under stress conditions. So far as we known this is the first study on the effects of *A. nodosum* pretreatment on olive fruits quality of two olive cultivars, 'Galega' the traditional cultivar usually used in rainfed orchards

and 'Arbequina', the Spanish cultivar more used in high density orchards, exposed to different irrigation levels. The results clearly evidence that biostimulant pretreatment promote olive fruit growth and quality, but Arbequina and Galega responded differently to this biostimulant and irrigation level (Figure 3.1.5)

As expected, the water deficit imposed (50%FC) for a long period (SP1 69 days and SP2 118 days) reduced the leaf water status in both cultivars. Similar results were reported for olive trees exposed to different water deficit levels (Araújo et al., 2019, 2021; Dias et al., 2018; Gonçalves et al., 2020; Martinelli et al., 2013; Silva et al., 2018), being a common response of this species to water deficit. Nevertheless, at the end of the development stage (SP2) the biostimulant influence under water deficit (50%FC B) was more visible for 'Arbequina', helping to maintain a water availability higher than the water deficit without biostimulant (50%FC 0 for SP2). These data suggest that biostimulant used under water deficit may have helped to improve the 'Arbequina' plants' water status. However, the response profile of fruits to the different factors, irrigation, and biostimulant, was different. Also at the same development stage, in 'Galega', the biostimulant favors fruit water maintenance under water deficit conditions (50%FC B), helping to maintain the water content to levels similar to the controls (100%FC). Furthermore, the positive contribution of the biostimulant to the FWC under well-watered conditions is also evidenced by the PCA (Figure 3.1.5). In 'Arbequina', the biostimulant effect on the fruit water availability under water deficit conditions was not evident. These data suggest that for 'Arbequina' the beneficial effects of biostimulant on tissue water availability are more notorious at the plant level (in leaves), while in 'Galega' are at the fruits. The application of biostimulant based on *A. nodosum* extracts in several species, such as hazelnut, soybean, and sweet cherry, also helped to preserve the leaf water status under water deficit conditions (Cabo et al., 2020; Rosa et al., 2021; Santaniello et al., 2017; Shukla et al., 2019). This response is mostly related to a positive effect of this biostimulant at the stomata level, inducing a reduction of the stomata aperture, and therefore lower water loss via transpiration (Santaniello et al., 2017).

Regarding fruit growth, the application of the biostimulant stimulated both fruit length and width in both cultivars, and this was particularly evident at the end of the development period independently of the irrigation level. Other works demonstrated that applying seaweed-based biostimulants in tomato plants increased the number of fruits and size, and in cauliflower the heart size (Ali et al., 2021). In olives, *A. nodosum* extracts enriched with nitrogen and boron improved productivity, oil content, and fruit weight (Chouliaras et al., 2009). Seaweed products contain several growth regulators (e.g., cytokinins, auxins, and gibberellins) and other compounds such as amino acids, betaine, and inorganic elements that promote cell growth, division, and expansion (Kulkarni et al., 2019; Pereira et al., 2020).

Concerning fruit sugars and polyols, the cultivar 'Galega' was generally more responsive to biostimulant. For this cultivar, the biostimulant increased the β -D-glucopyranose and α -D-galactopyranose pools under water deficit conditions at the end of the development stage (SP2), reaching levels very close to control plants (100%FC 0, Figure 3.1. 5). This increase of sugars in olive fruits in response to stress can represent a higher availability of energy necessary to complete the development process but can also contribute to osmoregulation to maintain cell turgor under water deficit (Silva et al., 2018). The high levels of water availability found in these fruits of this treatment (50%FC B) can support the putative osmolyte function of these sugars under stress. The accumulation of sugars was previously reported in olive fruits but only in response to a water deficit + heat condition (Valente et al., 2020). *Ascophyllum nodosum* also promoted the accumulation of sugars in some fruits, like strawberries and oranges (Ali et al., 2021; Drobek et al., 2019). This biostimulant seems to upregulate the genes related to the biosynthesis of sugars and down-regulate the genes involved in sucrose degradation (Ali et al., 2021; Shukla et al., 2019). Sugars play an important role as they are the precursors for the biosynthesis of olive oil (Marsilio et al., 2001) and the carbon source for the production of secondary metabolites responsible for the organoleptic characteristics during the fermentation (by microorganisms) of table olives (Issaoui et al., 2011). Therefore, using this biostimulant enhanced sugar levels mostly in the final stages of development potentially promoting a better organoleptic characteristic of table olives and an increase in the oil content.

Olive trees are very tolerant to drought and some polyols (e.g., mannitol and *myo*-inositol) are involved in stress protective mechanisms, such as osmolytes, or acting against reactive oxygen species (Cataldi et al., 2000). Several works demonstrated that these polyols accumulate in olive fruits in response to abiotic stresses, like drought, osmotic and salt stress (Conde et al., 2011; Martinelli et al., 2013). In the present work, *myo*-inositol seems more responsive (increase) to the biostimulant application. For the cultivar 'Galega', this effect was more evident under well-watered conditions, particularly at the end of the development process (SP2). In 'Arbequina', the biostimulant was able to maintain the levels of *myo*-inositol under water deficit condition (50%FC B) close to controls (100% FC 0), but only at the beginning of the development process (SP1), possibly helping to cope with the beginning of the stress condition. Concerning the D-mannitol, its relevance was evident under stress conditions (50% FC 0 and B), as indicated in the PCA (Figure 3.1.5), particularly in the cultivar 'Arbequina', highlighting its important role possibly as osmolyte. However, the stimulation of polyols production by biostimulants was reported in other studies, *A. nodosum* seems to modulate the expression of genes involved in the biosynthesis of these compounds (inositol and trehalose) (Shukla et al., 2019).

Fatty acids are one of the main components of olive fruits. The oleic acid, which is a healthy monounsaturated fatty acid, is usually present in high amounts in olive fruit and oil, followed by the polyunsaturated fatty acid linoleic acid, and the saturated fatty acids palmitic acid (Guo et al., 2018). In this work, the oleic acid was the most abundant fatty acid followed by palmitic, linoleic, and stearic acids a profile already reported for olive fruits (Gonçalves et al., 2020; Valente et al., 2020). The application of *A. nodosum* to plants favored the accumulation of oleic and palmitic acids in the olives, but only in the cultivar 'Arbequina', independently of the irrigation level, in the first stages of fruit development (SP1). However, this positive effect is not so clear with the progress of the development process (SP2). While the ratio between the monosaturated fatty acids and saturated fatty acids, an indicator of quality and stability against oxidative stress, in 'Arbequina' at SP2 was higher in the groups with biostimulant (100%FC B was 6.54 ± 1.41 and 50%FC B was 7.22 ± 0.76 , $p < 0.001$) than the groups without (100%FC 0 was 4.04 ± 0.41 and 50%FC 0 was 3.99 ± 0.12). This indicates that the biostimulant promoted the maintenance of the levels of the monounsaturated fatty acids in the stressful condition tested. *Ascophyllum nodosum* effects on fatty acids profile are very variable, and the factor cultivar/species also contribute to this variability. For instance, in almonds, no changes were observed in the levels of oleic, stearic, and palmitic acids, but linoleic acid contents decreased in response to *A. nodosum* application under well-watered conditions (Pascoalino et al., 2021). In hazelnuts, also palmitic and stearic acids were not affected by *A. nodosum* treatment (under well-watered conditions), however, the pool of oleic acid decreased while linoleic acid increased (Pascoalino et al., 2021).

Regarding the organic acids, quinic and citric acids were found in both cultivars. These compounds are involved in several pathways as intermediate products that can be affected by abiotic stressors (e.g., drought, heat). Moreover, organic acids levels influence the fruit' flavor and they can be used as indicators of ripeness, and act as antioxidants or/and osmolytes (Arslan and Özcan, 2011). The accumulation of quinic acid in olive fruits seems to be stimulated by the biostimulant. For the 'Galega' cultivar, the positive effect of *A. nodosum* started already to be notorious at the beginning of the fruit development (SP1) under both irrigation levels but is at the end (SP2) that it stands out namely under water deficit (50% FC B). In 'Arbequina' fruits, this effect started later (SP2) and in both irrigation conditions but was not so expressive as in 'Galega'. Since the quinic acid has antioxidant properties (Nicolì et al., 2019), the increase of the pool of this compound by *A. nodosum* may represent higher protection against stress conditions, particularly for the 'Galega' cultivar. For the antioxidant compound, citric acid, the biostimulant helped to maintain their levels under water deficit conditions (50% FC B similar to the 100%FC treatments), but only at the beginning of the development process in the cultivar 'Galega'.

All these data suggest that the effects of this type of biostimulants depend on the cultivar, irrigation condition, and exposure time. Even the stimulation of the production of some compounds by the biostimulant at the beginning of the development process may represent a benefit and can help to cope better with the stress condition until the end of the development process. Studies with *Arabidopsis* plants demonstrated that the stress protection induced by *A. nodosum* is achieved by readjustments in the pools of several compounds, like sugars, sugar alcohols, organic acids, and fatty acids (Shukla et al., 2019).

As reported in other studies (Ali et al., 2021; Shukla et al., 2019), biostimulant application under well-watered conditions can also benefit the olive fruits, increasing the growth of olives and the pool of important compounds that improve the quality. This was evident by the stimulation of the olive growth parameters and increase of the levels of *myo*-inositol and organic acids (butanedioic, palmitic, oleic, and linoleic acids) in 'Galega' fruits, and also the quinic acid and the β -D-glucopyranose in 'Arbequina' fruits, particularly at end of the development process.

The squalene was identified in olive fruits, and the level of this triterpene depends on several factors, such as the cultivar/species, climate conditions, and fruit ripening stage (Martinelli et al., 2012; Valente et al., 2020). Squalene is the precursor of β -sitosterol and acts as a scavenger and quencher of free radicals (Micera et al., 2020). Some studies in olives indicate that the levels of squalene drop along with the ripeness of the fruit (Ambra et al., 2017; Martinelli et al., 2012), however another study on the cultivars 'Arbequina' and 'Picual' showed a different pattern, the levels of this triterpene increased during the fruit maturation (Fernández-Cuesta et al., 2013). In general, it was observed a decrease in the levels of squalene in both cultivars tested during the ripening, which is in line with previous data for olive fruits (Martinelli et al., 2012). The effect of *A. nodosum* seems also more important under well-watered conditions (100%FC B) and occur first at the beginning of the development process of 'Arbequina' fruits (SP1) and later in 'Galega' fruits (SP2). Nonetheless, this positive effect of the *A. nodosum* may contribute to valorizing the nutritive value of these fruits.

Similar to the reported by Valente et al. (2020), the β -sitosterol was the only sterol identified in olive fruits of both cultivars. The biostimulant only affected positively the level of this sterol under well-watered conditions, at the end of the development process of 'Galega' fruits. Since plant sterols, such as sitosterol, stigmasterol, and campesterol, are important biomolecules precursors and act in plants similar to the cholesterol in animals (Ozkan et al., 2017), when is consumed by humans are benefic to the human health (e.g., can reduce total and LDL cholesterol) (AbuMweis et al., 2009). The increase of β -sitosterol levels by *A. nodosum* represents an improvement of the nutritional quality of olives.

Regarding 1-monopalmitin, a monoglyceride generally present in plants (Basas-Jaumandreu and de las Heras, 2019; Nitbani and Jumina, 2020), reduced with the ripeness of the fruits in both cultivars, however the biostimulant did not had a relevant role.

CONCLUSION

The effect of *A. nodosum* on olives' water content and metabolite profile during the development process depends on the cultivar, irrigation level, and fruit ripening stage. Concerning the effects of this biostimulant under water deficit conditions, the two olive cultivars adopted different response strategies. Regarding plant water status, the beneficial effects of biostimulant application under water deficit conditions were more evident in the cultivar 'Arbequina', while at the fruit tissue level were more notorious in the cultivar 'Galega'. Also, in the cultivar 'Galega', *A. nodosum* was very efficient for stimulating the production of quinic acid all over the fruit development process. However, in terms of fruit growth and sugars, the positive effect of the biostimulant was more pronounced at the end of the development stage. Contrarily, citric acid accumulation was more favored at the beginning of the development process. For the cultivar 'Arbequina', biostimulant favors fruit growth and quinic acid accumulation, particularly at the end of the development process. Concerning other compounds, such as *myo*-inositol and fatty acids (oleic and palmitic acids), the *A. nodosum* effect seems more evident at the beginning of the fruit development process. In conclusion, the use of *A. nodosum* under water deficit seems very positive, since it promotes fruit growth and metabolites readjustment, contributing to the improvement of the nutritional quality of olives. Moreover, even under well-watered growth conditions, the use of biostimulant could be also valuable to enrich fruits with some fatty acids, squalene, β -sitosterol, *myo*-inositol, and butanedioic acid in 'Galega' fruits or quinic acid and sugars (β -D-glucopyranose) in 'Arbequina' fruits.

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Chapter IV

Functional and metabolic responses to water deficit of an orchard under drought conditions



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Chapter IV is constituted by two subchapters that are works under field conditions.

The first subchapter is published in *Plants* and the second was submitted to the *Journal of the Science of Food and Agriculture*.

4.1. Antioxidant adjustments of olive trees (*Olea europaea*) under field stress conditions.

Araújo, M., Prada, J., Mariz-Ponte, N., Santos, C., Pereira, J. A., Pinto, D. C. G. A., Silva, A. M. S., Dias, M. C. (2021). Antioxidant adjustments of olive trees (*Olea europaea*) under field stress conditions. *Plants*, 10(4), 684. <https://doi.org/10.3390/plants10040684>

4.2. Metabolites' profile of fruit and oil of Cobrançosa olive trees under field stress conditions.

Araújo, M., Rodrigues, N., Santos, C., Pinto, D. C. G. A., Pereira, J.A. Silva, A. M. S., Dias, M. C. (2021) Metabolites' profile of fruit and oil of Cobrançosa olive trees under field stress conditions. *Journal of the Science of Food and Agriculture* (Submitted, Ref No.: JSFA-22-1030)

4.1. Antioxidant adjustments of olive trees (*Olea europaea*) under field stress conditions⁴

ABSTRACT

Extreme climate events are increasingly frequent, and the 2017 summer was particularly critical in the Mediterranean region. Olive is one of the most important species of this region, and these climatic events represent a threat to this culture. However, it remains unclear how olive trees adjust the antioxidant enzymatic system and modulate the metabolite profile under field stress conditions. Leaves from two distinct adjacent areas of an olive orchard, one dry and the other hydrated, were harvested. Tree water status, oxidative stress, antioxidant enzymes, and phenolic and lipophilic metabolite profiles were analyzed. The environmental conditions of the 2017 summer caused a water deficit in olive trees of the dry area, and this low leaf water availability was correlated with the reduction of long-chain alkanes and fatty acids. H_2O_2 and $\text{O}_2^{\bullet-}$ levels increased in the dry area trees, but lipid peroxidation did not augment. The antioxidant response was predominantly marked by GPOX activity that regulates the H_2O_2 harmful effect and by the action of flavonoids (luteolin-7-O-glucuronide) that may act as ROS scavengers. Secoiridoids adjustments may also contribute to stress regulation. This work highlights for the first time in olive trees the protective role of some metabolite under field drought conditions.

KEYWORDS

Climate change, Flavonoids, Secoiridoids, *Olea europaea*, Rainfed olive groves, Drought

⁴ Araújo, M., Prada, J., Mariz-Ponte, N., Santos, C., Pereira, J. A., Pinto, D. C. G. A., Silva, A. M. S., Dias, M. C. (2021). Antioxidant adjustments of olive trees (*Olea europaea*) under field stress conditions. *Plants*, 10(4), 684. <https://doi.org/10.3390/plants10040684>

INTRODUCTION

The Mediterranean basin is the world's leading region of olives and olive oil production (FAOSTAT, 2017). However, this area is increasingly facing climate change events characterized by combined extreme weather conditions (IPCC, 2018). The summer of 2017 was particularly critical to this region (including Portugal and Spain) due to prolonged water deficit periods accompanied by heatwaves and high UV-irradiance levels (Haworth et al., 2018).

Olive trees (*Olea europaea*) have outstanding phenotypic plasticity (e.g., Fernández et al., 2018). However, the nature of these climate change-associated extreme episodes and the predictions of their increase in frequency and intensity in the next decade (IPCC, 2018) represent a new threat to olive cultivation. This threat may potentially lead to significant losses in olive productivity and fruit and oil quality (Araújo et al., 2019; De Ollas et al., 2019; Dias et al., 2018, 2019a; Fernández et al., 2018; Valente et al., 2020).

