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The Olive Tree Genome

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The Olive Tree Genome

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Preface

Olive (*Olea europaea* L., subsp. *europaea*, var. *europaea*), a multifunctional long-living tree crop, is relevant not only for table olive and oil production, but also for shaping and protecting the landscape and for its impact on human nutrition and rural lifestyle.

It is usually accepted that olive has been primarily domesticated in the Levant. Then, three main clusters of the var. *europaea* inside the primary gene pools have been identified for the cultivated olive in Eastern, Central, and Western Mediterranean. These centers of diversity likely reflect crop diversification from East to West, but could also result from independent domestications.

Gene exchanges between wild (*Olea europaea* L., subsp. *europaea*, var. *sylvestris*, named as oleaster) and cultivated olive have played a major role in the diversification of the crop. In the Mediterranean area, where minimum winter temperatures do not usually fall below −7 °C, olive cultivation occupies 12 million hectares, representing about 95 % of total world olive cultivated area. Recently, its cultivation has spread to non-traditionally olive-growing countries, i.e., USA, Argentina, Chile, South Africa, and Australia, with intensive and super high-density systems, for which high productive, high oil producing, and low vigor varieties are required.

The *Olea* species belongs to the Oleaceae family that comprises nearly 25 genera and 600 species distributed in the temperate and tropical regions. In this family, plants are mostly evergreen trees, bushes, and vines, many of them producing essential oils in their flowers and fruits. The olive has a medium-sized genome (about 1.4 Gb), but the high number of chromosomes (n = 23), the large amount of the repetitive component (>70 %, made up by 30 % of tandem repeat sequences and 40 % of transposable elements), and the high level of heterozygosity have made very difficult the sequencing tasks and only a first draft of the olive genome sequence has recently been released.

The molecular bases underlying the phenotypic differences among cultivars still remain poorly understood. Nowadays, the acknowledged beneficial health properties of the extra-virgin olive oil and the ability of the species to produce under harsh conditions (e.g., drought stress) have provided new impulses for introducing innovation through olive genomics and breeding, leading to a deeper understanding of the biological processes underlying oil accumulation, polyphenol synthesis, adaptation to environmental constraints,

and response to threatening epidemics by biological agents. The 'omics' studies have particularly been useful to unravel the intricacy of main biochemical pathways and to characterize genes involved in the expression of complex traits.

Information about olive phylogeny, domestication, and relationships with related wild forms represents a fundamental prerequisite for the genetic improvement of the species, allowing for the introgression of important alleles from oleaster or from other *O. europaea* subspecies. The intercompatibility between cultivated olive and related forms has been analyzed for numerous subspecies, resulting compatible with the subsp. *cuspidata*, *laperrinei* and tetraploid *cerasiformis*, while a pre- or post-zygotic incompatibility has been observed in other cases (e.g., *ferruginea* and *Olea capensis*, respectively). The in vitro techniques now available may overcome these intercross limitations, opening the road toward new hybridization approaches.

Although the poor knowledge available on the genetic basis of the main olive characters, the lack of sound QTL markers, the limited experience on gene-transfer technologies, and the long generation interval, significant programs of genetic improvement may be undertaken profiting of the new information rising from biotechnology and genomics research. Harnessing innovations in these two research fields will help the development of fast-track breeding procedures, to improve important economical and agronomical traits, shorten the prefruiting period, and increase the selection efficiency of the designed new olive varieties through the cloning and genotyping of in vitro germinated embryos or developed seedlings.

Topics of this book cover the description of olive genetic resources, the classical and modern breeding methods for releasing new cultivars, the genotype/environment interactions determining the response to biotic and abiotic stresses, the fruit metabolism related to oil production and synthesis of health beneficial molecules, the mapping of genes and QTLs, the genome sequencing, and the transcriptomic and proteomic strategies pertinent to the development of molecular platforms and templates amenable to the precise and rapid genetic modifications using the recently developed genome-editing tools.

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Abbreviations

ABA	Abscisic acid
AChE	Acetylcholinesterase
ADH	Alcohol dehydrogenase
AFLP	Amplified fragment length polymorphism
ArMV	Arabis mosaic virus
ATT	Alcohol acetyl transferases
BCA	Biological control agent
BSseq	Bisulfite sequencing
CAP	Common Agrarian Policy
CDD	Cumulated degree day
cDNA	Complementary DNA
ChIPseq	Chromatine immunoprecipitation sequencing
CLRV	Cherry leaf roll virus
cM	centiMorgan
CMV	Cucumber mosaic virus
CoDiRO	Complesso del disseccamento rapido dell'Olivo
cpDNA	Chloroplast DNA
DAF	Days after flowering
DArT	Diversity arrays technology
dsRNAs	Double-stranded RNA
Ece	Saturated paste extract
Eco-TILLING	Targeting-induced local lesions in genomes
EFSA	Commission of the European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tag
EU	European Union
EVOO	Extra-virgin olive oil
FAD	Fatty acid desaturase
FAO	Food and Agriculture Organization
FsRIDL	Female-specific release of insects carrying a 'Dominant Lethal'
GBS	Genotyping by sequencing
GP	Gene pool
GSI	Gametophytic self-incompatibility
GWAS	Genome-wide association study
HD	High density
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid

IOGC	International olive genome consortium
IOC	International olive council
ISSR	Inter-simple sequence repeat
JERF	Jasmonate and ethylene response factor
LAI	Leaf area index
LA	Linoleic acid
LDR-UA	Ligation detection reaction-universal array
LG	Linkage group
LnA	Linolenic acid
LN	Liquid nitrogen
LOX	Lipoxygenase
MAS	Marker-assisted selection
MFO	Mixed function oxidase
MS	Murashige and Skoog (medium)
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
NGS	Next-generation sequencing
nsSNPs	Non-synonymous single nucleotide polymorphisms
OCWE	Olive crop wild relatives
OLF	Olive fly
OLRV	Olive latent ring spot virus
OLS	Olive leaf spot
OLV-3	Olive latent virus
OLYaV	Olive leaf yellowing-associated virus
OM	Olive medium-Rugini olive medium
OMMV	Olive mild mosaic virus
Ops	Organophosphates
OQDS	Olive quick decline virus
OSLV	Olive semi-latent virus
OWGB	Olive World Germplasm Bank
OYMDaV	Olive yellow mottle and decline-associated virus
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDO	Protected designation of origin
PEG	Polyethylene glycol
PGIP	Polygalacturonase-inhibiting protein
PGI	Protected geographic identification
PPM	Plant preservative mixture
PR	Pathogen-related protein
PrsS	Pistil S determinant
PSII	Photosystem II
PVS1	Plant vitrification solution 1
PVS2	Plant vitrification solution 2
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RIDL	Release of insects carrying a ‘Dominant Lethal’
RNAseq	RNA sequencing
ROS	Reactive oxygen species

RWC	Relative water content
SA	Salycilic acid
SCAR	Sequence characterized amplified region
SCR	S-locus cysteine rich protein
SHD	Super high density
SIT	Sterile insect technique
SLG	S-locus glycoprotein
SLRSV	Strawberry latent ring spot virus
SNP	Single nucleotide polymorphism
SRK	S-locus receptor kinase
SSH	Suppression subtractive hybridization
SSR	Simple sequence repeat
TDZ	Thidiazuron
TF	Transcription factor
TG	Taxon group
TNA	Total nucleic acid
TNV-D	Tobacco necrosis virus D
UNESCO	United Nations Educational, Scientific and Cultural Organization
VOCs	Volatile organic compounds
VOO	Virgin olive oil
VWO	Verticillium wilt of olive
Wt	Wild type
WUE	Water use efficiency
Xf	*Xylella fastidiosa*

Origin and Domestication

1

Guillaume Besnard

Abstract

The olive is the most iconic Mediterranean tree. The multiple uses of wild and cultivated olives make this species economically significant and a keystone of traditional Mediterranean agrosystems. The literature on its domestication is reviewed here, with a focus on the recent results on population, archaeobotanical, and genetic studies. Since the Late Tertiary, the olive distribution has been shaped by past climatic and geological changes as well as humans during prehistoric and historic times. It is usually accepted that olive has been primarily domesticated in the Levant. Three main gene pools are, however, identified for the cultivated olive in eastern, Central, and western Mediterranean. These centers of diversity likely reflect crop diversification from East to West but could also result from independent domestications. The breeding process is still ongoing, including areas outside of the native range where cultivated olives and wild relatives were introduced into the same regions. Gene exchanges between wild and cultivated olives have played a major role in the diversification of the crop. In the future, the in situ conservation of wild populations, locally endangered, should be essential to preserve the evolutionary potential of the cultivated olive.

1 Introduction

The cultivated olive (*Olea europaea* L. subsp. *europaea* var. *europaea*; Oleaceae) is the most iconic tree of the Mediterranean basin, and its omnipresence in agrosystems makes this species economically significant and a keystone of the traditional Mediterranean agriculture (Loumou and Giourga 2003). Today, hundreds of cultivated olive varieties are reported to produce both oil and/or table fruits (Bartolini et al. 2005), but a few major cultivars are usually exploited at a regional scale (e.g., Khadari et al. 2008). The first use of the olive is still hotly debated (e.g., Vossen 2007; Margaritis 2013). At the Copper and Bronze Ages, the primary utilization of olive oil is

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reported for light and body ointment with ritual significance, but its culinary use was found later and widespread during the early Roman epoch (Tardi 2014). The Mediterranean wild olive tree —usually named oleaster—[*O. europaea* subsp. *europaea* var. *sylvestris* (Mill.) Lehr] is also a source of wood and fodder for cattle (Margaritis 2013). The double nature of the olive as a wild element of the vegetation of the Mediterranean basin and as a crop was confounding for researchers addressing its domestication. It has been long supposed that the olive was not native to the Mediterranean basin and cultivars were introduced from adjacent regions [all Mediterranean spontaneous trees being seen as feral olives (e.g., Oliver 1868; Newberry 1937; Ciferri and Breviglieri 1942; Chevalier 1948; Turrill 1951)], but today this idea is categorically refuted and an autochthonous Mediterranean origin has been definitely demonstrated (e.g., Angiolillo et al. 1999; Besnard et al. 2001b; Terral et al. 2004; Carrión et al. 2010). Palaeobotanical, archaeological, historical, and molecular data have recently been accumulated, and a critical evaluation of this evidence allowed reconsidering the biogeography of the wild olive and the history of its cultivation (e.g., Terral et al. 2004; Carrión et al. 2010; Kaniewski et al. 2012; Besnard et al. 2013b; Díez et al. 2015). In this chapter, and based on a literature review, I propose a sequential history of the olive during the Quaternary, from the Late Pliocene to historical times.

2 Long Persistence and Diversification of Oleaster Populations in the Mediterranean Basin During the Pleistocene

Wild olives belong to the so-called olive complex in which six subspecies are recognized (Médail et al. 2001; Green 2002). These taxa are naturally distributed from South Africa to South Asia, in Saharan mountains, Macaronesia, and Mediterranean countries (Fig. 1). According to phylogenetic dating analyses, the most recent common ancestor of olive subspecies dates back to the Late Miocene or Early Pliocene (Besnard et al. 2009). An aridification of the Saharan region from the Late Miocene until present (De Menocal 1995; Schuster et al. 2006) may have contributed to reduce gene flow between North African and Tropical African olive populations. This may explain the early split in phylogenetic reconstructions between subsp. *cuspidata* and other subspecies (Besnard et al. 2007, 2009). The ancestor of the Mediterranean olive was thus probably present in the Mediterranean area during the Messinian Salinity Crisis about five to six million years ago (Gautier et al. 1994). Three distantly related plastid DNA lineages (namely E1, E2, and E3) were revealed in the Mediterranean olive (Fig. 2; Besnard et al. 2007, 2013b). Based on a fossil-calibrated dating, it was shown that their divergence may have started during the Late Pliocene or Early Pleistocene (Besnard et al. 2013b) with the establishment of the Mediterranean climate (Suc 1984).

The Pleistocene was characterized by climatic fluctuations punctuated by glacial and dry periods. In response to these climatic shifts, oleaster populations have experienced successive contractions and expansions. Today, eastern and western Mediterranean wild olive populations are genetically differentiated as a result of gene exchange limitations due to geographic distance and natural barriers (deserts, seas, or mountains) over long periods of time (e.g., Angiolillo et al. 1999; Besnard et al. 2001b, 2007, 2013a, b; Lumaret et al. 2004; Rubio de Casas et al. 2006; Breton et al. 2008; Belaj et al. 2010; Besnard and El Bakkali 2014; Díez et al. 2015). Based on nuclear, biparentally inherited markers, two main gene pools have thus been recognized in the eastern and Western/central Mediterranean basin. The initial pattern of genetic differentiation has, however, been considerably blurred due to gene flow from cultivated to wild olives. Phylogenetic patterns were also investigated on a few single copy genes that revealed divergent lineages of alleles (Besnard and El Bakkali 2014). Interestingly, these allelic lineages are mixed in oleasters, both in the eastern and western Mediterranean basin, suggesting that ancient admixture events have also occurred, possibly before historical times with recurrent gene flow breaks and

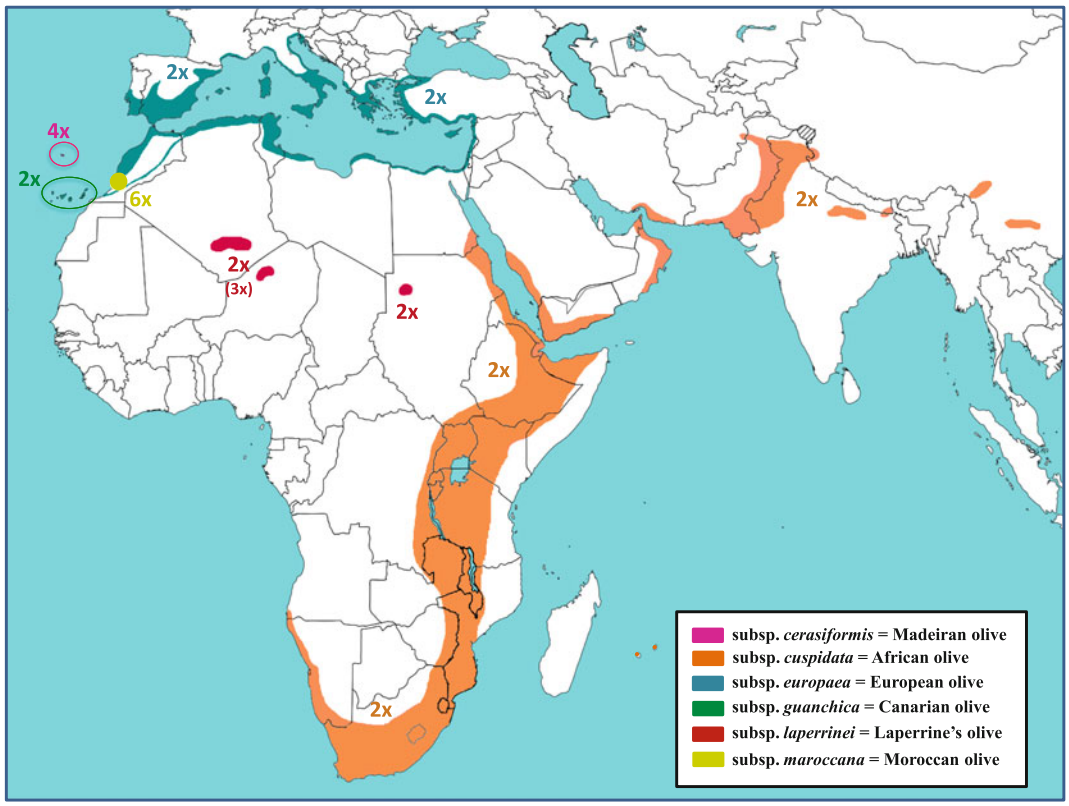

Fig. 1 Native distribution of the olive relatives (*Olea europaea* L.; according to Rubio de Casas et al. 2006). Six subspecies are currently recognized in the olive complex (Médail et al. 2001; Green 2002). They are usually considered as the primary genetic resources of the cultivated olive (Zohary 1994; Green 2002), but to date cross-compatibility has been reported only between diploids (e.g., Besnard et al. 2008, 2013a, 2014; Hannachi et al. 2009; Cáceres et al. 2015). Polyploidy level is indicated for each subspecies according to Besnard et al. (2008). Hexaploidy and tetraploidy were reported in subspecies *maroccana* and *cerasiformis*, respectively. A few triploids (ca. 3%) have also been revealed in the Lapperine's olive (Besnard and Baali-Cherif 2009)

reconnections due to past climatic changes. Phylogeographic patterns have been more deeply investigated using strictly maternally inherited genomes (i.e., mitochondria and plastids; Besnard et al. 2002). These organellar genomes are uniparentally transmitted and consequently more prone to genetic drift than nuclear genes (e.g., Schaal and Olsen 2000). Furthermore, polymorphism of the organellar DNA is disseminated only by seeds, and hence at shorter distance than nuclear DNA polymorphisms, which are also dispersed by pollen. Organellar DNAs are therefore very useful to reveal genetic patterns of strong differentiation and to study phylogeographic processes. In oleasters, most of plastid haplotypes (or chlorotypes) are confined to limited areas, while a few (also detected in cultivars: E1.1, E1.2, E1.3, E2.1, E2.2, E3.1, and E3.2; Fig. 2) have spread throughout the Mediterranean basin (Besnard et al. 2013b). Prior to the human spread of both oleasters and cultivated olives, the plastid lineage E1 was probably restricted to the East, from Greece to the Levant, while the plastid lineages E2 and E3 were specific to the West and Central parts. Today, three regional hot spots of plastid DNA diversity are identified in oleasters, namely the Levant (lineage E1), the Aegean region (lineage E1), and the Gibraltar Strait (lineages E2 and E3). The high genetic diversity found in these three areas might indicate that they

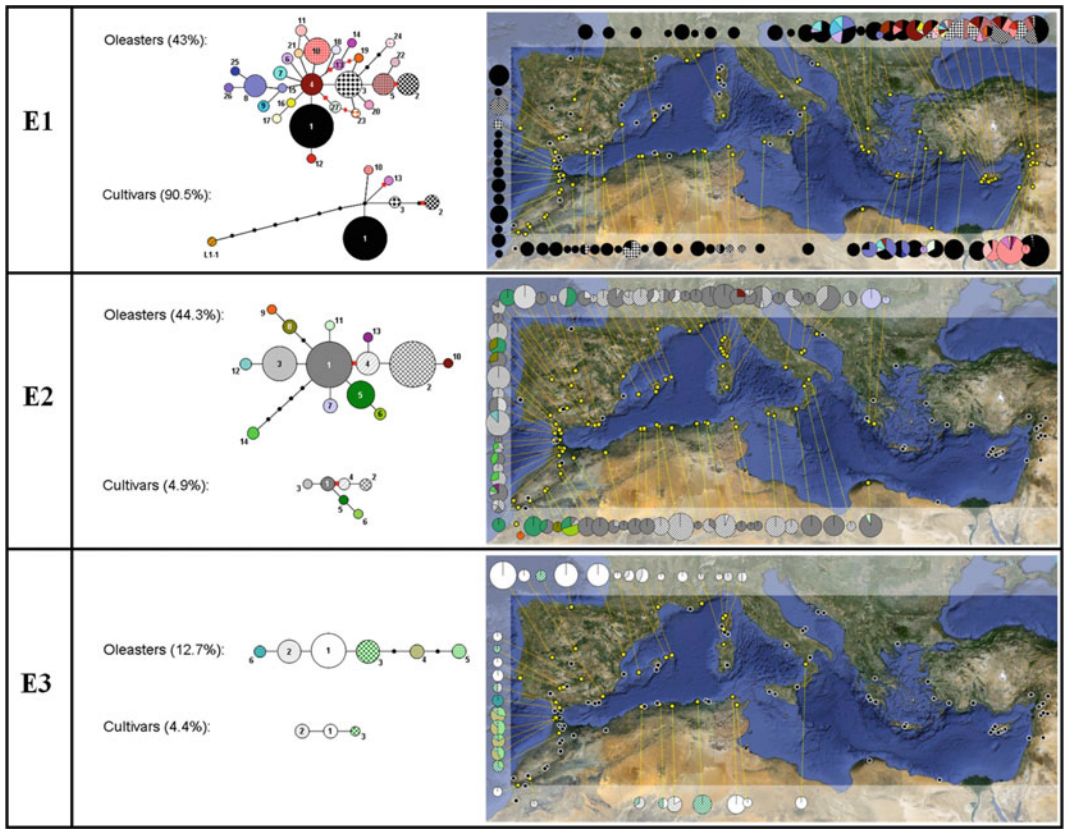

Fig. 2 Diversity of the three Mediterranean olive plastid lineages (namely E1, E2, and E3) reproduced from Besnard et al. (2013b). A total of 1797 trees (1253 oleasters and 534 cultivars) were characterized with 61 polymorphic plastid loci, especially multistate microsatellites (i.e., mononucleotide stretches) that are variable and informative in the olive (Besnard and Bervillé 2002). On the left, reduced median haplotype networks (Bandelt et al. 1999) for each lineage and for both wild and cultivated gene pools are shown. Each chlorotype is numbered and represented by a symbol with a definite color and/or motif. Chlorotype frequencies are proportional to symbol diameter. The missing, intermediate nodes are indicated by *small black points*. The frequency of each lineage in oleasters and cultivars is indicated in *brackets*. On the right, the geographical distribution of chlorotypes in oleaster populations is given. The size of pie charts is relative to the number of trees analyzed per location. For more details on the analyses, see Besnard et al. (2013b). In lineage E1, note that chlorotypes E1.1, E1.2, and E1.3 (the most frequent chlorotypes of cultivated olives) have spread on the whole Mediterranean basin. Their presence in non-cultivated olives from the western Mediterranean area is interpreted as an evidence of ferality (i.e., trees escaped from cultivation; Besnard et al. 2013b)

have acted as long-term refugia for the oleaster (Besnard et al. 2013b). Barriers to dispersal (e.g., Libyan Desert, Adriatic Sea, and Rechinger's Line) have probably limited long-distance dispersal of these lineages and prevented their complete admixture during post-glacial recolonization. A coalescent-based Bayesian approach indicated that the present diversification of the three Mediterranean lineages has started during the Middle Pleistocene or Early Late Pleistocene, long before the Last Glacial Maximum (Besnard et al. 2013b).

3 First Uses of the Oleaster During the Holocene and Early Evidence of Domestication in the Levant

Human exploitation of oleasters is attested by archaeobotanical data since the Upper Paleolithic and Early Neolithic (Kislev et al. 1992; Terral 1997; Terral et al. 2004; Carrión et al. 2010; Kaniewski et al. 2012; Zohary et al. 2012). The fossil record also shows that wild olive populations have progressively recolonized the Mediterranean area during the post-glacial period (Carrión et al. 2010). Notably, olive abundance in palynological records increased at the Holocene with human activities both in the East and West of the Mediterranean basin indicating that the expansion was associated with green oak deforestation (Carrión et al. 2010). This early use and further spread could have been mostly linked to an exploitation of oleasters for wood and/or fodder, which is usually regarded as a pre-domestication stage (Renfrew 1972; Margaritis 2013). Indeed, olive fruit production was very likely favored by a pruning effect, offering to humans the possibility to select trees with the highest agronomic values.

As the olive has been exploited by humans since prehistoric times, it is important to identify the reasons of its cultivation and to define the process of its domestication (i.e., that aims to optimize fruit production). Sedentary human communities probably established the first orchards of selected olive genotypes (in particular, with higher fruit set, bigger fruits, and higher oil content) to optimize olive production and meet a sudden increase of the local or regional demand due to human population growth. Olive domestication is also characterized by vegetative propagation of the most valuable individuals (Zohary et al. 2012). Such genotypes were probably also selected for their ability to grow in anthropogenic environments and their propensity to be multiplied (i.e., grafting and cuttings). It is, however, very likely that olive domestication has been a continuous process involving the selection of trees propagated using both vegetative and sexual reproduction as well as the reiterated cultivation of wild trees that presented the most interesting agronomic traits. Such practices still occur in some places, and the traditional exploitation of spontaneous forms can be observed in different places of the Mediterranean basin (e.g., Monastery of Stavrovouni, Cyprus; Rif Mountains, Morocco; Andalusian Mountains, Spain; G. Besnard, B. Khadari, and R. Rubio de Casas, pers. observ.).

Although the use of wild olives has been documented since the Late Paleolithic, it is commonly believed that cultivation of the tree postdates Neolithic grain agriculture (Galili et al. 1997; Carrión et al. 2010; Kaniewski et al. 2012; Zohary et al. 2012). Phylogeographic and population genetic studies demonstrated that cultivated olive mainly derives from the eastern oleaster gene pool (e.g., Besnard et al. 2001b, 2013a, b; Lumaret et al. 2004; Baldoni et al. 2006; Breton et al. 2008; Díez et al. 2015). In particular, both plastid and nuclear data sustain a major origin in the Near East (Fig. 3a). Three chlorotypes belonging to lineage E1 (i.e., E1.1, E1.2, and E1.3) characterize ca. 90 % of cultivars and are now observed in feral olives in the whole Mediterranean basin (Fig. 2; Besnard et al. 2013b). Based on the present distribution of E1 chlorotypes in oleasters and their phylogenetic relationships, Besnard et al. (2013b) have argued that the main chlorotypes of cultivated olives (i.e., E1.1, E1.2, and E1.3) originated in the northwest of the Fertile Crescent.

The olive oil trade has been developed during the Chalcolithic period in the Near East (Kaniewski et al. 2012). Based on this archaeological evidence, olive domestication is usually considered to have started then (Liphschitz et al. 1991; Galili et al. 1997), but an earlier cultivation cannot be excluded. In the northwestern Fertile Crescent, major human civilizations have emerged during the Neolithic and in particular the Pre-Pottery Neolithic B (PPNB; Edwards et al. 2004) that domesticated many crops and animals (Zeder 2011). It was hypothesized that these sedentary cultures might have also started domesticating the olive (Kaniewski et al. 2012; Besnard et al. 2013b).

