

# Chapter 11

## Metabolomics Connecting Genomes and Phenomes in Plant Molecular Breeding—A Special Focus on Mediterranean Agronomical Important Tree Species



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**Abstract** The interest in Mediterranean species has grown exponentially in recent decades, driven by their distinctive organoleptic properties and the high content of bioactive compounds beneficial to human health. This study presents a comprehensive review of current knowledge on the alterations in metabolomic profiles associated with the biosynthesis of functional metabolites in response to abiotic stressors, namely salinity, extreme temperatures, drought and UV exposure, in five agronomically significant Mediterranean tree species: olive (*Olea europaea* L.), cork oak (*Quercus suber* L.), pine tree (*Pinus pinaster* Ait. and *Pinus pinea* L.), and carob tree (*Ceratonia siliqua* L.). Furthermore, considering that the reliability of metabolomic data depends on the methodologies employed for sample preparation and analysis, we provide an extensive overview of the primary methods for comprehensive metabolome extraction and the analytical techniques utilized for subsequent analysis.

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## 11.1 Introduction

Plants, as sessile organisms, must cope with a diversity of environmental constraints that directly affect their growth and development. In agronomical systems, these stress factors can have devastating consequences on crop yield, consequently leading to significant economic losses. Environmental factors that could affect plant development include light, extreme temperatures (cold/freezing and heat), wind, water availability (flooding and drought), soil structure, air humidity, and nutrient availability, as well as biotic components, from pathogens to competitors (Tonsor et al. 2005).

Throughout evolution, plants have learned how to efficiently respond to environmental stresses by altering their molecular program at cellular level, thereby modulating gene expression patterns that consequently lead to changes in biochemical, physiological, and, in some cases, morphological features. Plant plasticity is recognized as an important agronomical trait that reflects the capacity of a plant genotype to efficiently alter its molecular cell program and adapt its growth and development when facing a particular stress condition (Cardoso and Arnholdt-Schmitt 2013). Comprehending the molecular mechanisms underlying this effective behavior holds significant importance for their implementation in plant breeding endeavors. Such insights contribute to the cultivation of more resilient genotypes capable of enduring the stresses induced by climate change, thereby fostering the adoption of more sustainable agricultural practices.

The metabolome, as the product of a complex gene expression network, protein interactions, and other regulatory mechanisms, is representative of a plant genotype growing under specific conditions. Its capacity to be modulated by environmental changes makes metabolomics a more accurate strategy for describing a plant phenotype's response compared to transcriptomics or proteomics (Fiehn 2002; Arbona et al. 2013; Pinu et al. 2019; Li et al. 2019). Due to its proximity to the phenotype and its heightened responsiveness to environmental influences, metabolomics stands as a pivotal tool for evaluating biochemical alterations in plants impacted by abiotic stress (Shulaev et al. 2008), enabling the delineation of mechanisms underlying plant tolerance to such stressors. Metabolomics, a research field that enables the detection and semi-quantification of both known and unknown metabolites, is currently in high demand owing to its broad spectrum of applications for plant metabolites (Salam et al. 2023). In this field, Mediterranean species, well adapted to cool, wet winters, and hot, dry summers (usually xerophytic species), are known for their ability to produce a high content of secondary metabolites. The olive tree (*Olea europaea* L.), cork oak (*Quercus suber* L.), pine tree (*Pinus pinaster* Ait. and *Pinus pinea* L.), and carob tree (*Ceratonia siliqua* L.) are significant agronomical species finely attuned to the Mediterranean climate. They demonstrate the capacity to synthesize a diverse

















range of metabolite compounds in response to various abiotic stresses, encompassing drought, salinity, extreme cold and heat, and UV radiation exposure (see Table 11.1).

The olive tree (*O. europaea* L.) is a pivotal evergreen species extensively cultivated within the Mediterranean basin (Anguita-Maeso et al. 2021). The high pharmacological potential of its leaves and the nutritional value of its fruits attribute enormous cultural and economic value to this fruit tree (Hashmi et al. 2015). Olive cultivation also plays an important ecological role for Mediterranean countries, increasing biodiversity, controlling erosion, and enhancing the retention of nutrients, water in the soil and atmospheric carbon (Fraga et al. 2021). The ideal soil conditions for olive tree cultivation include deep, sandy loam soils adequately enriched with nitrogen (N), phosphorus (P), potassium (K), and water, with pH levels ranging from 5.5 to 8.5. However, the olive tree can tolerate poor, dry, calcareous, and rocky soils (Fernández and Moreno 2000). The olive tree is regarded as the species most suited to the Mediterranean climate. Its optimal temperature range for vegetative development is between 10 and 30 °C. It requires periods of cool temperatures ranging from 0 to 7 °C for optimal development but cannot withstand temperatures below – 7 °C (Lockwood 2009). Although genotype-related variations exist, the olive tree is generally a drought-resistant (xerophytic) species (Bacelar et al. 2004). This resilience is attributed to various morpho-physiological and biochemical adaptations, which consequently influence its productivity (Bacelar et al. 2004). It also exhibits moderate tolerance to salinity, up to a maximum of 3.2 g/L NaCl (sodium chloride). This tolerance is primarily achieved through the capacity to sequester sodium chloride within the leaf cellular compartments and regulate the net import of sodium chloride into the aerial parts of the plant (Trabelsi et al. 2019). The degree of tolerance is, however, significantly dependent on the cultivar (Chartzoulakis 2005).

Similarly, to other trees in the Mediterranean region, all the components (leaf, fruit, flowers, wood) of the *O. europaea* L. can be exploited for a multitude of purposes. Several studies of the phytochemical composition of different olive tree components revealed a wide range of compounds, such as oleuropein, hydroxytyrosol, hydroxycinnamic acid derivatives, linoleic acids and phenolic compounds, among others (Ozturk et al. 2021) that contribute to their appreciation. Despite this wide range of studies, the way in which olive trees modulate their metabolite profile under multiple stress agents is far to be fully revealed.











The cork oak (*Q. suber* L.) is a perennial angiosperm indigenous to the Mediterranean botanical assemblage (Caudullo et al. 2017). In Portugal, this species is associated with a distinctive agrosilvopastoral system named “montado” and its most important bioresource, cork, plays an important role in the country’s economy (Peacock 2011). These “montado” systems, together with the cork oak, ensure soil protection, increased carbon dioxide retention and contribute to the regulation of the water cycle. Compact, loamy and sandy soils, with good aeration and overflooded, are the preferred soil texture for these trees (Vallejo et al. 2009). Owing to its high plasticity in the face of various abiotic stresses, this arboreal species demonstrates robust adaptation to the Mediterranean climatic conditions. The cork oak is considered a xerophytic tree (Ramírez-Valiente et al. 2010), that is also highly thermotolerant being able to withstand temperatures near 55 °C (Ghouil et al. 2003;

**Table 11.1** Metabolites identified in the different Mediterranean species when submitted to abiotic stresses

Stress	Metabolite	Regulation	Species	Organ	References
Drought	ROS	↑	Olive tree		Ben Abdallah et al. (2018) and Brito et al. (2018, 2019)
	Antioxidant enzymes	↑	Olive tree		Brito et al. (2019)
	Flavonoids	↑	Olive tree		Dias et al. (2020)
			Cork oak		Almeida et al. (2020)
			Maritime pine		de Miguel et al. (2016) and de Simón et al. (2017)
	Carotenoids and chlorophylls	↓	Olive tree		Silva et al. (2018)
			Carob tree		Gadoum et al. (2019)
	Ascorbate	↑	Olive tree		Brito et al. (2019)
	Glutathione				
	Abscisic acid				
	Oleuropein	↓	Olive tree		Dias et al. (2020)
	Lipophilic compounds	↓	Olive tree		Dias et al. (2018)
	Terpenes	↑	Cork oak		Almeida et al. (2020)
	Sugars	↑	Maritime pine		de Miguel et al. (2016)
			Carob tree		Gadoum et al. (2019)
Sterols	↑	Maritime pine		de Miguel et al. (2016)	
Carbohydrates	↑	Olive tree		Karimi et al. (2018)	
Proline	↑	Olive tree		Karimi et al. (2018) and Silva et al. (2018)	
		Cork oak		Hu et al. (2013) and Kwak et al. (2011)	











(continued)

