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DROUGHT AND THE OLIVE TREE IN A CHANGING CLIMATE: A MULTI-LEVEL RESPONSE CHARACTERISATION TO EXPLORE AND VALORISE ITALIAN CULTIVARS

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Abstract

The Mediterranean climate faces significant warming and reduced rainfall due to climate change. This will make droughts more frequent and severe, affecting agriculture. Higher temperatures can damage crops while lower rainfall increases the need for irrigation. Despite that irrigation has sustained agriculture in arid regions, competition for water is increasing. To adapt to current challenges, improvements in irrigation practices, adoption of agro-ecological techniques and use of drought-tolerant crops are essential to stabilise crop performance. The olive tree (*Olea europaea* L.) is one of the most widely cultivated crops in the Mediterranean. Although it is considered a drought-tolerant species, different cultivars do not respond equally to water stress. In this study, 18-month-old plants of three olive cultivars (Giarraffa, Leccino and Maurino) were grown in a growth chamber and monitored during one month of drought stress. 'Maurino' and 'Leccino' are mainly grown in central Italy and are derived from local oleasters, while 'Giarraffa' is locally adapted to Sicily and probably introduced from Spain or Morocco. Given their long-term adaptation to different environments, it was hypothesised to use them as representatives of different response patterns of the olive plant with the aim of evaluating and differentiating their drought tolerance. A multi-level approach was used to evaluate physiological changes, metabolic profiles, biochemical responses and anatomical observations. All cultivars showed significant physiological responses within the first two weeks of stress, while metabolomic and biochemical responses were more pronounced at the end of the stress. In particular, the cultivars exhibited different coping mechanisms: 'Giarraffa' adopted a "drought avoidance" strategy with early stomatal closure and investment in stem osmoregulation. In contrast, 'Maurino' adopted a "water-consuming" strategy with high initial water use and high investment in phenolic antioxidant compounds, which did not prevent a dramatic increase in electrolyte leakage. 'Leccino' shared the responses of the other two cultivars, maintaining a basal carbon fixation and investing in osmoprotectant molecules which confer drought tolerance. These findings highlight the importance of considering the timing and integration of observed responses within the plant. This study provides valuable knowledge on drought tolerance mechanisms in olive cultivars, supporting future breeding programs and water management strategies. Future research should investigate mature trees in field settings during the reproductive stage to assess impacts on fruit and oil quality.

Abstract

La regione mediterranea sta affrontando un significativo aumento delle temperature medie unito a una diminuzione delle precipitazioni dovuti al fenomeno globale del cambiamento climatico. Questo comporterà una maggiore frequenza e severità degli episodi siccitosi, che hanno ripercussioni disastrose sull'agricoltura. Queste condizioni richiederanno cospicui interventi di irrigazione, in un contesto in cui la risorsa idrica sarà sempre più richiesta anche da altri settori. Possibili soluzioni per stabilizzare le rese agricole sono individuabili nel miglioramento delle pratiche di irrigazione, nell'adozione di tecniche agro-ecologiche e nell'identificazione e utilizzo di cultivar tolleranti allo stress idrico. L'olivo (*Olea europaea* L.) è una delle colture più diffuse del Mediterraneo. Nonostante sia considerata una specie tollerante allo stress idrico, cultivar diverse non mostrano risposte univoche allo stress. In questo studio, piante di 18 mesi di tre cultivar di olivo (Giarraffa, Leccino e Maurino) poste in camera di crescita sono state monitorate durante un periodo di quattro settimane di assenza di irrigazione. 'Maurino', diffuso principalmente in Italia centrale, e 'Leccino', coltivato in tutto il mondo, sembrano derivare dalla locale oleastra, mentre 'Giarraffa', probabilmente introdotta dalla Spagna o dal Marocco, è coltivata principalmente in Sicilia. Dato l'adattamento delle tre cultivar a differenti condizioni ambientali, è stato ipotizzato che possano essere rappresentative di diverse strategie di risposta allo stress proprie dell'olivo, con lo scopo di individuarne la diversa tolleranza. Lo studio è avvenuto a livello fisiologico, metabolico, biochimico e anatomico. Le cultivar hanno mostrato significative variazioni fisiologiche nelle prime due settimane di stress, mentre più tardive sono state le risposte metaboliche e biochimiche. Le cultivar hanno inoltre mostrato diverse strategie di risposta: 'Giarraffa' ha adottato una strategia "evitante", con una precoce chiusura stomatica e investimenti in osmoprotettori soprattutto nel fusto. Al contrario, 'Maurino' è risultato traspirare copiosamente l'acqua disponibile e investire soprattutto in composti fenolici con proprietà antiossidanti, che però non sono stati efficaci nell'evitare un drammatico incremento dell'*electrolyte leakage*. 'Leccino' condivide alcune risposte di entrambe le altre cultivar, mantenendo livelli basali di fissazione del carbonio e investendo in osmoprotettori e proteine che possano risultare vantaggiose nella tolleranza allo stress. I risultati hanno evidenziato l'importanza di considerare il tempo dell'attuazione delle risposte e di valutare l'integrazione di più meccanismi di risposta all'interno della pianta. Questo studio contribuisce ad

approfondire la conoscenza delle strategie di risposta allo stress idrico mostrate da cultivar diverse, fornendo informazioni utili per i programmi di breeding e per le strategie di gestione della risorsa idrica. Ulteriore valore alla ricerca potrebbe scaturire dallo studio di individui maturi, in campo, durante la fase riproduttiva di crescita per poter valutare l'impatto dello stress sulla qualità del frutto e dell'olio.

Table of contents

1. General introduction **8**

1.1 Climate change and agriculture in the Mediterranean **8**

1.2 Olive tree: a valuable resource of the mediterranean area **10**

2. Objective and experimental setup of the study **14**

3. Cultivar responses to drought as assessed by morpho-physiological parameters **18**

3.1 Introduction to the chapter **19**

3.2 Material and Methods **21**

3.2.1 Soil Water Content 21

3.2.2 Relative Water Content of leaves and stems 21

3.2.3 Stomatal density 22

3.2.4 Leaf gas exchange and chlorophyll fluorescence 22

3.2.5 Electrolyte Leakage 23

3.2.6 Malondialdehyde content 24

3.2.7 Pigments quantification 24

3.2.8 Statistical analysis 24

3.3 Results **25**

3.1. Drought effects on plant water status and biochemical responses 25

3.2. Impact of water deficit on the photosynthetic process 30

3.3. Correlation 35

3.4 Discussion **36**

3.5 *Bulleted* conclusions **41**

3.6 Supplementary materials of Chapter 3 ..... **41**

4. Comparative metabolomics of leaves and stems: phenolic and lipophilic profiling reveal cultivar-specific response patterns **46**

4.1 Introduction to the chapter **47**

4.2 Material and Methods **49**

4.2.1 Phenolic and lipophilic compounds extraction 49

4.2.2 GC-MS analysis 49

4.2.3 UHPLC-MS analysis 50

4.2.4 Statistical analysis 51

4.3 Results **52**

4.3.1 Profile of phenolic secondary metabolites 52

4.3.2 Lipophilic compounds metabolite profiles 55
4.3.3 HCA of leaf metabolites 59
4.3.4 HCA of stem metabolites 62
4.3.5 PCA of leaf metabolite classes 63
4.3.6 PCA of stem metabolite classes 65
4.4 Discussion 66
4.4.1 The accumulation of phenolic metabolites in response to drought occurs primarily in the leaves 66
4.4.2 Drought stress causes changes in leaf and stem lipophilic metabolites 68
4.4.3 Cultivar-specific changes in metabolite profiles in response to drought 69
4.5 *Bulleted* conclusions 70
4.6 Supplementary material of Chapter 4 71
5. Proline, sugars and water-related proteins: the biochemical drivers of water management 83
5.1 Introduction to the chapter 84
5.2 Material and methods 87
5.2.1 Protein extraction 87
5.2.2. Electrophoresis and immunoblotting of leaf protein extracts (without Stain-Free technology) 88
5.2.3 Electrophoresis and immunoblotting of stem protein extracts (Stain-Free technology) 89
5.2.4 Proline content 90
5.2.5 High Performance Liquid Chromatography (HPLC) sugars analysis 90
5.2.6 Starch content 91
5.3 Results 91
5.3.1 Sugar levels in stems and leaves of olive cultivars 91
5.3.2 Starch content 95
5.3.3 Aquaporins 95
5.3.4 Dehydrins 97
5.3.5 Osmotin 100
5.4 Discussion 103
5.5 *Bulleted* conclusions 106
5.6 Supplementary materials of Chapter 5 107
6. A look at stem anatomy and lignin composition 108
6.1 Introduction to the chapter 109
6.2 Material and methods 111
6.2.1 Analysis of the lignin 111
6.2.2 Determination of lignin content 111
6.2.3 Analysis of lignin composition by 2D-NMR spectroscopy 111

6.2.4 Stem anatomy analyses 112

6.3 Results 113

6.3.1 Lignin content and composition 113

6.3.2 Xylem anatomy 116

6.4 Discussion 118

6.5 *Bulleted* preliminary conclusions 119

7. Appendix: Application of leaf-derived phenolic compounds to human umbilical vein endothelial cells (HUVECs): olive leaf extracts (OLEs) from the three cultivars differentially protect cells against oxidative stress 120

7.1 Introduction to the chapter 121

7.2 Material and methods 122

7.2.1 Materials 122

7.2.1 Determination of the Antioxidant Capacity and Polyphenols Content 122

7.2.2 Ferric Ion Reducing Antioxidant Power (FRAP) 123

7.2.3 Folin-Ciocalteu Method 123

7.2.4 Leaf Metabolite Extraction and UHPLC-MS analysis 123

7.2.5 Cell Viability Test by WST-1 Assay 124

7.2.6 ROS production 124

7.2.8 Permeation of antioxidants contained in OLE across excised rat intestine 124

7.2.9 Statistical analysis 125

7.3 Results 125

7.3.1 Antioxidant Capacity and Polyphenols Content 125

7.3.2 OLEs phenolic characterisation 127

7.3.3 Cell Viability Test 130

7.3.4 OLEs protective effect from oxidative stress 131

7.3.5 OLEs antioxidant activity as assessed by ROS production 132

7.3.6 Permeation of antioxidants contained in OLE across excised rat intestine 134

7.4 Discussion 134

8. How does the data fit together? Final Model, Conclusion, and Prospects 137

References 141

Chapter 1

General introduction

1.1 Climate change and agriculture in the Mediterranean

The Mediterranean Basin consists of the semi-enclosed Mediterranean Sea and the European, African, and Asian continents that border it. It is in a transition zone between mid-latitude and subtropical atmospheric circulation regimes, with significant topographic gradients. The Mediterranean climate has warm, dry summers and mild, wet winters. This seasonality is caused by the Azores High pressure system, also known as the North Atlantic Anticyclone, which moves northward in the summer and reduces rainfall. During the winter, the polar jet stream moves south, increasing rainfall, particularly in the northern and eastern regions. The IPCC report 2022 (Ali et al., 2022) uses the term "climate change hotspot" from Giorgi (2006) to describe this region, taking into account both its vulnerability to climate change and the density of its human societies and biodiversity-rich ecosystems. The report stated that since the 1980s, atmospheric warming in the Mediterranean has exceeded the global average, and future annual and summer warming rates are expected to be 20% and 50% higher, respectively, than the global annual average. Changes in precipitation patterns are closely related to temperature increases. Precipitation is expected to fall by 4% for every 1°C of global warming, while extreme precipitation events will increase in some Mediterranean areas. Over the last century, the Mediterranean has seen increased temperatures, less frequent rainfall, and decreased moisture availability (Lionello, 2012). **Figure 1.1** shows the trend of temperature anomalies over the last 150 years at regional and global scales.

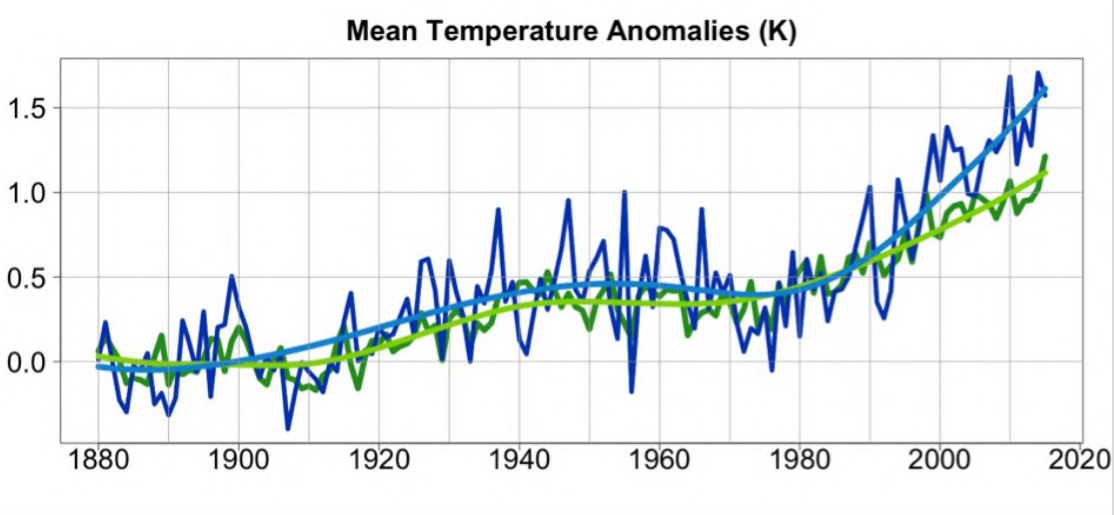

Figure 1.1. Warming of the atmosphere (annual mean temperature anomalies with respect to the period 1880-1899), in the Mediterranean Basin (blue lines, with and without smoothing) and for the globe (green line). In the Mediterranean region, average annual temperatures are now 1.4 °C higher than during the period 1880-1899, well above current global warming trends (Marini, 2018).

Reduced rainfall and higher temperatures increase the evaporative demand of all wet surfaces, resulting in decreased river runoff, streamflow, and groundwater recharge, all of which are directly related to drought. Drought is a natural hazard influenced by numerous hydroclimatic variables (such as precipitation, runoff, potential evapotranspiration, and soil moisture) (Mukherjee et al., 2018). Drought severity is determined by the duration, peak intensity, and recurrence interval. As a result of climate change in the Mediterranean, droughts will become more severe, frequent, and prolonged throughout the basin, directly affecting the agricultural sector (Ali et al., 2022). Several indices, including the Palmer Drought Severity Index (PDSI) (Palmer, 1965) and the Standardized Precipitation Evapotranspiration Index (SPEI) (Vicente-Serrano et al., 2010), can currently be used to calculate the severity of *agricultural* droughts. According to a model applied to European data (Van Passel et al., 2017), farms will lose between 8% and 44% of their land value due to climate change by 2100, depending on the climate models used to estimate long-term impacts. Moore and Lobell (2014) carried out another European study that examines farmers' adaptive potential, which is defined as the difference between short-term responses, where adaptation options are limited, and long-term responses, where significant adaptation processes can occur. Their findings indicate that if farmers implement effective adaptation strategies, most of Europe may see a limited increase in agricultural profits by 2040. However, the study confirms that, particularly in Mediterranean regions such as Spain, Greece, Portugal, southern France, and Italy, climate change may cause residual damage, even if farmers implement long-term adaptation strategies (Bozzola and Ravetti, 2017). Climate change has a negative impact on agriculture for two reasons: high temperatures affect crop phenology and reduce crop yields by shortening the growing season, while lower rainfall increases water demand, resulting in increased irrigation needs. Irrigation compensates for the water deficit that occurs when the amount of water available in the soil is less than the crop's water demand, which is determined by evapotranspiration (OECD, 2014). Until now, irrigation practices have enabled agriculture to thrive even in arid and semi-arid

areas, contributing to widespread and stable food production (Hanjra and Qureshi, 2010). Today, agriculture uses 70% of the world's available freshwater (Unesco, 2016). However, water used in agriculture competes with that used in emerging industrial sectors, as well as the consumption needs of an ever-growing population (Vergni and Todisco, 2011). These climatic and social factors will make water availability a constraint on agricultural productivity, with unavoidable consequences for food supply. For these reasons, it is important to focus on improving the efficiency of irrigation practices, adopting agroecological techniques, and promoting crop diversification, including the use of new varieties adapted to future climate scenarios (Fideghelli and Engel, 2011; Ali et al., 2022).

1.2 Olive tree: a valuable resource of the mediterranean area

The olive tree, a member of Oleaceae family and the genus Olea, is one of the most iconic and oldest cultivated plants in the Mediterranean basin, having always contributed to the agriculture, landscape, culture, and economy of this region. It originated in the Eastern Mediterranean and is historically attributed to the Greek tradition, as its development and adaptation for human use is believed to have first occurred in Greece (Kostelenos and Kiritsakis, 2017). Its fruits are used to produce olive oil, which is an important source of food, but also one of the most valuable exported goods of the Mediterranean region, representing 19% of the value of the world trade in vegetable oils for human consumption (Loumou and Giourga, 2003). The Mediterranean region is the leading producer of olive oil in the world, accounting for more than 95% of total olive oil production in 2019 (Leone et al., 2021). Three Mediterranean countries (Spain, Italy and Greece), along with Portugal, produce 99% of the EU's olive oil, the world's largest contribution, accounting for 69% of total production (Producing 69% of the World's Production, the EU Is the Largest Producer of Olive Oil - European Commission). Furthermore, its valuable leaves appear to have a potential use, primarily in the health sector: when used as traditional herbal medicines, they have demonstrated a possible beneficial effect on metabolism due to the presence of phenolic compounds, which are also known for their antioxidant activity (El and Karakaya, 2009).

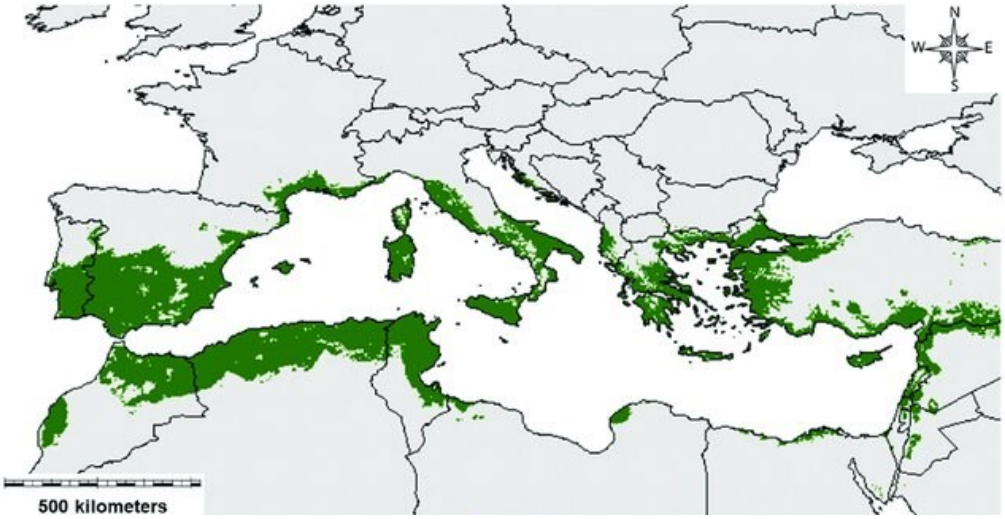

Figure 2.2. Distribution area of olive tree groves (Rodríguez Sousa et al., 2020).

The olive tree is well adapted to the Mediterranean climate and soil conditions (Brito et al., 2019). It can grow on calcareous and gravelly soils, but sandy soils are preferable (Brito et al., 2019). Temperature is the primary environmental factor limiting olive growing areas: optimal olive growing areas have an average annual temperature of 15-20°C, with a minimum of 4°C and a maximum of 40°C. Typically, the optimal temperature for olive vegetative growth ranges between 10°C and 30°C, although olive trees require a period of low temperature (0-7°C) to differentiate flowering buds. However, the minimum temperature should not fall below -7 °C, as this can severely damage trees, and even lower temperatures can kill them (Brito et al., 2019). Olive trees can tolerate a wide range of water availability: they can grow with 200 mm rainfall per year, but 1000 mm per year is optimal for commercial olive yield under rainfed conditions (Brito et al., 2019). Although olive trees have traditionally been rainfed, an increasing number of groves are irrigated (Carr, 2013; Duarte et al., 2021). Irrigation, particularly during the flowering season, helps to ensure a consistent crop of olives, which is beneficial to farmers. The need for irrigation has become even more urgent over the last decade, as rising temperatures have caused changes in rainfall patterns. The olive tree is the model woody plant used to study drought responses and tolerance because it can maintain a positive carbon balance by reaching very low leaf water potential under harsh conditions (Diaz-Espejo et al., 2018). It has also been studied under various environmental stress conditions, such as UV-B (Piccini et al., 2020) and salt stress (Rossi et al., 2015). Several traits, including small stomata, waxy leaf surfaces, narrow xylem vessels, non-photochemical quenching activation, and rapid osmotic adjustment,

have been identified as critical for drought tolerance (Fernández, 2014; Diaz-Espejo et al., 2018; Brito et al., 2019). However, the species *Olea europaea* includes a wide range of cultivar genotypes, suggesting that there are significant differences in drought responses (Bosabalidis and Kofidis, 2002; Sofo et al., 2004; Guerfel et al., 2009; Marino et al., 2021). These differences are related to the specific environment in which cultivars are grown and adapted, emphasizing the importance of studying olive plant diversity and promoting a more thorough characterisation of different stress tolerance mechanisms (Faraloni et al., 2011; Pierantozzi et al., 2013; Dias et al., 2018). The world's olive germplasm lists more than 2600 different varieties, with many local varieties and ecotypes contributing to the high biodiversity of olive trees (Muzzalupo, 2012). Italy has over 500 different types of olive trees registered in the National Olive Oil Register, making it a country rich in olive tree biodiversity. However, the number is likely to increase as analyses (mostly genetic) continue. These varieties differ not only in morphology, but also in yield and oil quality. 'Frantoio', 'Leccino', 'Taggiasca' and 'Coratina' are some of the most common and well known, but many others characterise different Italian regions. The comparison of different cultivars will enable the identification of both basic, ubiquitous plant tolerance mechanisms and potential interactions between different mechanisms for drought tolerance, as well as the identification of potential strategies to improve olive tree growth and productivity in water-stressed conditions. Three different olive cultivars grown in Italy, among the many listed in the Italian National Register, were studied for a thorough examination of their responses to drought stress. The cultivar Giarraffa, native to the arid region of Sicily, is tolerant to UV light (Piccini et al., 2021). The cultivar Leccino is widespread worldwide and shows good tolerance to drought, cold, and bacteria (*Xylella fastidiosa*) (de Pascali et al., 2019). When exposed to drought, the cultivar Maurino, which is native to Tuscany and is cold tolerant, exhibited a unique morphological trait (leaf wilting) (Claudio Cantini, personal communication). Based on a GBS-derived SNP catalogue of 94 Italian cultivars, it was proposed that 'Maurino' and 'Leccino' belong to a cluster population derived from local oleasters. 'Giarraffa', on the other hand, stands out from other Italian cultivars and was most likely introduced from Spain and Morocco (D'Agostino et al., 2018). Drought tolerance, or sensitivity, is a multifaceted concept that involves multiple areas of analysis. In addition to

morpho-physiological traits such as root depth and leaf morphology, genetic factors and environmental conditions also play a crucial role in determining a plant's ability to withstand drought. Understanding the complex interactions between these different factors is essential for developing resilient crop varieties that can thrive in water-limited environments. Researchers are exploring new strategies to improve drought tolerance in plants, including traditional breeding techniques and genetic engineering. This understanding of drought tolerance can help ensure food security in water-limited environments and combat unpredictable climate conditions. Most studies on olive trees and water deficits have focused on specific aspects such as the physiological response pattern (Ennajeh et al., 2008; Guerfel et al., 2009; Marino et al., 2014), metabolomic pathways (Dias et al., 2021; Azri et al., 2024), and the anatomy and biochemistry of the trees (Bosabalidis and Kofidis, 2002; Ennajeh et al., 2010; Gholami and Zahedi, 2019). Without a doubt, leaf responses are more extensively studied than stem responses. However, it is important to consider all parts of the tree when assessing its overall drought tolerance. Understanding the mechanisms of water transport and storage in the stems can provide valuable insights into how olive trees adapt to periods of water scarcity. By taking a holistic approach to studying drought tolerance in olive trees, researchers can gain a more comprehensive understanding of how olive trees cope with water stress. Indeed, a single approach can lead to a misunderstanding of tolerance as well as a partial understanding of the mechanism that allows a particular response to drought conditions. We hypothesized that the three cultivars would react differently to drought due to their long-term adaptation to different environments and genetic backgrounds. Our results aim to confirm this hypothesis, demonstrating the importance of considering multiple factors when studying plant responses to environmental stressors. During the three-year PhD project, fully drought-stressed plants were studied at multiple levels, including physiological, metabolomic, biochemical, and anatomical parameters, in a functionally oriented characterisation. Given the complex genetic background of olive cultivars and the intrinsic difficulty of correlating changes in gene expression to specific responses, we decided to postpone the genetic characterization of olive cultivars for the duration of this project. However, the genetic patterns that highlight the differences between cultivars can be investigated further in the future.

Chapter 2

Objective and experimental setup of the study

Given the value of olive trees in the Mediterranean area, as well as the region's vulnerability to climate change, the purpose of this study was to evaluate and differentiate the drought tolerance of three Italian olive cultivars (Giarraffa, Leccino, and Maurino). The study was carried out using certified 18-month-old olive trees (*Olea europaea* L., cultivars Leccino, Maurino, and Giarraffa) provided by "Spoolivi" (Società Pesciatina di Orticoltura, Pescia, PT, Italy). The plants were grown in 4 L pots (15x15x20 cm) using a substrate of 50% peat and 50% pumice (Tosca et al., 2021). When the plants arrived at the university laboratories, they were moved to a growth chamber with LED illumination for flowering and growth (TLED Secret Jardin-SRL AGOMOON, Manage, Belgium), which provided a photosynthetic photon flux of 450-550 $\mu mol\ m^{-2}\ s^{-1}$ at plants' leaves level. The photoperiod consisted of 12 hours of light and 12 hours of darkness. After one week of acclimation to the general environmental conditions with continuous watering, 20 plants of each variety were divided into two groups of 10 plants: CTRL (control) and DS (drought stress). The CTRL groups were fully watered (500 mL of water per week), whereas the DS groups were completely water-deprived for 4 weeks. The pots were rotated weekly to avoid positional effects (Pierantozzi et al., 2013). Temperature and humidity were measured hourly using the EBI 20-th1 datalogger (Ebro): temperature was 27.5°C and humidity 51.1% (data were averaged over the day and night). The minimum and maximum temperature values were 22.3°C and 31.8°C, respectively, while the minimum and maximum humidity values were 29.8% and 70.1%, respectively. The vapour pressure deficit (VPD, in kPa) was calculated from RH and temperature records. First, the saturated vapour pressure (SVP, in kPa) at each given temperature was calculated using the Magnus equation (Alduchov and Eskridge, 1996). VPD was then calculated from each RH and SVP using the standard equation $VPD = SVP \cdot (1 - \frac{RH}{100})$. The average VPD was 1.79 ± 0.45 kPa, with a minimum of 0.85 kPa and a maximum of 3.10 kPa. The experimental period involved an increasing water deficit divided into five time points: t0, t1, t2, t3, and t4, which corresponded to the beginning of withholding irrigation and the first, second, third, and fourth weeks of water deficit, respectively. Leaves were collected at all time points, while stems were collected from each

experimental group at t0, t2, and t4. **Figure 2.1** depicts an example of CTRL and DS plants from Giarraffa (**A**), Leccino (**B**) and Maurino (**C**) at the end of the experiment.

Figure 2.1. Control (left) and stressed (right) individuals of cultivars Giarraffa (A), Leccino (B) and Maurino (C) at the end of the experimental period (t4).

All analyses were carried out using the samples collected during the experiment. The study starts with a physiological analysis of cultivar responses (**Chapter 3**), which aims to understand how changes in water distribution and management may affect photosynthetic performance and oxidative status. In **Chapter 4**, the changes in lipophilic and phenolic metabolites in the stems and leaves of control and drought-stressed plants were evaluated using gas chromatography-mass spectrometry (GC-MS) and ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS), respectively. The analysis was carried out to identify metabolic differences that could explain the different physiological behaviour of the three cultivars. In **Chapter 5**, biochemical analysis is used to reveal the differential investment of the three cultivars in the accumulation of osmoprotectants (such as sugars and proline), as well as water-related proteins (osmotin, dehydrins and aquaporins). Finally, **Chapter 6** examines the anatomical and chemical characteristics of stems, which play an important role in plant growth, water transport, and carbohydrate storage. **Chapter 7** is an appendix resulting from a collaboration with the University of Pisa that investigates the potential antioxidant properties of phenolic extracts of olive leaves on human cells. Each chapter begins with a detailed review

of the parameters studied and their relationship to olive biology and drought responses. The last section (**Chapter 8**) contains a cumulative discussion aimed at bringing together all the aspects examined in the previous chapters. A final graphical descriptive model will help to summarise the conclusions. **Figure 2.2** shows the methods and experimental designs used to study the drought responses of the three olive cultivars. Detailed methods and a complete list of the analyses carried out are discussed further in each chapter.

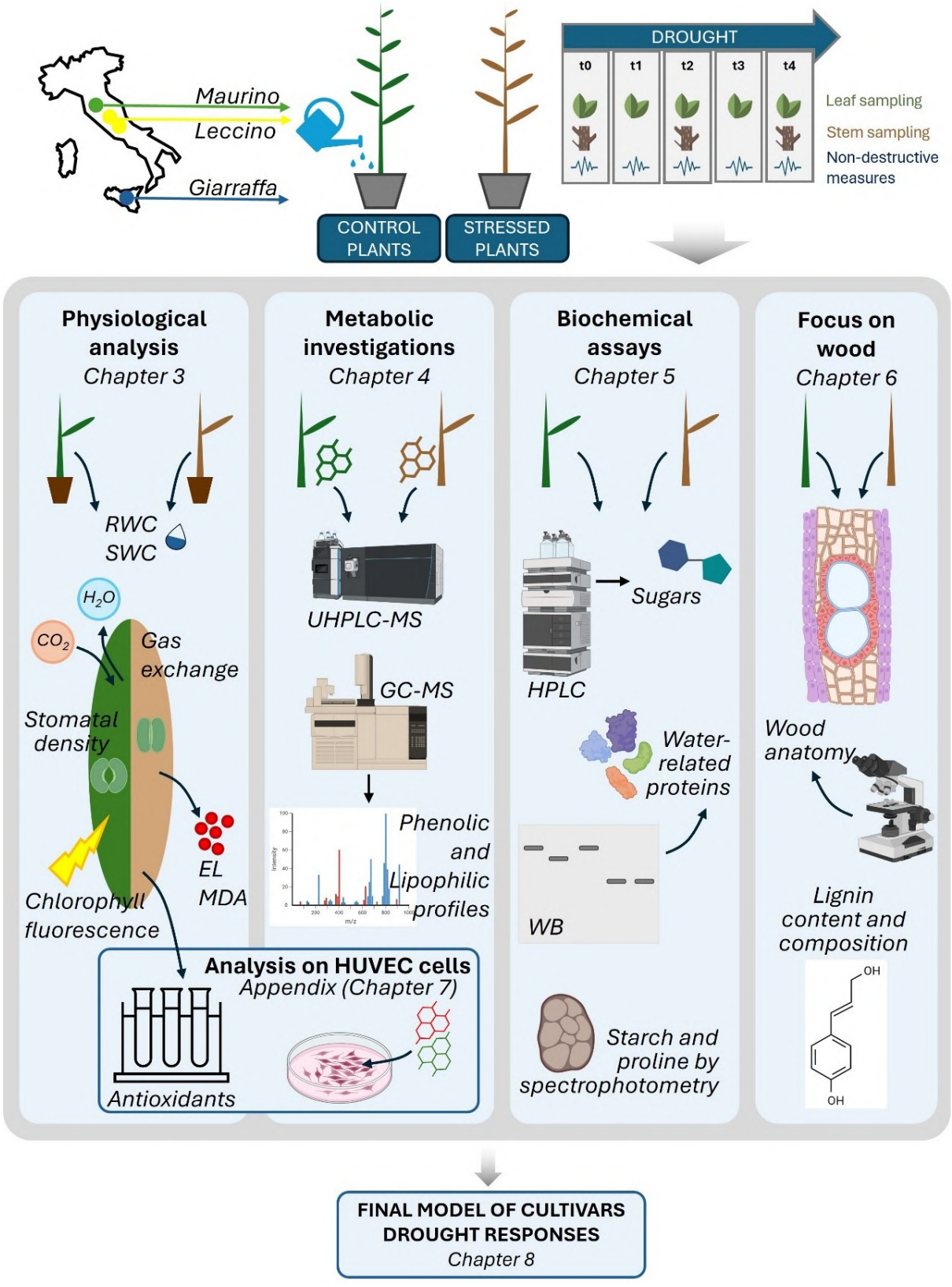

Figure 2.2. Summary diagram of both the experimental design and the analyses carried out, as well as their division into chapters in this thesis. More information about each specific approach can be found in the Materials and Methods section of each chapter.

Chapter 3

Cultivar responses to drought as assessed by morpho-physiological parameters

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3.1 Introduction to the chapter

The physiological approach helps to understand how plants respond to drought stress at various levels, from the leaf to the whole-plant levels. Physiological adaptations involve mechanisms that allow plants to respond to and survive droughts; thus, by examining physiological responses, we can gain an initial understanding of how cultivars cope with water scarcity and which major differences emerge among their response strategies.

When the amount of water lost through transpiration exceeds the amount taken up by the roots, the plant experiences a water deficit. To maintain cell turgor and reduce water loss, plants use the closing response of stomata (Brito et al., 2019), which results in a decrease in stomatal conductance (g_s). The olive tree is known to have a precise control in stomatal behaviour, likely more controlled by leaf hydraulic conductance than by hormonal signals (Hernandez-Santana et al., 2016; Rodriguez-Dominguez et al., 2016; Brito et al., 2019). Furthermore, in association with a decrease in g_s, a reduction in leaf water potential (Ψ) and relative water content (RWC) also takes place (Guerfel et al., 2009; El Yamani et al., 2019). Stomatal closure immediately reduces the amount of CO_2 in the substomatal cavity, which then slows the rate of photosynthesis; in most cases, photosynthesis is completely stopped by stomatal closure before metabolism is affected (Flexas et al., 2004). Increase mesophyll diffusion resistance to CO_2 has also gained importance as limitation to the photosynthetic process under stress conditions (Centritto et al., 2003; Flexas et al., 2004; Hoshika et al., 2022); for example, under salt stress conditions, changes in mesophyll conductance (g_m) have been shown to be as rapid as those in g_s (Centritto et al., 2003). However, as RWC, g_m and g_s significantly decline during drought, additional metabolic restrictions can occur (Lawlor, 2002). For example, Flexas and Medrano (2002) found that Ribulose-1,5-bisphosphate (RuBP) regeneration capacity decreases as ATP production declines. Furthermore, drought can impair carboxylation capacity by reducing Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content at low g_s values (< 0.01 mol H_2O m^{-2} s^{-1}) (Flexas et al., 2004). Reductions in g_s and g_m are a reversible adaptation to water deficit; however, when severe stress levels are reached, biochemical components of the photosynthetic apparatus may be compromised (Brito et al., 2019). Specifically, when the rate of light absorption exceeds the capacity for photosynthesis, irreversible photoinhibition occurs

(Petridis et al., 2012). In addition, a low transpiration rate caused by stomatal closure during drought conditions raises leaf temperature, potentially exacerbating photoinhibition (Kitao et al., 2000). Stomatal closure reduces the availability of CO_2, which slows down the Calvin-Benson cycle and thus reduces the consumption of NADPH and ATP (Chaves et al., 2009; Mishra et al., 2012). When water stress is accompanied by normal or intense light conditions that maintain high rates of the light phase, the reaction between excess electrons in the thylakoid membranes relative to the $NADP^+$ substrate and oxygen derived from water splitting results in the formation of reactive oxygen species (ROS), such as superoxide anion radicals (Smith et al., 2012). ROS can be harmful at high concentrations, causing membrane peroxidation as can be measured by malondialdehyde content (MDA content) and electrolyte leakage (EL). Excess electrons can be neutralised through the water-water cycle, where they are used to reduce oxygen and reform water (Smith et al., 2012), thus preventing ROS formation and allowing electron transport to continue (Baker and Rosenqvist, 2004). The water-water cycle activates a second protective mechanism for photosynthetic systems by creating a pH gradient that causes the enzyme violaxanthin de-epoxidase (VDE) to move from the lumen to the thylakoid membrane, which contains LHC-associated violaxanthin. VDE catalyses the conversion of violaxanthin to antheraxanthin and zeaxanthin; their lower energy state compared to chlorophyll allows them to receive excess energy from chlorophyll according to the gradient and dissipate it as heat (Smith et al., 2012). This process is known as non-photochemical quenching (NPQ), and it may be important for plant tolerance to water stress. In prolonged stress situations, NPQ may be insufficient, resulting in damage to photosystem II (PSII) (Baker and Rosenqvist, 2004). As a result, photosynthetic pigments may degrade and PSII efficiency may decline (El Yamani et al., 2019), according to the measurement of chlorophyll fluorescence of PSII, an effective and non-destructive technique for detecting PSII damage and photoinhibition levels (Zhou et al., 2015). This measurement can be used to calculate a variety of parameters, including electron transport rate (ETR), effective quantum efficiency of PSII (phiPSII), maximum efficiency of PSII (F_v/F_m), photochemical quenching (qP), and NPQ, too (Brito et al., 2019). Finally, carotenoids plays an important role in protection of photosystems, both as precursors of the xanthophyll biosynthetic pathway and antioxidants against ROS

(Pogson and Rissler, 2000; Jaleel et al., 2009). Finally, stomatal density was evaluated. Stomata are located on the lower surface of olive leaves (Bosabalidis and Kofidis, 2002). The stomatal system should maximize gas exchange while minimizing water loss (Zhong et al., 2020). According to some studies, higher stomatal density and smaller stomatal size corresponds to a more precise control of transpiration, based on the "flexible regulation" of stomatal closure (Bacelar et al., 2004; Boughalleb and Hajlaoui, 2011; Bertolino et al., 2019).

This chapter aimed to differentiate the drought tolerance of three Italian olive cultivars by studying mainly physiological and morphological parameters, supported by pigment content and lipid peroxidation, as well as to assess how changes in water distribution and management within the soil, stem, and leaf affected physiological responses.

3.2 Material and Methods

3.2.1 Soil Water Content

The Soil Water Content (SWC) was evaluated according to Bilskie (2001). Four soil samples were collected for each group and immediately weighed to obtain the wet mass (m_{wet}). Then, samples were put in the oven for 24 h at 105°C and then weighed again (m_{dry}). Finally, soil water content was calculated as:

$$SWC = (m_{wet} - m_{dry}) / m_{dry}$$

3.2.2 Relative Water Content of leaves and stems

The relative water content of leaves (leaf RWC) and stems (stem RWC) was calculated as described by El Yamani et al. (2019). Fully expanded and mature leaves at each time point were cut below the petiole and immediately placed in pre-weighed plastic tubes. The leaves were weighed along with the tubes to obtain fresh weight (FW). Stems were harvested only at t0, t2, and t4. For both leaves and stems, tubes were filled with distilled water and samples incubated for 24h at 4°C in the dark. Afterwards, leaves and stems were removed from the tubes and dried with paper towels to absorb excess water. The samples were weighed to determine the turgid weight (TW). Finally, the samples were placed in paper bags and heated in an oven at 80°C for 48 hours. The samples were weighed to determine the dry weight (DW). The RWC of leaves and stems was calculated as:

$$RWC\ (\%) = (FW - DW) / (TW - DW) \cdot 100$$

3.2.3 Stomatal density

Stomatal density was measured according to Xu et al. (2008). Briefly, 5 mature leaves per group were selected at t0, t2 and t4. The abaxial epidermis of the leaf was coated with clear nail polish. Once dried, the film was peeled off the leaf and placed on a slide over a drop of water. The samples were examined with the Zeiss Axiophot light microscope (Oberkochen, Germany). Six images were taken for each leaf sample (thus a total of 30 images per group). The images were analyzed with ImageJ. Stomatal density was then calculated as the number of stomata per leaf area.

3.2.4 Leaf gas exchange and chlorophyll fluorescence

The LiCor-6800 instrument (LICOR, Lincoln, USA) equipped with a leaf chamber fluorometer was used to assess gas exchange and chlorophyll fluorescence. Light-saturated net photosynthetic rate (A) and g_s were recorded throughout the experiment (t0 to t4). During the gas exchange measurements, photosynthetic active radiation (PAR) was set at 1600 µmol m^{-2} s^{-1} (Diaz-Espejo et al., 2007; Rodriguez-Dominguez et al., 2016), CO_2 at 400 ppm, block temperature at 28 °C and relative humidity at 60% inside a leaf cuvette. In addition, CO_2 assimilation rate curves against intercellular CO_2 concentration (i.e. A/Ci curves) were obtained at t2 by using the following 12 CO_2 concentration steps: 400, 200, 50, 100, 300, 400, 600, 800, 1000, 1200, 1400, 1600 ppm. A-Ci curves data were elaborated using the approach described by Ethier & Livingston (2004) to obtain maximum carboxylation efficiency (V_{cmax}) and maximum rate of electron transport (J_{max}). A/Ci and A/Cc curves are provided as supplementary material, **Figure S3.1**. This assumes that gm was constant throughout the CO_2 range. The V_{cmax} and J_{max} values were standardized at 25 °C using a temperature dependency of those parameters (Bernacchi et al., 2001). Michaelis-Menten constants for CO_2 (Kc) and O_2 (Ko) were derived according to the approach by Bernacchi et al. (2001). To calculate the gm values, the variable J method was applied for calculating the A/Cc curves as the use of an independent methodology should be preferable for preventing the propagation of errors or assumptions (Pons et al., 2009). The variable J method was applied to calculate g_m (Loreto et al., 1992) based on the point measurement of A at 400 ppm of Ca with fluorescence measurements:

$g_m = A / (Ci - \Gamma^* [ETR + 8 (A + Rd)] / [ETR - 4 (A + Rd)])$

where: Rd is the day respiration, which was obtained from the previous study on olive leaves (1.39 μmol m^{-2} s^{-1} (Marchi et al., 2007)), Γ* is the CO_2 compensation point to photorespiration, which was calculated using the Rubisco specificity factor estimated for evergreen woody species (Galmés et al., 2005). The fluorescence of chlorophyll was evaluated throughout the experiment (t0 to t4) by the same apparatus with the activated fluorometer (rectangular flash with a red target of 8000 μmol m^{-2} s^{-1}, a duration of 1000 ms, and the output rate of 100 Hz (Loriaux et al., 2013)). Light-adapted leaves were used to obtain the effective efficiency of PSII (ΦPSII) and the electron transport rate (ETR) according to Gilbert et al. (2012). The concentration of CO_2 at the chloroplast envelope (Cc) was estimated using the g_m value.

$Cc = Ci - A / g_m$

Relative photosynthetic limitations were calculated according to Grassi & Magnani (2005) as follows:

$L_s = (g_{tot} / [g_s / 1.6] \delta A / \delta Cc) / (g_{tot} + \delta A / \delta Cc)$

$L_m = (g_{tot} / g_m \delta A / \delta Cc) / (g_{tot} + \delta A / \delta Cc)$

$L_b = g_{tot} / (g_{tot} + \delta A / \delta Cc)$

where L_s, L_m and L_b are the relative limitations by stomatal diffusion, mesophyll diffusion and biochemical limitation, respectively. g_{tot} is the total CO_2 conductance ($g_{tot} = [(g_s / 1.6) g_m] / [(g_s / 1.6) + g_m]$), 1.6 is the ratio of the diffusion coefficients for water vapor to CO_2 and δA/δCc indicates an initial slope of A/Cc curves that was estimated using a range of 0-150 μmol m^{-2} s^{-1} of Cc. In addition, the maximum quantum yield of PSII (F_v/F_m) was evaluated on dark-adapted leaves covered with aluminium foil for at least 20 minutes. One fully expanded leaf per five to six plants per treatment were used for each gas exchange and fluorescence parameter.

3.2.5 Electrolyte Leakage

Cell membrane permeability to solutes was assessed by measuring electrolyte leakage (EL) according to ben Abdallah et al. (2018). Two leaf discs with 0.5 cm diameter were cut from a fresh leaf and placed inside capped tubes filled with 10 ml deionized water. The samples were incubated for 3 h at 37°C. Immediately after incubation, the conductivity of the solution was

measured to obtain the electrical conductivity E1 value. Then, the samples were heated at 95°C for 30 min before measuring the conductivity again (E2). The EL was calculated as:

$EL = E1 / E2 \cdot 100$

3.2.6 Malondialdehyde content

Malondialdehyde (MDA) content was used to quantify lipid peroxidation in leaves. Frozen leaves (0.1 g) were ground with 1.5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The samples were centrifuged at 10000 g for 10 min at 4°C. Then, 0.25 mL of the supernatant was mixed with 1 mL of 20% (w/v) TCA containing 0.5% (w/v) thio-barbituric acid for the positive control; the same supernatant was mixed with 1 mL of 20% (w/v) TCA alone for the negative control. The samples were incubated for 30 min at 95°C; then, the extracts were immediately cooled on ice and centrifuged (10000 g for 10 min at 4°C). Supernatants from positive and negative controls were read at 600, 532 and 400 nm in a microplate reader EnSpire (PerkinElmer). MDA equivalents were calculated according to Hodges et al. (1999) and then normalized to dry weight.

3.2.7 Pigments quantification

Chlorophyll a, chlorophyll b and carotenoids were extracted in pure acetone and quantified spectrophotometrically according to Lichtenthaler (1987). Briefly, 1.5 mL of cold 100% acetone was added to ground frozen leaves (500 mg). After 2 to 3 min of agitation, the samples were centrifuged (15 000 g, for 15 min, at 4°C) and the supernatants were collected in a new tube. The pellets were extracted again twice. The pool of extracts was then read at 662, 645 and 470 nm with a Shimadzu UV-1280 spectrophotometer. Pigment content was then normalized to dry weight.