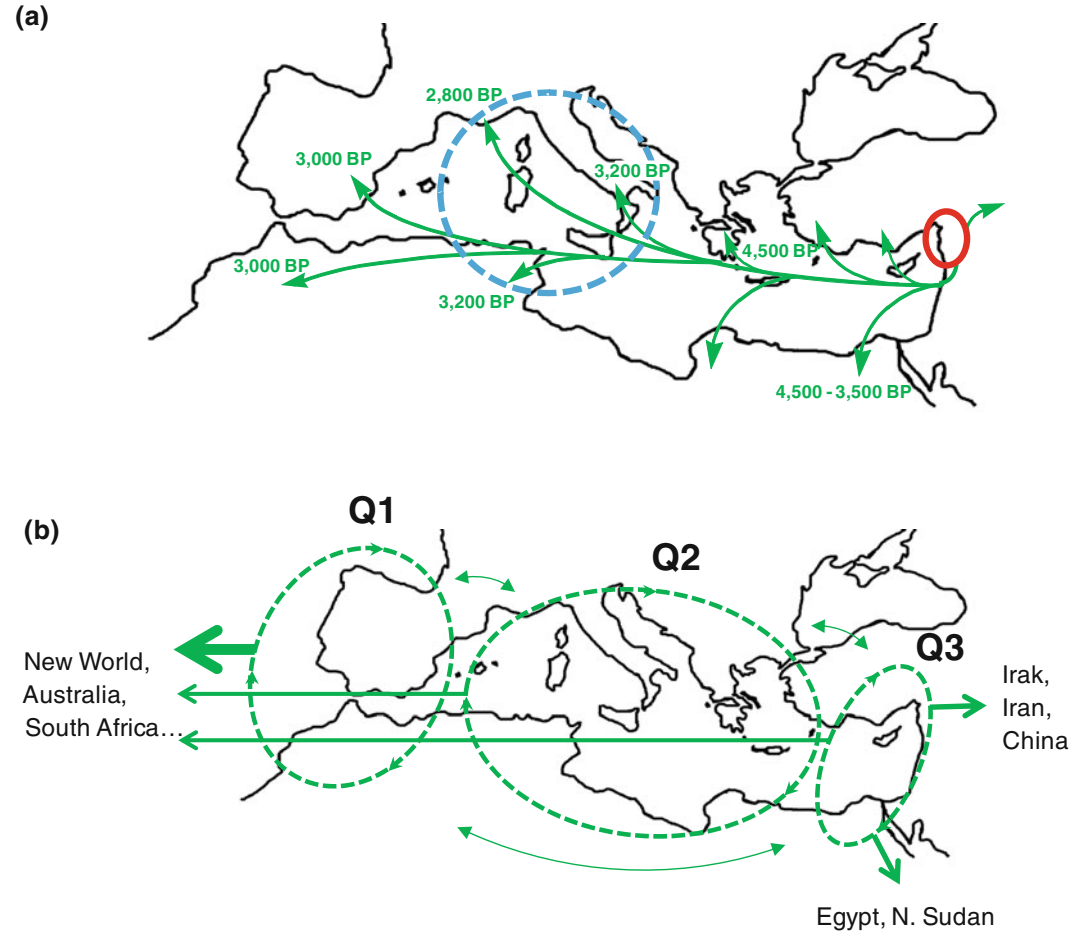

Fig. 3 Scenario on the primary domestication and secondary diversification of the olive [modified from Besnard and Rubio de Casas (2016)]. **a** The *red circle* indicates the region of initial domestication in the northern Levant during the Holocene, maybe during the Pre-Pottery Neolithic B period (Kaniewski et al. 2012; Besnard et al. 2013b). *Green arrows* indicate the subsequent human-mediated diffusion of the crop throughout the whole Mediterranean basin (approximate dates are given and deduced from archaeological data that attested for the development of oleiculture and olive oil trade; from Terral 1997). The *dotted blue circle* indicates a putative independent domestication in the Central Mediterranean as posited by Díez et al. (2015). **b** Three main regions (*dotted circles*) of cultivated olive diversification (with possible, but limited admixture) are recognized as inferred by genetic analyses (Haouane et al. 2011; Belaj et al. 2012; Díez et al. 2012, 2015; Besnard et al. 2013a). The three gene pools (Q1, Q2, and Q3) are named according to Díez et al. (2015). *Arrows* indicate the spread of each gene pool and notably out of the native area. A possible new diversification has occurred or is ongoing in these new areas (Hosseini-Mazinani et al. 2014), particularly in contact with other wild relatives (subsp. *cuspidata*) in Africa, Asia, or Australia (Besnard et al. 2014)

4 Secondary Diversification of the Crop Across the Mediterranean Basin Versus Multiple Independent Domestications

The olive oil trade was first developed in the Near and Middle East before becoming widespread across the whole Mediterranean basin (e.g., Kaniewski et al. 2012; Newton et al. 2014; Fig. 3a). Studies on the genetic diversity of cultivated olive revealed a structure in relation to the geographic origin of varieties and their different uses, i.e., oil or table fruits (Claros et al. 2000; Belaj et al. 2001; Besnard et al. 2001a; Owen et al. 2005; Marra et al. 2013; Linos et al. 2014; Yoruk and Taskin 2014; Biton et al. 2015). Based on comprehensive samplings, independent research teams also revealed that present olive cultivars belong to three main genetic pools that approximately match three geographic areas corresponding to the West (namely Q1), Center (Q2), and East (Q3) of the Mediterranean basin (Fig. 3b; Haouane et al. 2011; Belaj et al. 2012; Díez et al. 2012, 2015; Besnard et al. 2013a). Other studies have additionally reported structural details at a regional scale that could reflect the clustering of very closely related individuals that were selected locally (e.g., Khadari et al. 2003; Breton et al. 2008; Muzzalupo et al. 2014). From these results and studies, there is clear and unanimous evidence for multiple centers of diversity of the cultivated olive tree.

Several authors have argued that cultivated olive diversification occurred in westernmost regions not as the result of local independent domestication but as the consequence of hybridization among local oleasters or pre-domesticated forms and introduced cultivars (Besnard et al. 2001b, 2013a, b; Díez et al. 2015). Biton et al. (2012) reported hybrid vigor F1 in olive progenies, which might suggest that admixture between genetic pools may indeed potentially generate superior new genotypes. This scenario of a primary domestication event in the Levant followed by secondary diversification has recently been challenged by Díez et al. (2015), who suggested that an independent domestication (of Q2) could have also occurred in central Mediterranean. Besnard et al. (2013a) however showed, based on nuclear markers (microsatellites), that most Mediterranean cultivars were mainly assigned to the eastern oleaster genetic pool, while no cultivar was unambiguously assigned to the western one, even those with plastid lineages that originated from the western Mediterranean basin. This result supports that present elite cultivars either belong to the eastern genetic pool or are admixed forms. In addition, several teams have reported a significant excess of heterozygosity in cultivated olive (Díez et al. 2011; Besnard et al. 2014), which is congruent with the hypothesis of admixture-mediated diversification of the crop (i.e., of a single initial domestication followed by secondary domestication events). It is, however, important to note that other authors have reported an excess of homozygosity (e.g., Lumaret et al. 2004). This apparent incongruence could be explained by differences in the plant sampling and genetic markers used by different authors. Indeed, excessive homozygosity can be caused not only by the presence of null alleles on some loci, but also by the selection on some alleles (for instance, on isozyme loci; Lumaret and Ouazzani 2001). Conversely, the most likely cause for a global excess of heterozygosity such as the one revealed by studies using nuclear microsatellites (Díez et al. 2011; Besnard et al. 2014) is the maintenance of early generation admixed individuals but it could also be partly due to an accumulation of mutations on highly mutable loci in ancient genotypes (e.g., Baali-Cherif and Besnard 2005; Barazani et al. 2014).

Another relevant result reported by Díez et al. (2015) concerns the South Iberian group of cultivars (namely group Q1), for which they demonstrated a relatively recent origin following a strong genetic bottleneck. Using co-ancestry analyses, they identified two ancient varieties that could be the main progenitors of Q1. This means that the selection of the Q1 cultivar group was initially based on a very limited number of genotypes. This also demonstrates that the genetic basis of the current elite olive material can be locally reduced and that the selection of

cultivars could have been sometimes constrained by available genetic resources and not necessarily involved a major contribution of autochthonous oleasters.

5 New Opportunities for Crop Diversification Out of the Mediterranean Range

Wild diploid olive subspecies can be easily crossed with the Mediterranean olive and these taxa can thus be considered as primary genetic resources of the olive (e.g., Zohary 1994; Besnard et al. 2008; Hannachi et al. 2009; Klepo et al. 2013; Arias-Calderón et al. 2015; Cáceres et al. 2015). The long evolutionary history of the olive complex in contrasted environments over three continents (Fig. 1; Médail et al. 2001; Green 2002) makes that all wild olive taxa can be considered as a putative source of genes for the improvement of the cultivated olive (e.g., Lavee and Zohary 2011), notably for adaptations to new habitats and pathogen resistance (e.g., Arias-Calderón et al. 2015; Trapero et al. 2015). Non-natural contacts between the cultivated olive and non-Mediterranean wild relatives have been favored by humans with the diffusion of cultivars. Admixture between different olive subspecies has been observed both in the native and introduced ranges of *O. europaea* (Besnard et al. 2013a, 2014).

Within its native range, the cultivated olive historically spreads beyond the boundaries of the Mediterranean area, in particular in the Middle to Far East (from Iraq to SW China), but also in Saharan oases, the Canary Islands, and the Central Saharan mountains (Besnard et al. 2013a; Noormohammadi et al. 2014; Mousavi et al. 2014; Hosseini-Mazinani et al. 2014; Zhan et al. 2015). Contacts between the cultivated olive and the wild subspecies *cuspidata*, *guanchica,* or *laperrinei* have therefore potentially occurred. Diversification of crops by admixture with different closely related taxa has been already documented in fruit trees such apples and date palms (Cornille et al. 2012; Zehdi-Azouzi et al. 2015). In olive, early generation hybrids are rare but have been detected with nuclear microsatellites. In particular, the 'Dohkar' variety showed that hybridizations between Laperrine's and Mediterranean olives have occurred and contributed to cultivar diversification in the Maghreb (Besnard et al. 2013a).

During the last five centuries, the cultivated olive has been introduced into new regions, from the New World to Australia and New Zealand (e.g., Hobman 1993; Koehmstedt et al. 2011; Beghé et al. 2015). During crop diffusion, new genotypes were selected after supposedly uncontrolled crosses between cultivars and/or feral olives (e.g., Beghé et al. 2015). The wild African olive has also been introduced during the nineteenth century to Australia and New Zealand, and latter to Hawaii. Mediterranean and African subspecies have both naturalized in southeast Australia and have admixed in different places near Sydney and Adelaide (Cuneo and Leishman 2006; Besnard et al. 2014; Cornuault et al. 2015). In Southern Australia, promising genotypes were selected among naturalized olives (Sedgley 2004), and the possibility that these trees have been introgressed by subspecies *cuspidata* remains to be tested.

6 Concluding Remarks

The contribution of several disciplines was necessary to depict the processes of olive domestication, spread, and diversification. The olive now represents a case study of fruit tree domestication but its history is complex and several issues still need to be investigated. As mentioned above, a great part of the cultivated olive's genetic background came from the eastern Mediterranean. Such a situation could reflect that the primary domesticated gene pool harbors major alleles of domestication at some loci that have been maintained during the secondary diversification. The identity of major traits under selection is, however, not yet clearly identified and usually relates to fruit or vegetative traits but also to increased adaptation to cultivation. Deciphering this complex process of olive cultivar selection still represents an important challenge with

potential applications in the breeding of new varieties. Furthermore, the possibility of gene exchange between cultivars and local uncultivated olives (wild or feral) was an important feature of cultivated olive diversification that potentially allowed and allows the preservation of a high evolutionary potential in the crop (e.g., McKey et al. 2010). This might facilitate its adaptation to new environments and climates, as well as the breeding for specific agronomic traits (e.g., oil quality or disease resistance; Klepo et al. 2013; Arias-Calderón et al. 2015). This important link between wild and cultivated gene pools should be preserved in the Mediterranean basin (e.g., Díez et al. 2016). In situ and ex situ conservation strategies should thus be considered for wild olive populations, especially in the Near East where genuine oleasters have been reported to be rare and endangered (Lumaret and Ouazzani 2001).

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Olive Biology

2

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Abstract

The olive is a medium-sized evergreen tree, which integrates a unique set of morphological and developmental characteristics suited to the relatively dry, rustic conditions of its Mediterranean origin. Also particular to the olive tree are its numerous small fruits, which are rich in oil that is highly appreciated for both flavor and health benefits. The *Olea europaea* species includes both wild and cultivated forms, and both a long period of domestication and the perseverance of wild varieties provide a range of morphological variation, as does the developmental plasticity of this species. This chapter reviews the general growth and taxonomy of the olive tree and describes its vegetative and reproductive morphology and anatomy. Basic structural features of the trunk and branches, leaves, roots, flowers, fruits, and seeds are described. Current research is indicated for the structures discussed, and information provided regarding adaptive significance, environmental influences, and genetically based variability among cultivars or between wild and cultivated genotypes.

1 The Olive Tree

1.1 Description and Habit

The olive is a medium-sized evergreen tree, which grows natively in relatively dry, rustic conditions with a Mediterranean climate. Its numerous small fruits are rich in oil, which is highly appreciated for both flavor and health benefits. Numerous wild, cultivated, and feral genotypes exist, particularly in the Mediterranean Basin, where it is widely spread and emblematic of the diet and landscape. A long history of domestication, in which cultivars have been

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selected at multiple times and locations, has produced a range of tree architecture and fruit morphology. Tree characteristics such as vigor, stature, and crown density are genotype related, as is fruit size and shape. In addition, the olive tree is highly plastic in its development, so environmental conditions and agricultural management practices, in particular pruning and irrigation, impact greatly on both tree and fruit forms.

A unique set of characteristics is integrated into olive tree morphology and development. The olive is a woody plant, which may grow as a tree with a large central trunk that becomes characteristically gnarled and twisted in old trees, or as a multi-trunk, shrubby bush-like structure. The tree trunk and limbs have a high capacity for forming new lateral branches from numerous meristematic zones or axillary buds formed at the time of leaf initiation, and suckers sprout frequently around the collar at the trunk base. The olive tree's high branching capacity and the longevity of the axillary buds have further implications for its growth habit and agricultural management. Sprouting provides healthy regrowth following pruning or grazing and has been useful for vegetative propagation as well as contributing to the long endurance, sometimes multiple centuries, of individual trees. Heavy branching and the accompanying formation of numerous leaves also tend to produce a dense canopy which, for optimum fruit production, requires thinning to allow light penetration and reduce disease.

The olive tree is recognized as well adapted to the dry Mediterranean environments where it is native. Trunk and branches, leaves, fruits, and roots all contain structural features considered xerophytic adaptations, which aid in reducing water loss (see future sections). As a woody plant, the olive is characterized by a long juvenile period, as much as 10–15 years under natural conditions, until the tree is capable of forming flowers, the structures responsible for sexual reproduction. In its reproductive behavior the olive tree tends to relatively pronounced alternate bearing, in which years of heavy flowering and fruiting alternate with those with highly reduced crop levels. While these processes can be modified by environmental conditions and tree developmental physiology, there is a genetic component involved in the regulation of both the juvenile period length and the tendency to alternate bearing, with differences expressed among cultivars.

The fruit of the olive tree is unusual in the plant kingdom in that oil, in the form of fatty acids, is the major storage component. It is a drupe, in which the fleshy, oil-containing mesocarp or pulp surrounds a substantial, highly lignified endocarp or pit (see future sections). Olive fruits are consumed in the wild by birds and small mammals and under domestication by humans, principally as extracted oil but also as pickled or processed fruit.

1.2 Taxonomy

The olive, *Olea europaea* L., belongs to the Oleaceae, a medium-sized family comprising approximately 25 genera and 600 species distributed throughout temperate and tropical regions of the world (Besnard et al. 2009). The plants in this family are primarily evergreen trees and bushes along with a number of vines, many of which produce essential oils in their flowers or fruits. Other economically important genera of this family are *Fraxinus*, (ash), which is used for lumber, *Jasminum* (jasmine), *Ligustrum* (privet), *Phillyrea*, and *Syringa* (lilac), utilized for ornamental purposes, but only the olive is cultivated for its edible fruit.

Olive morphology is logically representative of the Oleaceae family, for which it is the type species. Oleaceae diagnostic characters are principally related to leaf and flower structure (Heywood 1985): Leaves are simple and generally occur opposite each other along the stem, or else pinnate, with opposite leaflets along the leaf axis. Flowers are frequently grouped in terminal or axillary inflorescences. The flowers are radially symmetrical and usually hermaphrodite, but sometimes unisexual. The calyx (sepal unit) and corolla (petal unit) are hypogenous, inserted below the ovary, with the sepals united in a

bell-like shape and the petals sometimes united. Generally, there are two stamens which are inserted on the petals. The ovary is superior, inserted above the petals and sepals, consistent with their hypogenous arrangement. Typically, the ovary originates from two carpels and is composed of one to two locules, each containing two ovules. Morphological distinction between the cultivated variety *O. europaea* subsp. *europaea* var. *europaea* and the wild *O. europaea* subsp. *europaea* var. *sylvestris* has been mainly based on the small fruit and pit size of the wild variety and is considered rather unprecise and a potential source of error (Ganino et al. 2006).

Following a thorough revision by Green (2002), the genus *Olea* is considered to include 33 species and nine subspecies. Six of these subspecies form the *Olea* subsection or complex: *O. europaea*, *O. cuspidata*, *O. laperrinei*, *O. maroccana*, *O. cerasiformis*, and O. *guanchica*. This complex extends through Macronesia (*O. cerasiformis*, and *O. guanchica*), Mediterranean (*O. europaea*), African (*O. maroccana*), and Asian (*O. cuspidata*) regions. Green's taxonomic scheme further recognizes the close relationship between the cultivated olive and its wild relative, often referred to *oleaster* or *silvestris*, as two varieties within the *Olea europaea* subspecies: *Olea europaea* subsp. *europaea* var. *europaea* and *O. europaea* subsp. *europaea* var. *sylvestris*.

Historically there have been many and differing opinions as to nomenclature and hierarchies within both the olive genus and species, particularly because of the use of morphological characters which often vary in relation to environmental conditions or phenological stage. For example, certain taxonomic confusion within the species seems to have arisen because one of the morphological characters used for taxonomic identification is leaf size, and the juvenile foliage of germinated seedlings or resprouted vegetation of heavily grazed cultivars is easily misinterpreted as the small leaves characteristic of the wild type. Molecular techniques are proving highly useful in clarifying taxonomic relationships in *O. europaea* (Green 2002; Baldoni et al. 2006; Besnard et al. 2008, 2009).

The wild *O. europaea* subsp. *europaea* var. *sylvestris* is considered to be the main wild progenitor of the cultivated olive, based on similar morphology and ecological requirements and ploidy level (Green 2002; Besnard and Rubio de Casas 2016). It is generally agreed that subsequent to domestication, introgressive hybridization between cultivated and wild populations in different locations has played an important role in the evolution of olive cultivars (see following chapters). There is debate, however, as to whether olive domestication was initially a single (Besnard and Rubio de Casas 2016) or multiple events (Díez et al. 2015).

2 Vegetative Morphology and Anatomy

2.1 Trunk and Branches

Olive tree architecture, the structural pattern of trunk and lateral shoot growth, shows high phenotypic diversity as well as ontogenetic modification as the plant matures (Rallo et al. 2008; Hammami et al. 2012; Ben Sadok et al. 2013). Evaluation of cultivars and of progenies in breeding programs have demonstrated clear genetic influence and heritability in olive architectural traits, along with a highly plastic response to environmental and management conditions. Young olive plants show a prevalent tendency to form a single-axis trunk. Lateral shoots sprout along the trunk from one or both of the two lateral buds present at each node. Branched and unbranched nodes are distributed vertically along the trunk in a scattered or irregular manner, although long areas of no branching are infrequent. Within this diffuse pattern there appear to be relatively equal tendencies for different dominant zones of more numerous and more vigorous branching: basitony near the trunk base, mesotony in the central region, acrotony in the upper or distal zone, or the absence of a dominant zone. Branch orientation is more frequently upright (orthotropic) than horizontal (plagiotropic), and while the majority of

branches are straight there are often substantial numbers, which bend upward or downward. Olive genotypes with a drooping or pendulous branching habit are considered to be of particular interest for hedgerow orchard systems (Rallo et al. 2008).

Over time, olive trees exhibit a continuous branching hierarchy, forming second order, third order, fourth order branches, and so on throughout the tree canopy. The branching system is monopodial, in which the main shoot and branch axes are maintained and their apices continue to be active, rather than being overtaken by lesser order lateral shoots. In older trees branching follows a proleptic pattern, in which new-shoot sprouting from dormant lateral buds occurs periodically, once a year. In contrast, young trees and regrowth following heavy pruning often present syllepsis, where multiple orders of branches develop in the same growth season due to reduced bud dormancy. Sylleptic branching in the olive has a significant genetic component and a strong role in early tree morphology (Ben Sadok et al. 2013).

Vigor is a morphological characteristic, which refers to overall plant growth rate and size. In cultivated olive varieties, vigor is considered to be an important agronomic component which is relevant to juvenile period length and variety choice for planting density and has noted genotypic tendencies (de la Rosa et al. 2006; Rallo et al. 2008; Farinelli and Tombesi 2015). Olive tree vigor may be classified qualitatively as high, medium, or low, as in the official Olive Oil Council cultivar passport criteria (Barranco et al. 2000), or measured as canopy volume, canopy surface parallel to the ground, or trunk diameter at a specified distance from the soil surface (Rallo et al. 2005). Although trunk growth is clearly influenced by multiple factors, a molecular marker for olive trunk diameter has been recently identified at the seedling stage (Atienza et al. 2014).

Young olive shoots are slender, and their anatomy is characterized by small xylem vessels, a large central pith, and a notable sclerenchyma ring composed of fiber bundles and sclereids internal to the phloem. Secondary xylem in the olive stem and trunk is diffusely porous, in which the relatively small-diameter vessels are distributed uniformly within the annual growth ring. The xylem tissue (Fig. 1) is also rich in fibers and contains a relatively low proportion of parenchymatic cells (Salleo et al. 1985; Rossi et al. 2013). In a 1-year-old stem, vessel diameters are typically lower than 40 μm, with 90 % in

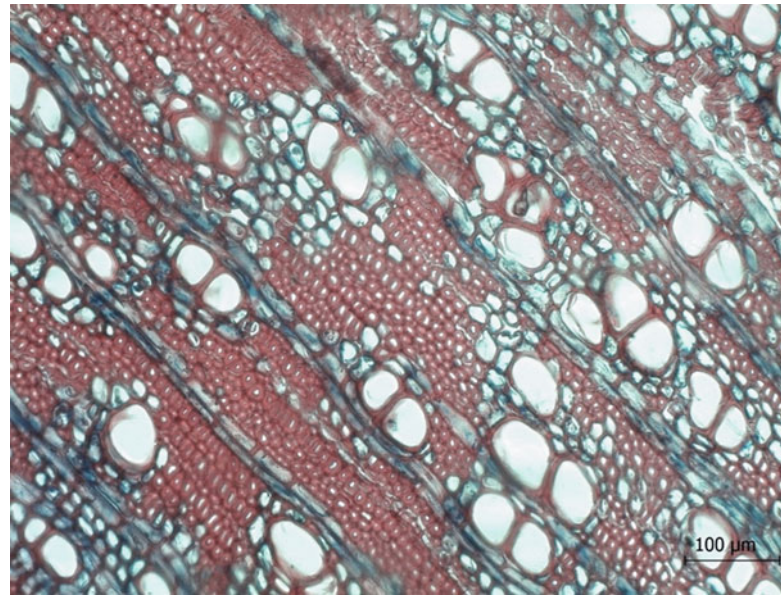

Fig. 1 Microphotograph of xylem tissue sampled within a yearly ring of an olive stem. Xylem vessels (*large* and *red*), numerous fibers (*small* and *red*), and both axial and ray parenchyma cells (*blue*) are visible (*photograph credit* Luca Sebastiani)

diameter classes around 20 μm (Lo Gullo and Salleo 1990). In mature olive plants the vessel diameters can vary among different years and according to water availably, indicating a strong capacity to respond to the environmental. Rossi and coworkers (2013) found that irrigated olive trees formed fewer small-diameter xylem vessels (<20 μm) than rainfed trees, while vessels with diameters higher than 20 μm were more abundant. These authors hypothesize that the narrow xylem vessels could contribute to olive tree tolerance of low water availability.

2.2 Leaves

The olive tree is evergreen and its leaves usually live for two to four seasons, although older leaves may remain on the tree longer. The small leaves are simple, with an elongated blade (3–9 cm long) and a very short petiole (approximately 0.5 cm). Blade shape varies from the slightly wider and more symmetrical elliptical form to lanceolate, where the width is greatest at the base and blade length is greater than six times its width. A thick, highly visible central vein divides the leaf in half along its length and protrudes along the lower surface. The leaves are positioned on the branches with decussate phyllotaxy: There are two opposite leaves (180° apart) at each node, alternating at right angles with those of the nodes above and below. Shoot growth producing new nodes and leaves may occur at any time of the year, depending on water supply, temperature, and solar radiation, but usually is only active or most active in spring and autumn (Connor and Fereres 2005).