Olive trees have evolved morpho-physiological adaptive mechanisms to cope with the Mediterranean climate (Sghaier et al., 2019). These include an efficient ability to control leaf transpiration (e.g., thick cuticle, high density of foliar tissue, and trichome layers), and to regulate stomata aperture, osmotic adjustment, and extract water from soils with low water potential (Fernández et al., 2018). However, the investment of olive energy resources (or shifts in metabolic pathways) in defense mechanisms compromise growth and yield (Petridis et al., 2012a). Both water deficit and heat may reduce olive water status, decrease stomata aperture, impair photosynthesis, and lead to oxidative stress (Araújo et al., 2018; Brito et al., 2018; Dias et al., 2019a, 2020a). The prevention of cellular oxidative damage (e.g., lipid, protein, pigments, and DNA) is one of the first stress tolerance mechanisms (Demidchik, 2015). To control the increase of ROS, olive plants activate the antioxidant battery, upregulating antioxidant enzymes [e.g., superoxide dismutase (SOD), catalase (CAT), glutathione reductase (Gr), ascorbate peroxidase (APX), and guaiacol peroxidase (GPOX)], and invest in the production of several antioxidant metabolites, like ascorbate, glutathione, carotenoids and phenolic compounds (Dias et al., 2019a, 2019b, 2020a; Koubouris et al., 2015).

Polyphenols are among the most significant antioxidants (Agati et al., 2011; Zhang et al., 2018). For instance, in response to drought or heat, olive plants increase flavonoids in leaves, especially the catechol B-ring substituted flavonoids (e.g., quercetin 3-glycoside), acting as protective antioxidants (Dias et al., 2019a). Flavonoids can accumulate in the leaf epidermis, acting more efficiently as UV shields, or they can be found near the cell compartments where ROS is generated (or transported to the sites of ROS generation), acting as ROS scavengers (Agati et al., 2013; Brunetti et al., 2013; Zhang et al., 2018). The secoiridoids, such as oleuropein, are also another group of polyphenols with a high antioxidant activity, which can

only be found in the Oleaceae family (Talhaoui et al., 2015). This family of compounds, together with flavonoids, has been reported to have a defensive role in olive and other Mediterranean species exposed to salt stress or high UV-B radiation (Dias et al., 2019a, 2020a; Petridis et al., 2012b; Rodriguez et al., 2015). Moreover, this family of compounds' abundance and the ratio strongly depend on the growing environmental conditions, species, cultivar, and organ. In addition to these families of metabolites, also lipophilic compounds related to cuticle wax (e.g., long-chain alkanes and ursolic acid) and with ROS scavenger capacity (e.g., thymol glycosides and ursolic acid), together with compounds associated with the maintenance of membrane integrity (e.g., oleic and palmitic acids) were identified and reported to contribute to olive adaptation to high UV-B radiation (Dias et al., 2018).

Field extreme stressful episodes may trigger physiological and biochemical responses different from those activated by mild stressing conditions (Jorge and António, 2017; Suzuki et al., 2014). Thus, understanding the changes in these metabolites' profiles under extreme climate change conditions will support the selection of more adapted cultivars and contribute to ensuring olive productivity and olive/oil quality in the following decades. Most studies on young olive plants used single (e.g., Petridis et al., 2012a; Dias et al., 2020) or combined (drought and heat) stressful conditions (Araújo et al., 2019; Sghaier et al., 2019) imposed in a controlled environment, which is easier to manipulate but does not mimic the complex environmental conditions of the field. Moreover, the source-sink relationships, determinant for photosynthetic acclimation process, in young potted plants are completely distinct from adult field-grown trees. Besides, potted plants in climate chambers or greenhouses are subjected to stronger effects (e.g., high temperatures in low or non-ventilated greenhouses) and growth conditions are far from what occurs in the field (Ogbaga et al., 2020). Thus, it is not reliable to compare potted plants' responses growing in climate chambers or greenhouse with those obtained under natural field experiments. Therefore, it is necessary to study olive tree stress responses under natural conditions to provide a real scenario of the stress-induced adjustments in trees and their impacts on olive productivity and oil quality. Persistent drought is particularly important in rainfed orchards and, alone or combined with other stressful conditions, causes agricultural production losses worldwide (Wani et al., 2016).

We hypothesized that adult field olives' physiological plasticity to stressful conditions in the field involves adjusting lipophilic and phenolic metabolic pathways, and the activation of antioxidant enzymes. Hence, a phenolic and lipophilic metabolite profiling and a throughout redox status analysis were conducted in leaves of *O. europaea* trees from an orchard exposed to drought conditions, as verified in the summer of 2017 in the Mediterranean region.

MATERIALS AND METHODS

Reagents and standards

Dithiothreitol (DTT), nitroblue tetrazolium (NBT), H₂O₂, titanium dioxide, dichloromethane, pyridine, *N,O*-bis(trimethylsilyl) trifluoroacetamide, trimethylsilyl chloride, sodium azide, thiobarbituric acid (TBA), methionine, catalase, peroxidase, cholesterol, maltose, octadecane, phenol, palmitic acid, sorbitol, ammonia and potassium phosphate buffer were purchased from Sigma-Aldrich (Saint Louis, USA). Tetracosane and 4-aminoantipyrine were purchased from Fluka (Bucharest, Romania). Sulfuric acid was purchased from Fisher Chemical (New Hampshire, EUA). Trichloroacetic acid (TCA) was purchased from BIOCHEM Chemopharma (France). Guaiacol and polyvinylpyrrolidone (PVP) were purchased from Acrós Organics. Triton-X and ascorbic acid were purchased from Panreac. Acetic acid, ethylenediaminetetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF) were purchased from VWR (Pennsylvania, EUA). Riboflavin, *n*-hexane p.a., methanol p.a., sodium carbonate (Na₂CO₃), dimethyl sulfoxide (DMSO), potassium sulfate and quercetin were purchased from Merck (Darmstadt, Germany). Luteolin and oleuropein were purchased from Extrasynthese (Genay, France).

Plant material and field experiment

The selected rainfed olive grove is located in the northeast of Portugal, Trás-os-Montes region, the producing region of the Protected Designation of Origin "Azeite de Trás-os-Montes", in Suçães-Mirandela (41° 30'39.5 "N 7° 15'28.1 "W). The grove consists of adult trees from the cultivar Cobrançosa (one of the most representative cultivars in Portugal) with about 30 years old. This olive grove is managed as dryland farming, following the integrated production guidelines for olive groves. This orchard is located in a region classified as Csb, according to the Köppen-Geiger climate division, and is characterized by a temperate climate with hot and dry summers, but with rainy winters (Instituto Português do Mar e da Atmosfera, 2017; Kottek et al., 2006; Peel et al., 2007). In 2017, between April and December, the Portuguese territory was under severe/extreme drought (Instituto Português do Mar e da Atmosfera, 2017). The average of the monthly precipitation from June to the end of September in the local of the orchard reached values below 30 mm (Figure 4.1.1 A), which is typical of a dry summer according to Köppen climate classification (Kottek et al., 2006; Peel et al., 2007). Also, the orchard's air temperature reached values around 38°C in June, and even during October, the maximum air temperature reached values above 30°C (Figure 4.1.1 A). Two distinct adjacent areas of the orchard were selected and followed from June to the end of September, one without water supply, with dried topsoil and olive trees with leaves and fruits

with visible symptoms of dehydration (dehydrated trees, dry area), and the other located near a groundwater supply providing constant water to olive trees (hydrated area). On the second day of October 2017, leaves (young and fully developed) from five trees of each area (Figures 4.1.1 B and C) were sampled from different parts of the trees, frozen in liquid nitrogen, and then stored at -80°C.

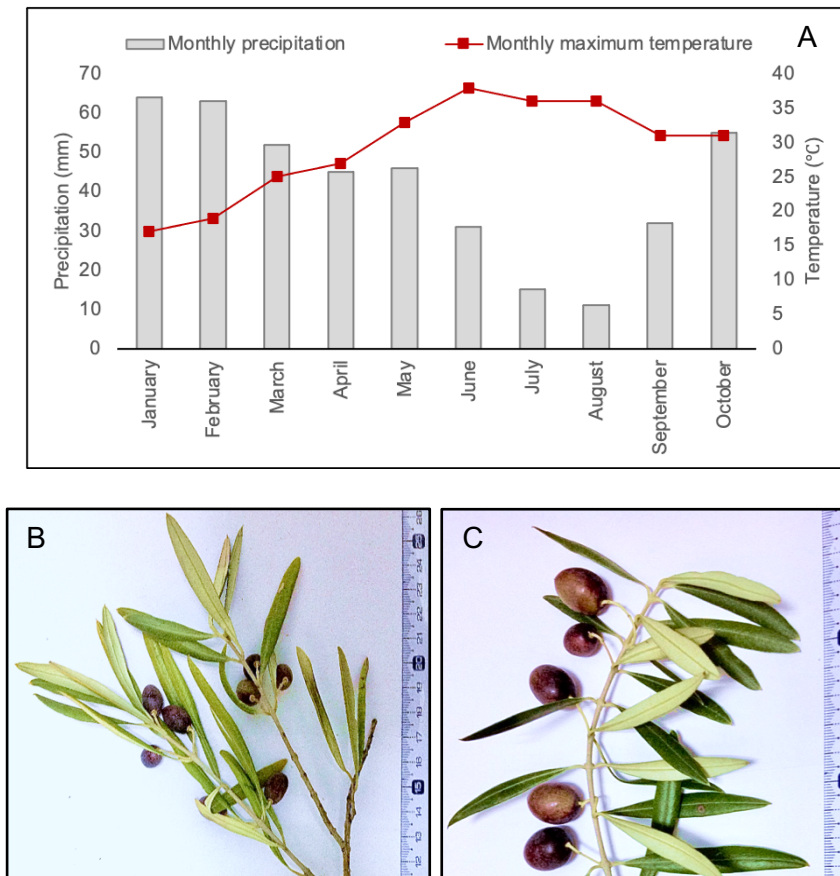


Figure 4.1.1 (A) Monthly average precipitation, monthly maximum air temperature from January to October of 2017 in the local of the olive orchard. (B) Branch of olive trees from the dry area; and (C) branch of olive trees from the hydrated area.

Determination of the tree water status

Tree water status was evaluated by measuring the leaf water content. Leaf fresh weight was measured, and then the leaf was dried at 40°C until constant weight. The leaf water content (LWC, %) was calculated as $((\text{fresh mass} - \text{dry mass}) / \text{fresh mass}) \times 100$.

Malondialdehyde (MDA) determination

To quantify the MDA (Dhindsa and Matowe, 1981), the powder of frozen leaves (100 mg) was homogenized with 1.5 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged for 10 min at 10000g, 4°C. Two aliquots containing 250 µL of supernatant were collected. One

aliquot was mixed with 1 mL of 20% (w/v) TCA, and to the other one with 1 mL of 20% (w/v) trichloroacetic acid and 0.5% (w/v) thiobarbituric acid (TCA+TBA). Both aliquots were placed in a hot bath (95°C) for 30 min, cooled, and centrifuged at 10000g (10 min at 4°C). Specific absorbance was measured at 532 nm and the non-specific absorbance at 600 nm. MDA was calculated by subtracting the non-specific absorbance from the specific absorbance and using the extinction coefficient of 155 mM⁻¹ cm⁻¹ (Dhindsa and Matowe, 1981). Data are presented as mean ± standard deviation (*n* = 9, per condition).

Antioxidant enzyme analysis

Frozen olive leaf powder was homogenized with 0.1 M phosphate buffer (pH 7), 0.5 M Na₂EDTA, 1% PVP (m/v), 1 mM PMSF, 0.2% Triton X-100 (v/v) and 2 mM DTT. The activity of the enzymes superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and guaiacol peroxidase (GPOX, EC 1.11.1.7) was measured in the supernatant. SOD was measured according to Agarwal et al. (2005). The reaction mixture contained 50 mM of potassium phosphate buffer (pH 7.8), 0.1 mM of EDTA, 50 mM Na₂CO₃, supernatant, 13 mM methionine, 63 mM nitroblue tetrazolium (NBT) and 2 µM riboflavin. The mixture was irradiated with fluorescent light (15 W) for 15 min. A non-irradiated mixture (blank) and a mixture without the illuminated supernatant (control) were prepared in parallel. The absorbance of the mixtures was read at 560 nm. One unit of SOD activity is the amount of enzyme necessary to inhibit the NBT reduction by 50% (per minute). To determine CAT activity (Teranishi et al., 1974), 500 µL of ultrapure water, 0.1 M potassium phosphate buffer (pH 7), 50 µL supernatant, and 6 mM H₂O₂ were mixed. After 5 min of the reaction starts, 1 mL of titanium reagent (1 g titanium dioxide and 10 g potassium sulfate were dissolved in 150 mL sulfuric acid) was added to stop the reaction. The mixture was centrifuged (10000g, 10 min at 4°C), and the absorbance was read at 415 nm. CAT activity was determined using a calibration curve prepared with known concentration of catalase (and R² = 0.98) (Teranishi et al., 1974). The method described by Nakano and Asada (1981) was used to measure the activity of APX. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 0.5 mM ascorbic acid and supernatant. The reaction was started with 0.1 mM H₂O₂, and the reduction of ascorbate was followed at 290 nm. The activity of APX was calculated using the molar extinction coefficient of ascorbic acid (ϵ = 2.8 mM⁻¹ cm⁻¹). To measure GPOX activity, the method described by Pütter (1974) was used. GPOX activity was measured by adding 100 mM phosphate buffer (pH 7), 15 mM guaiacol, and supernatant. The reaction was started with 3 mM of H₂O₂. The oxidation of guaiacol was followed at 470 nm, and the GPOX activity calculated using the tetraguaiacol molar extinction

coefficient ($\epsilon = 26.6 \text{ mM}^{-1}\text{cm}^{-1}$). Data are presented as mean $\pm$ standard deviation ($n = 9$, per condition).

Determination of superoxide radical ($\text{O}_2^{\bullet-}$) and H_2O_2 content

$\text{O}_2^{\bullet-}$ content was measured according to Chaitanya and Naithani (1994). Briefly, leaves (100 mg) were incubated with 2 mL of extraction buffer containing 0.01 M phosphate buffer (pH 7.8), 0.05% NBT dissolved in 100 mL of DMSO, and 10 mM of sodium azide. Samples were incubated for 1 h at room temperature with agitation. The samples were then centrifuged for 2 min at 13000g, and 1.5 mL of supernatant was incubated at 85°C for 15 min. After cooling, the absorbance was read at 580 nm. The content in H_2O_2 was measured as described by Zhou et al. (2006). Leaves (100 mg) were grounded in 1 mL precooled 5% TCA (w/v) and 0.15 g of activated charcoal. The mixture was centrifuged (12000 g, 15 min at 4°C), and then the pH of the supernatants adjusted to 8.4 with 17 M ammonia. The supernatant was divided into two aliquots, and 8 μg of catalase was added only to one aliquot. After 10 min at room temperature, the colorimetric reagent [10 mg of 4-aminoantipyrine, 10 mg of phenol, 5 mg of peroxidase (150 U mg^{-1}) dissolved in 50 mL of 100 mM acetic acid buffer (pH 5.6)] was added to both aliquots. After 10 min at 30°C, the absorbance was read at 505 nm. The amount of H_2O_2 was determined using a calibration curve prepared with known concentration of H_2O_2 () and $R^2 = 0.99$ (Zhou et al., 2006). Data are presented as mean $\pm$ standard deviation ($n = 9$, per condition).