3.2.8 Statistical analysis

Each analysis included at least five biological replicates (n = 5). For all the parameters recorded at different time points, the effects of the drought treatment (S), cultivar (C), and their interaction (C x S) were evaluated using a 2-way Repeated ANOVA analysis. For the MDA content, g_m, L_s, L_m, L_b, V_{cmax}, and J_{max} which were measured only at t2, the effects of the treatment (S), cultivar (S), and their interaction (C x S) were analysed by using a 2-way ANOVA. At each time-point, post-hoc analysis was performed by Tukey HSD test. ANOVA

and post-hoc tests were performed by the Systat 11 statistical package (Systat Software Inc., Richmond, CA, USA). At each time point, the bar graphs show the mean and the standard error of the recorded parameters and the significant differences according to the post-hoc test ($p < 0.01$). In the pie chart (**Figure 3.8**), the percentages shown are the averages of five values calculated independently for each limitation (L_s, L_m, L_b). Eight parameters were taken into account to create the correlogram (**Figure 3.9**). First, each parameter was expressed as the ratio of the DS group value to the CTRL group value at each time-point. Then Rstudio (ver. 4.2.2, R core team, 2022) was used for correlation according to the time course.

3.3 Results

3.1. Drought effects on plant water status and biochemical responses

This first section contains the results organized as follows. **Table 3.1** displays the variability of nine physiological parameters, as well as the significance of two factors (cultivar and treatment) and their mutual interaction as determined by ANOVA. Furthermore, only the data that showed a significant interaction between cultivar and treatment (C x S) were fully described.

Table 3.1. The effects of factors "cultivar" (C), "treatment" (S), and their interaction (C x S), as well as their statistical significance, on the following parameters: soil water content (SWC), relative water content of leaves (leaf RWC) and stems (stem RWC), stomatal density (SD), stomatal conductance (g_s), electrolyte leakage (EL), lipid peroxidation (as measured by malondialdehyde content, MDA), photosynthetic pigments content of chlorophyll a and b (Chl a+b) and carotenoids (Car). Each value represents the mean ± standard deviation.

	SWC (%)	leaf RWC (%)	stem RWC (%)	SD (n. mm^{-2})	g_s (mol m^{-2} s^{-1})	EL (%)	MDA (mmol kg^{-1} DW)	Chl a+b (µg mg^{-1} DW)	Car (µg mg^{-1} DW)
Cultivar (C)									
Giarraffa	89 ±52[a]	64.0 ±16.1	69.2 ±17.9	34.4 ±7.6[c]	0.086 ± 0.039[b]	26.3 ±12.5[b]	5.28 ±0.98[b]	3.93 ±0.82	0.70 ±0.15
Leccino	76 ±50[b]	60.5 ±15.1	66.7 ±17.0	38.4 ±5.0[b]	0.070 ± 0.040[c]	27.4 ±13.3[b]	3.78 ±0.50[c]	3.66 ±0.71	0.65 ±0.13
Maurino	76 ±49[b]	61.3 ±17.6	69.4 ±20.0	48.4 ±5.4[a]	0.112 ± 0.051[a]	37.7 ±21.1[a]	7.26 ±1.33[a]	3.49 ±1.93	0.63 ±0.32
p-value	**0.014**	**0.080**	**0.051**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.837**	**0.649**
Treatment (S)									
CTRL	117 ±26[a]	82.0 ±8.6[a]	88.0 ±2.1[a]	40.1 ±9.3	0.119 ± 0.037[a]	21.9 ±4.8[b]	4.93 ±1.67[b]	3.43 ±1.17	0.61 ±0.19
DS	26 ±21[b]	50.9 ±10.8[b]	64.4 ±9.8[b]	40.7 ±7.6	0.052 ± 0.029[b]	41.2 ±20.1[a]	5.94 ±1.72[a]	4.10 ±1.31	0.73 ±0.23
p-value	**< 0.001**	**< 0.001**	**< 0.001**	**0.203**	**< 0.001**	**< 0.001**	**0.004**	**0.116**	**0.143**
C *x* S									
p-value	**0.715**	**0.041**	**0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.422**	**0.636**	**0.824**

The first parameter we monitored was Soil Water Content (SWC), which was found to be strongly affected by DS treatments. 'Giarraffa' exhibited a higher value of SWC (89% compared to 76% of the other cultivars). However, the significance of the interaction (C x S) was not relevant, meaning that the SWC was not affected by the cultivar growing in the soil but only by treatment. The graph of SWC data is available as supplementary material, **Figure S3.2**. Drought stress also had a significant effect (*p-value* < 0.001) on leaf RWC data, but in this case also cultivar and the interaction C x S were significant (**Table 3.1**). As shown in **Figure 3.1**, the drought-stressed groups of 'Giarraffa' and 'Leccino' retained less water than 'Maurino' DS after the first week of stress. While the difference between control and stressed groups was significant for all cultivars at t1, 'Giarraffa' DS did not differ from its respective control one week later.

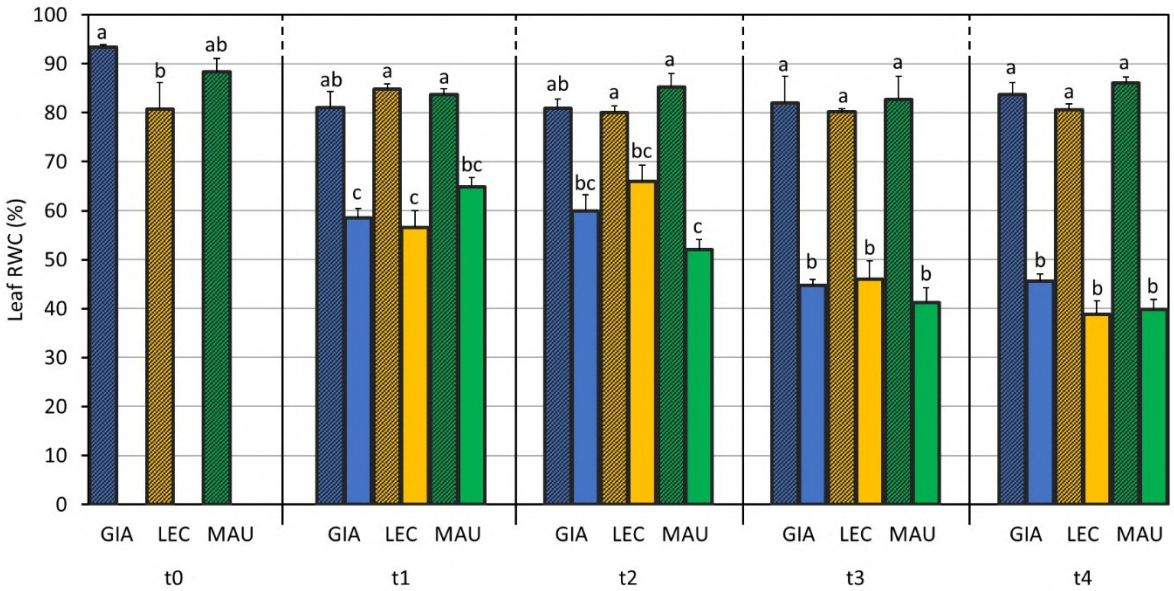

Figure 3.1. Leaf Relative Water Content (leaf RWC) measured in control and drought olive cultivars, from t0 to t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. Values for 'Giarraffa' (GIA) are in blue, 'Leccino' (LEC) in orange and 'Maurino' (MAU) in green. Striped bars indicate control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

Table 3.1 shows that, considering both CTRL and DS, the 'Giarraffa' stem contains more water than the cultivar Leccino (69.2% and 66.7%, respectively). The higher value recorded in the cultivar Maurino is probably due to the lack of samples at t4. Drought stress reduced stem RWC, resulting in 88.0% in controls and 64.4% in stressed samples. **Figure 3.2** shows that all cultivars lost stem water as stress progressed, with 'Maurino' DS having a lower and significantly

different stem RWC value than 'Giarraffa' DS, at t2. Stems stored more water than leaves as the stress increased, with 10% more water at t0 up to 20% at t4.

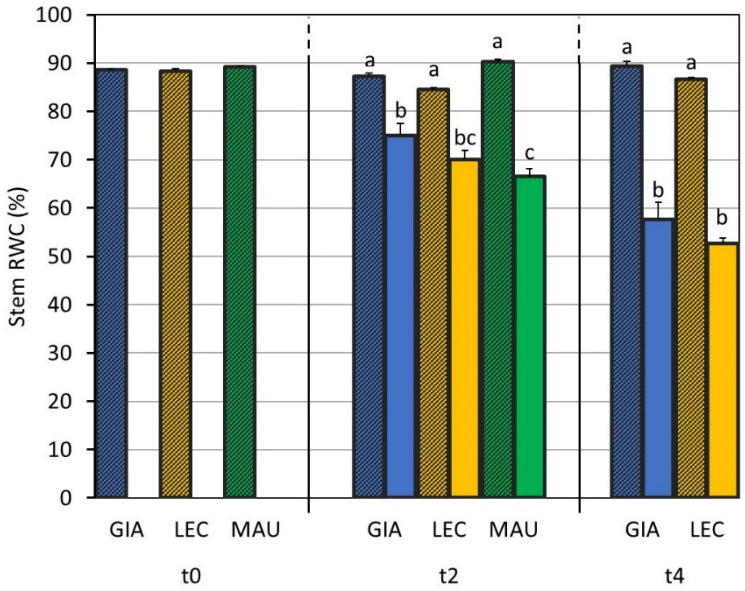

Figure 3.2. Stem Relative Water Content (stem RWC) measured in control and drought olive cultivars analysed at t0, t2, and t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. Values for 'Giarraffa' (GIA) are in blue, 'Leccino' (LEC) in orange and 'Maurino' (MAU) in green. Striped bars indicate control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment. MAU values at t4 are not shown due to technical problems.

Stomatal density (SD) affects the exchange rate of plants with the external environment; since the sampled mature full-expanded leaves had developed before the experiment, drought treatment had no effects on this parameter (**Table 3.1**). However, cultivar (C) had a significant impact on it, with the average number of stomata per mm^2 being 34.4 for 'Giarraffa', 38.4 for 'Leccino', and 48.4 for 'Maurino'.

The stomatal conductance, i.e. the water vapour flux through a leaf sample, showed a significant difference for each factor considered (C, S, C x S) (**Table 3.1**). The cultivar Maurino showed the highest values of g_s (0.112 mol m^{-2} s^{-1}) and it had a higher g_s even when comparing only the control groups of the cultivars, as shown in **Figure 3.3**. Drought stress had a strong effect (*p-value* < 0.001), and all the stressed groups had lower and significantly different g_s value compared to the controls from t2, but 'Giarraffa' DS already differed from the respective control at t1. The lowest value of g_s was reached by all stressed groups of the cultivars at t4.

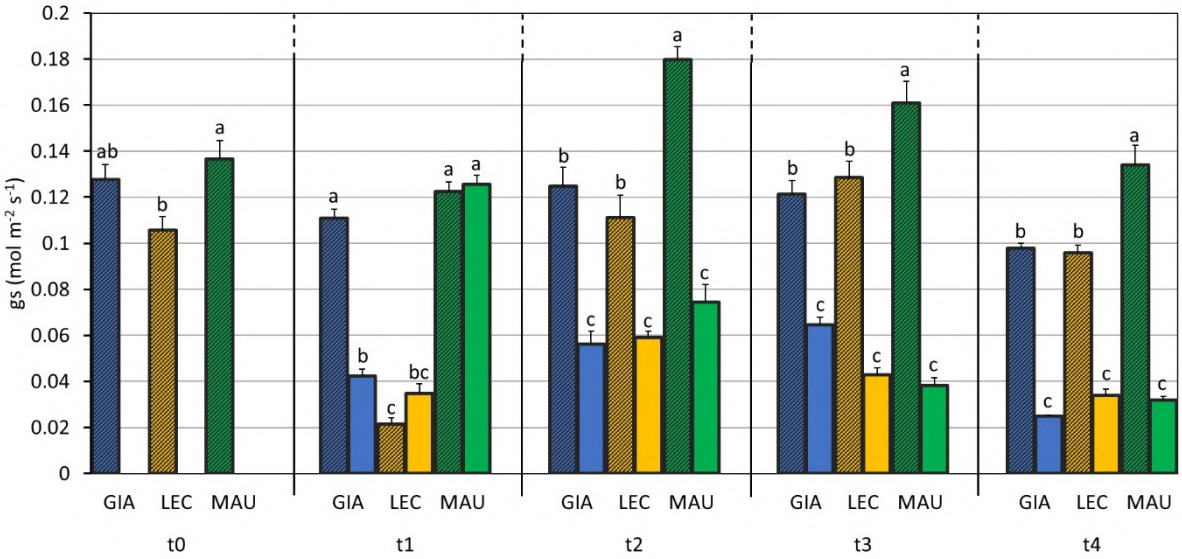

Figure 3.3. Stomatal conductance (g_s) in control and drought-stressed olive cultivars, from t0 to t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. The values for 'Giarraffa' (GIA) are in blue, those for 'Leccino' (LEC) in orange, 'Maurino' (MAU) in green. Strip bars refer to control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

Electrolyte leakage (EL) is the loss of electrolytes from cells or tissues, which can be caused by a variety of factors such as physical injury or disease. This parameter was found to be strongly influenced by the factors C, S, and their interaction (**Table 3.1**). As shown in **Figure 3.4**, EL increased progressively and significantly from t1 to t4, and the stress condition resulted in a two-fold increase in value in the stressed samples (41.2%) compared to the control samples (21.9%). 'Maurino' was the cultivar more strongly affected, showing a consistent increase in the EL value already at t2. After an additional week of stress (t3), 'Giarraffa' DS (40.2%) and 'Leccino' DS (42.6%) differed significantly compared to their respective controls. At t4, the stressed group of each cultivar scored the highest EL value.

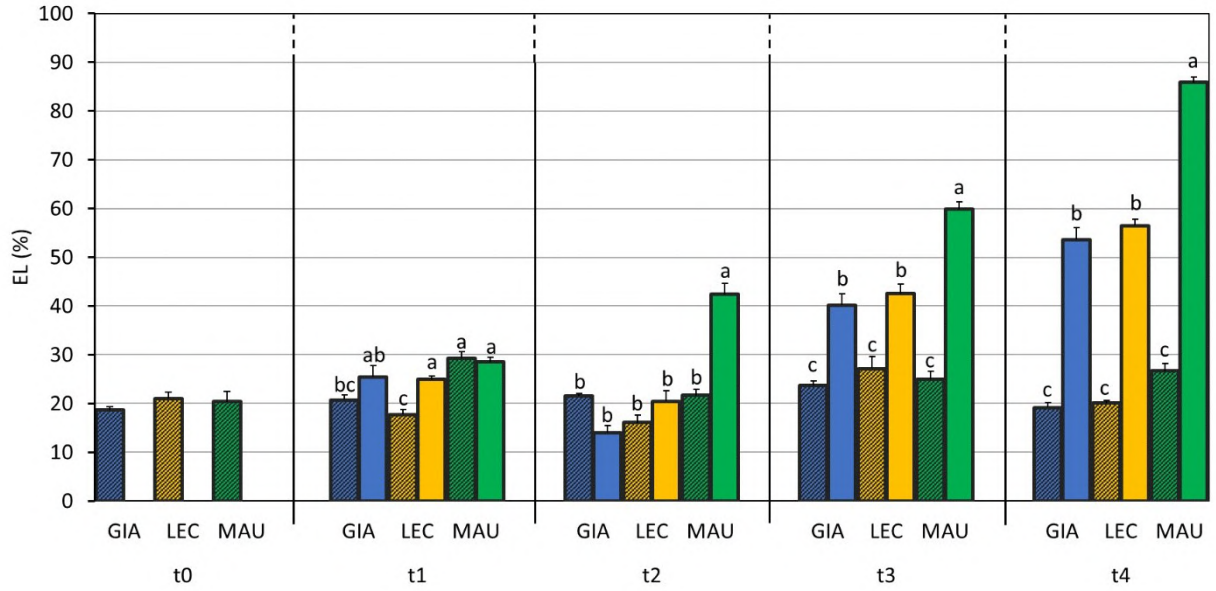

Figure 3.4. Electrolyte leakage (EL) in control and drought-stressed olive cultivars, from t0 to t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. The values for 'Giarraffa' (GIA) are in blue, those for 'Leccino' (LEC) in orange, 'Maurino' (MAU) in green. Strip bars refer to control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

Malondialdehyde (MDA) content is proportional to the intensity of lipid peroxidation and was analysed only at t2 for the sole purpose of saving leaves samples. The cultivar Leccino showed the lowest MDA content, followed by 'Giarraffa' (5.28 mmol kg^{-1} dw) and 'Maurino' (7.26 mmol kg^{-1} dw). Drought-stressed samples exhibited a slightly higher value than control samples, but no statistical difference was observed regarding the interaction C x S. MDA data has not been shown but made available as supplementary material, **Figure S3.3**. **Table 3.1** also shows the content of major photosynthetic pigments. Carotenoids (Car) and chlorophylls a+b (Chl a+b) were not affected by the variables studied and remained constant throughout the experiment (Supplementary Material, **Figures S3.4** and **S3.5**).

3.2. Impact of water deficit on the photosynthetic process

Table 3.2 shows the variability of ten photosynthetic parameters, as well as the significance of two factors (cultivar and treatment) and their mutual interaction, as determined by 2-way Repeated ANOVA. Similar to the previous section, only data where the interaction between cultivar and treatment (C x S) was significant were fully described.

Table 3.2. Effects of the factors "cultivar" (C), "treatment" (S), and their interaction (C x S) and their statistical significance on the following parameters: Net photosynthetic rate (A), effective efficiency of photosystem II (Φ PSII), maximum efficiency of photosystem II (F_v/F_m), electron transport rate (ETR), mesophyll conductance (g_m), stomatal limitation (L_s), mesophyll conductance limitation (L_m), biochemical limitation (L_b), maximum rate of carboxylation (V_{cmax}), maximum electron transport rate (J_{max}). Each value is the mean ± standard deviation.

	A (μmol m^{-2} s^{-1})	**Φ PSII**	**F_v/F_m**	**ETR (μmol m^{-2} s^{-1})**	**g_m (mol m^{-2} s^{-1})**	**L_s (%)**	**L_m (%)**	**L_b (%)**	**V_{cmax} (μmol m^{-2} s^{-1})**	**J_{max} (μmol m^{-2} s^{-1})**
Cultivar (C)										
Giarraffa	7.79 ± 3.87^{b}	0.149 ± 0.032^{b}	0.797 ± 0.048	100.6 ± 21.5^{b}	0.110 ± 0.043	44.5 ± 10.7^{a}	27.5 ± 10.4	28.0 ± 17.9	57.1 ± 13.4	74.9 ± 16.2^{ab}
Leccino	7.14 ± 3.78^{b}	0.154 ± 0.034^{ab}	0.797 ± 0.055	100.1 ± 19.3^{b}	0.139 ± 0.077	43.9 ± 5.2^{ab}	21.6 ± 8.0	34.5 ± 12.7	55.8 ± 12.1	87.4 ± 13.4^{a}
Maurino	10.66 ± 5.48^{a}	0.166 ± 0.039^{a}	0.800 ± 0.047	111.7 ± 26.1^{a}	0.112 ± 0.053	38.9 ± 11.6^{b}	27.5 ± 11.1	33.6 ± 14.7	49.1 ± 23.0	68.8 ± 19.4^{b}
p-value	**< 0.001**	**< 0.001**	**0.860**	**< 0.001**	**0.061**	**0.026**	**0.295**	**0.196**	**0.520**	**0.054**
Treatment (S)										
CTRL	11.38 ± 3.40^{a}	0.172 ± 0.031^{a}	0.823 ± 0.010^{a}	113.9 ± 19.6^{a}	0.156 ± 0.058^{a}	34.8 ± 6.1^{b}	21.9 ± 6.9^{b}	43.3 ± 6.1^{a}	52.8 ± 12.4	76.2 ± 17.9
DS	4.96 ± 3.46^{b}	0.137 ± 0.031^{b}	0.768 ± 0.061^{b}	91.9 ± 21.1^{b}	0.087 ± 0.037^{b}	50.7 ± 6.2^{a}	30.7 ± 11.4^{a}	18.6 ± 11.5^{b}	54.9 ± 21.9	75.2 ± 18.6
p-value	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.015**	**< 0.001**	**0.706**	**0.945**
C *x* S										
p-value	**< 0.001**	**0.001**	**0.091**	**0.034**	**0.732**	**0.067**	**0.140**	**0.271**	**0.419**	**0.058**

As shown in **Table 3.2**, Net CO_2 assimilation (A) is significantly influenced by cultivar, drought, and their interaction. The cultivar Maurino showed a higher value of A than 'Giarraffa' and 'Leccino', even when comparing the control groups (**Figure 3.5**). Drought stress generally reduced A, but the earliest reduction occurred in 'Giarraffa' DS at t1. From t2, the stressed groups of all cultivars significantly differed from their respective controls.

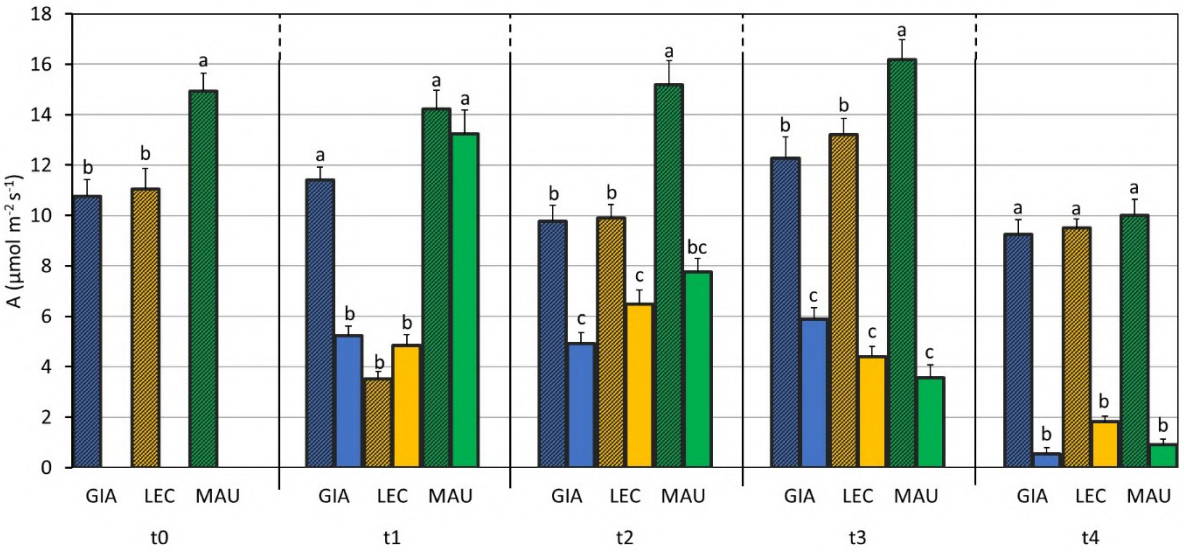

Figure 3.5. Net CO_2 assimilation (A) in control and drought-stressed olive cultivars, from t0 to t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. The values for 'Giarraffa' (GIA) are in blue, those for 'Leccino' (LEC) in orange, 'Maurino' (MAU) in green. Strip bars refer to control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

Figure 3.6 shows the actual PSII efficiency. It was found that ΦPSII was affected by cultivar, drought stress and their interaction (**Table 3.2**). The cultivar Leccino showed the first difference between control and stressed group at t1. From t2, the significant difference between control and stressed group appeared in the cultivar Maurino. Only at t4, 'Giarraffa' DS was significantly lower compared to its respective control.

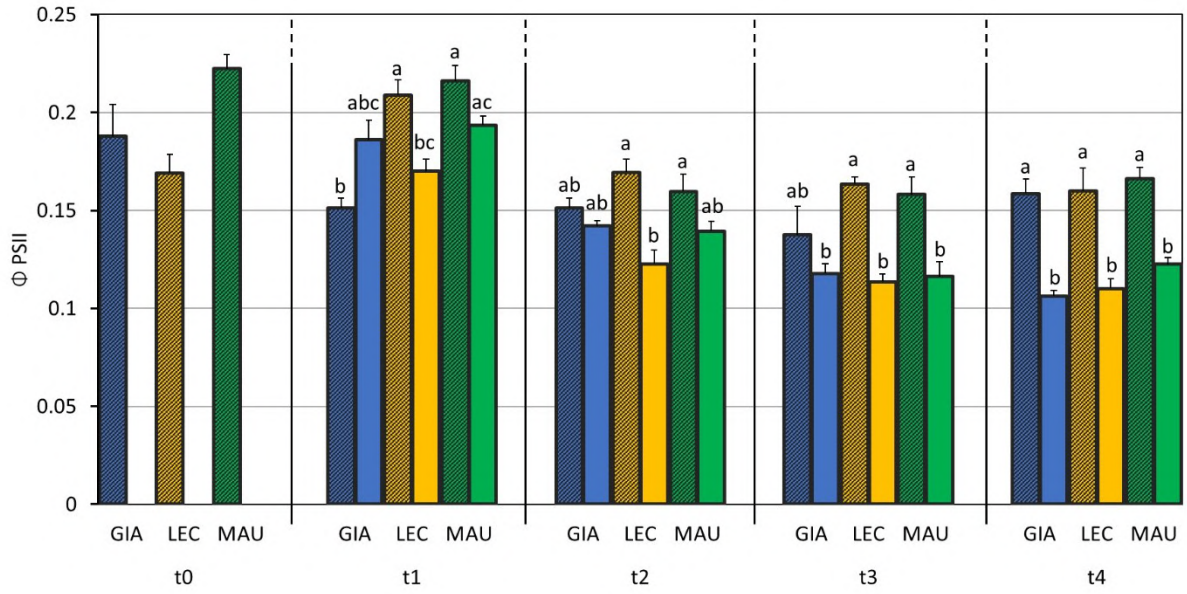

Figure 3.6. Effective efficiency of PSII (ΦPSII) in control and drought-stressed olive cultivars, from t0 to t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. The values for 'Giarraffa' (GIA) are in blue, those for 'Leccino' (LEC) in orange, 'Maurino' (MAU) in green. Strip bars refer to control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

In contrast, there were no differences in F_v/F_m among cultivars. However, drought stress affected the maximum efficiency of PSII, but the cultivars responded similarly in relation to the stress (C x S, *p-value* = 0.091). All cultivars showed a decrease in F_v/F_m starting from t3, with the lowest values in DS groups (0.68 for 'Giarraffa' DS, 0.69 for 'Leccino' DS, and 0.70 for 'Maurino' DS) at t4. The graph is available as supplementary material, **Figure S3.6**.

Conversely, cultivar (C), drought stress (S) and their interaction (C x S) significantly affected the ETR (**Table 3.2**). As shown in **Figure 3.7**, drought stress slowed down the ETR of all stressed groups, but 'Leccino' showed the first difference already at t2 ('Leccino' CTRL 110.7 µmol m^{-2} s^{-1}, 'Leccino' DS 82.6 µmol m^{-2} s^{-1}, $p < 0.01$), when the stressed groups of the other two cultivars are still comparable with their respective controls. A significant difference between CTRL and DS appeared in 'Maurino' at t3, and in 'Giarraffa' only at t4.

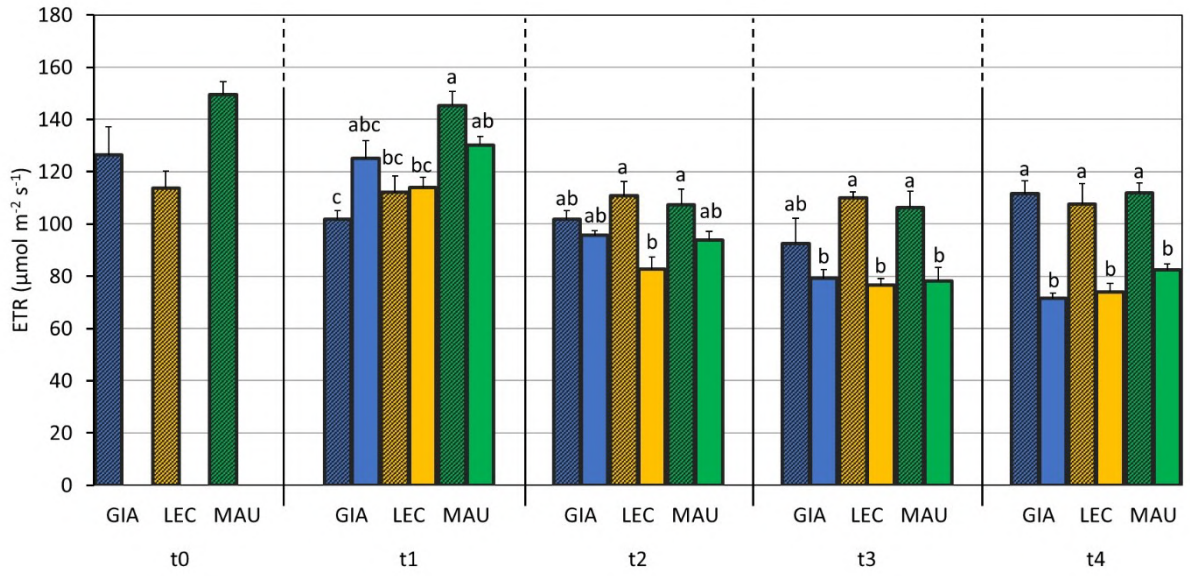

Figure 3.7. Electron Transport Rate (ETR) in control and drought-stressed olive cultivars, from t0 to t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. The values for 'Giarraffa' (GIA) are in blue, those for 'Leccino' (LEC) in orange, 'Maurino' (MAU) in green. Strip bars refer to control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

Table 3.2 shows no differences in mesophyll conductance among cultivars, despite a significant decrease in g_m caused by stress, resulting in 0.156 ± 0.058 mol m^{-2} s^{-1} in controls and 0.087 ± 0.037 mol m^{-2} s^{-1} in stressed groups. The graph of g_m calculated at t0, t2, and t4 is available as supplementary material, **Figure S3.7**.

As a result, drought stress significantly affected the stomatal (L_s), mesophyll conductance (L_m) and biochemical (L_b) limitation of the photosynthesis at t2 (**Table 3.2**). Specifically, after two weeks of stress, the contribution of L_s and L_m was 16% and 9% higher respectively in the stressed groups compared to the control, at the expense of biochemical limitation, which decreased. 'Giarraffa' and 'Maurino' showed a significant higher stomatal limitation in the stressed group compared to their respective controls, while the L_s in 'Leccino' DS and CTRL are similar, as shown in **Figure 3.8**. As suggested by the low biochemical limitation of the stressed groups, V_{cmax} and J_{max}, the maximum carboxylation rate and the maximum electron transport rate, respectively, did not seem to be significantly affected by the stress treatment (**Table 3.2**). The data are plotted in the Supplementary Material, **Figures S3.8** and **S3.9**.

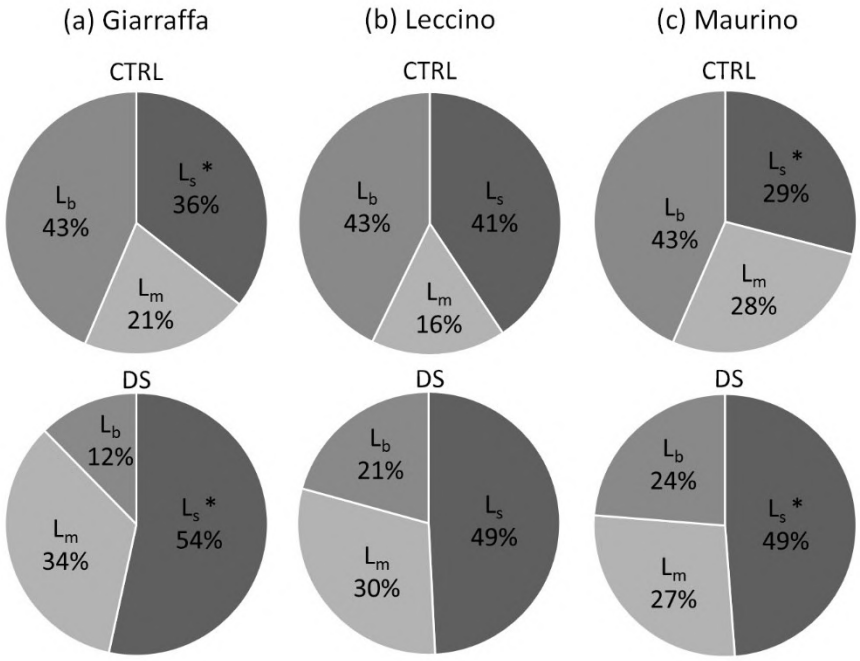

Figure 3.8. Percentages of stomatal limitation (L_s), mesophyll diffusion resistance (L_m) and biochemical limitation (L_b) on photosynthetic process in control and drought-stressed olive cultivars, calculated for 'Giarraffa' (A), 'Leccino' (B), and 'Maurino' (C) at t2. The percentages shown for each group are the means of five values calculated independently for each limitation (L_s, L_m and L_b). The asterisks denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc test. Only the significant difference between control and stressed samples of the same cultivar is highlighted.

3.3. Correlation

Figure 3.9 shows the correlogram generated for each cultivar. When the cultivars were examined together, they produced very similar results. Firstly, all parameters, with the exception of EL, show a positive correlation. Therefore, the main difference is the magnitude of the correlation. When examining the correlations between the water-related parameters (SWC, RWC and g_s), the cultivars behaved differently: the cultivar Maurino had the highest positive correlation between all these parameters. 'Leccino' showed the same high positive correlation between SWC and g_s, but the value was lower when leaf RWC was correlated with SWC and g_s. In cultivar Giarraffa, the correlations of both leaf RWC and SWC with g_s were low, while that between SWC and leaf RWC was higher than in 'Leccino'. Concerning the correlation between water-related parameters and photosynthesis, 'Maurino' showed the highest correlation between SWC, leaf RWC, g_s and A. The same strong positive correlation was found between A and SWC in 'Leccino'. The correlations between leaf RWC and A (in 'Leccino') and leaf RWC, SWC and A (in 'Giarraffa') are weaker. Moreover, ETR and ΦPSII

were strongly positively correlated with g_s in 'Maurino' and 'Leccino', while they were more positively correlated with leaf RWC in 'Giarraffa'.

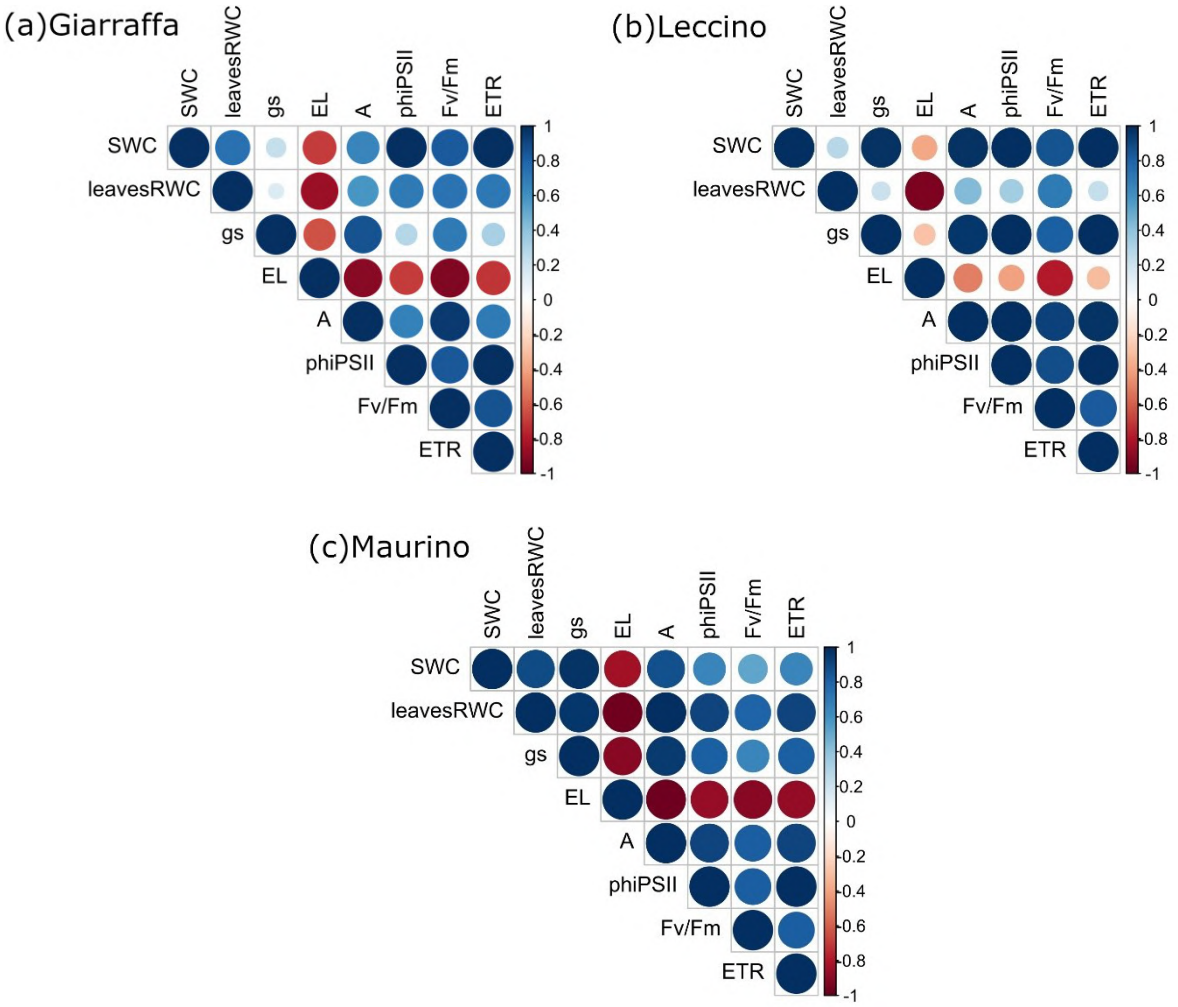

Figure 3.9. Correlogram of the 8 parameters evaluated at each time point in 'Giarraffa' (a), 'Leccino' (b), and 'Maurino' (c). Each parameter was expressed as the ratio of the DS group to the CTRL group at each time point and then correlated according to the time course (t1, t2, t3, t4). The size of the dots corresponds to the value of the correlation coefficient according to the right-positioned scale, while the color indicates the direction of change (blue for positive correlation, red for negative correlation).

3.4 Discussion

Olive trees are one of the most cultivated crops in the Mediterranean region and will face frequent droughts due to climate change. Understanding how water deficit affects olive cultivars differently will help select those that are best suited for future climate change (Ennajeh et al., 2010). This study examined inter-varietal differences in drought stress responses among

three olive cultivars in Italy (Maurino, Leccino and Giarraffa). The primary variables to be studied were plant water relations and photosynthetic traits. Relative water content analysis reveals the distribution of water within plants, while physiological analysis demonstrates how drought affects photosynthesis light reactions.

The first fundamental variable we examined was the amount of water in the soil, which could indicate how well different plants absorb water through their roots. After only one week of stress, all stressed plants had half the soil water content of the controls, indicating that the cultivars remove comparable amounts of water from the soil. This parameter did not distinguish between cultivars, which is consistent with the findings of other studies. In the study by Oddo et al. (2008), for example, the three Italian cultivars tested (Giarraffa, Biancolilla, and Nocellara del Belice) showed no significant differences, and the report indicates a 55% reduction from controls during the first week of stress. There were no discernible differences in the decrease in SWC in plants stressed for two and three weeks compared to plants stressed for one week. Melaouhi et al. (2021) studied the response of two-year-old 'Arbequina' and 'Empeltre' cultivars to mild (30% field capacity) and severe (50% field capacity) stress and found that both treatments resulted in a significant reduction in SWC, but no differences or interactions between cultivars or treatments and cultivars were observed. Using SWC as a benchmark, the current study confirmed that drought stress had an equal impact on all cultivar groups, with the severity directly related to the duration of the water shortage. Some differences emerge if the available water rather than soil water content is considered. In other-than-soil media (such as peat and pumice used in this case) available water in the pots is the difference between the volumetric soil water content determined at psi = −1 kPa and −10 kPa (Pardossi et al., 2009a). Data reported by Pardossi et al. (2009b) defined the range 57/39% of the volumetric soil water content as the available water for peat:pumice 1:1. Considering the gravimetric soil water content of this study, we assume that the control groups of the three cultivars have the maximum available water during the whole period. The stressed group of 'Leccino' and 'Maurino' approached the wilting point at t1, while 'Giarraffa' reached this state one week later. From t2 onwards, the water available to the stressed groups of all cultivars was very limited.

Although the cultivars retained the same amount of water in the soil, their transpiration rates varied consistently. First, each cultivar had a distinct default stomatal density. This morphological difference is perfectly reflected in a varying rate of stomatal conductance. The cultivar Maurino, for example, has the highest stomatal density and the highest stomatal conductance, even when grown in well-watered conditions, followed by 'Leccino' and then 'Giarraffa', which has the lowest stomatal density and the earliest decrease in stomatal conductance. Oddo et al. (2008), on the other hand, found that 'Giarraffa' retained high g_s values after seven days of drought stress (0.4 mol m^{-2} s^{-1}). In this case, 'Maurino' appeared to have stomatal characteristics that were less effective in preventing water loss when compared to the controls of the other two cultivars, but this did not result in an overall lower leaf Relative Water Content, except for t2. As suggested by Bosabalidis and Kofidis (2002), the higher stomatal density of 'Maurino' may allow more precise regulation of transpiration. Drought tolerance of plants is generally associated with their ability to maintain high leaf RWC under drought stress conditions (Flower and Ludlow, 1986). Given that stomatal conductance regulates plant water status (Jones and Tardieu, 1998), the simultaneous decrease in RWC and g_s in 'Giarraffa' at t1 results in a smaller difference between the stressed and control groups at t2 compared to the other two cultivars. On the contrary, in 'Leccino' and 'Maurino', stomatal conductance decreases one week later than leaf RWC, unlike what has been described by Boussadia et al. (2008), Guerfel et al. (2009), and Lawlor (2002). During periods of water scarcity, the stem could serve as a water reservoir (Traversari et al., 2018). At t2, however, the slightly higher water content in the stems of 'Maurino' under the control condition did not provide any support to counteract RWC loss in the leaves at the same time point. The differences between cultivars in water-related parameters became apparent after one or two weeks of stress but were completely lost as the stress level increased (at t3 and t4). Along with its capacity to maintain higher water contents, the rapid reduction in stomatal conductance and low stomatal density may allow 'Giarraffa' to use water resources more conservatively, making this cultivar interesting for future water management research.

Water stress generally reduces stomatal conductance, which decreases the quantity of CO_2 taken up by leaves, limiting net carbon assimilation (A) (Larcher, 2003) and thus affecting plant

growth. All the olive cultivars studied showed a decrease in parameter A, supporting the conclusion reached by Sofo et al. (2009). The authors of that case study looked at the cultivars Biancolilla and Coratina and found that ten days of total water depletion (roughly between t1 and t2 in the current study) resulted in a decrease in A of 11.8 to 3 mol m^{-2} s^{-1} and 14.1 to 1.1 mol m^{-2} s^{-1}, respectively. In the current study, drought-stressed 'Giarraffa' showed an early decrease in A at t1, coinciding with stomatal closure (as indicated by the decrease in g_s) and with a decrease in leaf RWC. Instead, both 'Leccino' and 'Maurino' cultivars showed a delayed decrease in A compared to the decrease in leaf RWC. Guerfel et al. (2009) used leaf water potential to assess plant water status in two olive cultivars ('Chemlai' and 'Chetoui') subjected to 21 days of drought. They found a relationship between leaf water potential and A (R2 0.96 for 'Chemlai' and 0.79 for 'Chetoui'), which can be compared to the behaviour of the stressed group of 'Giarraffa'. Mesophyll conductance is another important diffusive parameter that regulates the transfer of CO_2 from sub-stomatal cavities to the chloroplasts within a leaf (Larcher, 2003). The decrease in g_m was linked to a decrease in A in drought-stressed plants, which played a crucial role in reducing CO_2 availability in the mesophyll, as evidenced by a relative increase in mesophyll conductance limitation under water stress conditions. The underlying mechanisms regulating of g_m under water stress conditions are not fully understood and require further investigation. However, previous studies have reported that g_m may be affected by increasing abscisic acid (ABA) concentrations as the soil dries (Brunetti et al., 2019; Hoshika et al., 2022) or changes in leaf structure (such as cell wall thickness) as an acclimation response to drought (Clemente-Moreno et al., 2019).

According to Grassi and Magnani (2005), the contribution of stomatal, mesophyll conductance, and biochemical limitations can explain a decrease in net CO_2 assimilation under drought stress. In this study, limitations were calculated after two weeks of drought. The main factors limiting photosynthesis are stomatal and mesophyll conductance, confirming that diffusive limitations rather than biochemical impairments play a significant role under intermediate stress conditions (Flexas et al., 2004; Grassi and Magnani, 2005). In particular, water stress increased the overall contribution of stomatal limitation to photosynthesis in 'Giarraffa' and 'Maurino' stressed plants at t2. The lack of biochemical impairment was further demonstrated by the absence of

any effects on the maximum electron transport rate (J_{max}) and the maximum carboxylation capacity (V_{cmax}), at least up to t2, as found in drought-stressed *Q. ilex* by Hoshika et al. (2020, 2022) and in salt-stressed olive trees by Centritto et al. (2003). Chlorophyll fluorescence parameters provide more information on the functionality of the photosynthetic machinery during stress. The decrease in ETR and ΦPSII observed in 'Leccino' at t2 anticipates the PSII impairment shown by the strong reduction in F_v/F_m at t3. The cultivar Giarraffa, which shows the decrease in ETR and ΦPSII only at t4, seems to avoid photosystem impairments thanks to the earlier decrease in g_s and A (Maxwell and Johnson, 2000). The cultivar Maurino showed an intermediate pattern, revealing the first PSII impairments at t3.