**Table 11.1** (continued)

Stress	Metabolite	Regulation	Species	Organ	References	
			Carob tree		Gadoum et al. (2019)	
	Aminoacids	↑	Cork oak		Almeida et al. (2020)	
			Maritime pine		Araújo et al. (2011), Aranda et al. (2017) and de Simón et al. (2017)	
	Chiro-inositol	↑	Maritime pine		de Miguel et al. (2016)	
	Pinitol					
	Squayitol	↓				
	Pyruvate	↑	Maritime pine		Warren et al. (2011)	
	Salinity	Proline	↑	Carob tree		El-Kahkahi et al. (2015)
		Glycine	↑	Olive tree		Ben Abdallah et al. (2018)
		Betaine				
Trehalose						
Sucrose						
Myo-inositol						
Sugars		↑	Olive tree		Conde et al. (2011)	
			Cork oak		Peguero-Pina et al. (2009)	
			Carob tree		El-Kahkahi et al. (2015)	
		↓	Olive tree		Skodra et al. (2021)	
Mannitol	↑	Olive tree		Skodra et al. (2021)		
Simple phenols	↑	Olive tree				
	↓					
Flavonoids	↑					
Secoiridoids	↑					









(continued)












**Table 11.1** (continued)

Stress	Metabolite	Regulation	Species	Organ	References
		↓			
	Organic acids	↓			
	Carotenoids and chlorophylls	Early ↑; Later ↓	Cork oak		Peguero-Pina et al. (2009)
		↑	Carob tree		El-Kahkahi et al. (2015)
	Antioxidant enzymes	↑	Cork oak		de Oliveira et al. (2022)
	Total phenolic content		Maritime pine		Azevedo et al. (2009a, b)
	ROS				
Heat stress and radiation (ultraviolet and infrared)	Oleuropein	↑ in UVB radiation; ↓ in heat shock	Olive tree		Dias et al. (2019, 2020)
	Verbascoside	↑ in UVB radiation			Dias et al. (2020)
	Flavonoids				Dehghanian et al. (2022)
	Carotenoids and chlorophylls	↓ in heat shock and UVB radiation	Olive tree		Dias et al. (2019)
		↑ under UVC radiation	Carob tree		Costa-Pérez et al. (2023)
	Proline	↓ in heat shock and UVB radiation	Olive tree		Dias et al. (2019)

(continued)

**Table 11.1** (continued)

Stress	Metabolite	Regulation	Species	Organ	References
	Sugars	↑ under UVC radiation	Carob tree		Costa-Pérez et al. (2023)
Cold (chilling and freezing)	Soluble carbohydrates	↑	Olive tree		Ortega-García and Peragón (2009), Saadati et al. (2019, 2021a, b), Petruccelli et al. (2022), Jiang et al. (2023) and Rejšková et al. (2007)
	Unsaturated fatty acids				Matteucci et al. (2011), Saadati et al. (2021a) and Petruccelli et al. (2022)
	Proline				Saadati et al. (2019)
	Oleuropein				Ortega-García and Peragón (2009), Petruccelli et al. (2022) and Jiang et al. (2023)
	Malondialdehyde (MDA)				Wang et al. (2018), Saadati et al. (2019, 2021a, b), Petruccelli et al. (2022) and Jiang et al. (2023)
	Sugars	↑		Cork oak	
		Maritime pine		Cañas et al. (2015)	
	Malic and citric acid	↑	Cork oak		Passarinho et al. (2006)
	Quercitol	↑			
	Quinic acid	↓			

 Increase;  Decrease;  Leaves;  Aerial organs;  Roots;  Stems;  Needles;  Suspension cell culture;  In vitro shoot cultures;  Fruits;  Bark

Correia et al. 2013). The mechanisms behind these adaptive responses remain to be unraveled, although it is known that both temperature and drought have significant repercussions on the epigenome, metabolism and growth of these trees (Ghouil et al. 2003; Ramírez-Valiente et al. 2010; Chaves et al. 2011; Correia et al. 2013). Salt stress studies carried out on leaves and roots reveal high tolerance to salinity, with the activation of specific genes and metabolic pathways related to antioxidant defense system (Dias et al. 2022; de Oliveira et al. 2022). Recently, Almeida et al. (2020) identified key compounds in the cork oak related to drought stress, such as amino acids, terpenes and specific phenolic compounds. However, we still know very little about the mechanism behind this plasticity and resistance in the response to these stresses.

*Pinus* species (*Pinus* spp.) are resinous perennial trees constituting the most extensive genus within the *Pinaceae* family (Nobis et al. 2012; Faria and Rodrigues 2021). Pine trees are an important source of primary resources for forestry sectors, such as timber and resin production. Additionally, they possess ornamental value and offer edible products like pine nuts, as well as serve as a source of essential oils. Beyond their economic significance, these arboreal species also exert a substantial influence on the regulation of Mediterranean forest ecosystems (Jaouadi et al. 2021). Following the cork oak species, *Pinus* ssp. represents one of the largest families of trees in Portuguese forests, which are mainly constituted by populations of *P. pinaster* Ait. and *P. pinea* L.. Both native Mediterranean populations assume an important economic role, namely to produce pine nuts for consumption (*P. pinea*), wood, and resin (*P. pinaster* and *P. pinea*) (Jaouadi et al. 2021).

The *P. pinaster*, known as maritime pine, is not only economically important in the timber industry, but is also widely used for medicinal and nutritional purposes (Mármol et al. 2019). In addition to its economic significance, maritime pine (*P. pinaster*) serves a crucial ecological function, mitigating erosion in sandy terrains and hilly landscapes, conserving soil integrity owing to its vigorous growth and extensive root system (Alonso-Esteban et al. 2022), facilitating reforestation of barren farmlands, and shielding agricultural crops against salt-laden winds by functioning as a coastal windbreak in the western coastal regions of the Iberian Peninsula (Abad Viñas et al. 2016; Kurtca and Tumen 2020). By retaining in their needles and accumulating atmospheric contaminants (e.g. fluoranthene, phenanthrene, pyrene and fluorene) in their bark, they can act as biomonitors (Ratola et al. 2009; Alonso-Esteban et al. 2022). The maritime pine, due to its resistance to low and moderate fire, is an excellent solution for reforesting burnt forest areas (Aguar et al. 2021; Molina et al. 2021). It is well adapted to Mediterranean climate, since it prefers warm temperate zones, and is highly resistant to frost (Ramos et al. 2022; Alonso-Esteban et al. 2022) and drought (Sánchez-Salguero et al. 2018; Alonso-Esteban et al. 2022). It thrives on siliceous soils characterized by coarse textures, notably including dunes, rotten substrates, and sandy soils (Abad Viñas et al. 2016).

Stone pine (*P. pinea*), a genuine forest species due to its absence of domestication as an orchard crop (Mutke and Gil 2005), presently stands as one of the foremost economically, socially, and ecologically significant trees within the Mediterranean forest ecosystem (Sáez-Laguna et al. 2014). Pine nuts are the most sought-after



resource from these trees due to their high nutritional value (Nergiz and Dönmez 2004) and phytochemical constitution (Loewe-Muñoz and Noel 2021). Like maritime pine, stone pine plays an important ecological role in dune stabilization, soil protection, as a place of refuge for biodiversity and carbon sequestration. The stone pine is well adapted to the Mediterranean climate, although it is dendroclimatologically sensitive (Akkemik 2000; Ramos et al. 2022). It is an isohydric species that avoids drought by significantly reducing its root mortality, photosynthesis, biomass allocation, and stomata control (Oliveras et al. 2003; Mayoral et al. 2015; Zalloni et al. 2016). Furthermore, this species exhibits a notable tolerance to salt stress, capable of enduring salt concentrations of up to 100 mM (Khaldi et al. 2011). Regarding soil characteristics, stone pine prefers siliceous and sandy soils (Ramos et al. 2022). Currently, a considerable number of studies on the phytochemical composition of different components of *P. pinaster* and *P. pinea* can be found in literature (Sousa et al. 2018; Faria and Rodrigues 2021; Alonso-Esteban et al. 2022). Nevertheless, based on our review of the existing literature, studies associating the response to abiotic stress with alterations in the phytochemical profile of this species are solely identified for *P. pinaster*. These investigations underscore the significant involvement of certain secondary metabolites (e.g.: flavonoids, alkaloids, lignans, and glycosyloxyflavones) (de Simón et al. 2017; López-Hidalgo et al. 2023).