Lipophilic extract preparation and gas chromatography-mass spectrometry (GC-MS) analysis

Approximately 50 g (fresh weight) of leaf powder was mixed with 500 mL of *n*-hexane with stirring for 72 h and at room temperature. After the first extraction, the *n*-hexane was renewed, and an additional extraction in the same conditions was made. Then, the solvent evaporated to dryness with a rotary vacuum evaporator, and the extracts were used for the GC-MS analysis. For the silylation procedure, 200 μL of the extract solution (approximately 30 mg mL^{-1}), 200 μL of internal standard (tetracosane, 0.46 mg mL^{-1}), 50 μL of dichloromethane, 250 μL of pyridine, 250 μL of *N,O*-bis(trimethylsilyl) trifluoroacetamide and 50 μL of trimethylsilyl chloride were incubated in glass tubes in a water bath (70°C) for 30 min (Dias et al., 2019a). Each silylated extract was injected in a GC-MS QP2010 Ultra Shimadzu equipped with a FactorFour Capillary Column VF-5ms 30 m x 0.25 mm ID DF=0.25. The chromatographic conditions were as follows: carrier gas, He at a linear velocity of 40.0 cm s^{-1} ; column oven temperature, 70°C; injection temperature, 320°C; injection mode, split;

pressure, 76.1 kPa; total flow, 63.7 mL min⁻¹; column flow, 1.19 mL min⁻¹; purge flow 3.0 mL min⁻¹; split ratio, 1:50. The initial temperature was 70.0°C for 5 min, followed by an increase of 4°C up to 250°C and then 2°C up to 300°C for 5 min. The total program time was 80 min. The mass spectrometer conditions: ion source temperature, 200°C; interface temperature, 300°C; solvent cut time, 6.5 min; micro scan width, 0 µs; threshold, 0; detector voltage, relative to the tuning result, 0.1kV. The start time was 6.5 min and the end time at 80.0 min. The acquisition mode was scan, with event time at 0.10 s, the scan speed of 20000, the start m/z at 50.0, and the end m/z at 1000.0. The compounds were identified based on a direct comparison with the mass spectra database's libraries (NIST14 Mass spectral and WILEY Registry TM of Mass Spectra Data) and comparing the retention times and mass spectra of the standard compounds injected in the same chromatographic conditions of the samples. Calibration curves of pure compounds (cholesterol, maltose, octadecane, palmitic acid, and sorbitol) were used for the quantitative analysis. The correlation coefficient of the calibration curves was above 0.97. The pure compounds were injected (after silylation) in the same chromatographic conditions of the samples. At least four independent analyses were performed per condition.

Phenolic extract preparation and ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) analysis

Approximately 50 g (fresh weight) of leaf powder was mixed with 500 mL of methanol with stirring for 72 h and at room temperature. After the first extraction, the solvent was renewed one more time. Each extraction solvent was evaporated to dryness with a rotary vacuum evaporator, and the extracts were used for the UHPLC-MS analysis. For the UHPLC-MS analysis, the final concentration of samples was 15 mg mL⁻¹. Samples were filtered through a 0.2 µm Nylon membrane (Whatman) and injected in a UHPLC-MS (Thermo Scientific Ultimate 3000RSLC (Dionex)) equipped with a Dionex UltiMate 3000 RS diode array detector and coupled to a mass spectrometer (Dias et al., 2019a). The column used was a Thermo Scientific Hypersil Gold column (100 mm × 2.1 mm) with a particle size of 1.9 µm, and its temperature was maintained at 30°C. The mobile phase was composed of (A) acetonitrile and (B) 0.1% formic acid (v/v), both degassed and filtered before use. The flow rate was 0.2 mL min⁻¹. The solvent gradient was 5% of solvent B in the first 14 min, followed by 40% of solvent B for 2 min, 100% over 7 min, and 5% over 10 min. The injection volume was 2 µL. UV-Vis spectral. Data were gathered in a range of 230 to 517 nm. The mass spectrometer used was an LTQ XL linear ion trap 2D equipped with an orthogonal electrospray ion source (ESI). The equipment was operated in negative-ion mode with an electrospray ionization source of 5.00 kV and ESI capillarity temperature of 275°C. The full scan covered a mass

range of 50–2000 m/z. Collision-induced dissociation MS/MS and MSⁿ experiments were simultaneously acquired for precursor ions. The chromatograms obtained at 280, 240, and 230 nm were analyzed, and the qualitative profile was performed. The extract's phenolic compounds were identified based on UV–Vis spectra and MSⁿ spectra data with those closest available reference standards and data reported in the literature. The semi-quantification of the individual compounds in the plant extracts was performed by peak integration at 240 nm through the external standard-method. The detection and quantification limits (LOD and LOQ, respectively) were determined from the calibration curves. The calibration curves were determined by injecting pure standard compounds (luteolin, oleuropein, and quercetin). These compounds' concentrations guarantee each compound's quantification in the extracts by interpolation in the calibration curve. The correlation coefficients confirmed the linearity of the calibration curves ($R^2 > 0.95$). At least four independent analyses were performed per condition.

Statistical analysis

The student's *t*-test was used to evaluate the statistical significance between groups (hydrated and dehydrated). The significance level was 0.05. Pearson's correlation was analyzed. All statistical analyses were performed in the SigmaStat program for Windows, version 3.1. (Systat Software, San Jose, CA). Log₂ fold change of two groups' means was calculated using Microsoft® Excel for Mac (version 16.32).

RESULTS

Leaf water content and plant oxidative stress

The leaf water content (LWC) in the dry area trees was significantly lower than in the hydrated area, showing a percentage of 50.4±0.9 and 95.3±3.6, respectively.

Regarding oxidative stress, plants from the dry area presented significantly higher values of superoxide radical (O₂^{•−}) and H₂O₂ than plants in the hydrated area (Figures 4.1.2 A and B, respectively). The activity of SOD and CAT was significantly lower in the leaves of trees from the dry area (Figures 4.1.2 C and D, respectively), and the opposite (increased activity) occurred with the activity of GPOX (Figures 4.1.2 F). The activity of APX and the MDA content remained similar in both conditions (Figures 4.1.2 E and G, respectively).

Phenolic profile

Table 4.1.1 showed the phenolic profile and the fold changes in these metabolites. The profile of response of flavonoids and secoiridoids was heterogeneous, as, within each group, both negative and positive responses to the environmental conditions were observed.

Flavonoids were represented in the leaf samples by the quercetin-3-O-rutinoside, luteolin, luteolin-7-O-glucoside, luteolin-7-O-glucuronide, luteolin-4-O-glucoside (and its isomer), apigenin-7-O-glucoside, and apigenin-7-O-hexosyl rhamnosides. Leaves from the dry area showed a level of luteolin-4-O-glucoside (and its isomer), luteolin-7-O-glucoside, and apigenin-7-O-glucoside higher ($p \leq 0.05$) than the ones found in the hydrated area. The levels of apigenin-7-O-hexosyl rhamnosides and quercetin-3-O-rutinoside were similar ($p > 0.05$) in leaves from trees of both areas. The luteolin and luteolin-7-O-glucuronide levels were lower ($p \leq 0.05$) in leaves from the dry area trees than those from the hydrated area. The secoiridoids were represented by oleoside-11-methyl ester (and its isomer), oleuropein, oleuropein aglycone, and 6'-O-[8-hydroxy-2,6-dimethyl-2-octenoyloxy] secologanoside. Leaves from the dry area trees presented the levels of oleoside-11-methyl ester, oleuropein, and oleuropein aglycone higher ($p \leq 0.05$) than those of the hydrated area. Contrarily, the amount of oleoside-11-methyl ester (isomer) and 6'-O-[8-hydroxy-2,6-dimethyl-2-octenoyloxy] secologanoside were significantly lower in leaves from the trees in the dry area.

Oleuropein aglycone, oleoside-11-methyl ester (isomer), luteolin-4-O-glucoside, luteolin, and luteolin-7-O-glucuronide presented a higher amount of differences between leaves from both areas tested. While leaves in the dry area had a higher quantity of oleuropein aglycone and luteolin-4-O-glucoside than those found in the hydrated area. Oleoside-11-methyl ester (isomer), luteolin, and luteolin-7-O-glucuronide in leaves from the dry area were the ones whose quantities decreased more than those presented in the hydrated area.

Lipophilic profile

Table 4.1.2 showed the lipophilic profile and the fold changes of metabolites in dehydrated leaves concerning the hydrated ones. The profile of response of fatty acids, long-chain alkanes, and sterols was very similar, presenting a negative response characterized by a decrease of these metabolites. Contrarily, the response profile of carbohydrates and terpenes was positive, representing an increase of the respective metabolites.

Fatty acids were represented in the leaf samples by oleic, linoleic, palmitic, stearic, and α -linolenic acids. Leaves from the dehydrated trees presented lower ($p \leq 0.05$) levels of α -linolenic acid, oleic acid, linoleic acid, and palmitic acid compared to the hydrated ones' values. Stearic acid presented similar ($p > 0.05$) values in both sampling areas (hydrated vs. dehydrated trees). Terpenes were represented in the leaf samples by squalene, lupeol derivative, ursolic acid, and α - and β -amyrin. These compounds' levels were similar ($p > 0.05$) in the leaves of trees from hydrated and dry areas. Sorbitol and D-glucose were the only quantified polyol and sugar identified in leaves from trees of the hydrated and dry areas. Leaves from the dehydrated trees presented higher ($p \leq 0.05$) levels of sorbitol and D-glucose

than the hydrated ones. β -Sitosterol was the only sterol identified in olive leaves, and samples from the dry area presented less ($p \leq 0.05$) levels than samples from the hydrated one. Six long-chain alkanes were identified in the leaves. For the long-chain alkane 1, 3, 4, 5, and 6, dehydrated leaves showed lower ($p \leq 0.05$) levels than the hydrated ones.

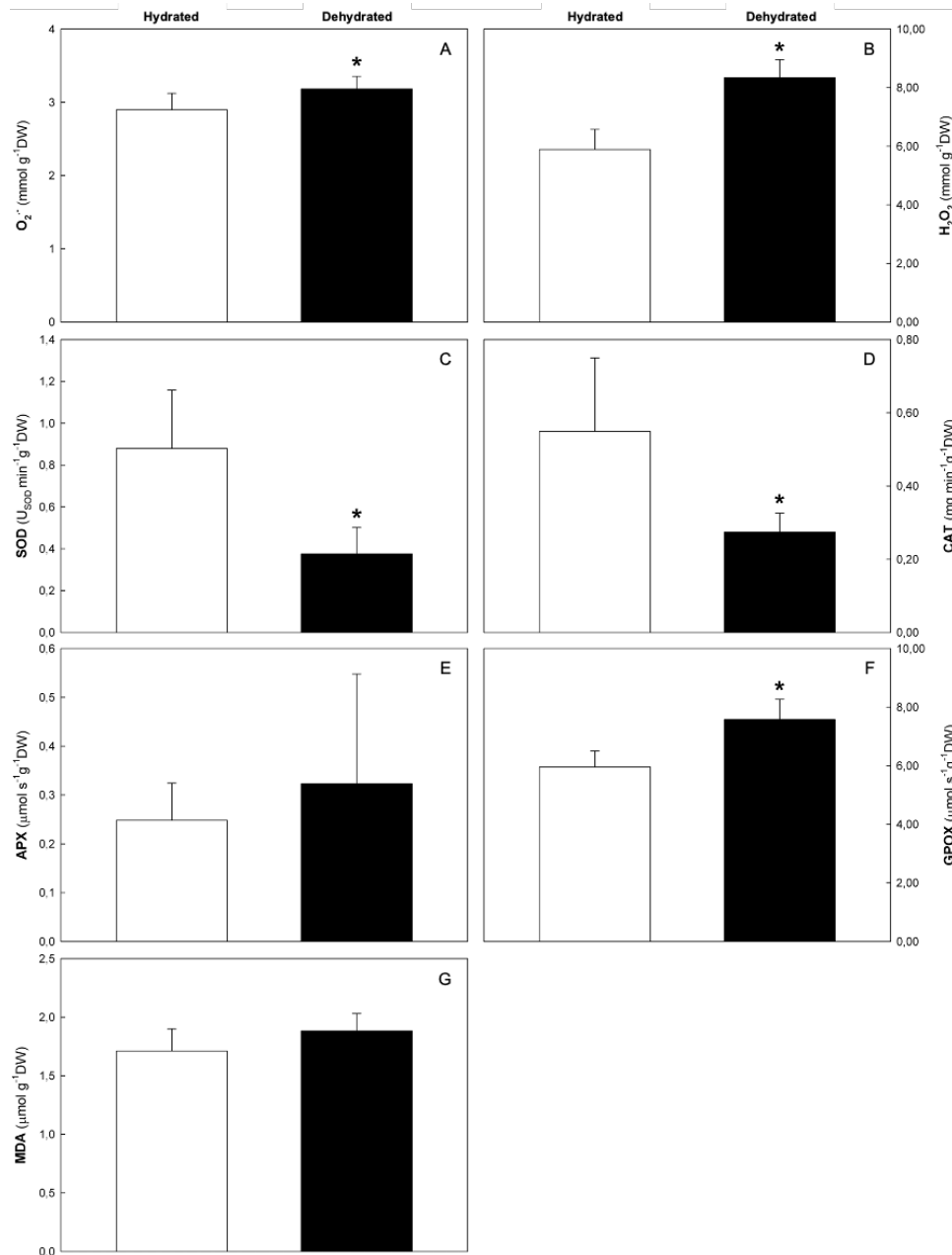


Figure 4.1.2 O_2^- (A), H_2O_2 (B), SOD (C), CAT (D), APX (E) and GPOX (F) activities, and MDA (G) contents in Cobrançosa' olive trees leaves from hydrated and dry areas. Values are mean $\pm$ standard deviation ($n = 9$). Asterisk (*) indicates significant differences ($p \leq 0.05$) between conditions.

Table 4.1.1 Phenolic profile of olive leaves from trees of the hydrated and dry areas.

Compound	RT (min)	M-H] ⁺ m/z	Main fragments	Hydrated leaves	Dehydrated leaves	Fold change
Flavonoids						
Apigenin-7-O-glucoside	9.8	431	385/223	1.860±0.063	2.693±0.116*	
Luteolin-7-O-glucuronide	11.0	461	415	2.147±0.111	1.296±0.046*	
Luteolin-7-O-glucoside	12.1	447	285	0.967±0.136	1.180±0.045*	
Apigenin-7-O-hexosyl rhamnosides	12.8	577	269	1.555±0.125	1.642±0.057	
Quercetin-3-O-rutinoside	13.0	609	301/300	6.233±0.163	6.341±0.040	
Luteolin-4-O-glucoside	13.4	447	285	3.121±0.333	6.222±0.301*	
Luteolin-4-O-glucoside (isomer)	13.9	447	285	1.041±0.065	1.349±0.055*	
Luteolin	15.7	285	285	1.825±0.107	0.939±0.099*	
Secoiridoids						
Oleoside-11-methyl ester	9.6	403	223/179	1.362±0.030	1.959±0.109*	
Oleuropein aglycone	10.3	377	197/153	0.348±0.018	1.329±0.066*	
Oleoside-11-methyl ester (isomer)	11.5	403	241/223	1.968±0.039	0.747±0.032*	
Oleuropein	14.4	539	377/307/275/345	1.049±0.089	1.304±0.060*	
6'-O-[8-hydroxy-2,6-dimethyl-2-octenoyloxy] secologanoside	15.3	557	371/227/185/389/165/183	0.878±0.053	0.681±0.084*	

Values are mean ± standard deviation ($n = 4$). Within the same line, the asterisk (*) indicates significant differences ($p \leq 0.05$) between conditions. Fold changes (Log_2 (dehydrated/hydrated leaves)) of metabolites in 'Cobrançosa' leaves.

Table 4.1.2 Lipophilic profile of olive leaves from trees of the hydrated and dry areas. Adapted from Araújo et al., 2021.

Compound	RT (min)	Hydrated	Dehydrated	Fold change		
		leaves (g kg ⁻¹ DW)	leaves (g kg ⁻¹ DW)	-0.46	0	0.89
Fatty acids						
Palmitic acid	39.3	1.808±0.076	1.570±0.121*			
Linoleic acid	43.0	1.412±0.008	1.214±0.006*			
α-Linolenic acid	43.2	2.200±0.229	1.597±0.064*			
Oleic acid	43.3	1.882±0.339	1.420±0.105*			
Stearic acid	43.8	1.552±0.098	1.386±0.205			
Terpenes						
Squalene	55.6	0.251±0.011	0.262±0.019			
β-Amyrin	68.8	0.276±0.021	0.286±0.030			
α-Amyrin	69.9	0.305±0.029	0.299±0.038			
Lupeol derivative	73.3	0.810±0.316	0.937±0.154			
Ursolic acid	73.9	0.357±0.084	0.450±0.104			
Long-chain alkanes						
Alkane 1	29.6	0.379±0.009	0.321±0.001*			
Alkane 2	57.7	0.651±0.052	0.647±0.049			
Alkane 3	60.2	0.414±0.012	0.380±0.007*			
Alkane 4	65.3	0.472±0.026	0.428±0.013*			
Alkane 5	67.9	0.961±0.131	0.909±0.072*			
Alkane 6	70.5	0.416±0.025	0.374±0.009*			
Carbohydrates						
D-Glucose	35.1	0.080±0.001	0.113±0.019*			
Sorbitol	35.9	0.230±0.002	0.427±0.097*			
Sterols						
β-Sitosterol	68.4	0.270±0.016	0.232±0.014*			

Values are mean ± standard deviation ($n = 4$). Within the same line, the asterisk (*) indicates significant differences ($p \leq 0.05$) between conditions. Fold changes (Log_2 (dehydrated/hydrated leaves)) of metabolites in Cobrançosa leaves.