Measuring pigment levels can help assess the health of a plant and determine whether it is under abiotic stress (Marino et al., 2014). Indeed, Dias et al. (2018) found that the cultivars Cobrancosa, Cordovil de Serpa, and Cordovil de Castelo Branco showed a decrease in chlorophyll and carotenoid content, most likely due to oxidative stress damage under drought stress. However, no stress-related differences in pigment content were observed in this study, indicating that pigment impairment was not present throughout the experiment. Marino et al. (2014) found similar results: after a dry summer without irrigation, 10-year-old olive trees ('Leccino') showed no significant changes in chlorophyll or carotenoid content. The lack of differences in lipid peroxidation between control and stressed plants in our case study indicates that damage to the lipid bilayer of cell membranes does not occur in the early stages of drought stress. Since no lipid peroxidation is observed (at least until t2), the increase in EL may be due to a preliminary response of the plants to drought stress rather than cell damage. According to Demidchik et al. (2014), EL is primarily caused by the efflux of K^+ through specific channels activated by ROS. K^+ release can cause programmed cell death (PCD) or decrease anabolism in favour of catabolic processes, resulting in energy release. All the cultivars studied increased their EL in response to stress, but 'Maurino' showed a stronger and faster response. The cultivar Maurino could theoretically achieve a faster EL response due to increased water loss and transpiration rate, at least until t2. The rapid reduction of gas exchange (as SWC and RWC decreased) has been shown to benefit the defence of photosynthetic processes under drought

stress in 'Giarraffa', which, despite having a lower A than its control, maintained a higher electron transport rate and effective photosystem II efficiency until the last week of the stress.

3.5 *Bulleted* conclusions

- The three Italian olive cultivars tested in this study responded differently to drought stress in growth chamber experiment, though not all parameters showed significant differences.
- The main physiological changes caused by drought discriminate the cultivars mainly at t1 and t2.
- 'Giarraffa' had the earliest stomatal response, but this was not the determining factor for significantly more water savings in the leaf. Compared to the other cultivars, this cultivar retains more water in the stem and soil.
- 'Maurino' showed highest g_s, both in control and drought stressed conditions.
- In the stressed samples, 'Giarraffa' and 'Leccino' showed significantly lower EL compared to 'Maurino'.

3.6 Supplementary materials of Chapter 3

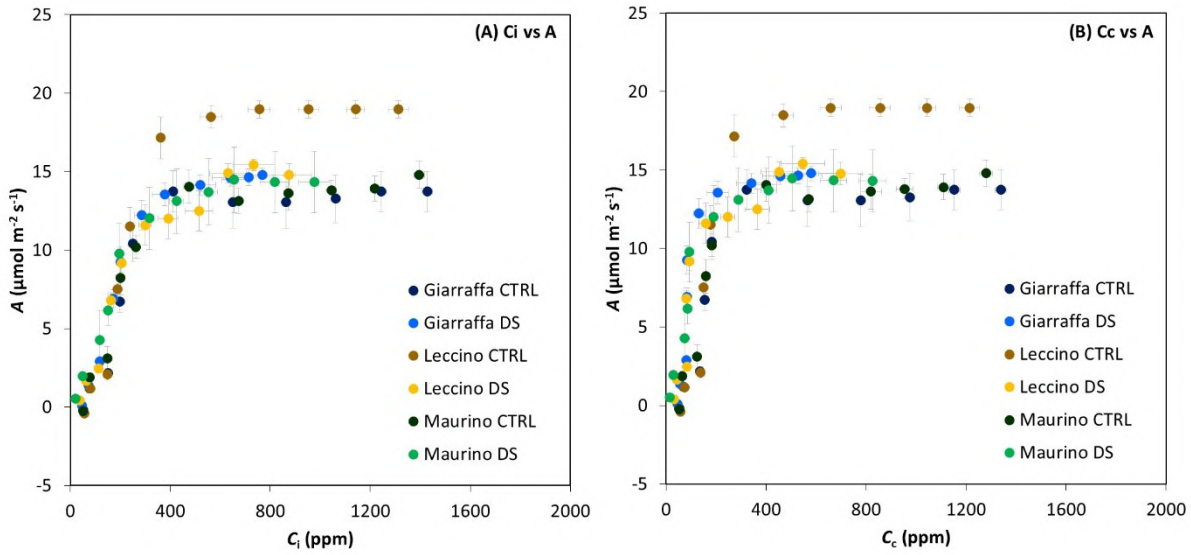

Figure S3.1. Responses of net photosynthetic rate (A) to the sub-stomatal concentration of CO2 (Ci) (A) or the concentration of CO_2 inside the chloroplast envelope (Cc) (B) for three olive cultivars subjected to well watered (CTRL) and drought stress (DS). The A/Ci curves were obtained by measuring A over eleven ambient CO2

concentration steps. Values of g_m derived from the variable J method were used to calculate the A/Cc curves (Ethier and Livingston, 2004). Each value is the mean ± standard error (n = 3 to 6).

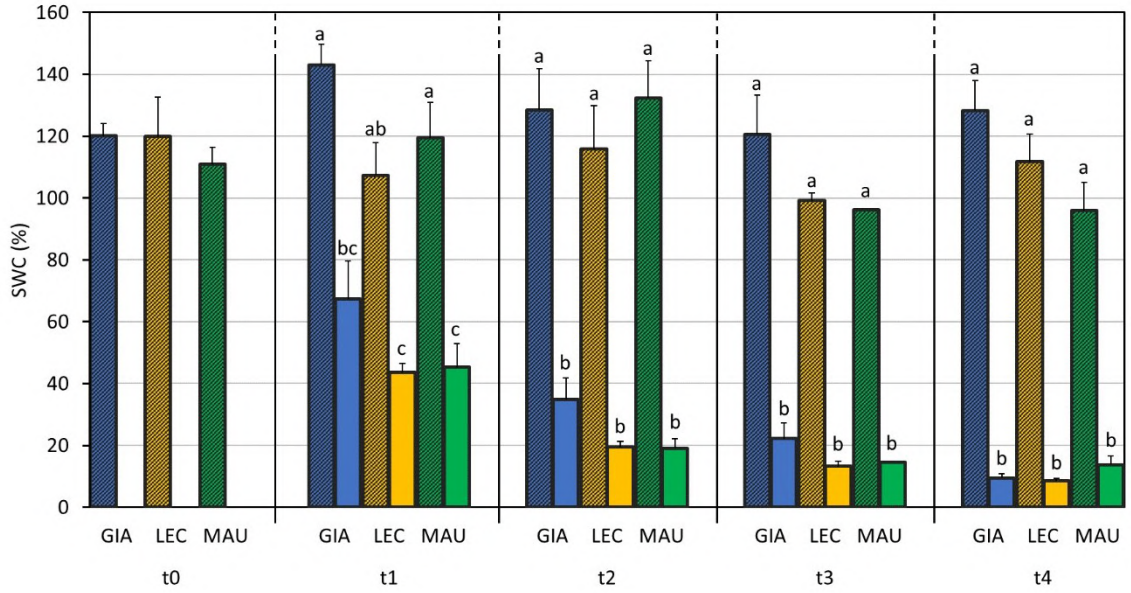

Figure S3.2. Soil Water Content (SWC) in control and drought-stressed olive cultivars from t0 to t4. At t0, stressed and control samples were still one group. The bars represent mean ± standard error. The values for Giarraffa (GIA) are in blue, those for Leccino (LEC) in orange, Maurino (MAU) in green. Strip bars refer to control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment. SWC decreased in drought-stressed samples as early as the first week of stress (t1); by this time, all cultivars were already showing significant differences between control and stress values. During the first three weeks of stress (t1 to t3), the SWC of Giarraffa DS (0.673, 0.350, 0.222, respectively) was always higher than that of Leccino (0.436, 0.194, 0.133) and Maurino (0.453, 0.191, 0.146). The exception was the last time point (t4), when Maurino DS retained more water (0.138) in the soil than Giarraffa (0.095) and Leccino (0.085).

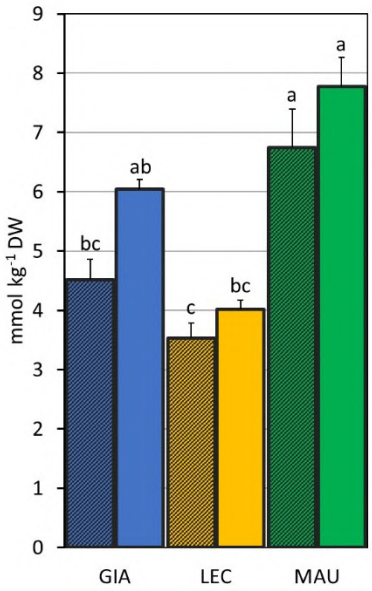

Figure S3.3. Malondialdehyde content (MDA) in control and drought-stressed olive cultivars, at t2. The bars represent mean ± standard error. The values for Giarraffa (GIA) are in blue, those for Leccino (LEC) in orange,

Maurino (MAU) in green. Strip bars refer to control samples Different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

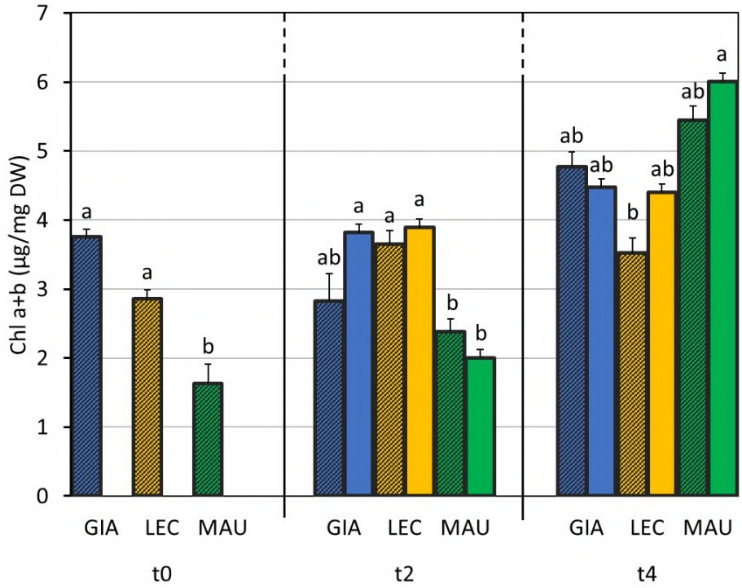

Figure S3.4. Chlorophyll a + b content in control and drought-stressed olive cultivars, at t0, t2, and t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. The values for Giarraffa (GIA) are in blue, those for Leccino (LEC) in orange, Maurino (MAU) in green. Strip bars refer to control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

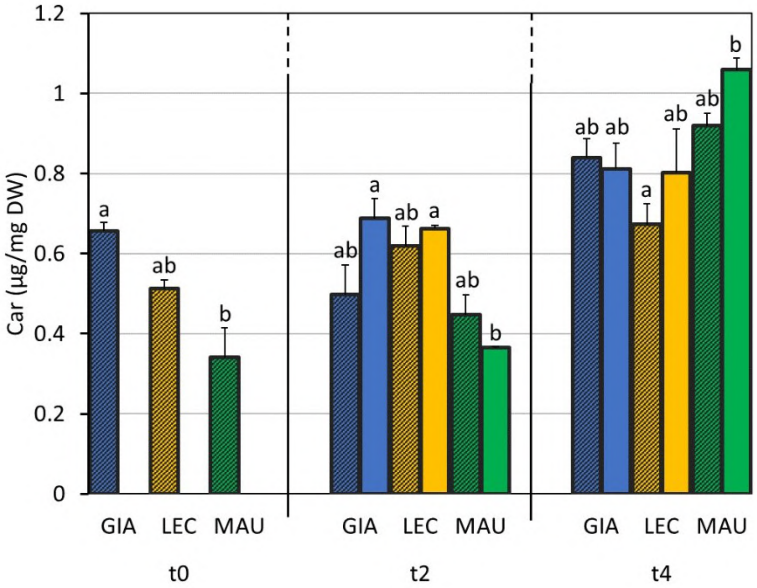

Figure S3.5. Carotenoids content in control and drought-stressed olive cultivars, at t0, t2, and t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. The values for Giarraffa (GIA) are in blue, those for Leccino (LEC) in orange, Maurino (MAU) in green. Strip bars refer to control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

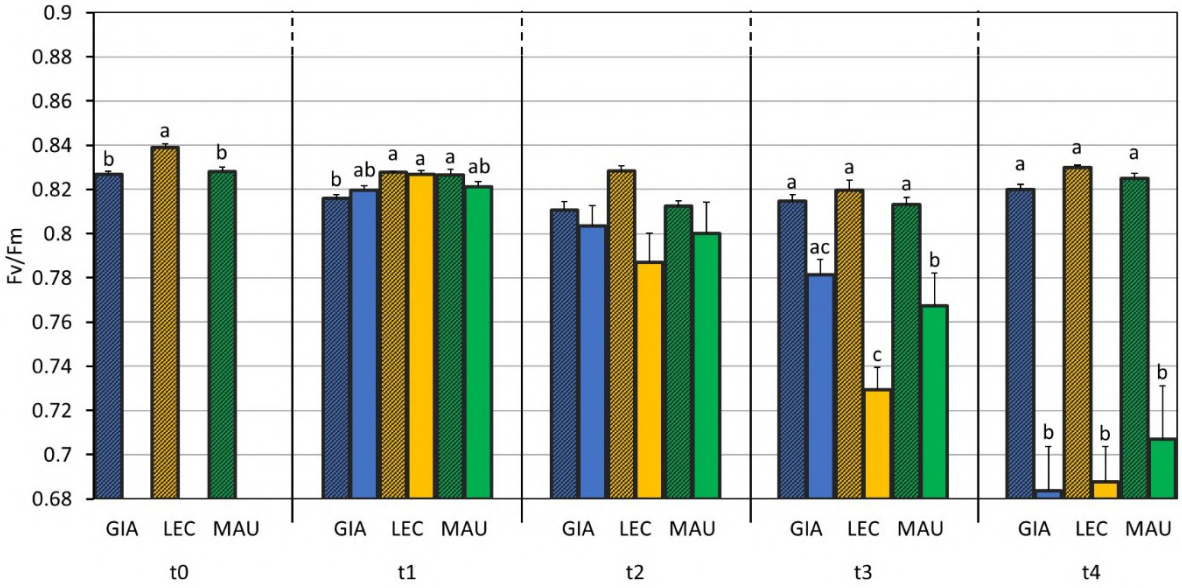

Figure S3.6. Maximum efficiency of PSII (Fv/Fm) in control and drought-stressed olive cultivars, from t0 to t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. The values for Giarraffa (GIA) are in blue, those for Leccino (LEC) in orange, Maurino (MAU) in green. Strip bars refer to control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

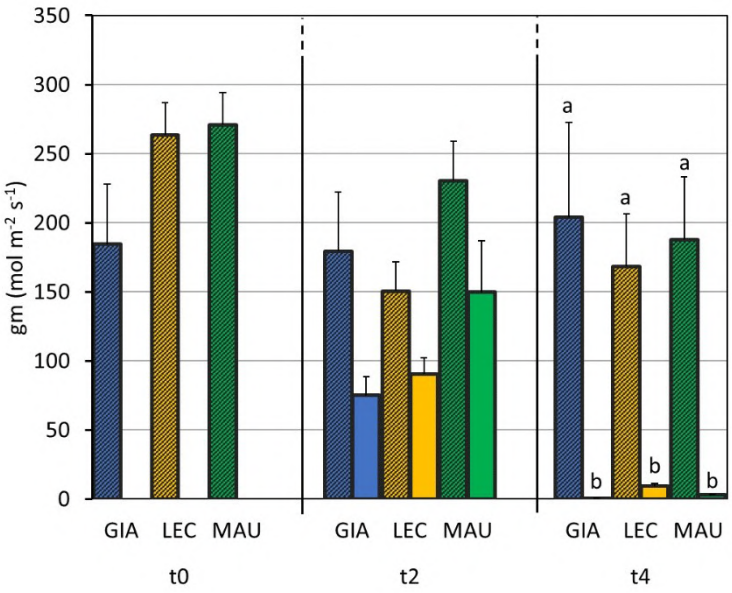

Figure S3.7. Mesophyll conductance (g_m) in control and drought-stressed olive cultivars, at t0, t2, and t4. At t0, the stressed and control samples were still one group. The bars represent mean ± standard error. The values for Giarraffa (GIA) are in blue, those for Leccino (LEC) in orange, Maurino (MAU) in green. Strip bars refer to control samples. Within each time point, different letters denote statistical significance (*p-value* < 0.01) according to Tukey's multiple post hoc tests considering both cultivar and treatment.

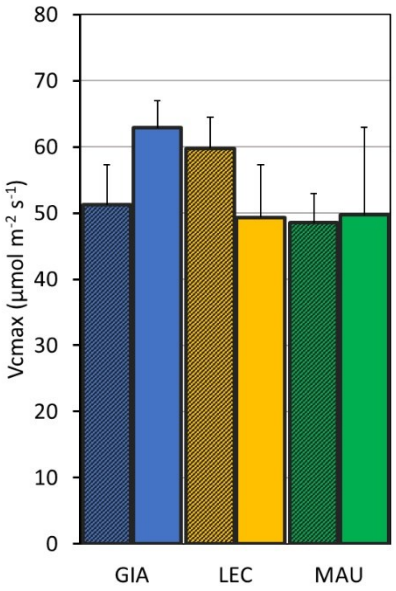

Figure S3.8. Maximum rate of carboxylation (V_{cmax}) in control and drought-stressed olive cultivars, at t2. The bars represent mean ± standard deviation. The values for Giarraffa (GIA) are in blue, those for Leccino (LEC) in orange, Maurino (MAU) in green. Strip bars refer to control samples. Under the monitored conditions, we observed no statistically significant difference between control and stressed samples for any of the cultivars analysed.

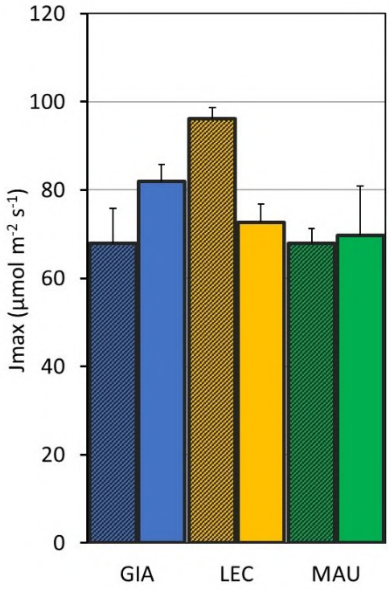

Figure S3.9. Maximum electron transport rate (J_{max}) in control and drought-stressed samples of the olive cultivars, at t2. The bars represent mean ± standard deviation. The values for Giarraffa (GIA) are in blue, those for Leccino (LEC) in orange, Maurino (MAU) in green. Strip bars refer to control samples. Under the monitored conditions, we observed no statistically significant difference between control and stressed samples for any of the cultivars analysed.

Chapter 4

Comparative metabolomics of leaves and stems: phenolic and lipophilic profiling reveal cultivar-specific response patterns

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This chapter is based on:

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4.1 Introduction to the chapter

Metabolic adaptations are part of the defence mechanisms against drought or stress in general. The metabolomic approach is critical for understanding cultivar-specific responses to drought stress because it provides insights into dynamic changes in metabolite profiles. It enables the identification of various metabolic adaptations that underpin the diverse responses of cultivars to drought. For example, sugar metabolism is known to be involved in the response and adaptation of olive trees to drought, including changes in the levels of simple sugars such as sucrose and glucose (Bacelar et al., 2007; Brito et al., 2019; Tsamir-Rimon et al., 2021). Drought stress promotes the accumulation of monosaccharides and disaccharides, which play an important role in drought tolerance; however, sucrose can also produce trehalose-6-phosphate and raffinose, which are equally important (Kumar et al., 2021). In addition to simple or complex carbohydrates, sugar alcohols such as mannitol and sorbitol (an isomer of mannitol) may accumulate in response to drought due to their role in osmotic adjustment (Mechri et al., 2015, 2020a). Thus, in addition to being an important source of energy in plants, carbohydrates can also be used for survival during stress conditions or physiological recovery during rehydration (Dias et al., 2021). In addition to primary metabolism, secondary metabolism helps olive trees adapt to stress conditions. Secondary metabolites are not directly involved in plant growth and development, but they serve a variety of functions in their interactions with the environment and include terpenoids, phenolics, and alkaloids. Secondary metabolites act as scavengers of reactive oxygen species (ROS) to protect plants from lipid peroxidation and oxidative damage under stress. Furthermore, secondary metabolites could systemically alert plant tissues to implement drought stress defence processes (Yadav et al., 2021). A growing body of literature describes the metabolomic approaches that have already been used to identify the primary stress-related metabolites in olive trees and to investigate the most sensitive pathways modulated by drought. Among the phenolic compounds, luteolin-7-*O*-glucoside, quercetin-7-*O*-rutinoside, apigenin-7-*O*-glucoside, chrysoeriol-7-*O*-glucoside, and oleuropein were found to accumulate in olive leaves, most likely to counteract the increase in ROS formation caused by stress (Petridis et al., 2012; Mechri et al., 2020b; Dias et al., 2021). Lupeol, a lipophilic terpenoid, has been shown to have antioxidant properties (Araújo et al., 2021; Dias

et al., 2021). Higher levels of terpenes and long-chain alkanes were associated with leaf cuticle thickness in drought-stressed olive plants (Piccini et al., 2022), whereas increases in saturated/unsaturated fatty acid ratio and sterol content were associated with low membrane permeability (Dias et al., 2021). The majority of studies in the literature on the metabolomics of drought-stressed olive plants concern the leaf system, but there are few studies on the variation of the content of lipophilic and phenolic compounds in drought-stressed olive stems (Mechri et al., 2020b). Jiménez-Herrera et al. (2019) found that the total content of triterpenes and the phenolic compounds oleuropein, hydroxytyrosol, and tyrosol were lower in stems than in leaves; they also reported a decrease in hydroxytyrosol, tyrosol, and total triterpenes and an increase in oleuropein in response to drought. Although the leaves are the primary target of drought stress, the role of stems in mitigating drought stress cannot be underestimated, as it serves as a resource and redistributor of water, limiting the damage caused by water scarcity. It is thus critical to study the metabolic behaviour of both leaves and stems in drought-stressed plants. This could help to uncover previously unknown mechanisms of olive plant adaptation to drought stress, as well as specific mechanisms that individual olive cultivars may have implemented by allocating specific secondary metabolites between the two organs.

In **Chapter 3**, the physiological responses of the three Italian olive cultivars to drought stress differed significantly. To summarize, 'Giarraffa' reduced stomatal conductance (g_s) during the first week of stress, whereas 'Maurino' (which already had a high g_s in the control groups) did so after two weeks. In contrast, 'Leccino' showed an intermediate response pattern. Differences in stomatal conductance and thus transpiration control did not result in significant differences in leaf water content, whereas stem and soil water content were higher in 'Giarraffa'. The differences between varieties under drought stress extended to different degrees of membrane damage; 'Maurino' showed the highest and earliest increase in electrolyte leakage, while 'Giarraffa' showed less electrolyte leakage. Given that 'Maurino' and 'Leccino' did not rapidly adapt stomatal conductance as a defence against membrane damage, we hypothesize and propose that 'Maurino' and 'Leccino' should have invested in other responses, such as metabolic changes, to signal drought and scavenge ROS formation and oxidative stress. In this chapter, we analysed the metabolic changes occurring in Leccino, Maurino, and Giarraffa

cultivars, both in control plants and in plants exposed to two and four weeks of drought stress. We used gas chromatography-mass spectrometry (GC-MS)-based profiling of lipophilic metabolites and ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS)-based profiling of phenolic metabolites. The analysis was performed on both leaves and stems in order to reveal metabolic differences that could explain the differing physiological behaviour of the three cultivars.

4.2 Material and Methods

4.2.1 Phenolic and lipophilic compounds extraction

Frozen olive leaves and stems harvested at t0, t2 and t4 from both control and stressed groups of cultivars 'Giarraffa', 'Leccino' and 'Maurino' were dried at 40°C for 7 days. The dried plant material was then finely ground in a mill to obtain a powder for metabolite extraction. The ground material was combined with n-hexane (1:10 w:v) at room temperature with magnetic stirring for 48 hours. The n-hexane was then decanted, and a second extraction was performed with fresh n-hexane for a further 24 hours. The combined n-hexane extracts were concentrated to dryness in a rotary evaporator under reduced pressure to give the crude extracts, which were air dried for one week. The pellet resulting from the n-hexane extraction was further air dried and then subjected to extraction with 50 mL of methanol to isolate phenolic compounds. The methanol extraction involved two cycles: a first cycle of 48 hours at room temperature with magnetic stirring, followed by removal of the methanol, and a second cycle of 24 hours with fresh methanol. The methanol extracts from both cycles were pooled and concentrated to dryness in a rotary evaporator under reduced pressure. Finally, the concentrated extract was air dried for two weeks.

4.2.2 GC-MS analysis

The extracted samples from the n-hexane extraction were weighed and prepared for silylation. In a glass tube, a 200 µL aliquot of the extract was mixed with 200 µL of tetracosane solution (0.5 mg mL^{-1}), 250 µL of pyridine, 250 µL of N,O-bis(trimethylsilyl)trifluoroacetamide and 50 µL of trimethylsilyl chloride. The mixture was then incubated at 70°C for 40 minutes. After the incubation period, 1 µL of the silylated extract was injected into a gas chromatography-mass

spectrometry (GC-MS) apparatus (QP2010 Ultra Shimadzu, Kyoto, Japan). The chromatography conditions followed the protocols described in Dias et al. (2019). Data were collected at a rate of one scan s^{-1} over a range of *m/z* 33–750. For identification of lipophilic compounds, chromatographic peaks were compared with entries in mass spectral databases such as the NIST14 Mass Spectral Library and the Wiley Registry® of Mass Spectral Data. In addition, comparison was made with mass spectra and retention times of pure compounds prepared and analysed under conditions similar to those of the samples. Calibration curves were established for quantification purposes using standard compounds representing the major families of compounds present in the extracts. These standards included maltose for sugars (y = 0. 0416x + 0.0117, R2 = 0.99), palmitic acid for fatty acids (y = 0. 0941x + 0.5236, R2 = 0.99), octadecane for alkanes (y = 0. 0942x + 0.061, R^2 = 0.99), octadecanol for alcohols (y = 0. 2322x + 0.0474, R^2 = 0.99), and cholesterol for sterols and terpenes (y = 0. 0596x + 0.0447, R^2 = 0.99). The detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves. Quantitative results were expressed as grams per kilogram dry weight (g/Kg DW) and are presented as the mean ± standard deviation of three independent analyses.

4.2.3 UHPLC-MS analysis

Methanol residues were weighed and resuspended in methanol to achieve a final concentration of 10 mg/mL. Next, 4 µL of the solution was injected into a Thermo Scientific Ultimate 3000RSLC Dionex (Waltham, MA, USA) equipped with a Dionex UltiMate 3000 RS diode array detector coupled with a mass spectrometer operating in negative ion mode. The analysis was performed using a Hypersil GOLD column (1.9 µm particle diameter, Thermo Scientific, Lenexa, KS, USA) as described in Dias et al. (2019). A mass range of 50.00–2000.00 m/z was covered. Compound identification was also performed as described in the same reference. UV-Vis spectral data were collected between 250 and 500 nm, and the chromatogram profile was recorded at 280 nm. A semi-quantitative analysis was performed using peak integration through the standard external method. The peaks were identified by comparing the retention times, UV-Vis spectra, and spectral data obtained from the reference compounds. The calibration curves were calculated using the reference compounds (quercetin for flavonoids and oleuropein for

secoiridoids) to determine the detection and quantification limits. The calibration curves were generated by injecting various concentrations of quercetin and oleuropein. The equation for quercetin was y = 4 × 106x − 390882 with an R^2 value of 0.99, where x represents the amount of the compound in mg/mL and y represents the peak area obtained in the chromatogram. The equation for oleuropein was y = 106x − 6948 with an R^2 value of 0.98, where x represents the amount of the compound in mg/mL and y represents the peak area obtained in the chromatogram. The detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves. The same conditions were used for the sample analysis. The compound concentration was measured in milligrams per gram of tissue dry weight. The mean ± standard deviation of three independent analyses per sampling time and treatment are presented.

4.2.4 Statistical analysis

A "group" is defined by the cultivar considered (Giarraffa, Leccino and Maurino), the irrigation treatment (control, CTRL or drought-stressed, DS) and the time of sampling (beginning of stress, t0, two weeks after the beginning, t2, four weeks after the beginning, t4). Each group was analyzed in triplicate. Data distributions were tested for normality using the Shapiro-Wilk normality test in R studio (ver. 4.2.2, R core team, Vienna, Austria, 2022). Repeated measures ANOVA was used to test the significance of each of the three factors (treatment, cultivar and organ) and their interactions. Post hoc analysis was performed using the Tukey HSD test when ANOVA showed $p \leq 0.05$. This statistical analysis was conducted using the Systat 11 statistical package (Systat Software Inc., Richmond, CA, USA). Due to the absence of the "MAU DS t4" group of stems, leaf and stem data were processed separately for the following analysis. This allowed not to exclude this group from the leaf data processing. For the following analyses, first, metabolite contents were normalized by Z-score and missing data were replaced by a value of 0.0001. Hierarchical clustering analysis (HCA) was performed on metabolites using Euclidean distance as the similarity metric and the complete linkage method between groups. The resulting heat maps and clustering were generated using Rstudio (ver. 4.2.2, R core team, Vienna, Austria, 2022) with the package "pheatmap" version 1.0.12. The observation inputs correspond to the variance of each group at t2 and t4, when the drought stress occurred. The

variables input were the classes of both phenolic and lipophilic compounds. The Principal Component Analysis (PCA) biplots were realized with Rstudio (ver. 4.2.2, R core team, Vienna, Austria, 2022) with the package "Factoextra" version 1.0.7.

4.3 Results

4.3.1 Profile of phenolic secondary metabolites

The phenolic profiles of olive leaves and stems were studied in three Italian olive cultivars (Leccino, Maurino, and Giarraffa) under both control and drought stress conditions. As a first general observation, 20 phenolic compounds (16 flavonoids and 4 secoiridoids) were identified: 6 in both organs, 3 in stems, and 10 in leaves only. Retention times, m/z, MS2 (m/z) fragments and mean ± standard deviations for each experimental group at t0 and t2 are summarized in **Tables S4.1** and **S4.2** for leaves, and in **Tables S4.3**, **S4.4**, and **S4.5** for stems (Supplementary Material). Data on phenolic compounds from leaves at t4 are listed in **Table 7.1**, where they have been used to characterize olive leaf extracts tested on human umbilical vein endothelial cells (HUVECs). **Tables 4.1**, **4.2**, and **4.3** show the repeated measures ANOVA results for phenolic compounds found in stem and leaf, leaf only, and stem only, respectively. Tables show that cultivar had a significant effect on all compounds examined (*p-value* < 0.05). Indeed, the cultivars studied contained significantly different amounts of the identified compounds, with some compounds found only in one cultivar (for example, fraxamoside was found only in the stem of 'Leccino'). Unlike the other two cultivars, in the leaf of 'Giarraffa' luteolin-7-*O*-rutinoside was not detected. Instead, another isomer of luteolin-7-*O*-glucoside was identified in this cultivar, namely isomer 1, but not in 'Leccino' and 'Maurino'. Considering the leaves, chrysoeriol-7-*O*-glucoside and luteolin-7-*O*-glucoside is.3 were found only in the cultivar Leccino. The "organ" parameter significantly influenced the amount of compounds found in both leaves and stems. For example, luteolin, apigenin, and oleuropein were more abundant in leaves, whereas luteolin-7-*O*-glucoside and dihydroquercetin were more abundant in the stems. It is worth noting that drought treatment affected most of the compounds found in the leaves (such as oleuropein aglycone, aldehyde form of decarboxyl elenolic acid, luteolin-7-*O*-rutinoside) as well as the ubiquitous compounds (luteolin-7-*O*-glucoside, oleuropein, luteolin,

and apigenin), but not the compounds found only in the stem. However, for dihydroquercetin, quercetin, apigenin-7-*O*-rutinoside is.1, and luteolin-7-*O*-glucoside is.3, which were unaffected by drought stress alone, the interaction between cultivar and treatment was significant. Only luteolin-7-*O*-glucoside was significantly affected by treatment, with no differences in cultivar-organ interactions.

Table 4.1. Repeated measures ANOVA table carried out with the contents (mg/g DW) of phenolic compounds found in both stem and leaf of three olive cultivars (Giarraffa, Leccino and Maurino) irrigated (CTRL) or exposed to drought (DS). Samples were taken at the beginning of the drought stress (t0) and two (t2) and four (t4) weeks after the start of water withholding. Each value represents the mean.

Compound	dihydroquercetin	luteolin-7-*O*-glucoside	oleuropein	chrysoeriol-7-O-glucoside	luteolin	apigenin
Cultivar (C)						
Leccino	3.733	3.647	1.451	0.576	3.633	3.427
Maurino	3.049	3.641	4.478	1.953	2.653	1.730
Giarraffa	2.817	6.847	0.944	1.555	1.971	2.243
p-value	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Organ (O)						
Leaf	2.942	3.871	2.981	1.729	4.474	3.626
Stem	3.457	5.552	1.602	0.994	1.031	1.307
p-value	*0.005*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Treatment (T)						
Control	3.182	4.437	1.35	1.312	2.965	2.582
Stressed	3.218	4.986	3.232	1.411	2.539	2.351
p-value	*0.832*	*0.046*	*<0.001*	*0.106*	*<0.001*	*<0.001*
Time						
t0	3.416	4.686	1.621	1.369	3.044	2.661
t2	3.322	4.616	1.796	1.437	2.715	2.535
t4	2.861	4.833	3.456	1.279	2.497	2.204
T x C						
p-value	*0.028*	*0.552*	*<0.001*	*0.007*	*<0.001*	*<0.001*
T x O						
p-value	*0.304*	*0.406*	*<0.001*	*0.438*	*<0.001*	*<0.001*
C x O						
p-value	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
T x C x O						
p-value	*0.716*	*0.096*	*<0.001*	*0.025*	*<0.001*	*<0.001*

Table 4.2. Repeated measures ANOVA table carried out with the contents (mg/g DW) of phenolic compounds found in the leaves of three olive cultivars (Giarraffa, Leccino and Maurino) irrigated (CTRL) or exposed to drought (DS). Samples were taken at the beginning of the drought stress (t0) and two (t2) and four (t4) weeks after the start of water withholding. Each value represents the mean.

Compound	oleuropein aglicone	aldehydic form of decarboxyl elenolic acid	luteolin-7-O-rutinoside	luteolin-7-0-glucoside is.1	apigenin-O-dideoxyhexoside-hexoxide	apigenin-7-0-rutinoside is.1	apigenin-7-0-rutinoside is.2	luteolin-7-0-glucoside is.3	apigenin-7-O-rutinoside is.3	diosmetin
Cultivar (C)										
Leccino	0.207	3.205	2.822	0	2.791	5.03	3.074	0	2.154	2.765
Maurino	1.221	0.684	4.217	0	2.243	2.869	2.396	2.600	2.483	3.117
Giarraffa	0.937	0.512	0	2.104	1.809	3.147	2.359	2.075	1.907	2.469
p-value	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.019*	*<0.001*	*<0.001*	*<0.001*	*0.006*	*<0.001*
Treatment (T)										
Control	0.674	1.821	1.857	0.669	2.26	3.675	2.558	1.557	2.135	2.991
Stressed	0.930	1.113	2.836	0.734	2.302	3.689	2.662	1.560	2.228	2.576
p-value	*<0.001*	*0.046*	*<0.001*	*<0.001*	0.008	*0.594*	*<0.001*	*0.912*	*0.449*	*<0.001*
Time										
t0	0.639	1.823	1.852	0.667	2.273	3.687	2.569	1.594	2.132	2.98
t2	0.785	1.266	1.817	0.716	2.253	3.644	2.594	1.596	2.253	2.657
t4	0.942	1.313	3.370	0.722	2.317	3.715	2.665	1.557	2.159	2.714
C x T										
p-value	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.054*	*<0.001*

Table 4.3. Repeated measures ANOVA table carried out with the contents (mg/g DW) of phenolic compounds found in the stems of three olive cultivars (Giarraffa, Leccino and Maurino) irrigated (CTRL) or exposed to drought (DS). Samples were taken at the beginning of the drought stress (t0) and two (t2) and four (t4) weeks after the start of water withholding. Each value represents the mean.

Compound	quercetin-3-*O*-glucoside	fraxamoside	quercetin
Cultivar (C)			
Leccino	2.33	2.249	1.126
Maurino	1.459	0	0.746
Giarraffa	2.324	0	0.781
p-value	*<0.001*	*<0.001*	*<0.001*
Treatment (T)			
Control	2.021	0.812	0.878
Stressed	2.055	0.687	0.891
p-value	*0.819*	*0.692*	*0.679*
Time			
t0	2.303	0.853	1.024
t2	2.276	0.868	1.015
t4	1.535	0.528	0.613
C x T			
p-value	*0.761*	*<0.001*	*0.006*

4.3.2 Lipophilic compounds metabolite profiles

Similar to phenolic compounds, the lipophilic profiles of olive leaves and stems from three cultivars (Leccino, Maurino, and Giarraffa) were studied under control and drought stress conditions. A total of 30 lipophilic compounds were identified, including sterols and terpenes, sugars, alcohols, fatty acids, and alkanes. Leaves and stems had distinct profiles, with 12 compounds found in both leaves and stems, 12 in leaves, and 6 in stems only. Retention times and mean ± standard deviations for each experimental group at t0, t2 and t4 are summarized in **Tables S4.6**, **S4.7**, **S4.8** for leaves and in **Tables S4.9**, **S4.10**, and **S4.11** for stems (Supplementary Material). **Tables 4.4**, **4.5**, and **4.6** show the results of the repeated measures ANOVA for lipophilic compounds found in both stem and leaf, leaf only, and stem only, respectively. Cultivar had a significant effect on all identified compounds except for sugar turanose in stems. Similarly, alpha-tocopherol was not found in the leaves of Maurino, while phytol was found only in the leaves of Giarraffa. The "organ" parameter had a significant effect on the compound profile of both stems and leaves. In fact, the leaves had higher levels of alpha-

D-mannopyranose, alpha-D-sorbitol, alpha-D-glucose, palmitic acid, stearic acid, alpha-monopalmitin, alpha-monopalmitin derivatives, lupeol derivatives, and ursolic aldehyde than the stems. Only oleic acid and alpha-linolenic acid were more abundant in the stems. Ursolic acid was the only compound for which no organ, treatment, or interaction had a significant impact. Other compounds that were not significantly affected by drought stress included alpha- and beta-amyrin, two long-chain alkanes in leaves, and turanose in stems. However, treatment, cultivar, and organ interactions lose significance for D-glucose, whereas drought becomes significant for alpha-amyrin and the second long chain alkane found in the cultivar by treatment interaction.

Table 4.4. Repeated measures ANOVA table carried out with the contents (mg/g DW) of lipophilic compounds found in both stem and leaf of three olive cultivars (Giarraffa, Leccino and Maurino) irrigated (CTRL) or exposed to drought (DS). Samples were taken at the beginning of the drought stress (t0) and two (t2) and four (t4) weeks after the start of water withholding. Each value represents the mean.

Compound	α-D-mannopyranose	D-glucose	D-sorbitol	palmitic acid	α-linolenic acid	oleic acid	stearic acid	α-monopalmitin	α-monopalmitin derivative	lupeol derivative	ursolic acid	ursolic acid aldeyde
Cultivar (C)												
Leccino	0.132	0.178	0.873	7.107	2.626	6.621	6.732	6.251	6.640	0.516	0.922	0.507
Maurino	0.131	0.140	0.635	6.272	1.629	4.871	5.638	5.131	5.755	0.568	0.672	0.517
Giarraffa	0.068	0.157	0.82	8.956	7.365	2.978	8.644	8.242	8.627	1.132	1.186	0.305
p-value	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.010*	*<0.001*
Organ (O)												
Leaf	0.126	0.205	0.963	9.357	2.937	4.794	8.759	8.215	8.330	1.051	1.319	0.5
Stem	0.095	0.112	0.588	5.534	4.809	4.852	5.250	4.867	5.685	0.426	0.534	0.387
p-value	*<0.001*	*0.024*	*<0.001*	*0.003*	*<0.001*	*0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.219*	*<0.001*
Treatment (T)												
Control	0.115	0.179	0.792	7.299	3.335	4.771	6.906	6.404	6.917	0.831	0.911	0.425
Stressed	0.111	0.138	0.757	7.591	4.412	4.875	7.103	6.679	7.098	0.646	0.942	0.462
p-value	*<0.001*	*0.004*	*0.012*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.612*	*<0.001*
Time												
t0	0.115	0.163	0.763	7.194	4.011	4.625	6.744	6.183	6.815	0.825	0.890	0.451
t2	0.111	0.147	0.690	7.844	4.411	5.139	7.217	6.721	7.208	0.850	0.854	0.508
t4	0.106	0.166	0.873	7.298	3.197	4.705	7.053	6.720	6.999	0.541	1.036	0.372
T x C												
p-value	*<0.001*	*0.063*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.578*	*<0.001*
T x O												
p-value	*<0.001*	*0.063*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.907*	*<0.001*
C x O												
p-value	*<0.001*	*0.082*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.779*	*<0.001*
T x C x O												
p-value	*<0.001*	*0.212*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.170*	*<0.001*

Table 4.5. Repeated measures ANOVA table carried out with the contents (mg/g DW) of lipophilic compounds found in the leaves of three olive cultivars (Giarraffa, Leccino and Maurino) irrigated (CTRL) or exposed to drought (DS). Samples were taken at the beginning of the drought stress (t0) and two (t2) and four (t4) weeks after the start of water withholding. Each value represents the mean.

Compound	linoleic acid	oleic acid derivative	neophytadiene	phytol	squalene	α-amyrin	β-amyrin	alpha tocopherol	LCAlkane 1	LCAlkane 2	LCAlkane 3	LCAlkane 4
Cultivar (C)												
Leccino	7.161	7.193	0.536	0.515	0.694	0.727	0.755	0.752	0.976	1.289	1.809	1.202
Maurino	6.851	6.850	0.690	0	0.651	0.731	0.750	0	1.095	1.443	1.970	1.144
Giarraffa	3.569	0.000	0.654	0	0.000	1.129	1.262	0.224	1.614	2.202	2.926	2.057
p-value	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Treatment (T)												
Control	6.064	4.860	0.814	0.215	0.460	0.814	0.959	0.240	1.264	1.686	2.340	1.501
Stressed	5.656	4.502	0.440	0.129	0.437	0.911	0.886	0.410	1.193	1.603	2.129	1.434
p-value	*<0.001*	*<0.001*	*<0.001*	*0.008*	*0.009*	*0.153*	*0.052*	*<0.001*	*0.029*	*0.207*	*0.122*	*0.399*
Time												
t0	4.297	4.308	0.740	0.196	0.405	0.814	0.914	0.224	1.207	1.615	2.282	1.410
t2	4.557	4.573	0.600	0.193	0.433	0.919	0.922	0.235	1.229	1.653	2.253	1.435
t4	8.727	5.162	0.540	0.125	0.508	0.753	0.931	0.517	1.250	1.665	2.170	1.557
C x T												
p-value	*<0.001*	*<0.001*	*<0.001*	*0.019*	*<0.001*	*0.031*	*0.133*	*<0.001*	*0.007*	*0.042*	*0.144*	*0.176*

Table 4.6. Repeated measures ANOVA table carried out with the contents (mg/g DW) of lipophilic compounds found in the stems of three olive cultivars (Giarraffa, Leccino and Maurino) irrigated (CTRL) or exposed to drought (DS). Samples were taken at the beginning of the drought stress (t0) and two (t2) and four (t4) weeks after the start of water withholding. Each value represents the mean.

Compound	pentadecan-1-ol derivative	palmitic acid derivative	palmitic acid derivative	turanose	monostearin	stigmast-5-ene
Cultivar (C)						
Leccino	0.398	5.484	5.271	0.078	6.026	0.777
Maurino	0.292	3.478	3.318	0.137	4.478	0.520
Giarraffa	0.554	6.083	5.982	0.14	6.634	0.748
p-value	*<0.001*	*<0.001*	*<0.001*	*0.252*	*<0.001*	*<0.001*
Treatment (T)						
Control	0.330	4.554	4.384	0.320	5.334	0.652
Stressed	0.499	5.479	5.330	0.105	6.092	0.711
p-value	*<0.001*	*<0.001*	*<0.001*	*0.418*	*<0.001*	*<0.001*
Time						
t0	0.436	4.795	4.573	0.174	5.789	0.726
t2	0.504	5.525	5.331	0.104	6.233	0.761
t4	0.304	4.73	4.667	0.078	5.116	0.558
C x T						
p-value	*<0.001*	*<0.001*	*<0.001*	*0.320*	*<0.001*	*<0.001*

4.3.3 HCA of leaf metabolites

HCA was used to group phenolic and lipophilic compounds in relation to control and drought-stressed cultivars at both time points. HCA of leaf metabolites (**Figure 4.1**) revealed two major clusters (clusters 1 and 2), each with two additional subclusters (1a, 1b, 2a, 2b). The sub-cluster 1a consisted of 8 phenolic compounds that were very abundant in the cultivar Maurino; in particular, the metabolites luteolin-7-*O*-rutinoside, dihydroquercetin and oleuropein were very abundant in "MAU DS t4", whereas apigenin-7-*O*-rutinoside is.3 was highly enriched in "MAU DS t2". The metabolites of this cluster (especially chrysoeriol-7-*O*-glucoside and luteolin-7-*O*-glucoside is.3) were much less abundant in 'Leccino' regardless of the time point and treatment. Furthermore, metabolites that accumulated in 'MAU DS t4' (including apigenin-7-*O*-rutinoside is.3) were less abundant in all 'Giarraffa' groups than the other cultivars. The sub-cluster 1b contained 10 metabolites (mainly lipophilic compounds except for the flavonoid diosmetin) whose content was significantly lower in 'Giarraffa' and in 'MAU DS t4' regardless of the time point and treatment. In particular, 'GIA DS t2' had a very low content of D-glucose and D-

sorbitol. Metabolites from this cluster accumulated preferentially in all other groups of ‘Leccino’ and ‘Maurino’ (except for 'MAU DS t4').