Olive leaf anatomy and surface characteristics (Fig. 2) are typical of drought-resistant sclerophyllous vegetation, including small size, thick cuticle, high stomatal density, high pubescence, and compact mesophyll cells (Bacelar et al. 2004). The upper surface of the leaf is dark green and glossy, covered with a thick cuticle. The lower surface appears gray or pale in color due to the presence of numerous multicellular trichomes in the form of umbrella-shaped peltate scales, forming a dense protective layer. The stomata

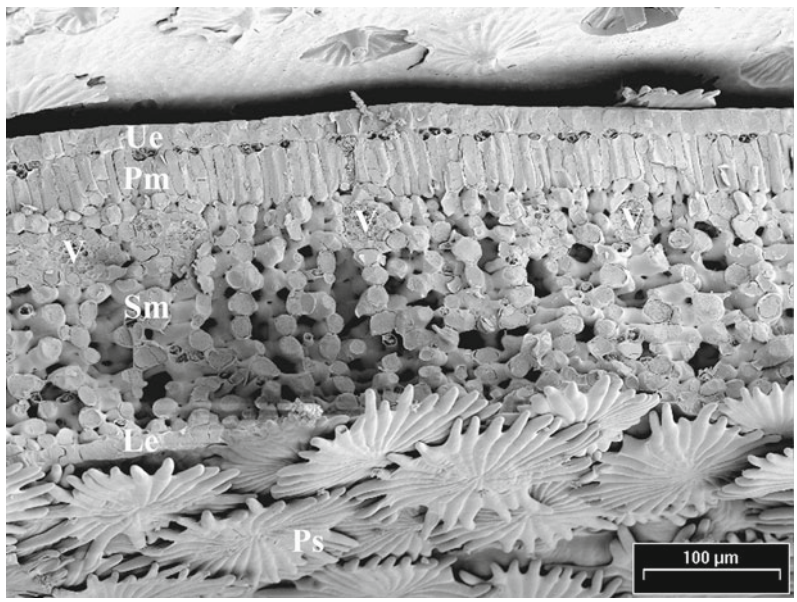

Fig. 2 Cryo-SEM image of frozen-hydrated young leaf (cv. Leccino), freeze fractured transversally showing cell types. At the figure, top, the upper epidermis (Ue) and palisade mesophyll (Pm); in the center, the spongy mesophyll (Sm) traversed by veins (V); and below, the lower epidermis (Le). The lower surface is covered by numerous overlapping trichomes (Ps, peltate scales) (*photograph credit* Antonio Minnocci)

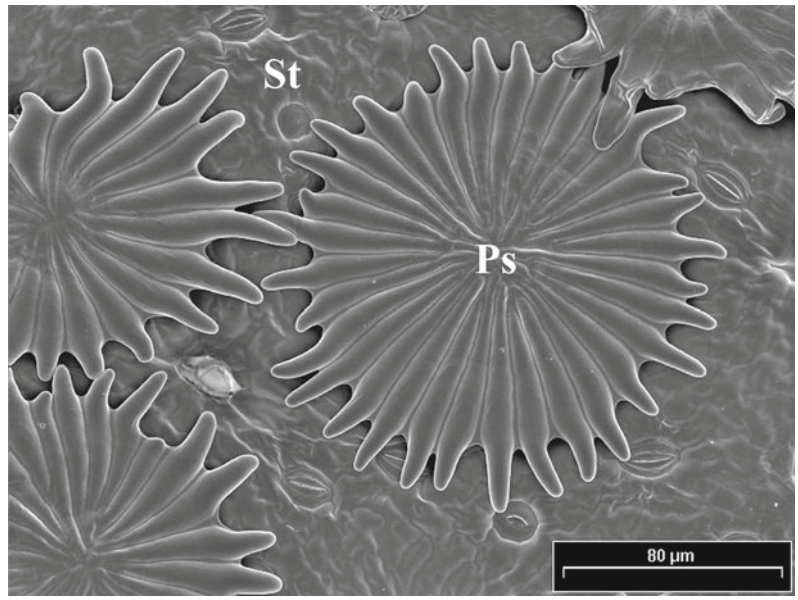

Fig. 3 Cryo-SEM image of frozen-hydrated young leaf (cv. Leccino), lower surface. The trichomes (Ps, peltate scales) and the stomata (St) they will eventually cover are in early stages of development (*photo credit* Antonio Minnocci)

form only on the underside of the leaf, where they are shielded by the peltate trichomes or scales (Fig. 3). Internally, the upper zone of the leaf mesophyll is composed of palisade parenchyma consisting of several layers, usually two to three, of compact, relatively elongated cells. The spongy mesophyll, composing the lower portion of mesophyll, is characteristically replete with large intercellular spaces. Directly below the lower leaf epidermis, between that tissue and the spongy mesophyll, is frequently a second palisade or pseudo-palisade parenchyma, consisting of a continuous single layer of elongated cells, although shorter and less densely packed than the principal palisade tissue (Chartzoulakis et al. 1999; Bacelar et al. 2004; Moreno-Alías et al. 2009). Within the leaf, where the palisade parenchyma and spongy mesophyll meet, is a dense reticulate network of secondary veins, and throughout both mesophyll layers are numerous long, slender branched sclereids (Arzee 1953). These latter structures contribute to the characteristic toughness of the olive leaf, also considered a typical structural feature of leaves adapted to dry environments.

Olive leaf size and structure vary in relation to genotype and development, and in response to growth conditions. Leaves of juvenile plants tend to be smaller and to have a lower length-to-width ratio, less peltate trichomes, and no second palisade parenchyma layer (Moreno-Alías et al. 2009). Leaves from wild genotypes are also frequently small and more elliptical in form. Under water stress, cell density increases for all leaf tissues, and the cuticle is substantially thicker (Chartzoulakis et al. 1999).

2.3 Roots

Olive tree root morphology varies with growth conditions, including soil characteristics, nutrition, and water, and with the origin of the plant as a seedling or cutting; however little information is available with respect to cultivar differences or genetic control. Seedlings produce a single long, primary taproot, whereas in rooted cuttings the multiple roots tend to branch more profusely and closer to their junction with the stem.

Anatomically, the olive tree root presents attributes related to withstanding water stress and like the shoot shows the capacity to rapidly modify its structure in relation to water availability (Rapoport 2010; Tataranni et al. 2015). Root hairs, the elongated lateral outgrowths of

the epidermal cells which are responsible for absorption, are relatively short but numerous. The endodermis is the cell layer located within the root between the cortex and vascular cylinder and contains a prominent Casparian strip on its radially oriented cell walls. The substantial suberin composition of the Casparian strip is impermeable to water and forces the water movement through the endodermal cell protoplasts, providing the metabolically controlled hydraulic gates for the entry of water into the plant vascular system. The endodermis of olive roots is well differentiated and furthermore has demonstrated the capacity to undergo additional suberization in response to exposure to water (Lo Gullo et al. 1998) and salinity stress (Rossi et al. 2015).

The external cell layers of the cortex, those that lie directly below the epidermis, also exhibit structural modifications to create a hypodermis or exodermis. The hypodermal cells undergo specialized secondary wall differentiation, which includes thickening and deposition of suberin, acting as an additional barrier to water movement and protecting the root from water loss. In fact, under water-limiting conditions olive roots produce added hypodermal cell layers and increased suberization hypodermal as well as endodermal cell walls (Tataranni et al. 2015).

3 Reproductive Structures and Development

3.1 Inflorescence and Flower

In the year prior to bloom, the inflorescences originate as buds in the leaf axils of new shoots. The buds (Fig. 4) are small, highly reduced shoots composed of 4–5 nodes, and each with two oppositely positioned leaf primordia (Fabbri and Alerci 1999; de la Rosa et al. 2000). All of the buds are structurally identical and basically vegetative; that is, they do not present any morphological differences which distinguish between vegetative and reproductive buds (Pinney and Polito 1990; Andreini et al. 2008). Subsequent bud fate, depending on a complex combination of endogenous and exogenous factors, will follow one of four pathways: (1) development of an inflorescence (reproductive bud), (2) growth of a shoot branch, (3) continue dormant, and (4) death and abscission. The lack of microscopically visible differences between buds with reproductive and vegetative fates has long been an obstacle for the investigation on olive tree flowering controls. Molecular tools offer great potential for identifying vegetative and reproductive buds and providing a means of advancing in this area (Amasino 2010).

Fig. 4 Olive axillary bud (*left*) and early reproductive and vegetative sprouting (*center* and *right*). The axillary bud is a relatively undifferentiated structure composed of opposite pairs of leaf primordia. In reproductive sprouting (*center*), the peduncle axis elongates, the leaf primordia form small bracts, and inflorescence development occurs within the bracts. Vegetative sprouting (*right*) begins with elongation of the branch axis and the external leaf primordia, which will develop into the first pair of leaves (*photograph credit* Franco Castillo-Llanque)

Reproductive differentiation proceeds the following spring under adequate environmental conditions in buds, which have received the necessary induction and initiation stimuli. Olive flowers are borne on 1.5–4.0 cm long inflorescences that develop from the lateral buds or directly at the shoot apex. The paniculate inflorescence has a central axis or rachis, on which individual flowers or secondary flower-bearing axes (rachillas) arise at a series of bilaterally symmetrical branching points. One inflorescence may contain from 10 to 35 flowers, of which only one or a few will set fruit. The number of flowers in an inflorescence is influenced by the cultivar and growing conditions, including the position of the inflorescence on the tree and the fruiting shoot.

The olive flowers are small and have actinomorphic (regular) symmetry and hypogenous arrangement, in which the masculine and accessory flower organs are attached in successive whorls below the ovary base. The sepals are fused to form a small green conical calyx at the flower base. Within the calyx are four white symmetrically arranged petals, joined at their base, composing the corolla. The androecium, the male reproductive system, consists of two stamens, each with a relatively large, plump bright yellow anther, which are attached to the petal bases by a short filament. In the flower center is the gynoecium, the female reproductive system, a single pistil composed of a long, two-lobed stigma, a short white style and a small round green ovary (Fig. 5).

The elongated, two-lobed stigma surface is composed of multicellular papillae and is classified as wet type because of the secretion it produces, a heterogeneous substance including carbohydrates, lipids, and proteins (Serrano et al. 2008). Below the papillae lies a funnel-shaped receptive tissue, which is continuous with and similar in structure to the columnar transmitting tissue in the center of the solid style (Suarez et al. 2012). The ovary (Fig. 6) has two locules or cavities, derived from its two-carpel origin. Each locule contains two ovules, each joined to the upper part of the placental separation between the locules. Olive ovules are anatropous and during development undergo a turning movement, which orients the micropyle, the embryo sac's entranceway for the pollen tube, upward toward the style (King 1938). Fertilization of one of the four ovules is required for fruit development.

Within the olive ovule, macrogametophyte differentiation follows a bisporic pattern in which two of the four megaspore nuclei produced during meiosis abort and two survive, generating an 8-celled embryo sac (Extremera et al. 1988). The olive ovule is tenuinucellate and unitegmic, signifying that the nucellus, the ovule tissue where the embryo sac forms, is thin at ovule maturity and is surrounded by only a single integument. Full development of the ovule occurs in the three weeks prior to flowering, at which time the embryo sac is mature and capable of fertilization. Differentiation is sometimes incomplete and no embryo sac forms within the ovule, only a

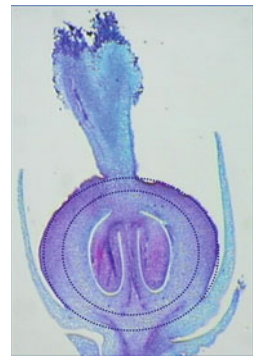

Fig. 5 Microphotograph of a longitudinal section of olive gynoecium, composed of a single stigma, style, and ovary. The upper stigma surface has received pollen grains. The funnel-shaped receptive tissue of the upper style is continuous with the stigma papillae. Indicated between *circles*, the mesocarp or future pulp forms the outer part of the ovary. Within the ovary endocarp, one ovule is visible in each of the two locules (*photograph credit* Hava Rapoport)

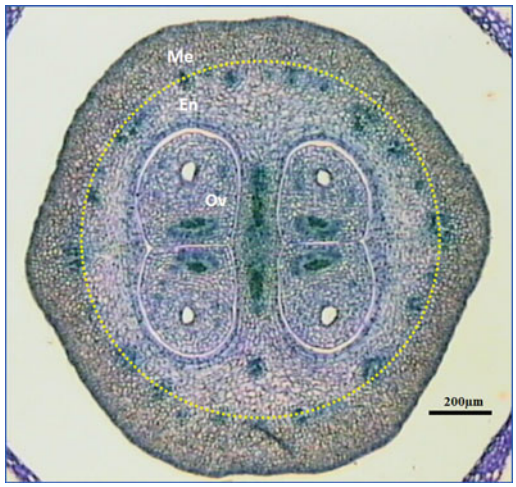

Fig. 6 Microphotograph of a central transverse section of olive ovary. A ring of vascular bundles, indicated by the *dotted line*, lies between the ovary mesocarp (Me) and endocarp (En). Each of the two locules contains two ovules (Ov); in this ovary, all four ovules are fully developed, evidenced by the round clear areas (*photograph credit* Ester García-Cuevas)

residual atrophied nucellus at the base of a long micropylar canal. Such incomplete ovule development was first identified in the non-fruiting ornamental cultivar Swan Hill, but also occurs in fruiting cultivars to varying degrees, clearly associated with genotype (Rallo et al. 1981; Moreno-Alías et al. 2012). Fertilization of the undeveloped ovules is not possible, but, usually other, fully differentiated and competent ovules are also present in the ovary, allowing fertilization to proceed. That factor, along with the olive tree's tendency to produce numerous flowers, means that in normal, good flowering years the presence of undeveloped ovules will not likely cause a drop in fruit production (Rapoport and Rallo 1991).

The olive tree sexual system is andromonoecious, with two sexually different kinds of flowers present together within the same inflorescence: (1) hermaphroditic or bisexual flowers, with both male and female reproductive organs, and (2) staminate, with only male reproductive parts. The hermaphroditic flowers are also referred to as perfect flowers, and the staminate flowers, imperfect. Staminate flower development involves varying degrees of pistil abortion, and a residual atrophied pistil is often visible within the center, but stamen development and pollen production are similar in both flower types (Cuevas and Polito 2004). The proportion of imperfect flowers varies according to cultivar and is also influenced by environmental conditions, such as temperature, mineral nutrition, and water availability, and substrate competition among flowers when flower number is high. The critical role of substrate supply in the differentiation of the two flower types is evidenced by the much higher starch accumulation in perfect than staminate flowers (Reale et al. 2009). Within the inflorescence, there appear to be preferred positions for perfect flower formation, likely related to positional priorities for substrate supply (Cuevas and Polito 2004; Seifi et al. 2008).

The olive anthers produce large quantities of pollen (Fig. 7) which as well as being critical for fertilization of the flowers is also a source of allergic reactions. Pollination is anemophilous, by wind, and the pollen may travel over very large distances, a process recently confirmed by molecular paternity testing. Olive cultivars express varying degrees of self- and inter-incompatibility.

3.2 Fruit

The olive fruit is a drupe, an indehiscent fleshy fruit composed of three principal tissues, the endocarp, mesocarp, and epi- or exocarp, each of which follows a very distinct form of growth and differentiation during fruit development. Together they form the pericarp, initiated as the ovary wall. At bloom, the ovary contains a ring of vascular bundles which lies between the mesocarp and endocarp (Fig. 6), at this time composed of small parenchymatic cells (King 1938; Rallo and Rapoport 2001; Rosati et al. 2012). Observations of the olive ovary cells and tissues have shown that ovary size and cell number at bloom are highly correlated with cultivar fruit size (Rosati et al. 2009, 2012) and that ovary size differences among cultivars are mainly due to cell number in both the endocarp and mesocarp (Rosati et al. 2011).

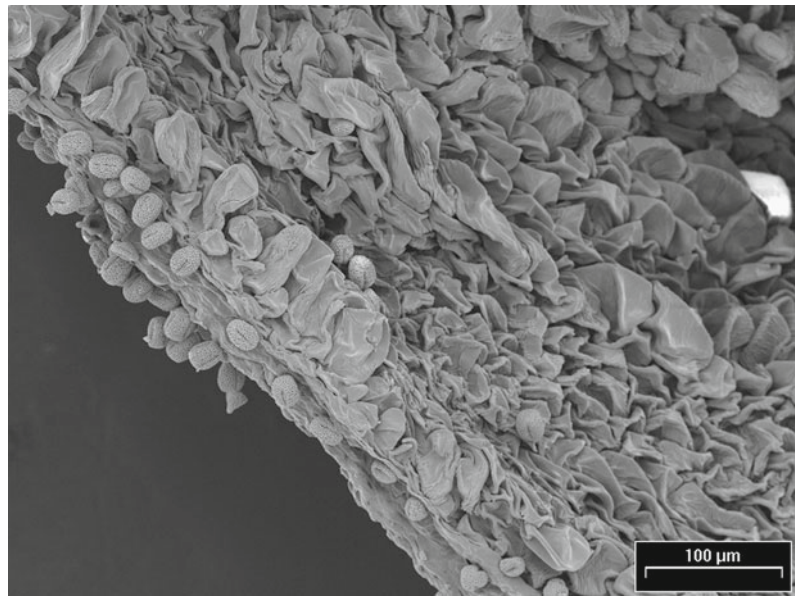

Fig. 7 Cryo-SEM image of an olive anther with numerous pollen grains (*photograph credit* Antonio Minnocci)

The endocarp is the fruit pit, a hard, woody structure, which surrounds the seed in the fruit center. During fruit growth the endocarp cells undergo the differentiation process of sclerification, in which a thick secondary wall rich in lignin is deposited, eventually filling the entire cell and obliterating its living contents (Fig. 8). As the endocarp grows, sclerification is initiated in successively increasing numbers of its cells (Hammami et al. 2013), while the cells which previously started sclerification continue to undergo lignin deposition and cell wall thickening. Physical hardening intensifies after maximum endocarp size is reached, the time when the majority of endocarp cells have initiated sclerification and can no longer expand nor undergo division. Afterwards hardening continues, as do cell sclerification and the associated endocarp dry weight increase (Hammami et al. 2013; Rapoport et al. 2013).

Exterior to the endocarp is the mesocarp (Fig. 9), or fruit pulp, the major and edible part of the olive fruit, and the site for oil metabolism and storage. Oil deposition, for many years mistakenly thought to occur in the large, aqueous vacuole of the mesocarp cells, takes place in the cytoplasm, a process consisting of the formation and coalescence of small oil bodies to become increasingly larger oil droplets (Rangel et al. 1997). The mesocarp is a fleshy tissue composed of parenchyma cells, whose capacity for cell division and expansion drives the long-term growth of this tissue. The highest rate and

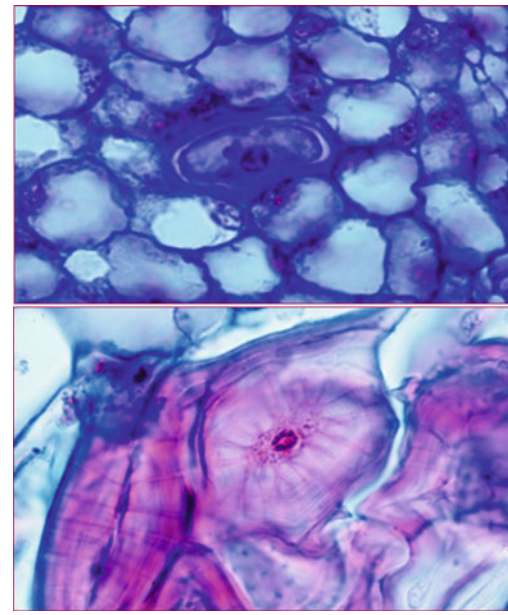

Fig. 8 Sclerification of olive endocarp cells which produces pit hardening: a thick secondary wall rich in lignin is formed (*upper photo*) which progressively thickens, eventually obliterating the cell contents (*lower photo*) (*photo credit* Hava Rapoport)

amount of olive fruit mesocarp cell division occur in the period immediately following bloom, although some cell division continues at a reduced rate throughout fruit development (Hammami et al. 2011). Cultivar differences in fruit size depend principally on cell number, not cell size, but the seasonal pattern of cell division intensity is consistent for olive cultivars with a wide range of fruit size (Hammami et al. 2011).

The epicarp or exocarp, the fruit skin, constitutes the external fruit tissue. The exocarp is principally composed by the epidermis, the external layer of cells with its thick protective cuticle. In an attempt to clarify different views as to whether any or how many subepidermal cell layers also constitute part of the exocarp, recent studies of cell dimensions (Hammami and Rapoport 2012) indicate that approximately five layers of subepidermal cells, although continuous with and similar in structure to mesocarp cells, show structural and developmental patterns more unified with the epidermis. Scattered across the exocarp are lenticels, derived from ovary stomata which become atrophied early in fruit growth and are subsequently covered with the cuticle. The lenticels are most visible during mature green stages of fruit growth as light-colored specs, and their size and frequency are characters used in cultivar identification (Barranco et al. 2000).

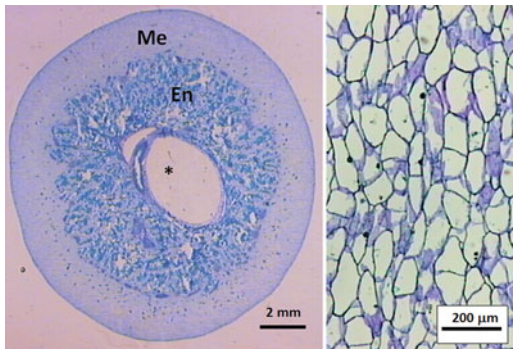

Fig. 9 Microphotograph of a central transverse section of olive fruit (cv. Manzanilla de Sevilla) 8 weeks after bloom (*left*) and detail of parenchymatous mesocarp at fruit maturity (*right*). At 8 weeks the mesocarp (Me) is expanding and the endocarp undergoing sclerification; within the endocarp the locule containing the growing seed has expanded (*). (*photo credit* Ester García-Cuevas)

The fruits of cultivated olive varieties are 1–4 cm long, 0.5–2 cm in diameter, and can weigh as much as 10 g fresh weight, 2.5 g dry weight. Fruit form ranges from spherical to ellipsoidal or elongated, with varying degrees of asymmetry and a rounded or pointed apex. While there is a tendency for the cultivars with smaller fruits to be destined for oil production and those with larger fruits for table use, many are of double aptitude and size is not a strict determinant of use. Fruits of wild genotypes are commonly much smaller (length 0.5–1.5 cm, width less than 1 cm, and weight less than 2 g) and have a much higher proportion of endocarp as compared to cultivated varieties.

3.3 Seed and Embryo

Within the endocarp, the olive fruit contains one, sometimes two, or very rarely three elongated seeds, a characteristic which seems to be associated with the cultivar. The seed coat, derived from the single ovule integument, is thin, tough, and permeated by numerous vascular strands. The embryo is straight, with long, flat, spatula-shaped cotyledons, and fills most of the seed volume. Between the embryo and seed coat is a small amount of endosperm, rich in starch, proteins, and oil (Alché et al. 2006).

Immediately following fertilization the multicellular endosperm is formed and commences rapid growth, accompanied by notable expansion of the ovule and differentiation of ovule vascular tissues. In contrast, embryo development is delayed, and after approximately 3–4 weeks a single-celled proembryo, connected to a long filamentous suspensor, appears at the tip of the now endosperm-filled embryo sac. Embryogenesis follows the classical sequence from multicellular embryo proper to globular and then heart-shaped embryo, and 2 months after bloom the two cotyledons are clearly formed. At 4–5 months, the mature olive embryo contains a well-differentiated root apex at the tip of a short embryo axis, and appreciable provascular strands and storage substances in the axis and cotyledons. Between the cotyledons, however, the

shoot apex is flat, or sometimes slightly domed, with a subtending zone of intense metabolic activity but no other visible differentiation or plumule formation (Germanà et al. 2014).

As the fruit matures, physiological dormancy is imposed on the seed, with factors apparently located in the endosperm as well as the embryo itself (Germanà et al. 2014). Thus olive seed germination procedures require removing the mechanical barrier of the hardened pit, and cold stratification to remove physiological dormancy. Among tested genotypes, optimum germination was consistent with stratification at 14 °C for 30 days, although germination rate was highly related to genotype (Morales-Sillero et al. 2012).

At maturity, numerous olive fruits contain empty pits with no seed present. It appears that embryo and subsequently seed abortion are responsible for this phenomenon, evidenced by the consistent observation of growing fertilized ovules in young fruits, the occurrence of aborted embryos at two months, and by the enlargement of one of the ovary locules in the same manner as when a seed is present; that is, the locule expands to accommodate the growing fertilized ovule (Rapoport 2010). Early embryo abortion could possibly be related to post-zygotic incompatibility factors.

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Olive Genetic Resources

3

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Abstract

As one of the most important and ancient fruit crops in the Mediterranean Basin, olive is characterized by a huge genetic patrimony, represented by cultivated and wild germplasm, ancient trees and related forms. The richness of this germplasm represents an unusual case among horticultural crops, due to species longevity, lack of new better performing genotypes, and the millennial tradition of cultivation. Focusing on a wide spectrum of

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genetic resources, their conservation, characterization, and management, this chapter tries to give an insight into the achievements and the necessities of this type of works in olive. Knowledge of existing diversity among the olive genetic resources is essential to maximize their conservation, safeguard, and exploitation.

1 Current Status of Olive Genetic Resources

Olive (*Olea europaea* subsp. *europaea*), the most iconic tree crop species of the Mediterranean area, has maintained a very rich genetic heritage, represented by the cultivated form (var. *europaea*) (Bartolini 2008; Belaj et al. 2010; Haouane et al. 2011; Diez et al. 2015), the wild trees (var. *sylvestris*) (Baldoni et al. 2006; Besnard et al. 2013), and the related subspecies (Green 2002). In addition, ancient olive trees have revealed to represent further important and valuable genetic resources (Diez et al. 2011; Barazani et al. 2014; Lazovic et al. 2016).

The cultivated olive germplasm, unlike other fruit trees, has not suffered significant genetic erosion, maintaining almost intact its entire variability, represented by a very high number of cultivars. The cultivated germplasm is estimated to include more than 1200 clonally propagated cultivars, maintained in over 100 regional, national, and international collections, and cultivated in 54 countries (Bartolini 2008). Over two-thirds of the olive cultivars are present in the southern European countries: 538 in Italy, 272 in Spain, 88 in France, and 52 in Greece (Bartolini et al. 1998; Khadari et al. 2003; Barranco et al. 2005). In addition, a rich genetic patrimony is still preserved *on farm* in traditional olive-growing countries and outside Europe, such as in Syria, Turkey, Tunisia, Morocco, and Iran (Belaj et al. 2003a; Noormohammadi et al. 2007; Khadari et al. 2008; Fabbri et al. 2009; Fendri et al. 2010; Isk et al. 2011; Hosseini-Mazinani et al. 2014). The use of seedlings as rootstocks, or their spreading and further selection in the traditional area of cultivation and in countries where olive cultivation has been recently introduced, such as Argentina, Australia, Brazil, California, China, Colombia, Mexico, and Uruguay, might have contributed to a 'new-emerging' genetic variability (Sedgley 2000; Caballero et al. 2006; Kohemested et al. 2010; Soleri et al. 2010; Do Val et al. 2012; Beghé et al. 2015; Zhan et al. 2015). However, the consistency of the olive germplasm remains controversial, and other data report the existence of more than 2600 different olive varieties (FAO 2010; Muzzalupo et al. 2014). The large number of cultivars, the presence of synonyms (different names for the same cultivar) and homonyms (the same name for different cultivars), and the lack of information on many local varieties and ecotypes make particularly difficult the description and classification of olive varieties (Fabbri et al. 2009; Bracci et al. 2011).