The carob tree (*Ceratonia siliqua* L.) is a perennial evergreen species indigenous to the Mediterranean region, increasingly acknowledged for its significant ecological and socio-economic contributions. It is currently esteemed as one of the most valuable trees in fruit and forestry sectors across various industries (Damani et al. 2020; Zagoub et al. 2023). This species is highly resistant to drought (xerophytic), attributed to morphological and physiological adaptations including leaf area reduction, leaf curling, and enhanced root system development (Gadoum et al. 2019; Ozturk et al. 2021). It is also a very salinity-tolerant tree, withstanding up to 2 g/L of NaCl, which makes this species more resilient to salinity than pistachio, date palm and olive trees (Correia et al. 2010). Carob tree thrives in temperate and subtropical climates and exhibits tolerance to hot and humid conditions along coastal areas. It is considered a thermophilic and heliophile species but is highly susceptible to cold (Dahmani et al. 2023). In addition to these features, the carob tree is also used for soil restoration purposes, as it can fix atmospheric nitrogen and is ideal for growing in calcareous soils (Dahmani et al. 2023; Zagoub et al. 2023). Furthermore, it demonstrates resilience to impoverished soils, including sandy, clayey, and rocky substrates, within a pH range spanning from 6.2 to 8.6. However, it does not thrive in acidic and excessively humid soil conditions (Ait Chitt et al. 2007). The carob tree is also recognized as a multipurpose species, as all its components, including leaves, flowers, fruit, wood, and roots, can be utilized (Zagoub et al. 2023). Studies of the phytochemical composition of various components of the carob tree show that this tree possesses a wide range of compounds, some of which are recognized as having great medicinal and therapeutic value, such as coumaric and gallic acids, kaempferol and quercetin (Dahmani et al. 2023). Despite the extensive number of research on the chemical composition of various components of the carob tree, few studies have directly linked these compounds to the tree's ability to withstand abiotic

factors (e.g.: drought, salinity, and radiation) (El-Kahkahi et al. 2015; Gadoum et al. 2019; Costa-Pérez et al. 2023).

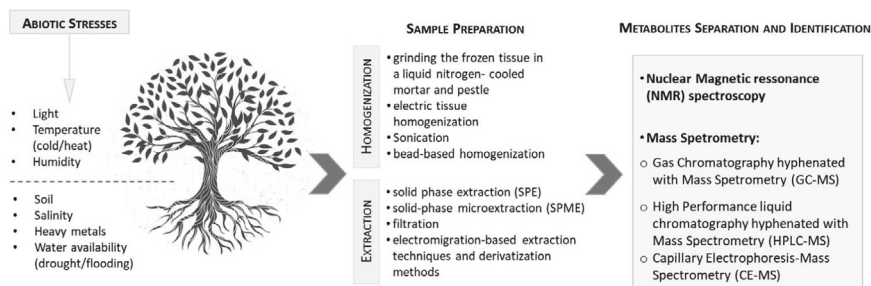
The huge coverage of the metabolome poses a considerable challenge owing to the diverse array of physicochemical properties exhibited by the molecules identified across various plant species. For the identification of metabolomic profile and quantification of target metabolites, different techniques could be selected. Spectroscopic (NMR) and chromatographic (LC, GC), and also the hyphenation of those techniques, and more recently, comprehensive analytical techniques (Patel et al. 2021, Ma et al. 2021). In addition to selecting the most suitable technique, the most critical step on this procedure involves sample preparation, that requires a previous optimization step considering the effect of some endogenous and exogenous factors. A revision on the methods commonly used for sample preparation and analysis are here presented previous to the description of the metabolites identified in each selected Mediterranean species upon the most serious abiotic stresses on this region.

## 11.2 Analytical Techniques Applied in Metabolomic Analysis of Plant Samples

### 11.2.1 *Methods Used on Sample Preparation*

Commonly, sample preparation is a key step in analytical methodologies since it provides a previous step of pre-concentration and clean-up of the compounds under study. In metabolomics, sample preparation should consider minimal procedures that encompasses remarkable advantages such as simplicity and speed, but also no selectivity, ensuring a deeper coverage of the studied metabolites. Among all the sample preparation methods (Fig. 11.1), the solvent extraction is the most used in plant tissues, providing a higher concentration of the metabolites in the extracts. However, some drawbacks could be ascribed, like the requirement of a step to physically break up the tissue, which is very time-consuming contributing to a less high throughput. In fact, in the traditional methods is greatly applied the disruption of tissues by means of grinding the frozen tissue in a liquid nitrogen-cooled mortar and pestle. It is labour-intensive and susceptible to cross contamination. More recently, improvements of these extraction techniques are being introduced, including electric tissue homogenization, sonication, or a bead-based homogenization (Wu et al. 2008; Vuckovic and Pawliszyn 2011).

Despite the huge applicability of minimal sample preparation, it lacks for the efficient removal of some matrix components, such as residual proteins and salts. It could compromise the detection and sensitivity of some chromatographic techniques (namely LC-ESI-MS) by competing with the target signal, owing to the ion suppression effect and the saturation of the detector, affecting column reproducibility and its lifetime. To overcome these limitations, more selective sample preparation methodologies have been developed, thus enabling cleaner extracts and



**Fig. 11.1** Schematic procedure followed for metabolome analysis including the most applied techniques or sample preparation and metabolites separation and identification

addressing some issues related to matrix effects, increasing column lifetime and overall method robustness (Vuckovic 2012). Moreover, in specific applications based on targeted metabolomics focused on low abundant metabolites, or even untargeted metabolomics-based approach that aims to detect trace amounts of contaminants in complex biological matrices, it is crucial to promote selective extractions. Indubitably, to improve the detection limits of trace metabolites, an effective clean-up procedure based on selective sample methodology is mandatory. Concretely, selective sample preparation methodologies encompass solid phase extraction (SPE) and solid-phase microextraction (SPME), filtration, electromigration-based extraction techniques, and derivatization methods (for non-volatile metabolites). SPE is one of the most used methodology due to its effectiveness on the removal of several components, like residual proteins and salts, and thus minimizing ion suppression effect. Within SPE, remarkable pre-concentration of the sample can be performed since there are available a huge diversity of sorbents showing different retention mechanisms. Then, the choice of the most adequate sorbent, attending to the metabolites in study for untargeted and targeted metabolomics, enables to reach high levels of selectivity. Therefore, improvements on reproducibility and lifetime of column are also achieved, which are relevant parameters for analytical applications (Vuckovic 2012). More recently, it has been shown that SPME could be a promising alternative to SPE. Moreover, SPME is directly compatible with LC-MS and GC-MS injections, which is highly advantageous (Bojko et al. 2014).

## 11.2.2 Techniques Used on Metabolites Separation and Identification

### 11.2.2.1 Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic resonance (NMR) spectroscopy is largely used on metabolomic applications due to its high reproducibility and cost efficiency. Nevertheless, it

requires that the compounds have a concentration at micromolar range, which is higher than the required for mass spectrometry (MS) techniques. Therefore, it is also used as complementary technique to GC-MS and LC-MS. In fact, the major limitation of NMR is its significantly lower sensitivity compared to MS (Markley et al. 2017), so it is not suitable for the analysis of a huge numbers of low-abundance metabolites. Several approaches have been explored to overcome this drawback, including the introduction of higher field magnets, cryogenically cooled probes, as well as other emerging techniques like high-temperature superconducting oils, microcoil-NMR probes, and hyperpolarization (David and Rostkowski 2020). NMR is a straightforward and largely automated, non-destructive technique. Despite the quick and easy analysis, in NMR could occur the signal overlapping in the complex mixture of plant extracts during metabolite profiling (Kumar et al. 2017).

### 11.2.2.2 Mass Spectrometry (MS)

Mass spectrometry plays a crucial role on separation sciences and therefore is a valuable tool in high-throughput metabolomics owing to its high sensitivity and wide range of covered metabolites, becoming the technique of choice in metabolomic studies, and is often coupled with chromatographic techniques. Indeed, recent developments and improvements in mass accuracy enable to extend the range of metabolites that can be analysed by MS, achieving an enhancement on the accuracy of compound's identification (Zhang et al. 2012; Patel et al. 2021).

#### Gas Chromatography Hyphenated with Mass Spectrometry (GC-MS)

GC-MS is a core technique as an analytical method for metabolomic studies, providing a platform for non-targeted efficient and reproducible analysis. Particularly, in GC-MS technique, the separation is reached by the use of a GC column, but the compounds under analysis must be volatiles. If not, a derivatization step is mandatory to produce volatile compounds, which are directly separated and quantified by GC-MS. There are two major forms of ionization used in GC-MS that comprises of electron ionization (EI) and chemical ionization (CI). However, most GC-MS methods in metabolomics utilize EI. More recently, the introduction of GC systems equipped with a time-of-flight mass spectrometry (TOF-MS) analyser is more suitable for metabolic profiling since it provides higher mass accuracy, faster acquisition times, and improved deconvolution for complex mixtures (Patel et al. 2021). Indeed, within a single run with minimum consumption of samples and reagents, an accurate peak identification is performed through the standard retention times and mass spectra (Zhang et al. 2012; Patel et al. 2021). Moreover, GC-MS system provides a huge number of well-established libraries, which is crucial for metabolites identification (Segers et al. 2019).