The sub-cluster 2a contains 6 phenolic compounds but only 2 lipophilic compounds. The metabolites of this sub-cluster were primarily accumulated in the cultivar ‘Leccino’ regardless of time point or treatment, though 'GIA DS t2' contained a high amount of alpha-tocopherol. In contrast, the compounds in this subcluster had lower content in both ‘Giarraffa’ and ‘Maurino’. The 14 leftmost compounds in sub-cluster 2b were all lipophilic compounds, with the exception of the flavonoid luteolin-7-*O*-glucoside is.1. The metabolites of this cluster accumulated in higher amounts in ‘Giarraffa’ (regardless of time point and treatment) than in the other two cultivars; in fact, their content was particularly low in 'MAU DS t4'. The HCA revealed a clear separation of leaf lipophilic and phenolic compounds, which were mostly grouped into distinct clusters.

Cultivar clustering analysis (including time points and treatments) revealed two major sub-clusters (B1 and B2) of cluster B and an orphan group containing only “MAU DS t4” (cluster A), which behaved completely differently from the other groups. In particular, it showed a very low content of all long-chain alkanes and other metabolites belonging to the sterol and terpene class, whereas a huge amount of many phenolic compounds of sub-cluster 1a were found, especially oleuropein, dihydroquercetin and luteolin-7-*O*-rutinoside. Sub-cluster B1 included the Giarraffa cultivar, which differed from the other two cultivars in the higher content of long-chain alkanes, sterols and terpenes and the lower amount of some fatty acids such as oleic and linoleic acid. Within sub-cluster B1, the metabolic composition of the ‘Giarraffa’ control differed from the metabolic profile of ‘Giarraffa’ under drought stress at t2 and t4, especially for the lower amount of D-glucose, neophytadiene and D-sorbitol. Sub-cluster B2, on the other hand, included both ‘Maurino’ and ‘Leccino’, which behaved much more similarly to each other than to ‘Giarraffa’, especially with regard to lipophilic compounds (sub-clusters 1b and 2b). The ‘Maurino’ control and the drought-stressed samples at t0 were further separated from the drought-stressed ‘Maurino'’ at t2, where there was a higher amount of apigenin-7-*O*-rutinoside is.3, oleuropein aglicone and luteolin-7-*O*-glucoside is.2. Similarly, in the case of cultivar Leccino, the control and stressed samples at t0 differed significantly from the drought-stressed

samples at t2 and t4, which showed lower amounts of diosmetin and dihydroquercetin. As a result, the groups in cluster B were more similar within the cultivar than across treatment conditions. In particular, sub-cluster B1 distinguished 'Giarraffa' from the other two cultivars. Only within each cultivar cluster did the control groups form a separate cluster than the stressed groups.

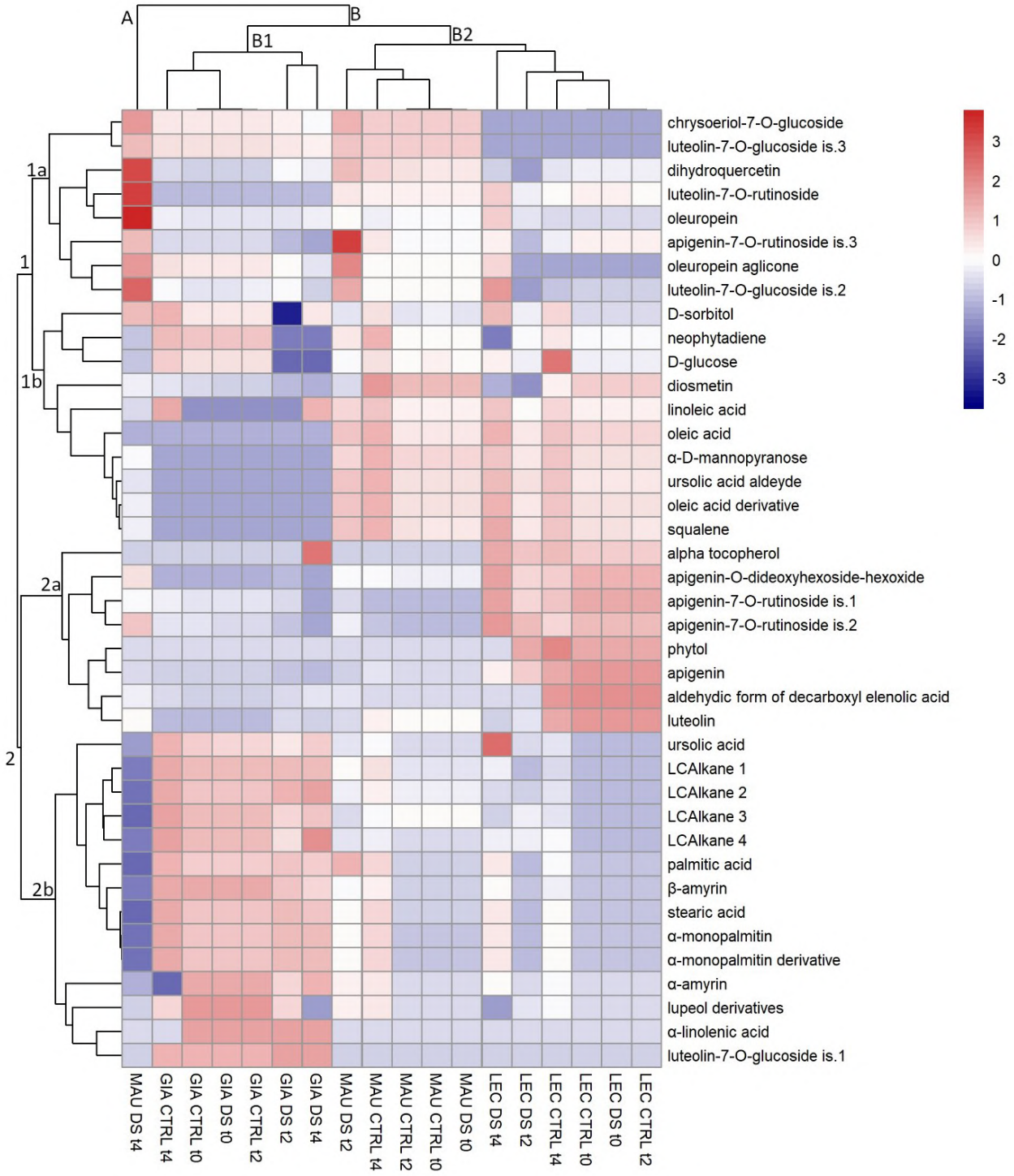

Figure 4.1. Heat map of metabolites extracted from the leaves of olive cultivars Giarraffa (GIA), Leccino (LEC) and Maurino (MAU) irrigated (CTRL) or exposed to drought (DS). Samples were taken at the beginning of the

drought stress (t0) and two (t2) and four (t4) weeks after the start of water deprivation. Hierarchical clustering is shown for both metabolites (left) and experimental groups (top). The red and blue colors correspond to higher and lower relative metabolite amounts normalized according to Z-score.

4.3.4 HCA of stem metabolites

HCA was also used to assess the pattern of stem metabolites (**Figure 4.2**). The analysis divided the compounds into two main clusters (1 and 2). The sub-cluster 1a consisted of two flavonoids (luteolin and quercetin-3-*O*-glucoside). Their amount was unchanged or increased in the irrigated samples of 'Leccino' as well as in "GIA DS t4" and "MAU DS t2". Two flavonoids (luteolin-7-*O*-glucoside is.2 and apigenin) and two lipophilic compounds (stearic acid and ursolic acid) formed the sub-cluster 1b. They were more abundant in the Giarraffa cultivar regardless of time and treatment, but especially in "GIA DS t4" and in "MAU DS t4". The sub-cluster 2a contained oleuropein, quercetin and chrysoeriol-7-*O*-glucoside, phenolic compounds whose content decreased in the experimental groups of 'Giarraffa' and 'Leccino' but increased in the cultivar 'Maurino', especially in 'MAU DS t2'. The sub-cluster 2b contained 15 lipophilic compounds, with higher content in the stressed groups of 'Maurino' at t2, 'Giarraffa' and 'Leccino' at t4, and also in 'LEC CTRL t4'. Exceptions were neophytadiene and stigmast-5-ene (which decreased strongly in 'LEC DS t4') and LCAlkane 4, which decreased in 'MAU DS t2'. The clustering of stem lipophilic and phenolic metabolites was less clear than that of leaf compounds, with the exception of the 15 lipophilic compounds.

The clustering of the experimental group of cultivars showed two distinct clusters. Cluster A consisted of two sub-clusters, the first of which (A1) included 'Giarraffa', which showed high levels of ursolic acid, luteolin-7-*O*-glucoside is.2 and apigenin. The second sub-cluster (A2) included 'Maurino' and 'Leccino'. Both showed low levels of many fatty acids, such as linoleic acid, sugars and other metabolites belonging to sub-cluster 2b. Finally, cluster B included four experimental groups, three of which were exposed to drought stress. Interestingly, the 'Maurino' group, after 2 weeks of stress, was grouped together with the stressed 'Leccino' and 'Giarraffa' groups, stressed by 4 weeks of drought. All of them had higher amounts of the metabolites of sub-cluster 2 and, overall, of some other compounds (such as luteolin, quercetin-3-*O*-glucoside, neophytadiene) belonging to the other sub-clusters of metabolites.

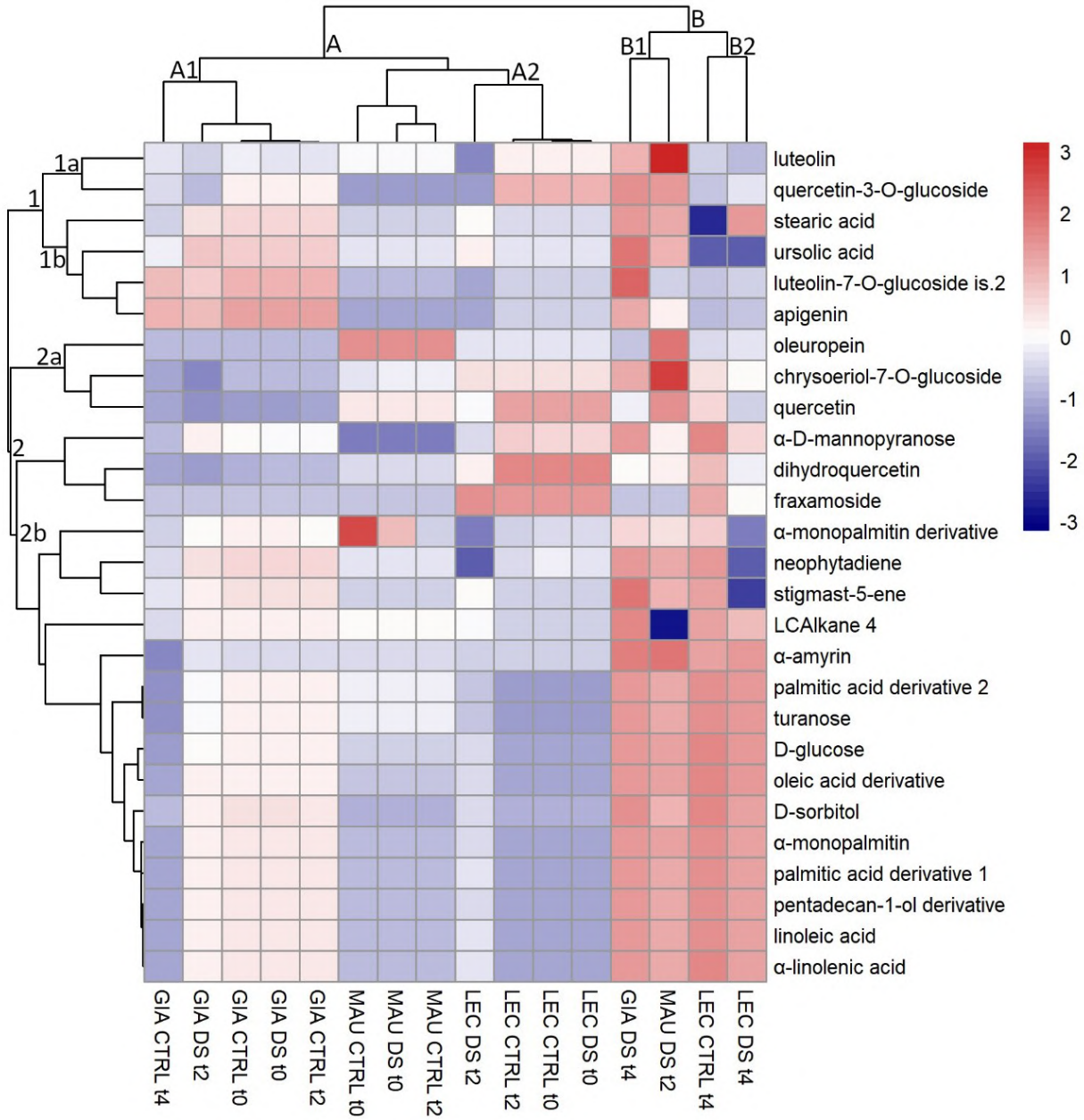

Figure 4.2. Heat map of metabolites extracted from the stems of olive cultivars Giarraffa (GIA), Leccino (LEC) and Maurino (MAU) irrigated (CTRL) or exposed to drought (DS). Samples were taken at the beginning of the drought stress (t0) and two (t2) and four (t4) weeks after the start of water withholding. Hierarchical clustering is shown for both metabolites (left) and experimental groups (top). The red and blue colors correspond to higher and lower relative metabolite amounts normalized according to Z-score.

4.3.5 PCA of leaf metabolite classes

PCA biplots (**Figure 4.3**) were performed to highlight the differences between the experimental groups and to identify the classes of leaf metabolites that best contributed to group separation. Principal components 1 (PC1) and 2 (PC2) together accounted for 74.6% of the variation in the leaf data. As shown in **Figure 4.3A**, all experimental groups of the Giarraffa cultivar were

distinct from the other two cultivars and distributed in a restricted area, corresponding to the class of alkanes. On the other hand, the 'Leccino' and 'Maurino' groups were less distinct than 'Giarraffa' because their distributions partially overlapped. However, 'Leccino' was more associated to the classes of alcohols and sugars, while 'Maurino' was best related to the classes of secoiridoids, sterols and terpenes, and fatty acids. However, when considering the treatment exposure of the experimental groups (**Figure 4.3B**), the control and stressed samples did not have a clearly defined distribution area. This means that drought stress elicited a wide range of metabolic responses in the experimental groups, resulting in no unique or few common metabolic responses to drought stress in the leaves of the three cultivars.

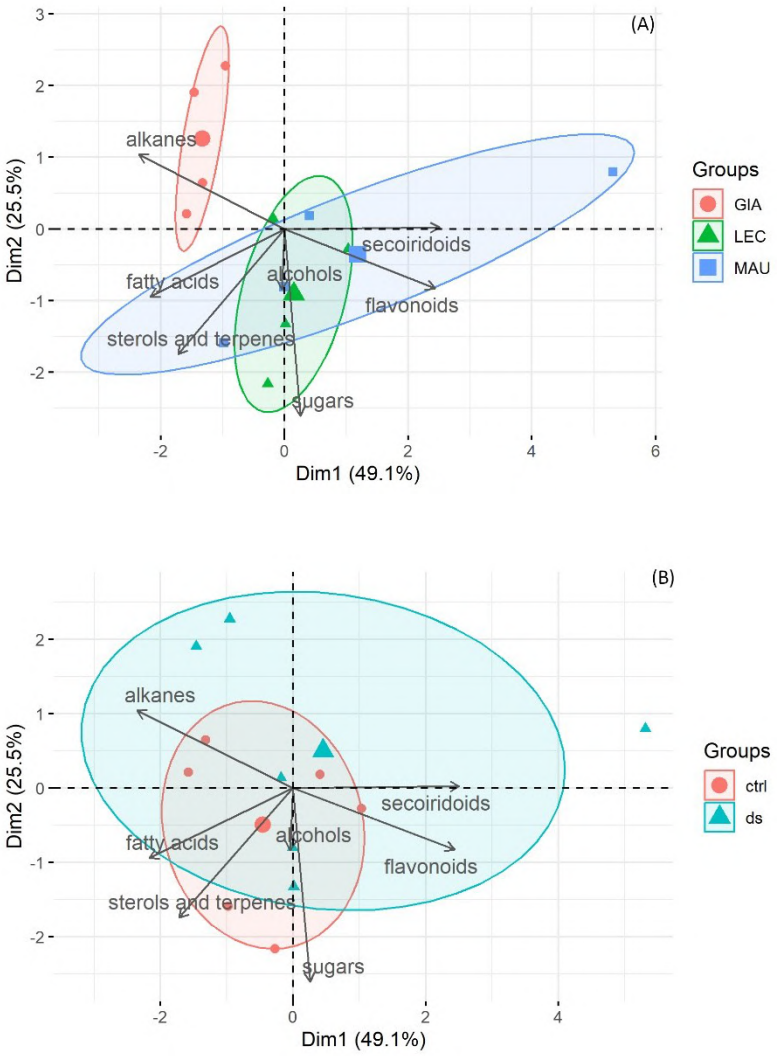

Figure 4.3. PCA-biplots of principal component 1 (Dim1) and principal component 2 (Dim2) including both observations (experimental groups) and loadings (metabolites classes) of leaf samples. A) Observations were shown as cultivars (GIA, Giarraffa; LEC, Leccino; MAU, Maurino); B) Observations were shown as treatments (ctrl, control; ds, drought stressed).

4.3.6 PCA of stem metabolite classes

Figure 4.4 shows PCA biplots with the stem metabolite classes. PC1 and PC2 accounted for a significant 75.3% of the total variation in the stem data, indicating that these two components capture much of the information in the dataset. Similar to the leaf results in **Figure 4.3**, the cultivars separated based on the distribution of stem compounds (**Figure 4.4A**). This separation was more pronounced than that observed between the control and drought treatments (**Figure 4.4B**). The cultivar distribution was primarily resolved by PC1. The secoiridoids class was the main contributor to this component, while flavonoids and alkanes have negative loadings. This suggests that the class of secoiridoids had a significant influence on the distribution of cultivars along PC1. Two cultivars, 'Leccino' and 'Giarraffa', showed a similar and partially overlapping distribution. However, 'Leccino' was well associated to the negative loadings of five lipophilic classes, namely fatty acids, sterols and terpenes, sugars, and alcohols. In contrast, the cultivar Maurino showed a different distribution. The distribution area of stem samples from both the stressed and control groups is large, with some overlap. This overlap indicated that there is no common metabolic response pathway to drought in the stems of the three cultivars.

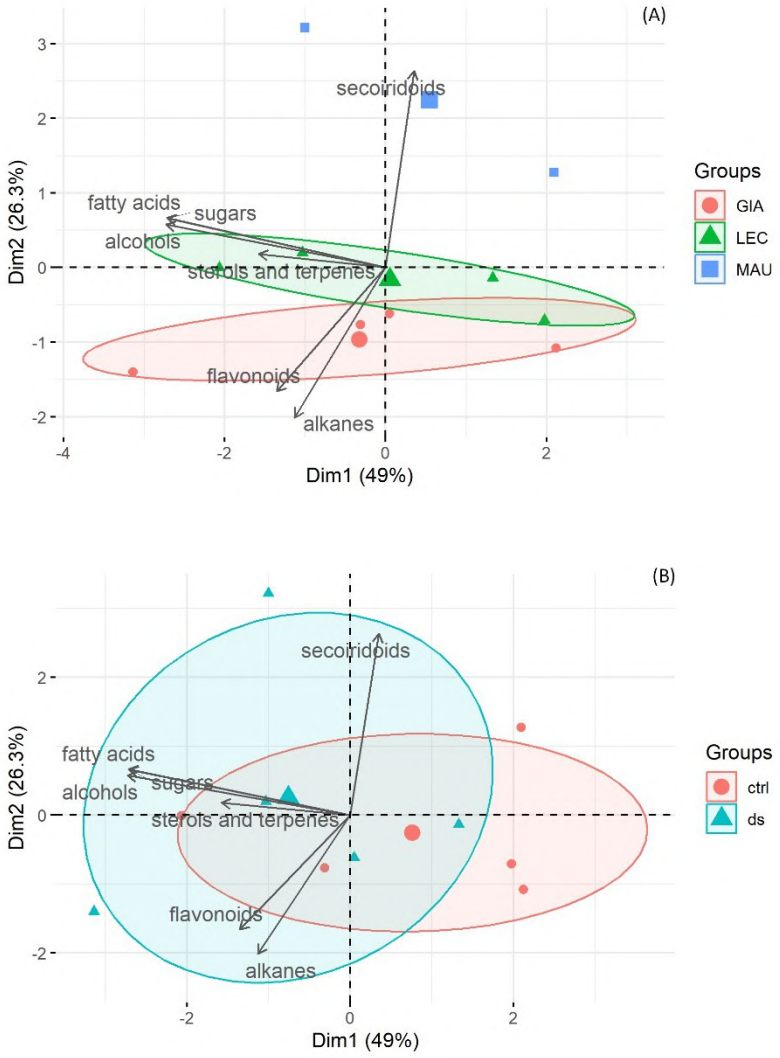

Figure 4.4. PCA-biplots of principal component 1 (Dim1) and principal component 2 (Dim2) including both observations (experimental groups) and loadings (metabolites classes) of stem samples. A) Observations were shown as cultivars (GIA, Giarraffa; LEC, Leccino; MAU, Maurino); B) Observations were shown as treatments (ctrl, control; ds, drought stressed).

4.4 Discussion

4.4.1 In response to drought, phenolic metabolites accumulate primarily in leaves

The metabolomic approach revealed information about the quality and quantity of primary and secondary metabolites found in the leaves and stems of three Italian olive cultivars (Giarraffa, Leccino, and Maurino) that were either well irrigated or subjected to drought stress for two and four weeks, respectively. In this study, we focused on both lipophilic and phenolic compounds; the latter are secondary metabolites known for their antioxidant activity, specifically the ability to act as a defence against reactive oxygen species (Morelló et al., 2005). All three olive

cultivars studied contain phenolic compounds, and most of them increase in response to drought stress only in the leaf. The shikimic acid pathway in photosynthetic cells synthesizes phenolic compounds (Del Río, 2003), and the leaf is the primary metabolic source for plants, resulting in the accumulation of primary and secondary plant products in this organ (Ryan et al., 2002). Accumulation of higher levels of phenolic compounds in leaves compared to stems was found also in the Spanish olive cultivar 'Picual' (Del Río, 2003; Jiménez-Herrera et al., 2019). Groundnut trees exposed to drought accumulated phenolic compounds in both leaves and stems in a genotype-dependent manner; however, the total content and rate of increase were higher in leaves compared to stems (Aninbon et al., 2016). Flavonoids are often the most abundant type of phenolic compound identified. They are a large group of phenolic compounds with two benzene rings connected by a three-carbon bridge, which can typically form a third ring (Kumar and Pandey, 2013). Flavonoids have traditionally been classified into "effective antioxidants" (orto-dihydroxy B-ring substituted, such as luteolin and quercetin derivatives) and "poor antioxidants" (mono-hydroxy B-ring substituted, such as apigenin derivatives) based on their ability to donate electrons or hydrogen atoms (Agati et al., 2012). Flavonoids are particularly known to respond to UV-B stress conditions (Ryan et al., 2002; Agati et al., 2012; Dias et al., 2021; Piccini et al., 2022); however, they can also accumulate in the leaves of olive trees exposed to drought stress conditions (Mechri et al., 2020b). It was found that the non-toxic and highly soluble flavone-7-glucoside forms of luteolin and apigenin increased under drought stress conditions, whereas the aglycone forms, such as luteolin and apigenin, were found at lower levels under stress conditions compared to the respective controls. As glycosylation substitutes hydroxyl groups of flavonoids, it reduces their antioxidant activity and allows them to accumulate in the vacuole. The glycoconjugates of flavones synthesized from aglycone forms can accumulate and deplete apigenin and luteolin.

Although olive stems have a lower diversity of flavonoids, they contain more quercetin and luteolin derivatives than leaves, which, due to their high antioxidant capacity, can compensate for the lower flavonoids content of stems. The other class of phenolic compounds detected were the secoiridoids, which are coumarin-like compounds related to the iridoids. Phenolic secoiridoids, like oleuropein, have an oleoside moiety derived from terpene synthesis esterified

with a phenolic moiety via a branch in the mevalonic acid pathway (Ryan et al., 2002). Ortega-García and Peragón (2009) identified oleuropein as the main stem compound extracted in the methanol fraction, while Işin et al., (2012) found that oleuropein levels increased in olive leaves with severe water deprivation. In this experiment, we found that oleuropein and oleuropein aglycone were accumulated in response to drought stress, especially in leaf tissue, in contrast to Jiménez-Herrera et al. (2019), who found that oleuropein was accumulated in olive stem tissue rather than leaf tissue in response to drought.

4.4.2 Drought stress causes changes in leaf and stem lipophilic metabolites

Like phenolic compounds, leaf tissue contains more primary metabolites than stems. However, higher levels of unsaturated acids, such as alpha-linolenic acid and oleic acid, were detected. Two palmitic acid derivatives were found only in olive stems, which could be attributed to the lower concentration of palmitic acid (a saturated acid) in the stem than the leaf. In contrast to the phenolic profiles, the treatment had a significant impact on the profile of primary metabolites in both the stem and the leaf. Drought primarily increased the content of fatty acids (with the exception of linoleic acid), which can act as membrane reinforcement against peroxidation (Piccini et al., 2022; Yin et al., 2024) or as an energy source for stress recovery (Dias et al., 2021). The lower sugar levels available during stress may be due to a reduction in the photosynthetic process or an increased use of energy to cope with stress. Furthermore, drought stress decreased sterols content, which may be related to the conversion of sterols into steryl esters, which were linked to membrane reinforcing in drought-tolerant cultivars (Rogowska and Szakiel, 2020), or simply an increase in membrane fluidity due to the stress (Dias et al., 2021). Unlike previous studies on the UV-B stress response, drought stress did not significantly increase the content of long-chain alkanes. These compounds are involved in cuticle wax thickness, which can be useful in coping with solar radiation (Piccini et al., 2022) and even water loss (Bacelar et al., 2004). This was not the case in our study. Sorbitol, a mannitol isomer, is an important osmoprotectant agent in olive trees. This polyol did not accumulate during drought stress, but it was found in high concentrations in the leaf, possibly supporting cell turgor and/or acting as an antioxidant. Because no differences were found, the antioxidant properties of triterpene and lupeol derivatives were most likely unnecessary after

drought stress. The levels of these molecules have been found to be unaffected by stress (Dias et al., 2021), with variations occurring only during recovery.

4.4.3 Cultivar-specific changes in metabolite profiles in response to drought

Although the data collected allowed the identification of the most responsive molecules and their different accumulation in the olive leaf and stem, the three cultivars studied displayed distinct drought response metabolic patterns. Lipophilic compounds respond more consistently than phenolic compounds within each cultivar, regardless of drought treatment. The two sub-clusters 2b of **Figures 4.1** and **4.2** of leaf and stem metabolites, which contain most of the lipophilic compounds, clearly distinguish Giarraffa from the other two cultivars, regardless of treatment. 'Giarraffa' leaves have a high concentration of long chain alkanes, which may be linked to the thickness of the epicuticular wax, allowing this cultivar to avoid excessive water loss. Furthermore, the abundance of palmitic and stearic acids, sterols, and terpenes may allow 'Giarraffa' to maintain good membrane fluidity without over-permeability (Dias et al., 2021). The potential accumulation of wax on the leaf surface, combined with the physiological responses of 'Giarraffa' under drought stress (**Chapter 3**), indicates that this is a typical "drought-avoiding" cultivar (Fang and Xiong, 2015). Water deficiency in 'Giarraffa' reduces stomatal conductance relatively early, resulting in increased stem water content and prolonged soil water availability. Another water-saving strategy of 'Giarraffa' is the accumulation of the osmoprotectant D-sorbitol in leaves exposed to two weeks of drought stress. In addition, 'Giarraffa' had the lowest level of lipid peroxidation and membrane damage, as measured by electrolyte leakage assay in **Chapter 3** (**Figures 3.4**). As a result, there was no need to adjust flavonoid and secoiridoid pools in response to drought, indicating that oxidative stress was low (Agati et al., 2012). However, 'Giarraffa' showed a significant decrease in D-glucose, indicating stomatal closure and a lower photosynthetic rate. 'Giarraffa' also showed higher levels of alkanes and fatty acids under UV-B conditions, but we did not observe a corresponding accumulation of flavonoids (Piccini et al., 2022); this is most likely due to the early physiological response implemented by 'Giarraffa' (**Chapter 3**), which allows this cultivar to avoid drought and reduce oxidative stress. In contrast, the metabolic profile of 'Maurino' after four weeks of drought stress revealed unusual levels of phenolic and lipophilic compounds,

placing it in a separate cluster. The high levels of malondialdehyde and electrolyte leakage suggest that the reduction in lipophilic compounds in both leaves and stems may be due to oxidative stress-induced damage to cellular components (**Chapter 3**). Surprisingly, the stressed group of 'Maurino' accumulated flavonoids and secoiridoids, which may aid in the regulation of oxidative stress in both the stem and the leaves. However, unlike *Amaranthus tricolor* (Sarker, 2018) and *Zea mays* (Li et al., 2021), which showed higher MDA and EL levels but lower flavonoid content, the antioxidant response of 'Maurino' did not allow it to avoid oxidative stress damage. Agati et al. (2012) proposed one possible explanation: flavonoid accumulation as an oxidative stress response occurs primarily in stress-sensitive individuals under severe stress conditions, when the first line of defense against ROS (antioxidant enzymes) is compromised. The metabolite response of 'Leccino' is intermediate. Unlike 'Giarraffa', 'Leccino' contains few long-chain alkanes, sterols, and terpenes and, like 'Maurino', the main fatty acids are oleic and linoleic. However, under drought stress, it exhibits heterogeneous phenolic profile changes. Secoiridoids accumulate only after four weeks of drought, while changes in the flavonoid pool occurred only for a few of them, such as luteolin and apigenin-7-*O*-rutinoside and glucoside, at the expense of a decrease in apigenin and luteolin levels. However, the phenolic profile of this cultivar was associated with lower antioxidant capacity than the other two cultivars under drought stress conditions, as shown by Ferric Ion Reducing Antioxidant Power analysis (**Figure 7.1**).

4.5 *Bulleted* conclusions

- The analysis of the phenolic and lipophilic profiles of the three Italian olive cultivars exposed to drought stress revealed a cultivar-specific response to drought.
- The cultivars Maurino and Leccino responded more similarly than Giarraffa.
- 'Maurino' showed the higher antioxidant response and the greater decrease in most lipophilic compounds, indicating a "drought-stressed" profile.
- 'Giarraffa' did not increase flavonoid and secoiridoid pools but showed higher levels of cell wall and cuticle wax components than the other cultivars.

- ‘Giarraffa’ is thought to have a “drought avoidance” pattern, as demonstrated by physiological analyses (**Chapter 3**).

4.6 Supplementary material of Chapter 4

Table S4.1. Phenolic profile (mg/g DW) of the leaves of the Giarraffa (GIA), Leccino (LEC), and Maurino (MAU) cultivars measured under control (CTRL) conditions and exposed to drought stress (DS) before the start of water deprivation (**t0**). The mean values ± standard deviation (n = 3–4) are presented. Rt—retention time; nd—not detected; is.—isomer.

Rt (min.)	Compound	[M-H] −(m/z)	MS^2 (m/z) Fragments	GIA CTRL	GIA DS	LEC CTRL	LEC DS	MAU CTRL	MAU DS
	secoiridoids								
10.3	oleuropein aglicone	377	197/153	1.052 ± 0.136	1.026 ± 0.139	nd	nd	0.878 ± 0.011	0.876 ± 0.010
10.8	aldehydic form of decarboxyl elenolic acid	215	197/153/ 171/ 185	0.406 ± 0.073	0.411 ± 0.084	4.498 ± 0.396	4.501 ± 0.404	0.561 ± 0.100	0.559 ± 0.105
14.4	oleuropein	539	377/307/275	1.183 ± 0.207	1.180 ± 0.225	nd	nd	2.469 ± 0.026	2.464 ± 0.023
	flavonoids								
11.9	dihydroquercetin	303	285/177/ 125	2.407 ± 0.113	2.412 ± 0.098	2.779 ± 0.074	2.783 ± 0.055	3.263 ± 0.050	3.271 ± 0.033
12.1	luteolin-7-*O*-rutinoside	593	447/285	nd	nd	2.724 ± 0.013	2.722 ± 0.012	3.830 ± 0.144	2.837 ± 0.067
12.1	luteolin-7-*O*-glucoside is.1	447	287/285	2.003 ± 0.030	1.997 ± 0.008	nd	nd	nd	nd
12.4	apigenin-*O*-dideoxyhexoside-hexoxide	449	269	1.812 ± 0.023	1.809 ± 0.018	2.842 ± 0.014	2.842 ± 0.013	2.167 ± 0.002	2.167 ± 0.002
12.8	apigenin-7-*O*-rutinoside is.1	577	269	3.293 ± 0.018	3.296 ± 0.022	5.201 ± 0.073	5.194 ± 0.085	2.570 ± 0.006	2.570 ± 0.007
13.0	apigenin-7-*O*-rutinoside is.2	577	269	2.436 ± 0.183	2.420 ± 0.117	3.085 ± 0.035	3.083 ± 0.036	2.194 ± 0.029	2.198 ± 0.016
13.3	luteolin-7-*O*-glucoside is.2	447	285	3.454 ± 0.090	3.486 ± 0.007	3.017 ± 0.154	3.002 ± 0.058	3.930 ± 0.025	3.932 ± 0.027
13.5	chrysoeriol-7-*O*-glucoside	461	299/446	2.154 ± 0.058	2.151 ± 0.061	nd	nd	2.784 ± 0.025	2.783 ± 0.019
13.9	luteolin-7-*O*-glucoside is.3	447	285	2.158 ± 0.144	2.156 ± 0.108	nd	nd	2.489 ± 0.007	2.488 ± 0.007
15.7	luteolin	285	285	2.515 ± 0.050	2.513 ± 0.025	7.731 ± 0.177	7.737 ± 0.185	4.571 ± 0.007	4.552 ± 0.017
16.7	apigenin-7-*O*-rutinoside	577	269	1.990 ± 0.015	1.994 ± 0.033	2.263 ± 0.014	2.258 ± 0.039	2.145 ± 0.008	2.146 ± 0.009
17.4	apigenin	269	269/225	2.496 ± 0.049	2.497 ± 0.054	6.477 ± 0.099	6.473 ± 0.096	2.720 ± 0.008	2.720 ± 0.007
17.8	diosmetin	299	284	2.526 ± 0.067	2.521 ± 0.054	3.112 ± 0.014	3.112 ± 0.018	3.305 ± 0.014	3.303 ± 0.009

Table S4.2. Phenolic profile (mg/g DW) of the leaves of the Giarraffa (GIA), Leccino (LEC), and Maurino (MAU) cultivars measured under control (CTRL) conditions and exposed to drought stress (DS) for two weeks (**t2**). The mean values ± standard deviation (n = 3–4) are presented. Rt—retention time; nd—not detected; is.—isomer.

Rt (min.)	Compound	[M-H] −(m/z)	MS^2 (m/z) Fragments	GIA CTRL	GIA DS	LEC CTRL	LEC DS	MAU CTRL	MAU DS
	secoiridoids								
10.3	oleuropein aglicone	377	197/153	1.000 ± 0.144	0.811 ± 0.012	nd	nd	0.878 ± 0.011	2.020 ± 0.007
10.8	aldehydic form of decarboxyl elenolic acid	215	197/153/ 171/ 185	0.416 ± 0.095	0.611 ± 0.062	4.505 ± 0.413	0.704 ± 0.049	0.557 ± 0.110	0.801 ± 0.079
14.4	oleuropein	539	377/307/275	1.177 ± 0.247	0.512 ± 0.074	nd	1.208 ± 0.636	2.459 ± 0.027	3.731 ± 0.321
	flavonoids								
11.9	dihydroquercetin	303	285/177/ 125	2.418 ± 0.096	2.894 ± 0.060	2.787 ± 0.044	1.920 ± 0.035	3.280 ± 0.017	3.767 ± 0.560
12.1	luteolin-7-*O*-rutinoside	593	447/285	nd	nd	2.720 ± 0.011	1.920 ± 0.070	2.845 ± 0.017	3.417 ± 0.185
12.1	luteolin-7-*O*-glucoside is.1	447	287/285	1.991 ± 0.044	2.302 ± 0.006	nd	nd	nd	nd
12.4	apigenin-*O*-dideoxyhexoside-hexoxide	449	269	1.806 ± 0.021	1.875 ± 0.010	2.842 ± 0.016	2.602 ± 0.058	2.167 ± 0.003	2.228 ± 0.009
12.8	apigenin-7-*O*-rutinoside is.1	577	269	3.299 ± 0.053	3.173 ± 0.042	5.188 ± 0.097	4.438 ± 0.132	2.569 ± 0.008	3.196 ± 0.017
13.0	apigenin-7-*O*-rutinoside is.2	577	269	2.403 ± 0.069	2.311 ± 0.043	3.081 ± 0.038	3.052 ± 0.086	2.202 ± 0.004	2.517 ± 0.049
13.3	luteolin-7-*O*-glucoside is.2	447	285	3.518 ± 0.078	3.725 ± 0.065	2.987 ± 0.252	2.115 ± 0.065	3.933 ± 0.029	5.708 ± 0.030
13.5	chrysoeriol-7-*O*-glucoside	461	299/446	2.147 ± 0.065	2.057 ± 0.039	nd	nd	2.783 ± 0.016	3.447 ± 0.016
13.9	luteolin-7-*O*-glucoside is.3	447	285	2.154 ± 0.126	2.023 ± 0.059	nd	nd	2.488 ± 0.007	2.750 ± 0.036
15.7	luteolin	285	285	2.510 ± 0.049	3.519 ± 0.020	7.743 ± 0.213	3.666 ± 0.096	4.532 ± 0.039	3.458 ± 0.010
16.7	apigenin-7-*O*-rutinoside	577	269	1.999 ± 0.051	1.813 ± 0.040	2.253 ± 0.064	1.802 ± 0.046	2.146 ± 0.010	3.505 ± 1.793
17.4	apigenin	269	269/225	2.497 ± 0.059	2.331 ± 0.060	6.469 ± 0.096	5.102 ± 0.180	2.720 ± 0.007	2.627 ± 0.009
17.8	diosmetin	299	284	2.516 ± 0.042	2.332 ± 0.072	3.111 ± 0.022	2.102 ± 0.041	3.300 ± 0.008	2.579 ± 0.005

Table S4.3. Phenolic profile (mg/g DW) of the stems of the Giarraffa (GIA), Leccino (LEC), and Maurino (MAU) cultivars measured under control (CTRL) conditions and exposed to drought stress (DS) before the start of water deprivation (**t0**). The mean values ± standard deviation (n = 3–4) are presented.

Rt (min.)	Compound	[M-H] −(m/z)	MS^2 (m/z) Fragments	GIA CTRL	GIA DS	LEC CTRL	LEC DS	MAU CTRL	MAU DS
	secoiridoids								
14.4	oleuropein	539	377/275	0.779 ± 0.125	0.776 ± 0.137	1.410 ± 0.280	1.412 ± 0.284	3.893 ± 0.704	3.891 ± 0.694
15.4	fraxamoside	537	223/375	nd	nd	2.557 ± 0.554	2.560 ± 0.551	nd	nd
	flavonoids								
11.9	dihydroquercetin	303	285/177/ 125	2.992 ± 0.544	2.994 ± 0.541	5.630 ± 1.205	5.615 ± 1.215	3.425 ± 0.592	3.423 ± 0.602
12.8	quercetin-3-*O*-glucoside	463	301	2.336 ± 0.378	2.336 ± 0.379	2.517 ± 0.384	2.518 ± 0.383	2.057 ± 0.226	2.054 ± 0.231
13.5	chrysoeriol-7-*O*-glucoside	461	299/446	1.028 ± 0.134	1.027 ± 0.138	1.159 ± 0.129	1.159 ± 0.129	1.088 ± 0.063	1.092 ± 0.054
13.6	luteolin-7-*O*-glucoside	447	285	9.841 ± 1.608	9.844 ± 1.667	4.284 ± 0.715	4.308 ± 0.740	3.565 ± 0.533	3.566 ± 0.532
15.5	quercetin	301	285/257	0.751 ± 0.036	0.752 ± 0.035	1.272 ± 0.103	1.272 ± 0.107	1.049 ± 0.052	1.050 ± 0.052
17.4	apigenin	269	149	2.134 ± 0.293	2.131 ± 0.287	1.195 ± 0.090	1.195 ± 0.088	0.944 ± 0.028	0.944 ± 0.030
15.8	luteolin	285	285	1.109 ± 0.122	1.106 ± 0.110	1.199 ± 0.076	1.198 ± 0.086	1.152 ± 0.062	1.151 ± 0.066

Table S4.4. Phenolic profile (mg/g DW) of the stems of the Giarraffa (GIA), Leccino (LEC), and Maurino (MAU) cultivars measured under control (CTRL) conditions and exposed to drought stress (DS) for two weeks (**t2**). The mean values ± standard deviation (n = 3–4) are presented. Rt—retention time; nd—not detected.

Rt (min.)	Compound	[M-H] –(m/z)	MS^2 (m/z) Fragments	GIA CTRL	GIA DS	LEC CTRL	LEC DS	MAU CTRL	MAU DS
	secoiridoids								
14.4	oleuropein	539	377/275	0.773 ± 0.149	0.687 ± 0.171	1.141 ± 0.289	1.306 ± 0.242	3.889 ± 0.684	4.382 ± 0.870
15.4	fraxamoside	537	223/375	nd	nd	2.562 ± 0.549	2.646 ± 0.553	nd	nd
	flavonoids								
11.9	dihydroquercetin	303	285/177/ 125	2.997 ± 0.538	2.718 ± 0.625	5.600 ± 1.225	4.025 ± 0.841	3.421 ± 0.612	4.036 ± 0.735
12.8	quercetin-3-*O*-glucoside	463	301	2.336 ± 0.381	2.120 ± 0.444	2.519 ± 0.381	2.036 ± 0.251	2.054 ± 0.230	2.591 ± 0.324
13.5	chrysoeriol-7-*O*-glucoside	461	299/446	1.025 ± 0.142	0.961 ± 0.164	1.159 ± 0.130	0.866 ± 0.088	1.097 ± 0.058	1.406 ± 0.112
13.6	luteolin-7-*O*-glucoside	447	285	9.848 ± 1.726	8.711 ± 2.143	4.332 ± 0.765	2.662 ± 0.410	3.568 ± 0.532	4.287 ± 0.623
15.5	quercetin	301	285/257	0.753 ± 0.035	0.723 ± 0.042	1.271 ± 0.111	0.969 ± 0.094	1.050 ± 0.052	1.326 ± 0.124
17.4	apigenin	269	149	2.128 ± 0.281	1.935 ± 0.336	1.196 ± 0.086	0.908 ± 0.059	0.945 ± 0.033	1.557 ± 0.253
15.8	luteolin	285	285	1.102 ± 0.102	1.032 ± 0.118	1.197 ± 0.095	0.857 ± 0.051	1.150 ± 0.069	1.818 ± 0.178

Table S4.5. Phenolic profile (mg/g DW) of the stems of the Giarraffa (GIA), Leccino (LEC), and Maurino (MAU) cultivars measured under control (CTRL) conditions and exposed to drought stress (DS) for four weeks (**t4**). The mean values ± standard deviation (n = 3–4) are presented. Rt—retention time; nd—not detected.

Rt (min.)	Compound	[M-H] –(m/z)	MS^2 (m/z) Fragments	GIA CTRL	GIA DS	LEC CTRL	LEC DS
	secoiridoids						
14.4	oleuropein	539	377/275	0.723 ± 0.096	0.924 ± 0.131	1.238 ± 0.232	1.333 ± 0.294
15.4	fraxamoside	537	223/375	nd	nd	2.191 ± 0.396	0.979 ± 0.245
	flavonoids						
11.9	dihydroquercetin	303	285/177/ 125	2.823 ± 0.373	3.914 ± 0.483	4.854 ± 0.931	3.757 ± 0.773
12.8	quercetin-3-*O*-glucoside	463	301	2.213 ± 0.260	2.606 ± 0.308	2.158 ± 0.276	2.230 ± 0.426
13.5	chrysoeriol-7-*O*-glucoside	461	299/446	0.996 ± 0.107	1.249 ± 0.078	1.005 ± 0.119	1.122 ± 0.258
13.6	luteolin-7-*O*-glucoside	447	285	9.301 ± 1.178	13.66 ± 2.081	3.751 ± 0.583	4.407 ± 0.857
15.5	quercetin	301	285/257	0.762 ± 0.024	0.946 ± 0.048	1.106 ± 0.083	0.866 ± 0.052
17.4	apigenin	269	149	2.050 ± 0.187	2.091 ± 0.230	1.019 ± 0.064	1.147 ± 0.101
15.8	luteolin	285	285	1.100 ± 0.070	1.376 ± 0.117	1.029 ± 0.068	0.976 ± 0.069

Table S4.6. Lipophilic profile (mg/g DW) of the leaves of the Giarraffa (GIA), Leccino (LEC), and Maurino (MAU) cultivars measured under control (CTRL) conditions and exposed to drought stress (DS) before the start of water deprivation (**t0**). The mean values ± standard deviation (n = 3–4) are presented. Rt—retention time; nd—not detected.