The wild olive, also known as oleaster, coexists with the cultivated form in the same areas around the Mediterranean Basin. Wild olive trees have colonized diverse environments characterized by semiarid climatic conditions with different altitudes, vegetative communities, and soils, including those with extreme levels of drought, low temperatures, and salinity (Baldoni et al. 2006; Klepo et al. 2013). Various studies have revealed the presence of genuine wild olive forests (Lumaret et al. 2004; Belaj et al. 2007), with high levels of diversity (Baldoni et al. 2006; Breton et al. 2006; Belaj et al. 2007, 2010; Erre et al. 2010). Wild olives may be a useful source of variability to introduce into the cultivated olive traits such as biotic (Colella et al. 2008; Trapero et al. 2015; Arías-Calederón et al. 2015) and/or abiotic stress resistance (Murillo et al. 2005; Aranda et al. 2011), as well as to improve the olive oil health value and taste (Baccouri et al. 2008, 2011; Hannachi et al. 2008). As far as

presently known, there is no immediate reason for concern about demographic bottlenecks in wild olive populations (Belaj et al. 2007).

In addition to the wild and cultivated form, other five *O. europaea* subspecies are also recognized as primary resource for the cultivated olive (Green 2002; Brito et al. 2008). They have been distinguished into the following: (a) *laperrinei*, present in the Saharan massifs; (b) *cuspidata*, spread in Africa, from South Africa to southern Egypt, and in Asia, from Arabia to northern India and southwest China; (c) *guanchica*, only recognized in the Canary Islands; (d) *maroccana*, only known in southwestern Morocco; and (e) *cerasiformis*, present in Madeira islands (Green 2002). The analysis of ploidy level on samples of the different subspecies revealed that most of them are diploid, as all wild and cultivated olive, also sharing the same chromosome number ($2n = 2x = 46$) (Bitonti et al. 1999). While the subspecies *cerasiformis* and *maroccana* were reported as tetraploid and hexaploid, respectively (Brito et al. 2008; Besnard et al. 2008), in subsp. *laperrinei* has been recently shown the coexistence of two ploidy types (diploid and triploid) (Besnard and Baali-Cherif 2009; Besnard and Rubio de Casas 2016). The olive-related subspecies have shown high genetic diversity by means of both nuclear and cytoplasmic DNA markers (Baali-Cherif and Besnard 2005; Besnard et al. 2007, 2011, 2013; García Verdugo et al. 2009).

The survival of very ancient olive trees has been reported throughout the entire Mediterranean area (Michelakis 2002; Baldoni et al. 2006; Erre et al. 2010; Beghé et al. 2011; Diéz et al. 2011; Arnan et al. 2012; El Bakkali et al. 2013; Barazani et al. 2014; Bernabei 2015) and beyond (Hosseini-Mazinani et al. 2014), with some examples also found in countries where the

Fig. 1 The olive world germplasm collection at IFAPA (OWGB, CAP-UCO-IFAPA), Cordoba (Spain), accounting for more than 900 accessions. Olive trees in the field may be phenotyped for any trait of interest

olive cultivation has been introduced in the past 500 years (Soleri et al. 2010). The ancient olives are the living proof of the very long tradition of olive cultivation, and in most cases, their genotyping has revealed strong differences with varieties presently known (Barazani et al. 2014; Diez et al. 2011; Erre et al. 2010; Lazović et al. 2016). Thus, in addition to their historical and cultural significance, old olives offer horticultural interest due to their genetic potential, constituting an unexploited reservoir of olive diversity (Fig. 1).

2 Cultivated Germplasm

2.1 Features and Conservation of Cultivated Germplasm

The reasons leading to the high genetic variability of cultivated olive are manifold and mainly related to its allogamous and prevalently self-incompatible nature, the long tradition of cultivation, the survival of empirically selected varieties, the lack of turnover with new genotypes, and the great tree longevity (Baldoni and Belaj 2009; Beghé et al. 2011). Thus, each country, region, or valley of the Mediterranean Basin has maintained its own local traditional varieties, pollinators, ecotypes, and feral trees, in a patchwork of microenvironments and growing systems (Barranco and Rallo 2000; Bracci et al. 2009; Erre et al. 2010; Marra et al. 2013). Nevertheless, in the very recent years, the features of olive germplasm diversity have been subject to some drastic changes. In fact, in some areas of olive cultivation, in order to reduce the production costs and increase the yield, many traditional orchards characterized by local varieties have experienced a remarkable transformation into new intensive planting, with mechanical harvesting, advanced horticultural technologies, and controlled management, favoring the reduction in cultivars to those able to fulfill the requirements of these new intensive systems (Lavee 2013). Among the most important varieties that contribute to more than 90 % of total production are included the following: In Spain, olive-growing areas are dominated by the cultivars 'Picual,' 'Hojiblanca,' and 'Arbequina' (Belaj et al. 2010), being the last one the cultivar of choice worldwide. In Italy, the major cultivars are more numerous, including the main 'Coratina,' followed by 'Peranzana,' 'Ogliarola,' 'Carolea,' 'Frantoio,' 'Leccino,' and some others. In Greece, 'Koroneiki' is the main oil-producing cultivar, while Portugal and Morocco are, respectively, dominated by the cultivars 'Galega' and 'Picholine Marocaine' (Gemas et al. 2004; Martins-Lopes et al. 2007; Khadari et al. 2008; Fabbri et al. 2009). Despite the richness of olive varietal patrimony, only a limited number of them contribute to most of the olive oil and table olives production; meanwhile, most of the varieties only play a very local game and are represented by a few trees. The globalization of olive oil, table olives, and plant market, within and beyond the borders of Mediterranean area (Rehman et al. 2012), may boost the tendency to further reduce the cultivar diversification in modern olive orchards. In addition, in the near future, it is expected the spread into intensive plantations of new cultivars obtained by breeding (Rallo et al. 2008), likely causing the ultimate detriment of olive variability.

All the aforementioned factors may lead to a real erosion of the cultivated olive germplasm through progressive abandonment, substitution, and finally loss of autochthonous and local cultivars (Fabbri et al. 2009; Belaj et al. 2010; Haouane et al. 2011). Nevertheless, the traditional olive cultivars represent an important and yet poorly known genetic patrimony. Local cultivars could represent an imperative source of diversity against new and unforeseen climatic changes and in case of outburst of new pests and diseases, such as the case of *Xylella fastidiosa* (Abbott 2016; Almeida 2016). The exploration, collection, conservation, characterization, and evaluation of olive genetic resources are necessary steps against the genetic erosion risk and toward their efficient use in breeding programs (Barranco and Rallo 2000; Barranco et al. 2005; Bartolini 2008; Belaj et al. 2010).

Traditionally, olive cultivars have been maintained in ex situ field collections, which are aimed at acquiring, maintaining, documenting, assessing, and making accessible the genetic diversity of the crop, thus representing essential tools for any breeding program and to avoid and/or minimize the loss of total variation (Caballero et al. 2005, 2006; Bartolini 2008). In this sense, in the last twenty years, within the EU Resgen projects, the International Olive Council (IOC) has promoted a networking of National Germplasm Banks representing 22 different countries, focusing on sampling, cataloging, and conserving local olive cultivars in each partner country (Barranco et al. 2000, 2005; Trigui et al. 2002, 2006; Moutier et al. 2004; Mendil and Sebai 2006; Muzzalupo et al. 2010). An important methodological achievement of these projects was the establishment of a common record card for morphologically characterizing and cataloging olive cultivars (Barranco et al. 2000, 2005). As a main result, around 1100 accessions were collected and characterized so far in 2011 (http://www.internationaloliveoil.org/resgen/index.html). However, the use of different criteria of sampling, the lack of representativeness of plant material, the unequal efforts on cultivar identification and characterization among partners, and the presence of several collections per country (Caballero et al. 2006; Haouane et al. 2011) have, at least partially, reduced the effectiveness of this intervention. The establishment of three international germplasm collections, also supported by the same network, may supplement and further improve the work carried out by national collections.

The acknowledgment and/or creation of three World Olive Germplasm Banks, in Cordoba (Spain), Marrakech (Morocco), and Izmir (Turkey), aimed at protecting the olive genetic patrimony of all olive-growing countries and as insurance policy against potential risks due, for example, to pest and disease attacks, catastrophic events, or political limitations, which can hardly happen at three locations simultaneously. The conservation and further evaluation of the world olive diversity in different environments may also provide useful information for the scientific community and olive-growing industry (Figs. 2 and 3).

The Olive World Germplasm Bank of Cordoba (Spain) was established more than 45 years ago at the Center of the Agricultural, Fishery, Food and Organic Farming Research and Training Institute (IFAPA), 'Alameda del Obispo,' representing the first international attempt of conservation and management of the olive germplasm through a FAO-INIA project and with the IOC support (Del Río 1994; Caballero et al. 2005, 2006; IOC 2011). During these years, the collection has been enlarged with the acquisition of new accessions collected from national and international prospecting surveys, as well as with samples provided by different scientific institutions, including the EU-IOC Resgen partners (Barranco and Rallo 2000; Belaj et al. 2003a; Barranco et al. 2005; Caballero et al. 2005, 2006). The OWGB of IFAPA is continuously enriched with additional olive genotypes, particularly from the eastern Mediterranean countries, in order to improve their representativeness (Belaj et al. 2012, 2013; De la Rosa et al. 2015). In 2015, the collection, that is, now managed by a network of Andalusian institutions, providing new resources and facilities, accounted 900 accessions from 25 countries (De la Rosa et al. 2015; Belaj, unpublished data), more than half of which have already been characterized and identified by means of morphological descriptors and/or molecular markers (Belaj et al. 2004a, 2012; Barranco et al. 2005; Atienza et al. 2013; Trujillo et al. 2014). The plant material maintained in this collection is representative of all Mediterranean olive-producing countries The experience acquired at the OWGB of Cordoba and the progress in the knowledge about the olive germplasm have been of great help for the setting up of the other two international germplasm collections.

The second IOC international germplasm bank was established in Morocco in 2003 at the INRA (National Institute of Agronomic Research) experimental orchard of Tessaout, 70 km from Marrakech, by introducing previously characterized genetic resources from 14

Fig. 2 The OWGB at INRA—Marrakech (Morocco), presently including about 590 olive varieties. First trees were planted in 2003

Fig. 3 OWGB at the Olive Research Institute (Izmir, Turkey). Rootstock trees under preparation

Mediterranean countries (Haouane et al. 2011; El Bakkali et al. 2013). Extended to around seven hectares, the OWGB of Marrakech presently includes 591 accessions and the introduction of additional genotypes is ongoing.

The Olive World Germplasm Collection of Izmir (Turkey) is located at the Experimental Station of the Olive Research Institute, in Kemalpasa District. The allocated area for the collection occupies 26 ha, with two randomized blocks and two trees in each block. The implementation project started in 2012, and since then, numerous varieties have been incorporated into the collection over the years, reaching a number of 187 varieties from 13 different countries so far (Gurbuz, personal communication).

2.2 Characterization and Management of Cultivated Germplasm

Characterization of genotypes in olive germplasm collections is a very complex task due to the richness of cultivated olive germplasm, the confusion on cultivar denominations, the potential presence of intra-cultivar clonal variants, and the unbalanced organization of cultivar collections in different olive-growing countries (Belaj et al. 2004a; Baldoni et al. 2009; Bracci et al. 2011; Haouane et al. 2011). This reinforces the need to use efficient and reliable tools to distinguish, unambiguously, between cultivars and to clarify cases of synonyms and homonyms. Organization of prospecting surveys, protocols for collecting plant material, plant propagation, and growing, and assays for identifying and characterizing each genotype represent some of the challenging tasks that collection curators must face on a daily basis (Barranco et al. 2000, 2005; Barranco and Rallo 2000). Traditionally, genotype description has been based on morphological characterization, and the continuous efforts made to simplify this task (Ganino et al. 2006) have led to the adoption, by the International Union for the Protection of New Varieties of Plants (UPOV), of a common record card with 27 descriptors related to tree, leaf, fruit, and endocarp traits (Barranco et al. 2000, 2005). The use of these parameters has proved to be very useful to distinguish olive cultivars and detecting many cases of identity or diversity (Cantini et al. 1999; Barranco et al. 2000, 2005; Trigui et al. 2002, 2006; Moutier et al. 2004; Mendil and Sebai 2006; Strikiç et al. 2010; Ribeiro et al. 2012; Cordeiro et al. 2013; Muzzalupo et al. 2014).

In spite of the great utility for identification purposes, morphological description of olive cultivars remains an incomplete approach to classify each variety. Besides, morphological data are prone to environmental conditions and plant developmental stage (Belaj et al. 2001; Fabbri et al. 2009; Gomes et al. 2012). However, among the aforementioned morphological traits, those related to the endocarp have been recognized as the most effective, due to their high discrimination capacity, low sensitivity to environmental conditions, capacity of long conservation, and easiness of exchange among collections (Fendri et al. 2010; D'Imperio et al. 2011; Trujillo et al. 2014). Recently, image analysis has been suggested as an alternative method to allow a fast and automatic data capture of main plant organs (Vanloot et al. 2014) (Fig. 4).

2.3 Molecular Tools as a Means of Genotyping of Olive Varieties

The application of molecular tools has greatly overcome the limitations of morphological description (D'Imperio et al. 2011; Gomes et al. 2012; Rotondi et al. 2003; Trujillo et al. 2014). In olive, in fact, more than for other crop species, a high number of works was conducted for the molecular analysis of varieties, reflecting, to some extent, the history of molecular markers development. Advantages and disadvantages of different markers have been largely reviewed (Hatzopoulos et al. 2002; Belaj et al. 2003b; Ganino et al. 2006; Muzzalupo et al. 2009; Fabbri et al. 2009; Bracci et al. 2011; Gomes et al. 2012), evidencing that main molecular

Fig. 4 The international collection of olive varieties in Lugnano in Teverina (Terni, Italy), held by CNR, Umbria Region, and 3A-PTA. Trees were planted in 2014

techniques include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and, more recently, single-nucleotide polymorphism (SNP). RFLP, RAPD, and AFLP techniques (De la Rosa et al. 2003) have been largely superseded by dinucleotide SSRs, which represent to date the most popular markers in olive (Sefc et al. 2000; Carriero et al. 2002; Cipriani et al. 2002; De la Rosa et al. 2002; Sarri et al. 2006; Baldoni et al. 2009; Belaj et al. 2012; Muzzalupo et al. 2014; Trujillo et al. 2014). More recently, special attention is being paid to the development and use of SNP markers (Reale et al. 2006; Consolandi et al. 2007; Muleo et al. 2009; Hakim et al. 2010; Belaj et al. 2012; Kaya et al. 2013; Biton et al. 2015). Due to their abundance along the genome, coupled with the increase in information on olive genome sequence, SNPs are expected to become the markers of choice in the near future. In addition to the above-mentioned techniques, DArT (Diversity Arrays Technology) markers have provided a practical and cost-effective whole-genome fingerprinting without the need for sequence information, offering an interesting alternative for olive germplasm evaluation (Belaj et al. 2012; Dominguez et al. 2012a; Atienza et al. 2013) (Fig. 5).

2.4 Molecular Identification of Cultivars

Most of molecular studies in olive have been devoted to olive cultivar identification in the last 20 years. Molecular data have been used for the identification of olive cultivars coming from different regional olive groves (Poljuha et al. 2008; Rony et al. 2009; Albertini et al. 2011; Beghé et al. 2011; Linos et al. 2014; Fernandez and Martí et al. 2015), or national (Hagidimitrou et al. 2005; Fendri et al. 2010; Brake et al. 2014; Muzzalupo et al. 2014) and international collections (Belaj et al. 2001, 2004a, 2012; Sarri et al. 2006; Haouane et al. 2011; Atienza et al. 2013; Trujillo et al. 2014). The map of the identification works in olive does not only include main olive-producing countries, such as Italy (La Mantia et al. 2005;

Fig. 5 Fruiting shoots of some varieties at WOGBs of Cordoba and Marrakech

Muzzalupo et al. 2009, 2014; Cantini et al. 2008; Alba et al. 2009; Bracci et al. 2009; Corrado et al. 2009; Rotondi et al. 2011; Marra et al. 2013), Spain (Belaj et al. 2001, 2004b, 2010), Greece (Hagidimitrou et al. 2005; Roubos et al. 2010; Linos et al. 2014), Turkey (Kaya et al. 2013), Morocco (Khadari et al. 2008), and Tunisia (Hannachi et al. 2008; Fendri et al. 2010; Abdelhamid et al. 2013). Identification studies, by molecular markers, also include other Mediterranean countries, such as Portugal (Gemas et al. 2004; Martins-Lopes et al. 2007), France (Khadari et al. 2003), Israel (Barazani et al. 2014; Biton et al. 2015), Algeria (Dominguez-Garcia et al. 2012b; Abdessemed et al. 2015), Egypt (El Saied et al. 2012; Hegazi et al. 2012), Syria (Belaj et al. 2003a), Jordan (Hdeib and Hassawi 2010; Rawashdeh et al. 2009; Brake et al. 2014), Palestine (Basheer-Salimia et al. 2010; Obaid et al. 2014), Lebanon (Rony et al. 2009; Chalak et al. 2015), Albania (Belaj et al. 2003c), Slovenia (Bandelj et al. 2004), Croatia (Strikic et al. 2009), and Montenegro (Lazović et al. 2016). Outside the boundaries of Mediterranean area, molecular fingerprinting of olive cultivars has been performed in USA (Koehmstedt et al. 2010; Soleri

et al. 2010), Australia (Guerin et al. 2003; Rehman et al. 2012), Argentina (Torres and Prenol 2014), Brazil (Do Val et al. 2012), Colombia (Beghé et al. 2015), China (Zhan et al. 2015), Iran (Noormohammadi et al. 2007; Mousavi et al. 2014), and Iraq (Harbi et al. 2012), on varieties usually derived from the Mediterranean area, kept in ex situ collections and with doubtful identity (Fig. 6).

The molecular characterization of olive accessions in ex situ collections represents a powerful tool to distinguish different genotypes and to recognize duplications and errors, thus providing new identification means to the curators. Among the difficulties in olive cultivar identification, naming of cultivars represents one of the most important because, historically, it has been based on common morphological traits (particularly of the fruit), toponyms, or practical use of the varieties (Barranco et al. 2000; Belaj et al. 2001).

The use of molecular markers has shed light on the presence of many cases of homonymy in numerous collections, and in all cases, it has been demonstrated that generic names of olive cultivars include different genotypes (Belaj et al. 2003a, c; Noormohammadi et al. 2007; Fendri et al. 2010; Atienza et al. 2013; Trujillo et al. 2014). In order to distinguish among different genotypes carrying the same name, it has been suggested to add to the general name also the site of main diffusion (e.g., 'Manzanilla de Sevilla,' 'Manzanilla de Jaén,' and 'Manzanilla Cacereña') (Barranco and Rallo 2000).

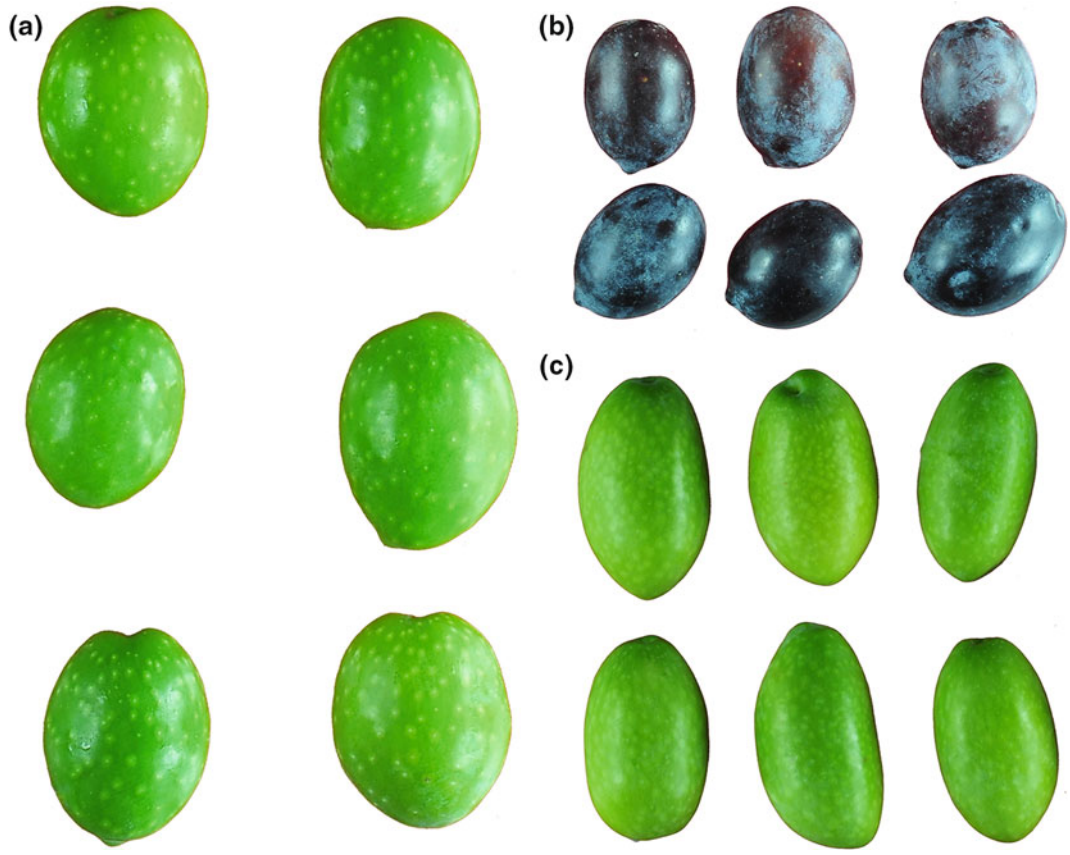

Fig. 6 Fruits from different varieties (Carolea, Grossa di Spagna, and Itrana) taken for image analysis

To ascertain the presence of duplicates in germplasm collections, it is as important as verifying and save as much variants as possible. The presence of redundant germplasm, i.e., accessions that slightly differ from the main profile of each cultivar, is often frequently within olive germplasm banks. Although at lower level than in other fruit trees species (0.2–0.3 % of pairwise comparisons), potential redundancies have been found by using SSRs (Haouane et al. 2011; Trujillo et al. 2014) and DArTs (Atienza et al. 2013) in the international olive germplasm collections of Marrakech and Cordoba (Fig. 7).

The scarce genetic differentiation found between some pairs of cultivars very likely excludes the possibility of sexually reproduced trees (Diez et al. 2011; Atienza et al. 2013), but other reasons might explain these potential redundancies (Atienza et al. 2013; Trujillo et al. 2014). Among them, the existence of synonymy cases might be a plausible cause of such low genetic differentiation between cultivars. Synonymy cases may include cultivars from the same country (Belaj et al. 2004a, b, c; Bracci et al. 2009; Fendri et al. 2010; Erre et al. 2010; Houane et al. 2011), as well as pairs or groups of cultivars from neighboring countries, likely reflecting a continuous migration and human displacement of olive cultivars in the Mediterranean Basin and beyond (Barranco et al. 2000; Besnard et al. 2001; Belaj et al. 2002, 2004; Soleri et al. 2010; Atienza et al. 2013; Trujillo et al. 2014). The presence of synonymy cases in olive germplasm collections may be a direct consequence of the use of ambiguous denominations of olive cultivars (Besnard et al. 2001; Belaj et al. 2001, 2004; Fendri et al. 2010; Trujillo et al. 2014). As a means of better management of synonymies, it has been suggested the naming of synonymy groups after the name that the variety has in its wider and original area of cultivation (Trujillo et al. 2014).

Another possible explanation of the presence of redundant germplasm should be attributed to the accumulation of somatic mutations that may occur in long-living and clonally propagated plants as olive (Belaj et al. 2004c; Diez et al. 2011). Somatic mutations are most likely to occur in highly variable and neutrally evolving genomic regions such as dinucleotide microsatellite regions, most widely used for the identification purposes in olive. Possible somatic mutations have been reported by means of different molecular markers in a wide range of varieties in Italy (Cipriani et al. 2002; Muzzalupo et al. 2010; Caruso et al. 2014), Spain (Belaj et al. 2004c; Diez et al. 2011; Ninot et al. in press), Croatia (Strikic et al. 2009, 2011), Portugal (Gemas et al. 2004; Martins-Lopes et al. 2009), Turkey (Ercisli et al. 2011; Ipek et al. 2012), and Montenegro (Lazović et al. 2016). However, taking into account the size of the olive genome of about 1500 Mb (Cruz et al. 2016; Loureiro et al. 2007) and the low rate of mutations, the chances to detect differences due to mutational events by the analysis of a very limited number of microsatellite regions are considered very low. In order to detect the eventual point mutations, different strategies should be undertaken, including the development of new and more informative markers (De la Rosa et al. 2013), or the resequencing of the

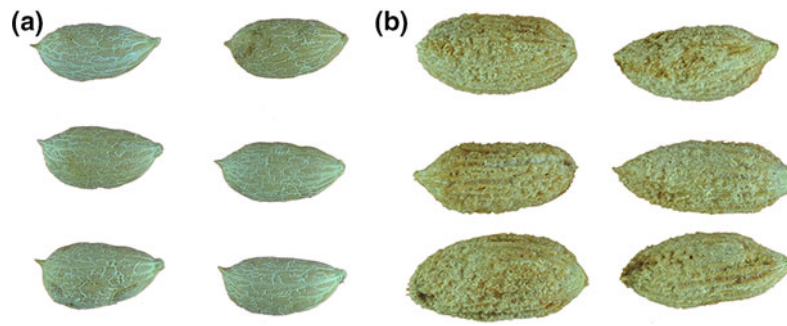

Fig. 7 Stones from varieties Frantoio and Gordal Sevillana

entire genome of doubtful varietal cases (Belaj et al. 2004c; Barazani et al. 2014; Lasoviç et al. 2016). A further step to solve this problem is then related to the relationship between somatic mutations and phenotypic variation, bearing in mind that a different genotype could only be considered as a distinct cultivar with a distinct phenotypic or agronomic performance (Barranco et al. 2005; Atienza et al. 2013; Trujillo et al. 2014), as well as for being uniform and stable (UPOV 2002; Diez et al. 2011). In most cases, somatic mutations would have no effect on phenotypic diversity (Belaj et al. 2004c). In such cases, Trujillo et al. (2014) suggested to consider the accessions as molecular variants, representing just cases of intra-cultivar variability (Fig. 8).