DISCUSSION

The frequency of climate change events is increasing, and the year 2017 was particularly atypical in several regions of the Mediterranean, with scarce precipitation and prolonged heatwaves. From April to December 2017, Portugal was under severe/extreme drought conditions (Instituto Português do Mar e da Atmosfera, 2017), particularly the region of Trás-os-Montes and Alto Douro, one of the reference regions of olive production. In this region, olive groves are mainly under dryland farming conditions.

A general overview of the oxidative damages and antioxidant enzymes response, and lipophilic and phenolic profile adjustment occurred in trees from the dry area of the orchard compared to the ones from the hydrated area was presented in Figure 4.1.3.

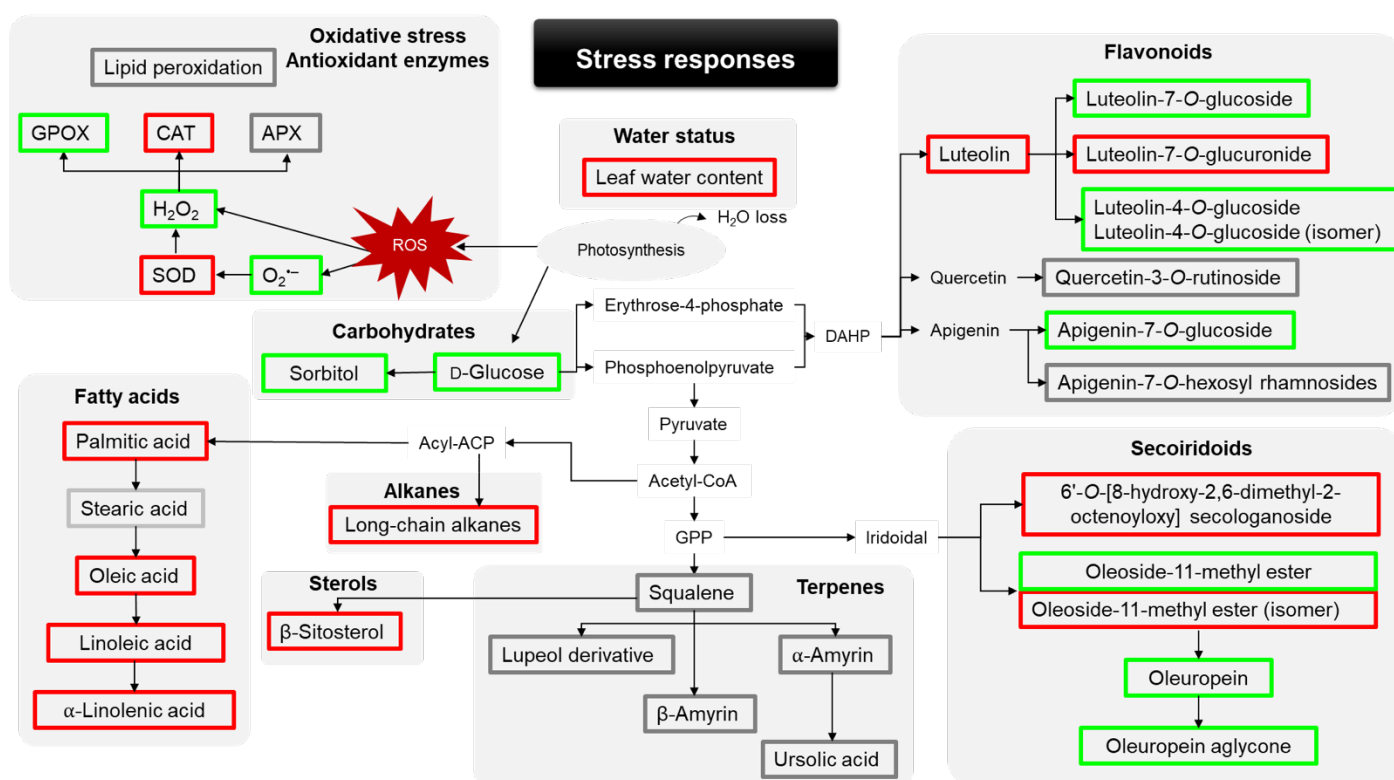


Figure 4.1.3 General overview of the responses of olive trees to field conditions. Parameters analyzed are present in boxes surrounded by a green, red, and grey square that indicates a significant increase, decrease, and no significant difference in the abiotic stress response, respectively. Between black arrows occurs multiple reactions. DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; ACP, acyl carrier protein; GPP, geranyl pyrophosphate.

Native in the Mediterranean ecosystems, olive trees have evolved characteristics of protection against water loss through tight control of the stomata aperture, a high-density foliar tissue, and thick cuticle and trichome layers (Bacelar et al., 2007a; Petridis et al., 2012a). However, our study reveals that the absence of precipitation in this region in 2017, coupled with high temperature, induced dehydration symptoms in olive trees, corroborated by the lower

leaf water content observed in dry area trees. Interestingly, our data showed that leaf water availability correlated positively with both long-chain alkanes (long-chain alkanes 1, 3, 5 and 6: $r = 0.78-0.96$, $p \leq 0.02$, Figure 4.1.4) and fatty acids (linoleic acid: $r = 0.994$, $p \leq 0.001$; palmitic acid: $r = 0.808$, $p = 0.015$; α -linolenic acid: $r = 0.879$, $p = 0.004$; Figure 4.1.), which are main components of the leaf wax cuticle that also confers a barrier to limit water loss (Bianchi et al., 1992; Mihailova et al., 2015; Pierantozzi et al., 2013). Thus, the stress conditions decreased the accumulation of some wax cuticle components, lowering water loss protection. Some studies in olive reported that drought tolerance implied an increase of the cuticle thickness (Brito et al., 2018) and the number of wax components like long-chain alkanes (Pierantozzi et al., 2013). In line with these findings, we suggest that the orchard's prolonged exposure to the extreme meteorological conditions of 2017 influenced Cobrançosa trees' responses, leading to a profile different from Brito et al. (2018). Furthermore, the decrease of long-chain alkanes and fatty acids in response to the stress conditions can also represent a metabolic shift to promote osmoprotectant agents (e.g., carbohydrates and polyols) or/ and antioxidants (e.g., secoiridoids and flavonoids, Figure 4.1.3).

The pool of two important osmoprotectant agents, sorbitol (an isomer of mannitol, the main polyol usually found in olive (Conde et al., 2011)) and D-glucose, increased under the stress condition, a typical response of olive trees to drought and heat stress (Araújo et al., 2019; Conde et al., 2011). However, the accumulation of these osmotic agents was not sufficient to maintain cell turgor in trees of the dry area (correlation between LWC vs D-glucose $r = -0.826$, $p = 0.012$; and LWC vs sorbitol $r = -0.861$, $p = 0.006$; Figure 4.1.4). Besides osmolytes, these compounds also represented a carbon and energy source available under stress conditions, and sorbitol seems to act as a defense compound against photo-oxidative damages (Conde et al., 2011; Dias et al., 2018).

The stress condition enhances some ROS levels ($O_2^{\bullet-}$ and the H_2O_2) in olive leaves. However, this increase did not induce lipid peroxidation (a key endpoint to cell damage), supporting that this species had a tolerance mechanism to cope with the imposed stress condition (Araújo et al., 2019; Dias et al., 2018, 2020a). Some lipophilic compounds' contribution to membrane protection in Cobrançosa trees exposed to stress also deserves further studies. For instance, despite being lower under stress, the ratio of unsaturated/saturated fatty acids remained similar (hydrated leaves: 1.6 and dehydrated leaves: 1.4), possible contribution to support the membrane integrity as also suggested by Dias et al. (2018).

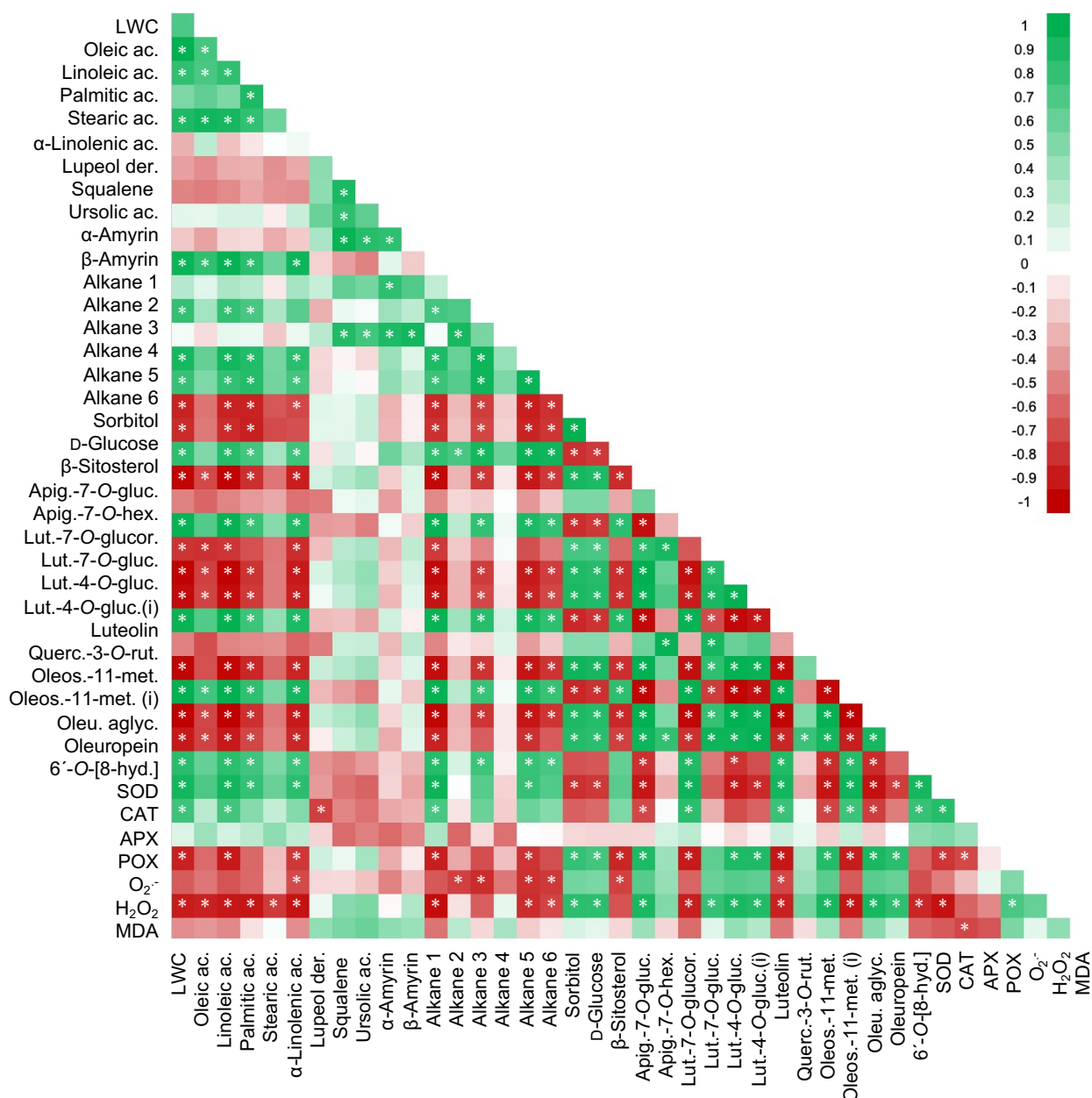


Figure 4.1.4 Color rectangles are correlation coefficients; green means a positive correlation coefficient, and red means a negative correlation coefficient. Significant differences ($p \leq 0.05$) are indicated by a white asterisk (*). Alkane, long-chain alkane; Lut.-7-O-gluc., luteolin-7-O-glucoside; Lut.-7-O-glucor., luteolin-7-O-glucuronide; Lut.-4-O-gluc., luteolin-4-O-glucoside; Lut.-4-O-gluc.(i), luteolin-4-O-glucoside (isomer); Querc.-3-O-rut., quercetin-3-O-rutinoside; Apig.-7-O-gluc., apigenin-7-O-glucoside; Apig.-7-O-hex., apigenin-7-O-hexosyl rhamnosides; 6'-O-[8-hyd.], 6'-O-[8-hydroxy-2,6-dimethyl-2-octenoyloxy] secologanoside; Oleos.-11-met., oleoside-11-methyl ester; Oleos.-11-met. (i), oleoside-11-methyl ester (isomer); Oleu. aglyc., oleuropein aglycone; ac., acid; Lupeol der., lupeol derivative.

The enzymatic and non-enzymatic (e.g., phenolic compounds) antioxidant systems also play an important role in maintaining oxidative damage levels under abiotic stresses (Dias et al., 2018, 2020a; Petridis et al., 2012a). The antioxidant enzymes are usually the first line of defense against ROS's excess (Mittler, 2017). In Cobrançosa trees, the lower stimulation

of APX and CAT activities under those stress field conditions suggested that H_2O_2 is used by GPOX that reduces its putative harmful effect. This is also corroborated by the strong positive correlation between GPOX and H_2O_2 ($r = 0.714$, $p = 0.047$, Figure 4.1.4). Contrarily to other works that studied olive plants under controlled environmental conditions and during a short-term stress experiment (Dias et al., 2020a; Koubouris et al., 2015), our data showed that SOD activity in Cobrançosa leaves was not stimulated at the moment of sampling (October 2017). Bacelar et al. (2007b) also reported an upregulation of the GPOX activity in olive trees (cv. Cobrançosa) growing under field conditions in response to water deficit, while the SOD activity decreased and the O_2^- accumulated in leaves resulting in oxidative membrane damages. In turn, Sofo et al. (2008, 2005) showed that in young potted olive plants from the cultivar Corantina, SOD, APX, CAT and GPOX activities increased as the severity of the water deficit increased. Data also show that additional sources of H_2O_2 may include the oxidation of fatty acids, glycolate, and the oxidation of xanthine/ hypoxanthine to uric acid (Koubouris et al., 2015; Saxena et al., 2016; Song et al., 2014). For instance, during the oxidation steps of α -linolenic acid, it occurs the generation of H_2O_2 and jasmonic acid (Mata-Perez et al., 2015), a key phytohormone and signaling molecule that regulates abiotic stress responses and modulates plant growth and development (Per et al., 2018). In Cobrançosa, fatty acids decreased in general, particularly the strong negative correlation between α -linolenic acid and H_2O_2 ($r = -0.922$, $p = 0.001$, Figure 4.1.) supports our hypothesis. Concerning the other ROS measured, the excess of O_2^- generated under stress conditions could be detoxified by other antioxidant compounds (Mittler, 2017) and/or via the enzyme SOD and then converted in H_2O_2 and used mostly by the enzyme GPOX, as described above.

Besides the referred antioxidant strategies, the role of flavonoids and secoiridoids as antioxidants in olive trees was also very representative (Dias et al., 2019a, 2020a). The leaf phenolic's profile of Cobrançosa leaves was very similar to other olive cultivars (Dias et al., 2020a; Ortega-García and Peragón, 2010). Within the flavonoids, quercetin-3-O-rutinoside, and some luteolin derivatives such as luteolin-4-O-glucoside and luteolin-7-O-glucuronide, and the secoiridoids oleoside-11-methyl ester and oleuropein were the most representative in Cobrançosa leaves and possible related to this species' high tolerance to stress (Dias et al., 2020a; Gonçalves et al., 2020). The flavonoids with the catechol B-ring structure, such as luteolin-7-O-glucuronide, were recognized as "effective antioxidants" and responded to the stress condition by reducing their levels. This response suggested using these flavonoids into neutralizing radicals and forming flavonoids radicals (Agati et al., 2011), or the reduction/inactivation of the enzymes that catalyse their synthesis (Grignon-Dubois and Rezzonico, 2012). The oxidative reactions resulting from the flavonoid radicals formed may conjugate with/or stimulate other antioxidant pathways such as the ASC/GSH cycle (Pereira

et al., 2013; Petrusa et al., 2013). Considering that flavonoids comprised a wide group of compounds with multiple antioxidant functions, they were distributed by different cells and cellular compartments (Petrusa et al., 2013). For instance, Agati et al. (2013) proposed a mechanism of flavonoids transport from the place of their biosynthesis to several cellular centers where ROS were generated, and/or even the transport of H_2O_2 by flavonoids to sites where they were neutralized (e.g., by a system consisting of GPOX/flavonoids/ASC). In olive leaves, the strong correlations between H_2O_2 vs GPOX ($r = 0.714$, $p = 0.047$, Figure 4.1.4) and H_2O_2 vs luteolin-7-O-glucuronide ($r = -0.877$, $p = 0.004$; Figure 4.1.4) can also support these findings. Additionally, other flavonoids also changed in Cobrançosa leaves in response to the stress condition. For instance, luteolin pool decrease was correlated to the increase of luteolin-4-O-glucoside ($r = -0.962$, $p \leq 0.001$, Figure 4.1.4) and its isomer ($r = -0.913$, $p = 0.002$, Figure 4.1.4), and luteolin-7-O-glucoside ($r = -0.708$, $p = 0.049$, Figure 4.1.4), suggesting that luteolin was used to the synthesis of these derivatives. The accumulation of these luteolin derivatives and the flavonoid apigenin-7-O-glucoside possibly represent an improved availability of defense/protective compounds under stress.