Rt (min.)	Compound	GIA CTRL	GIA DS	LEC CTRL	LEC DS	MAU CTRL	MAU DS
	sterols and terpenes						
34.0	neophytadiene	0.966 ± 0.019	0.963 ± 0.014	0.608 ± 0.011	0.611 ± 0.008	0.647 ± 0.014	0.648 ± 0.014
42.1	phytol	nd	nd	0.588 ± 0.002	0.589 ± 0.001	nd	nd
55.4	squalene	nd	nd	0.613 ± 0.011	0.608 ± 0.004	0.601 ± 0.011	0.605 ± 0.007
68.0	β - amyrin	1.321 ± 0.102	1.324 ± 0.212	0.689 ± 0.010	0.689 ± 0.013	0.731 ± 0.0315	0.733 ± 0.038
69.0	α - amyrin	1.419 ± 0.419	1.442 ± 0.408	0.647 ± 0.006	0.646 ± 0.000	0.677 ± 0.011	0.675 ± 0.013
72.8	lupeol derivatives	2.311 ± 0.534	2.307 ± 0.547	0.679 ± 0.010	0.679 ± 0.001	0.654 ± 0.011	0.655 ± 0.011
73.4	ursolic acid	1.689 ± 0.593	1.682 ± 0.547	0.831 ± 0.027	0.830 ± 0.023	1.085 ± 0.107	1.083 ± 0.120
73.6	ursolic acid aldeyde	nd	nd	0.672 ± 0.018	0.668 ± 0.036	0.731 ± 0.029	0.730 ± 0.036
	sugars						
35.5	α - D - mannopyranose	nd	nd	0.173 ± 0.006	0.173 ± 0.002	0.196 ± 0.010	0.194 ± 0.010
37.7	D - glucose	0.250 ± 0.009	0.251 ± 0.008	0.188 ± 0.003	0.188 ± 0.002		
	alcohols						
36.3	D - sorbitol	1.092 ± 0.034	1.094 ± 0.039	0.814 ± 0.010	0.815 ± 0.013	0.875 ± 0.094	0.871 ± 0.007
63.0	α - tocopherol	nd	nd	0.670 ± 0.010	0.675 ± 0.011	nd	nd
	fatty acids						
39.2	palmitic acid	11.393 ± 0.318	11.392 ± 0.298	7.411 ± 0.087	7.416 ± 0.150	7.750 ± 0.668	7.752 ± 0.535
42.8	linoleic acid	nd	nd	6.472 ± 0.030	6.468 ± 0.010	6.419 ± 0.008	6.420 ± 0.003
42.9	α - linolenic acid	10.450 ± 0.019	10.497 ± 0.043	nd	nd	nd	nd
43.1	oleic acid	nd	nd	7.372 ± 0.415	7.374 ± 0.548	6.635 ± 0.005	6.635 ± 0.023
43.2	oleic acid derivative	nd	nd	6.505 ± 0.057	6.508 ± 0.0778	6.417 ± 0.006	6.417 ± 0.004
43.7	stearic acid	10.865 ± 0.057	10.867 ± 0.072	7.011 ± 0.109	6.989 ± 0.090	7.739 ± 0.100	7.236 ± 0.185
45.5	α - monopalmitin	10.283 ± 0.031	10.283 ± 0.036	6.469 ± 0.036	6.464 ± 0.014	6.544 ± 0.033	6.549 ± 0.041
50.7	α - monopalmitin derivative	10.420 ± 0.028	10.420 ± 0.050	6.540 ± 0.010	6.537 ± 0.009	6.701 ± 0.098	6.704 ± 0.096
	alkanes						
57.6	long chain alkane 1	1.603 ± 0.097	1.606 ± 0.107	0.897 ± 0.012	0.896 ± 0.003	1.122 ± 0.088	1.117 ± 0.104
62.6	long chain alkane 2	2.109 ± 0.219	2.107 ± 0.226	1.193 ± 0.050	1.191 ± 0.047	1.544 ± 0.138	1.545 ± 0.169
67.7	long chain alkane 3	2.931 ± 0.737	2.929 ± 0.760	1.630 ± 0.141	1.625 ± 0.062	2.289 ± 0.343	2.286 ± 0.208
73.0	long chain alkane 4	1.993 ± 0.448	1.999 ± 0.189	1.004 ± 0.021	1.003 ± 0.076	1.230 ± 0.111	1.229 ± 0.065

Table S4.7. Lipophilic profile (mg/g DW) of the leaves of the Giarraffa (GIA), Leccino (LEC), and Maurino (MAU) cultivars measured under control (CTRL) conditions and exposed to drought stress (DS) for two weeks (**t2**). The mean values ± standard deviation (n = 3–4) are presented. Rt—retention time; nd—not detected.

Rt (min.)	Compound	GIA CTRL	GIA DS	LEC CTRL	LEC DS	MAU CTRL	MAU DS
	sterols and terpenes						
34.0	neophytadiene	0.961 ± 0.012	nd	0.613 ± 0.006	0.604 ± 0.011	0.649 ± 0.027	0.772 ± 0.002
42.1	phytol	nd	nd	0.590 ± 0.001	0.571 ± 0.002	0.597 ± 0.012	nd
55.4	squalene	nd	nd	0.604 ± 0.008	0.588 ± 0.007	0.610 ± 0.004	0.798 ± 0.005
68.0	β - amyrin	1.327 ± 0.367	1.187 ± 0.045	0.689 ± 0.017	0.688 ± 0.081	0.735 ± 0.044	0.905 ± 0.030
69.0	α - amyrin	1.425 ± 0.399	1.149 ± 0.127	0.645 ± 0.006	0.678 ± 0.050	0.674 ± 0.014	0.946 ± 0.050
72.8	lupeol derivatives	2.302 ± 0.686	1.523 ± 0.092	0.678 ± 0.010	0.785 ± 0.057	0.658 ± 0.016	1.246 ± 0.045
73.4	ursolic acid	1.675 ± 0.511	1.474 ± 0.006	0.829 ± 0.049	1.098 ± 0.133	1.082 ± 0.136	1.114 ± 0.043
73.6	ursolic acid aldeyde	1.348 ± 0.570	nd	0.662 ± 0.017	0.732 ± 0.063	0.128 ± 0.048	0.883 ± 0.026
	sugars						
35.5	α - D - mannopyranose	nd	nd	0.173 ± 0.002	0.165 ± 0.005	0.192 ± 0.022	0.192 ± 0.002
37.7	D - glucose	0.252 ± 0.008	nd	0.188 ± 0.004	0.179 ± 0.008	0.215 ± 0.026	0.197 ± 0.002
	alcohols						
36.3	D - sorbitol	1.095 ± 0.045	nd	0.817 ± 0.022	0.884 ± 0.055	0.868 ± 0.108	0.861 ± 0.018
63.0	α - tocopherol	nd	nd	0.680 ± 0.013	0.731 ± 0.074	0.669 ± 0.017	nd
	fatty acids						
39.2	palmitic acid	11.39 ± 0.28	11.47 ± 0.09	7.422 ± 0.218	7.203 ± 0.149	7.754 ± 0.421	12.26 ± 5.20
42.8	linoleic acid	nd	nd	6.466 ± 0.018	6.135 ± 0.005	6.422 ± 0.010	8.323 ± 0.004
42.9	α - linolenic acid	10.50 ± 0.10	10.74 ± 0.02	6.384 ± 0.034	nd	nd	nd
43.1	oleic acid	nd	nd	7.376 ± 0.681	6.611 ± 0.021	6.635 ± 0.045	9.070 ± 1.052
43.2	oleic acid derivative	nd	nd	6.511 ± 0.148	6.177 ± 0.016	6.417 ± 0.003	8.333 ± 0.015
43.7	stearic acid	10.84 ± 0.139	11.14 ± 0.071	6.967 ± 0.129	6.798 ± 0.077	7.233 ± 0.269	8.904 ± 0.043
45.5	α - monopalmitin	10.28 ± 0.042	10.66 ± 0.015	6.459 ± 0.009	6.229 ± 0.027	6.554 ± 0.063	8.415 ± 0.015
50.7	α - monopalmitin derivative	10.42 ± 0.072	10.76 ± 0.041	6.533 ± 0.018	6.350 ± 0.046	6.707 ± 0.112	8.470 ± 0.132
	alkanes						
57.6	long chain alkane 1	1.610 ± 0.118	1.594 ± 0.066	0.894 ± 0.0139	0.926 ± 0.042	1.113 ± 0.126	1.236 ± 0.023
62.6	long chain alkane 2	2.105 ± 0.234	2.233 ± 0.080	1.190 ± 0.089	1.297 ± 0.113	1.545 ± 0.230	1.552 ± 0.057
67.7	long chain alkane 3	2.926 ± 0.806	2.656 ± 0.0725	1.620 ± 0.195	2.127 ± 0.277	2.283 ± 0.490	1.908 ± 0.135
73.0	long chain alkane 4	2.001 ± 0.477	1.760 ± 0.046	1.002 ± 0.137	1.364 ± 0.179	1.229 ± 0.133	1.255 ± 0.017

Table S4.8. Lipophilic profile (mg/g DW) of the leaves of the Giarraffa (GIA), Leccino (LEC), and Maurino (MAU) cultivars measured under control (CTRL) conditions and exposed to drought stress (DS) for four weeks (**t4**). The mean values ± standard deviation (n = 3–4) are presented. Rt—retention time; nd—not detected.

Rt (min.)	Compound	GIA CTRL	GIA DS	LEC CTRL	LEC DS	MAU CTRL	MAU DS
	sterols and terpenes						
34.0	neophytadiene	1.034 ± 0.002	nd	0.778 ± 0.007	nd	1.066 ± 0.221	0.361 ± 0.002
42.1	phytol	nd	nd	0.753 ± 0.003	nd	nd	0.359 ± 0.004
55.4	squalene	nd	nd	0.604 ± 0.008	0.588 ± 0.007	0.610 ± 0.004	0.798 ± 0.005
68.0	β - amyrin	1.327 ± 0.367	1.187 ± 0.045	0.689 ± 0.017	0.688 ± 0.081	0.735 ± 0.044	0.905 ± 0.030
69.0	α - amyrin	nd	1.358 ± 0.613	0.841 ± 0.022	0.905 ± 0.010	0.996 ± 0.028	0.417 ± 0.002
72.8	lupeol derivatives	1.579 ± 0.355	nd	1.026 ± 0.065	nd	1.294 ± 0.071	0.547 ± 0.040
73.4	ursolic acid	1.922 ± 0.587	1.397 ± 0.219	1.169 ± 0.086	1.228 ± 0.331	1.261 ± 0.083	0.628 ± 0.058
73.6	ursolic acid aldeyde	nd	nd	0.861 ± 0.037	0.950 ± 0.060	1.000 ± 0.065	0.380 ± 0.008
	sugars						
35.5	α - D - mannopyranose	0.276 ± 0.003	nd	0.224 ± 0.004	0.217 ± 0.003	0.246 ± 0.013	0.122 ± 0.008
37.7	D - glucose	0.290 ± 0.003	nd	0.428 ± 0.326	0.225 ± 0.007	0.263 ± 0.018	0.131 ± 0.009
	alcohols						
36.3	D - sorbitol	1.327 ± 0.023	1.073 ± 0.010	1.172 ± 0.050	1.287 ± 0.126	1.106 ± 0.090	1.282 ± 0.173
63.0	α - tocopherol	nd	1.343 ± 0.353	0.813 ± 0.022	0.841 ± 0.175	nd	0.447 ± 0.019
	fatty acids						
39.2	palmitic acid	12.10 ± 0.204	11.20 ± 0.118	9.179 ± 0.093	10.08 ± 0.359	10.72 ± 0.347	4.524 ± 0.113
42.8	linoleic acid	10.99 ± 0.003	10.43 ± 0.004	8.189 ± 0.005	9.237 ± 0.238	9.624 ± 0.012	3.899 ± 0.004
42.9	α - linolenic acid	nd	10.64 ± 0.028	nd	nd	nd	4.001 ± 0.007
43.1	oleic acid	11.21 ± 0.047	nd	8.599 ± 0.030	10.19 ± 1.359	9.793 ± 0.053	nd
43.2	oleic acid derivative	nd	nd	8.268 ± 0.006	9.191 ± 0.032	9.625 ± 0.003	3.892 ± 0.004
43.7	stearic acid	11.67 ± 0.132	10.91 ± 0.082	8.797 ± 0.062	9.589 ± 0.067	10.29 ± 0.217	4.287 ± 0.070
45.5	α - monopalmitin	11.09 ± 0.027	10.48 ± 0.012	8.273 ± 0.012	9.169 ± 0.014	9.720 ± 0.046	3.950 ± 0.014
50.7	α - monopalmitin derivative	11.25 ± 0.048	10.61 ± 0.028	8.405 ± 0.029	9.278 ± 0.032	9.832 ± 0.086	3.999 ± 0.023
	alkanes						
57.6	long chain alkane 1	1.684 ± 0.021	1.589 ± 0.125	1.068 ± 0.014	1.176 ± 0.078	1.389 ± 0.103	0.592 ± 0.029
62.6	long chain alkane 2	2.297 ± 0.158	2.360 ± 0.435	1.448 ± 0.076	1.413 ± 0.242	1.740 ± 0.188	0.731 ± 0.057
67.7	long chain alkane 3	3.245 ± 0.398	2.871 ± 0.742	1.992 ± 0.133	1.860 ± 0.268	2.149 ± 0.356	0.903 ± 0.089
73.0	long chain alkane 4	2.215 ± 0.505	2.371 ± 1.068	1.451 ± 0.118	1.388 ± 0.248	1.381 ± 0.248	0.538 ± 0.026

Table S4.9. Lipophilic profile (mg/g DW) of the stems of the Giarraffa (GIA), Leccino (LEC), and Maurino (MAU) cultivars measured under c ontrol (CTRL) conditions and exposed to drought stress (DS) before the start of water deprivation (**t0**). The mean values ± standard deviation (n = 3–4) are presented. Rt—retention time; nd—not detected.

Rt (min.)	Compound	GIA CTRL	GIA DS	LEC CTRL	LEC DS	MAU CTRL	MAU DS
	sterols and terpenes						
67.4	stigmast-5-ene	0.731 ± 0.002	0.730 ± 0.003	0.720 ± 0.009	0.720 ± 0.008	0.726 ± 0.012	0.727 ± 0.013
71.7	lupeol derivatives	0.581 ± 0.003	0.582 ± 0.001	0.370 ± 0.026	0.0365 ± 0.027	0.356 ± 0.005	0.356 ± 0.003
73.0	ursolic acid	0.647 ± 0.006	0.647 ± 0.003	0.490 ± 0.010	0.490 ± 0.007	0.604 ± 0.006	0.603 ± 0.005
73.2	ursolic acid aldeyde	0.595 ± 0.005	0.593 ± 0.002	0.354 ± 0.006	0.353 ± 0.005	0.356 ± 0.006	0.356 ± 0.003
	sugars						
35.5	α - D - mannopyranose	0.139 ± 0.001	0.139 ± 0.001	0.097 ± 0.006	0.091 ± 0.002	0.088 ± 0.001	0.087 ± 0.000
37.5	D - glucose	0.145 ± 0.005	0.143 ± 0.003	0.087 ± 0.006	0.088 ± 0.003	0.087 ± 0.001	0.087 ± 0.001
51.4	turanose	0.147 ± 0.006	0.145 ± 0.003	0.096 ± 0.013	0.092 ± 0.006	0.349 ± 0.453	0.218 ± 0.226
	alcohols						
36.2	D - sorbitol	0.663 ± 0.006	0.662 ± 0.005	0.782 ± 0.031	0.784 ± 0.024	0.354 ± 0.005	0.353 ± 0.003
44.8	pentadecan-1-ol derivative	0.563 ± 0.006	0.562 ± 0.002	0.391 ± 0.000	0.389 ± 0.003	0.355 ± 0.005	0.354 ± 0.002
	fatty acids						
39.1	palmitic acid	6.475 ± 0.005	6.475 ± 0.002	4.693 ± 0.006	4.691 ± 0.009	5.440 ± 0.013	5.440 ± 0.023
42.7	α - linolenic acid	6.063 ± 0.005	6.064 ± 0.003	3.535 ± 0.006	3.533 ± 0.001	3.973 ± 0.013	3.971 ± 0.017
42.9	oleic acid	6.109 ± 0.003	6.109 ± 0.003	3.621 ± 0.009	3.620 ± 0.005	4.016 ± 0.012	4.013 ± 0.010
43.5	stearic acid	6.303 ± 0.005	6.306 ± 0.004	4.244 ± 0.041	4.243 ± 0.032	4.816 ± 0.006	4.814 ± 0.005
45.3	α - monopalmitin	6.107 ± 0.005	6.105 ± 0.003	3.631 ± 0.001	3.632 ± 0.001	4.066 ± 0.005	4.066 ± 0.005
45.6	palmitic acid derivative 1	6.207 ± 0.005	6.207 ± 0.003	3.854 ± 0.011	3.851 ± 0.004	4.325 ± 0.025	4.325 ± 0.026
49.3	palmitic acid derivative 2	6.116 ± 0.006	6.117 ± 0.003	3.513 ± 0.006	3.514 ± 0.005	4.090 ± 0.010	4.089 ± 0.010
50.5	α – monopalmitin derivative	6.724 ± 0.012	6.724 ± 0.003	4.410 ± 0.000	4.415 ± 0.021	6.093 ± 0.030	6.089 ± 0.035
54.5	monostearin	6.759 ± 0.010	6.756 ± 0.007	4.447 ± 0.015	4.442 ± 0.019	6.163 ± 0.082	6.168 ± 0.050

Table S4.10. Lipophilic profile (mg/g DW) of the stems of the Giarraffa, Leccino, and Maurino cultivars measured under control (CTRL) conditions and exposed to drought stress (DS) for two weeks (**t2**). The mean values ± standard deviation (n = 3–4) are presented. Rt—retention time; nd—not detected.

Rt (min.)	Compound	GIA CTRL	GIA DS	LEC CTRL	LEC DS	MAU CTRL	MAU DS
	sterols and terpenes						
67.4	stigmast-5-ene	0.729 ± 0.004	0.733 ± 0.007	0.719 ± 0.011	0.720 ± 0.008	0.727 ± 0.016	0.940 ± 0.034
71.7	lupeol derivatives	0.582 ± 0.033	0.522 ± 0.000	0.361 ± 0.041	0.494 ± 0.008	0.355 ± 0.001	0.696 ± 0.002
73.0	ursolic acid	0.647 ± 0.020	0.642 ± 0.035	0.490 ± 0.011	0.596 ± 0.020	0.601 ± 0.016	nd
73.2	ursolic acid aldeyde	0.592 ± 0.022	0.612 ± 0.027	0.352 ± 0.005	0.481 ± 0.022	0.355 ± 0.002	0.696 ± 0.004
	sugars						
35.5	α-D-mannopyranose	0.139 ± 0.004	0.132 ± 0.000	0.085 ± 0.002	nd	0.087 ± 0.000	0.171 ± 0.000
37.5	D-glucose	0.141 ± 0.003	0.135 ± 0.000	0.089 ± 0.000	0.107 ± 0.000	0.087 ± 0.000	0.171 ± 0.000
51.4	turanose	0.142 ± 0.004	0.135 ± 5.321	0.087 ± 0.000	nd	0.087 ± 0.000	0.171 ± 0.00
	alcohols						
36.2	D-sorbitol	0.660 ± 0.024	0.705 ± 0.002	0.786 ± 0.023	0.566 ± 0.005	0.353 ± 0.000	0.692 ± 0.000
44.8	pentadecan-1-ol derivative	0.560 ± 0.008	0.546 ± 0.003	0.387 ± 0.006	0.489 ± 0.011	0.353 ± 0.000	0.691 ± 0.000
	fatty acids						
39.1	palmitic acid	6.476 ± 0.094	6.304 ± 0.002	4.688 ± 0.014	5.531 ± 0.015	5.439 ± 0.033	8.191 ± 0.039
42.7	α-linolenic acid	6.064 ± 0.171	5.754 ± 0.007	3.531 ± 0.006	4.743 ± 0.006	3.970 ± 0.024	7.639 ± 0.001
42.9	oleic acid	6.110 ± 0.185	5.775 ± 0.006	3.619 ± 0.001	4.823 ± 0.007	4.010 ± 0.012	7.639 ± 0.001
43.5	stearic acid	6.309 ± 0.093	6.141 ± 0.003	4.242 ± 0.029	5.170 ± 0.008	4.811 ± 0.007	8.024 ± 0.030
45.3	α-monopalmitin	6.103 ± 0.154	5.822 ± 0.001	3.634 ± 0.002	4.786 ± 0.003	4.066 ± 0.013	7.639 ± 0.001
45.6	palmitic acid derivative 1	6.206 ± 0.144	5.945 ± 0.002	3.848 ± 0.006	4.935 ± 0.006	4.325 ± 0.028	7.891 ± 0.035
49.3	palmitic acid derivative 2	6.117 ± 0.154	5.834 ± 0.004	3.515 ± 0.013	4.790 ± 0.005	4.089 ± 0.011	7.639 ± 0.001
50.5	α-monopalmitin derivative	6.724 ± 0.190	6.380 ± 0.007	4.420 ± 0.043	5.268 ± 0.003	6.085 ± 0.078	8.378 ± 0.026
54.5	monostearin	6.753 ± 0.204	6.381 ± 0.005	4.438 ± 0.038	5.290 ± 0.015	6.174 ± 0.082	8.364 ± 0.083

Table S4.11. Lipophilic profile (mg/g DW) of the stems of the Giarraffa (GIA), Leccino (LEC), and Maurino (MAU) cultivars measured under c ontrol (CTRL) conditions and exposed to drought stress (DS) for four weeks (**t4**). The mean values ± standard deviation (n = 3–4) are presented. Rt—retention time; nd—not detected.

Rt (min.)	Compound	GIA CTRL	GIA DS	LEC CTRL	LEC DS
	sterols and terpenes				
67.4	stigmast-5-ene	0.635 ± 0.035	0.932 ± 0.028	0.882 ± 0.093	0.901 ± 0.006
71.7	lupeol derivatives	0.418 ± 0.054	0.876 ± 0.061	0.756 ± 0.000	nd
73.0	ursolic acid	0.510 ± 0.056	0.970 ± 0.049	0.870 ± 0.083	0.807 ± 0.005
73.2	ursolic acid aldeyde	0.393 ± 0.045	0.880 ± 0.089	nd	nd
	sugars				
35.5	α - D - mannopyranose	0.086 ± 0.001	0.184 ± 0.001	0.190 ± 0.004	nd
37.5	D - glucose	0.091 ± 0.000	0.188 ± 0.001	0.193 ± 0.006	0.178 ± 0.000
51.4	turanose	0.087 ± 0.002	0.184 ± 0.001	0.194 ± 0.007	nd
	alcohols				
36.2	D - sorbitol	0.511 ± 0.003	0.954 ± 0.008	0.990 ± 0.177	0.776 ± 0.004
44.8	pentadecan-1-ol derivative	0.360 ± 0.002	0.730 ± 0.015	nd	0.732 ± 0.006
	fatty acids				
39.1	palmitic acid	4.419 ± 0.004	8.373 ± 0.008	8.635 ± 0.254	8.341 ± 0.009
42.7	α - linolenic acid	3.616 ± 0.002	8.019 ± 0.005	8.364 ± 0.055	7.877 ± 0.002
42.9	oleic acid	4.098 ± 0.004	8.252 ± 0.009	8.541 ± 0.184	8.192 ± 0.004
43.5	stearic acid	3.638 ± 0.002	8.041 ± 0.003	8.350 ± 0.043	7.921 ± 0.002
45.3	α - monopalmitin	3.803 ± 0.002	8.132 ± 0.000	8.419 ± 0.094	8.026 ± 0.003
45.6	palmitic acid derivative 1	3.653 ± 0.001	8.056 ± 0.001	8.363 ± 0.053	7.933 ± 0.002
49.3	palmitic acid derivative 2	4.371 ± 0.007	8.715 ± 0.004	8.865 ± 0.425	8.661 ± 0.008
50.5	α – monopalmitin derivative	4.406 ± 0.009	8.751 ± 0.011	8.862 ± 0.422	8.674 ± 0.006

Chapter 5

Proline, sugars and water-related proteins: the biochemical drivers of water management

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5.1 Introduction to the chapter

As described in the previous chapters, olive trees, like other plants, respond to drought in a complex manner involving morphological, physiological and metabolic changes. Morphological and physiological analyses provide information on the overall plant response, whereas biochemical and molecular analyses reveal how plants implement response mechanisms. The study of biochemical responses to drought is difficult due to the potential involvement of numerous metabolic and enzymatic factors, such as the activity of water stress proteins, sugar metabolism, and levels of osmoprotectants (such as proline), all of which may contribute significantly to olive plants' tolerance to water deficit (Oguz et al., 2022). In this chapter, we focused on molecules that can influence water management within plant organs, as well as molecules required to prevent non-native protein aggregation and maintain protein functional conformation during cell dehydration. These molecules include proteins, sugars, and the amino acid proline. Dehydrins, also known as group II late embryogenesis abundant (LEA) proteins, are hydrophilic, thermostable, disordered stress proteins with a high concentration of charged amino acids. They are produced in response to cold and drought stress in a wide range of organisms, including higher plants, algae, yeast, and cyanobacteria. Late embryogenesis abundant (LEA) proteins accumulate in vegetative tissues during normal growth and in response to cellular dehydration caused by stresses such as drought, low temperature, and salinity, when they serve a protective function (Banerjee and Roychoudhury, 2016). The link between dehydrin and drought responses is further supported by abscisic acid, which accumulates in cells during dehydration and enhances dehydrin synthesis (Tiwari et al., 2019). Dehydrin protection is expressed in a variety of functions, including increasing water retention, increasing chlorophyll content, maintaining the photosynthetic machinery, activating ROS detoxification, and promoting the accumulation of compatible solutes (Riyazuddin et al., 2022). Osmotin is a multifunctional protein of the pathogenesis-related (PR) protein family, specifically the PR-5 family, which is a large family of defense proteins with antimicrobial properties that provide tolerance to a variety of abiotic and biotic stresses (Manghwar and Hussain, 2022). Under abiotic stresses such as drought, salt, and cold, osmotins or osmotin-like proteins (OLPs) induce the expression of genes involved in proline biosynthesis and act as

osmoprotectants themselves, providing enzyme protection and protein chaperone functions (Hakim et al., 2018). Osmotins also play a role in chlorophyll protection, which helps to preserve photosynthetic processes during drought (Viktorova et al., 2019).

Additionally, aquaporins (AQPs) are important transmembrane channel proteins that play a critical role in the plant defence response to drought stress. They regulate plant water relations and participate in water and nutrient transport (Gautam and Pandey, 2021). Aquaporins play a role in the uptake of water by plant leaves in arid and semi-arid regions that are severely affected by drought by regulating transpiration rate and hydraulic conductivity (Vignesh and Palanisamy, 2021). Aquaporins thus play an important role in the regulation of water levels in the plant system. This protein family is divided into five different subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), small basic intrinsic proteins (SIPs), nodulin-26-like intrinsic proteins (NIPs), and X intrinsic proteins (XIPs) (Ren et al., 2021). The PIP family is the most abundant in living cells associated with long-distance transport tissues (Secchi et al., 2017). They also help transport small neutral molecules like glycerol, urea, metalloids, carbon dioxide, nitrogen, hydrogen peroxide, and ammonia (Kourghi et al., 2018). PIPs play an important role in regulating ion homeostasis and water levels in response to environmental stresses.

Proline is an amino acid that has shown effectiveness in improving drought tolerance in various plants by acting as an osmoprotectant and assisting plants in maintaining cellular water balance during drought stress (Ghosh et al., 2022). It also plays a critical role in scavenging reactive oxygen species produced during drought conditions, thus protecting cellular structures from oxidative damage (Ozden et al., 2009). Furthermore, proline has been shown to regulate the expression of stress-responsive genes, thereby improving plant resilience in drought-prone environments (Pham et al., 2020). There is a large body of literature focusing on proline levels in olive trees exposed to drought stress (Ahmed et al., 2009, 2016; Bacelar et al., 2009; Boughalleb and Mhamdi, 2011; Karimi et al., 2018; Melaouhi et al., 2021), but higher proline levels are not always associated with greater drought tolerance and this relationship requires further investigated.

Sugars are one of the most important molecular components in drought tolerance. Starch synthesis is reduced under water deficit stress, and remaining starch is hydrolysed and converted to sucrose. This boosts the accumulation of monosaccharides and disaccharides, which are essential components of drought tolerance. Sucrose can produce trehalose 6-phosphate, which is recognized as one of the most important sugars for drought tolerance. Furthermore, sucrose can combine with galactosides to form raffinose, a trisaccharide that consistently reduces water loss and improves drought resistance. Under water deficit stress, sucrose content increased and starch content decreased in olive leaves of various cultivars; accumulation of sucrose, in turn, can inhibit further photosynthetic activity through feedback mechanisms (Bacelar et al., 2009; Brito et al., 2019). Sucrose can be compartmentalized, but it is also converted into simpler compounds like glucose, mannitol, and fructose (Ahmadipour et al., 2018). Accumulation of the latter compounds results in a decrease in the water potential of leaf cells, which increases tolerance to water deficit (Dichio et al., 2003; Chehab et al., 2009). The leaves of two-year-old olive plants exposed to water deficit stress showed an increase in soluble carbohydrates (Karimi et al., 2018). It should be noted that the increase in carbohydrates, particularly mannitol and glucose, may be cultivar dependent (Lo Bianco and Scalisi, 2017); it can also be influenced by the severity of water deficit stress and the duration of the treatment. As a result, sugars are not only necessary for plant energy, but they also act as osmoprotectants, increasing water storage capacity (Secchi and Zwieniecki, 2016). Mannitol, a compatible solute, has been shown to accumulate in algae and higher plants during water scarcity and may play a role in drought tolerance. However, not all plants are known to produce mannitol. While mannitol appears to play an important role in increasing plant drought tolerance, the precise mechanisms and efficacy may differ depending on the plant species and the severity of the drought. Mannitol levels in olive trees were found to increase under moderate drought stress, but the effects were cultivar dependent (Lo Bianco and Scalisi, 2017).

In this chapter, we examined the levels of aquaporins, osmotins, and dehydrins that regulate water balance, as well as sugars (including mannitol), starch, and proline. The biochemical differences between the three cultivars were investigated in two different organs (the leaf and the stem). Following the previous physiological characterization of the cultivars' response to

drought and the effects of stress on metabolite profiles, the molecules involved in water management and compartmentalization within the plants were assessed. These molecules play a role in the water and carbon balance of plants. The hypothesis was that 'Leccino', which had lower antioxidant pools than 'Maurino' and higher stomatal conductance than 'Giarraffa' and 'Maurino' (which had the highest transpiration rate) relied on osmoprotectants to keep water within plant tissues.

5.2 Material and methods

5.2.1 Protein extraction

Olive leaves and stems from all experimental groups sampled at t0, t2 and t4 were used for stem analysis. The olive leaf is a very recalcitrant tissue, which makes protein extraction a long and difficult process. For this reason, we excluded t0 from leaf protein analysis and we preferred to obtain extracts from all time points during stress exposure, hypotising and relying on the comparison between drought stressed and control samples at each time point being sufficient. Since olive tissues are recalcitrant to protein extraction, the method described by Wu et al. (2014) was followed with some modifications. Briefly, for each experimental group, 3 samples of 0.3 g stem/leaf powder were prepared. To each sample, 1.8 mL of 10% TCA/acetone and 20 µL of DTT were added, and after 30 seconds of vortexing, the samples were left at -20 °C for 20 minutes. After centrifugation (15 000 rcf, 5 min, 4 °C), the supernatants were discarded and the pellets were washed three times with 80% acetone. After the final wash, the pellets were left at room temperature under vacuum to allow the solvents to evaporate completely. To the pellets, 0.8 mL SDS extraction buffer, 8 µL DTT 1M and 0.8 µL protease inhibitors were added to resuspend the proteins. The samples were allowed to stand for 1 hour at room temperature with gentle agitation. After centrifugation (15 000 rcf, 10 minutes, RT), the supernatants were collected in new tubes and divided into a number of tubes sufficient to contain a maximum of 0.8 mL of supernatants per tube. The same volume of Tris-buffered phenol was added to each tube. Samples were vortexed for 3 minutes and then centrifuged at 15 000 rcf, 5 minutes, RT. The phenolic upper phases were collected in new tubes (max 0.4 each) and 1.5 mL of ammonium acetate in methanol was added. After brief vortexing, the samples were left at −20

°C for 2 hours. After centrifugation (15 000 rcf, 10 minutes, 4 °C), the supernatants were discarded and a small spot of white pellet was visible. The pellets were washed twice with 80% acetone and then left under vacuum for 2/3 minutes. 100 µL Laemmli buffer was added to the first tube of each experimental group and heated at 95 °C for 5 minutes to resuspend the pellets. The same Laemmli buffer was then transferred to the second tube of the same experimental group and heated to resuspend the pellet present, and so on with all the tubes of the same experimental group.

Traditional methods of protein extract quantification (such as the 2-D Quant Kit or Bradford assay) were ineffective, most likely due to interfering substances present in olive leaf and stem extracts. To address these issues, we used two distinct procedures for leaves and stems, depending on the temporal availability of various imaging systems: Fluor-S (Bio-Rad) at the start of my PhD and ChemiDoc (Bio-Rad) in the second part. The latter can make use of the Stain-Free technology (Bio-Rad), which overcome the need of a precise protein quantification prior to gel loading.

5.2.2. Electrophoresis and immunoblotting of leaf protein extracts (without Stain-Free technology)

All protein extracts were initially separated on 10% bis-tris SDS-PAGE (Hachmann and Amshey, 2005) at pH 6.5-6.8 with all blue standard (Bio-Rad) as a molecular weight marker. The run lasted approximately 45 minutes at 200 V and used XT MOPS (Bio-Rad Laboratories, Hercules, CA, USA) as the running buffer. The proteins in each lane were quantified using ImageJ (https://imagej.net/ij/index.html), and a normalization factor was calculated for each extract to calculate the loading volume containing the same amount of protein for immunoblotting. Another electrophoresis (same conditions) was performed with the normalized volume of extracts and an unstained protein standard (Bio-Rad) as the molecular weight marker. Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Segrate, Italy) was used to transfer proteins to nitrocellulose membranes. Transfer was performed at 1.3 A, 25 V, for 5 minutes. The membranes were placed in 5% Blotting-Grade Blocker solution (Bio-Rad), 0.1% Tween 20 (Bio-Rad) in Tris-buffered saline (TBS) for one hour. The membranes were washed twice with TBS for 5 minutes each time. After removal of TBS, primary antibodies anti-osmotin

(Agrisera AS19 4336 rabbit polyclonal 1:1000), anti-dehydrin (Agrisera AS07206A rabbit polyclonal 1:1000), and anti-aquaporin (Agrisera AS09 489 rabbit polyclonal 1:1000) were added. The membranes were incubated for 1 hour with shaking and then washed twice with 1X TBS. The goat anti-rabbit secondary antibody (Bio-Rad 1706515 1:3000) was added and the membranes were incubated for one hour and then washed twice with 1X TBS. The immunological reaction was visualised using Clarity and Clarity Max Western ECL Multimagers (Bio-Rad Laboratories, United States). Images of the blots were acquired using a Fluor-S device (Bio-Rad Laboratories, Segrate, Italy) and analysed using Bio-Rad Quantity One software (Bio-Rad, Hercules, California 94547, USA) for relative quantitative assessment of band intensity subtracted from background (expressed as integrated density).

5.2.3 Electrophoresis and immunoblotting of stem protein extracts (Stain-Free technology)

Proteins were separated on polyacrylamide gels prepared with the TGX Stain-Free FastCast Acrylamide Kit 12% (Bio-Rad). Unstained protein standards (Bio-Rad) were used as molecular weight reference. The run was performed at 200 V for 45 minutes using TGS running buffer (Tris/Glycine/SDS buffer TGS 1X) (Bio-Rad). Finally, the gel was placed into the ChemiDoc MP Imaging System (Bio-Rad) and subjected to the Stain-Free Gel procedure (590/110 UV Trans) with auto optimal exposure for 45 seconds. For immunoblotting, proteins were transferred to a nitrocellulose membrane using the Trans-Blot Turbo instrument (Bio-Rad). Transfer was performed at 1.3 A, 25 V, for 5 minutes. The membrane was visualized in ChemiDoc with the Stain-Free Blot command (auto-optimized exposure) to verify proper transfer. The membranes were placed in 5% Blotting-Grade Blocker solution (Bio-Rad), 0.1% Tween 20 (Bio-Rad) in Tris-buffered saline (TBS) for one hour. The membranes were washed twice with TBS for 5 minutes each time. After removal of TBS, primary antibodies anti-osmotin (Agrisera AS19 4336 rabbit polyclonal 1:1000), anti-dehydrin (Agrisera AS07206A rabbit polyclonal 1:1000), and anti-aquaporin (Agrisera AS09 489 rabbit polyclonal 1:1000) were added. The membranes were incubated for 1 hour with shaking. The membranes were then washed twice with TBS for 10 minutes each time. Secondary antibody (goat anti-rabbit, IgG Star-Bright Blue 700, Bio-Rad, code 12004162, 1:2500) was added and the membranes were incubated for one hour. Finally, the membranes were loaded into ChemiDoc and total proteins

were visualized by selecting Stain-Free Blot, Auto-Optimal Exposure. The immunological reaction was visualized using the StarBright B700 Blot command (auto optimal exposure). Quantification of immunoblot signals was performed using Bio-Rad Image Lab software (Bio-Rad, Hercules, California 94547, USA). Each membrane was visualised in the Stain-Free Blot channel, and the lane with the highest protein abundance was selected as the reference. This lane was used to normalise the proteins present in all other lanes of the same membrane. The intensity of the immunoblotting signal in each lane was then normalised according to the relative protein content.

5.2.4 Proline content

Proline content was determined according to Khedr et al. (2003). Briefly, 100 mg of frozen leaf and stem powder was mixed with 1.5 mL of 3% sulfosalicylic acid and centrifuged at 10 000 rcf for 10 min at 4 °C. An aliquot of the supernatant (100 µL) was incubated (1 h at 100 °C) with 2 mL glacial acetic acid and 2 mL acidic ninhydrin (1.25 g ninhydrin heated to dissolution in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid). The samples were immediately cooled on ice, 1 mL of toluene was added. After vortexing, the samples were allowed to stand until the two phases separated. The absorbance of the upper phase (chromophore containing toluene) was read at 520 nm. The proline content was determined from a calibration curve for D-proline ($y = 13.945x + 0.006$, $R2 = 0.99$). Five leaf or stem samples for each experimental group were taken. Results show mean ± standard error.

5.2.5 High Performance Liquid Chromatography (HPLC) sugars analysis

Sugars (sucrose, fructose, glucose and mannitol) were analysed in leaves and stems of all experimental groups sampled at t0, t2 and t4, except for the stem samples of 'Maurino' at t4. Sugars were quantified according to Piccini et al. (2020), with some modifications. 0.2 g (fresh weight, FW) of stem and leaf powder were dried at 70 °C for 36 and 24 h to obtain the dry weight (DW) of stem and leaf samples, respectively. After the addition of 1 mL of distilled water, samples were homogenised using a tissue-lyser (2 minutes at 50 Hz). Homogenised samples were incubated at 70 °C for 10 minutes and then placed in an ultrasonic bath for 15 minutes. After centrifugation at 16 000 rcf for 5 minutes, supernatants were collected, and pellets were extracted a second time. The supernatants were filtered (0.45 µm) and 20 µL of the

extracts were injected. Analysis was performed on a Waters Sugar-Pak I ion exchange column (6.5 x 300 mm) at 90°C. MilliQ water (pH 7) was used as the mobile phase at a flow rate of 0.5 mL/min. The total run time was 30 minutes. Each sugar was identified using a Waters 2410 refractive index detector and by comparing the retention times with those of the standards (8 minutes - sucrose, 10 minutes - glucose, 11 minutes - fructose, 13 minutes - mannitol). The curve area of each peak was plotted against that of the standards for quantification. The results are expressed as the mean ± standard error of three replicates in mg/g DW.

5.2.6 Starch content

Starch content was measured according to the method proposed by Loppi et al. (2021). Briefly, 100 mg (FW) of stem and leaf powder were dried at 70 °C for 36 and 24 h to obtain the dry weight (DW) of stem and leaf samples, respectively. Powder was homogenised with ultra turrax and 4 mL of dimethyl sulfoxide (DMSO) and 0.5 mL of 8 M HCl were added. After an incubation period of 30 minutes at 60 °C, 0.5 mL of 8 M NaOH was added. Each sample was brought to the final volume of 10 mL with deionized water. After a centrifugation at 4000 rpm for 5 minutes, 0.5 mL of the supernatants were collected in new tubes. To each tube, 2.5 of Lugol solution (HCl 0.05 M - 0.03% I_2, 0.06% KI) was added and left to react for 15 minutes. Absorbance of the samples was read at 605 nm with a UV-Vis spectrophotometer (UV-1280, Shimadzu) and the starch was quantified using a calibration curve (0.4 - 0.0025 mg/mL) prepared with soluble starch (Sigma-Aldrich). Results show the mean ± standard error of three replicates in mg/g DW.

5.3 Results

5.3.1 Sugar levels in stems and leaves of olive cultivars

We compared the glucose levels in the leaves (**Figure 5.1A**) and stems (**Figure 5.1B**) of three olive cultivars under control (CTRL) and drought stress (DS) conditions at t0, t2, and t4. In all experimental groups examined, the stem had significantly lower glucose levels than the leaves. The glucose content in stems ranged from 5 to 15 mg/g DW, whereas leaves ranged from 40 to 80 mg/g. After 4 weeks of stress (t4), the glucose in the stem decreased significantly in the 'Leccino' and 'Giarraffa' stressed group compared to the corresponding control group. The

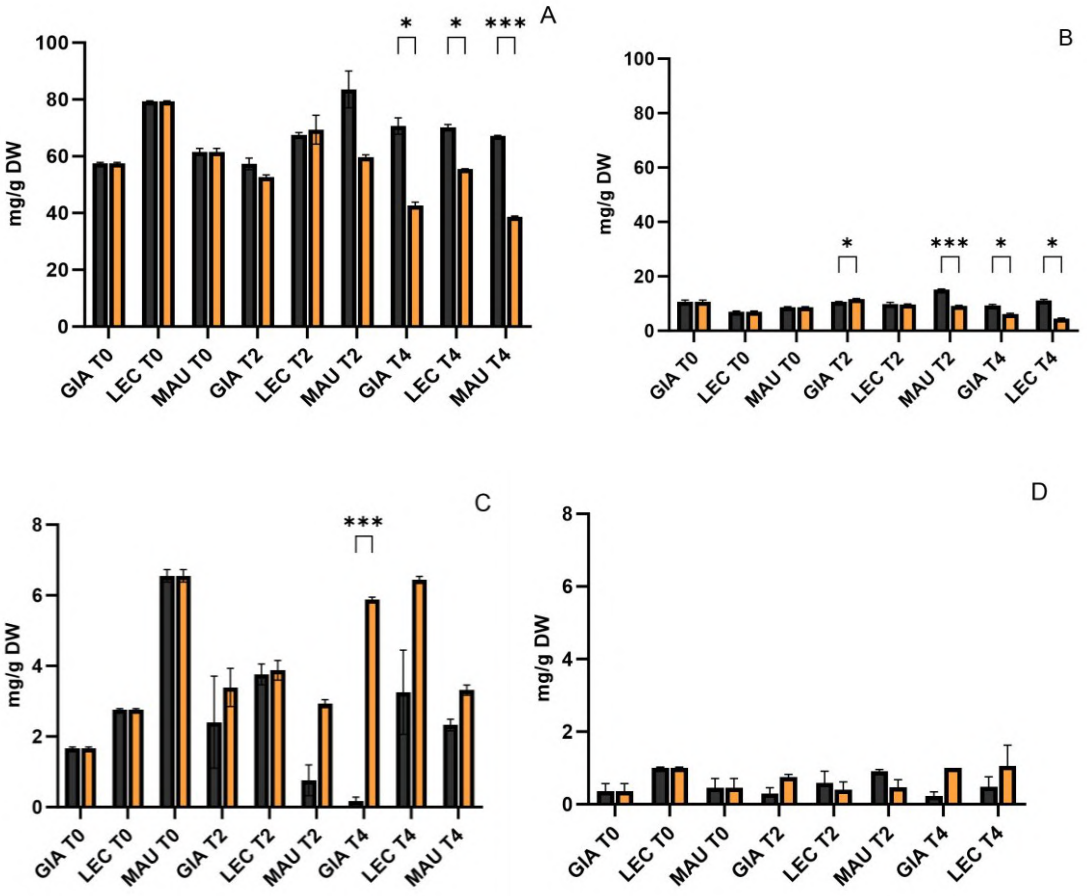
same difference emerged in 'Maurino' at t2. At the same time point, a first decrease in the glucose level of 'Maurino' DS leaves was observed, but it was not significant. At t4, leaves of all varieties showed a strong and significant difference in glucose levels between control and drought-stressed samples.

Different results were obtained with fructose. **Figure 5.1** depicts the fructose levels in both the leaves (**C**) and stems (**D**). As with glucose, fructose levels in stems were significantly lower than in leaves. In fact, the fructose level in stems ranged between 1-2 mg/g, whereas the fructose level in leaves was more consistent at 1-6 mg/g. In the stems, no significant differences were found between control and stressed samples for any of the cultivars, but in 'Giarraffa' and 'Leccino' DS a higher fructose level can be observed compared to their controls. The leaf samples generated more heterogeneous results. First, the fructose levels in the cultivars varied at t0, with 'Maurino' having three times the levels of 'Giarraffa'. At t4, 'Giarraffa' showed the only significant difference between the control and drought-stressed groups. However, the fructose content was higher in the drought-stressed samples of all the cultivars compared to the control. A similar trend was shown by the fructose content in the stems at t4.

The sucrose content was examined subsequently. The sucrose levels in the stems (**Figure 5.1F**) ranged from 1 to 10 mg/g, whereas the sucrose content in the leaves (**Figure 5.1E**) was always less than 5 mg/g. Sucrose levels in the stem were similar between control and stressed samples for all three cultivars at various time points. At t2, the only discernible differences were in 'Giarraffa', where the drought-stressed sample had slightly lower sucrose levels than the control, and in 'Maurino', where sucrose levels were higher and significantly different in the drought-stressed sample than in the control. In contrast, at t4, the cultivars Giarraffa and Leccino showed no significant differences between control and stressed samples. At t4, there was a significant difference in leaves sucrose level of 'Maurino' and 'Leccino' under drought stress and their respective controls. 'Giarraffa', on the other hand, showed no metabolic variation.

Mannitol is a sugar alcohol that acts as an osmoprotectant in plants, specifically olive trees. The control groups of the three cultivars had similar mannitol concentrations in both stem and leaf, around 20 mg/g DW. In stems (**Figure 5.1H**), 'Giarraffa' showed a slight increase in mannitol

in the stressed groups at t2 and t4, while the stressed group of 'Leccino' was lower than the control at t4. The differences were prominent in the leaves (**Figure 5.1G**). When exposed to drought stress, leaves of all cultivars had a higher mannitol content starting at t2. At t4, drought-stressed samples had nearly double the mannitol content of the controls.