Molecular markers have evidenced scarce genetic differentiation among morphologically similar cultivars (Barranco et al. 2005), or among those collected in the same or close geographical areas, which may indicate the existence of prospecting redundancies in the germplasm collections (Atienza et al. 2013), or a closer parental relationship among varieties growing in the same site (Besnard et al. 2001; Baldoni et al. 2006).

Molecular analysis has also been able to identify the cases of errors (within and among accessions) in different stages of plant material conservation and management (Atienza et al. 2013; Belaj et al. 2003a; Haouane et al. 2011; Trujillo et al. 2014), due to mislabeling in the receptor and/or donor collections, propagation mistakes, as well as planting and replanting mistakes of the cultivars. In such cases, the curators should undertake activities to correct the detected errors, as well as replanting the correct cultivars. For such a purpose, accurate passport data are needed as genetic fingerprinting can detect inconsistencies within and between collections but cannot pinpoint when they may have occurred. In order to prevent and/or reduce the occurrence of different types of errors in the collections, discrimination of new accessions by means of molecular markers before their introduction into the collections or at early stages of tree growth may be of great help (Belaj et al. 2003a; Amokrane 2010; Dominguez-Garcia et al. 2012a).

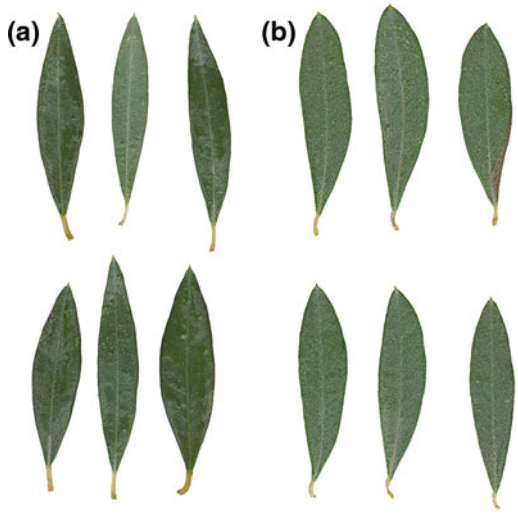

Fig. 8 Leaves from varieties Nocellara del Belice and Ottobratica

The decision on how to handle possible redundant germplasm is not an easy task. The combined use of current and new molecular data, along with morpho-agronomical evaluations at all levels, would be very useful to reduce and discard the possible redundancies. However, before the removal of duplicates, the curators should verify if (a) the accession does not belong to a unique genotype, (b) the accession is already conserved at its maximum safety, (c) it does not represent a highly demanded accession by breeders or other researchers, (d) it does not create disruption in the collection, and (e) there is an urgent need of alternative planting sites for the incoming new accessions. Thus, it should be necessary to integrate all the information to critically re-examine the composition of the collections and improve the management strategies (Atienza et al. 2013; Trujillo et al. 2014).

Cultivar fingerprinting should finally allow comparisons among collections, very useful for the authentication process, i.e., to guarantee that a cultivar distributed worldwide corresponds to the original cultivar growing in its area of origin (Trujillo et al. 2014). An accession is considered to be authentic if it matches the DNA and/or endocarp profiles of samples coming from donor collections or from prospecting surveys in the

area of origin of the putative cultivar to which it belongs (Barranco et al. 2000, 2005; Trujillo et al. 2014). The conservation of a reference collection of endocarps has proved to be a useful tool for this purpose in the World Olive Germplasm Bank of Córdoba (Barranco et al. 2005; Caballero et al. 2006; Trujillo et al. 2014). Although it should be an essential prerequisite for exchanging plant material, in order to avoid the extended confusion among denominations and true-to-type cultivar names (Bartolini 2008), to date, authentication is a pending task in most olive collections around the world (Trujillo et al. 2014). This may be mainly due to the lack of voucher samples (endocarp and/or DNA) of each accession in the collections, the scattered and partial characterization in many olive-growing countries, the displacing or loss of many local old cultivars from their autochthonous areas, as well as the incomplete or inaccurate passport data of the accessions (Trujillo et al. 2006, 2014; Atienza et al. 2013).

Although molecular markers play an important and active role at its improvement, management of olive germplasm collections remains a complex and costly task. Thus, the practical utility of any molecular approach for germplasm management is partly determined by the ability to differentiate between large numbers of accessions. The greatest challenges for the identification of cultivars by means of molecular markers is to reduce the risk of classifying two random accessions under the same molecular profile (Belaj et al. 2004a). In this sense, various studies have tried to determine the minimum number of markers that can reliably identify a large set of cultivars with very low confusion probabilities. Thus, the combination of three SSR markers was considered very effective for the discrimination of a large number of cultivars (Sarri et al. 2006); meanwhile, Haouane et al. (2011) found that three out of 12 SSR loci were able to distinguish about 80 % of the 505 defined genotypes under study (Fig. 9).

Besides the important results obtained by several independent studies at the characterization

Fig. 9 Wild olive bushes growing in a natural area

and identification of olive cultivars, the comparison of data between different collections and laboratories is not a straightforward task. In this regard, the use of a common set of 11 very high discrimination SSR markers has been proposed for the study of olive genetic resources (Baldoni et al. 2009). The use of the selected markers and the application of a common strategy for data comparison would allow data convergence, a preliminary condition for genotype identification. However, the comparison and convergence among different sets of SSR data still persists as a hard task, considering that size discrepancies among alleles at the same locus obtained in different laboratories may reach up to 5–6 base pairs (Doveri et al. 2008; Baldoni et al. 2009; Beghé et al. 2015). Based on their high discrimination capacity, the use of three nested sets of SSR has also been proposed for an efficient and progressive identification of olive cultivars in germplasm banks, whereas the use of 17 SSR loci allowed to distinguish between all accessions under study (Trujillo et al. 2014). On this respect, an ongoing European project (BeFOre—Bioresources for Oliviculture, 2015–2019), involving 15 countries all over the world, is aimed at aligning common protocols for both molecular and morphological characterization of olive genetic resources at international level.

Recent advances in olive sequence information have enabled the use of sequence repositories such as ESTs as a source of long core repeat SSRs (De la Rosa et al. 2013; Muñoz-Mérida et al. 2013). However, even if SSR with longer repeats were easier to analyze, their variation revealed to be lower (De la Rosa et al. 2013). Based on the sequence of genes or other genome regions, wide sets of SNP markers are being identified and used for genotyping purposes (Kaya et al. 2013; Biton et al. 2015). They could be applied at high-throughput scale (thousands of markers available from single runs) and could be screened at a single centralized platform, thus allowing for a rapid genotyping of a large number of accessions and for comparing data from all germplasm collections.

Molecular markers, employed for the identification of olive varieties, are directly applicable to the analysis of DNA derived from olive oil. In fact, in the last years, many efforts have been dedicated to improve the methods of DNA extraction from olive oil as well as to identify the suitable and reliable markers to distinguish different cultivars in a blended oil, thus revealing its varietal composition (Consolandi et al. 2008; Agrimonti et al. 2011; Bracci et al. 2011; Rossi et al. 2012; Baldoni et al. 2013; Ben Ayed et al. 2013). Some efforts to identify fruits of table olive cultivars have also been performed as well (Pasqualone et al. 2013; Raieta et al. 2015). On the contrary, rare efforts have been dedicated to the identification of nursery plant material for true-to-type nursery stock certification (Belaj et al. 1999; Doveri et al. 2008; Rehman et al. 2012).

2.5 Genetic Diversity and Relationships Within the Cultivated Germplasm

Systematic fingerprinting of olive germplasm collections is providing a detailed knowledge of the amount and distribution of genetic diversity within the species. Knowledge on the genetic diversity among olive cultivars is essential to maximize the exploration and use of the germplasm resources and for the long-term success of breeding programs.

High level of polymorphism, genetic diversity values, and allelic variations have been revealed by means of different molecular markers in olive tree at regional or very local level (Cantini et al. 2008; Beghé et al. 2011; Delgado-Martinez et al. 2012; Marra et al. 2013; Fernandez and Martí 2015), as well as at national (Hagidimitrou et al. 2005; Muzzalupo et al. 2009; Belaj et al. 2010; Fendri et al. 2010; Linos et al. 2014; Brake et al. 2014; Kaya et al. 2013; Abdessemed et al. 2015; Lazović et al. 2016) and Mediterranean levels (Belaj et al. 2002; Owen et al. 2005; Sarri et al. 2006; Haouane et al. 2011; Atienza et al. 2013; Trujillo et al. 2014). Similar to other woody perennial outbreeding species which maintain most of their variation within a population, most of the olive diversity is attributable to differences

among cultivars within each region, country, or wider area (Belaj et al. 2003b, 2012). Thus, breeders in each olive-growing country may rely on the autochthonous olive genetic resources for the design of breeding programs, since there is a sufficient genetic variability among native cultivars (Belaj et al. 2002). However, there is still a strong need to improve the knowledge on the level and distribution of variability (Fig. 10).

Development of gene-based strategies will enable the screening of olive germplasm for the presence of specific alleles. The increasing information on the transcriptome and genome sequences (Muleo et al. 2012; Muñoz-Merida et al. 2013; Carmona et al. 2015; Alagna et al. 2016; Cruz et al. 2016) will allow for the identification and characterization of important genes involved in agronomic and productive traits (Alagna et al. 2009; Yanik et al. 2013; Cultrera et al. 2014; Iaria et al. 2016), biotic and abiotic stress resistance (Bazakos et al. 2012; Gómez-Lama Cabanás et al. 2015; Guerra et al. 2015), and important development characters, such as juvenility (Fernandez-Ocaña et al. 2010; Jiménez-Ruiz et al. 2015). This unprecedented knowledge on the variation at genome level will finally offer new opportunities for diversity evaluation, population analysis, and genome-wide association studies.

Molecular markers are important tools to analyze the genetic relationships among varieties with different geographical origin (Besnard et al. 2001; Belaj et al. 2003a, 2010, 2012). Clustering of olive cultivars according to their putative geographical distribution has been revealed at regional (Belaj et al. 2003b; Marra et al. 2013; Muzzalupo et al. 2009; Isk et al. 2011; Kaya et al. 2013; Linos et al. 2014) and large-scale level (Besnard et al. 2001; Belaj et al. 2002, 2010, 2012; Baldoni et al. 2006; Haouane et al. 2011; Dominguez-García et al. 2012a; El Bakkali et al. 2013; Muzzalupo et al. 2014). Three main

Fig. 10 Ancient olive tree in Fasano (Brindisi, Apulia, Italy)

gene pools have been described in the Mediterranean Basin, as well as evidences of a diversification of cultivated olive from east to west Mediterranean (Haouane et al. 2011; Belaj et al. 2012; Besnard et al. 2013; El Bakkali et al. 2013). The clustering of cultivars originating from the same or nearby geographical areas likely suggests that multilocal selection and breeding of olive cultivars occurred in each area of present diffusion (Besnard et al. 2001; Belaj et al. 2002, 2012; Owen et al. 2005; Dominguez-Garcia et al. 2012a). Local adaptation can explain some differences between accessions and could be of great interest for olive breeding.

Correlation between olive cultivars and their usage have also been observed (Hagidimitriou et al. 2005; Brake et al. 2014; Abdessemed et al. 2015; Biton et al. 2015). Thus, a multilocal selection of olive cultivars with common selection pressure toward the large fruit size and the enhancement of olive oil content might have occurred during the olive domestication process (Besnard et al. 2001; Belaj et al. 2002; Hagidimitriou et al. 2005; Breton et al. 2006).

Knowledge on the genetic similarity among genotypes may facilitate the efficient sampling and utilization of germplasm resources by identifying unique or very distinctive gene pools, overrepresentations or gaps of cultivars from certain geographical areas, and the need to evaluate phenotypic variability on a restricted set of genotypes (Haouane et al. 2011; Belaj et al. 2012; El Bakkali et al. 2013; Trujillo et al. 2014).

The development of core collections has been suggested as a means of reducing the gap between the available diversity in olive germplasm collections and its use (Haouane et al. 2011; Belaj et al. 2012; El Bakkali et al. 2013). Chosen to maximize the genetic diversity of olive collections in a reduced number of accessions, these core collections could facilitate the study of the variability and correlation of morphological and agronomical traits in comparative trials. Additionally, they represent an ideal set of genotypes for supporting ongoing efforts of olive genomics and sequencing, validation of new molecular markers, and exploration of their linkage with agronomic traits of interest for breeders.

2.6 Phenotyping and Agronomical Performance of the Olive Varieties

The ex situ conservation of the genetic resources allows for the agronomic evaluation of many olive cultivars in the same environmental conditions, giving thus the opportunity for a general view of their diversity (Caballero et al. 2005, 2006). In general, there is a delay between sample collection and its agronomic evaluation, due to the time (at least six years after planting) required for the trees to reach maturity and the need of multiannual systematic measurements of each trait (Del Río et al. 2005). In fact, in comparison with the proliferation of morphological and molecular studies performed on olive germplasm, there is still limited information on their agronomical behavior. However, agronomical traits of olive cultivars have been evaluated relating to vigor, production, phenology and fruit characters (Caballero et al. 2005, 2006; Ozkaya et al. 2006; Taamalli et al. 2006; Hannachi et al. 2008; Trentacoste and Puertas 2011; Di Vaio et al. 2013), oil content and composition (Beltrán et al. 2004, 2016; Alba et al. 2012; Ruiz Dominguez et al. 2013), disease resistance (Lopez-Escudero and Mercado Blanco 2011; García-Ruíz et al. 2014), and abiotic stress tolerance (Alcantara et al. 2003).

Agronomical evaluation studies are being performed in the three international olive germplasm collections, but most of them are still referred to the OWGB of Cordoba, due to the long tradition and the recent establishment of the other two. High levels of diversity have been found for all the agronomic traits under evaluation (Caballero et al. 2005, 2006). The results of such evaluations are very important to identify cultivars with outstanding agronomical performance, for their use in olive production and as potential parents in future cross-breeding programs (Rallo et al. 2008; Lavee 2013). Taking into account the low number of tree replicates

(2–4) per cultivar and the fact that some agronomic and quality traits are environmentally dependent, the evaluation of the olive varieties within germplasm collections should be considered as a preliminary step before a complete wide bioagronomical evaluation. In this sense, the establishment of comparative trials in different agroclimatic conditions and with many replicates is the most efficient way to determine the best-suited varieties for each specific area (De la Rosa et al. 2013). The information obtained in comparative trials may be very important for olive growers and for the diversification of olive plantations with cultivars previously not assessed in different environments, thus contributing to a better use of genetic resources. However, comparative trials have not been extensively realized until recent years and published results are scarce (Del Río et al. 2005; Dabbou et al. 2011).

One of the challenges in the near future should be to link the information obtained by molecular and morphological descriptors with data on the phenotypic profile of olive cultivars, in order to provide a complete understanding of the diversity available and the ways it could be best managed and used. The integration of

Fig. 11 Ancient olive tree in Vuves (Crete, Greece)

present and future information on olive cultivars and the establishment of common strategies of their study and evaluation are the mandatory tasks in order to build a universal database of olive genetic resources (Fig. 11).

3 Wild Olives

Oleasters (*Olea europaea* subsp. *europaea* var. *sylvestris*, named oleaster) represent the wild form of olive, naturally occurring along most of the Mediterranean shores, growing within the maquis or as isolated bushes or trees. They are propagated by seeds and usually disseminated by birds. Wild olive populations may be present in forest areas associated with other typical Mediterranean species, such as *Quercus ilex*, *Pistacia lentiscus,* and *Phillyrea angustifolia*. Genuine wild olives, corresponding to natural populations evolving without any human intervention (or with very restricted impact), can be found in those ecological habitats far from agroecosystems (Lumaret et al. 2004; Breton et al. 2006; Belaj et al. 2007, 2010). Besides, wild plants can be easily confused with the feral forms, which can be defined as secondary sexual derivatives of the cultivated olive or issued from hybridization between cultivated trees and oleasters. They can be also found in the same habitats, especially in open areas not far from cultivated fields (Lumaret et al. 2004; Baldoni et al. 2006; Belaj et al. 2010). Therefore, wild olive can be considered as a complex ranging from genuinely wild to feral forms.

Oleasters mainly differ from cultivated olive by the presence of smaller fruits with less fleshy mesocarp and usually lower oil content (Belaj et al. 2011), and seedling juvenility may last for several years (Pritsa et al. 2003). Hence, morphological differences between wild and domesticated olives are quite vague and it may be difficult to distinguish the two forms.

In recent years, the patterns of genetic variation and the relationships between wild forms have been evaluated by allozymes, genomic, and cytoplasmic DNA markers (Lumaret et al. 2004; Mariotti et al. 2010; Belaj et al. 2007, 2011; Besnard et al. 2011), as well as between wild and cultivated olives at a narrow scale (Bronzini de Caraffa et al. 2002; Baldoni et al. 2006; Breton et al. 2006; Belaj et al. 2010; Erre et al. 2010; Hannachi et al. 2008, 2009; Diez et al. 2011), or over the whole Mediterranean Basin (Besnard et al. 2002, 2013; Lumaret et al. 2004). These studies have evidenced the persistence of genuine wild olives in the western Mediterranean area (Lumaret et al. 2004; Baldoni et al. 2006; Breton et al. 2006; Belaj et al. 2007), northern Levant, Cyprus, and Turkey (Besnard et al. 2013; Yoruk and Taskin 2014) and a clear distinction between eastern and western Mediterranean oleasters (Besnard et al. 2002; Lumaret et al. 2004; Breton et al. 2006; Rubio de Casas 2006). The distinction between true oleasters and feral forms has been based on geo-ecological parameters (Lumaret et al. 2004) or on molecular markers (Baldoni et al. 2006; Breton et al. 2006; Belaj et al. 2007, 2010). It is difficult to identify clear-cut genetic boundaries between genuine wilds and feral germplasm. Different studies indicated that reproductive isolation of wild olives is highly unlikely and that genetic material seems to be exchanged frequently among different populations (Rubio de Casas et al. 2006; Belaj et al. 2007). A high genetic differentiation between supposedly genuine wild olives and olive cultivars has been reported in Corsica (Bronzini de Caraffa et al. 2002), Morocco (Lumaret et al. 2004), Italy (Baldoni et al. 2006), Spain (Belaj et al. 2010; Diez et al. 2011), Tunisia (Hannachi et al. 2008), and Turkey (Yoruk and Taskin 2014). Besides, genetic diversity and relationship studies between oleasters and olive cultivars have also contributed to elucidate the history of domestication of olive cultivars (Kaniewski et al. 2012; Besnard et al. 2013; Diez et al. 2015; Besnard and Rubio de Casas 2016; see Chap. 4 and the references therein).

The majority of studies carried out in wild olive populations did not have a direct implication on conservation of wild olive genetic resources and their use for breeding purposes. Traditionally considered of low agroforestry

value (Mulas and Francesconi 1999), wild olives may represent an interesting gene pool to enrich the genetic basis of cultivated material as they may contribute a wider allelic richness (Lumaret et al. 2004) and genetic variability (Baldoni et al. 2006; Breton et al. 2006; Belaj et al. 2007, 2010; Erre et al. 2010). In this sense, although still scarce (Baldoni and Belaj 2009), the in situ evaluation of wild olive trees in Italy (Mulas and Francesconi 1999), Tunisia (Hannachi et al. 2009; Baccouri et al. 2011; Dabbou et al. 2011), and Spain (Belaj et al. 2011) has given some insights into the potential value of oleasters as a source of morpho-agronomical interesting traits. Being found in a high diversity of environments, altitudes, and soils, wild olives may be a very important source of resistance to important diseases, such as Verticillium wilt (Colella et al. 2008; Bubici and Cirulli 2012; Trapero et al. 2015; Arías-Calderón et al. 2015) and other biotic (Ciccarese et al. 2002; Mkize et al. 2008; Sesli et al. 2010) and abiotic stresses (Mulas 1999; Meddad-Hamza et al. 2010; Aranda et al. 2011). In addition, some studies have evidenced the breeding potentiality of oleasters for olive oil quality (Baccaouri et al. 2008, 2011; Hannachi et al. 2008), productivity, and fruit set (Hannachi and Marzouk 2012), as well as oil quality. On the other hand, ongoing climate change and the shortage of water resources raise the need of future selection and breeding works in olive tree to look for cultivars resistant to drought and other adverse environmental conditions. And, in this context, wild olives may represent an useful source of genetic variability.

Despite all these characteristics, further studies on the real potential value of oleasters are needed (Baldoni and Belaj 2009), in order to increase the possibility to introduce novel and superior alleles into cultivated varieties. Some selection works based on the use of feral olive populations as another alternative for olive breeding are being carried out in Australia (Sedgley 2000; Guerin et al. 2003) and Spain (Klepo et al. 2013, 2014). The combined use of both, cultivated and wild genotypes in olive breeding programs, could boost the heterosis in the resulting progenies (Biton et al. 2012) and could shorten the juvenile period of their descendants (Klepo et al. 2013, 2014).

To better profit by the resource of olive wild relatives, an ex situ collection of oleasters coming from populations naturally growing in adverse and heterogeneous ecological conditions is being carried out at IFAPA, Centre 'Alameda del Obispo,' Cordoba, Spain (Belaj et al. 2010). A first agronomical evaluation for traits such as fruit size and fruit oil content has been conducted (De la Rosa et al. 2013).

Finally, although there is no immediate reason for concern about demographic bottlenecks in wild olive populations, a strong and continuous gene flow between wild and cultivated germplasm may lead to a slow but steady genetic erosion of the genuine oleaster gene pool. This reinforces the need of continuous efforts on prospecting, analyzing, and monitoring native Mediterranean forests that should help to identify additional genuine oleasters.

4 Ancient Olives

The genetic patrimony of olive, including several major and minor varieties and wild olives, is further enriched by the ancient trees still conserved in situ. The extraordinary longevity of the species and its high adaptation to different environmental constraints have allowed the survival of numerous monumental olives. These trees hold main genetic, agronomical, naturalistic, landscape, and historical importance. The distribution of these olives spreads around the Mediterranean Basin up to Middle East. Thousand plants are still present in Italy and Greece, followed by Spain, Portugal, Near- and Middle East countries, but their number is decreasing every year due to numerous environmental and human threats (Pannelli et al. 2010; Diez et al. 2011; Barazani et al. 2014).

The study of monumental olives could help to find variants showing strong adaption, to clarify the origin and diffusion of olive varieties and to rediscover the paleo-varieties cultivated in the past and now abandoned. They could represent reservoirs of genetic characteristics,

which could help to face present and future cultivation problems due to climate change, pollution, and reduction in resources, and should be valorized as local production at high regional value (Cicatelli et al. 2013), landscape additional rate (Meilleur and Hodgkin 2004), eco-tourist icons, and suitable bioindicators (Moriondo et al. 2013). These trees should undergo an overall survey and a molecular and agronomical evaluation. Molecular characterization of many samples has demonstrated that these ancient trees often represent unknown genotypes (Pannelli et al. 2010; Diez et al. 2011) frequently with high constant fruit production and considerable oil quantity and quality (Cicatelli et al. 2013) and with a valuable tolerance to abiotic stresses.

Territorial prospecting surveys are the first step to locate the most relevant ancient trees, through territorial scanning, reports of historical documents and maps analysis, or interviews to local olive growers. The second step is the geographical localization of each tree by GPS (Global Positioning System) and collecting general information on plant status, including plant diseases, tree size (canopy height, trunk diameter, number of trunks, etc.), and soil and climate characteristics of the site. Finally, a thorough morphological characterization is followed by the molecular analysis of samples from the canopy and from the trunk base, in order to analyze also the possible rootstocks, in case of grafted plants (Michelakis 2002; Pannelli et al. 2010; Diez et al. 2011; Barazani et al. 2014). In fact, recent researches on pools of ancient olives have demonstrated that grafting practices have been widely applied in the past, especially in the Middle East, by using seedlings, preselected clonal rootstocks, or oleasters (Baranzani et al. 2014), at a lower extent in the western part of Mediterranean Basin or in central Italy (Pannelli et al. 2010; Diez et al. 2011).

A main problem in studying ancient olive trees is related to estimating their age. Difficulties rely on the peculiar wood growth, the rapid decay of the original trunk, and the development of numerous new trunk bundles. For this reason, dendrochronology may not be applied, but only used as complementary information to detect the plant age (Arnan et al. 2012). This method cannot individuate the real age of olive trees even with the most accurate imaging analyses (Cherubini et al. 2013). Age determination by radiocarbon (^{14}C) dating may be helpful in establishing the age of most ancient wood along main trunk, but ^{14}C analysis should be supported by a thorough evaluation of a number of parameters (environmental, climatic, genetic, etc.), using algebraic formulas that will provide an estimate of the true age of the plant. The literature regarding the radiocarbon dating individuated a maximum wood's age for an olive tree (still alive) of 635 ± 35 years at Garden of Gethsemane (Israel) (Bernabei 2015), and by using the specific algebraic formulas, the most ancient olive tree was found in Umbria (center of Italy) with an estimated age of 975 ± 150 years (Pannelli et al. 2010). Another innovative approach tried to apply 3D model in order to individuate, as much accurately as possible, the dimension and volume of the ancient tree, but more interesting results will be available when this technique will be applied year by year, in order to establish the tree's growth incremental model, as suggested by the authors (Maravelakis et al. 2012). Anyhow, the evaluation of age for ancient olives cannot be related only to trunk dimension, but must take into consideration other parameters, such as genotype vigor, days of seasonal growing, and climatic and soil conditions.

The conservation efforts are linked to rural development (Altieri et al. 1987); thus, in situ/on-farm conservation of ancient olive trees could represent a valuable way to preserve this important genetic source for the future generations. In addition, considering the potentiality of the ancient olive trees, the action of safeguard in situ/on-farm should be mandatory for all of them. Taking into account the parameters such as stress resistance, adaptation to environmental constraints, productivity, estimated age, and olive oil quality, the most interesting genotypes should be safeguarded as original mother plants, then propagated and conserved and evaluated in ex situ collections and field trials.