### High Performance Liquid Chromatography Hyphenated with Mass Spectrometry (HPLC-MS)

Like in GC-MS, the HPLC-MS hyphenated technique is commonly used for metabolite characterization in complex biological samples and also structural information in the field of metabolomics. Indeed, both techniques are often used on the characterization of unknown endogenous or exogenous metabolites. As in GC-MS, HPLC-MS comprises a first step of separation that is performed in the liquid column but, generally, without the need to derivatize compounds prior to the analysis. In fact, HPLC separations are better suited for the analysis of labile and nonvolatile polar and nonpolar compounds in their native form. To obtain a more detailed chemical composition of complex samples and the fully metabolites identification, are being employed soft ionization approaches in the MS mode, as atmospheric pressure ionization (API) and electron spray ionization (ESI) ionization tools, making MS more robust for a daily use. Thus, LC-MS measurement constitutes the basis for subsequent data processing and multivariate data analysis. Nowadays, LC-MS emerges as a powerful tool to metabolomic studies owing to its high sensitivity, robustness, and quantitative reproducibility. In fact, nontargeted metabolomics analysis based on LC-MS has been thoroughly tested, validated, and applied on the screening and identification of novel metabolic biomarkers, which is particularly relevant in several fields (Zhang et al. 2012; Segers et al. 2019; Patel et al. 2021).

### Capillary Electrophoresis-Mass Spectrometry (CE-MS)

CE-MS is another analytical technique mostly used on metabolomic studies for evaluating a diversity of ionic metabolites based on their charge and size ratio. Within small injection volumes, a fast and high resolution of charged compounds is obtained, assessing metabolites characterization based on mass fragmentation. Since this technique does not require derivatization, it is more straightforward, although presents a low sensitivity and reproducibility, poor migration and lack of libraries to help in metabolites identification. Despite all the constraints, CE-MS seems an appropriate platform for studying metabolites, in part due to the low volume of separation, which is particularly relevant for the study of plant metabolome (Patel et al. 2021).

## 11.3 Metabolomics Unrevealing Plant Phenotypes Upon Abiotic Stress Constrains

In the context of climate change, fluctuations in precipitation, resulting in either water flooding or reduced water availability (drought and salinity), along with extreme temperature events such as cold/freezing conditions and heatwaves associated with

varying radiation quality/intensity, contribute to diverse forms of abiotic stress. These stresses exert detrimental effects on global agriculture and food production. Investigating the metabolic responses induced by abiotic stresses in various crop species can enhance our understanding of plant resistance mechanisms. This knowledge is pivotal for the development of strategies aimed at mitigating stress, including the breeding of crops with enhanced resistance to abiotic stressors (Carrera et al. 2021). One approach to increase this knowledge is to elucidate the fundamental central metabolic pathways that potentially play a critical role in modulating plant growth, development, and responses to stress under adverse conditions. The activation of specific metabolic pathways channels precursors and intermediates towards the synthesis of bioactive molecules, antioxidants, signaling compounds, intermediates for cell structure biosynthesis, or storage compounds. Considering the metabolome as the equilibrium among defense, signaling, and damage, metabolites can be utilized to evaluate plant tolerance to specific stress conditions (Mata et al. 2016).

### **11.3.1 Drought**

Drought, as one of the most prevalent and severe abiotic stresses, significantly impacts arid and semi-arid Mediterranean ecosystems. It has a detrimental impact on various physiological and biochemical parameters, as well as on plant growth and development (Qiao et al. 2011). To survive water stress, plants employ a regulatory mechanism to maintain a balance between their physiology, morphology and metabolism. Plant reactions to water shortage stress manifest at various levels, ranging from individual leaves to the whole plant, requiring the creation of a new cellular equilibrium and leading to changes in both primary and secondary metabolic processes (Mata et al. 2016). This section offers an in-depth examination of how water deficits impact the metabolomic profiles of Mediterranean species, such as the olive tree, cork oak, pine tree, and carob tree. Metabolite plasticity plays a crucial role in understanding the specific mechanisms by which these species cope with drought stress.

#### **11.3.1.1 Olive**

Although the olive tree is well-adapted to drought conditions (Bacelar et al. 2007; Brito et al. 2018), significant metabolomic alterations are observed in its protective processes. Under drought stress, reactive oxygen species (ROS) levels rise in olive leaves due to excess accumulated energy, amplifying photo-oxidative effects. Other studies highlighted ROS as mediators of signal transduction in plant responses to water deficit stress (Venkateswarlu and Shanker 2012; Brito et al. 2019). The intensity of the stress is a crucial factor determining the physiological consequences for the plant and its ability to recover. Drought-tolerant olive cultivars, when subjected to mild and moderate water deficits, increase the concentration of enzymatic antioxidants such as ascorbate peroxidase, catalase, superoxide dismutase, and glutathione

reductase, as well as non-enzymatic antioxidants including phenolic compounds, tocopherols, carotenoids, ascorbate, and glutathione. However, severe drought stress disrupts the balance between ROS production and the antioxidant defense system, causing damage to lipids, proteins, carbohydrates, and pigments. Also, biosynthesis and accumulation of abscisic acid (ABA), considered the main stress hormone, is stimulated by drought (Brito et al. 2019). Another indicator of oxidative stress in drought-affected olive trees is the reduction of chlorophylls and carotenoids in leaves, suggesting that drought conditions promote the degradation of these pigments due to oxidative damage, while increasing levels of anthocyanins and proline (Silva et al. 2018).

In a recent study, Dias et al. (2021) characterized the phenolic and lipophilic metabolite profiles of olive plants subjected to a 30-day water deficit (WD) and subsequent recovery. They identified and quantified 11 phenolic compounds, with flavonoids and secoiridoids being particularly notable. Regardless of the treatment, olive leaves are rich in a specific type of flavonoids, the o-dihydroxy B-ring flavonoids, characterized by having a great capacity to neutralize ROS, potentially contributing to the high stress tolerance of this species (Dias et al. 2020). Secoiridoids, namely the most abundant component oleuropein, also significantly decrease its accumulation in case of water deficit. Regarding lipophilic compounds, 22 compounds were identified, including fatty acids, sterols, long-chain alkanes, terpenes, and carbohydrates. Notably, long-chain alkanes, the main components of olive leaf cuticular wax (Mihailova et al. 2015), showed a significant reduction in leaves after water stress, which could affect water loss control (Dias et al. 2018). This finding aligns with reduced water status, photosynthetic efficiency, and increased lipid peroxidation observed in young olive plants under water deficit in a previous study (Silva et al. 2018). Martinelli et al. (2011) reported increased expression of two oxidosqualene cyclase genes, responsible for specific steps in the triterpenoid biosynthetic pathway, in olive fruit from water-stressed trees. Regarding the recovery period, Dias et al. (2021) highlighted the relevance of lipophilic compounds, observing an increase in sterol levels. The catabolism of fatty acids and carbohydrates likely provides the necessary energy for reestablishing plant performance, and the metabolic pathways of sterols, long-chain alkanes, and terpenes may shift to produce compounds with more significant stress protection roles. According to Karimi et al. (2018), carbohydrates also play a role in osmotic adjustment in *O. europaea* under water deficit conditions, with their concentrations increasing. This study also indicated that higher drought tolerance in olive trees was associated with higher proline concentrations in leaves.

### 11.3.1.2 Cork Oak

The *Q. suber* is a xerophytic species, appearing as high resistant upon drought stress (Ramírez-Valiente et al. 2014). Understanding the adaptive strategies of this species under limited water availability is crucial. Due to the prolonged life cycles of trees and limited data availability (Neale and Kremer 2011; Rodrigues et al. 2021),

metabolomic research in forest trees is inherently time-consuming. Almeida et al. (2020) conducted an untargeted metabolomic analysis to identify potential metabolic biomarkers and pathways associated with drought tolerance in *Q. suber*, both in short- and long-term scenarios. During the initial phase of water stress, pathways involved in flavone and flavonol biosynthesis, the pentose phosphate pathway, ABC transporters, and alanine, aspartate, and glutamate metabolism were notably active, but these activities decreased significantly one month after water stress. Also, flavonoids such as epigallocatechin 3-gallate and 3,5-digalloylquercetin were detected only during short-term drought conditions, indicating their role in early defense mechanisms and reactive oxygen species detoxification. In contrast, flavonoid glycosides were associated with long-term water stress, contributing to plant osmoregulation. Additionally, the compounds leonuridine and tarennoside, which are terpenes, were newly linked to long-term drought stress responses (Almeida et al. 2020).