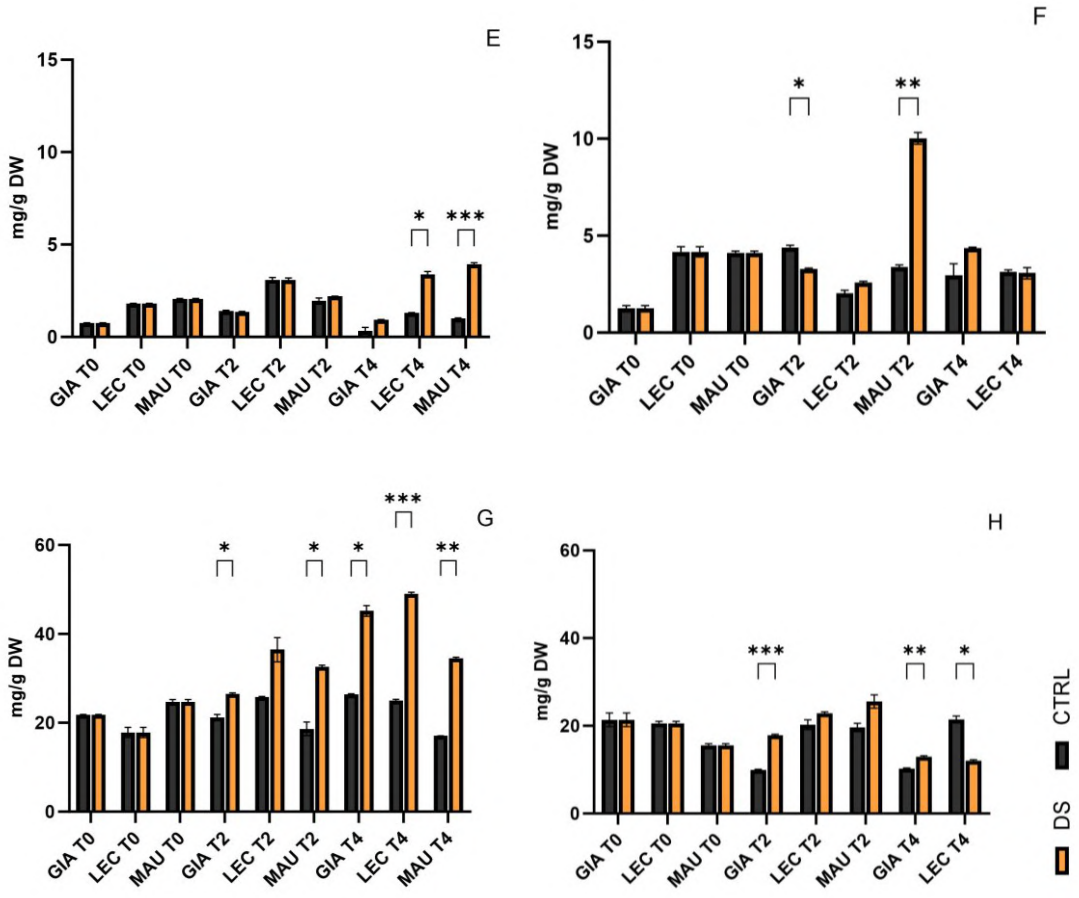


Figure 5.1. Levels of sugars detected by HPLC in Giarraffa (GIA), Leccino (LEC) and Maurino (MAU) under control (CTRL, black) and drought stress (DS, orange) conditions. A) glucose in leaf; B) glucose in stem; C) fructose in leaf; D) fructose in stem; E) sucrose in leaf; F) sucrose in stem; G) mannitol in leaf; H) mannitol in stem, expressed in mg g^{-1} tissue dry weight (DW). For each column, values are given as mean ± standard error. Asterisks (*) indicate significant differences between control and treated groups (* *p-value* < 0.05, ** = *p-value* < 0.01, *** = *p-value* < 0.001).

Proline levels in the samples analysed ranged from 10 to 40 µg/g DW (**Figures 5.2**). In stems (**Figure 5.2B**), the proline content is heterogeneous between the control and stressed groups of the three cultivars, but the only significant difference was found in 'Giarraffa' at t2, when the proline content in the drought-stressed sample was significantly lower than in the control. At t4, the results remained difficult to interpret, as both 'Giarraffa' and 'Leccino' showed deviations from the control values that were not statistically significant. In leaves (**Figure 5.2A**), 'Giarraffa' and 'Maurino' showed no difference between control and drought-stressed samples, whereas 'Leccino' showed higher and significantly different proline content in the stressed samples. Except for 'Maurino', the stressed samples in all three cultivars increased slightly at t4. Overall, the results showed no significant differences between control and

drought-stressed samples, implying that proline is not an osmoprotectant that is specifically used by the three cultivars under the experimental conditions to which they were exposed.

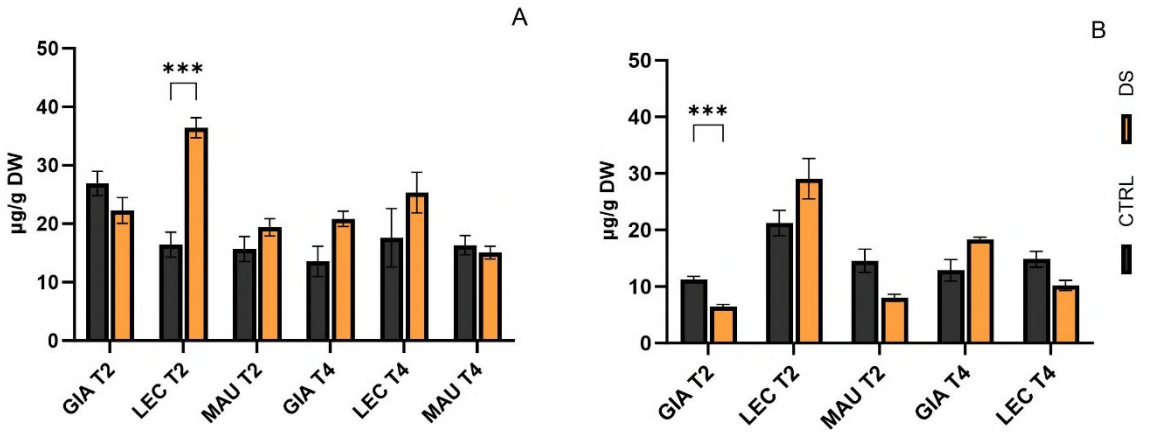


Figure 5.2. Proline content in leaf (A) and stem (B) of Giarraffa (GIA), Leccino (LEC) and Maurino (MAU) cultivars under control (CTRL, black) and drought stress (DS, orange) conditions. Contents are expressed as µg g^{-1} tissue dry weight (DW). For each column, values are expressed as mean ± standard error. Asterisks (*) indicate significant differences between control and treated groups (*** = *p-value* < 0.001).

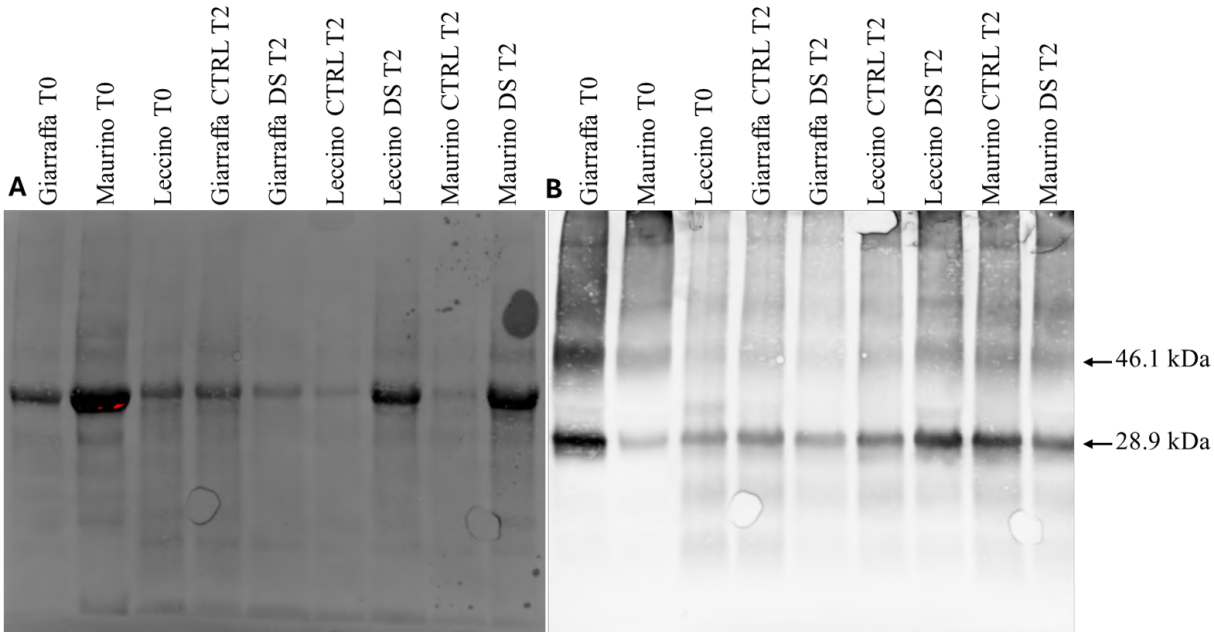
5.3.2 Starch content

The availability of simple sugars, such as sucrose, is determined by both photosynthetic activity and starch catabolism. As a result, starch levels in leaves and stems were also measured; in leaf samples, starch was determined just before the lamps were turned off (before night) and in the morning (at dawn). The control and drought-stressed groups had very similar starch levels in all cultivars, with the exception of 'Leccino' and 'Maurino' leaves sampled early in the morning, where the DS groups had significantly lower starch levels than the controls. Overall, a decreasing trend in starch content over time can be observed, particularly in stems and leaves collected at dawn. This could be due to environmental conditions that do not promote starch accumulation. For these reasons, the starch content was not further discussed, and the graphs are included in the Supplementary Material of this chapter (**Figure S5.1**).

5.3.3 Aquaporins

The blot in **Figures 5.3B** and **5.3D** show the accumulation of aquaporins (AQPs) in the stems of 'Giarraffa', 'Leccino' and 'Maurino' during the experiment. Bands were quantified relative to the total protein content of the corresponding lanes, which had been previously normalized

against a standard lane (**Figures 5.3A** and **5.3C**). Blots revealed three or more bands, but only the two most distinct bands (46.1 and 28.9 kDa) were used for quantification. Densitometric analysis (**Figure 5.3E**) revealed a highly heterogeneous accumulation of these proteins across the experimental groups. Compared to the other cultivars, 'Giarraffa' had an extremely high AQP content at t0. However, the content decreased in subsequent time points. The AQP content of this cultivar was lower in stressed stem samples at t2, but higher at t4 than in controls. At both t2 and t4, the stressed groups of the cultivar Leccino accumulated significantly more AQPs than the respective controls. In contrast, 'Maurino' accumulated a lesser amount AQPs in the stressed groups compared to the controls at both time points.



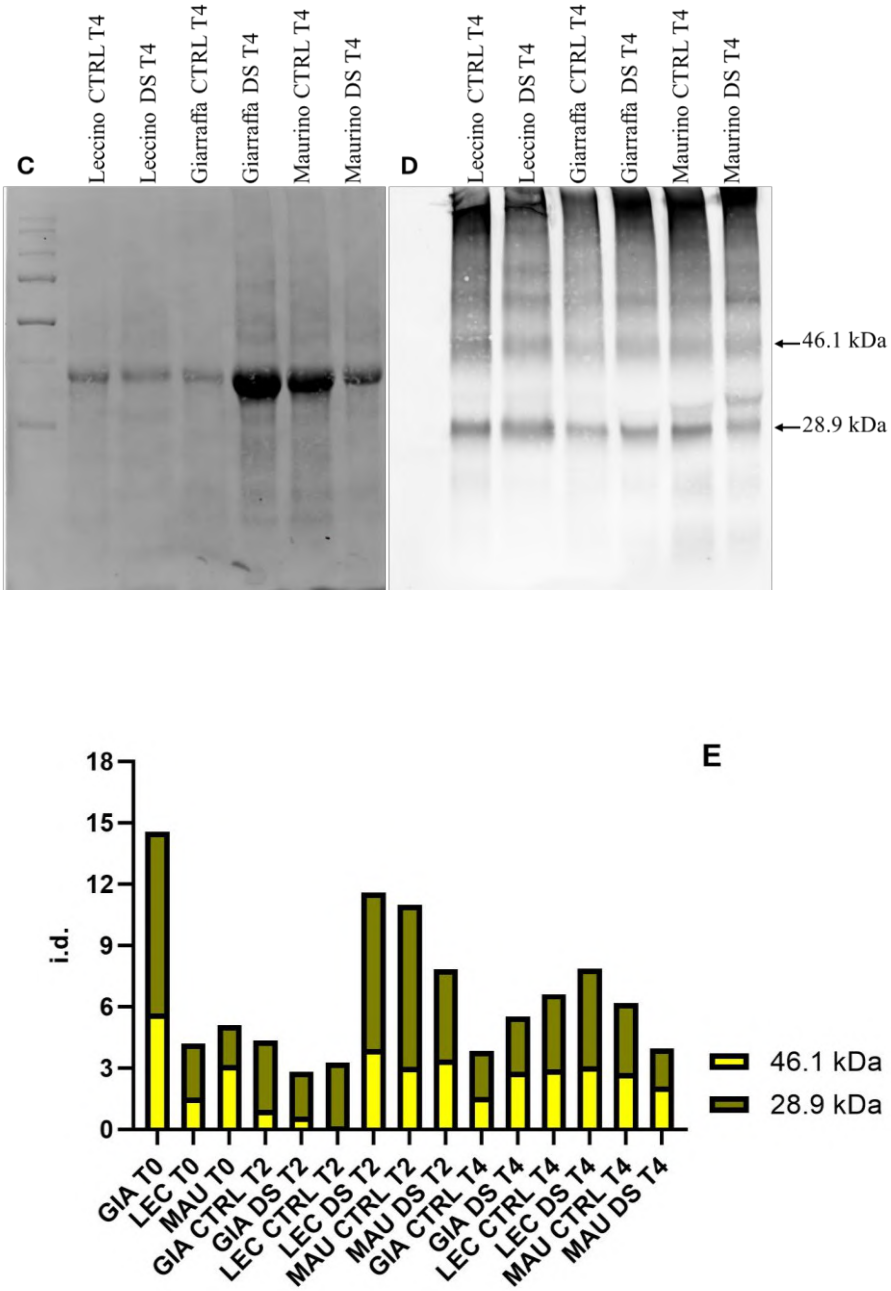


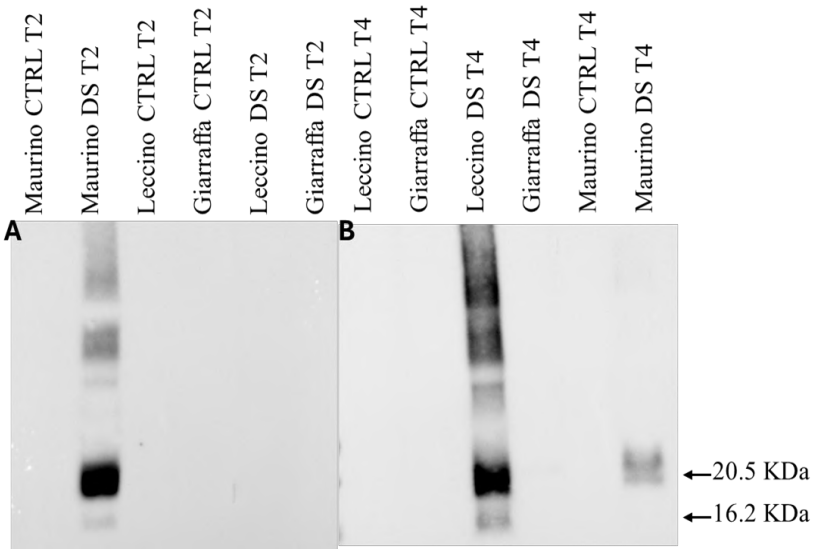
Figure 5.3. Aquaporin content in stems of Giarraffa (GIA), Leccino (LEC) and Maurino (MAU) cultivars under control (CTRL) and drought stress (DS) conditions, at the beginning of stress (t0), after two weeks (t2) and after four weeks (t4) of stress. (**A**) and (**C**) Stain-free membranes used for relative quantification of blotting; (**B**) and (**D**) immunoblotting with anti-aquaporin antibodies of the above labelled experimental groups; (**E**) relative quantification of blotting expressed as integrated density (i.d.). Note that panels A and B refer to t0-t2 samples, whereas panels C-D refer to t4 samples.

5.3.4 Dehydrins

Figures 5.4A and **5.4B** show a dehydrin blot on leaves. There was no stain-free membrane, and quantification was performed using the same amount of total protein in each lane for all

experimental groups. Two polypeptides with molecular weights of 20.4 and 16.3 kDa were detected in the leaves and quantified. The results for leaves (**Figure 5.4C**) are unambiguous: dehydrins were found only in two of the three cultivars, and all the groups with dehydrins had been subjected to drought stress. Maurino DS showed a significant increase in dehydrins at t2, and the proteins were still present at t4, albeit at lower levels. The stress group of Leccino did not accumulate dehydrins significantly until late at t4. No dehydrins were found in the Giarraffa cultivar.

Figures 5.5B and **5.5D** show the accumulation of dehydrins in the stems of 'Giarraffa', 'Leccino', and 'Maurino' throughout the course of the experiment. Each band was quantified relative to the lane's total protein content in comparison to a standard lane (**Figures 5.5A** and **5.5C**). In contrast to the leaves, three polypeptide bands with molecular weights of 13.1, 16.2, and 20.5 kDa were selected for quantification. The densitometric analysis (**Figure 5.5E**) revealed that the accumulation of dehydrins in the stems followed three distinct response patterns unique to each cultivar. Only the 'Maurino' cultivar accumulated these proteins in drought-stressed groups at t2 and t4. 'Giarraffa' and 'Leccino' adopted the opposite approach: the stressed group of 'Giarraffa' accumulated high levels of dehydrins at t2 but showed a slight decrease at t4 compared to the controls. 'Leccino' DS, on the other hand, showed a decrease at t2 compared to the controls and a significant increase at t4.



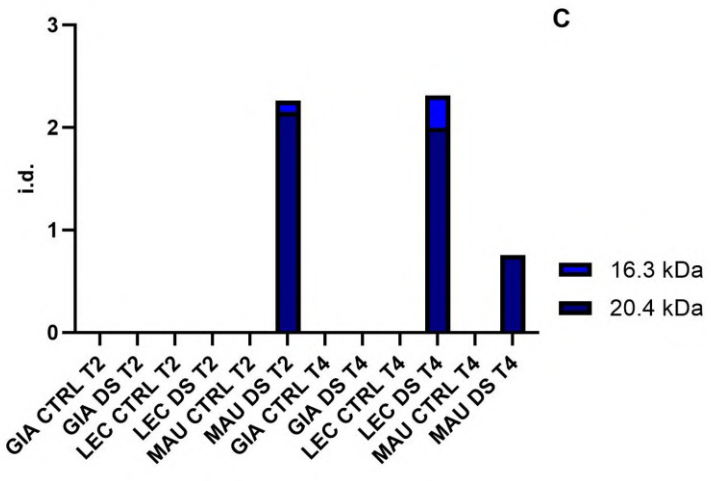
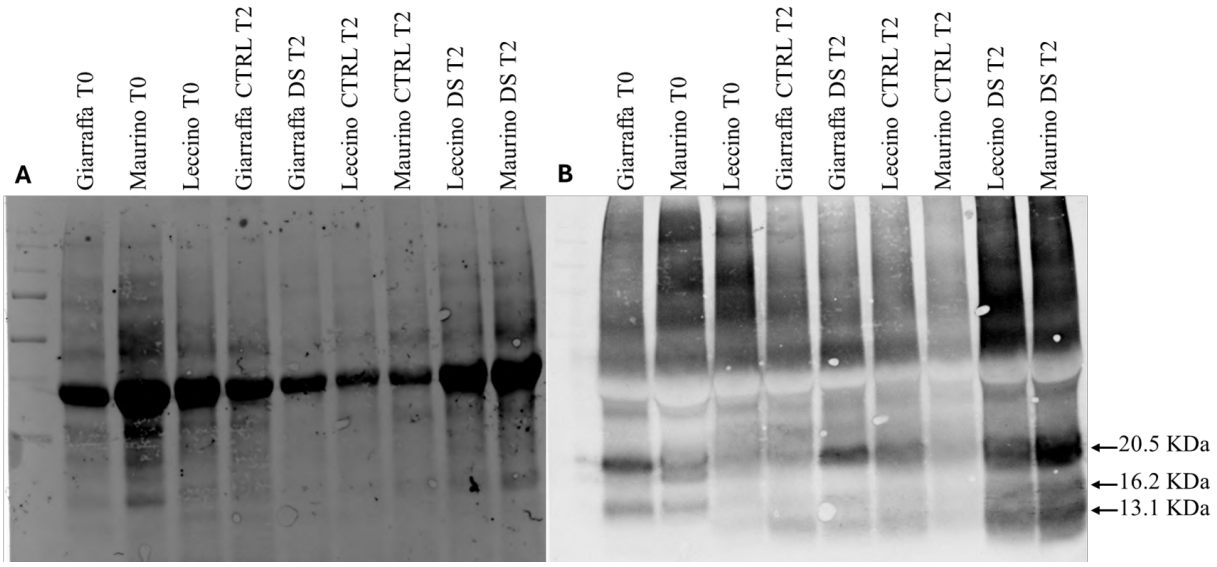


Figure 5.4. Dehydrin levels in leaves of Giarraffa (GIA), Leccino (LEC) and Maurino (MAU) cultivars under control (CTRL) and drought stress (DS) conditions, after two (t2) and four (t4) weeks of stress. (**A**) and (**B**) Immunoblotting with anti-dehydrin antibodies of the above labelled experimental groups; (**C**) relative quantification of blotting expressed as integrated density (i.d.).



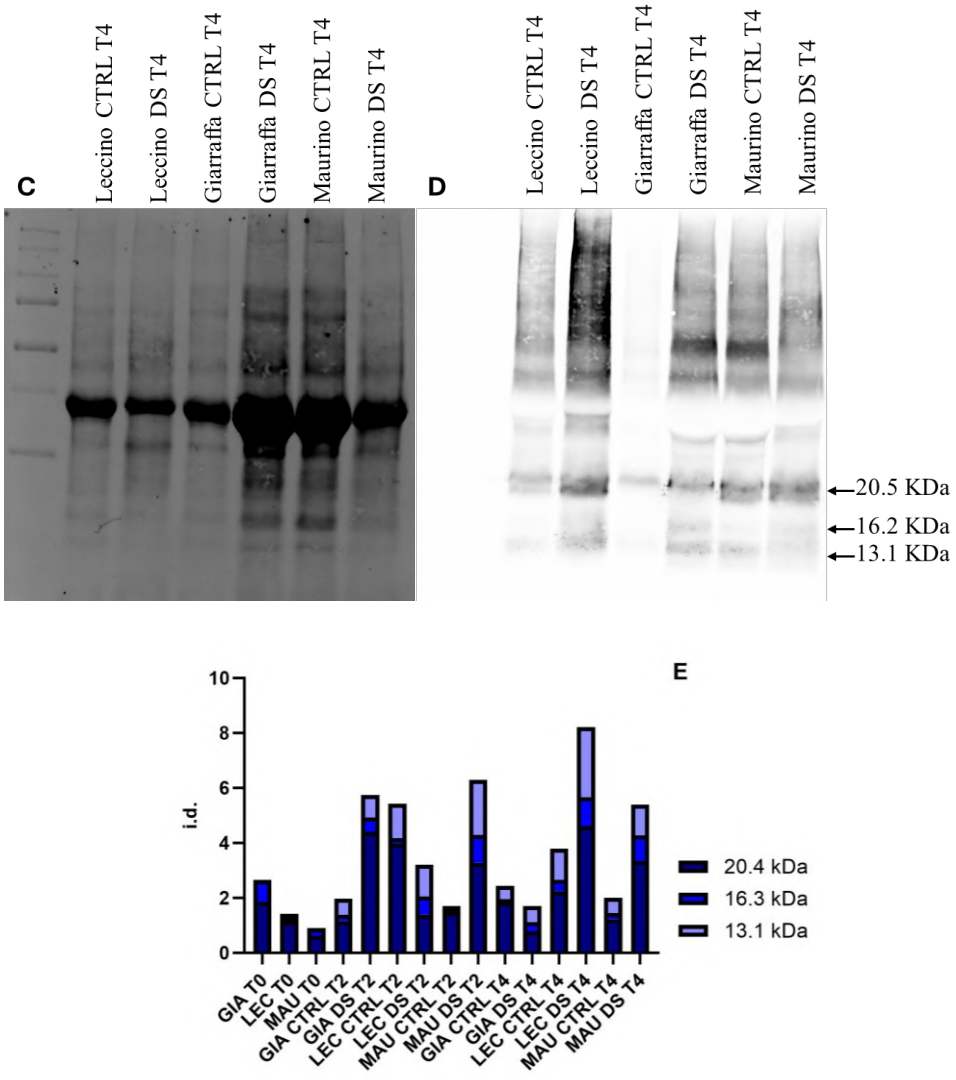


Figure 5.5. Dehydrin levels in stems of Giarraffa (GIA), Leccino (LEC) and Maurino (MAU) cultivars under control (CTRL) and drought stress (DS) conditions, at the beginning of stress (t0), after two weeks (t2) and after four weeks (t4) of stress. (**A**) and (**C**) Stain-free membranes used for relative quantification of blotting; (**B**) and (**D**) immunoblotting with anti-dehydrin antibodies of the above labelled experimental groups; (**E**) relative quantification of blotting expressed as integrated density (i.d.). Note that panels A and B refer to t0-t2 samples, whereas panels C-D refer to t4 samples.

5.3.5 Osmotin

The blots in **Figures 5.6A** and **5.6B** show the levels of osmotin detected in the leaves of three cultivars that were or were not exposed to drought stress. Two isoforms were identified at 30.1 and 28.3 kDa, though they were sometimes difficult to distinguish. The accumulation of osmotin (**Figure 5.6C**) was clearly correlated with drought, as it was higher at t4 in all stressed groups and cultivars. However, osmotin levels varied, with 'Giarraffa' having the lowest levels and 'Maurino' DS having the highest. At t2, the responses varied by cultivar. The stressed

'Giarraffa' group had lower osmotin levels than the control group, but this protein increased to t4. 'Leccino' and 'Maurino' behaved similarly, with 'Leccino' showing a consistent increase in this protein at both t2 and t4, whereas 'Maurino' increased osmotin content at t4 while remaining nearly identical to the control at t2.

Finally, the blots in **Figures 5.7B** and **5.7D** show the accumulation of osmotin in the stems of the three cultivars. As for the other proteins studied in the stems, the quantification of each band (**Figure 5.7E**) was carried out relative to the total protein content of the lane compared to a lane selected as a standard (**Figures 5.7A and 5.7C**). The content of osmotin in the stems of stressed 'Giarraffa' plants started to decrease at t2 and persisted until t4. On the other hand, 'Leccino' exhibited a different behaviour, with osmotin levels decreasing at t2 compared to controls but increasing in drought-stressed plants at t4. 'Maurino' exhibits a pattern of response similar to 'Leccino'. Again, the cultivar Giarraffa made a different temporal use of the protein osmotin than 'Leccino' e 'Maurino'.

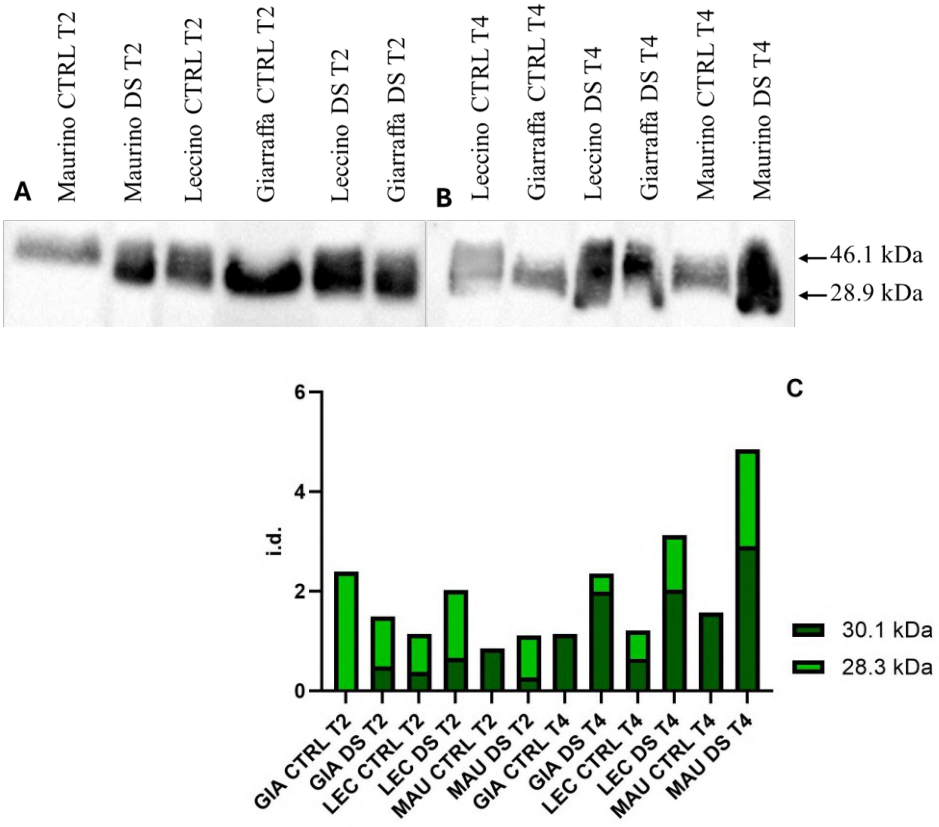


Figure 5.6. Osmotin levels in leaves of Giarraffa (GIA), Leccino (LEC) and Maurino (MAU) cultivars under control (CTRL) and drought stress (DS) conditions, after two (t2) and four (t4) weeks of stress. (**A**) and (**B**)

Immunoblotting with anti-osmotin antibodies of the above labelled experimental groups; (**C**) relative quantification of blotting expressed as integrated density (i.d.).

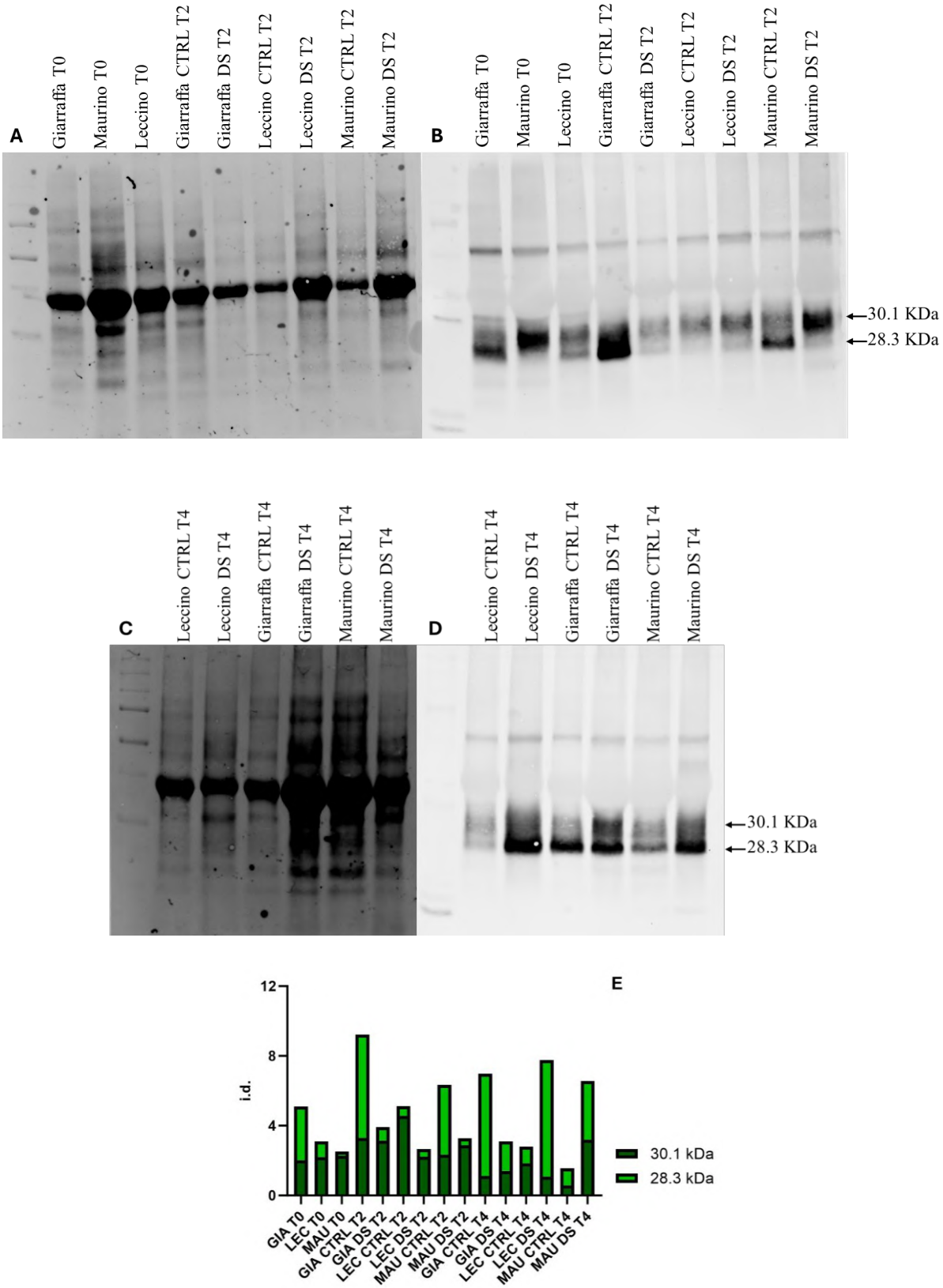


Figure 5.7. Osmotin levels in stems of Giarraffa (GIA), Leccino (LEC) and Maurino (MAU) cultivars under control (CTRL) and drought stress (DS) conditions, at the beginning of stress (t0), after two weeks (t2) and after

four weeks (t4) of stress. (**A**) and (**C**) Stain-free membranes used for relative quantification of blotting; (**B**) and (**D**) immunoblotting with anti-osmotin antibodies of the above labelled experimental groups; (**E**) relative quantification of blotting expressed as integrated density (i.d.).

5.4 Discussion

Sugars produced by photosynthesis are a source of available carbon that can be used to provide energy for plant growth and productivity or for osmoregulation to maintain cell turgor under various abiotic stresses (Deslauriers et al., 2014). If drought can affect carbohydrate accumulation by modulating specific gene expression in plant cells (such as genes encoding sucrose synthase, invertase, hexokinase, fructokinase), regulation of soluble carbohydrate concentrations is part of plant adaptation to water deficit (Yang et al., 2019). Among the sugars analysed, glucose was the most abundant in the leaves of the three cultivars but drought stress caused a decrease in glucose in both stems and leaves of all cultivars at t4. This means that a sufficient period of water deficit is required to induce a statistically significant decrease in glucose levels in drought-stressed samples of all olive cultivars. Glucose is consumed for several reasons: (1) it can be stored as starch, (2) it can be used in cellular respiration, and (3) it can be converted to other sugars (Atkin et al., 2000). Since starch content was measured and no significant accumulation was found, hypothesis 1 can be rejected. Although no data on cellular respiration were collected, the use of glucose to support cellular respiration under drought stress conditions may have some advantages, such as consumption of O_2 and removal of excess redox equivalents from the chloroplasts (Atkin and Macherel, 2009). Along with the decrease in glucose, fructose accumulation was also observed in leaves exposed to drought, especially in the Giarraffa cultivar (at t4). The increase in fructose can provide a substrate for the synthesis of secondary metabolites such as lignin and phenolic compounds (Rosa et al., 2009). The same trend of sugar accumulation was also observed in olive leaves exposed to UV-B radiation (Piccini et al., 2020).

In plant cells, sucrose can be broken down by either sucrose synthase or invertase to form fructose + UDP-glucose or fructose + glucose. The breakdown of sucrose by sucrose synthase provides energy conservation, while invertase is less conservative; therefore, sucrose synthase is preferred under stress conditions to conserve energy, while invertase is used when

ATP is needed more (Stein and Granot, 2019). The cultivars differed in sucrose accumulation in response to drought: only the stressed groups of 'Leccino' and 'Maurino' showed an increase in sucrose levels in the leaf (at t4), while a dramatic increase was recorded only in the stem of 'Maurino' (at t2). Sucrose is the main transportable form of photosynthetic products and is found to be higher in those cultivars that retain stomatal conductance and carbon assimilation of stressed groups for a longer time during drought exposure (**Figures 3.3** and **3.5**). Sucrose is generally considered an anti-stress molecule; in the leaf, sucrose plays an important role in stabilizing membranes and macromolecules (Rejšková et al., 2007). In the stem, it has been reported that an increase in sucrose content of parenchyma cells and the apoplast may allow the tissue water storage capacity to maintain xylem transport capacity and allow embolism removal by "refilling" water-deprived xylem vessels (Secchi and Zwieniecki, 2016; Secchi et al., 2017). Therefore, the large accumulation of sucrose in the stem of drought-stressed 'Maurino' can be interpreted as an attempt to counteract the cavitation that occurred in plants not prone to stomatal closure (Sevanto et al., 2014).

Fructose is the starting point for the biosynthesis of mannitol (Stoop et al., 1996). Mannitol is a polyol abundant in olive trees and other species (such as celery and carrots) that helps the cell regulate osmotic potential, maintain turgor, and stabilize macromolecules against ROS (Stoop et al., 1996). All cultivars showed an increase in mannitol in both leaves and stems at t4. However, in the stems of 'Giarraffa', mannitol started to accumulate already at t2, which probably helps this cultivar to conserve water in this plant organ (**Figure 3.2**). On the contrary, mannitol decreased in the stem of 'Leccino' at t4. This cultivar was the only one to show an increase in another osmoprotectant, proline, which accumulated in both leaves and stems at t2 and could play the same role as mannitol in 'Giarraffa', helping the Leccino cultivar to maintain a higher water content in both stem and leaf than 'Maurino' (**Figures 3.1** and **3.2**). The variations in carbohydrates and proline under drought stress conditions reported in the literature suggest a cultivar-specific response in olive trees. De Pascali et al. (2022) found a decrease in glucose in both 'Leccino' and 'Cellina di Nardò', but only the latter showed a decrease in fructose and sucrose. The total carbohydrate content increased in the wood of the Tunisian cultivars 'Picholine' and 'Meski'. However, only the more drought sensitive 'Meski' showed a

decrease in leaf carbohydrate content, while 'Picholine' had a higher mannitol content (Chehab et al., 2009). A greater accumulation of this alcohol sugar was also found in the drought-resistant cultivar Chemlai compared to the more susceptible Meski (Ennajeh et al., 2009). A study on the effect of water availability on Tunisian olive cultivars showed that water deficit affected shoot elongation and photosynthetic performance, except for the cultivar Chemlali, which maintained a better plant water status, higher antioxidant defence mechanisms and higher proline content (Ahmed et al., 2009). Boughalleb and Mhamdi (2011) found that a greater accumulation of proline in 'Chemlali' and 'Zalmati' cultivars was associated with a better maintenance of plant water status and biomass production compared to 'Chetoui'. It is thus clear that a significant accumulation of sugars and proline is related to the cultivar's osmoregulation and water retention capacity in water-stressed conditions. In drought-stressed samples, the main difference between 'Giarraffa', 'Leccino', and 'Maurino' was stem sugar content.

Protein accumulation, on the other hand, showed significant differences in leaves and stems of all cultivars. Overexpression of aquaporins of the PIP family in xylem-associated living cells was found to be related to facilitation of water flow towards embolized vessels (following the osmotic gradient) (Secchi and Zwieniecki, 2016; Secchi et al., 2017). The drought-stressed stems of 'Leccino' accumulated the most aquaporins (both at t2 and t4), whereas the cultivar Maurino, which had the highest sucrose content, had lower aquaporin content compared to the control. Given the scarcity of data on the distribution of sucrose and aquaporins within the stems, we can only speculate on a link to the phenomenon of the refilling of embolised xylem vessels. Accumulation of aquaporin may also be related to the increase in stem conductivity already observed under drought stress (Massenti et al., 2022). Despite an initial increase in dehydrins in the stressed stems of 'Giarraffa' at t2, these proteins were only detected in the stressed leaves of 'Maurino' (at t2 and t4) and 'Leccino' (only at t4); they also accumulated in the stems of these cultivars at t4. These proteins intervene specifically during drought exposure and are associated with a reduction in oxidative damage (i.e. a reduction in malondialdehyde content and electrolyte leakage) and with water retention capacity through the accumulation of compatible solutes (Riyazuddin et al., 2022). However, the leaves of 'Maurino' were extremely exposed to electrolyte leakage and hypothetical oxidative damage both at t2 and t4 (**Figure 3.4**).

Since both ‘Leccino’ and ‘Maurino’ showed a later decrease in gas exchange rates than their control, the accumulation of dehydrins could be attributed to an effort to retain water during high transpiration rates under drought conditions. Accumulation of these proteins was also found in the most drought-sensitive of the four tomato varieties studied by Conti et al. (2022). Similarly, osmotin accumulated in the stressed leaves and stems of ‘Maurino’ and ‘Leccino’ at t4; ‘Giarraffa’ also had higher levels of this protein in stressed leaves than in its respective control. Again, osmotin was found in the most drought sensitive tomato cultivar in the study by Conti et al. (2022). The literature on olive osmotin suggests that increased expression of the osmotin gene may confer drought resistance. Olive plants that express the tobacco osmotin gene were found to be more resistant to cold stress, suggesting that osmotin may also be involved in the response mechanism to drought, which is closely related to cold stress (D’Angeli and Altamura, 2007). However, in this case, the accumulation of dehydrins and osmotin may be more related to “drought stress occurrence” than to tolerance, because the most "drought avoidant" cultivar Giarraffa, invests less in the accumulation of these proteins.

5.5 *Bulleted* conclusions

- Glucose decreased in stressed leaves and stems of all cultivars, probably used in cellular respiration and/or converted to other sugars (such as UDP-glucose and fructose).
- The increase in leaf mannitol is a common response of the stressed groups of all three cultivars.
- ‘Giarraffa’ preferentially accumulates mannitol, while ‘Leccino’ accumulates proline in stressed stems.
- The increase in sucrose content and the accumulation of aquaporins in the stressed stems of ‘Leccino’ and ‘Maurino’ may be related to the ability to remove embolisms by "refilling" the xylem vessels.
- Accumulation of dehydrins and osmotin occurs mainly in ‘Leccino’ and ‘Maurino’, which maintain higher gas exchange rates compared to ‘Giarraffa’.

5.6 Supplementary materials of Chapter 5

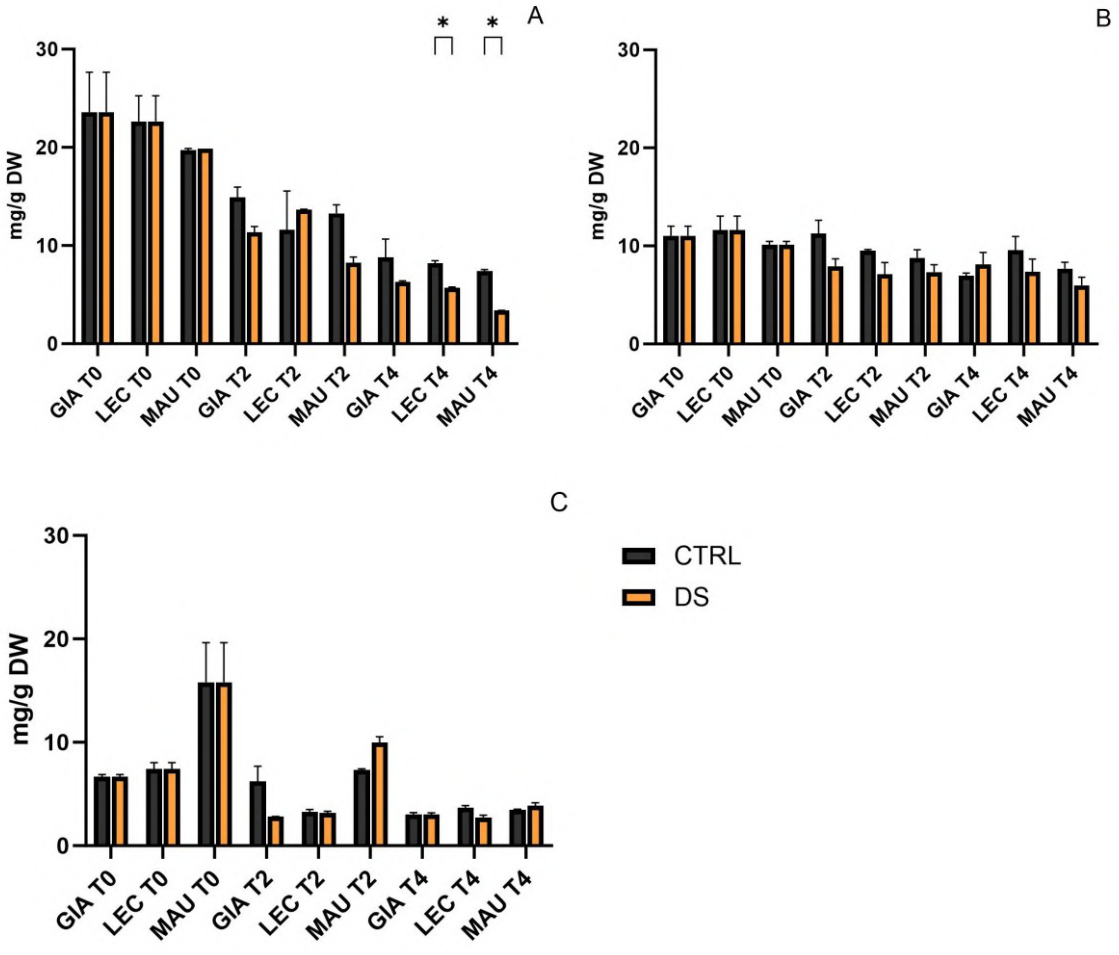


Figure S5.1. Starch content in leaf sampled at dawn (A), before night (B) and stem (C) of Giarraffa (GIA), Leccino (LEC) and Maurino (MAU) cultivars under control (CTRL, black) and drought stress (DS, orange) conditions. The contents are expressed as mg g^{-1} tissue dry weight (DW). For each column, values are expressed as mean ± standard error. Asterisks (*) indicate significant differences between control and treated groups (* = *p-value* < 0.05)

Chapter 6

A look at stem anatomy and lignin composition

Contributors to this chapter:

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6.1 Introduction to the chapter

Woody stems were included as the final variable in the characterization of olive cultivars under drought stress. Wood plays an important role in plant growth, leaf phenology, tree biomechanics, water transport, and carbohydrate storage (Lima et al., 2018). The xylem transports approximately 99% of the water from roots to photosynthetic tissues (Secchi and Zwieniecki, 2016). Xylem vessels are long, hollow tubes with finite dimensions that die upon maturity. They are characterized by thick, lignified cell walls that can withstand high negative pressure. In the olive tree, wood generally consists of less than 20% vessels, with the rest occupied by fibres, which give the stem strength, and parenchyma, a tissue that stores water and solutes (Venturas et al., 2017). Drought affects the xylem when water loss through transpiration exceeds water uptake by the roots, resulting in high xylem tension, embolism formation, and loss of conductivity, which is the cause of hydraulic failure mortality (Sevanto et al., 2014). Thus, xylem vulnerability to drought-induced cavitation is one of the most important factors affecting plant productivity and survival (Venturas et al., 2013). Cavitation vulnerability/resistance has been linked to both hydraulic (wood density and xylem anatomy) and chemical wood properties (lignin content and composition). While higher wood density is typically associated with greater cavitation resistance (Hoffmann et al., 2011; Savi et al., 2019), recent research does not agree on the best xylem anatomical traits for drought resistance. A long-held assumption is that wide and long vessels transport water more efficiently (Hagen-Poiseuille law), but they are also more vulnerable to drought-induced embolism (Venturas et al., 2013). On the other hand, many small vessels ("vascular redundancy") offer greater protection against drought-induced conductivity losses (Venturas et al., 2013). However, not all experimental observations support this, and because climate-vessel size relationships can also be determined by climate-plant size (dryland plants are typically shorter and have narrower vessels) (Olson and Rosell, 2013), conduit diameter has been suggested as a poor proxy for drought-induced embolism resistance (Lens et al., 2022). However, xylem morphology is always a balance of efficiency and safety (Venturas et al., 2017).