Molecular identification of some ancient olive trees has demonstrated that some of them were clonally propagated, meaning that they probably represent the remnants of ancient olive cultivations, since hundreds years ago (Pannelli et al. 2010; Diez et al. 2011).

Their spreading and survival under cultivation or in natural conditions may not be considered random, but it represents the result of centuries of experience in their productive behavior or a high degree of environmental compatibility (Pannelli et al. 2010). The outstanding performance of these trees makes their agronomical evaluation and possible use in olive breeding programs especially useful (Diez et al. 2011), as well as creating an international database and catalog, in order to locate the hotspot areas of ancient olives and to increase the cooperation between plant genetic resource managers and plant conservation communities (Meilleur and Hodgkin 2004), in order to avoid losing forever this priceless olive germplasm.

5 Concluding Remarks on Olive Genetic Resources

Olive represents an unusual case among horticultural crops, and its germplasm could constitute a particularly rich source of variability to be directly used or maintained for future breeding.

A better knowledge, management, and exploitation of cultivated, wild, and ancient trees are still needed, establishing common protocols for the molecular identification and for cultivar naming, whereas accurate phenotyping and morpho-agronomical evaluation of plants at germplasm collections and establishment of comparative trials to evaluate the agronomical performance are also envisaged.

Populations of wild relatives, widely diffused in the same areas of cultivation or close to them, represent a potential source of variability for olive breeding to face new agricultural challenges and ongoing climate change.

Combined application of high-throughput genotyping and phenotypic evaluation will allow performing more effective genome-wide association studies and able to identify genomic determinants of important agronomic traits, thus assisting and speeding up the genetic improvement efforts of the crop.

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Olive Growing in a Time of Change: From Empiricism to Genomics

4

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Abstract

Since its beginning, the olive crop has been a long-lived agricultural system in the Mediterranean Basin being well adapted to this area. Traditional olive growing, still prevalent in most producing areas, is characterized by low tree density and rainfed orchards with low yield and manually harvested. The traditional olive growing technology is local, diverse, and empirically based. New high density, irrigated, and mechanically harvested orchards has been progressively planted since the end of World War II. These plantations produce high crops at low costs, but they reduce the diversity of cultivars, increase the demand of inputs and the risk of environment unbalances. The expansion and intensification of olive growing, and the perception of olive oil and table olives as healthy foods, have largely increased the production of these products. However, the intensification and expansion of olive growing to new regions is also raising some concerns related to genetic erosion, the adaptation of cultivars, the spread of biotic agents, the scarcity of water, and the increase of soil erosion, among others. New technological advances in olive growing and breeding, and the development of new disciplines such as genomics promise to be of outstanding role to guarantee the conservation and sustainable use of the olive genetic diversity and the rational use of natural resources.

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1 Introduction

The origin of olive growing is associated with the discovery of vegetative propagation using cuttings in the Middle East about 5500 years ago. Since that time, the crop has expanded westward along both shores of the Mediterranean Basin (Besnard et al. 2013; Díez et al. 2015). The expansion of Phoenicians to Central and Western

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Mediterranean coasts, approximately 3000 years ago, spread the olive growing across the Mediterranean Basin. This crop was well established by Roman times as witnessed by the treatises of Pliny and Columella. Olive oil exports from Andalusia towards the rest of the Roman Empire are well documented by 'Mount Testaccio,' a rubbish dump remain of oil amphorae, which was found in Rome. The subsequent changes in olive growing and in the demand of its main products, oil and table olives, have been discontinuous, with periods of expansion and contraction associated with demographic changes and political factors. For instance, the expansion of olive growing in American colonies in the sixteenth century was arrested by the Spanish Policy, which tried to promote the export of olive oil from Spain to the American colonies, in the seventeenth and eighteenth centuries. Nowadays, a significant olive growing expansion is taking place in America and Australia.

2 Olive Growing in the World: Production, Consumption, and Trade

According to the International Olive Council (IOC 2016), currently there are more than 10.3 million ha of olive orchards in more than 40 countries; the majority of this surface (97.9 %) is localized in the Mediterranean Basin. In this area, traditional olive groves and new olive orchards represent the basis of the current olive oil and table olive industries. However, in new olive producing countries, such as Australia or Argentina, intensive orchards, which are irrigated and highly mechanized, represent most of the new planted areas.

The expansion and intensification of olive growing and the perception of olive oil and table olives as healthy foods have increased the production and consumption of these products worldwide. In the last 25 years, the production of olive oil and table olives in the world has increased from 1874 to 2881 thousand tons and from 960 to 2564 thousand tons, respectively. In the current global market scenario, the olive oil, the only vegetable oil obtained from a fruit by mechanical processes, represents less than 4 % of total vegetable oils, resulting as a high-value 'commodity' mainly consumed in the producing countries and in the richest nations.

According to the IOC, the EU countries lead in olive oil production, and among them, Spain is the leading producing country with about 1.4 million tons of olive oil, which represents about 50 % of world production. This country has increased the area dedicated to the olive tree with new plantations, which are irrigated, specialized, and fully mechanized. Italy, with 470,000 tons of olive oil (campaign 2015–2016), of which about 60 % is Extra Virgin Olive Oil (EVOO), is the second largest producer of olive oil, ahead of Greece (310 thousand tons) and Portugal (99 thousand tons).

Among other IOC partners, some Mediterranean countries have lately increased their production. For instance, Tunisia is currently producing an average of 190 thousand tons of olive oil per year; Turkey 160 thousand tons, and Morocco 120 thousand tons. In the latter country, since 2008 a deep technological modernization process promoted by a governmental program called 'Maroc Vert' is ongoing in the olive oil sector. This program has led to an improvement in quantity and quality of the olive oil production and to an increase of the exports, which are currently around 100,000 tons/year.

According to the Council (IOC 2016), the global consumption of olive oil, in the period between 2011 and 2016, was approximately 3 million tons. The main consumers are the EU countries, which absorb about 1.6 million tons of olive oil, corresponding to 55 % of the total consumption. Italy is the first olive oil consumer in the world, with an average over the last five years of 585,000 tons (20 %), followed by Spain, with 515,000 tons (17 %), Greece 158,000 tons (5 %), and France 106,000 tons (3.5 %).

Among non-European countries, the major consumers of olive oil are the USA with approximately 308,000 tons, followed by Syria 170,000 tons and Turkey 124,000 tons. Japan is

currently developing a surprisingly high interest for olive oil; its average annual consumption of olive oil has increased from only 4 thousand tons in 1991 to 60,000 tons in 2016. Furthermore, because of the growing interest of consumers in functional foods that promotes health, in the last four years, the consumption of such category of olive oil has increased at a rate of around 1 % per year.

Over the past five years oil, exports worldwide have accounted for more 800 thousand tons. Spain and Italy are the largest exporters, with export shares in 2015–2016 of 31 and 29 %, respectively. Tunisia is the third exporting country (15 %), followed by Portugal and Greece. Morocco, the USA, and Palestine have doubled their export in the last five years.

Italy is also the largest importer with a share of 40 % of the value of world imports in 2014. Other major importing countries are the USA (15 % of world imports), France (6 %), Spain (6 %), the UK (4 %), Germany (4 %), Portugal (4 %), Japan (3 %), and Australia (2 %). EU imports the largest amount of olive oil from Tunisia (50 % of EU imports), Turkey (22 %), Syria (15 %), and Morocco (9 %). Japan and Canada import almost all the olive oil that they consume (96 %) from the EU.

3 Plantations

Nowadays, the gradual change from empirical based practices to progressively scientific based technologies represents an outstanding innovation for olive growing. This change will summarize the transformation from traditional groves to new olive orchards (Rallo et al. 2013).

3.1 Traditional Orchards

Several patterns characterize the traditional olive groves in Mediterranean countries. The large size of the trees, a common feature in many plantations, constitutes a difficulty for harvesting the crop. Besides that many plantations are more than 50 years old, and some others more than 100. Grove longevity represents a major constraint to social and economic changes that are taking place nowadays because obsolescence becomes a common feature in many olive groves.

The olive tree is very well adapted to the Mediterranean climate, which is characterized by a mild and short winter and a long, dry, and hot summer. Olive has developed adaptation mechanisms to summer water stress insuring the survival of the trees at the expense of reducing crop load. Up to recent years, olive orchards were restricted to marginal soils, many of them on sloping lands, without possibility of irrigation. These orchards were characterized by low yields. The increasing demand for olive oil and table olives has traditionally been achieved by planting more olives in new and less productive soils. Because of this reason, while more than one million hectares of olive trees were planted in Spain between 1888 and 1972, the yield per hectare decreased.

Harvesting olives has been a highly intensive hand-labor task. More than 70 % of the total labor demand in an olive orchard today is required just for harvesting. Traditionally, even today, olive harvest has provided the principal source of work in many rural areas. This is a major reason why olive expansion has been historically associated with periods of demographic increase. The progressive concentration of plantations in many olive-producing zones of the Mediterranean world has triggered a temporary labor demand to attend olive harvesting. Temporary migration during the 2–4 months of olive harvest and cultural celebrations associated with the end of the harvest are common features in the olive world. However, the unemployment rates during the rest of the year, the biennial trend of the olive tree crop, and the cyclic crisis of oil prices have been considered a major cause of social instability in zones with olive monoculture.

Most of the traditional olive growing technology is local and empirically based; the cultivars and the pruning practices are clear examples. Olive cultivars in almost all the Mediterranean countries are locally selected

individuals within open-pollinated seedlings that have been vegetatively propagated by farmers for many centuries. As a rule, the spread of most cultivars was limited to their supposed area of origin. In Spain, for example, 24 cultivars accounted for most of the cultivated areas, spreading just to contiguous areas. Only two main cultivars, 'Manzanilla de Sevilla' and 'Empeltre', both propagated by grafting, were largely cultivated out of their original area of diffusion. Thus, farmers have used the best performing cultivars among the many selected in most olive growing areas. Also pruning practices, either to train or renew the trees, as much as to increase fruit size for table olives, are empiric, local, and diverse. Reducing tree size to facilitate the harvest has been a common feature of many pruning practices. However, different approaches have led to several local pruning strategies in different growing areas.

3.2 The Crisis of Traditional Orchards

After the World War II, migration towards cities reduced rural population in olive growing areas of Southern Europe. First in Italy and afterward in Spain, Portugal, and Greece scarcity and increasing cost of labor became a major economic problem in the olive groves of these countries. Furthermore, between the 1950 and 1970, the low yield of the plantations and the concurrence in the markets of other vegetable oils from annual crops such as soybean, sunflower, and rape, which are cheaper than olive oil, triggered a crisis in the Northern Mediterranean olive oil industry. Public programs to reconvert olive groves aimed at increasing yield and reducing cost by mechanical harvesting was held first in Italy and afterward in Spain, Portugal, and Greece. Also the birth and enlargement of the current UE and its Common Agrarian Policy (CAP), that subsidized olive oil production, promoted a revived olive industry. Since that time profound and probably historical changes have transformed the new olive plantations in the UE countries.

3.3 High Density Mechanically Harvested Orchards

Italy was the first European country to propose the intensification and mechanization of olive groves after the World War II. Since the beginning of the 1950s, new intensive olive plantations were established.

The current spread of highly appreciated olive oils from local cultivars has relied on the set up of intensive orchards due to the suitability of almost all the cultivars to this plantation system. In contrast, only a handful of olive cultivars adapt to the Super High Density (SHD) or super intensive plantation systems, which requires cultivars having particular architecture and horticultural traits. The intensive system is characterized by a planting density of 200–400 plants/ha, obtained by spacing the plants 5–6 × 4–5 m or 7–8 × 6–7 m, depending on the cultivar vigor, canopy architecture, and the agronomical conditions of the plot (soil fertility, water availability, length of the annual growth season, etc.). The polyconic vase is the recommended tree shape to harvest the crop with trunk shakers that are equipped with an umbrella interceptor. The polyconic vase consists of a single trunk of 100–120 cm height, with three or four main articulated branches, well distributed and forming a 30°–35° angle with respect to the vertical axis. For the intensive planting system, drought tolerance and disease resistance should be taken into account to select the most convenient cultivar (Marra et al. 2016).

In traditional rainfed olive orchards water availability is related to tree density that limits the interception of solar radiation and constrains the crop and oil yield. By the 1950s, drip irrigation was the most efficient way to increase yield in Californian and Israeli orchards. A steady increase in drip irrigation occurred since the 1970s and early 1980s in Southern European countries. For instance, in Spain the area of irrigated olive plantations raised from 101,000 ha in 1981 to 749,000 ha in 2015. Currently, most of the new olive orchards are irrigated according to a deficit irrigation strategy.

The new irrigated, high-density orchards are the main cause of the increase of the average annual oil production in Spain from 466,000 tons in 1981–1985 to 1,250,000 tons in 2011–2015. Oil production has also risen in Portugal from 32,000 tons in 1981–1985 to 70,000 tons in 2011–2015 because new plantations following this model were allowed by the UE in 1998.

In the first half of the 1990s, a new planting system for olive, known as Super High Density or super intensive system and consisting in a continuous narrow hedgerow (>1500 trees per ha), was developed first in Spain and then worldwide (Rius and Lacarte 2015). Since 1993 the expansion of this system has been exponential, and currently more than 100,000 ha have been planted in many countries. This system requires a high initial investment (>6000 €/ha) and an irrigation dosage of 150–250 mm/year; it produces the earliest crop (>1000 kg of oil/ha) at the third year after planting and more than 1800 kg of oil/ha from the 5th to the 9th or 10th year. These orchards are mechanically harvested by a straddle harvester, which may collect up to 3–4 ha per day reducing drastically the recollection costs. Initially, the major problem of SHD systems for olive was the management of the adult orchards over 9–10 years old. However, new experimental data have shown that accumulated production increase with tree density (from 780 to 2560 trees/ha) when the height and width of the hedgerow is controlled by biennial topping, annual lateral pruning, and deficit irrigation (Díez et al. 2016a). In the future, simulation modeling of oil production in SHD systems may contribute to design efficient hedgerows and to select their orientation according to the geographical site (Connor and Gómez-del-Campo 2013; Connor et al. 2014).

New harvester designs have also allowed the establishment of new olive orchards with densities in the range of 450–800 trees per ha. This system, known as High Density (HD), consists in an irrigated continuous large hedgerow (4.00 m height and 3.00 m width). These orchards would probably last longer than SHD orchards; however, the insufficient porosity restricts the penetration of the incident solar radiation into the canopy, limiting the Leaf Area Index (LAI) as leaves are only located in the canopy external surface. Also difficulties in the mobility of the big sized harvesters may limit their operations even in flat areas. Low vigor cultivars or dwarfing rootstocks, able to extend the time of maximum crop, became a major objective for breeders since the beginning of this system (see chapter of Rugini and De Pace in this book).

4 The Future of World Olive Oil Consumption: Improving Oil Quality

4.1 The Health Concern

In 400 BC, Hippocrates claimed that 'food is our medicine,' alluding to the importance of the active role of certain foods against chronic diseases, which now account for 46 % of global disease, and cause more than 34 million deaths/year in the world. The Mediterranean diet, which has become synonymous of proper nutrition and fitness (Henríquez Sánchez et al. 2012), has been recently declared World Intangible Cultural Heritage from United Nations Educational, Scientific and Cultural Organization (UNESCO), and it represents one of the healthiest models of diet known today. The benefits of the Mediterranean diet are due to the foods that make it up. Among these foods, the virgin olive oil and especially the EVOO assume particular importance; especially because of the antioxidant and anti-inflammatory actions and the lipid control in the blood carried out by some molecules present in the oil. In order to show the beneficial effects of olive oils for human health, the EU, through the Commission of the European Food Safety Authority (EFSA) has approved health claims, reported in reg. 432/2012, regarding foods with a high content of monounsaturated fatty acids (oleic acid), alpha-tocopherol (vitamin E), and phenolic compounds, the latter exclusively present in olive oil.

The healthy nature of EVOO is therefore much more complex than that provided by the rules of the Codex Alimentarius or the

International Olive Council (IOC 2016; IOOC 2008), which support a commercial segmentation based on the degree of hydrolytic alteration (free acidity) and oxidation (peroxide number and constant spectrophotometric) and an absence of sensory defects. In contrast an EVOO is considered good for health if it is characterized by a high oleic acid content, a high content of α-tocopherol and biophenols (particularly hidroxytyrosol) (Tuck et al. 2002) (see chapter of Servili et al. in this book).

The food market that promotes the well-being and the health of the consumer is an opportunity for further expansion of oil consumption, both in rich and in developing countries, considering the high social and health costs generated by the high incidence of chronic diseases. The consumption growth rate of the EVOO will depend, at least in part, from the effectiveness of the promotional programs, which must be specific to the different countries, and to the ability to segment the supply of oil in order to meet the different consumer needs.

4.2 The Global Market Challenge

The spread of a food market that promotes the health of the consumer and the scientific confirmation of the healthy properties of certain groups of molecules present in the olive oil have contributed to the growth in oil consumption and demand. An increase in oil consumption is linked to economic growth worldwide, given the higher cost of sales of high-quality olive oils, and the ability of different countries to segment the supply of oil, in order to meet the different requirements of consumers. A significant part of the olive oil market is facing a multiplicity of consumers, who pay more attention to the selling price but less to the health properties and sensory characteristics of the oils. This part of olive oil market must respond to the expectations and the average buyer's taste, who do not particularly like oils with marked fruity, bitter, and/or spicy flavors.

For this product segment, the imperative is to produce at low costs through the adoption of technologically advanced cropping systems that allow high productivity and low costs of production, particularly for harvesting. Currently, the olive high density mechanical harvested orchards (see above) address these requirements. While cultivar availability for intensive orchards (150–400 trees/ha) does not limit the available large diversity of cultivars, only few ones are available for SHD orchards (>1500 tree/ha). Thus, oil diversity requires enlarge the cultivars planted or alternatively blended with oils from other cultivars.

The selection of EVOO at low cost, in the production context, is strongly affected by the bottling industry monopoly, since currently few companies own the brands of greater value, making the market uncompetitive. The market of high quality olive oils fits more to small and medium size enterprises that represents the ones able to adopt marketing strategies to convince consumers, i.e., the product value should meet specific hedonistic needs and/or nutritional-health benefits, for which they are willing to pay a higher price. To gain access to this market segment, based on the diversification of the product, entrepreneurs should focus on factors that allow differentiating their product based on quality attributes, related to varietal diversity, the particular environmental, and cultural conditions of the different olive-growing districts—Protected Designation of Origin (PDO) and Protected Geographic Identification (PGI)—or by implementing biological or environmentally friendly production practices. The extent of that market to extra Mediterranean countries, with little experience in the olive industry, will greatly depend on communication skills and marketing strategies. The increase of olive oil production in new countries should not be seen as a threat, but rather as an opportunity to create new retail space for high-quality oils, through direct knowledge of the plant and its products by consumers.

5 The Sustainability Challenge

The adaptation of the olive tree to different environments is crucial given the current expansion of this crop to new climatic areas and the need to foresee the possible effects of climate change in the Mediterranean Basin (Ponti et al. 2014).

Two major factors are critical: a) the global warming and b) the water availability:

Global warming may adversely affect the adaptation of many traditional and/or introduced cultivars due to scarce flowering and uneven time of bloom. This disequilibrium is associated with insufficient chilling to release potential reproductive buds from dormancy and low setting due to high temperatures at bloom. The effects of global warming in the Mediterranean Basin may be anticipated evaluating the olive cultivars adaptation to new olive growing areas of America (Aybar et al. 2015).

The scarcity of water in most olive growing areas is the major constrain for olive crop in many regions. On the other hand, the irregular extreme rainfall is the cause of soil erosion that stands as the major environmental threat for olive plantations in diverse and large growing areas. Therefore, the use of deficit irrigation for water use efficiency (WUE) in irrigated plantations and the implementation of soil temporal cover crops, or natural vegetation to limit soil erosion, represent efficient management practices for environment sustainability (Orgaz and Fereres 2010; Pastor 2010) (see chapter of Sebastiani et al. in this book).

The use of models based on genotypic traits for designing ideo-typing varieties able to face specific environmental conditions (Cesaraccio et al. 2004; De Melo-Abreu et al. 2004) might have the potential to assist breeding programs in the generation of cultivars able to cope with water scarcity, reduce soil erosion, and lessen the use of chemical products. These models will be also helpful to maximize the effectiveness of expensive comparative trials by better focusing the critical points to test the adaptation of the cultivars.

6 Propagation, Genetic Resources, and Breeding

Propagation methods have also changed since the 1980s. The propagation of semi-hardwood and softwood cuttings under mist (Hartmann and Loreti 1965) has promoted the continuous expansion of the nursery industry worldwide. Nowadays traditional methods of propagation have been substituted by efficient industrial nursery techniques able to produce millions of plants in a short time. For instance, in Spain the nursery industry capacity exceeded 30 million plants per year in 2007. The propagation of plants carried out by farmers has practically disappeared and the trade of olive plants is becoming global. As a consequence, guaranteeing true to type and pathogens free cultivars is mandatory in this time of global interchange of plant material. Moreover, the spread of *Verticillium dahliae* since the 1970s and, recently, the outbreak of *Xilella fastidiosa* in Puglia, emphasize the need for the immediate establishment of a Plant Certification Program worldwide (see chapter of Corrado et al. in this book).

The development of the nursery industry has been associated with the standardization of the propagated cultivars. The cultivar diversity has been drastically reduced in the new olive plantations. For example, in Spain few olive cultivars: 'Arbequina,' 'Picual,' and 'Arbosana' for oil, 'Manzanilla de Sevilla' for table olives, and 'Hojiblanca' for both oil and table olives, account for most of the produced plants. Few more cultivars ('Koroneiki', 'Leccino', and 'Frantoio') are eventually planted in some new orchards. Finally, some local cultivars associated with PDO and DGI are also planted in a limited surface. This trend also seems to affect the nursery production in other countries.

For the first time, the risk of genetic erosion has dramatically appeared in olive as consequence of cultivar standardization (Díez et al. 2016b). The conservation of olive germplasm becomes a priority in all olive growing countries. Since 1994 the IOC leads a collaborative Network of Banks of Olive Germplasm currently

including 22 countries. The authentication of their olive accessions, which is the compulsory first step for their evaluation and sustainable use, is on development (Trujillo et al. 2014).

In the current production systems, for the development of the olive oil industry is fundamental to search for new cultivars and/or rootstocks with the sustainable characteristics previously mentioned and suited to: (a) the complete mechanization of farming operations; (b) ensure a constant and abundant crop production; and (c) produce oils with special chemical and sensory characteristics. Classical methods of genetic improvement such as clonal selection within main cultivars and induced mutagenesis have not proved successful (Rugini et al. 2016). Since the 1960s several olive breeding programs were developed in different countries. Most of these programs were based on planned cross breeding, selection within the progenies and cloning. They were oriented to obtain cultivars for olive oil production, for table olives, or both. Up to date, the programs from Israel, Italy, and Spain have yielded just 14 new releases. In Spain, there are ongoing programs to obtain new cultivars for oil production and table olive cultivars with the following characteristics: earliness of bearing, high oil yield, oil and table olive quality, resistance to bruising in table olives, suitability to different plantation systems, and resistance to *Verticillium* wilt and other diseases. Methodological improvements allowed the development of protocols to force the growth of the plants in order to shorten their juvenile period; also, the establishment of early and simplified selection criteria (Moral et al. 2013; Trapero et al. 2015) (see also chapter of Rugini and De Pace in this book). As a result, four new advanced selections from the UCO-IFAPA breeding program of Cordoba (Spain), which are high yielding under different growing systems, are currently under registration (Rallo et al. 2016). In the years to come, more breed selections will be released in different countries.

7 The Role of Genomics in the Future of the Olive Growing

The recent publication of a reference genome for olive is a landmark result that will doubtlessly open new avenues for olive research (Cruz et al. 2016). It will facilitate the incorporation of new genomic tools to breeding, which are already generating promising results in other fruit crops such as apple, peach, or citrus (Kumar et al. 2012, 2013). Modern genomics tools will help to characterize olive genetic resources and to accelerate the long breeding cycles of this crop. The application of genomics is not only devoted to save time, but also economic resources; while the cost of phenotyping is likely to remain relatively static or even increase in the future, the cost of genotyping has gone down considerably over time, and this trend is expected to continue (McClure et al. 2014).

The primary use of genomics in breeding is marker-assisted selection (MAS) for traits controlled by major genes or quantitative trait loci (QTLs). By means of MAS, genetic markers that are either known to cause a phenotype, or are strongly linked to the causal genetic variant, can be genotyped at the seedling stage allowing a prediction about the phenotype of the adult plant.

Most applications of MAS to date are for simply inherited traits mostly related to disease resistance to pathogens and to the quality of some crop products (Francia et al. 2005; McClure et al. 2014). However, the application of MAS to complex traits, which are normally related to critical traits such as yield, yield stability, and adaptation, is not straightforward. Molecular markers have been successfully associated with QTLs in many fruit crops such as apple, peach, cherry, or grape—for a complete review see Badenes and Byrne (2012)—or recently in olive (Atienza et al. 2014). However, their direct application in breeding has been limited. Difficulties in manipulating these traits are derived

from their genetic complexity, principally the number of genes involved, the interactions between genes (epistasis), and environment-dependent expression of genes. The selection of correctly sized populations for mapping is the starting point to obtain reliable and compressive data from QTL analysis (Francia et al. 2005). It is increasingly necessary to planned breeding programs, based on 'gene pools,' using the available biotechnology and molecular techniques (see chapter of Rugini and De Pace in this book).

The availability of a reference genome opens new possibilities to characterize these interactions and perform genome wide association mapping to accelerate the breeding process. In the near future, it will be also possible to describe particular interactions among genes during development and to determine the genome-wide distribution of DNA methylation and histone modifications with techniques such as bisulfite sequencing (BSseq) and ChIPseq (Díez et al. 2014). With these technological advances, olive is about to enter in a new phase focused on detailing variation among species, tissues, and cell types that will be crucial for adaptation to changing environments.