Besides flavonoids, one of the main secoiridoids, oleuropein, also had an important antioxidant role, the catechol group in the A-ring had the capacity to scavenging free radicals (Hassen et al., 2015; Kruk et al., 2005). Similar to the described for other olive cultivars exposed to abiotic stress (Petridis et al., 2012a, 2012b) oleuropein accumulation was related in some way to antioxidant availability protection. Other compounds biosynthetically related to oleuropein were also responsive to the stress conditions. For instance, the increase of oleoside-11-methyl ester pool may result from the oleuropein degradation ($r = 0.924$, $p = 0.001$, Figure 4.1.4) by esterases (Gutierrez-Rosales et al., 2010; Tsimidou, 2013). In addition, the decrease of oleoside-11-methyl ester isomer may represent that it is being used to produce more oleuropein, supporting the increased levels of oleuropein (oleuropein vs. oleoside-11-methyl ester isomer $r = -0.876$, $p = 0.004$, Figure 4.1.4). Other secoiridoids, such as 6'-O-[8-hydroxy-2,6-dimethyl-2-octenoyloxy] secologanoside decreased in Cobrançosa leaves under stress, but its role in plants remains unclear, putatively regulating stress response (Karioti et al., 2006).

Our data was in line with other *O. europaea* studies, where the antioxidant pattern of response to abiotic stressors was based on the accumulation of lipophilic protective compounds, activation of antioxidant enzymes, and increase of phenolic compounds (Dias et al., 2020a, 2020b, 2019a, 2018). However, it should be considered that the involvement and contribution of specific antioxidant metabolites to plant stress response seems to vary according to the type of abiotic stress and cultivar. For instance, in Galega plants exposed to high UV-B, oleuropein (and its derivatives) play a more critical role than flavonoids and

hydroxycinnamic acid derivatives by acting as signaling molecules and ROS scavengers (Dias et al., 2020a). However, these plants also showed an essential contribution of the enzymes SOD, CAT, GPOX, and APX to maintain membrane integrity (Dias et al., 2020a).

CONCLUSIONS

The environmental conditions of the summer of 2017 induced water deficit in olive trees of the dry area of the orchard. This lower leaf water availability was associated with the loss of long-chain alkanes and fatty acids levels, which were the main components of wax cuticles that protect from water loss. Moreover, the dry area trees showed high ROS levels (H_2O_2 and $O_2^{\cdot-}$), but the olive antioxidant system was effective in controlling oxidative damages, preventing the augment of lipid peroxidation. This olive antioxidant response was mainly due to the upregulation of GPOX to reduce the H_2O_2 harmful effect with the complement of some flavonoids (luteolin-7-O-glucuronide), also acting as ROS scavengers. The dynamic response of some secoiridoids (e.g., 6'-O-[8-hydroxy-2,6-dimethyl-2-octenoyloxy] secologanoside and oleoside-11-methyl ester isomer decrease, and oleuropein and its aglycon increase) in trees of the dry area remains unclear, possible regulating stress response. This study demonstrated the leaf metabolomic adjustment in olive trees for the first time under a real scenario of field drought conditions. Like to the reported in controlled environment experiments and extreme weather conditions, olive remarkable physiological plasticity was highlighted here. The high ability of physiological and metabolite adjustment under field stress will provide the necessary capacity to overcome and survive the extreme climatic conditions expected in the Mediterranean region in the following decades. The impacts of these weather conditions on fruits and oil quality are also a matter of concern and are under study.

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4.2. Metabolites' profile of fruit and oil of Cobrançosa olive trees under field stress conditions⁵

ABSTRACT

The Mediterranean basin is the world leading region for olive production, but it is highly prone to extreme episodes associated with the ongoing climate change. How these climate events affect olivegroves and their products' quality remains unknown. We aim to study the metabolomic and sensorial effects of drought summer on olive fruit and oil quality. Fruits from a dry and hydrated olive groves were harvested in autumn and fruits' water content, oil yield and quality, and fruit and oil metabolite profiles were analysed. Fruits from the dry area presented low water availability, reduced levels of palmitic, palmitelaidic and linoleic acids, and increased stearic acid, carbohydrates, β -sitosterol, α -tocopherol, oleuropein, oleoside, and elenolic acid glucoside. Oil yield was lower in the dry area, but the levels of unsaturated fatty acids, β -sitosterol, oleacein and some sensory sensations increased. Climate events can reduce oil yield but stimulate the accumulation of bioactive compounds that improve oil quality.

KEYWORDS

Climate change, flavonoids, secoiridoids, *Olea europaea*, drought, sensorial quality, olive metabolome

⁵ Araújo, M., Rodrigues, N., Santos, C., Pinto, D. C. G. A., Pereira, J.A. Silva, A. M. S., Dias, M. C. (2021) Metabolites' profile of fruit and oil of Cobrançosa olive trees under field stress conditions. *Journal of the Science of Food and Agriculture* (Submitted, Ref No.: JSFA-22-1030)

INTRODUCTION

Olea europaea L. is a pivotal crop in the Mediterranean basin, the leading world region in olive production. Since 2001 the area occupied by olives orchards in the Mediterranean increased $\approx 25\%$, reaching in 2019 around 10.5 Mha (FAOSTAT, n.d.), which demonstrates a demand for olives/olive oil by consumers. However, the Mediterranean basin and its agriculture are particularly threatened by climate change, with extreme combined episodes (IPCC, 2019). For instance, in the summer of 2017, the Mediterranean region suffered prolonged drought and heatwaves (Haworth et al., 2018), and that year is considered one of the warmest years on record for Europe (“Climate bulletins | Copernicus,” n.d.). These weather extreme events are predicted to be more frequent and intense in the following decades (IPCC, 2019), representing losses in productivity of traditional crops and chain of value, including in olive orchards (Fraga et al., 2020).

Besides being used in the Mediterranean diet, olive products (fruits and oil) show enormous benefits for human health and cosmetics, which are well documented. Olive oil and fruits are rich in fatty acids and phenolic compounds, carbohydrates, sterols, terpenes, proteins, minerals, and vitamins (Dias et al., 2018, 2019, 2020; Guo et al., 2018). The levels of these compounds have a significant influence on the organoleptic properties of olive oil and fruits. The healthy monounsaturated fatty acid (MUFA), oleic acid, and the polyunsaturated fatty acid (PUFA), linoleic acid, are present in high amounts in both fruit and oil (Guo et al., 2018). Other important fatty acids, such as saturated fatty acids (SFA), palmitic and stearic acids, are present in high amounts in these olive products (Guo et al., 2018). Also, olive fruits and oil have several phenolic compounds (e.g., secoiridoids and flavonoids) known to have benefits for human health (Guo et al., 2018), like in preventing cancer and cardiovascular diseases (Guo et al., 2018; Valente et al., 2020). Besides that, phenolic compounds help prolong the olive fruits' shelf life (Thielmann et al., 2017) due to their known antioxidant activities and organoleptic characteristics in olive fruits and oil (Velázquez-Palmero et al., 2017). Within the secoiridoids, the oleuropein, which can scavenge reactive oxygen species, is one of the most abundant phenolic compounds found in all olive plant organs, particularly in response to stress (Mechri et al., 2019). Regarding flavonoids, luteolin has an essential role as this compound has a catechol group on its B-ring, which can detoxify H_2O_2 and H_2O_2 -generated hydroxyl radicals (Dias et al., 2021). Olive fruits also contain sugars (e.g., glucose) and polyols (e.g., mannitol) that provide energy for metabolic adjustments and are precursors for the biosynthesis of olive oil (Marsilio et al., 2001). Several factors might influence the quantity of phenolic compounds present in olive oil, for instance, the olive cultivar, fruit maturation, environmental factors (e.g., drought, heatwaves, UV), climate, altitude, soil type, and cultivation practice (López-Huertas et al., 2021). Additionally, the crushing of olive fruit

and malaxation process for oil extraction can have a considerable impact on the quality of olive oil due to the release of phenolic compounds and the formation of volatile compounds (Servili et al., 2015). During these processes, several biochemical reactions occur involving several enzymes (e.g., β -glucosidase, lipoxygenase) (Servili et al., 2015; Velázquez-Palmero et al., 2017) that act on the phenolic compounds (β -glucosidase) and fatty acids (lipoxygenase). For instance, lipoxygenase catalyses the oxygenation of the PUFAs (e.g., linoleic acid) into volatile molecules that influence the oil flavour and aroma. At the same time by the action of β -glucosidase, oleuropein is hydrolysed into oleuropein aglycone and this into elenolic acid and hydroxytyrosol (Thielmann et al., 2017), which products influence the bitter and pungent taste of olive oil (Velázquez-Palmero et al., 2017).

Although the olive tree has developed morpho-physiological adaptative mechanisms to cope with the Mediterranean climate, it remains unclear how the extreme weather events influence olive fruit and oil quality. In particular, despite its phenotypic plasticity under drought or heat conditions (Mousavi et al., 2019), the impact of these abiotic stressors on the chemical profile of fruits and consequently on the olive oil chemical and sensorial quality remains poorly understood. For example, water deficit seems to increase oleuropein (Mechri et al., 2019) and tyrosol levels in fruits (Mechri et al., 2019), and high temperature can induce fatty acid desaturases, which might change the profile of olives fatty acids (Mousavi et al., 2019). However, water deficit did not affect the olive oil free acidity, peroxide value, and UV absorbances (Caruso et al., 2014). Unveiling the cascade of reactions that these abiotic factors induce on the olive trees' performance and fruit quality will help to predict the future of oliviculture in the future climate change scenarios.

To our knowledge, this is the first study in a real scenario that evaluates the effects of water deficit in the lipophilic and phenolic profiles of fruits and oil, as well as in the oil quality in adult olive trees of one of the most important Portuguese cultivars, 'Cobrançosa'. We hypothesise that climate conditions, particularly water deficit, induce an adjustment of important lipophilic and phenolic compounds that alter olives and olive oil nutritional profiles and increase oil quality. Our previous study conducted in the summer of 2017 in an olive grove (cv 'Cobrançosa') with trees growing under well-watered (hydrated area) and water-deficit conditions (dryland area) demonstrated that drought stress drastically modulated the stress metabolism (Araújo et al., 2021). Here, we aim to focus on the effects of this drought stress period on the olive fruit and oil quality. For that, fruits from trees of both areas, hydrated and dry, were collected, and fruits' water content, oil yield, fruit, and oil lipophilic and phenolic profiles, as well as oil quality parameters and oxidative status, were evaluated.

MATERIAL AND METHODS

Study site and plant material conditions of harvest

The selected olive grove for this study consists of olive trees about 30 years from the cultivar 'Cobrançosa', a highly representative cultivar in Portugal. The grove is located in Suções-Mirandela (41°30'39.5"N 7°15'28.1"W, northeast of Portugal) and is managed as dryland farming, as is this region's standard olive crop management. The climate is characterised as dry and hot summers and rainy winters (Peel et al., 2007). In 2017, the Mediterranean area was affected by extreme heatwaves, and Portugal was under severe/extreme drought between April and December (Instituto Português do Mar e da Atmosfera, 2017). From June to the end of September, the monthly average precipitation reached values below 30 mm [typical of a dry summer according to Köppen climate classification (Peel et al., 2007)] and the monthly air temperature reached values were above 30 °C (Araújo et al., 2021). The selected grove was monitored from June to the end of September. During this period of time, two adjacent distinct areas were selected: one without water supply had olive trees with visible dehydration symptoms in leaves and fruits (dehydrated trees, dry area); the other area had a groundwater supply (hydrated area) and the olive trees had leaves and fruits without signs of dehydration. During the first week of October, olive fruits with perfect sanitary conditions from five different olive trees were collected from the two study areas. Olive fruit water content was determined, and the fruits were used for metabolites analysis (lipophilic and phenolic profiles). Olive fruits were also used to extract olive oil and evaluate oil quality parameters, oxidative status, and metabolites analysis (lipophilic and phenolic profiles).

Fruit water content (FWC)

Olive fruit's fresh weight was determined, and then fruits were dried at 40 °C until constant weight. The fruit water content was calculated and expressed as a percentage (%).

Olive oil

Olive fruits processing for oil extraction

Olive fruits were processed in the first 24 h after handpicking in a pilot extraction plant with an Abencor system (Comercial Abengoa S.A., Sevilla, Spain). This system consists of three units: a mill, a thermobeater, and a pulp centrifuge. Olive milled from each plant (5 plants per condition) were weighted to about 1.2 kg, and 40 g of talc was added. After weighing, the olive milled was put in the thermobeater for 30 min at 25 °C, where malaxation took place, and after this, the pulp was centrifuged. The olive oil was separated by decantation, and the

amount of oil was evaluated. The oil was dehydrated with anhydrous sodium sulphate, filtered through Whatman no.4 paper, and stored in 100 mL dark bottles.

Chemical and sensorial quality parameters determination

Free acidity (FA, in % of oleic acid), peroxide value (PV, in mequiv O₂ kg⁻¹), coefficients of extinction at 232 nm (K₂₃₂) and 270nm (K₂₇₀), and the respective ΔK values were analysed following the European Union standard methods [Commission Regulation (EEC) No 2568/91 of 11 July 1991 Annexes II and IX].

Six months after oil extraction, olive oil samples were subjected to organoleptic analysis following the methods and standards adopted by the International Olive Council (IOC) for sensory analysis of olive oils, namely COI/T.20/Doc. no. 15/Rev. 6 and COI/T.30/Doc. no. 17. Each sample (5 samples per condition) was subjected to the judgment of a trained panel who classified the olfactory sensations, gustatory–retronasal sensations, and final olfactory–gustatory sensations using a modified test sheet following the recommendations of IOC with a scale from 0 (no perceived sensation) to 10 (highest perceived sensation). For olfactory sensations, the following attributes were evaluated: olive fruitiness, apple, banana, tomato, dry fruits, fresh grass, cabbage, tomato leaves, and harmony. Concerning gustatory–retronasal sensations were evaluated the olive fruitiness, sweetness, bitterness, pungent, apple, banana, tomato, dry fruits, fresh grass, cabbage, tomato leaves, and harmony. A final olfactory–gustatory sensation for each sample was also considered using the same scale for complexity and persistency. Complexity increases with the number and intensity of the perceived sensations. Persistency is related to the sensation' persistence of the on the mouth along the time.

Oxidative stability

Oxidative stability (OS) was measured (Rodrigues et al., 2020) using the 743 Rancimat (Metrohm CH, Switzerland). Olive oil (3 g) was heated at 120 ±1.6 °C and, filtered, cleaned, and dried air was incorporated at a rate of 20 L h⁻¹. The resulting volatiles were collected in water, and the consequent increase of water conductivity was continuously measured. The required time to reach the conductivity inflection was recorded and was assumed as the OS value.

Radical scavenging activity – DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The antioxidant activity of olive oil was analysed by measuring their capacity of scavenging DPPH[•], according to Rodrigues et al. (2020) (Rodrigues et al., 2020). Briefly, 1 mL of olive oil in ethyl acetate (10%, W/V) was mixed with 4 mL of 0.1 mM DPPH[•] (prepared in ethyl acetate). After vigorously shaken for 10 s, the mixture was kept in the dark for 30 min, and the absorbance was read at 515 nm (Thermo Fisher Scientific, GENESYS™ 10 UV-vis, USA) against a blank solution.