Primary and secondary metabolism are critical in *Quercus suber*'s drought responses. Similar to other species (Hamanishi et al. 2015; Gagné-Bourque et al. 2016; Todaka et al. 2017), amino acids L-isoleucine and L-phenylalanine accumulated significantly during short-term stress, whereas acetyl amino acids such as tryptophan and L-aspartic acid were prominent in long-term stress responses (Almeida et al. 2020). The accumulation of amino acids under different drought conditions is linked to their synthesis, activation/inhibition, and the degradation or decomposition of proteins (Di Martino et al. 2003; Ford et al. 2011). Proline, an important osmoprotectant, is specifically involved in drought tolerance under severe stress conditions (Kwak et al. 2011; Hu et al. 2013). Equally important as studying adaptive strategies is understanding the recovery mechanisms post-drought. Recovery capacity is a critical trait in drought response and determines the distinction between susceptible and resistant phenotypes (Chaves et al. 2009). The flavone and flavonol biosynthesis pathway was active during recovery from water stress, responsible for producing flavones that act as antioxidants under stress (Nishiyama et al. 1994). Isoscoparine, a flavonoid in the flavone subclass, appears to play a significant role in the recovery process (Almeida et al. 2020).

### 11.3.1.3 Maritime Pine and Stone Pine

Despite the ecological and economic importance of both *P. pinea* and *P. pinaster*, most studies about metabolomic alterations related to water stress have focused in *P. pinaster*. The metabolic comparison between these two species when subjected to drought was performed by Perdiguero et al. (2013), a study in which the expression pattern of 1124 genes, presumed to be involved in the response to severe and prolonged water stress, was analyzed. They found a significant induction of genes related to carbohydrate metabolism and secondary metabolism, including those involved in the synthesis of hormones such as ethylene, 1-aminocyclopropane-1-carboxylate synthase, and jasmonate, as well as genes related to the transport of



sugars, anions, and amino acids. Although both species exhibited gene induction, *P. pinea* demonstrated a more rapid and intense transcriptional response to drought compared to *P. pinaster*.

Regarding metabolomic alterations in *P. pinaster* under drought stress conditions, a set of induced key metabolites that distinguished water-stressed samples from well-watered ones included increased levels of chiro-inositol and pinitol, and decreased levels of sequoyitol in roots, stems, and needles (both adult and juvenile) (de Miguel et al. 2016). These cyclitols serve crucial cellular functions as osmoregulators and oxygen radical scavengers. Sugars such as sucrose, fructose, glucose, raffinose, ribose, erythronate, and xylonate, as well as pyruvate, also increased in response to drought, predominantly in roots (Warren et al. 2011). Additionally, amino acids from the aspartate and glutamate families increased in both aerial organs and roots, likely due to protein degradation under stress conditions (Araújo et al. 2011).

Metabolites response to water stress depend on the organ analyzed. De Miguel et al. (2016) demonstrated that in *P. pinaster*, while a high number of flavonoids were detected in aerial organs, the glutathione pathway was primarily induced in roots. This suggests that different antioxidant mechanisms are activated in roots and aerial organs. There are some metabolites that increase in roots compared to aerial organs, like sugars (such as rhamnose, arabinose, or related polyols as erythritol and arabitol) and aromatic amino acids or related acids (as quinate and shikimate and aspartate and glutamate family). These findings highlight the role of roots as early sensors of water stress, with stems and needles showing fewer metabolic changes in response to drought.

Free sterols, which are components of the membrane lipid bilayer, de Miguel et al. (2016) showed a significant increase in roots and stems of *P. pinaster*. This increase promotes the reduction of water loss in the cell and could be a mechanism that decreases the rate of water permeation (Navari-Izzo et al. 1993).

As with the other species here presented, there are key secondary metabolites associated with drought tolerance in *P. pinaster*, playing a protective role in the adaptation and survival of this species. Pinitol is crucial for osmoregulation under drought conditions, with increased levels observed in *P. pinaster* needles during drought (de Miguel et al. 2016). In *P. pinaster* needles, this osmotically active substance increases under water stress conditions (de Simón et al. 2017). Besides pinitol, also amine and amino acid levels increased in needles and leaves in drought conditions (Du et al. 2016; Aranda et al. 2017; de Simón et al. 2017), serving as osmotically active metabolites involved in reactive oxygen species (ROS) scavenging, membrane protection, and providing respiratory substrates during stress (Araújo et al. 2011; Selmar and Kleinwächter 2013). Glycolysis-TCA cycle and the metabolites involved (such as branched-chain amino acids) showed increased activity in response to water deficit, indicating a drought-influenced metabolic rate.

In response to water stress, *P. pinaster* needles activate ROS scavenging mechanisms to mitigate elevated ROS levels, leading to the overproduction of antioxidant compounds and up-regulation of ascorbate, shikimate/phenylalanine, and phenylpropanoid metabolic pathways, as well as the catabolism or mobilization of diterpenoid acids to other tissues (de Simón et al. 2017).

Needle ontogeny significantly influences the variation in polyphenol concentrations under conditions of water scarcity. Flavonoid polyphenols, with many physiological roles in plants such as antioxidant activity, increased their synthesis and accumulation in response to water deficit. A considerable increase in neutral diterpenes due to drought stress was observed in juvenile needles, while mono- and sesquiterpenes predominantly accumulated in adult needles (de Simón et al. 2017).

#### 11.3.1.4 Carob Tree

The carob tree (*Ceratonia siliqua* L.) is an agro-sylvo-pastoral species of significant environmental and socio-economic interest (Gharnit et al. 2001). Carob products have gained recognition in the human food industry, particularly in the Mediterranean regions, due to their abundant bioactive compounds and their potential to serve as a substitute for cocoa. Concentrated primarily in coastal, semi-arid, and arid zones, the carob tree is drought-resistant and exhibits morpho-physiological traits and genetic adaptations to climatic variations (Ait Chitt et al. 2007), serving as a valuable tool for comprehending acclimation mechanisms and showing great potential for implementing conservation approaches in drought-prone forest ecosystems. Similarly to other species discussed here, Gadoum et al. (2019) showed an increase in proline and soluble sugars in the leaves of distinct carob tree ecotypes under water deficit conditions. The involvement of these compounds in osmotic adjustment helps maintain cell turgor at low water potentials, preserving photosynthetic activity and protecting cellular membranes and proteins during water deficit (Lawlor and Cornic 2002; Ashraf and Harris 2004; Farooq et al. 2008). Also, a notable decrease in the concentration of chlorophyll and carotenoids in *C. siliqua* leaves was observed (Gadoum et al. 2019). Carobs can employ other strategies to cope with water stress, such as associating with arbuscular mycorrhizal fungi (AMF). In a recent study, Boutasknit et al. (2020) demonstrate that AMF can enhance drought resistance in carob trees at an early stage by increasing organic solutes (soluble sugars and protein content) and enhancing defense responses against oxidative damage in leaf tissues during prolonged water stress and recovery conditions.

#### 11.3.2 Salinity

Salinity is a major abiotic stress characterized by the accumulation of salts, primarily sodium (Na<sup>+</sup>) and chloride (Cl<sup>-</sup>) ions, in the rhizosphere [reviewed by (Chele et al. 2021)]. This accumulation of salts comes from agricultural malpractices, improper irrigation techniques, and pollution that, combined with the effects of climate change, lead to increased soil salt concentrations due to evaporation (Chele et al. 2021). The adverse effects of salinity are first perceived by the root system beginning with osmotic or water stress, which reduces the root's ability to absorb water, followed by ionic toxicity caused by nutritional imbalances in the cytosol, hormonal imbalances,

and increased susceptibility to pathogen infection [reviewed by (Singh et al. 2022)]. Plants employ various physiological and biochemical mechanisms to adapt to salinity, including changes in morphology, anatomy, and biochemical pathways, depending on factors such as species, plant age and size, and the duration and intensity of stress exposure (Acosta-Motos et al. 2017).