8 Conclusion

In summary, it is increasingly necessary to plan breeding programs, based on 'gene pools' and using the new techniques that are becoming available for olive. Strategies such as marker-assisted selection, rescue of embryos, the generation of dihaploids, somatic hybridization genetic transformation, will become essential to assist the classical breeding programs. The incorporation of these techniques will accelerate the process to obtain new olive cultivars with valuable traits and the capacity to react to eventual threads such as *X. fastidiosa*, *Verticillium* wilt, or climate change. To that end the role of genomics promises to be outstanding.

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Genetic Mapping and Detection of Quantitative Trait Loci

5

Luciana Baldoni, Bouchaib Khadari and Raul De La Rosa

Abstract

Olive tree is a long-living woody species with similar genomic and phenotypic constraints to other perennial fruit crops. However, compared to apple, grape, and peach, genomic investigations for designing innovative breeding strategies are still limited to only preliminary research in this species. In this chapter, we aim to describe the studies on genetic mapping and underline the most promising investigations and initiatives to build a Mediterranean network suitable for establishing robust marker-trait associations through QTL mapping and association studies. These tools should serve to finally implement new breeding programs driven by marker-assisted breeding.

1 Current Status of Genetic Mapping in Fruit Tree Crops

Perennial fruit crops are grown mainly with the single-genotype clonal cultivars derived from vegetative propagation over millennia. Indeed, clonal propagation maintains over time the identical allelic gene combinations and phenotypes. On the contrary, by sexual reproduction, traits can disappear in seedlings, even for hybrids developed from crossing between elite cultivars, as most of fruit species are highly heterozygous. However, perpetual propagation of ancient pre-bred cultivars, as is the case in olive, prevents new allele combinations to arise, thus affecting the plant's ability to face the forthcoming challenges. Olive production, in fact, has to deal with new scenarios, such as climate change, sourcing health-related compounds, and arising consumer

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demands for quality products with low agrochemical inputs. For instance, pathogens associated with fruit crops continue to evolve, while cultivars remain unchanged, forcing the increasing use of agrochemicals or loosing entire production areas due to attack of new pathogens to which traditional varieties are susceptible.

A future with sustainable and safe fruit production relies on the generation of new phenotypes through breeding that become now mandatory for sustainable cropping systems (Collard and Mackill 2008).

Selection of newly bred cultivars is a long-, cost-, and time-consuming procedure for many fruit crops, mainly due to the long juvenile and unproductive period of those species (Badenes and Byrne 2011). Trees have a large size and an extended juvenile phase, with fruit bearing taking up to 3–8 years before starting. For instance, an apple breeding program in Germany began with 52,000 seedlings and, after 26 years of evaluation, only three cultivars have been selected (Ignatov and Bodishevskaya 2011). Because of the large amount of time, space, and money necessary for growing and evaluating fruit trees, early seedling screening through molecular markers to distinguish between desirable and undesirable genetic profiles is essential (Leon et al. 2004, 2016).

The use of molecular tools should improve the identification of the best parental genotypes and shorten the selection process, allowing to reduce costs and time to obtain new genotypes (Edge-Garza et al. 2015). In fact, the use of molecular technologies has offered new opportunities to develop early selection strategies in many fruit crops (Martínez-García et al. 2013; Montanari et al. 2013; Bink et al. 2014; Muranty et al. 2015; Serra et al. 2016).

This screening process is known as marker-assisted selection (MAS), and it is widely considered as suitable tool for woody perennial fruit crops (Dirlewanger et al. 2004; Di Gaspero and Cattonaro 2010).

Most of traits of interest, such as fruit quality and disease resistance, are quantitatively inherited traits controlled by multiple loci. For these traits, MAS relies first on the establishment of robust marker-trait associations, or quantitative trait loci (QTL), through genetic mapping based on hybrid populations or through association mapping studies, based on unrelated genotype populations (Cappellin et al. 2015; Muranty et al. 2015). During the last decade, several studies on genetic and QTL mapping have been published on perennial fruit crops, offering large perspective to design new phenotypes through breeding (Mahanil et al. 2012; Emanuelli et al. 2010).

The use of molecular breeding in olive should help the identification of the best parental lines and increase selection efficiency, allowing to reduce costs and time to obtain new genotypes.

2 Genetic Mapping in Olive

Linkage analysis is based on the estimate of the recombination frequency between markers or between markers and loci affecting a trait. In fruit trees as olive, the use of multigeneration families is hindered by the long generation time; thus, full-sib families deriving from the crossing of two parental varieties are commonly used for this purpose (Doucleff et al. 2004; Fernández-Martínez et al. 2012; Sun et al. 2015). Linkage analysis is conducted separately for each parent, with a strategy named as two-way 'pseudo-testcross' mapping strategy (Grattapaglia and Sederoff 1994) (Table 1).

2.1 Mapping with Conventional Markers

The first linkage map of the olive was constructed in 2003 (De la Rosa et al. 2003) using dominant markers such as 279 random amplified polymorphic DNAs (RAPDs) and 304 amplified fragment length polymorphisms (AFLps), and just a few codominant ones, such as restriction fragment length polymorphisms (RFLPs) and simple-sequence repeats (SSRs). A progeny of 95 individuals derived from the cross of two highly heterozygous cultivars, Leccino (female self-incompatible parent) and Dolce Agogia (male parent), was used by applying the

Table 1 List of all genetic mapping projects performed in the olive species

Parental genotypes	Progeny number	Markers (N. total mapped)	LOD threshold	Recombination fraction	N. mapped markers	% distorted loci	Covered distance (cM)	N. major linkage groups	Mean markers distance	Consensus map coverage and marker distance	Authors
♀ 'Leccino' ♂ 'Dolce Agogia'	95	AFLPs, RAPDs, RFLPs, SSRs	4	0.30	249 236	16.8 %	2765 cM 2445 cM	22 27	13.2 cM 11.9 cM	–	De la Rosa et al. (2003)
♀ 'Frantoio' ♂ 'Kalamata' Consensus	104	RAPDs, SCARs, SSRs	3	0.49	92 89	19.5 %	798 cM 759 cM	27 23 15	12.3 cM 11.5 cM 10.2 cM	879 cM 10.2 cM	Wu et al. (2004)
♀ 'Olivière' ♂ 'Arbequina' Consensus	147	AFLPs, SSRs, ISSRs	6	0.40	222 219 450	19.0 %	2210 cM 1996 cM	36 31 26	11.2 cM 10.3 cM 8.7	2148 cM 4.77 cM	Khadari et al. (2010); Ben Sadok et al. (2013a, b); Essalouh et al. (2014)
♀ 'Picholine Marocaine' ♂ 'Picholine du Languedoc' Consensus	140	SSRs, AFLPs, ISSRs, RAPDs, SCAR	6	0.40	175 170 345	–	1547 cM 1428 cM 2366 cM	40 38 49	8.06 cM	2366.4 cM 8.06 cM	El Aabidine et al. (2010)
♀ 'Picual' ♂ 'Arbequina' Consensus	91	DArTs, SSRs	4	–	422 613	10 % 24 %	1205.1 cM 1639.3 cM	23 23	9.64 cM 8.04 cM	–	Dominguez-Garcia et al. (2012)
♀ 'Gemlik' ♂ 'Edincik Su' Consensus	121	SNPs, SSRs, AFLPs	9	0.30	10,941 22 272	32.4 % 39.3 %		25		3049 cM 0.54 cM	Ipek et al. (2016)
'Koroneiki' selfing	63	SNPs	4	0.35	1597	–		23		1189.7 cM	Marchese et al. (2016)

Information on parental genotypes, progeny number, type of markers used, LOD threshold, recombination fraction, number of mapped markers, percentage of markers showing segregation distortion, covered distance, number of major linkage groups obtained, marker distance and, where applicable, coverage, and marker distance of consensus map

pseudo-testcross strategy. Parental individuals and their cross-progeny were scored for polymorphic-dominant and codominant markers. Mapping markers included those present in one parent, absent in the other and segregating in 1:1 ratio in the progeny, as in a testcross-progeny, generating two data sets, one for each parent.

Those markers present in both the parents and segregating in 3:1 ratio in the progeny (because heterozygous at those loci) were not included in the screening, because it was impossible to distinguish heterozygous from homozygous progenies, and could not be used by the MapMaker software to join the parental maps (Lincoln et al. 1993). Only those codominant markers present in both the parents and simultaneously segregating in the progeny were used for mapping.

A total of 249 markers (110 RAPDs, 127 AFLPs, 8 RFLPs, and 3 SSRs) was linked in the cv. Leccino map, resulting in 22 major linkage groups and 17 minor groups (less than four markers) and covering a total distance of 2765 cM (mean distance between adjacent markers 13.2 cM), whereas 236 markers (93 RAPDs, 133 AFLPs, 6 RFLPs, and 4 SSRs) were grouped in the Dolce Agogia map, with 27 major linkage groups, three minor groups with a coverage of 2445 cM (marker distance of 11.9 cM). All the markers were homogeneously distributed in all linkage groups. The AFLP markers showed a high level of segregation distortion (16.8 % at 5 % level of probability), but the inclusion of skewed markers in the olive maps did not affect the arrangement of total markers, resulting uniformly distributed along the linkage groups.

The obtained maps did not merge into the 23 linkage groups expected for the olive species, probably due to the low number of markers, the small population size, the non-random sampling of the genome, or the hot spots of recombination. Despite the progeny segregated for some characters, such as tree habit (Fig. 1) or ripening time (Fig. 2), no QTLs were located on these maps.

Two parental and an integrated linkage maps were constructed (Wu et al. 2004) based on RAPD, SCAR (sequence-characterized amplified region), and SSR markers on a F_1 full-sib family of 104 individuals from a cross between cultivars Frantoio (female self-compatible parent) and Kalamata (male parent), again using the pseudo-testcross strategy.

Testcross markers segregating in 1:1 ratio and intercross markers segregating in 3:1, 1:2:1, or 1:1:1:1 ratios were used for map construction.

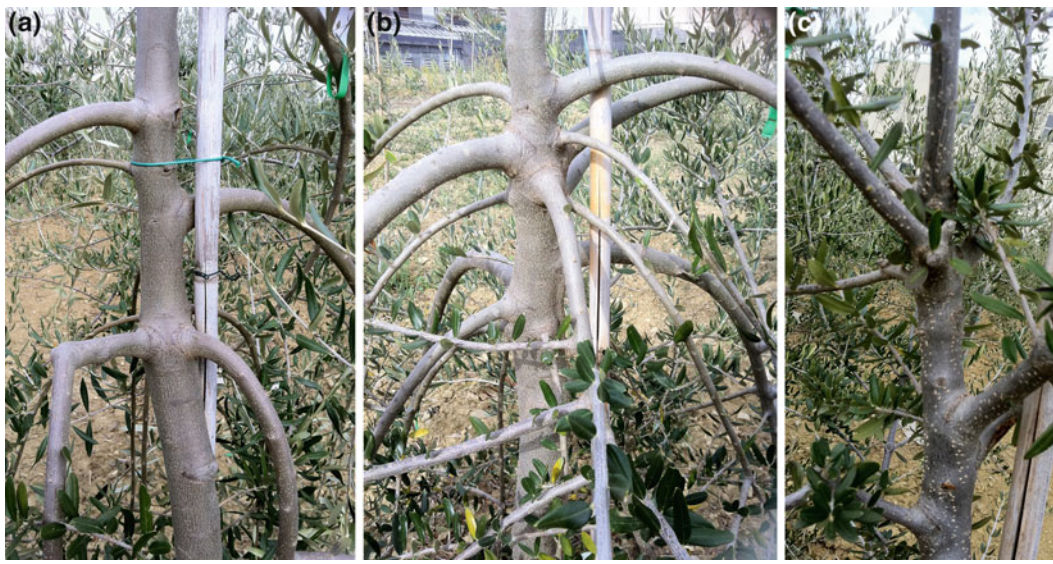

Fig. 1 Seedlings of the cross-progeny Leccino x Dolce Agogia used for genetic mapping showing segregation for the tree habit. Panel **a**: pendolous branches, Panel **b**: horizontal branches, Panel **c**: upright branches

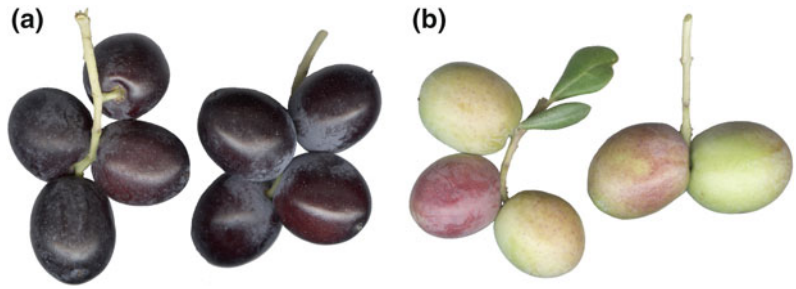

Fig. 2 Fruits from the cross-progeny Leccino x Dolce Agogia showing segregation for ripening time. Panel **a**: early-ripening fruits, Panel **b**: late-ripening fruits

Two separate maternal and paternal linkage maps were established using 118 and 126 testcross and intercross markers, respectively. Twenty-seven linkage groups were mapped for 'Frantoio,' only including 92 loci at an average distance between loci of 12.3 cM, covering 798 cM of the genome. Twenty-three linkage groups were mapped for 'Kalamata,' covering 759 cM of the genome, with 89 loci and an average distance between loci of 11.5 cM. Due to the low number of markers, most of the linkage groups were made up of only two or three markers. Patterns of skewed segregating markers (19.5 %) were consistent with those reported in the mapping population investigated by De la Rosa et al. (2003).

After merging the maps of 'Frantoio' and 'Kalamata,' a consensus map was obtained, with 101 loci and 15 major groups, covering 879 cM of the genome, and an average distance between loci of 10.2 cM. A SCAR marker resulted in linkage with the olive peacock disease resistance trait. No other markers were linked to the agronomic traits because at the time of data publication the progeny was still in the juvenile phase.

In 2010, a new linkage map was produced (Khadari et al. 2010) with a 147 full-sib 'Olivière' x 'Arbequina' cross-progeny used in a two-way pseudo-testcross configuration by the analysis with 352 AFLPs, 15 ISSRs (inter-simple-sequence repeat) and 44 codominant SSR markers against the few ones used in the previous maps (De la Rosa et al. 2003; Wu et al. 2004). Thirty-six linkage groups were obtained for 'Olivière,' defining 2210.2 cM map coverage and an average marker spacing of 11.2 cM. The paternal map contained 31 linkage groups and covered a distance of 1966.2 cM, with an average marker distance of 10.3 cM. The consensus map, made up by 42 linkage groups, included 436 markers at a mean distance of 8.7 cM, for a total coverage of 3823.2 cM.

The consensus map derived from this study, although not yet saturated, represents a significant step toward the construction of a solid and efficient map for identification of QTL markers. The phenotypic characterization of this progeny should provide useful information to detect QTLs related to tree growth and architecture.

A second new map has been generated in the same year on 140 cross-progeny 'Picholine Marocaine' (female parent) x 'Picholine du Languedoc' (male parent) cultivars (El Aabidine et al. 2010) by the use of 47 SSRs, 509 AFLPs, 27 ISSRs, 8 RAPDs, and one SCAR markers, according to the same strategy previously used in all other mapping initiatives. The maternal map spanned 1547.40 cM and was built on 40 linkage groups including 175 markers, while the paternal map included 170 markers clustering into 38 linkage groups and covering 1428.00 cM. The consensus map covered 2366.4 cM and included 345 markers clustering into 49 linkage groups with a mean distance between two adjacent loci of 8.06 cM. The map included the largest number of SSR markers (47 markers) ever analyzed. Despite the SCAR marker considered to be linked to peacock disease resistance in olive (Wu et al. 2004) was mapped onto one linkage group and the progeny resulted in segregation for resistance to olive leaf peacock disease, no QTLs for this trait were identified.

A mapping population derived from the 'Picual' x 'Arbequina' cross was used to build a new map based on diversity arrays technology (DArT) and SSR markers (Dominguez-Garcia et al. 2012). A total of 1630 DArT and 38 SSR markers was used for mapping analysis, making available 422 and 613 markers for the construction of 'Picual' and 'Arbequina' maps. Also in this case, no QTLs were mapped.

On the same cross-population 'Olivière' x 'Arbequina' previously constructed (Khadari et al. 2010), new genomic and expressed sequence tag (EST)-derived SSR markers were added (Essalouh et al. 2014). The EST sequences were derived from olive fruits of the cultivar 'Istrska belica' sampled during the whole period of fruit development. Three sets of 85, 64, and 94 new informative SSRs were made available for constructing parental 'Olivière' and 'Arbequina' maps and for the integrated one. Based on a previous genetic map (Khadari et al. 2010) and using a new set of 94 SSR loci, a new more saturated genetic map was constructed on the 147 hybrid genotypes (Ben Sadok et al. 2013a, b). A total of 450 markers was mapped in the 'Olivière' x 'Arbequina' integrated map including 103 total SSRs. These markers were assigned to 26 linkage groups. The integrated linkage map was 2148.4 cM, representing an observed genome coverage of 86.9 %. The average marker spacing was of 4.77 cM, with a considerable improvement in map saturation and linkage group definition. As a result, 25 linkage groups covering a total of 1745.3 cM and 21 linkage groups covering 1597.6 cM were obtained for the female 'Olivière' and male 'Arbequina' parental maps, respectively. In comparison with the previously developed maps in olive (De la Rosa et al. 2003; Wu et al. 2004; Khadari et al. 2010; El Aabidine et al. 2010; Dominguez-Garcia et al. 2012), the genetic map constructed by Ben Sadok et al. (2013a, b) includes the largest number of molecular markers so far, with the highest marker density.

2.2 Mapping with High-Throughput Markers

All the genetic maps available on olive until 2015 were based on low-coverage markers, such as SSRs, or on non-sequence-based markers, such as AFLPs and DArTs, but the rapid evolution of sequencing technologies has made available new high-throughput markers. Among them, single-nucleotide polymorphism (SNP) represents the most effective, because these markers are evenly distributed along the genome, highly numerous, highly polymorphic, codominant, and have potential functional effect.

Genotyping-by-sequencing (GBS), a SNP identification method based on next-generation sequencing (NGS) technologies, has been demonstrated to be useful for the identification of a high number of SNP markers and the construction of high-density genetic linkage maps.

In a recent study, a total of 10,941 SNPs was identified in an olive full-sib F_1 cross-progeny of 121 individuals derived from the cultivars 'Gemlik' and 'Edincik Su' using GBS and de novo SNP discovery (Ipek et al. 2016). The SNP markers segregated 38 % in the maternal parent, 37 % in the paternal one, and 25 % markers segregated in both the parents. A very high level of segregation distortion was observed, reaching 32.46 % in the maternal line and 39.33 % in the paternal parent. These markers were not included in the map.

Markers located on the map included 5736 SNPs, 21 SSRs, and 203 AFLPs, composing 25 linkage groups of the integrated map, covering 3049 cM of the genome, with a mean distance between adjacent markers of 0.53 cM, thus representing the first well-saturated genetic linkage map in olive.

Another genetic map was derived from the SNP analysis performed by GBS approach on a population derived from the selfing of cv. Koroneiki (Marchese et al. 2016). 1597 SNP markers

were mapped, covering a total genetic distance of 1189.7 cM over 23 linkage groups. Other 6658 SNPs were linked on the 23 linkage groups identified, but their order remained undetermined. The linkage map produced will serve as a useful resource for the study of tree habit and vigor traits segregating in the progeny.

3 QTL Mapping

Many olive tree traits of economic importance, such as those related to fruit quality and productivity, are quantitative traits, and loci affecting such traits are called QTLs. A statistical method that combines linkage analysis with a statistical model of phenotypic values of a trait referred to as QTL analysis is applied for mapping QTLs and estimating their effects.

The segregating population derived from the cross 'Olivière' x 'Arbequina' cultivars (Khadari et al. 2010) has served to investigate the existence of genetic determinism for reproductive behavior in olive tree, with particular reference to the balance between production and vegetative growth (Ben Sadok et al. 2013a, b). Tree yield was assessed annually, as well as plant growth, plant habit, flowering, fruiting, and production irregularities (e.g., alternate bearing). Based on a new genetic map, QTLs with small effects were detected, revealing multigenic control of the studied traits, most of them linked to alleles from 'Arbequina.' Most QTLs were associated with flowering traits.

In a progeny derived from the cross, 'Picual' x 'Arbequina,' previously mapped by Dominguez-Garcia et al. (2012), fruit-related and plant vigor traits, representing key factors for olive cultivation, were analyzed during two seasons (Atienza et al. 2014). Some QTLs for oil-related traits were located in two linkage groups of the 'Arbequina' map, explaining about 20–30 % of the phenotypic variability. The QTLs for fruit dry weight and for pulp/stone ratio were detected in three and two linkage groups, respectively, explaining about 15–20 % of phenotypic variance. Five additional QTLs were detected in the map of 'Picual.' A QTL for fruit weight explaining around 12–15 % of variability was identified in a linkage group of cv. Picual, as well as another one related to trunk diameter and explaining 16 % of phenotypic variation. Interactions among QTLs for the same trait were also investigated. The limited population size may have led to an overestimation of QTL effects and underestimation of QTL number. Data were also affected by seasonal variations; thus, the effects of QTLs should be further confirmed, and the position of the QTLs detected in a single season should be further validated.

Tree architecture is a critical trait in olive crop because it directly affects planting density, yield, and pruning, thus playing the main role for modern cultivation practices in this species. Little is known on the genetic determination of plant architecture in olive (González-Plaza and Hulak 2016; Hammami et al. 2011), but a recent work, carried out by microarray analysis and expression analysis of candidate transcripts on seedlings derived by the cross of varieties showing different architecture phenotypes, allowed to identify the first genes associated with plant architecture in olive (González-Plaza et al. 2016).

Recently, first results have been presented on mapping some QTLs for traits related to the fruit, such as ripening time, stone-flesh detachment, and flesh firmness, on the progeny 'Memecik' x 'Uslu' varieties (Ates 2016).

4 Association Mapping Studies

The approach adopted for locating genes that underlie a plant trait may shift from pedigree-based linkage studies to population-based association studies (Chen et al. 2014; Myles et al. 2009).

Genome-wide association studies (GWAS), in fact, require to perform whole genome scans with large sets of SNP markers directly on phenotypically characterized genetic resources in order to detect linkage disequilibrium (LD) between markers and traits. As the LD detected in an association study is the result of thousands of

recombinations and not just the few possible events occurring in a pedigree, the responsible genes can be mapped more accurately by GWAS than by genetic mapping approaches (Li et al. 2010; Sauvage et al. 2014). Furthermore, other approaches are now available, such as the scanning of haplotype variations of a single marker, that may be even more powerful at detecting significant associations.

Even if association mapping could be an effective approach for identifying marker-trait associations in fruit crop genotypes, without the development of mapping populations, at present this approach has been poorly applied in fruit crops (Iwata et al. 2013; Khan and Korban 2012). In olive, the first study has been recently published, where 96 olive genotypes were used to examine marker-trait associations by the use of SNP, AFLP, and SSR markers and five yield-related traits. By using different approaches, some significant associations were detected for fruit and stone weight (Kaya et al. 2016). However, number of genotypes and markers should be dramatically increased in order to precisely identify mutations responsible of the traits (Khadari et al. 2014).

5 Concluding Remarks

Results on QTL mapping in olive represent an important step toward the application of MAS in current and future breeding programs, but additional studies on these fields should be developed in the next years.

Several hybrid populations exist with common parents, offering the possibility to build a consensus genetic map based on different hybrid populations and hence to validate QTL detection.

The first olive genome sequence recently published (Cruz et al. 2016) and the numerous other ongoing initiatives (Muleo et al. 2012; Unver et al. 2016) are harnessing the genomic era for olive research and promoting the use of NGS data for GWAS.

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Host Response to Biotic Stresses

6

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Abstract

In olive, several biological processes, including those related to drupe maturation and oil production, are adversely affected by biotic stress. Pesticides are an important, valuable input in modern oliviculture, still central to secure yield and safeguard olive oil quality. However, concerns over the effects of plant protection products on the environment, non-target organisms, and human health prompt the development and implementation of more integrated control strategies. Functional genomics for biotic stress tolerance is a promising area that needs to be explored to increase olive tolerance and reduce reliance on pesticides. A number of studies have recently described, on a genome-wide scale, the participation of genes in olive response to different biotic stresses. Moreover, genes involved in stress tolerance and related signaling networks have been also identified. This chapter presents recent advances in olive molecular response to its major biotic stresses (insects, fungi, bacteria, and viruses). This topic is presented in a larger context that includes the main biological features of the major olive biotic stressors.

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1 Introduction

The olive tree is inextricably linked to every aspect of human life, especially for Mediterranean people. In Greek mythology, the olive was a gift from Athena to the people of Attica, who named their capital city after her. For all religions in the area, Judaism, Christianity, and Islam included, olive oil was revered as the light that illuminated the darkness of temples and houses. Olive trees have always been a source of heat, food, and medicinal compounds.

Today, olive oil is recognized as one the most typical elements of the Mediterranean diet. Biotic stress is an important determining factor of olive oil quality. Many stresses exert a direct and indirect effect on a number of olive parameters, which is assumed to be mainly detrimental. However, it is likely that the interaction between the olive and its biotic stressors can shape the compositional parameters of the drupes in a much more complex way, contributing with both positive and negative reinforcement of features that are under a complex genetic control (Atkinson et al. 2011). As biotic stress can be considered unavoidable in olive, understanding the complex molecular response to stress is important to develop suitable strategies that minimize impact on yield and maximize the amount of compounds that improve olive oil quality. To this aim, "omics" studies based on large-scale and high-throughput methods provide previously inaccessible information on several aspects of plant biology, including the interaction between plants and their enemies. The ever-increasing speed, throughput, and affordability of next-generation sequencing (NGS) approaches have revolutionized the way we can study biological interactions, allowing a level of resolution and depth that was unreachable by earlier tools. However, the full potential of current technologies can be unleashed in the presence of good reference genomes. Many issues have made very difficult the sequencing of the olive genome (Muleo et al. 2012) and a first draft of the genome of the cv. Farga was recently released (Cruz et al. 2016). The genome of the wild olive (*Olea europaea* var. *sylvestris*) was also sequenced and assembled, by the IOGC International Consortium (Unver et al. 2016).