Total phenols content

The total phenols content of olive oil was extracted according to Rodrigues et al. (2020) (Rodrigues et al., 2020). Olive oil (2.5 g) was diluted with 2.5 mL of *n*-hexane (1:1; v/v). To the mixture, 2.5 mL of methanol/water (80:20; v/v) was added, and the mixture was centrifuged for 5 min at 5000 g. Two phases formed, and the methanolic phase was extracted. The same procedure was repeated three times. To the methanolic extract (1 mL) was added Folin-Ciocalteu reagent (1 mL), 7.5% Na₂CO₃, and ultrapure water (7 mL). The homogenised mixture was stored overnight in the dark. The absorbance was read at 765 nm, and the concentration of phenols was calculated using a calibration curve with caffeic acid in methanol.

Metabolites extract preparation and analysis

Extracts preparation

Olive fruits were destoned and grounded in a small olive mill. Each sample's approximately 50 g (fresh weight) was mixed with 500 mL of *n*-hexane with stirring for 72 h at room temperature. The *n*-hexane was renewed, and an additional extraction in the same conditions was made. The solvent was evaporated to dryness using a rotary vacuum evaporator, and the extracts were used for the gas chromatography-mass spectrometry (GC-MS) analysis. To extract phenolic compounds, 500 mL of methanol was added to the olives dry pellet resulting from the hexane extraction. Two cycles of 72 h each at room temperature were made. Methanol was evaporated to dryness using a rotary vacuum evaporator, and the extracts were used for ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) analysis.

The olive oil obtained was also prepared before chromatography analysis. For GC-MS analysis, 20 mg of olive oil was homogenised with 200 µL of dichloromethane. For phenolic analysis, 5 mg of olive oil was mixed with 12 mL of *n*-hexane and then 5 mL of methanol/water (80:20; v/v), after which the mixture was shaken for 10 min, and centrifuged at 5000 rpm for

20 min at 4 °C to separate the two phases. After this, the methanolic phase was recovered, and the same procedure was repeated three times. The solvent was evaporated to dryness using a rotary vacuum evaporator and the extract was used for UHPLC-MS analysis (Negro et al., 2019).

Gas chromatography-mass spectrometry and ultra-high-performance liquid chromatography-mass spectrometry analysis

For GC-MS analysis, 200 µL of the extracts (fruits 40 mg mL⁻¹; oil 20 mg mL⁻¹), 200 µL of internal standard (tetracosane, 0.46 mg mL⁻¹), 50 µL of dichloromethane, 250 µL of pyridine, 250 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide and 50 µL of trimethylsilyl chloride were mixed in a glass tube and incubated at 70 °C for 30 min. The silylated extracts were then injected in a GC-MS QP2010 Ultra Shimadzu equipped with a FactorFour Capillary Column (VF-5ms 30 m x 0.25 mm ID DF=0.25) and the chromatography conditions were performed as described by Araújo et al. (2021). At least four independent analyses per condition were performed.

For the UHPLC-MS analysis, extracts from fruits (40 mg mL⁻¹) and oil (30 mg mL⁻¹) were resuspended in pure methanol, filtered through a 0.2 µm membrane (Whatman) and injected in an UHPLC-MS [Thermo Scientific Ultimate 3000RSLC (Dionex)] equipped with a Dionex UltiMate 3000 RS diode array detector coupled to a mass spectrometer. The chromatography conditions were performed as described by Araújo et al. (2021). The chromatograms obtained were analysed at 280, 240 and 230 nm. The phenolic compounds of the extracts were identified based on UV–Vis spectra and MSⁿ spectra data with those of closest available reference standards and data reported in the literature. The semi-quantification of the individual compounds in the plant extracts was performed by peak integration at 240 nm, through the external standard method. At least four independent analyses per condition were performed.

A semi-quantitative analysis was performed. The area of each peak was determined in all chromatograms obtained from the gas and liquid chromatography analysis. The peak areas sum was considered 100%.

Statistical analysis

The student's t-test was used to analyse the statistical significance between groups (hydrated and dehydrated) at a significant level of 0.05. When the variance failed, a non-parameter test (Mann-Whitney) was performed. Pearson's correlations for fruit parameters

and oil parameters were analysed. All statistical analysis were performed using SigmaPlot for Windows version 3.1 (Systat Software, USA).

RESULTS

Olive fruit

Water content

The fruits from the dry area had significantly less water content than those from the hydrated area, representing $34.5 \pm 0.5\%$ and $48.7 \pm 3.8\%$, respectively.

GC-MS profile

Fruits from trees in the dry area presented higher content of carbohydrates, namely α -D-mannopyranose, D-mannitol, and D-glucose ($p \leq 0.05$, Figure 4.2.1 A). In olive fruits, fatty acids are represented by palmitelaidic, palmitic, linoleic, oleic, palmitoleic, and stearic acids (Figure 4.2.1 B). Stearic acid was the only fatty acid that presented higher amounts ($p \leq 0.05$) on olive fruits from the dry area. Oleic and palmitoleic acids showed similar values ($p > 0.05$) in both conditions. Palmitelaidic, linoleic and palmitic acids were lower ($p \leq 0.05$) in dry area olive fruits. Squalene (Figure 4.2.1 C) showed no differences ($p > 0.05$) between conditions, while β -sitosterol (Figure 4.2.1 D) and α -tocopherol (Figure 4.2.1 E) increased ($p \leq 0.05$) in olive fruits from the dry area.

UHPLC-MS profile

Only secoiridoids were identified in olive fruit in 'Cobrançosa' trees from hydrated and dry areas. Secoiridoids are represented by desoxyelenolic acid, 1- β -D-glucopyranosyl acyclodihydroelenolic acid (GPADHEA), oleoside, elenolic acid glucoside, elenolic acid (EA), and oleuropein (Table 4.2.1). Only GPADHEA, EA, and oleuropein showed differences between conditions ($p \leq 0.05$). While GPADHEA and EA reduced their content in fruits from the dry area, oleuropein increased its content in the same condition.

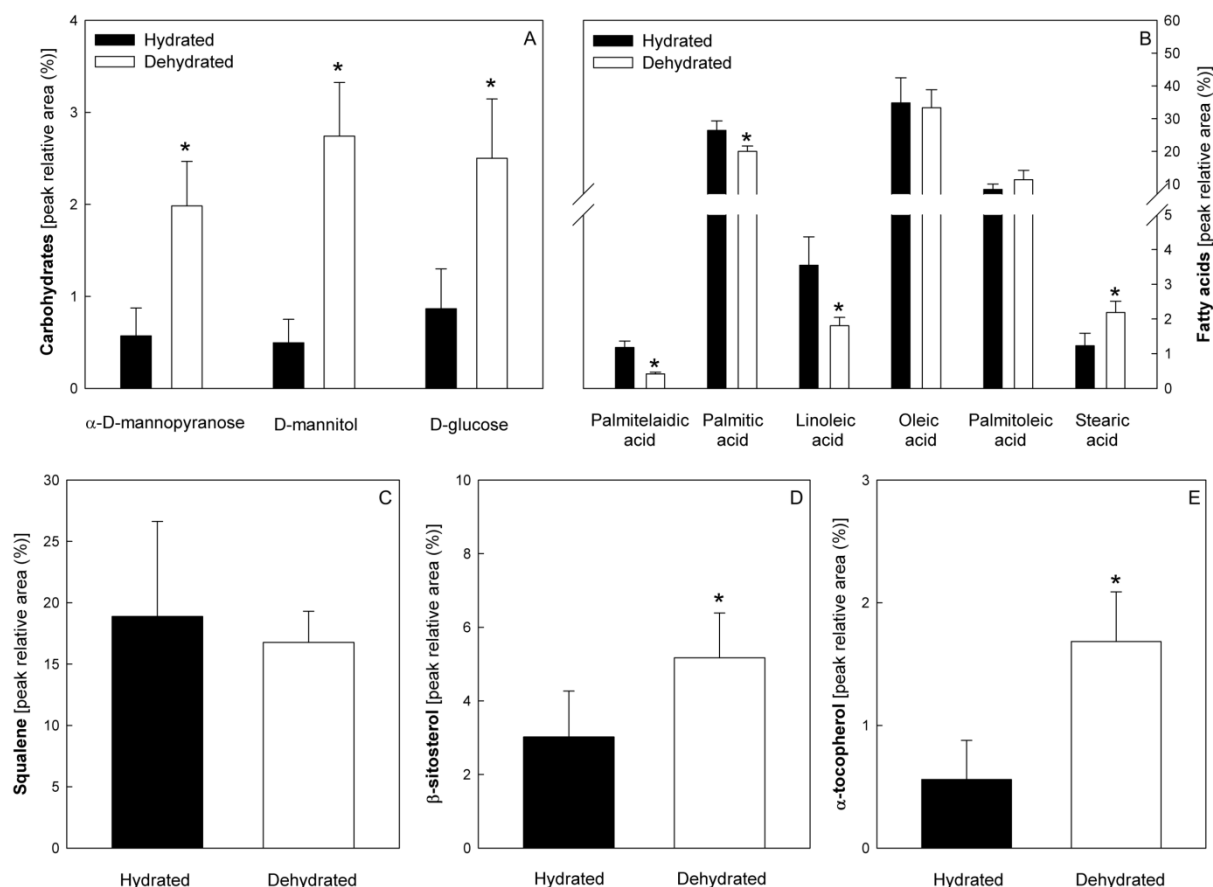


Figure 4.2.1 Olive fruit lipophilic profile of 'Cobrançosa' trees from the hydrated and dry areas. Carbohydrates (A), fatty acids (B), squalene (C), β-sitosterol (D) and α-tocopherol (E). Values are mean ± standard deviation ($n = 4$). Asterisk (*) indicates significant differences ($p \leq 0.05$) between conditions.

Table 4.2.1 Olive fruit phenolic profile of 'Cobrançosa' trees from the hydrated and dry areas.

RT (min)	Compound	<i>m/z</i>	<i>MS</i> ²	Hydrated area [peak relative area (%)]	Dry area [peak relative area (%)]
1.80	desoxyelenolic acid	225	179	11.73 ± 3.79	19.33 ± 10.70
5.80	1-β-D-glucopyranosyl acyclodihydroelenolic acid	407	389/375/357/313	60.67 ± 2.72	30.44 ± 4.98*
8.64	oleoside	389	345/209/121	1.38 ± 0.12	17.73 ± 2.93*
9.55	elenolic acid glucoside	403	371/223/179	0.96 ± 0.11	20.24 ± 1.97*
13.48	elenolic acid	241	139/127/95/209/101/165/223	23.48 ± 2.78	3.29 ± 2.19*
14.49	oleuropein	539	377/307/275/509/507	1.77 ± 0.32	8.48 ± 2.83*

Values are mean ± standard deviation ($n = 3$). Asterisk (*) indicates significant differences ($p \leq 0.05$) between conditions. *m/z* is presented in negative mode [M-H].

Olive oil

Yield

The olive oil yield extracted from olive plants in the dry area was significantly lower ($42 \pm 32 \text{ mL kg}^{-1}$ of fruits) than in the hydrated area ($190 \pm 13 \text{ mL kg}^{-1}$ of fruits).

Quality parameters

Free acidity, peroxide value, K_{232} , total phenols, and oxidative stability showed no significant differences ($p > 0.05$) between oils from trees in hydrated and dry areas (Figure 4.2.2 A). The coefficient K_{270} and ΔK increased ($p \leq 0.05$) in olive oil from the dry area (Figure 4.2.2 A). Also, in the olive oil from this condition, the radical scavenging activity was decreased ($p \leq 0.05$, Figure 4.2.2 A). The trained panel perceived 9 olfactory sensations and 12 gustatory-retronasal sensations (Figure 4.2.2 B). Olive oils from the dry area showed higher ($p \leq 0.05$) olfactory sensations scores of “apple”, “banana” and “dry fruits” in relation to oils from the hydrated area. Other olfactory sensations like “fruitiness”, “tomato”, “tomato leaves” and “green” were less perceived ($p \leq 0.05$) in the dry area oils. The olfactory sensation “cabbage” was not found in oils from the dry area plants. Harmony was similar ($p > 0.05$) in oils from both conditions. As in olfactory sensations, attributes like “apple”, “banana” and “dry fruits” as well as “sweet” in gustatory-retronasal sensations showed higher values ($p \leq 0.05$) in olive oils extracted from the dry area plants. These olive oils also showed lower ($p \leq 0.05$) gustatory-retronasal sensations on the fruitiness, bitter, pungent, green tomato, and tomato leaves compared to olive oils from the hydrated area plants. Also, oils from the dry area showed no gustatory-retronasal sensation of cabbage ($p \leq 0.05$). Harmony was similar ($p > 0.05$) in oils from both conditions. Concerning the olfactory-gustatory sensations, oils showed significant differences ($p \leq 0.05$) in complexity and persistence. The hydrated area oils were evaluated as 7.46 ± 0.35 , while dry area oils were 6.56 ± 0.44 in complexity. Regarding persistency, hydrated area oils were evaluated as 8.28 ± 0.42 while those from the dry area were 7.84 ± 0.37 .

GC-MS profile

Fatty acids are represented by palmitic acid, *cis*-vaccenic, linoleic, oleic, and stearic acids in samples of both conditions. In both conditions every fatty acid, except linoleic and oleic acids, were similar ($p > 0.05$). Both linoleic and oleic acids increased ($p \leq 0.05$) their content in oils from the dry area (Figure 4.2.3 A). The content in monopalmitin was similar in both conditions ($p > 0.05$, Figure 4.2.3 B). While α -tocopherol (Figure 4.2.3 E) was only found in oils from the hydrated area. Squalene reduced ($p \leq 0.05$, Figure 4.2.3 C) its content in dry

area oils while β -sitosterol increased ($p \leq 0.05$, Figure 4.2.3 D) its content in the same condition.

UHPLC-MS profile

Oils from both conditions (hydrated and dry areas) presented simple phenolics, flavonoids, and secoiridoids (Table 4.2.2). Tyrosol and hydroxytyrosol represented the simple phenolics. Hydroxylated product of the decarboxymethyl elenolic acid, elenolic acid, 10-hydroxy-decarboxymethyl oleuropein aglycon, oleacein, hydroxy-oleuropein aglycon, and oleuropein aglycon represented the secoiridoids. Luteolin represented the flavonoids. Both oleacein and hydroxylated product of the decarboxymethyl elenolic acid increased ($p \leq 0.05$) on oils from the dry area. Luteolin and oleuropein aglycon reduced ($p \leq 0.05$) their content in oils from the dry area. The other phenolics were similar ($p > 0.05$) between conditions.

Parameter	Hydrated area	Dry area	A
Free acidity (%)	0.29 ± 0.02	0.27 ± 0.02	
Peroxide value (mequiv O ₂ kg ⁻¹)	6.81 ± 0.69	6.65 ± 0.83	
K ₂₃₂	1.85 ± 0.18	1.68 ± 0.16	
K ₂₇₀	0.17 ± 0.03	$0.21 \pm 0.01^*$	
ΔK	-0.005 ± 0.00	$-0.016 \pm 0.00^*$	
DPPH [•] (%)	82.99 ± 0.70	$79.79 \pm 0.78^*$	
Total phenols (mg CAE kg ⁻¹)	320.84 ± 34.45	352.75 ± 45.47	
Oxidative stability (h)	17.93 ± 3.82	14.36 ± 1.98	

Values are mean $\pm$ standard deviation ($n = 5$). Asterisk (*) indicates significant differences ($p \leq 0.05$) between conditions.