Metabolomics, as a technique to understand the complex metabolite changes underlying plant responses to salinity, can provide significant strategies for optimizing crop productivity and quality. Several metabolomic studies on traditional Mediterranean plants species showed changes in primary and secondary metabolite profiles in response to salinity stress, with sugars and osmolytes the most altered primary metabolites in osmotic-stressed plants (Conde et al. 2011; Ben Abdallah et al. 2018). Additionally, a perturbation of secondary metabolites (like phenolic compounds and oleuropein) has also been detected in response to salinity and drought stresses (Conde et al. 2011). These metabolite changes will be described in detail in this section.

### 11.3.2.1 Olive

Olive trees are considered moderately tolerant to salinity, with responses that are genotype-dependent and influenced by the maturity level of the tree (Chartzoulakis 2005). However, there is limited information available on the changes in both primary and secondary metabolism in olive tissues subjected to salt exposure. Abdallah and co-workers documented the accumulation of specific primary metabolites, termed osmolytes (including proline, glycine betaine, trehalose, sucrose, and myo-inositol), in the leaves of olive trees exposed to elevated salinity conditions (Ben Abdallah et al. 2018), while Conde and colleagues demonstrated that sugars, such fructose, glucose, and mannitol constitute the primary components of olive tissues, supplying carbon and energy under salt stress conditions (Conde et al. 2011). In a recent investigation, a notable array of metabolites from primary metabolism exhibited alterations in NaCl-treated olive trees in a tissue-specific manner: the levels of various sugars (as glucose, sucrose, lactose, cellobiose and melibiose) were reduced in roots and increased in leaves exposed to NaCl. This difference in sugar levels between leaves and roots leads to the hypothesis that NaCl in olive roots promotes the transport of sugars from roots to leaves to ensure sufficient soluble sugar content in the stressed leaves (Skodra et al. 2021).

Mannitol, the predominant carbohydrate in olive tree leaves and an important sugar in the olive fruit pulp, functions as an osmoprotectant of olive tissues during salinity stress (Tattini et al. 1993; Conde et al. 2007). In a recent study, Skodra and colleagues observed the accumulation of mannitol in both roots and leaves in response to salinity (Skodra et al. 2021).

Regarding secondary metabolites, phenolic compounds, as simple phenols, secoiriods, and flavonoids, were extremely affected in roots and leaves after NaCl application. In general, these compounds decreased in roots and increased in leaves when

olive tissues were treated with NaCl. The decrease in several organic acids associated with the tricarboxylic acid (TCA) cycle, such as succinic, malic, and citric acid, across different olive tissues, may represent an energy conservation strategy in olive plants, indicative of a transition from growth-oriented processes to protective mechanisms (Skodra et al. 2021).

In olive trees, salinity promotes alterations in oleuropein metabolism, one of the main secondary metabolites in olive leaves (Ben Mohamed et al. 2018). This is reflected in Skodra and co-workers study by the accumulation of hydroxyl-decarboxylmethyl eleuropein aglycone and the increased expression of  $\beta$ -glucosyltransferase (GTF), which converts oleuropein aglycone into oleuropein, particularly in new leaves (Skodra et al. 2021).

### 11.3.2.2 Cork Oak

Despite the economic importance of this species, there remains limited understanding regarding its phenotypic plasticity and resilience in the face of escalating soil salinity. In a recent study, de Oliveira et al. (2022) investigated the effects of acute salinity episodes on photosystem II (PSII) functionality and oxidative status in *Q. suber* leaves. The authors observed that salinity did not lead to an accumulation of proline, which can be justified by the short trial duration and, similarly to drought stress in this species. Longer exposure periods to salinity stress, such as up to one month, merit exploration. Soluble sugars, namely glucose and sucrose, increased during short salinity stress suggesting roles in osmotic adjustment, regulation of reactive oxygen species (ROS) signaling, and protection of ROS-producing and scavenging pathways in *Q. suber*. Chlorophyll and carotenoid levels rose 24 h post-salinity stress, indicating the species' adaptive capacity to high osmotic and ionic stress, and providing photoprotection (Peguero-Pina et al. 2009). However, a decline in these pigments was noted 6 days after salinity stress, suggesting a differential response over time. Antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), constitute crucial defense mechanisms against ROS overproduction in various plant species under salt stress (Ma et al. 2020). In *Q. suber*, 24 h and 6 days after saline stress, the total enzyme activities of CAT, SOD, and APX increased, acting in the control of H<sub>2</sub>O<sub>2</sub> levels in *Q. suber* leaves (de Oliveira et al. 2022).

So far, data in the literature can help in the understanding of the response to the increasing soil salinization; however, future investigations in *Q. suber* subjected to prolonged salinity stress are needed and should also consider a longer exposure to stress.

### 11.3.2.3 Maritime Pine and Stone Pine

*Pinus pinaster*, as a key species for reforestation efforts, has been cultivated in dunes of coastal areas, being exposed to salt spray (Azevedo et al. 2009a). As a consequence, this specie has a high tolerance to saline environments, a strong indication of its adaptation to saline environments. Despite its ecological relevance, there are few studies about the molecular mechanisms underlying saline stress tolerance in this species. Indeed, responses to saline stress were analyzed in suspension cell culture subject to different NaCl concentrations, where the results confirm the activation of ROS-scavenging mechanisms, with an increase in ROS levels and lipid peroxidation (Azevedo et al. 2009b), which is regarded as an indicator of substantial oxidative stress and arises as a result of reactive oxygen species (ROS) production (Petersen 1999). In another species, elevated lipid peroxide levels were linked to sensitivity to salt stress (Masood et al. 2006; Koca et al. 2007). Superoxide dismutase (SOD) enzymatic activity is hypothesized to safeguard cells through the scavenging of  $O_2^{\cdot-}$ , thereby mitigating the production of hydroxyl radicals ( $HO^{\cdot}$ ) and their associated oxidative damage (Noctor and Foyer 1998). In *P. pinaster*, SOD activity increased after 12 h of saline stress exposure (Azevedo et al. 2009b). Enhanced superoxide dismutase (SOD) activity has been regularly documented in various species in response to salt stress (Masood et al. 2006; Koca et al. 2007).

The use of in vitro suspension cultures may contribute to a deeper understanding of the molecular aspects of saline stress resistance. Nevertheless, further investigations are required at the plant level to elucidate the correlation between salt stress, reactive oxygen species (ROS) homeostasis, and the impact of salt exposure on the photosynthetic apparatus.

### 11.3.2.4 Carob Tree

Carob seems to develop successfully in saline soils. Most studies about the effect of salt stress in carob are related to water-use strategies and morphological and nutrient changes (Correia et al. 2010; Cavallaro et al. 2016; Damani et al. 2020). Regarding metabolite levels, El-Kahkahi et al. (2015) demonstrated that chlorophyll content was significantly reduced, and soluble sugars and proline are accumulated in leaves under salt stress of seven populations of carob.

## 11.3.3 Heat Stress and Radiation (Ultraviolet and Infrared Radiation)

Climate change is altering weather patterns, leading to changes in light and temperatures regimes while also causing an increase in drought prevalence (Blunden and Boyer 2021). For terrestrial plants, light is not only an important source of energy,

but it is also an important medium for transferring information from the environment to plants. The solar spectrum is the main source of radiation that reaches the earth's surface, and the three relevant bands along this spectrum are ultraviolet (280–400 nm), visible light (400–700 nm), and infrared wavelengths (700–2500 nm). Of the total solar radiation that reaches earth's surface, approximately 92% is composed of infrared radiation and visible light, while ultraviolet radiation accounts only 8%. The ultraviolet radiation (UV radiation), invisible to human eye but felt as heat, can be further divided in UV-A (320–400 nm), UV-B (280–320 nm), and UV C (100–280 nm). UV C radiation is almost absorbed by the earth atmosphere and the remaining UV-A and UV-B reach the surface, being the wavelengths below 290 nm almost undetectable at earth's surface (Schäfer and Nagy 2006). In natural conditions, when plants receive more solar radiation, the temperature on the plant's surface tends to increase. This makes it difficult to distinguish the effects of UV radiation from the effects of temperature in plants. In this context, the effects of temperature and UV radiation on plants are often examined together in the bibliography.

All the wavelengths from the electromagnetic spectrum that reach the earth surface have an impact on the growth and crop production. Plants present sophisticated mechanisms to detect and interpret information from surrounding environment, including variations in the electromagnetic spectra. By detecting the variation in light environment, plants can optimize their growth and development, either through an effect on photosynthesis or by influencing photomorphogenesis. In the Mediterranean region, there have been several significant extreme warm episodes in the last decade with high irradiance (about 3000 sunshine hours per year) and high temperature (Blunden and Boyer 2021).