The chemical properties of wood are primarily related to lignin, a structural polymer found in wood along with cellulose and hemicellulose. Lignin adds strength, rigidity, and hydrophobicity

to cell walls and allows vascular tissue to withstand the pressure of water transport (Choi et al., 2023). Lignin is primarily composed of three hydroxycinnamyl alcohols (p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol), which give rise to p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units in the polymer (Choi et al., 2023). Lignin deposition necessitates a significant and irreversible carbon input that is tightly controlled (Choi et al., 2023). Lower lignin content is usually correlated with lower wood density and increased xylem embolism susceptibility (Lima et al., 2018). Indeed, drought conditions have been shown to increase total lignin content by increasing deposition in new vessels or further deposition in existing vessels (Choi et al., 2023). Lima et al. (2018) found a strong link between the S:G ratio and susceptibility to embolism. The monomer S is primarily found in the fibres wall, and S-rich lignin is more hydrophilic and flexible due to fewer cross-links between monomers. In contrast, the rigid and hydrophobic G-rich lignin is mostly found in conduit walls, where it confers the ability to withstand the tension of sap ascension. Due to this property, a higher S:G ratio in lignin has been found to confer greater resistance to embolism (Lima et al., 2018).

The literature on xylem anatomy and water deficit has primarily focused on other species, such as oak and pine (Corcuera et al., 2004; Castagneri et al., 2017; Martin-Benito et al., 2017; Giberti et al., 2023); little is known about olive xylem and drought. Olive wood is diffusely porous, with vessels evenly distributed throughout the annual ring, high in fibres and low in parenchyma. It is characterized primarily by small diameter vessels, which have low hydraulic conductivity and are thought to increase the plant's resistance to drought-induced cavitation (Sebastiani, 2011). Studies on *Xylella fastidiosa* infection of xylem vessels (De Benedictis et al., 2017; Sabella et al., 2019; Petit et al., 2021) have reported varying vessel diameters for different olive cultivars (e.g., ‘Leccino’, ‘Cellina di Nardò’, and ‘Ogliastra Salentina’). However, these differences represent an initial structure of cultivars, which can affect the water management of plants. Given the higher stomatal density and conductance in ‘Maurino’, as well as the lower stem and leaf water content under drought stress conditions, this cultivar may be more susceptible to drought-induced cavitation. The objective of this chapter is to examine both the morphological and chemical properties of the wood to identify recurring characteristics that may give the cultivar an advantage in withstanding conductance loss. This study additionally

contributes to the less studied morphological characterization of xylem traits in young olive cultivars.

6.2 Material and methods

6.2.1 Analysis of the lignin

The analyses were carried out by Jorge Rencoret and José C. del Río at the Instituto de Recursos Naturales y Agrobioloía de Sevilla (Spain). A brief description of the methods used is provided below. The stem samples of the three cultivars, CTRL and DS, at t4 were freeze-dried and manually debarked with a cutter, and the corresponding xylems were pre-ground in a Restch MM400 mill (Retsch, Haan, Germany). The pre-ground samples were then extracted twice with 80% ethanol and once with acetone in an ultrasonic bath for 30 minutes each, centrifuged, and the supernatant removed. The extractive-free samples were finely ball-milled in a Restch PM-100 planetary mill (Retsch, Haan, Germany) with a 50 ml agate jar and agate balls (10 × 10 mm) at 600 rpm for 2 hours (alternating 10 min of pause every 20 min of milling) for further analysis.

6.2.2 Determination of lignin content

The lignin content was determined using a recently developed protocol (Lu et al., 2021). Briefly, 5 mg of extractives-free sample was placed in a 4 mL glass vial and 1 mL of 72% H_2SO_4 containing cysteine (0.1 g/mL) was added. The mixture was screw capped, sonicated for 2-3 minutes, and stirred at room temperature until fully dissolved (60 min). To achieve adequate UV absorbance (< 1.0) at 283 nm, the solution was diluted with deionized water to 50 mL in a volumetric flask. The lignin content was calculated using the Beer-Lambert formula and an absorption coefficient of 11.23 g^{-1} L cm^{-1}. The measurements were taken in triplicate.

6.2.3 Analysis of lignin composition by 2D-NMR spectroscopy

Whole cell walls of olive stems were analyzed by 2D-NMR at "gel-state" using the previously published method (Kim et al., 2008; Rencoret et al., 2009). In brief, approximately 60 mg of finely milled, extractives-free samples were swelled in 0.6 mL of DMSO-d6. 2D HSQC NMR spectra were recorded at 300 K on a Bruker AVANCE III 500 MHz instrument equipped with a cryogenically cooled 5 mm TCI gradient probe with inverse geometry, at the NMR facilities of

the General Research Services of the University of Seville (SGI-CITIUS). The HSQC experiments were carried out using the Bruker standard pulse programs "hsqcetgpsisp2.2" with the following parameters: spectra were acquired from 10 to 0 ppm in F2 (1H) using 1000 data points for an acquisition time of 100 ms and an interscan delay of 1 s, and from 200 to 0 ppm in F1 (13C) using 256 increments of 32 scans, for a total experiment time of 2 h 34 min. The 1JCH used was 145 Hz. Processing used typical matched Gaussian apodization in 1H and a squared cosine bell in 13C. The central solvent peak was used as an internal reference (δC/δH 39.5/2.49). 2D-NMR cross-signals were assigned by literature comparison (Rencoret et al., 2009, 2019b). The volume integrals of the HSQC correlation peaks were semiquantitatively analysed using Bruker's Topspin 3.5 processing software. In the aromatic/unsaturated region, the correlation signals of H2,6, G2 and S2,6 were used to estimate the content of the respective H-, G- and S-lignin units (as signals H2,6 and S2,6 involve two proton-carbon pairs, their volume integrals were halved). The different inter-unit linkages were quantitated via the volume integrals of the Aα, Bα, Cα, and Fα correlation signals, corresponding to chemically analogous C/H with similar 1JCH coupling values. The relative abundance of cinnamyl alcohol end-groups (I) were estimated by integration of the signal Iγ, whereas the abundance of cinnamaldehyde end-groups (J) was determined by integrating the signal Jβ and comparing it with Iβ, as detailed in Rencoret et al. (2019).

6.2.4 Stem anatomy analyses

Three 1-cm-long stem samples were collected at 40 cm from the collars of two plants in each experimental group. The samples were cut into thin slices and immersed in 1.5 mL of 3% glutaraldehyde for two hours. The samples were then washed three times for 10 minutes each with 2 mL of cacodylate buffer. Samples were then dehydrated with increasing concentrations of ethanol (EtOH): 2 minutes in 2 mL 10% EtOH, 10 minutes in 2 mL 30% EtOH, 15 minutes in 2 mL 50% EtOH, 1 hour in 2 mL 70% EtOH, and 1 hour in 2 mL pure EtOH. The samples were then immersed in 2 mL of solution alcohol and resin (Technovit 7100, Heraeus Kulzer, Germany), with an increasing volume of resin, and kept in the dark with slight agitation for the following time: 2 hours in a 3:1 (v:v) solution; overnight in a 1:1 (v:v) solution; 24 hours in a 1:3 (v:v) solution; and 24 hours in pure resin. Finally, the samples were embedded in resin.

Transverse sections were cut with a rotary microtome. The sections were stained with cresyl violet acetate (0.16% in water) (Rossi et al., 2006). Anatomical observation was led with a optical microscope (Olympus BX51 coupled with Olympus DP-software). Approximately 16 images per cultivar of the first woody ring before the differentiating cells area of the cambium were analysed using ImageJ's "Analyse Particles" tool (size: 50 - infinity; circularity: 0.00-1.00) to obtain the lumen Feret diameters and area, and total xylem area. For each image, the following parameters were calculated (Sperry and Saliendra, 1994; Giovannelli et al., 2019):

- Vessel density (VD, n./mm^{-2}): number of xylem vessel per area of the xylem.
- Vessel diameter (d_m, µm): average of the xylem Feret diameters.
- Vessel area/xylem area (A_v, %): percentage of vessel lumen area/xylem area ratio.
- Hydraulic weighted vessel diameter (D_H, µm), calculated as

$$D_H = {d_m}^5 / {d_m}^4$$

- Theoretical specific xylem hydraulic conductivity (Kst, kg s^{-1} m^{-1} MPa^{-1}), calculated as

$$K_{st} = \frac{\pi \cdot \rho}{128 \cdot A_{image} \cdot \eta} \cdot \Sigma {d_m}^4$$

Where ρ is the density of water (998.2 Kg m^{-3} at 20 °C); η is the viscosity of water (1.002 × 10^{-9} MPa s at 20 °C), A_{image} is the area of the analysed image (m^2) and d_m is the vessel diameter.

6.2.5 Statistical analysis

Differences in the frequency distribution of vessel lumen diameters of the three cultivars were assessed using the chi-squared test. Significant differences for the parameters VD, d_m, A_v, D_H, K_{st} of the three cultivars were calculated using one-way ANOVA followed by Tukey HSD post hoc test. All analyses were carried out using R studio.

6.3 Results

6.3.1 Lignin content and composition

The lignin content of the stems of 'Giarraffa', 'Leccino', and 'Maurino' exposed to drought stress at t4 was determined and compared to the respective controls (**Table 6.1**). The three olive cultivars had similar lignin content (about 23% of the total wood composition in lignin, cellulose, and hemicellulose), with only minor differences between them. As a result, the lignin content of drought-stressed stems was very similar to that of control stems. However, the lignin

content in the drought stressed stems of 'Leccino' decreased slightly (from 23.0 to 22.3%). Nevertheless, no significant differences were found.

Table 6.1. Lignin content in the stems of different olive trees under drought stress (DS) compared with their respective controls (CTRL). Lignin content is expressed as percentage of the total wood composition (lignin, cellulose, and hemicellulose).

Giarraffa		Leccino		Maurino	
CTRL	*DS*	*CTRL*	*DS*	*CTRL*	*DS*
23.3 ± 0.1	23.4 ± 0.1	23.0 ± 0.1	22.3 ± 0.1	22.7 ± 0.2	22.8 ± 0.2

The structural modifications that may have occurred in the lignin of olive trees during drought stress were studied "in situ" using 2D-HSQC NMR experiments at the gel state, without the need for prior lignin isolation. **Figure 6.1** shows the HSQC spectra (δC/δH 50–125/2.4–7.8) of drought-exposed and non-exposed cultivars. The spectra of different olive stems revealed signals from carbohydrates (grey color) and lignin. The main substructures identified in the spectra are also shown. The aromatic region of the HSQC spectra revealed the aromatic rings of various guaiacyl (G) and syringyl (S) lignin units, including Cα-oxidized S-lignin units (S′). After increasing the threshold, only a small signal for p-hydroxyphenyl (H) units was detected. In the aliphatic-oxygenated region of the spectra, typical signals from the Cα/Hα, Cβ/Hβ, and Cγ/Hγ correlations of the different inter-unit linkages (β−O−4′ alkyl-aryl ethers, A; β–5′ phenylcoumarans, B; β–β′ resinols, C; β–1′ spirodienones, F), and cinnamylaldehyde (I) and cinnamyl alcohol (J) end-groups, were observed. The yellow tables in **Figure 6.1** show a semi-quantitative estimate of the main structural characteristics of lignin of the various stems based on signal integration in the HSQC, such as the relative abundances of the different interunit linkages and cinnamyl end-groups, as well as the relative abundances of the lignin units (H, G, and S). There were no significant differences in the composition and structure of lignin among the three olive cultivars. The three cultivars had similar compositions, with 'Leccino' lignin having a slightly higher content of S-units and thus a higher S/G ratio (2.54) than 'Giarraffa' and 'Maurino' lignin (S/G of 2.30). The three cultivars had similar abundances of inter-unit

linkages and cinnamyl-end groups, with a predominance of β-O-4′ aryl ethers (83-85% of all linkages) and lower amounts of phenylcoumarans (4%), resinols (7-8%), and spirodienones (4-5%). Cinnamyl alcohol and cinnamaldehyde end-groups made up 6% and 3-4% of the total sidechains, respectively. Finally, the NMR data revealed that the composition and structure of lignin in stressed stems from all cultivars are similar to those in controls. This clearly shows that drought stress has no effect on lignin composition or structure in olive stems, at least at this stage of development.

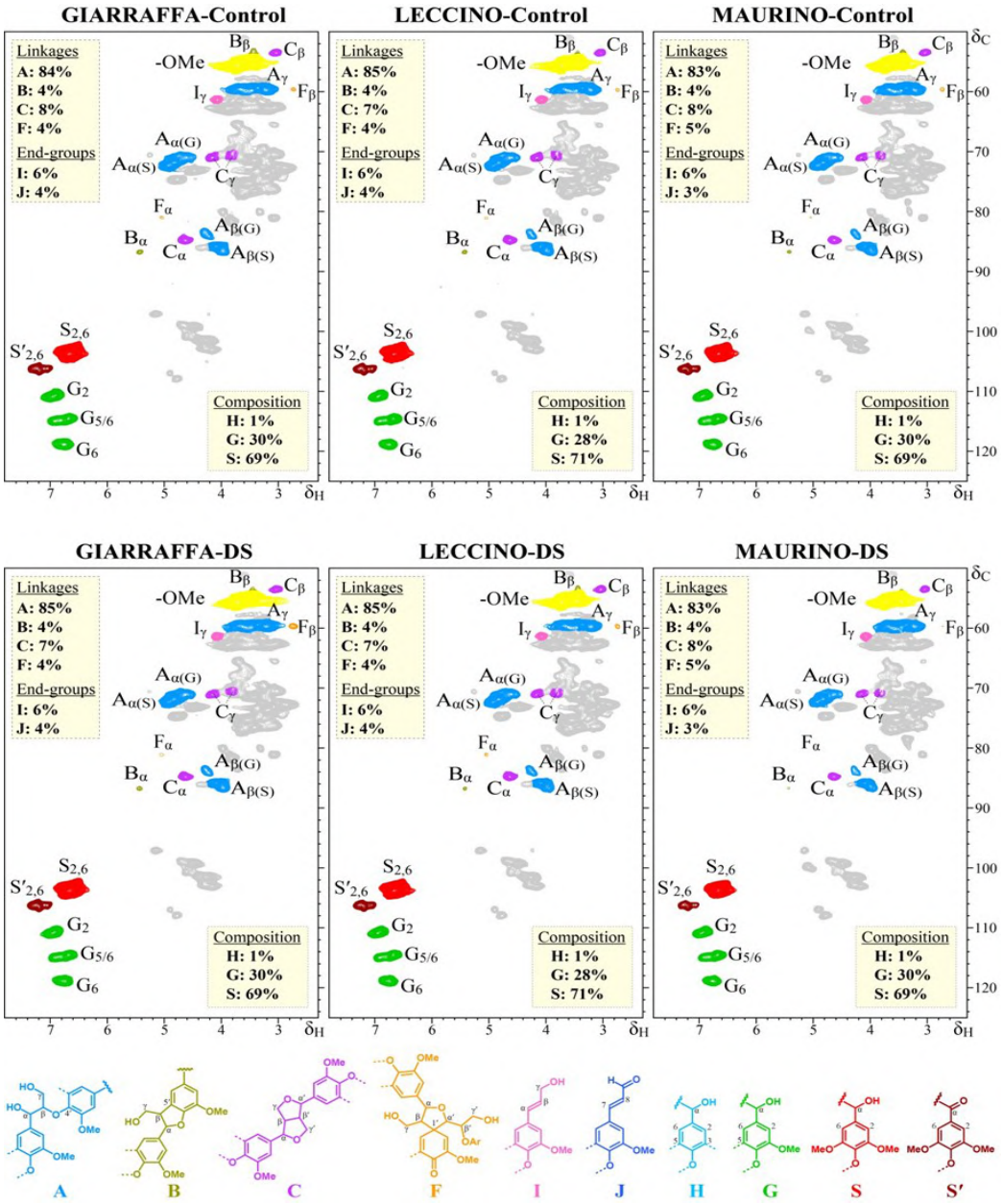

Figure 6.1. 2D-HSQC NMR spectra of stems of the three olive cultivars (Giarraffa, Leccino, Maurino) that have undergone drought stress (DS), (bottom); and of their respective stem controls, (top). The main lignin structures identified are also shown. **A**: β-*O*-4′ alkyl-aryl ethers; **B**: β-5′ phenylcoumarans; **C**: β-β′ resinols; **F**: β-1′ spirodienones; **I**: cinnamyl alcohol end-groups; **J**: cinnamaldehyde end-groups; **H**: *p*-hydroxyphenyl units; **G**:

guaiacyl units; **S**: syringyl units; **S′**: Cα-oxidized syringyl units. Semi-quantitative estimation of lignin units and linkages are indicated in the yellow boxes. Composition is expressed in molar percentages (H+G+S=100%); end groups are expressed as a fraction of the total lignin inter-unit linkage types A-F.

6.3.2 Xylem anatomy

Figure 6.2 shows cross sections of the phloem (ph), cambial zone cells (cz) with thin cell walls and small radial diameters, and xylem (x). The xylem area contains parenchyma rays identified by their longitudinal disposition (r), fibres (f) with thick cell walls and very small lumina, and paratracheal parenchyma (p), which is similar to the xylem but smaller and has a hexagonal cell wall under polarised light. Xylem vessels were typically found in groups of two or three cells.

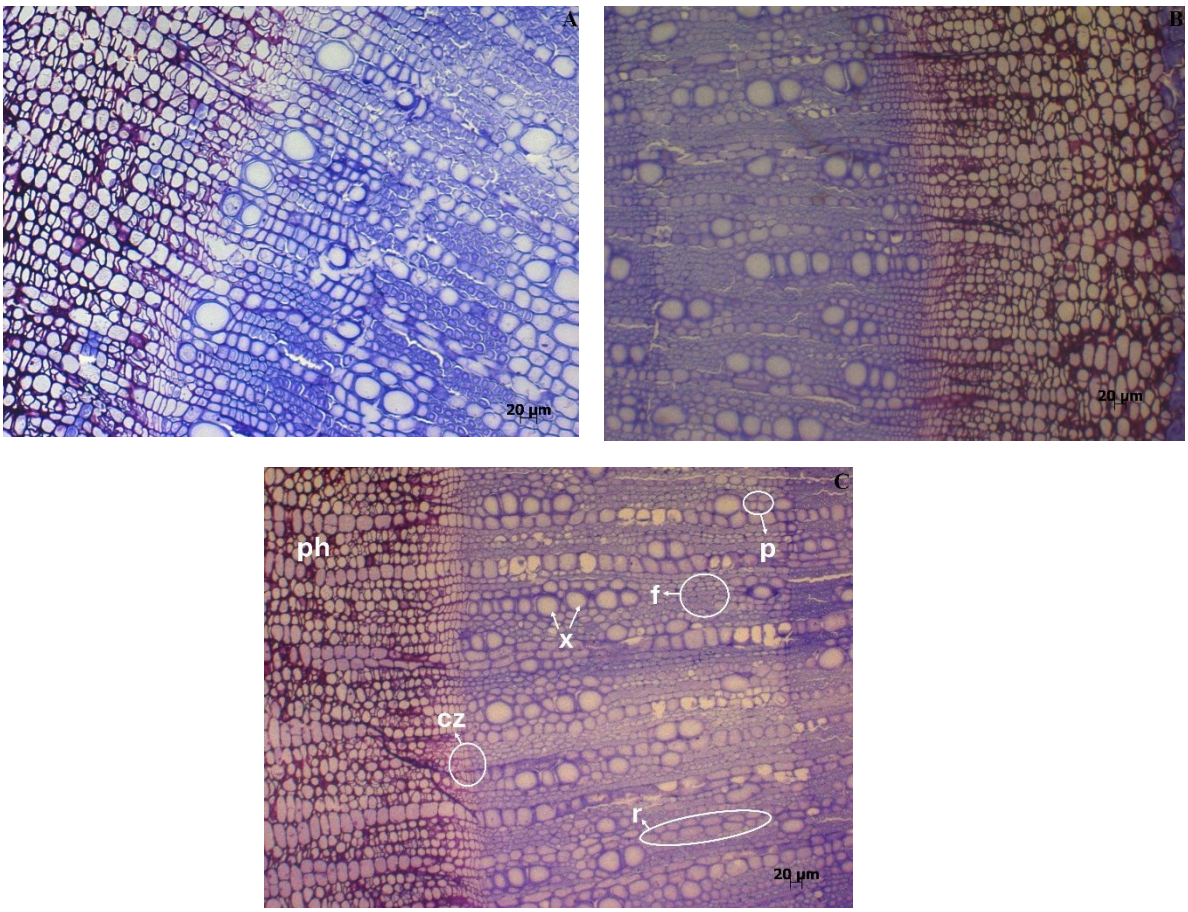

Figure 6.2. Stem cross section of *Olea europaea* cultivar 'Giarraffa' (**A**), 'Leccino' (**B**), and 'Maurino' (**C**). ph: phloem, x: xylem vessels; cz: cambial zone; r: parenchyma ray; f: fibres; p: paratracheal parenchyma.

The distribution of xylem diameters, divided into 5 µm classes (**Figure 6.3**), showed that few vessels had a diameter > 40 µm, with the main frequencies being between 20 and 35 µm. In 'Giarraffa', many vessels had a diameter between 20 and 25 µm, while in 'Leccino' and 'Maurino', the class 25-30 µm was primarily represented. Compared to 'Giarraffa', 'Leccino' and 'Maurino' had more smaller vessels (10-15 µm in diameter). However, no significant

differences were found between the cultivars. **Table 6.2** lists the morphological and hydraulic data obtained from the cross-section elaboration. The cultivars showed no significant difference in vessel density, but 'Maurino' had more vessels per unit area than 'Leccino' and 'Maurino'. Again, 'Maurino' had a lower average vessel diameter than the other two cultivars. The lumina of the vessels occupied a higher percentage of the xylem area in 'Maurino' than in 'Giarraffa', which also had the lowest hydraulic diameter (significantly different from 'Leccino'). The theoretical hydraulic conductivity showed no significant differences, but was lower in 'Giarraffa', higher in 'Maurino', and intermediate in 'Leccino'.

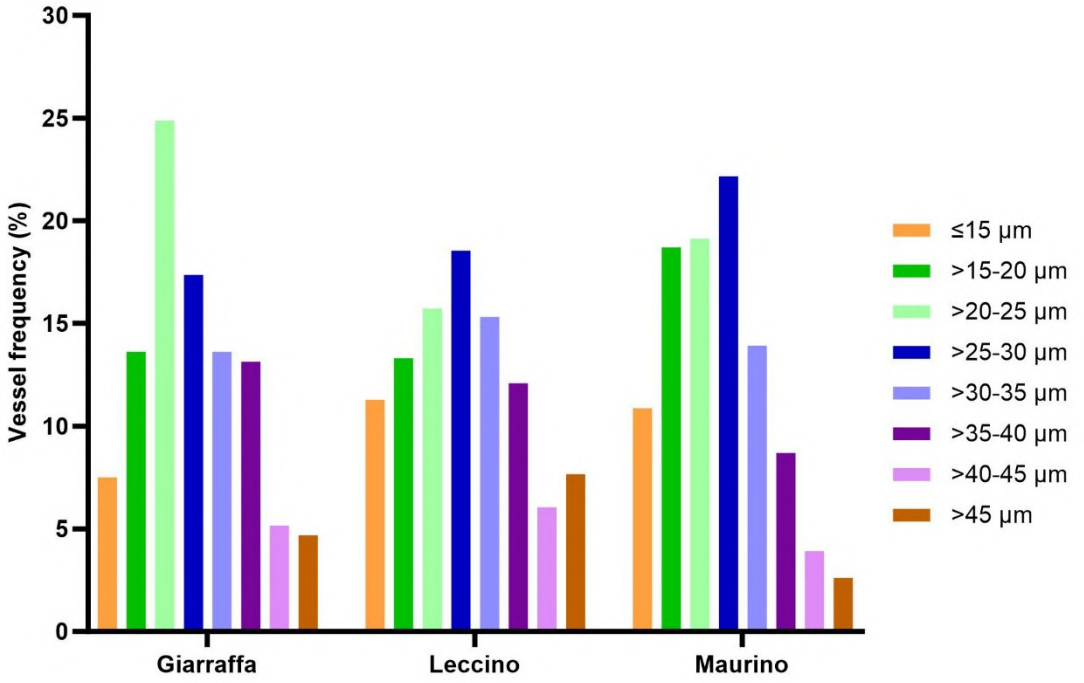

Figure 6.3. Frequency distributions (number of vessels per 5 µm diameter class) of vessel lumen diameters in the three olive cultivars.

Table 6.2. Mean values (± standard error) of morphological and hydraulic traits in the three olive cultivars. VD: vessel density, d_m: average vessel diameters, A_v: total vessel area to total xylem area ratio, D_H: hydraulic diameter, K_{st}: theorical hydraulic conductivity. Different letters denote statistical significance (*p-value* < 0.05) according to Tukey's multiple post hoc test.

	morphological traits			hydraulic traits	
cultivar	**VD** (n. mm^{-2})	**d_m** (µm)	**A_v** (%)	**D_H** (µm)	**K_{st}** (kg s^{-1} m^{-1} MPa^{-1})
Giarraffa	211.1 ± 27.9	30.0 ± 0.8^{a}	6.0 ± 0.7^{b}	30.5 ± 1.7^{b}	2.6 ± 0.4
Leccino	237.1 ± 32.4	28.7 ± 0.7^{a}	8.2 ± 0.5^{ab}	36.7 ± 1.4^{a}	3.7 ± 0.3
Maurino	251.6 ± 30.3	26.1 ± 0.7^{b}	8.7 ± 1.1^{a}	32.3 ± 1.3^{ab}	3.5 ± 0.6

6.4 Discussion

The chemical and anatomical properties of olive wood are important because they influence water transport along the stem and can be affected by drought (Lima et al., 2018; Choi et al., 2023), which in turn affects carbon availability. The analysis of the chemical characteristics included the determination of the lignin content and the lignin monomer composition. Lignin content of approximately 23% of total wood composition was also found in branches from an olive grove in Spain (Garcia-Maraver et al., 2013) and in branches of the Spanish cultivar Picual (Rencoret et al., 2019a). The lignin composition was consistent with that reported by Rencoret et al. (2019a), who found that the percentage of G and S monomers was greater than that of H monomers, which was around 1%. These parameters did not differ significantly between cultivars, except for 'Leccino', which diverged slightly. The drought-stressed stem of this cultivar had a lower lignin content whereas, regardless of treatment, 'Leccino' had a higher composition of S monomers, resulting in a higher S:G ratio than the other two cultivars. The potential weakening of the conduits due to lower lignin content could have been compensated by the increase in the S:G ratio, which confers resistance to embolism (Lima et al., 2018).

In terms of drought stress, the anatomy of control and stressed stems did not differ significantly. This is reasonable given that the xylem sampled for these analyses was collected on the final day of the experiment (which lasted 30 days). In *Picea mariana*, cell enlargement and secondary cell wall deposition take about 30 days (Buttò et al., 2019). Therefore, only about one ring of single xylem cells was fully mature at the onset of water deficit, making any differences from the controls impossible to detect. What can be assessed are the genotypic differences between the three cultivars, which serve as a baseline for the wood properties of each cultivar. For this reason, anatomical studies were performed on a pooled sample of half control and half stressed group images. Unlike the chemical characteristics, the three cultivars differed anatomically. The frequency distribution revealed that only a few vessels exceeded 35 µm in diameter, while 'Leccino' and 'Maurino' had more smaller vessels (≤ 15 µm), as observed in cultivar Leccino by Sabella et al. (2019) and Walker et al. (2023). In terms of morphological characteristics (VD, d_m, A_v), 'Maurino' had higher vessel density and vessel smaller average

diameter, resulting in a greater percentage of xylem vessel area per tissue area than the other two cultivars. Overall, the vessel density found in the three cultivars was lower than that observed in other studies on olive trees (Bacelar et al., 2007; Sabella et al., 2020). The theorical hydraulic conductivity (K_{st}) is an important parameter in water balance studies and has a negative correlation with whole-plant water flow resistance; higher K_{st} can be achieved by increasing vessel diameter and density (Gleason et al., 2012). Although the anatomical features of 'Maurino' indicate an increase in K_{st}, no significant differences were found. We can only hypothesise that VD and A_v in 'Maurino', combined with the high stomatal density (**Table 3.1**), contribute to sustain the higher stomatal conductance of this cultivar than the others. However, due to the variability of the anatomical observations, many sample sections must be examined to reveal statistical differences between the cultivars. For the time being, the findings presented in this chapter provide preliminary information about the wood properties of these young olive cultivars.

6.5 *Bulleted* preliminary conclusions

- Neither genotype nor stress determined differences in wood chemistry (lignin content and composition).
- 'Maurino' had a higher area vessel/area xylem ratio but a smaller average vessel diameter.
- 'Leccino' had the highest hydraulic diameter.

Chapter 7

Appendix: Application of leaf-derived phenolic compounds to human umbilical vein endothelial cells (HUVECs): olive leaf extracts (OLEs) from the three cultivars differentially protect cells against oxidative stress

Contributors to this chapter:

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This chapter is based on:

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7.1 Introduction to the chapter

This chapter includes a study on the pharmaceutical application of phenolic compounds extracted from olive leaves on human cells, which was carried out in collaboration with the Department of Pharmacy of the University of Pisa. As the olive tree is one of the most widespread plants, every year a large quantity of olive leaves is harvested and discarded together with the olive fruits. For every litre of olive oil produced, about 6 kg of leaves are discarded (Avraamides and Fatta, 2008), usually burned or ground and spread on the fields (Guinda et al., 2015). However, olive leaves have been reported to be a rich source of bioactive compounds (Selim et al., 2022). The antioxidant activity of olive leaf extracts (OLEs) is due to the presence of polyphenols (Tarchoune et al., 2019), making them an ideal candidate in the medical, cosmetic and pharmaceutical fields (Erbay and Icier, 2010). For example, the protective effects of OLEs against oxidative stress have been found in endothelial cells (De la Ossa et al., 2019), in renal cells exposed to cadmium (Ranieri et al., 2019) and in bronchial epithelial cells affected by cystic fibrosis (Allegretta et al., 2023). Oleuropein is the most abundant compound found in OLE (Ortega-García and Peragón, 2009) and is thought to be primarily responsible for its pharmacological effects. However, OLE contains a wide variety of flavonoids, which are the most abundant group of polyphenols in olive leaves. Therefore, the use of whole extracts may provide greater antioxidant capacity and health benefits than isolated compounds due to the synergistic effects of all the polyphenols present (Borjan et al., 2020).

In plants, polyphenols are involved in tolerance to various abiotic stresses, such as heat, drought, flood, light, salinity, heavy metals (Šamec et al., 2021)… and drought, as described in detail in Chapter 4 and also reported in several studies (Ennajeh et al., 2009, p.; Jiménez-Herrera et al., 2019; Mechri et al., 2020b). The increase in total polyphenol content as a consequence of drought stress contributes to an increase in the antioxidant properties of the extracts (Cesare et al., 2021). However, the phenolic profile of OLE varies depending on the origin and variety of the plant material (Irakli et al., 2018). Based on the different metabolic responses found in the three cultivars (as reported in Chapter 4), in this chapter OLEs from ‘Giarraffa’, ‘Leccino’ and ‘Maurino’, exposed or not to four weeks of drought stress, were investigated in terms of antioxidant properties and profile, protection against oxidative stress of HUVECs cells and

intestinal permeation. HUVECs are an excellent model to study a wide range of diseases, such as cardiovascular and metabolic diseases, therefore several studies have evaluated antioxidant molecules from natural products on HUVECs through in vitro experiments related to vascular dysfunction (Medina-Leyte et al., 2020). In this chapter, the thorough analysis of the antioxidant capacity of OLEs from the three Italian olive cultivars was carried out in order to identify the one with the most relevant effects on HUVEC cells. The outcome of this research was to highlight the high value of the agricultural by-product of olive leaves as a nutraceutical compound.

7.2 Material and methods

7.2.1 Materials

Olive leaf extracts of the Giarraffa (OLE-G), Leccino (OLE-L) and Maurino (OLE-M) varieties and the extracts obtained from the same cultivars subjected to water deficit (OLE-GS, OLE-LS, OLE-MS) were collected at Life Sciences Department of the University of Siena, Siena (SI), Italy. The HUVEC cells were purchased from Lonza (Basel, Switzerland). MCDB 131 Medium was purchased from Gibco-Thermo Fischer (Waltham, Massachusetts, USA). Fetal Bovine Serum (FBS), L- Glutamine and heparin sodium salt from porcine intestinal mucosa were purchased from Sigma-Aldrich (Darmstadt, Germany). Human FGF-basic and Human EGF (Animal Free) were purchased from Peprotech (Waltham, Massachusetts, USA). Cell proliferation reagent (WST-1) was provided by Roche diagnostic (Mannheim, Germany). The fluorescent probe 2,7-dichloro-dihydro-fluorescein diacetate, acetyl ester (CM-H2DCFDA) was provided by Invitrogen (Carlsbad, CA, USA). Gallic acid (GA), ferrous chloride, and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany).

7.2.1 Determination of the Antioxidant Capacity and Polyphenols Content

The extracts were prepared following the procedure described by De la Ossa et al. (2021) slightly modified. In detail, frozen leaves (1 g) were ground in liquid nitrogen and the powder resuspended in 3 mL of 70% acetone. Samples were homogenised with Ultra-Turrax T-25 basic (IKA®-Werke GmbH & Co. KG, Staufen im Breisgau, Germany) for 3 min and sonicated with an ultrasonic bath (Transsonic T 460/H Elma, Singen, Germany) for 20 min. The homogenate

was centrifuged at 4000 × g for 5 min at 4 °C. The supernatants, which contained the antioxidant extracts, were collected and used for the antioxidant power and polyphenols content determination. Another aliquot of the same supernatants was filtered through a 0.45 µm cellulose acetate membrane filter (Sartorius, Göttingen, Germany), frozen, and freeze-dried 48 h (freeze dryer LIO 5P, 5pascal, Italy). The lyophilized olive supernatants were used for the Cell Viability Test by WST-1 Assay, ROS production analysis, and permeation of antioxidants contained in OLE across the excised rat intestine.

7.2.2 Ferric Ion Reducing Antioxidant Power (FRAP)

FRAP method was carried out to determine the antioxidant capacity (Benzie and Strain, 1996). For each reaction, 20 µL of extract was mixed with 2040 µL of 300 mM acetate buffer pH 3.6, 200 µL of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine), and 200 µL of 20 mM $FeCl_3$. After 1 h-incubation at 37 °C, the absorbance of samples was measured using a UV-Vis spectrophotometer (wavelength set at 563 nm). The absorbance values were interpolated on a standard curve using known ferrous sulfate solutions. The antioxidant power of each group was expressed in mmol of ferrous chloride equivalent per 100 g of matter.

7.2.3 Folin-Ciocalteu Method

The total polyphenols content was determined by the Folin-Ciocalteu colorimetric assay (Singleton and Rossi, 1965). For each reaction, 500 µL of extract was mixed with 3950 µL of distilled water, 250 µL of Folin-Ciocalteu reagent, and 750 µL of a sodium carbonate saturated solution (Na_2CO_3) for each reaction. After a 30-minute incubation at 37 °C, the absorbance of each sample was measured at 795 nm using a UV-Vis spectrophotometer. The absorbance value was interpolated using a standard curve of a known gallic acid solution. Total phenolic content was measured in milligrams of gallic acid equivalents (GAE) per 100 g of matter.

7.2.4 Leaf Metabolite Extraction and UHPLC-MS analysis

Extraction and analysis of phenolic compounds are described in detail in sections 4.2.1 and 4.2.3.

7.2.5 Cell Viability Test by WST-1 Assay

The viability of HUVEC was assessed by the WST-1 assay. For this purpose, 2×104 cells per well were seeded in 96-well plates and placed in an incubator for 24h at 37 °C and 5% CO_2. Fifteen hours after seeding the cells were incubated for 4 hours with the OLEs at concentrations in the 1-50 µg/mL range. The samples to be tested were dissolved in fresh medium and filtered through 0.45 µm cellulose acetate filter prior contact with the cells. Following the 4-hour treatment the cells were washed with fresh medium to completely remove any residual extract, then incubated at 37 °C for 2h with WST-1 reagent diluted 1:10. The amount of formazan produced was evaluated at 450 nm. After washing, some of the cells were subjected to oxidative stress by incubating them for 1 hour with 500 µM H_2O_2. In this case the WST-1 reagent was added after completely removing H_2O_2 from the wells through a suitable washing.

7.2.6 ROS production

ROS production was evaluated as previously described Cesare et al. (2021). HUVECs during the last 30 minutes of treatment with OLEs or H_2O_2 were incubated with the fluorescent probe 2,7-dichloro-di-hydro-fluorescein diacetate, acetyl ester (CM-H2DCFDA) dissolved in PBS at a concentration of 10 µM in the dark at room temperature. At the end of the experiment, ROS production was detected measuring the increase in fluorescence at excitation of 488 nm and emission of 510 nm.

7.2.8 Permeation of antioxidants contained in OLE across excised rat intestine

A well-known procedure (Zambito et al., 2009; Zambito and Colo, 2010) authorized by the scientific-ethical committee of the Italian University and the Ministry of University and Research was carried out. Briefly, under veterinary supervision, the intestinal mucosa was excised from non-fasted male Wistar rats having a weight between 250-300 g. Longitudinal strips were obtained from the intestine by cutting, rinsed to remove luminal contents and then mounted in Ussing-type cells with an exposed surface area of 0.78 cm^2, while keeping the underlying muscle layer. After 20 minutes of equilibration at 37 °C, OLE-G, OLE-GS, OLE-M, or OLE-MS dispersed in phosphate buffer pH 7.4 (0.13 M) was added to the apical chamber. The experiment was performed with OLEs at the same extract concentration of 3 mg/mL and

the same GAE content of 0.16 mg/mL. Three mL of fresh phosphate buffer pH 7.4 (0.13 M) was added to the acceptor chamber. To ensure oxygenation of tissue and stirring, both compartments were bubbled with a mix of Clioxicarb (95% O_2 plus 5% CO_2 mix). The transport of OLEs from apical to basolateral was studied. At 30 min intervals of a total of 150 min, 1 mL of sample was withdrawn from the acceptor and replaced with fresh pre-thermostated medium. The amount of antioxidant molecules permeated was determined by analysing the samples by the Folin-Ciocalteau method.

7.2.9 Statistical analysis

All data are expressed as mean ± standard deviation (SD). When not stated otherwise, six independent replicates were performed for each experiment. Data were tested for normality of distribution using the Shapiro-Wilk test. Significant differences between the extracts analysed were determined by one-way ANOVA. When ANOVA showed $p \leq 0.05$, a post-hoc test (Bonferroni correction) was performed. $p \leq 0.05$ was considered to indicate a significant difference.

7.3 Results

7.3.1 Antioxidant Capacity and Polyphenols Content

Figure 7.1 shows the antioxidant capacity of leaves collected at different time points. After two weeks of stress, the first difference between OLE-G and OLE-GS appeared (**Figure 7.1a**). However, as the drought stress progressed, the antioxidant capacity increased in all cultivars: OLE-GS, OLE-LS and OLE-MS were significantly different from their respective controls at t4. At this time point, OLE-GS and OLE-MS showed a higher antioxidant value (22.6 mmol Fe^{2+} $(100\ g)^{-1}$ and 21.3 mmol Fe^{2+} $(100\ g)^{-1}$, respectively) compared to OLE-LS (12.2 mmol Fe^{2+} $(100\ g)^{-1}$).

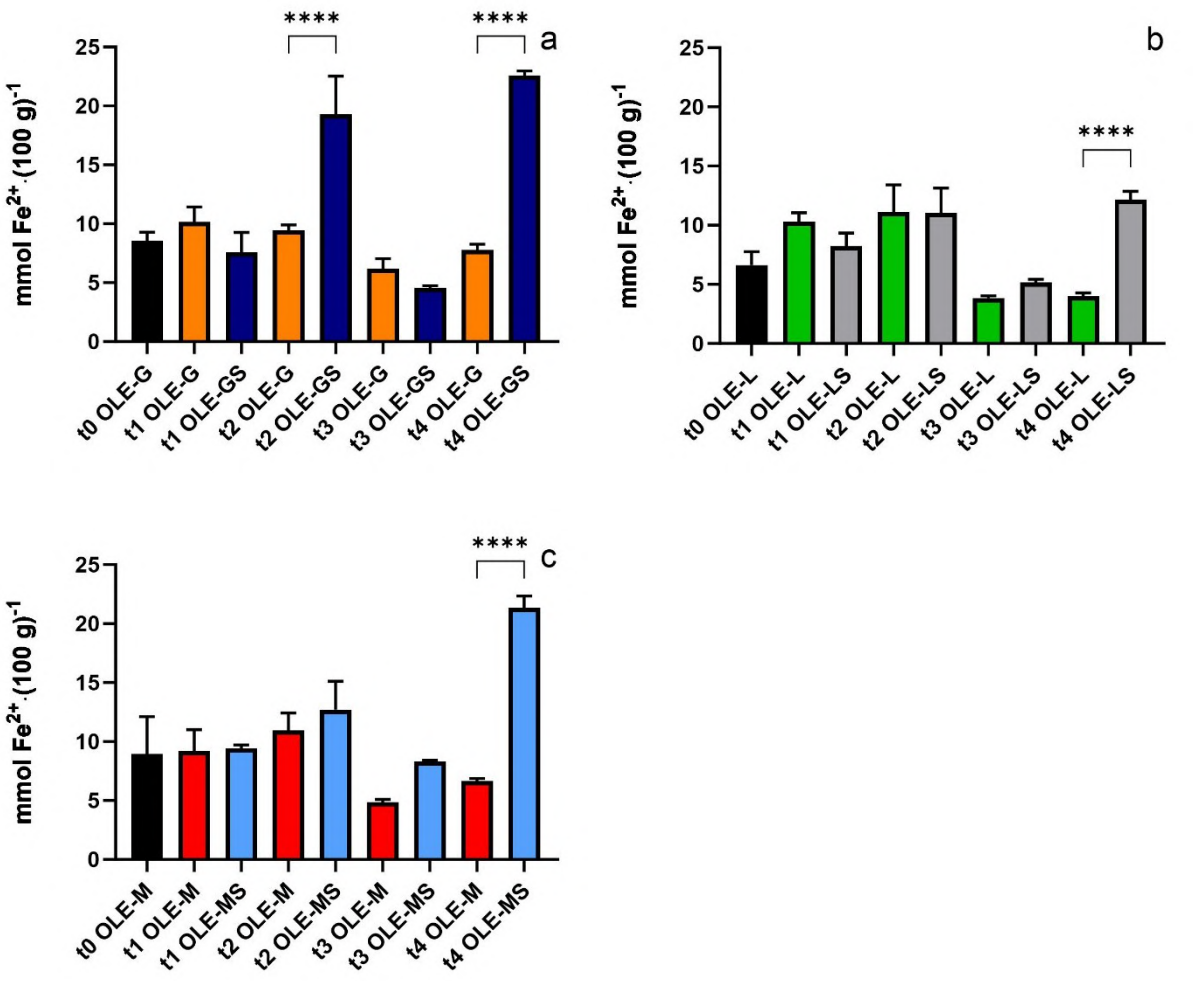

Figure 7.1. Antioxidant Capacity of (a) OLE-G and OLE-GS; (b) OLE-L and OLE-LS; (c) OLE-M and OLE-MS, from t0 to t4. Asterisks indicate a statistically significant difference (****: $p < 0.001$) between the stressed group and its respective control. In each panel, unstressed and stressed samples are indicated by different colors, regardless of the time of analysis.