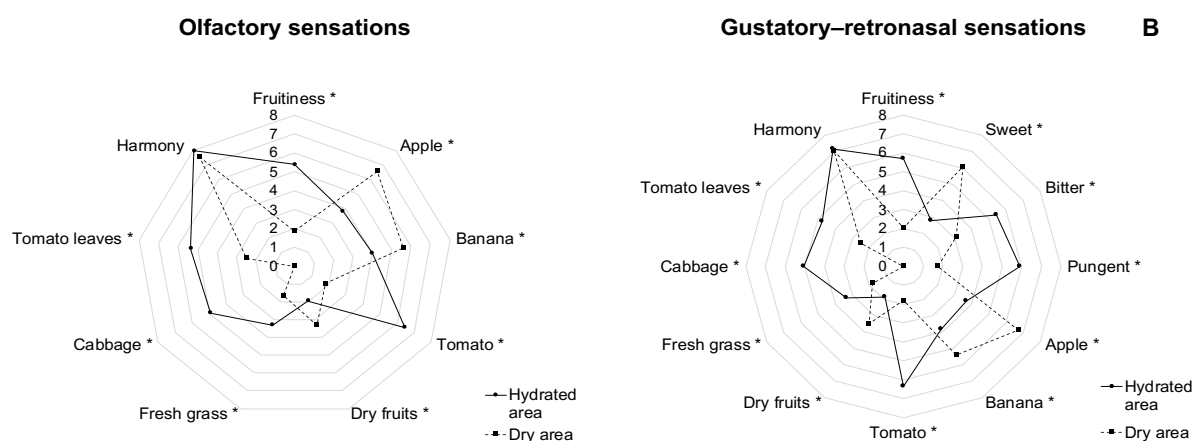


Figure 4.2.2 Quality parameters, antioxidant activity, total phenols and oxidative stability (A) and sensory analysis (B) of 'Cobrançosa' olive oils extracted from trees in the hydrated and dry areas. Values are mean $\pm$ standard deviation. Asterisk (*) indicates significant differences ($p \leq 0.05$) between conditions.

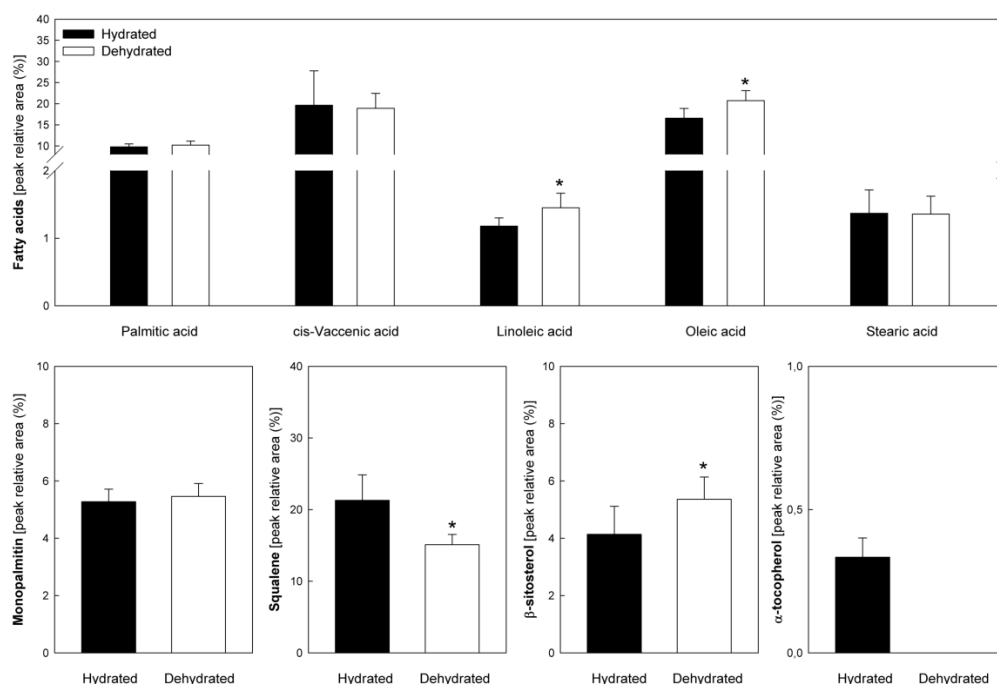


Figure 4.2.3 Olive oil lipophilic profile from 'Cobrançosa' olive trees in the hydrated and dry areas. Fatty acids (A), monopalmitin (B), squalene (C), β-sitosterol (D) and α-tocopherol (E). Values are mean ± standard deviation ($n = 5$). Asterisk (*) indicates significant differences ($p \leq 0.05$) between conditions.

Table 4.2.2 Olive oil phenolic profile from 'Cobrançosa' olive trees in the hydrated and dry areas.

R_t (min)	Compound	m/z	MS^2	Hydrated [peak relative area (%)]	Dehydrated [peak relative area (%)]
1.5	Tyrosol	137	137/119	14.58 ± 0.94	17.90 ± 3.57
4.4	Hydroxytyrosol	153	123/153	4.51 ± 1.66	2.65 ± 1.23
6.3	Hydroxylated product of the decarboxymethyl elenolic acid	199	181/155/111	0.40 ± 0.13	$1.08 \pm 0.18^*$
8.6	Elenolic acid	241	139/127/95/ 101/165/209	6.30 ± 1.38	7.75 ± 4.55
14.4	10-hydroxydecarboxymethyl oleuropein aglycon	335	199/181/155	16.83 ± 1.84	15.00 ± 2.08
15.8	Luteolin	285	285/151	2.79 ± 0.16	$1.32 \pm 0.46^*$
16.2	Oleacein	319	181/199/137 /155/111	12.12 ± 2.65	$27.99 \pm 8.49^*$
17.2	Hydroxyoleuropein aglycon	393		12.69 ± 1.63	11.35 ± 2.48
18.8	Oleuropein aglycon	377	307/275	30.48 ± 2.37	$15.22 \pm 4.75^*$

Values are mean ± standard deviation ($n = 4$). Asterisk (*) indicates significant differences ($p \leq 0.05$) between conditions. m/z is presented in negative mode $[M-H]^-$.

DISCUSSION

Olive fruit

The Mediterranean Basin is already under the pressure of more intense, frequent, and longer extreme climatic events, like heat waves (IPCC, 2019). The extreme climatic events verified in Europe in 2017, 2020, and 2021 are examples of the ongoing climate change ("Climate bulletins | Copernicus," n.d.). We demonstrated previously (Araújo et al., 2021) that the environmental conditions of the high temperatures and dryness observed during the summer of 2017 induced a reduction of the leaf water availability in olive trees of the dry area of the orchard, together with an upregulation of the plant antioxidant system that was able to control oxidative lipid damages. In the present work, we observed that besides this dehydration of the leaves from the dry area (Araújo et al., 2021), fruit water content was also decreased, presenting a low water availability. A similar response was observed in the fruits' water availability from field trees exposed to water deficit for the cultivars 'Cobrançosa', 'Frantoio', 'Corregiola', 'Leccino' and 'Koroneiki' (Gonçalves et al., 2020; Zeleke et al., 2012). Fruit water content is considered an important factor since a decrease in water availability can affect fruit and oil quality, and the efficiency of extraction during processing (Zeleke et al., 2012).

The olive tree is particularly tolerant to water deficit. Some sugars and polyols (e.g., mannitol and *myo*-inositol) are involved in stress protective mechanisms (Araújo et al., 2021; Martinelli et al., 2013), promoting osmoregulation avoiding tissue dehydration. The accumulation of carbohydrates in olive leaves and fruits in response to abiotic stress (e.g., drought and heat) was reported (Araújo et al., 2019; Gonçalves et al., 2020; Valente et al., 2020). Our data are also in line with those findings, as fruits from the dehydrated area showed higher levels of D-mannitol, α -D-mannopyranose, and D-glucose. Moreover, the strong negative correlation between carbohydrates and FWC (correlation of FWC with: α -D-mannopyranose, $r = -0.87$, $p = 0.005$; D-mannitol $r = -0.90$, $p = 0.002$; and D-glucose $r = -0.84$, $p = 0.009$) support that the accumulation of carbohydrates was influenced by the reduction of fruit water availability. Despite this response, carbohydrates accumulation seems not to be used or is insufficient to maintain cell turgor since fruit water content decreases. According to Valente et al. (2020), carbohydrates accumulation in olives exposed to drought + heat can increase energy availability and even reduce carbohydrates use in other metabolic processes, like oil synthesis (Marsilio et al., 2001).

The most abundant fatty acid found in olive fruits of 'Cobrançosa' was the MUFA oleic acid, followed by the SFA palmitic acid. This profile is similar to the reported previously for 'Cobrançosa' (Gonçalves et al., 2020; Valente et al., 2020) and other cultivars (Guo et al., 2018). Comparing the levels of fatty acids in olives from both areas, hydrated and dry, our

data showed that the water deficit stress decreased the pool of palmitic, palmitelaidic, and linoleic acids and increased the stearic acid. Despite this, the ratios between MUFA and SFA (an indicator of oil quality and stability against oxidative damage; Valente et al., 2020) (Valente et al., 2020) in olives from the hydrated and dry areas were similar (1.74 ± 0.32 and 2.13 ± 0.44 , respectively). Similar results were previously obtained by other authors (Gonçalves et al., 2020; Valente et al., 2020), proving that 'Cobrançosa', even under stressful conditions, can maintain high amounts of MUFA, keeping their health properties.

Stearic acid is the precursor of oleic and linoleic acids (He et al., 2020). The increase of stearic acid in the dry area may justify the decrease of linoleic acid. Some reports indicate that high temperatures increase the quantity of saturated fatty acids like stearic acid (Ahumada-Orellana et al., 2018). The increase of the stearic acid can be associated with a mechanism to counteract the water deficit associated with heat. Sánchez-Rodríguez et al. (2019) (Sánchez-Rodríguez et al., 2019) for the cultivar Manzanilla also associated the increase in the levels of stearic acid with the high water deficit stress imposed on the trees. The levels of the triterpene squalene can vary within cultivars, fruit ripening stage, and stress conditions (e.g., water deficit) (Ambra et al., 2017; Valente et al., 2020). Squalene is an important compound as it is the precursor of β -sitosterol (Micera et al., 2020), is involved in the regeneration of α -tocopherol from tocopheroxyl radical (Ambra et al., 2017), and acts as a scavenger and quencher of free radicals (Micera et al., 2020). Although no differences were found in the levels of this compound in trees from both areas tested, a tendency was observed to decrease the amounts of squalene in trees from the dry area, possibly increasing β -sitosterol and α -tocopherol, as both compounds increased in dry area fruits. Additionally, α -tocopherol is the most representative tocopherol in the composition of the olive fruit, acting as a lipid radical scavenger (Ambra et al., 2017). In plants, α -tocopherol is responsible for protecting photosynthetic system against oxidative stress caused by high-light and temperature exposure and functions as a lipid antioxidant, stabilizing the membrane by reducing membrane fluidity (Spicher et al., 2017). Here, using 'Cobrançosa' cultivar, it was verified that the levels of α -tocopherol were higher in the fruits from the dry area. These increased levels might be related to the decrease of squalene observed in these fruits of the dry area and/or may represent a protective measure against the high temperatures noticed in the 2017 summer to protect the photosynthetic system (Spicher et al., 2017).

Many phenolic compounds are found in olive fruits from the 'Cobrançosa' cultivar, GPADHEA and EA being the most representative compounds. Water deficit induced a decrease of the levels of both compounds (correlations between FWC vs GPADHEA, $r = 0.949$, $p = 0.003$ and FWC vs EA, $r = 0.882$, $p = 0.020$). GPADHEA compounds likely originate from reduction reactions of EA (Rubio-Senent et al., 2015), but their function is still unknown.

EA is a product of the hydrolyses of oleuropein (Figure 4.2.4), by β -glucosidase, into oleuropein aglycone and, this compound, into EA and hydroxytyrosol (Thielmann et al., 2017). Interestingly, other phenolic compounds, the oleoside, EA glucoside, and oleuropein, respond differently to water deficit stress, increasing their pools. Moreover, oleuropein, the compound responsible for the fruit bitterness, is a strong antioxidant (potent free radical scavenger), and water deficit increases olive fruits' oleuropein content. This response was also described in other studies using 'Cobrançosa' and 'Picual' cultivars (Gonçalves et al., 2020; Jiménez-Herrera et al., 2019). However, it seems that other abiotic stress, like high temperature, when coupled with water deficit, can have an opposite effect, reducing the levels of oleuropein in fruits from young 'Cobrançosa' plants growing in pots under controlled conditions, as demonstrated by Valente et al. (2020) (Valente et al., 2020).

Olive oil

Trees from the dry area produced much less olive oil than those in the hydrated area, demonstrating that water availability is important to oil production (Ramos and Santos, 2010; Servili et al., 2007). According to the European Union on characteristics of olive oil (EU regulation number 2568/91), oils from both conditions can be classified as Extra-Virgin Olive oils (Figure 4.2.2) according to their acidity, peroxide value, and UV absorbances. It was not observed differences in free acidity, peroxide value, and K_{232} between the oils from the dry and hydrated areas. However, oils from the dry area showed higher oxidation (K_{270}), probably due to a higher amount of secondary oxidation products that absorb at this wavelength (270 nm) (Malheiro et al., 2011). This may have influenced the lower antioxidant capacity (DPPH) in oils from the dry area.

As occurred in olive fruits, in olive oil oleic acid was the major fatty acid found under water deficit stress. A similar response profile was also observed for the PUFA linoleic acid. The levels of the other fatty acids found in 'Cobrançosa' fruits remained identical in both areas. This resulted in similar values of the ratio MUFA/SFA in oils from the dry area (3.35 ± 0.83) and from the hydrated area (3.35 ± 0.91), giving insights that water deficit stress does not affect oil quality and stability and may improve 'Cobrançosa' oil nutritional and health properties.

As observed before in fruits, squalene in oil decreased their content in dry area trees. This reduction may be associated with the increase in β -sitosterol. Or, it may be related to the activity of squalene as a ROS scavenger (Micera et al., 2020; Valente et al., 2020). α -Tocopherol was only detected in oils from trees of the hydrated area, supporting studies in other cultivars, where drought stress reduced (or did not influence) the levels of α -tocopherol in olive oils (Palese et al., 2010; Tovar et al., 2002). In the present work, despite the high levels

found in the fruits, no α -tocopherol was found in oils from the dry area, which can be related to the oxidation of the oil.

Interestingly, although the physicochemical analysis of the oils from the hydrated and dry areas was similar, the olfactory sensations were quite different between the conditions tested. For instance, oils from the dry area showed higher sensations of apple, banana, and dry fruits. These sensations (apple and dry fruits) were previously attributed to the “*terroir*” of this region (Rodrigues et al., 2020). In the oils from the hydrated area, predominated the sensations of tomato leaves, cabbage, fresh grass, tomato, and fruitiness. Regarding gustatory-retronasal sensations, the same pattern as olfactory sensations was found. In oils from the dry area prevailed the sensations of sweet, apple, banana, and dry fruits, while in the ones from the hydrated area the predominant sensations were of fruitiness, bitter, pungent, tomato, fresh grass, cabbage, and tomato leaves. The significant gustatory sensations, bitter and pungent, in oils of the hydrated area may be attributed to the high levels of the secoiridoid oleuropein aglycon and other phenolics not detected here, such as oleuropein (Visioli et al., 2002). The sweet sensation present in the oils of the dry area might be attributed to the breakage of the glycosidic bond in the oleuropein compound that originates oleuropein aglycon and glucose (that was not detected in the lipophilic profile) (Guo et al., 2018).

Another important antioxidant compound in the ‘Cobrançosa’ oils is luteolin, but the stress imposed in the dry area repressed its production, supporting the data for leaves in the same trees (Araújo et al., 2021). It is noteworthy that the stress condition augmented oleacein, a known antioxidant and anti-inflammatory phenolic compound. Oleacein (Figure 4.2.4) is formed when β -glucosidase catalyses the hydrolysis of oleuropein and ligstroside to form the aglycone forms, these reactions contribute to the formation of oleacein and also oleocanthal (not detected). These reactions may have contributed to forming the hydroxylated product of the decarboxymethyl elenolic acid, which also increased in consequence of water deficit stress.

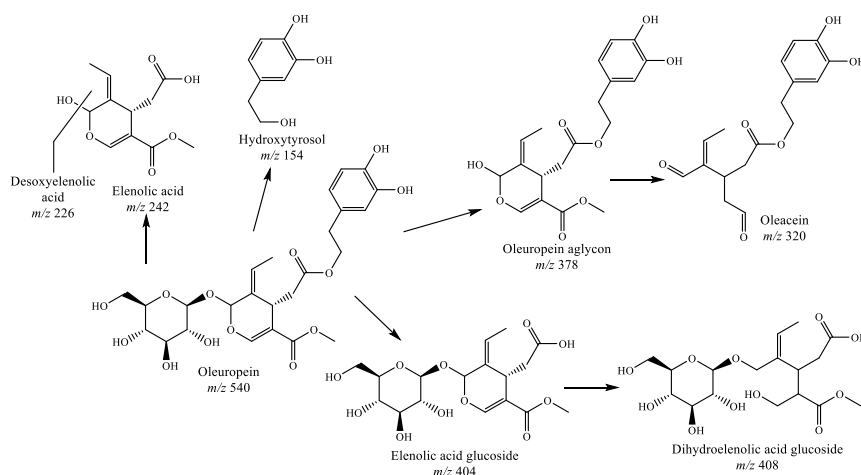


Figure 4.2.4 Chemical structures of the oleuropein and derivatives.