Despite UV radiation being a damaging inducing source of stress in plants, it can trigger various metabolic pathways that can positively influence crop productivity (Wargent and Jordan 2013; Mansour et al. 2022). In plants, UV-A and UV-B photoreceptors (UVR 8) activate numerous transcriptional changes with a crucial role in activating various protective mechanisms (Rai et al. 2021). The synthesis of metabolites with UV radiation protection activity, the biosynthesis of metabolites with antioxidant properties, and the modification of plant cuticular waxes composition to enhance reflectivity in plant's surface, are the major plant's adaptive mechanisms to cope with high UV exposure. In this review we will predominantly focus on the impact of UV and red and far-red radiation on plants metabolites profile.

### 11.3.3.1 Olive

In olive fruits, phenols contribute to the organoleptic characteristics, flavour, and colour of olive oil and, like in vines, these compounds are associated with positive health benefits. During olive ripening many UV absorbing compounds, such as phenols, decrease, and olive oil loses its bitterness and pungent taste, leading to a mild flavour profile (Gambacorta et al. 2010). Under natural conditions, the presence of phenolic compounds in olive pulp plays a significant role in enhancing the oxidative stability of olive oil. These compounds serve as potent protectors preventing

the olive oil from oxidative damage induced by external factors such as radiation and temperature (Araújo et al. 2021). Also, olive leaves are an abundant source of phenol compounds, being often used to obtain high added value compounds to be incorporated into dietary supplements and functional foods (Şahin and Bilgin 2018). Although olive trees are among the best-adapted species in the Mediterranean region, the impact of UV radiation in olive trees has been also pointed as a dangerous source of stress to olive fruits (Piccini et al. 2020, 2021). The content of phenolic compounds in olive leaves has been related to adaptive mechanisms to face climatic conditions, particularly radiation and temperature (Lorini et al. 2021). Heat shock (40 °C for 2 h) reduced the levels of verbascoside, oleuropein and luteolin-7-*O*-glucoside in olive leaves, while the increase in UVB radiation enhanced the production of verbascoside, oleuropein, and lutein-7-glucoside, as well as flavonoids and hydroxycinnamic acid derivatives in the fruits (Dias et al. 2018, 2020). Phenols in plant tissues may condense into more complex molecules such as tannins, lignin precursors, and flavonoids, which could also act as antioxidants. In olive leaves, tannins have been reported to function as a non-enzymatic antioxidant defence mechanism effectively protecting the fruits from oxidative damage caused by UVB radiation (Dehghanian et al. 2022).

Monoterpene, secoiridoid, and flavonoid levels are highly influenced by UV-B and heat shock, whereas sterols, triterpenes, and fatty acids are less responsive. UV-B specifically stimulated oleuropein levels, suggesting it may act as a protective antioxidant under these conditions (Dias et al. 2018). More recently, Dias et al. (2020) demonstrated that heat stress (40 °C) or UV-B shock induced changes in the physiological status of olive and promoted some metabolite adjustments. Both treatments led to a reduction in proline content, chlorophyll, and carotenoid levels. Other metabolites, such as anthocyanins and squalene, responded exclusively to heat stress or UV-B stress, respectively, aiding in the control of oxidative damage. Apart from these groups of metabolites, also lipophilic compounds connected to cuticle wax and those associated with membrane integrity maintenance were discovered to play an important role in olives adaptation to high radiation (Dias et al. 2018).

### 11.3.3.2 Cork Oak

Despite being a xerophytic species, *Q. suber* exhibited reduced sugar accumulation in its roots when exposed to a combination of UV radiation and water stress, indicating decreased plant UV sensitivity under low water availability conditions. In this chapter, despite the focus on the metabolomic changes that occur in the characteristic species of the Mediterranean, the majority of studies in the literature regarding the effects of high temperatures and radiation, encompass a wide variety of cork oak species. Indeed, another species exhibited an accumulation of phenolic compounds after the plants had been exposed to artificial UVB supplementation (Antonelli et al. 1998) and accumulation of condensed and hydrolysable tannins produced at high temperature (Top et al. 2017).

### 11.3.3.3 Maritime Pine and Stone Pine

Maritime pine is one of the most important forest species in the Mediterranean basin and often endures hotter droughts, meaning it may withstand particularly severe conditions (de Miguel et al. 2011). Although several studies have examined the effects of temperature on *Pinus* spp., revealing that the metabolome is characterized by an increase in cytokinins, flavone, flavonol, and diterpenoid biosynthesis metabolites (Escandón et al. 2017, 2018), there are few studies on *Pinus* species with agronomic interest in the Mediterranean region. Recently, Lopez-Hidalgo et al. (2023) evaluated *P. pinaster* metabolome in response to combined drought and heat stress. Needles from plantlets subjected to the highest temperature (40 °C) and drought conditions displayed increased accumulation of flavonoids, flavones (such as tribuloside), and isoflavonoids (like dolineone), along with elevated levels of terpenoids and polyketides, which assist the tree in coping with environmental stress.

The low winter temperatures and the snow cover, make the north coniferous forests more susceptible to UV radiation than the Portuguese pine forest (Laakso and Huttunen 1998). Beneficial effects of UVC radiation were referred by Reglinski et al. (2013) who found that the irradiation of *Pinus* spp. seedlings with UVC resulted in an enzyme-oxidative response, with an increase of peroxidase (POX) and polyphenol oxidase (PPO) activity, and consequently a higher resistance to drought stress. More recently, Pascual et al. (2017) observed a progressive accumulation of phenols and carotenoids in the needles under moderate UVB dosage, and a decrease in carotenoid with long UV stress exposure. Also, the synthesis of heat shock proteins and enzymes like Malate-dehydrogenase, fructose-biphosphate and Triose-phosphate-isomerase have been associated to UV stress exposure.

Understanding the UV-stress adaptive mechanisms in plants as well as the molecules involved in such mechanisms will provide valuable data to be integrated into breeding programmes aiming to select UV tolerant genotypes.

### 11.3.3.4 Carob Tree

Carob tree has shown an increase in phenols and tannins after being supplemented with UVB radiation. In addition, plants with a higher concentration of tannins, exhibited a protective anti-herbivorous mechanism, strengthening the view that this is a quite tolerant and well adapted specie to Mediterranean climate conditions (Grammatikopoulos 1999). The levels of antioxidant compounds, chlorophylls, carotenoids, and sugars, have been also increased in carob explant under UVC radiation during in vitro culture (Costa-Pérez et al. 2023). According to these authors, enhancing the carob antioxidant system by increasing the accumulation of secondary metabolites will improve the micropropagation processes of carob.



### 11.3.4 Cold stress

Cold stress in plants represents a substantial environmental challenge, and it manifests in two main categories: “chilling stress”, that is characterized by low temperatures typically ranging from 0 to 15 °C, and “freezing stress”, which is the result of temperatures dropping below the freezing point (0 °C). While both forms of cold stress can significantly impact plant well-being, chilling stress is generally considered less detrimental to plant health than freezing stress. These stressors impact plant physiology, metabolism, growth, and development in various direct and indirect ways, ultimately resulting in a decrease of crop productivity (Petruccelli et al. 2022).

#### 11.3.4.1 Olive

The olive tree, a subtropical species, is highly sensitive to cold. When air temperature, the main environmental factor restricting olive tree growth, falls below the optimal range (20–30 °C), a rapid reduction in productivity is observed. Chilling temperatures can slow down essential metabolic processes, disrupting respiration and photosynthesis, altering protein production, and modifying membranes, leading to shifts in enzyme activity, decreased permeability of membranes, leakage of solutes, and dehydration of cells. Despite these changes, olive trees demonstrate remarkable resilience to chilling temperatures, with no irreversible harm to plant development. Contrastingly, freezing temperatures can severely impair metabolic efficiency and physiological processes, and cause irremediable injuries to organs and tissues, culminating in the plant’s death. Ice crystal formation within the plant’s tissues results in a decrease of apoplastic water potential, drawing water from the plant’s interior, culminating in cellular dehydration. The degree of frost damage in olive trees is influenced by multiple factors, such as the season of the low temperatures, plant’s growth stage, plant age (younger plants show higher vulnerability), the hardening/dehardening process, or the varying endurance of different plant parts (Petruccelli et al. 2022).