The polyphenol content of each group during the experimental period is shown in **Figure 7.2**. The polyphenol content increased after four weeks of drought stress, when all the stressed groups differed significantly from their respective control. As shown in Figure 2a, the polyphenol content in OLE-GS already increased at t2 (39.4 mg GAE $(100\ g)^{-1}$), but the highest value was reached at t4 (52.2 mg GAE $(100\ g)^{-1}$). Despite the different antioxidant capacities, the polyphenol content in OLE-GS, OLE-LS, and OLE-MS were very similar at t4 (51.3, 49.9, 50.3 mg GAE $(100\ g)^{-1}$, respectively).

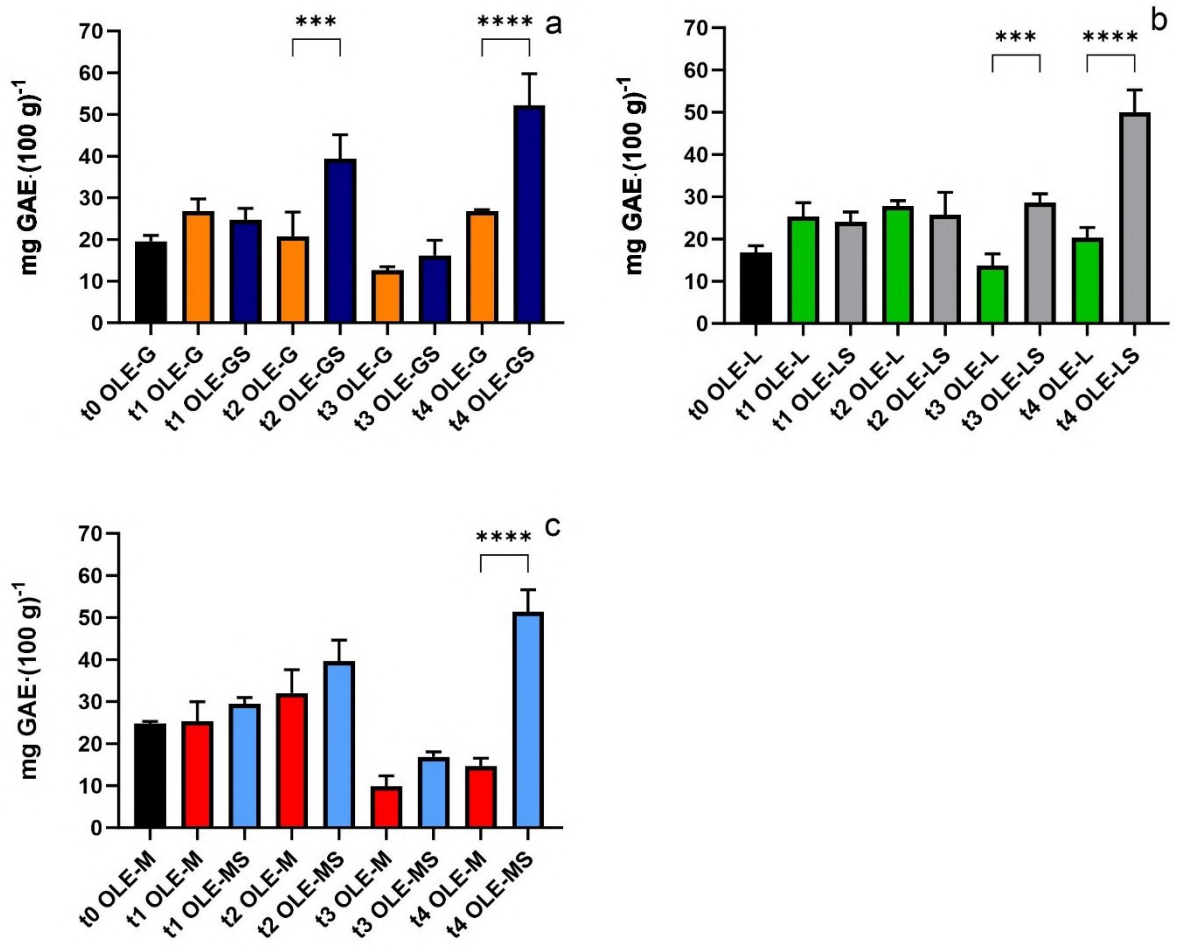

Figure 7.2. Polyphenols content of (a) OLE-G and OLE-GS; (b) OLE-L and OLE-LS; (c) OLE-M and OLE-MS, from t0 to t4. Asterisks indicate a statistically significant difference (****: $p < 0.001$; *** $p < 0.001$) between the stressed group and its respective control.

The strongest response, both in terms of antioxidant capacity and polyphenol content, was observed after four weeks of stress. Therefore, for all cultivars, leaves collected at t4 were selected for UHPLC characterization and analysis on human cells.

7.3.2 OLEs phenolic characterisation

In total, sixteen compounds were quantified: three secoiridoids and thirteen flavonoids. There were some differences between the extracts of the three olive varieties. In OLE-L, only one secoiridoid (aldehyde form of decarboxyl elenolic acid) could be detected, whereas the flavonoids chrysoeriol-7-*O*-glucoside and luteolin-7-*O*-glucoside could not be detected in either OLE-L or OLE-LS. In the same retention time (12.1 min), the extracts from 'Giarraffa' contained luteolin-7-O-rutinoside instead of 7-O-glucoside, as occurred in the extracts from 'Leccino' and 'Maurino'. The total amount of phenolic compounds is higher in OLE-LS (46.1 mg/g DW) and OLE-MS (79.1 mg/g DW) compared to their respective controls (40.7 mg/g

DW in OLE-L and 39.9 mg/g in OLE-M). In OLE-LS the increase is mostly due to the higher amount of detected secoiridoids (10.0 mg/g DW) compared to OLE-L (4.3 mg/g DW). OLE-MS showed a higher detected amount of both secoiridoids (27.7 mg/g DW) and flavonoids (51.4 mg/g DW) compared to OLE-M (3.2 mg/g DW and 36.7 mg/g DW, respectively). Conversely, OLE-GS showed a decrease in the total amount of phenolic compounds detected (29.4 mg/g DW) compared to OLE-G (33.2 mg/g DW) due to a lower amount of flavonoids detected (30.4 mg/g DW in OLE-G, 26.7 mg/g DW in OLE-GS).

The extracts from OLE-MS presented the highest ($p < 0.05$) amounts of oleuropein and oleuropein aglicone, dihydroquercetin, luteolin-7-*O*-rutinoside and chysoeriol-7-*O*-glucoside, and OLE-M the highest ($p < 0.05$) level of diosmetin (Table 1). In turn, OLE-L showed the highest ($p < 0.05$) content of aldehyde form of decarboxyl elenolic acid, and OLE-LS the highest ($p < 0.05$) levels of apigenin-*O*-dideoxyhexoside-hexoside, apigenin-7-*O*-rutinoside and luteolin-7-*O*-glucoside (**Table 7.1**).

Table 7.1. Phenolic profile (mg/g DW) of OLE-G, OLE-GS, OLE-L, OLE-LS, OLE-M, and OLE-MS extracts. Values are mean± standard deviation (n = 3–4). Rt—retention time; Nd—not detected; is. —isomer. For each compound, different letters denote significant difference ($p < 0.05$) between the values.

Rt (min.)	Compound	[M-H] –(m/z)	MS^2 (m/z) Fragments	OLE-G	OLE-GS	OLE-L	OLE-LS	OLE-M	OLE-MS
	Secoiridoids								
10.3	**Oleuropein aglicone**	**377**	**197/153**	1.140 ± 0.109^{b}	0.596 ± 0.025^{d}	nd	1.244 ± 0.022^{b}	0.875 ± 0.024^{c}	1.797 ± 0.005^{a}
10.8	**Aldehydic form of decarboxyl elenolic acid**	**215**	**197/153/ 171/ 185**	0.475 ± 0.118^{b}	0.752 ± 0.076^{b}	4.314 ± 0.681^{a}	0.708 ± 0.033^{b}	0.653 ± 0.184^{b}	0.973 ± 0.014^{b}
14.4	**Oleuropein**	**539**	**377/307/275**	1.228 ± 0.276^{c}	1.377 ± 0.106^{c}	nd	8.095 ± 0.494^{b}	1.661 ± 0.845^{c}	24.897 ± 1.353^{a}
	Flavonoids								
11.9	**Dihydroquercetin**	**303**	**285/177/ 125**	2.506 ± 0.145^{c}	2.722 ± 0.032^{c}	2.655 ± 0.014^{c}	2.391 ± 0.204^{c}	3.430 ± 0.048^{b}	5.279 ± 0.089^{a}
12.1	**Luteolin-7-*O*-rutinoside**	**593**	**447/285**	nd	nd	2.584 ± 0.015^{d}	4.261 ± 0.167^{b}	3.008 ± 0.046^{c}	10.366 ± 0.125^{a}
12.1	**Luteolin-7-*O*-glucoside is.1**	**447**	**287/285**	2.029 ± 0.070	2.305 ± 0.171	nd	nd	nd	nd
12.4	**Apigenin -*O*-dideoxyhexoside-hexoxide**	**449**	**269**	1.826 ± 0.023^{d}	1.726 ± 0.016^{d}	2.628 ± 0.015^{b}	2.992 ± 0.015^{a}	2.250 ± 0.025^{c}	2.479 ± 0.174^{b}
12.8	**Apigenin-7-*O*-rutinoside is.1**	**577**	**269**	3.471 ± 0.125^{c}	2.350 ± 0.050^{e}	4.797 ± 0.090^{b}	5.364 ± 0.071^{a}	2.688 ± 0.023^{d}	3.621 ± 0.047^{c}
13.0	**Apigenin-7-*O*-rutinoside is.2**	**577**	**269**	2.485 ± 0.102^{c}	2.099 ± 0.107^{d}	2.849 ± 0.036^{b}	3.296 ± 0.199^{a}	2.283 ± 0.041^{c}	2.979 ± 0.037^{b}
13.3	**Luteolin-7-*O*-glucoside is.2**	**447**	**285**	3.737 ± 0.163^{bc}	3.035 ± 0.462^{bc}	2.762 ± 0.233^{c}	6.135 ± 0.250^{a}	4.039 ± 0.139^{b}	7.161 ± 0.638^{a}
13.5	**Chrysoeriol-7-*O*-glucoside**	**461**	**299/446**	2.198 ± 0.099^{c}	1.669 ± 0.007^{d}	nd	nd	2.840 ± 0.112^{b}	4.111 ± 0.127^{a}
13.9	**Luteolin-7-*O*-glucoside is.3**	**447**	**285**	2.215 ± 0.145^{b}	1.741 ± 0.063^{c}	nd	nd	2.506 ± 0.173^{ab}	2.878 ± 0.187^{a}
15.7	**Luteolin**	**285**	**285**	2.668 ± 0.018^{c}	3.098 ± 0.290^{c}	7.160 ± 0.197^{a}	3.109 ± 0.014^{c}	4.905 ± 0.119^{b}	4.546 ± 0.200^{b}
16.7	**Apigenin-7-*O*-rutinoside**	**577**	**269**	2.000 ± 0.004^{c}	1.651 ± 0.078^{d}	2.086 ± 0.034^{c}	2.262 ± 0.019^{b}	2.339 ± 0.073^{b}	2.619 ± 0.046^{a}
17.4	**Apigenin**	**269**	**269/225**	2.612 ± 0.041^{d}	2.010 ± 0.077^{e}	5.982 ± 0.089^{a}	3.956 ± 0.042^{b}	2.884 ± 0.012^{c}	2.701 ± 0.010^{d}
17.8	**Diosmetin**	**299**	**284**	2.637 ± 0.028^{c}	2.283 ± 0.049^{d}	2.877 ± 0.020^{b}	2.277 ± 0.005^{d}	3.537 ± 0.047^{a}	2.674 ± 0.014^{c}

Figure 7.3 shows the heat map of the fold changes of the phenolic metabolites extracted from the three cultivars. In cultivar Giarraffa, drought stress caused, in general, a decrease of the phenolic content of OLE-GS/OLE-G, except for the secoiridoids aldehyde form of decarboxyl elenolic acid and oleuropein and the flavonoids dihydroquercetin, luteolin-7-O-glucoside is.1 and luteolin, which slightly increased. In contrast, a more heterogeneous response profile was observed in OLE-LS/OLE-L, with a similar number of compounds increased and decreased by drought stress. The phenolic pool of 'Maurino' was mostly increased by water deficit, with a large increase of oleuropein and luteolin-7-*O*-rutinoside.

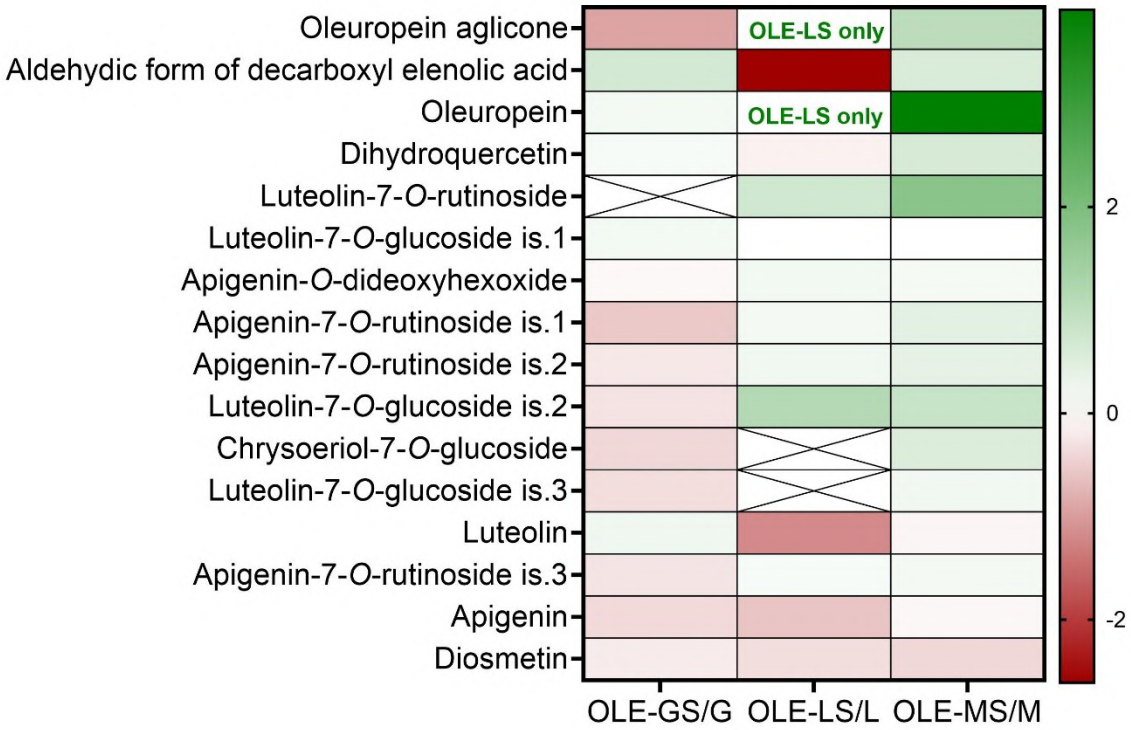

Figure 7.3. Heat map of the fold changes (log_2 (stressed/control)) in phenolic metabolites of Giarraffa (OLE-GS/OLE-G), Leccino (OLE-LS/OLE-L), and Maurino (OLE-MS/OLE-M).

7.3.3 Cell Viability Test

Figure 7.4 shows the viability of HUVEC treated with increasing concentrations of OLE-G and OLE-GS (**Figure 7.4a**), OLE-L and OLE-LS (Figure 4b) and OLE-M and OLE-MS (**Figure 7.4c**). The data appearing in Figure 4a show that OLE-G and OLE-GS are cytotoxic at concentrations above 10 µg/mL. **Figures 7.4b** and **7.4c** show that the olive leaf extracts of the Leccino and Maurino varieties have a low cytotoxicity at any of the concentrations tested. Therefore, the concentration of 10 µg/mL was chosen to carry out the subsequent experiments.

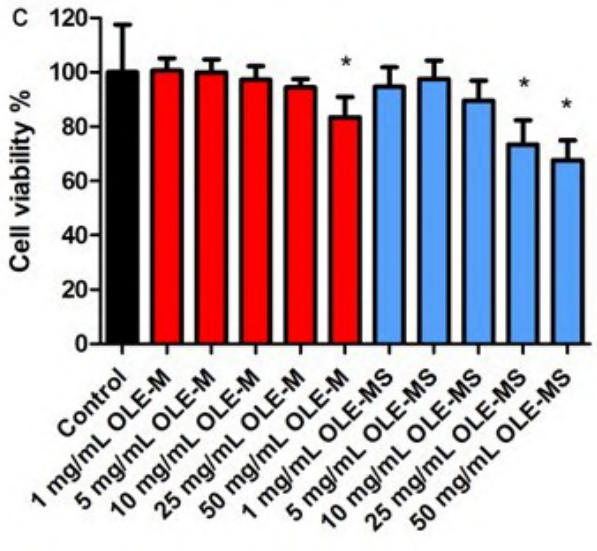

Figure 7.4. HUVECs viability after 4 h of incubation with: (a) OLE-G and OLE-GS; (b) OLE-L and OLE-LS; (c) OLE-M and OLE-MS in culture medium. Data are expressed as % viable cells referred to 100% control (untreated cells). Data are reported as mean ± SD (n = 6). *, significantly different from Control ($p < 0.05$).

7.3.4 OLEs protective effect from oxidative stress

With this test the influence of the treatment with OLEs on the viability of HUVECs after oxidative stress induced by H_2O_2 was evaluated. The OLEs were obtained from plants either subjected or not to water deficit stress. **Figure 7.5** shows data on the protection of HUVECs from oxidative damage after 2 hours of pretreatment with OLEs at a non-toxic concentration of 10 µg/mL, and subsequent treatment with 500 µM H_2O_2 for 1 hour. The data show that oxidative stress significantly reduced the number of viable cells compared to control (cells with medium only). Pretreatment of HUVECs with all the extracts under study at a concentration of 10 µg/mL reduced H_2O_2 cytotoxicity significantly. Apparently, the extracts from the Giarraffa and Maurino varieties subjected to water deficit stress (OLE-GS and OLE-MS) are significantly more effective than the corresponding extracts from olive trees not subjected to water deficit stress (OLE-G and OLE-M).

Figure 7.6 shows data on the protection of HUVECs from oxidative damage after 2 hours of pre-treatment with OLEs and gallic acid (GA) at the same polyphenol concentration of 0.5 µg/mL, and subsequent treatment with 500 µM of H_2O_2 for 1 hour. Also in this case, oxidative stress significantly reduced the number of viable cells compared to the control (cells with medium). HUVEC cells pre-treatment with all extracts at a concentration of 0.5 µg/mL of GAE showed a significant protective activity against the H_2O_2 oxidative damage, compared to GA pre-treatment. However, there are no statistical differences between the various OLEs tested.

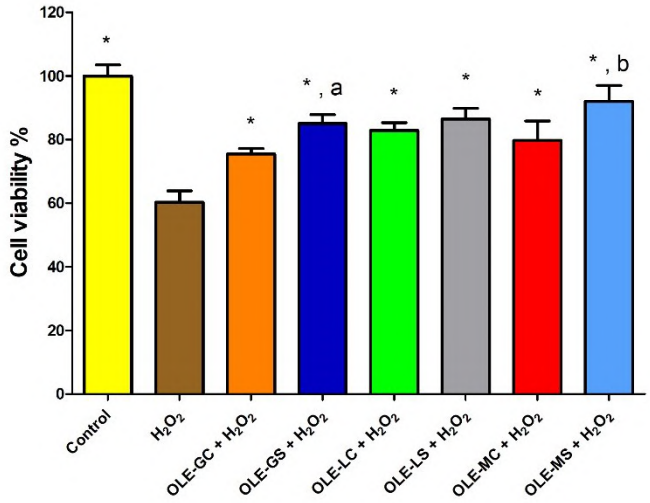

Figure 7.5. HUVECs viability after 2 h pre-treatment with OLE-G, OLE-GS, OLE-L, OLE-LS, OLE-M and OLE-MS (10 μg/mL) in culture medium, and subsequent 1 h treatment with 500 μM H_2O_2. Data are expressed as % viable cells compared to negative control (H_2O_2). *, significantly different from H_2O_2 ($p < 0.05$); a, significantly different from OLE-G ($p < 0.05$); b, significantly different from OLE-M ($p < 0.05$).

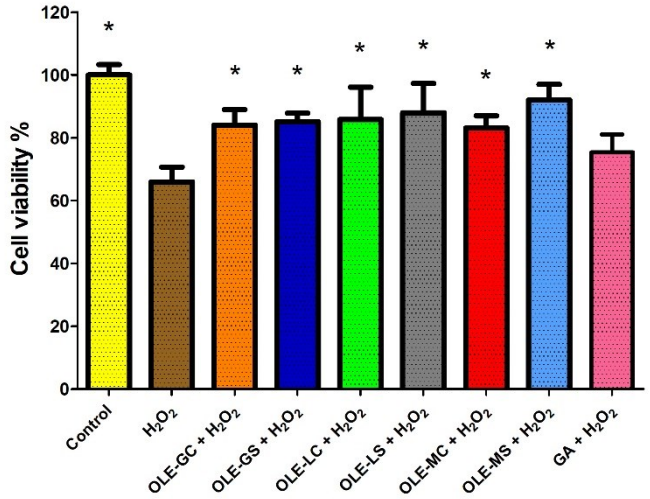

Figure 7.6. HUVECs viability after 2 h pre-treatment with OLE-G, OLE-GS, OLE-L, OLE-LS, OLE-M, OLE-MS and GA (0.5 μg/mL GAE) in culture medium, and subsequent 1 h treatment with 500 μM H_2O_2. Data are expressed as % viable cells compared to negative control (H_2O_2). *, significantly different from H_2O_2 ($p < 0.05$).

7.3.5 OLEs antioxidant activity as assessed by ROS production

Figure 7.7 shows the data for ROS production in HUVECs pretreated or not with the OLEs of interest and then subjected to oxidative stress. As can be seen, ROS production after cell treatment with H_2O_2 is significantly higher than ROS production in cells incubated with control (plain medium). All extracts tested, except OLE-M, can significantly reduce ROS production compared to cells treated with H_2O_2. Apparently, OLE-GS and OLE-MS can significantly reduce ROS production, more effectively than OLE-G and OLE-M, respectively. On the other hand, no significant differences are observed between OLE-L and OLE-LS. The results obtained with this test are in perfect agreement with those reported in Figure 4. These results

can be attributed to the fact that OLE-GS and OLE-MS have a significantly higher content of polyphenols and antioxidants than all the other extracts and are not significantly different from each other.

ROS level was also evaluated in HUVECs with OLEs at the same GAE concentration (0.5 µg/mL GAE). GA was also tested as a positive control (**Figure 7.8**). All extracts tested significantly reduced ROS level compared to H_2O_2 treated cells. Surprisingly, ROS in OLE-GS treated cells was significantly lower than that in OLE-G and GA treated cells. This result demonstrates that extracts from olive leaves subjected to water deficit stress contain antioxidant molecules with greater reducing power than those extracted from non-stressed olive trees.

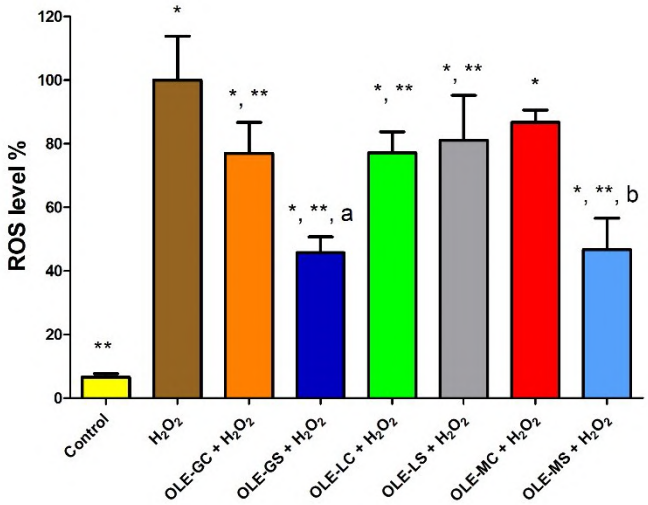

Figure 7.7. Reactive oxygen species (ROS) level in HUVECs treated with OLE-G, OLE-GS, OLE-L, OLE-LS, OLE-M, and OLE-MS (10 µg/mL) in culture medium, and subsequent treatment with 500 µM H_2O_2 for 1 h. Data are expressed as % ROS production on the basis of cells simply treated with H_2O_2. *, Significantly different from Control ($p < 0.05$); **, Significantly different from H_2O_2 ($p < 0.05$); a, significantly different from OLE-G ($p < 0.05$); b, significantly different from OLE-M ($p < 0.05$).

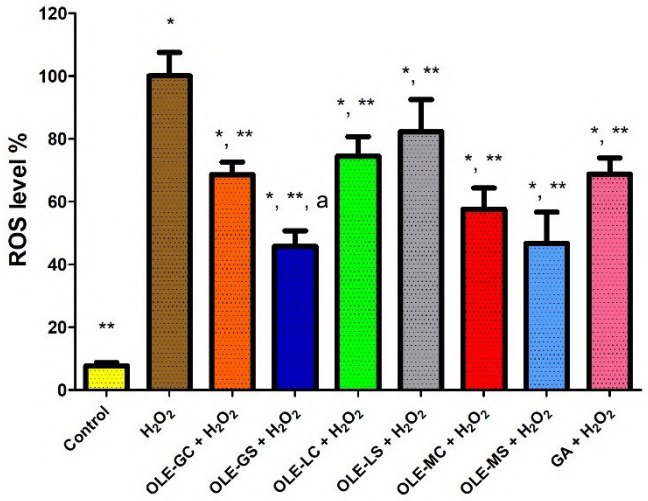

Figure 7.8. Reactive oxygen species (ROS) production in HUVECs treated with OLE-G, OLE-GS, OLE-L, OLE-LS, OLE-M, OLE-MS and GA (0.5 µg/mL GAE) in culture medium, and subsequent treatment with 500 µM H_2O_2 for 1 h. Data are expressed as % ROS production compared to 100% (cell treated with H_2O_2). *, Significantly

different from Control ($p < 0.05$); **, Significantly different from H_2O_2 ($p < 0.05$); a, significantly different from OLE-G and GA ($p < 0.05$).

7.3.6 Permeation of antioxidants contained in OLE across excised rat intestine

The epithelium of excised rat jejunal tract was selected among known ex vivo intestinal models for studies of the permeability of antioxidant molecules because its tight junctions are similar in size and number to those of the human jejunum (Legen et al., 2005). Only the OLEs extracted from the Maurino and Giarraffa varieties that showed a greater antioxidant ability than the Leccino were tested for permeation through the intestinal epithelium. The OLEs were tested keeping constant either the concentration of the extract or the amount of polyphenols present in the extracts expressed in mg of GAE per mL. **Figures 7.9a** and **7.9b** report the percentage of antioxidant molecules permeating through the intestinal epithelium over time. As can be seen, all the OLEs tested have the same permeation profile, regardless of the concentration or amount of antioxidant molecules applied. However, by comparing the data in **Figure 7.8a** with those in **Figure 7.8b**, the OLEs obtained from the Giarraffa variety have a significantly higher permeability than those obtained from the Maurino variety. These results, together with those shown in **Figure 7.8**, allow us to conclude that the OLEs from the Giarraffa variety are more permeable and, in particular, those obtained from plants subjected to water deficit stress have a higher antioxidant activity.

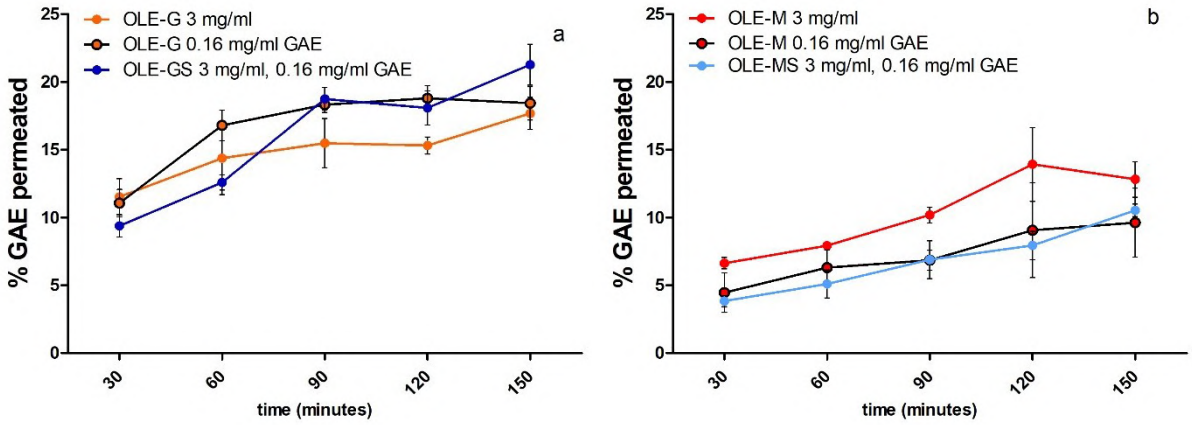

Figure 7.9. Data on the permeation of antioxidant molecules contained in OLE-G and OLE-GS a) and in OLE-M and OLE-MS b) in phosphate buffer, pH 7.4, 0.13 M, at the same concentration of extract (3 mg/ml) or at the equivalent gallic acid concentration (GAE) of 0.16 mg/mL across the excised jejunal rat epithelium ($n = 3$).

7.4 Discussion

Olive leaves are an unavoidable by-product of olive oil production, accounting for 25% of the dry weight of the total pruning residue (more than 6 kg/L of olive oil produced) (Espeso et al., 2021). Olive leaves are known to be rich in phenolic derivatives, mainly consisting of simple phenols, flavonoids, and secoiridoids, which may have various beneficial biological effects thanks to their antimicrobial, antioxidant, antiviral, and cardioprotective properties (Borjan et

al., 2020). The accumulation of phenolic compounds is a well-known response to various abiotic stresses. As drought will be one of the major challenges in the Mediterranean region, research on cultivar-specific antioxidant properties under controlled or stressful conditions may be useful to identify which cultivars have the highest antioxidant content and potential health benefits, thus turning olive leaves from agricultural waste into a health or pharmaceutical product.

In this work, the antioxidant effects of olive leaves extracts of the olive cultivars 'Giarraffa', 'Leccino' and 'Maurino' are compared with those of the same leaves subjected to water deficit stress.

As also reported in other studies (Petridis et al., 2012; Denaxa et al., 2020), drought stress of olive plants increased their antioxidant responses. In the present study, the antioxidant capacity (FRAP) and total phenols (TPC) reached their highest levels in the drought-stressed plants at the end of the experimental period, after four weeks of total water deprivation. At this time point, in OLE-GS and OLE-MS, the increase in the antioxidant capacity response is reflected by an accumulation of polyphenols. OLE-LS maintained a lower antioxidant capacity, although the polyphenol content increased. In fact, the antioxidant activity of an extract depends not only on the quantity of the polyphenols, but also on the type and the synergistic interactions that occur (Xie et al., 2015). The chemical structure heavily determines the antioxidant properties of phenolics: catechol moieties, multiple hydroxyl groups and conjugation with electron donating groups at the 4-position of the aromatic ring are factors that positively influence antioxidant activity (Benavente-García et al., 2000; Xie et al., 2015). UHLC-MS characterization of the phenolic compounds present in the leaf extracts revealed differences between cultivars and on the basis of water supplementation. Oleuropein, a 3,4-dihydroxyphenylethanol (hydroxytyrosol) ester with a-glucosylated elenolic acid, is commonly reported as the main component of olive phenolic extracts (Ennajeh et al., 2009; Xie et al., 2015; De la Ossa et al., 2019) and it is well known for its pharmacological effects related to its free-radical scavenging properties (Borjan et al., 2020). In this study, oleuropein was found as the main component of leaf phenolic extracts in OLE-MS and OLE-LS. According to the antioxidant capacity calculated for single phenolic compounds by Xie et al. (2015) and Benavente-García et al. (2000), OLE-MS is rich of high-performance compounds, such as oleuropein, dihydroquercetin, and flavon-7-glucosides of both luteolin and apigenin, with a probable consequent decrease in the free form of these flavones. Similarly, OLE-LS showed an increase in the content of these compounds compared to OLE-L; however, the constant level of the highly antioxidant-performance dihydroquercetin and the lower amount of the phenolics'

increase under stress conditions can have contributed to the lower FRAP response of OLE-LS compared to OLE-MS. Synergistic effects should be taken into account when considering the results of OLE-GS. The lower levels of all phenolic compounds detected in OLE-GS compared to OLE-G (**Figure 7.3**) do not justify its higher antioxidant capacity (**Figure 7.1a**). As suggested by Dias et al. (2019), this may be due to several reasons: it is possible that the stress condition increased the use in radical scavenging more than it affected the phenolic synthesis, in which case the antioxidant phenolic molecules did not increase in quantity. Alternatively, the molecules detected could have been in combination with some other antioxidant compounds not detected by LC-MS analysis.

The antioxidant activity of olive leaves extract was evaluated on HUVEC cells. All OLEs showed no cytotoxicity at the concentrations analysed (1-50 μg/mL), however it can be observed in **Figure 7.4** that the viability decreased slowly with increasing concentration especially with OLE from Giarraffa subjected or not to water deficit stress. Similar results were found by De La Ossa et al. (2021) and indicate that a high concentration of polyphenols present in olive extract exerts a cytotoxic effect, whereas a low concentration increases cell proliferation, as already demonstrated (Babich and Visioli, 2003; De Leonardis et al., 2007). Olive leaves have scavenging activity against multiple ROS and could display cardiovascular protection. The ability of polyphenols presents in OLEs to inhibit ROS production was evaluated in this study. All OLEs tested, and in particular OLE from Giarraffa and Maurino subjected to water deficit stress were able to reduce ROS production compared to untreated cells. Considering that these extracts have a higher antioxidant activity and polyphenols content than the others, these differences could be related to a synergic effect between the antioxidant compounds present (Vogel et al., 2015). Therefore, the protective effect of OLEs could be related to a determined concentration and combination of antioxidants present in the extract.

Finally in this study, we evaluated the ability of antioxidants present in OLE from Giarraffa and Maurino to cross the excised intestinal wall. As reported in **Figure 7.8b** OLE from Giarraffa has a significantly higher permeability than Maurino. In fact, a permeation of total polyphenols at 20% circa of the applied dose (0.16 mg/mL GAE) was observed in OLE from Giarraffa whereas that from Maurino was about 10%. These results indicate that OLE from Giarraffa is more permeable through the intestine compared to OLE from Maurino probably because it depends on the permeation ability of the single molecules contained. Indeed, various factors should be considered in the study of the transport of bioactive compounds in the intestine, such as concentration and degradation processes (Cuffaro et al., 2023).

Chapter 8

How does the data fit together? Final Model, Conclusion, and Prospects

Olive trees are an economically and culturally valuable crop in the Mediterranean, with many varieties grown. Different varieties have evolved effective physiological, biochemical, and morphological tolerance mechanisms to help them adapt to their specific environment. Climate change now poses a greater threat to crop growth and productivity than ever before, with droughts expected to become more severe and frequent, particularly in Mediterranean climate change hotspots. The use of various methods to evaluate the responses and drought tolerance of different cultivars is critical in determining the cultivar(s) best suited to future climate scenarios, both for breeding programs and water-saving agricultural practices. In this study, three olive cultivars were tested under drought conditions: 'Giarraffa', from the arid region of Sicily, 'Leccino', an Italian cultivar that is more widely distributed, and 'Maurino', grown primarily in Tuscany and central Italy. A multi-level approach, including physiological monitoring, metabolomic analysis, biochemical assays, and anatomical observations, was used to assess the plant water status and management under deficit conditions, as well as to investigate potential mechanisms underlying the observed responses. When interpreting the data obtained, it is important to remember that the water deficit tests were carried out on 18-months old plants and for a well-defined period of time (four weeks). Consequently, all results must be interpreted in the context of the plants studied and the period of stress, and extrapolation of results to more mature plants and periods of greater stress should be made with caution.

The results gathered from the analyses can be used to draw some broad conclusions about olive and drought stress. First, all the significant physiological changes that distinguished the cultivars occurred between the first and second weeks of stress. At t3 and t4, the cultivars behaved significantly more similarly than at t1 and t2, except for the metabolomic and biochemical responses, which differed primarily at t4. Second, leaf water content appeared to be extremely sensitive to drought, declining in all cultivars after one week of stress, with a significant decrease at t3. The parameter was unsuitable to differentiate the cultivars; monitoring leaf water potential instead of only relative water content will add information on the physiological relationship between plant water status and transpiration rate, as well as the iso-anisohydric behaviour of the cultivars (Diaz-Espejo et al., 2018).

To summarize the main mechanisms of drought stress defense (especially in terms of time and intensity), a model has been assembled and shown in **Figure 8.1**, which attempts to capture and consolidate the complex adaptation strategies. As with all models, it has limitations and only highlights the most obvious results, but it is a quick way of highlighting the differences between

the three cultivars. It is possible to identify two distinct patterns in ‘Giarraffa’ and ‘Maurino’, with ‘Leccino’ falling in the middle. ‘Giarraffa’ showed the most reliable “water saving” strategy. The stomatal conductance of this cultivar decreased within one week. As a result, stomatal density was lower compared to other cultivars. A thick waxy leaf surface can also reduce water loss; ‘Giarraffa’ had the highest concentration of long-chain alkanes and other lipophilic compounds in the leaves, which could be attributed to cuticle components; the robust pool of lipophilic compounds was maintained throughout the experiment. This strategy had a visible effect on stem and soil water content, which were higher than in the other two cultivars during the first two weeks of stress. Because stomatal closure can cause oxidative stress, ‘Giarraffa’ showed an increase in antioxidant capacity earlier than ‘Leccino’ and ‘Maurino’, but the pool of phenolic compounds decreased in the leaves and, except for t4, in the stem. It is possible that the antioxidant power was due to a synergistic effect of the molecules present, or to compounds that were not detected. It is worth noting that only non-enzymatic defence mechanisms were examined; the study of enzyme-based mechanisms (including the analysis of superoxide dismutase, catalase, and peroxidases) was originally planned but not carried out due to time constraints. The stem accumulated most of the biochemical resources, with an increase in dehydrins and mannitol occurring as early as t2. ‘Giarraffa’ had many aquaporins, which accumulated in stressed stems only at t4, possibly to release the water stored in this plant organ. These reactions were sufficient to maintain PSII efficiency until t4, with ‘Giarraffa’ being the last cultivar to exhibit a decrease in photosynthetic efficiency.

On the other hand, ‘Maurino’ is considered the most "water consuming" cultivar. It had the highest stomatal conductance of the controls, and its stressed leaves maintained a stomatal conductance similar to the controls until the second week of stress. The high transpiration rate was made possible by high stomatal density and increased xylem vessel area and density. The stem of this cultivar had the lowest water content already at t2. To keep the gas exchange active, 'Maurino' uses carbon resources to maintain a higher and earlier water status than the other cultivars. Both in the stem and in the leaf, we found an increase in the levels of dehydrins, glucose, sucrose, and phenolic compounds, which could act as antioxidants to counteract the ROS produced by the high electrolyte leakage beginning at t1. Despite these reactions, 'Maurino' exhibited a decrease in PSII efficiency at t3, increasing electrolyte leakage, and a dramatic decrease in lipophilic compound pools at the end of the experimental period.

‘Leccino’ shared some responses with ‘Maurino’ and others with ‘Giarraffa’, as evidenced by the stem water content being intermediate between the two cultivars. ‘Leccino’ started closing the stomata one week after ‘Giarraffa’, but it showed the earliest decrease in PSII efficiency

and the same timing and amount of electrolyte leakage as ‘Giarraffa’. Like ‘Maurino’, it has invested in water-related proteins (osmotin and dehydrins), but unlike the other cultivars, it has a high concentration of aquaporins. The phenolic compound pattern is interesting: the flavonoid pool decreased, as in ‘Giarraffa’, whereas the secoiridoid pool increased, as in ‘Maurino’. ‘Leccino’ was the only cultivar that invested in proline at t2, contrary to what has been reported in the literature about the late appearance of this osmoprotectant.

More generally, these results suggest that ‘Giarraffa’ adopts a “drought avoidance” strategy. The "drought avoidance" strategy consists of effective and timely stomatal control in response to soil water scarcity. This strategy appears to be beneficial during short periods of water scarcity because it preserves the plant for future recovery. However, long-term stress may have a negative impact on carbon fixation. Drought-tolerant species can maintain a positive carbon balance through maintaining longer basal gas exchange rates. This is made possible by investment in osmoregulatory capacity, which increases turgor during severe dehydration (Forner et al., 2018). This appears to be the case with ‘Leccino’. Finally, ‘Maurino’ appears to be severely dehydrated as a result of its high-water consumption.

Olive tree and drought is a fertile and fruitful research area; this study adds a new piece to the wall of response characterization, highlighting particularly the timing of the observed responses and the relationship between different areas, which are frequently studied separately. Based on this experience, physiology reveals more about the overall state of the plant while masking long-term responses and adjustments within the plant's organs. Given the important correlations between irrigation regimes and fruit and oil quality (Caruso et al., 2014; Perpetuini et al., 2018), the next steps may inevitably shift to a field study focusing on the reproductive stage and using this study as a benchmark. Indeed, the data collected during the doctoral research period show that the plant's response to water scarcity stress is multifactorial, involving numerous metabolic, physiological, and molecular mechanisms. Plants respond in a variety of ways that are not always completely understood. The evidence that three different olive cultivars exhibit temporally and quantitatively different mechanisms suggests adaptational plasticity, which is critical for the plant's survival in very different environments. A better understanding of the complexities of olive plants' responses to water deficit will undoubtedly necessitate a series of subsequent analyses, such as addressing the plants' diverse genetic background and conducting a broad-spectrum proteome analysis of individual organs. Deciphering changes in cell wall polysaccharide structure and composition may also reveal processes of cell/tissue adaptation to drought stress. Not to be ignored is the importance of evaluating all of this in terms of plant productivity, and thus the quality and quantity of their final product, olive oil.

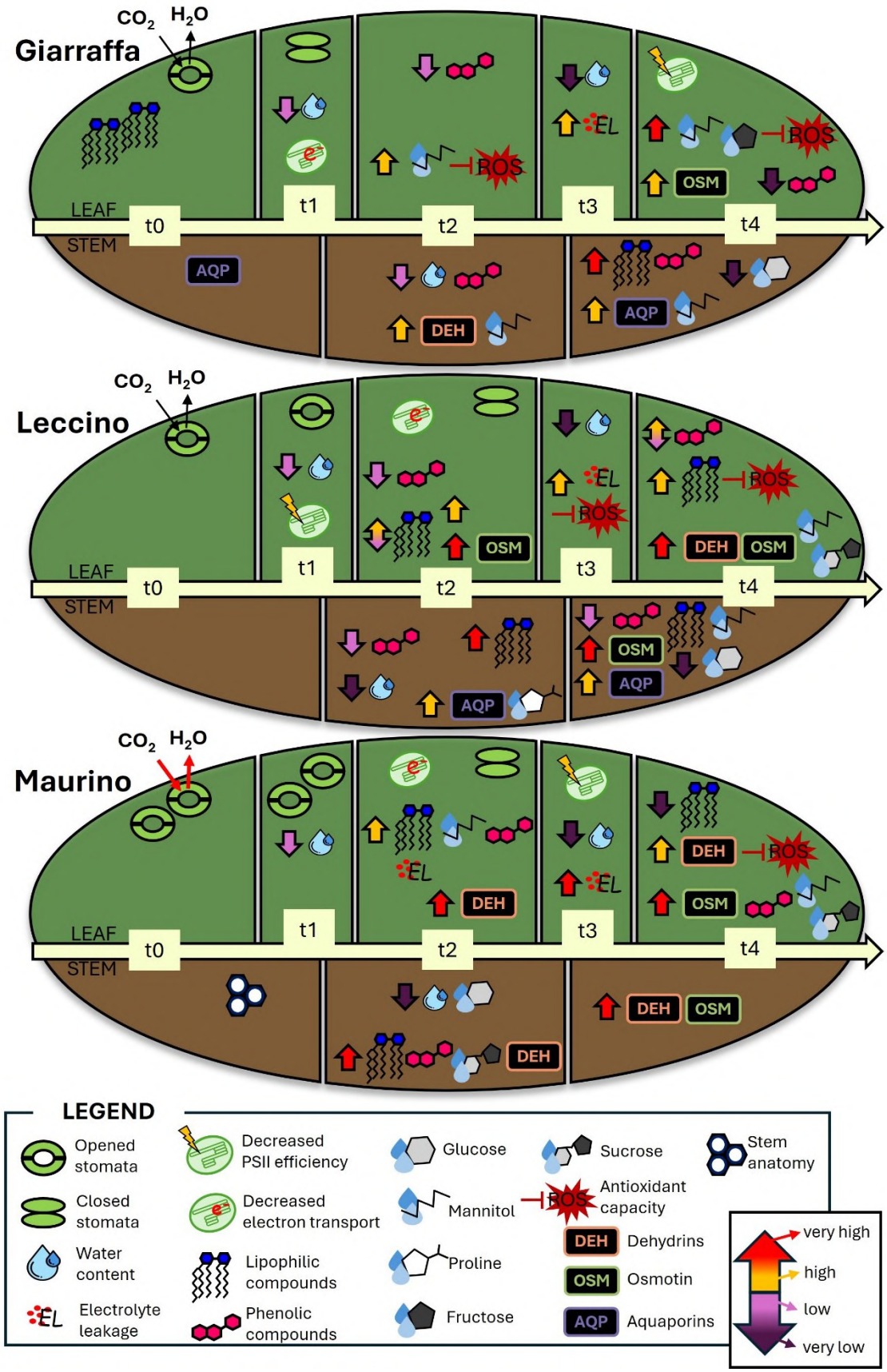

Figure 8.1. Graphical synthesis of the most sensitive parameters analyzed and their changes as a function of drought stress intensity and cultivar. The colored arrows indicate the unit of variation of the stressed sample compared to the control. A legend is provided at the bottom to help interpret the symbols.

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