CONCLUSIONS

The extreme weather events of the 2017' summer reduced the water availability in fruits, despite the accumulation of sugars and polyols. The water deficit stress condition did not affect one of the main fatty acids in 'Cobrançosa' fruits, oleic acid, but reduced other important fatty acids, like palmitic, palmitelaidic, and linoleic acids and increased SFA stearic acid. Additionally, water deficit stress increased the levels of other compounds in olive fruits, as in the case of β -sitosterol and α -tocopherol, and polyphenols, like oleuropein, oleoside, and EA glucoside. Regarding the olive oil, water deficit reduced the oil yield but increased the levels of compounds, such as unsaturated fatty acids (oleic acid and linoleic acid), β -sitosterol, and the secoiridoid oleacein. Despite these adjustments in fatty acids, the unchanged ratio MUFA/SFA suggest that the stress condition did not impact oil quality and stability. Also, water deficit stress promoted some olfactory sensations (like apple, banana, and dry nuts) and gustatory-retronasal sweet in this oil. This study demonstrates that extreme climate events possibly reduce oil yield but stimulate the accumulation of some important bioactive compounds that improve its nutritional value.

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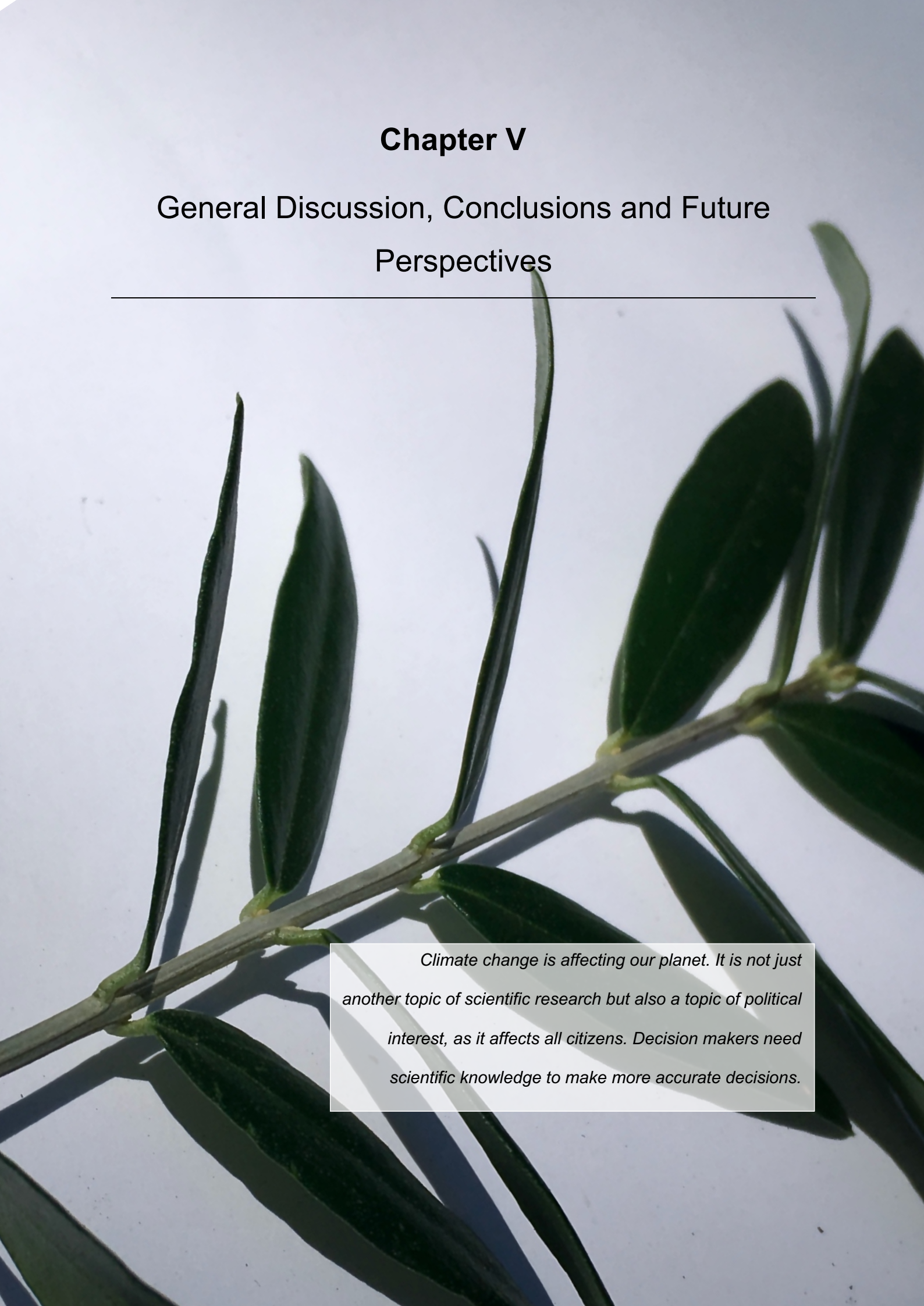
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Chapter V

General Discussion, Conclusions and Future Perspectives



Climate change is affecting our planet. It is not just another topic of scientific research but also a topic of political interest, as it affects all citizens. Decision makers need scientific knowledge to make more accurate decisions.

“Climate change is no longer some far-off problem; it is happening here, it is happening now”

Barack Obama

Former president of the United States of America (2009 to 2017)

As the former USA president said, climate change is happening now. We need to act yesterday. Drought and extremes in temperature are likely to occur more frequently due to climate change, affecting our economies due to the reduction in agricultural production (IPCC, 2019, 2013).

Considering the impact of climate change on Mediterranean traditional crops, this thesis aimed to contribute to the understanding of how the olive tree, a crop crucial to the socio-economy and cultural-landscape heritage of the Mediterranean, deals with drought and heat alone and combined. As the young phase of plants is the one where plants would struggle more to be able to recover and it is one of the most delicate phases of development, in this thesis we chose to also study young olive trees, besides field adult trees.

From controlled conditions...

Unveiling olive responses under greenhouse conditions

The effects of an episode of heat shock in young olive trees from the cultivar ‘Arbequina’, a cultivar improved for intensive production but that requires intensive irrigation at the groves (subchapter 2.1.) were evaluated. When this Ph.D. project was designed (in 2016) most state of the art on the abiotic stress on olive trees was focused on drought alone, being less known the impact of heat on this species and much even less known the effect of the combination of heat and drought. Interestingly, an analysis of the climatic data of IPMA in these last five years showed that the temperature has been increased ~1°C during these years (almost 2°C in 2022). Thus a single heat episode at 40°C for 2h is realistic, and we showed it was sufficient to induce moderate changes in the one-year-old-plants (Araújo et al., 2018). The subchapter 2.1 also contributed to the state of the art regarding the behavior of cultivars on their relative water content, gas exchange, carbohydrates content, cell membrane permeability and lipid peroxidation immediately after heat exposure. It was observed that only 2h at 40°C was sufficient to reduce plant water status and photosynthesis and induced

stomatal closure. This treatment does not induce changes in the carbohydrate contents (total soluble sugars and starch) nor in the membranes (no changes in lipid peroxidation or membrane damages).

This data support that even a species like olive, adapted to the Mediterranean climate, with xerophytic traits, starts to struggle with high temperature as Fraga et al., (2021) suggest that this effect is sensed even in juvenile trees (young stages of development). In fact, it was demonstrated that even a short period of heat stress induced functional shifts on young olive trees at the photosynthetic level. Moreover, this study also contributed to know that, despite leading to multiple physiological and molecular changes, the condition of 2h at 40°C was not sufficient to cause severe damages in young olive trees as no damages were observed in membranes; and that young olive trees are able to overcome a short heat shock episode.

From this first study, several questions emerged. For instance, what is young olive trees' physiological and metabolic response in a more realistic scenario of combined heat and drought stress? Are the young olive trees able to recover after the stress removal? So, the subchapter 2.2. aimed at answering to some of those questions. In the subchapter 2.2. a controlled experiment combined the heat shock (2h, 40°C) studied previously with drought (well-irrigated vs non irrigated) to mimic grove conditions, and used the same cultivar ('Arbequina') to minimize the variable of cultivars. The battery of parameters included responses at photosynthetic level, redox status and defence, and defence proteins (e.g., dehydrins, heat-shock proteins and aquaporins) and were monitored during stress and after its removal (recovery) (Araújo et al., 2019). As expected by other studies in olive trees (Bacelar et al., 2007a, 2007b, 2006), the non-irrigation treatment reduced some photosynthetic parameters whilst increased the amounts of carbohydrates, which may be due to the shift of using carbohydrates as osmoprotectants in the case of water shortage (Araújo et al., 2019; Bacelar et al., 2006). Interestingly, the trees which were previously exposed to water deficit suffered less effects of the heat shock than those who were well-irrigated the whole experiment. For instance, the net CO₂ assimilation rate had a pronounced drop in the group well-irrigated with heat shock, similar results were verified for chlorophyll *b*. Regarding the tested protective proteins expression, both dehydrins and heat shock proteins increased in the non-irrigated treatment, particularly in the treatment with heat, perhaps to neutralize the loss of membrane integrity and the lipid peroxidation increase. This represents an advance to state of the art in olive trees as a similar mechanism was previously proposed for other species (tomato and tobacco) as a plant strategy to counteract the effects of stress (Halder et al., 2018; Ré et al., 2016). Interestingly, the young olive trees were able to recover from all treatments after a short period, 10 days post-stress, and the aquaporins seem to have an important role in the recovery, as they increased their expression in both treatments. Another key point of

this study, is that the pre-exposure to water deficit may have promoted some tolerance mechanisms allowing plants to cope better with further stress episodes and olive trees have the plasticity to recover (Abdallah et al., 2017; Araújo et al., 2019; Dias et al., 2018).

Overall, the research of the Chapter II contributed to answer (at least in some extent) the Objective 1, regarding the knowledge of key physiological and molecular processes that change in olive trees when exposed to heat and/or drought, and if the chosen conditions were apparently not sufficiently severe to cause irreversible damages in young olive trees (as no damages were observed in membranes). Also, the objective is achieved by demonstrating that the young olive trees are able to overcome a short heat shock episode.

Influence of water stress and biostimulant on fruit development in young plants of two cultivars

With the acquired knowledge gained in Chapter II, sustainable solutions to reduce the drought and heat effects were studied, and eventually these strategies could also facilitate the recovery period. Nowadays it is urgent to address the issue of water consumption at the olive sector. Among the available strategies, it was studied how a biostimulant (based on the algae *Ascophyllum nodosum*) influenced the olive fruit development and its lipophilic profile (subchapter 3.1). This choice was grounded on the concept that this algae biostimulant is an credible alternative to the overuse of phytopharmaceuticals and fertilizers, thus well aligned with the F2F strategy, and on the other hand their effects on olives and olive groves remain underexplored. For this study the behavior of the Portuguese 'Galega' and the Spanish 'Arbequina' were compared because they are used in the traditional/dryland and intensive agricultural practices, respectively. Groups of plants (treated vs non treated with the biostimulant and, irrigated to 100% of field capacity vs only irrigated to 50% of field capacity) were used to study the impact of biostimulants and drought on the fruit development stages, using two sampling times. The results are innovative by showing the advantages for the producers regarding the use of these biostimulants. It was also demonstrated that the biostimulant effects on the olives' water content and metabolite profile depends on the cultivar, the irrigation level and the fruit ripening stage. For instance, *A. nodosum* application improved the leaf water status in 'Arbequina' under low irrigation. It increased the fruit growth and quinic acid accumulation, particularly at the end of the development process. In contrast, at the beginning of fruit development, the effect of biostimulants was more notorious in *myo*-inositol, oleic and palmitic acids. Contrarily, in 'Galega', the biostimulant stimulated the quinic acid production along the fruit development. Also, even under well water conditions, the

biostimulant can have a positive input as promoted some fatty acids, squalene, β -sitosterol, *myo*-inositol, and butanedioic acid in 'Galega' fruits or quinic acid and sugars (β -D-glucopyranose) in 'Arbequina' fruits. However, these results were obtained with five applications of the biostimulant, and a study of only one or two applications in similar conditions should be considered, as five applications may be too expensive to producers. Also, the impact of this biostimulant may have in the soil and plants' microbiome, and how and how much impact the presence biostimulant metabolites may have in the soil microbiome. In summary, these promising results open new questions that deserve further studies, for example to clarify (i) the minimal number of applications in groves to ensure benefits and their costs to the producer, (ii) the impacts on the soil biodiversity, (iii) if the effect of the biostimulants is still beneficial in orchards under drought and heat waves.

Despite the new questions arising from work developed in the Chapter III, it is well evidenced that this chapter contributed to the advance of state of the art regarding Objective 2, by showing that an algae biostimulant is beneficial in increasing the resistance of olives to extreme conditions of drought and heat, towards providing sustainable solutions to improve orchards resilience. These results not only demonstrate some positive effects but that also under non stressfull conditions, this biostimulant apparently improves the fruit size and quality.

... to field conditions

Functional and metabolic responses to water deficit of an orchard under drought conditions

Going from the laboratory to the field increases the complexity of the assays (the targeted variables are often not controlled, and the plant faces simultaneously multiple abiotic and biotic stressors that may influence the responses) and repeating the assays in different years is usually recommended. Nonetheless, the approach was use the atypical dry year of 2017 to study, in a grove (with irrigated and non-irrigated sectors), the functional plasticity of adult trees ('Cobrançosa') to the extreme conditions of that year, to understand if heat and/or drought affect the performance of olives and fruits, regarding the phenolic (e.g., oleuropein) and lipid profiles of olive leaves and fruits/oil, and if those conditions also influence the oil quality both at the physicochemical and sensorial levels. Interestingly, it was demonstrated that leaves from non-irrigated sector despite having higher H_2O_2 and $O_2^{\cdot-}$ levels, suffered no

increase in lipid peroxidation, probably due to an adjustment of the antioxidant response (e.g., GPOX activity) and by the action of flavonoids (luteolin-7-O-glucuronide), and eventually, of the secoiridoids (which response was dynamic) that may act as ROS scavengers (Araújo et al., 2021). A key advance beyond state of the art, regards the demonstration of the leaf metabolomic plasticity of adult trees in a real scenario of drought. The data also show the olive's remarkable physiological and metabolomic plasticity to adjust to the field conditions. This plasticity will provide the necessary capacity to overcome and survive the extreme climatic conditions expected in the Mediterranean region in the following decades.

Finally, the same grove was used to analyze drought summer's metabolomic and sensorial effects on olive fruit and oil quality and their metabolite profile. It was demonstrate here that the extreme weather events of 2017 summer reduced the water availability in fruits, despite the accumulation of sugars and polyols. Drought did not influence the main fatty acids (oleic acid), reduced other fatty acids (e.g., palmitic, palmitelaidic, and linoleic acids) and increased SFA stearic acid. Drought also increased the levels of other compounds in olive fruits and polyphenols, like oleuropein. Drought reduced the oil yield but increased the levels of unsaturated fatty acids (oleic acid and linoleic acid), β -sitosterol, and the secoiridoid oleacein. Despite these fatty acids plasticity, the ratio MUFA/SFA was maintained, showing no effects on oil quality and stability. Finally, it was covered a pivotal aspect of the value of the olive/oil chain: the impact of drought and heat on olfactory sensations and demonstrated that the extreme climate events reduced oil yield but stimulated the accumulation of some important bioactive compounds that improve its nutritional value. Thus, Chapter IV addressed this Third Objective, by using the exceptional conditions of a grove in Trás-os-Montes with irrigated and non-irrigated sectors, and with Cobrançosa trees of the same age in both sectors, to collect samples in the atypic summer of 2017 (extreme dryness and heat) and analyze the behavior of the plants in both sectors.

Briefly,

This Ph.D. thesis besides studying different cultivars ('Arbequina', 'Cobrançosa' and 'Galega'), explored different development stages (young trees and adult trees) and conducted assays in both controlled and field conditions with different abiotic stresses implemented (drought and heat). With these different arrangements of scenarios, we were able to contribute to olive new scientific further. The studies presented here may help develop new sustainable agricultural practices to control the impacts of climate change.

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