By investigating how cellular components and metabolite pools change in different olive cultivars, researchers can understand how these trees respond to cold stress and classify the cultivars based on their tolerance levels (Petruccelli et al. 2022). Several authors have highlighted adjustments in primary and secondary metabolic mechanisms of olive trees exposed to cold stress.

Studies have reported an increase in soluble carbohydrates during cold acclimation and emphasised their role as osmolytes to protect cells against freezing injury (Ortega-García and Peragón 2009; Saadati et al. 2019, 2021a, b; Petruccelli et al. 2022; Jiang et al. 2023). The accumulation of carbohydrates raises the cryostability of cell membranes which is an important requirement for cold tolerance (Saadati et al. 2019, 2021a). Gulen et al. (2009) presented a cultivar-dependent response to cold: sucrose content, total soluble sugars, and reducing sugars of olive leaves exhibited

varying increases according to the cultivar's cold hardiness (tolerant cultivars showed a significant increase, whereas cold acclimated sensitive ones remained unchanged or showed a slight increase in comparison to their non-acclimated counterparts). Thus, the capability of different cultivars to accumulate carbohydrates under cold stress is, at least, partially responsible for their different cold tolerances (Saadati et al. 2019). Rejšková et al. (2007) reported that the content of total endogenous carbohydrates increased in shoot segments exposed to chilling temperatures. Mannitol and raffinose family oligosaccharides (RFO) proportions increased, whereas the proportion of sucrose remained changed. These authors believe that this increase of RFO prevented the crystallization of sucrose, contributing substantially to its protective potential, therefore fulfilling some of the RFO functions under stress.

Another primary metabolic pathway also associated with cold response in olive is fatty acid metabolism. Changes in unsaturated fatty acid and lipid-protein composition have been identified as a mechanism to protect membrane fluidity against cold stress. It has been observed that greater amounts of unsaturated lipids lead to higher tissue tolerance to cold stress (Saadati et al. 2021a; Petruccioli et al. 2022). Matteucci et al. (2011), through NMR analysis of epi-mesocarp extracts, confirmed that, in drupes under cold conditions, the changes in unsaturated fatty acids are genotype-dependent. These authors reported increases of unsaturated fatty acids in a cold-sensitive genotype, while the cold-tolerant cultivar showed no significant changes in amount or composition of fatty acids upon cold stress. This higher production of unsaturated fatty acids was suggested to be a strategy of homeoviscous adaptation involving storage lipids, directly tied to the genotype's susceptibility to cold. According to Jiang et al. (2023), cold hardiness in olive relies more on the reserves of fatty acid than on their transformation. It appears that cold-tolerant genotypes do not require further homeoviscous adaptation, and that is why they show no notable changes (Matteucci et al. 2011).

Jiang et al. (2023) also described alterations in amino acid biosynthesis in olive leaves. Their non-targeted metabolome analysis by LC-MS/MS showed that cold stress restricted the accumulation of L-glutamic and N-acetyl-DL-glutamic acids, and, in the more cold-resistant cultivar, reduced the content of L-arginine as well. Furthermore, this study depicted a decline in reduced glutathione levels along with a rise of oxidized glutathione in the cold-sensitive cultivar, while the cold-resistant cultivar maintained their equilibrium, leading these authors to state that and the balance between various forms of glutathione contributes to cold tolerance. Numerous studies have also revealed increases in proline contents after cold exposure. Research seems to indicate that cold-tolerant cultivars accumulate higher amounts of free proline than cold-sensitive cultivars (Saadati et al. 2019).

Cold stress has an impact on olive trees' secondary metabolism as well. Plants appear to gain cold hardiness by producing secondary metabolites during cold acclimation. These metabolites are critical in activating a defence response to low temperature stress. Studies have shown that the total phenolic content in cold-acclimated olive trees have increased (Cansev et al. 2012; Saadati et al. 2021a, b; Petruccioli et al. 2022). Ortega-García and Peragón (2009) pointed out oleuropein as the primary phenolic compound detected in their HPLC analysis of olive leaves extracts. In

this study, moderate and heavy cold stress resulted in higher levels of oleuropein. This compound is involved in cold stress response as part of an antioxidant protection mechanism against oxidative and dehydration induced injuries. An abundant oleuropein accumulation is believed to promote cold tolerance (Ortega-García and Peragón 2009; Petruccioli et al. 2022; Jiang et al. 2023). Malondialdehyde (MDA) is also a secondary metabolite that has its content increased in olive plants under cold stress, not because of any protective behaviour, but as a result of lipid peroxidation due to ROS induced damage. Cold-sensitive genotypes tend to have higher concentrations of MDA. Therefore, in this case, the maintenance of low levels of MDA accumulation is thought to contribute positively to the improvement of cold resistance of olive cultivars (Wang et al. 2018; Saadati et al. 2019, 2021a, b; Petruccioli et al. 2022; Jiang et al. 2023).

#### 11.3.4.2 Cok Oak

A study on *Q. suber* monitored the seasonal changes in the concentrations of the main soluble organic compounds by <sup>13</sup>C-NMR analysis of leaf extracts (Passarinho et al. 2006). The results demonstrated a strong correlation between leaf composition and climate. During the autumn–winter season, when the temperatures are low, the levels of fructose, glucose, and sucrose increased. The concentrations of malic and citric acid also increased slightly over this season. Sugars and organic acids are generally stored in plant tissues to serve as solutes for osmotic adjustment and maintaining cation balance. In this analysis, quercitol was identified as the second most common organic metabolite, following quinic acid, whose concentration pattern strongly aligned with variations in air temperature and sunlight exposure. This compound decreased in concentration with the drop of temperature, while quercitol had its concentration sharply increased. Since quercitol is known to have a cryoprotective action on cell membranes, the authors propose that this increase of organic metabolites, particularly quercitol, could provide protection to *Q. suber* leaves against cold stress.

#### 11.3.4.3 Maritime Pine and Stone Pine

As with the other species previously mentioned, *Pinus* species also exhibited alterations in metabolite profiles in reaction to cold conditions. The NMR metabolite profiles of *P. pinaster* needles (Cañas et al. 2015) indicated a pattern of accumulation of metabolites such as sucrose, with possible impact in acclimation to winter conditions and response to environmental cues.

In another *Pinus* species with no agronomic interest in the Mediterranean region, an increase of soluble sugars, proline content, MDA (Lu et al. 2022) and total lipids, fatty acids, phospholipids, and unsaturated polymethylene-interrupted fatty acids ( $\Delta^5$ -UPIFA) (Makarenko et al. 2014; Nokhsorov et al. 2019) was reported in needles

of pines exposed to low-temperature stress. Increasing the concentration of intracellular solutes is a way for plants to maintain the water balance, reducing the water potential and improving the cell's capacity to absorb water. Therefore, this raise in osmoregulatory substances suggests an attempt to adjust the permeability of cells and increase cold tolerance.

Another indicator of plant adaptation to cold stress is lipid accumulation. It is believed that a high absolute content of these compounds, especially their unsaturated forms, is important for resisting low temperatures (Nokhsorov et al. 2019). The raise of unsaturated fatty acids level promotes an increase in membrane fluidity that allows the maintenance of cell structure integrity (Makarenko et al. 2014). In this regard, Nokhsorov et al. (2019) highlighted the increase of phosphatidylcholine content in the development of cryoresistance in plant tissues as mechanism to maintain cell membranes stable, assuring their survival while ice forms outside the cell.

## 11.4 Conclusions and Future Perspectives

Despite the progress in plant metabolomics for abiotic stress research, additional studies are essential to elucidate the mechanisms underlying plant resilience to environmental constraints that could be further applied in plant breeding. Food security in the face of climate change must consider the development of more resilient genotypes that can more efficiently cope with the severity of environmental constraints. Mediterranean species, as xerophytic species, are well-adapted to some abiotic stress factors such as drought, salinity, and heat; but few studies have been conducted to identify the mechanisms involved in stress resistance. Further studies identifying the molecular mechanisms underlying metabolome production upon abiotic stresses will contribute to improving abiotic stress-tolerant genotypes, either through conventional breeding or genetic engineering approaches involving the overexpression of key genes in secondary metabolite biosynthetic pathways. The development of some advanced analytical techniques, such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) boosted the study of metabolomes. Additionally, the integration of multiple analytical techniques or detectors in online or parallel analyses represents a promising approach, significantly enhancing metabolite coverage and raising quantification limits. These advanced methodologies enable precise monitoring of fluctuations in endogenous metabolite concentrations, thereby providing insights into plant responses to environmental conditions.

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