The application of genomic technologies to study olive stress response has been limited by the lack of adequate genomic information. For this reason, as in many plant species, early studies in olive focused on the gene expression analysis of selected genes (Botella et al. 2005; Giannoulia et al. 2007). More recently, efforts were produced to identify, at a larger scale, genes involved in the response to stresses and to describe the network of the multiple signaling pathways involved in olive resistance (Corrado et al. 2012; Gómez-Lama Cabanás et al. 2014, 2015; Leyva-Pérez et al. 2015; Alagna et al. 2016). As many proteins involved in plant resistance against biotic stress are direct gene products, the majority of studies in olive focused on the transcriptome, with particular emphasis on the polyadenylated coding RNAs. Although not all genes induced or repressed in response to a biotic stress necessarily have a direct effect on pest or pathogen performance, studies of responsive genes can yield suitable candidates for further functional investigation. In addition, transcriptomics studies have highlighted the main signaling pathways and metabolic routes activated in response to stress.

The identification of stress-related proteins and secondary metabolites using proteomics and metabolomics are probably the most employed complementary approaches to transcriptome-based gene discovery, although not many studies were performed in this area. Only recently, analysis of some components of the metabolome following biotic stress has been carried out in drupes (Alagna et al. 2016).

This chapter includes an overview of the major biotic stressors of the olive and provides examples of the use of genomics to understand the molecular basis of olive response. This chapter also includes examples of the discovery of genes associated with or involved in olive resistance. This information paves the way for

the development of new integrated strategies to increase stress resistance and for the molecular improvement of the olive tolerance to specific antagonists.

2 The Major Entomological Enemy: The Olive Fly

Bactrocera oleae (Rossi) (Diptera: Tephritidae), the olive fly (OLF), is a strictly monophagous insect pest infesting the fruits of cultivated and wild *O. europaea*. The female fly lays eggs inside ripe and unripe fruits. Hatching larvae feed on the olive pulp, boring galleries inside the fruit mesocarp. OLF infestation makes table olives unmarketable and deteriorates the quality of olive oil (Gucci et al. 2012). Given this tight relationship, the expansion of the OLF is exclusively restricted to the cultivation zone of the olive tree. Population analysis of OLF from different parts of the world showed three separate genetic groups, Pakistan, Africa, and the Mediterranean (Nardi et al. 2005), with Africa considered the likely center of origin. The OLF Mediterranean group was further divided into three genetic groups (Western, Central, and Eastern Mediterranean groups) (Augustinos et al. 2005; Zygouridis et al. 2009). The gradual decrease of fly variability from the Middle East to the Iberian Peninsula indicated a westward expansion of the species, most likely associated with the expansion of the olive cultivation in the Mediterranean (van Asch et al. 2012). A similar East-to-West pattern of expansion was observed in samples from Turkey (Dogaç et al. 2013). Nardi et al. (2010) suggested that most of the evolutionary history of OLF preceded the domestication of cultivated olives and took place on wild olives. In recent years, OLF has also invaded California (Rice 2000; Rice et al. 2003). Genetic analyses of the invasion pointed at an Eastern Mediterranean origin of the flies (Zygouridis et al. 2009).

B. oleae overwinters as adult, as larva in the fruit or as pupa in the soil. The fly is best adapted to develop in the autumn period: A lack of ovarian maturation during late spring and early to mid-summer can be observed (Fletcher et al. 1978; Tzanakakis 2003). The reproductive dormancy ends when suitable fruits become available, usually starting from mid or late summer (Tzanakakis and Koveos 1986). Drupes and temperature determine the number of generations that can be completed before the natural reproduction stop during winter. The number of OLF generations per year varies according to different factors: geographical region, agronomic and climatic conditions, olive canopy microclimate, availability, and quality of the fruits (Gutierrez et al. 2009; Malheiro et al. 2015a). Fruits become susceptible to OLF when the endocarp begins to harden, usually during summer. A single female of *B. oleae* can lay about 10–20 eggs a day and between 200 and 500 eggs in a lifetime (Burrack and Zalom 2008; Burrack et al. 2011). Typically, one egg is laid in an olive, allowing the larvae a direct access to food just after emergence. Once ready for pupae formation, larvae open an exit hole in the olive epicarp and either escape from the fruit to pupate in the soil or pupates inside the fruit and open an exit hole for the adult (Fig. 1). The physiological time scale on which *B. oleae* evolves (from egg to adult), expressed and approximated as cumulated degree-day (CDD), is 379.015 (Crovetti et al. 1982).

2.1 Cultivar Susceptibility to Olive Fly

Different levels of susceptibility are present in olive cultivars (Iannotta et al. 1999, 2006a, b, 2007; Iannotta and Scaliercio 2012; de Alfonso et al. 2014). Malheiro et al. (2015a) summarized the infestation levels from different olive cultivars around the Mediterranean Basin and California and grouped the main stimuli involved in the choice mechanism of olive fly females in three groups: physical, chemical, and molecular. Numerous studies confirmed the importance of fruit size and volume, fruit epicarp parameters, such as elasticity and firmness, and fruit color. OLF prefers large fruits, greener comparatively to ripened fruits, and lower skin elasticity and higher skin firmness (Neuenschwander et al.

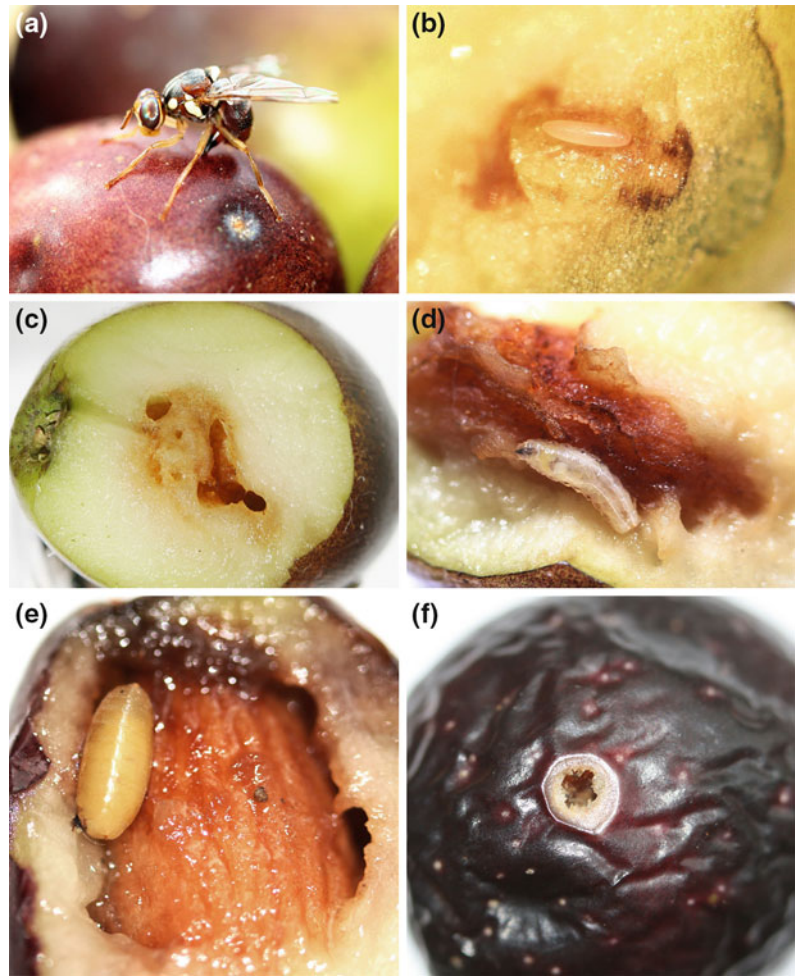

Fig. 1 *Bactrocera oleae* (Rossi). Egg-laying female (**a**); newly deposited egg (**b**); feeding galleries (**c**); intermediate larval stage (**d**); puparium in damaged drupe (**e**); emergence hole (**f**)

1985; Vlahov 1992; Katsoyannos and Kouloussis 2001; Gonçalves et al. 2012; Rizzo et al. 2012; Malheiro et al. 2015b). In relation to chemical parameters, the epicarp compounds may attract or repel OLF females. Cuticular waxes and ammonia may exert opposite effects, respectively, repellent or attractant, such as several volatiles emitted by fruits and leafs. For example, (E)-2-hexenal exerts a repellent action while the olive leaf volatiles toluene and ethylbenzene, stimulated oviposition in the OLF (Scarpati et al. 1993; Lo Scalzo et al. 1994; Scarpati et al. 1996). Another olive volatile α-copaene, a sesquiterpene, is present in higher amounts in more susceptible olive cultivars, promoting *B. oleae* oviposition (de Alfonso et al. 2014). Oleuropein, the main phenolic compound in drupes and leaves, is involved in the defense mechanism against OLF. A higher level of enzymatic hydrolysis of oleuropein is reported for less susceptible olive cultivars inhibiting the early development of OLF (Spadafora et al. 2008; Iannotta and Scaliercio 2012). Laboratory assays with olive leaves essential oils from cultivars with different susceptibilities showed different physiological response of adults OLF dependent from oils chemical composition (Malheiro et al. 2015a). The same authors analyzed leaf volatiles of three cultivars and reported a significantly lower infestation degree and

higher volatile amounts of cv. Cobrançosa than other two cultivars, with a probable deterrent effect for oviposition (Malheiro et al. 2015c, 2016). Among the investigated volatiles, toluene showed a general increase during fruit maturation and positive correlation with olive fly infestation levels (Malheiro et al. 2016). It has also been shown that olive flies are attracted by chemical cues emitted by epiphytic bacteria, which probably contribute to host location (Scarpati et al. 1996; Sacchetti et al. 2007, 2008).

2.2 Olive Fly Control: From Insecticides to SIT and Beyond

The olive fly can reduce more than 30 % of the olive oil production, for an estimated loss of more than 800 million dollars (Mazomenos 1989; Bueno and Jones 2002). During the last fifty years, the control of the fly has been based on chemical insecticides, mainly organophosphates (OPs), pyrethroids, and, more recently, Spinosad. Spinosad is an insecticide based on compounds derived from *Saccharopolyspora spinosa*. Besides the negative impact on the environment, the inconsiderate use of insecticides increases the occurrence of pesticide resistance. The resistance mechanism has been extensively studied for organophosphates, revealing the occurrence of three mutations in the acetylcholinesterase (AChE), the target gene of the insecticide. Two are point mutations in the catalytic gorge of the enzyme (Vontas et al. 2002). The third is a small deletion located in the carboxyl terminal of the enzyme (Kakani and Mathiopoulos 2008; Kakani et al. 2011). Pyrethroid resistance implicates an elevated level of the P450 mixed function oxidases (MFOs), enzymes involved in insecticide detoxification (Margaritopoulos et al. 2008). Spinosad resistance indicates the involvement of several immune system loci (Sagri et al. 2014a). The reduction of reliance on pesticides for crop protection necessitates the development of novel environmentally friendly methods of insect control. Alternative control methods include mass trapping, natural enemies and, the sterile insect technique (SIT). These strategies are not always adequate to control *B. oleae* populations and infestation. Attractive compounds may be used in mass trapping programs, to lure olive fruit flies into traps (Haniotakis et al. 1991; Noce et al. 2009) or to artificial surfaces treated with chemo-sterilantor persistent insecticides, such as that obtained with the *attract and kill* strategy (Broumas et al. 2002; Bueno and Jones 2002; Petacchi et al. 2003). Recently, the bioinsecticide Spinosad has been incorporated into a bait formulation to spray with large droplets (4–5 mm) on minimal parts of the upper tree canopies with limited environmental impact (Yokoyama 2015).

A wide range of natural enemies, mainly parasitoids (e.g., *Bracon celer*, *Eupelmus urozonus*, *Eurytoma martelli*, *Pnigalio agraules*, *Psyttalia concolor*, *Psyttalia lounsburyi,* and *Utetes africanus*) live at the expenses of OLF larvae in different geographical ranges (Boccaccio and Petacchi 2009; Daane and Johnson 2010; Daane et al. 2015). The presence of a high number of OLF parasitoids led to the hypothesis that olive may have evolved indirect defense responses by modulating emitted volatiles to attract natural enemies of OLF (Alagna et al. 2016). However, classical biological control programs for this insect pest have been implemented in several countries without significant success (Daane and Johnson 2010; Hoelmer et al. 2011; Wang et al. 2011). Other potential biological control factors may include the disruption of the relationship between OLF and its endosymbiont, *Candidatus Erwinia dacicola* (Capuzzo et al. 2005; Estes et al. 2012).

In Tephritidae, SIT has been proven reasonably successful. The SIT is an alternative species-specific control approach, whose principle is based on mass rearing, sterilization by irradiation, and subsequent release of the sterilized insects (Knipling 1955). The reproduction of the target population is therefore blocked, since mating between the released sterile males and the wild females leads to offspring reduction. Initial efforts to use the SIT for OLF control in the 1970s were unsuccessful (Economopoulos et al. 1978; Economopoulos and Zervas 1982;

Economopoulos 2002). Mixed sex releases[1] as well as factors of the fly biology are the main problems that lead to poor field performance (reviewed in Estes et al. 2012). They represent unsolved issue that need to be tackled to improve SIT succesful rate.

Genetic engineering provides alternatives to classical SIT. In the medfly *Ceratitis capitata*, the most studied member of the Tephritidae family, substantial progress resulted in transgenic fly lines capable of male-only releases, early embryonic lethality of the progeny between released laboratory males and wild females, and fluorescent marking of the responsible transgene (Gong et al. 2005; Schetelig et al. 2009; Ogaugwu et al. 2013). Such efforts were possible due to extensive classical genetic analysis of the medfly that led to accelerated development of the appropriate modern molecular and genomics tools. On the other hand, the lack of classical genetic tools for OLF (e.g., morphologically stable mutants) makes the early steps of this effort very challenging. Nonetheless, molecular and genomics approaches have now overcome the need for classical genetic analysis and have renewed the interest for OLF SIT.

A breakthrough in *B. oleae* molecular biology was achieved in 2006, when the insect was successfully transformed (Koukidou et al. 2006), generating hopes for the development of a biotechnology-based strategy for its suppression. A significantly improved SIT variant, "Release of Insects carrying a Dominant Lethal" (RIDL; Thomas et al. 2000) had gained ground, mainly because it circumventes the need for sterilization using irradiation. Instead, the transgenic reproductive sterility was achieved by males carrying a dominant lethal gene and killing the offspring in the field. A new transgenic strain was developed based on female-specific RIDL (FsRIDL) (Ant et al. 2012). This method uses sex-alternate splicing sequences from sex determination genes which results in sex-specific-engineered lethality of females at late larval and pupal stages, allowing male-only production and mortality of female progeny in the field. Further studies in medfly engineering resulted in the development of a transgene-based female-specific lethality system for early embryonic sexing (Ogaugwu et al. 2013). This strategy provides a more cost-effective sexing in SIT programs, since the elimination of the fly larval and pupal stages increases the efficiency in the procedure of mass rearing. Such an endogenous effective lethal system for OLF is under way, since the transcriptome analysis of the insect led to the identification of the appropriate genes (the early embryonic *serendipity-α* locus and the pre-apoptotic *head involution defective* gene (Sagri et al. 2014b).

The lowering cost of NGS technologies made it possible the sequencing of several *B. oleae* transcriptomes that focus either on detoxification gene families (Pavlidi et al. 2013), Spinosad resistance (Sagri et al. 2014a), or genes involved in development, reproduction, or olfaction (Sagri et al. 2014b). Since OLF is not a model organism, the overall premise of such analyses is to obtain tools that would lead to novel approaches for its control. The analysis of complex life-history traits, such as mate- or oviposition-choice, fertility or fecundity, now become feasible and may offer the desired alternative approaches. For example, the reproductive and the olfactory systems are of great research interest. The first system is involved in successful mating and egg development while the second controls the basic insect behavior, including the interactions with potential mates, food sources, and appropriate oviposition sites. A possible manipulation of a mechanism regulating these systems would severely affect the insect's fertility thus reducing its destructive ability.

The OLF biology has entered into the molecular era following the recent submission of its genome sequence to GeneBank (GCF_001188975.1). This effort was a combination of sequencing techniques

[1]In original SIT, both male and female insects are released, particularly because the distinction between male and female pupae is practically unfeasible. Released females, however, although sterile, sting fruits with their ovipositors, which generates a source of secondary bacterial or fungal infections at the sting site. Furthermore, co-released sterile females may also cause the sterile males to court these co-released females instead of seeking out wild females.

(Illumina short reads, Illumina mate pairs, and PacBio long reads), as well as a de novo transcriptome assembly with Illumina RNA sequencing. Further exploration of genomic data will enhance our knowledge of genome structure and function, offering access to many dynamic aspects of the biology of this pest. This will form the basis for future research that would i) provide important insights into fundamental biological questions (such as the interaction with the host plant or the evolution within the Tephritidae), ii) elucidate important mechanisms (such as reproduction, olfaction, or insecticide resistance) and iii) offer novel targets for OLF control.

3 Major Viral and Bacterial Enemies

3.1 Olive-Infecting Viruses and Viral Diseases

Olive-infecting viruses. The number of virus-infecting olive trees has increased with time. Currently, 15 different viruses belonging to nine genera in eight families have been identified (Table 1). Four (e.g., the *Olive latent ringspot virus* (OLRV), the *Olive leaf yellowing-associated virus* (OLYaV), the *Olive latent virus 3* (OLV-3), and the *Olive mild mosaic virus* (OMMV), a recombinant between OLV-1 and TNV-D, seem to be specific to olive since they have not been found so far in other host(s) (Cardoso et al. 2005). Whether *Olive vein yellowing-associated virus* (OVYaV), *Olive yellow mottle and decline-associated virus* (OYMDaV), and *Olive semilatent virus* (OSLV) are also host-specific remains to be established. Virus infections have been recorded in 22 different countries (Table 2). Since worldwide systematic surveys have not been carried out, it is reasonable to expect that the virus list will increase following more extensive investigations in countries where the olive industry is expanding (e.g., Argentina, India, China, Australia, New Zealand). The average infection rate, calculated on over 2000 samples of various geographical origins, analyzed in Italy and other countries, approximates 60 %. Such a high infection level apparently does not reflect on olive yield in an equally severe manner. Based on current knowledge, it seems possible to conclude that a viral etiology can be attributed with reasonable confidence only to the affections denoted "Bumpy fruits" and "Leaf yellowing complex," the latter consisting in a foliar discolorations ranging from chlorosis to bright yellowing. Although both diseases appear to have a detrimental impact on the yield, growth rate [OLYaV (Cutuli et al. 2011)], and rooting ability ("Bumpy fruits"), actual losses have not been quantified. A recent analysis of the "Frantoio" and

Table 1 Diseases and associated recognized viruses

Disease and associated virus	Mechanical transmission	Graft transmission	Country and year of record
Bumpy fruits (SLRSV)	+	+	Italy (1986), Portugal (1992)
Olive vein yellowing (OVYV)	+	−	Italy (1994)
Olive leaf yellowing (OLYaV)	−	+	Italy (1996)
Olive yellow mottling and decline (OYMDaV)	+	+	Italy (1996)
Leaf chlorosis, fasciation and deformation of the shoots (OLV-1)	+	−	Portugal (2000)
Leaf and fruit deformation, leaf yellowing (CLRV)	Putative viral agent identified by RT-PCR		Croatia (2011)
Vein banding (TMV)	+	+	Italy (1996)
Vein clearing (OSLV)	+	−	Italy (1996)

+ = Positive transmission, − = Transmission negative or not done

Table 2 Olive-infecting viruses and their geographical distribution

Virus	Taxonomic position (family, genus)	Country and year of first record
Strawberry latent ringspot virus (SLRSV)	*Secoviridae* (genus to be determined)	Italy (1979), Portugal (1990), Spain (1998), USA (2001), Egypt (2001), Turkey (2004), Lebanon (2005), Syria (2005), Croatia (2007), Tunisia (2009), Albania (2009)
Arabis mosaic virus (ArMV)	*Secoviridae*, *Nepovirus*	Italy (1979), Portugal (2000), Egypt (2001), USA (2001) Lebanon (2005), Syria (2005)
Cherry leafroll virus (CLRV)	*Secoviridae*, *Nepovirus*	Italy (1981), Portugal (1990), Spain (1998), Croatia (2011), USA (2001), Egypt (2001), Lebanon (2005), Syria (2005), Tunisia (2009)
Olive latent ringspot virus (OLRSV) Cucumber mosaic virus (CMV)	*Secoviridae*, *Nepovirus* *Bromoviridee*, *Cucumovirus*	Italy (1983), Portugal (1990), Syria (2005), Tunisia (2009) Italy (1983), Portugal (1993), Spain (1998), USA (2001), Syria (2005), Tunisia (2009), Algeria (2011), Australia (2011), France (2011), Cyprus (2011), Chile (2011), Israel (2011), Morocco (2011)
Olive latent virus 1 (OLV-1)	*Tombusviridae*, *Necrovirus*	Italy (1984), Jordan (1994), Turkey (1996), Portugal (2000), USA (2001), Egypt (2001), Lebanon (2005), Syria (2005), Tunisia (2009)
Olive latent virus 2 (OLV-2) Olive latent virus 3 (OLV-3)	*Bromoviridae*, *Oleavirus* *Tymoviridae*, *Marafivirus*	Italy (1984), Lebanon (2005), Syria (2005), Tunisia (2009) Italy (2009), Portugal (2009), Greece (2009), Malta (2009), Tunisia (2009), Lebanon (2009), Syria (2009), Turkey (2009)
Tobacco necrosis virus D (TNV-D)	*Tombusviridae*, *Necrovirus*	Portugal (2002, 2004)
Olive mild mosaic virus (OMMV)	*Tombusviridae*, *Necrovirus*	Portugal (2005)
Olive leaf yellowing-associated virus (OLYaV)	*Closteroviridae*, (genus to be determined)	Italy (1996), Albania (2006), Spain (2006), Croatia (2007), Israel (1999), Egypt (2001), Lebanon (2005), USA (2001), Syria (2005), Tunisia (2009), Cyprus (2011), Chile (2011), Australia (2011), Greece (2011), France (2011), Algeria (2011), Palestine (2011), Morocco (2011)
Olive vein yellowing-associated virus (OVYaV)	*Alphaflexiviride*, *Potexvirus*	Italy (1995)
Tobacco mosaic virus (TMV)	*Virgaviridae*, *Tobamovirus*	Italy (1996)
Olive semilatent virus (OSLV)	Unclassified	Italy (1996)
Olive yellow mottling and decline associated virus (OYMDaV)	Unclassified	Italy (1996)

"Ascolana tenera," two CLRV-infected Italian cultivars grown in Croatian Istria, disclosed that the presence of this virus affects the oil of "Frantoio" by decreasing the yield from 10.9 to 7.6 % and the quality, by lowering the amount of *o*-diphenols and the oleic/linoleic acid ratio (Godena et al. 2011). However, a better evaluation of the detrimental effects of virus infections

on the quality and quantity of the produced fruits and oil will be possible when sanitized clonal selections will be tested in comparative field trials with their infected mother stocks.

Little is known on the epidemiology of olive-infecting viruses. The assessment of virus spread in orchards, if any, is made virtually impossible by the widespread lack of visible symptoms in infected trees. Furthermore, some vectors (e.g., the dorylamoid nematode *Xiphinema diversicaudatum* that transmits SLRSV and ArMV) do not prosper under the climatic conditions found in most of the areas where olives are grown, whereas other vectors (e.g., aphids that are potential CMV vectors) rarely, if ever, colonize olives. In addition, several other viruses (OLV-2, OLV-3, and all those of the "Leaf yellowing complex") do not have recognized vectors. Thus, the only evidence currently available on the actual or potential virus spread in nature is limited to the three olive-infecting members of the genus *Necrovirus* (OLV-1, TNV-D, OMMV). These were experimentally shown to be picked up by the host in the absence of fungal vectors [OLV-1 (Martelli et al. 1996)] or to be transmitted by *Olpidium brassicae* [OMMV (Varanda et al. 2011) and, likely, TNV-D (Felix and Clara 2001]. Thus, except for the established cases of fungus-mediated transmission through the soil, the intervention of other vectors does not seem to be supported by two relevant notions: (i) the generalized and internationally high incidence of the infections, which could only be explained by the presence and activity of the same vectors in widely separated geographical areas, i.e., an unlikely condition to occur; and (ii) the erratic distribution of infected plants in the field, which does not conform to common vector-generated patterns. It seems more plausible that nurseries are the main centers for virus accumulation and subsequent dissemination through trading of their productions. Field surveys revealed a geographical distribution of the virus, consistent with the concept that the main source of infection is represented by propagative material.

Seeds represent another recently discovered source of infection. The presence of OLV-1 was ascertained in the seeds of cv. "Verdeal Alentejana" in Portugal (Lobão et al. 2002) and in cv. "Oliva rossa" in Italy, with an incidence of 82 % in the latter (Saponari et al. 2002a). Seeds of the same variety were infected by CLRV up to 90 % (Saponari et al. 2002b). The infection rate was lower in the seedlings, but still significant, i.e., 36 % (OLV-1) and 41 % (CLRV). Thus, an additional but still little explored mechanism exists, whereby viruses can spread with seeds in natural environments and in agricultural crops with seedlings used as rootstocks.

Most olive-infecting viruses (13 out of 15) are mechanically transmissible to a range of herbaceous hosts using tissue extracts from various organs (flowers, young leaves or drupes, succulent roots). Nevertheless, because of its low sensitivity, the use of manual transmission can hardly be recommended for assessing the sanitary status of olive selections. Thus, the current protocols for virus detection are not based on biotests (mechanical transmission to herbaceous hosts is unreliable and there are no differential woody indicators available) nor on immuno-enzymatic assays, which are also unreliable, but on nucleic acid-based techniques (Albanese et al. 2012).

Serology does not seem always an effective technique for the identification of olive-infecting viruses. For instance, ELISA was successfully applied for SLRV and CMV detection from field samples in Portugal and Spain but not in Italy, except following sample manipulations for virus concentration increase. The unsatisfactory outcome of ELISA applications has prompted the use of nucleic acid-based diagnostic techniques such as: (i) molecular hybridization of crude sap extracts, or denatured dsRNAs, or total nucleic acid (TNA) extracts with virus-specific riboprobes; (ii) one or more of the many RT-PCR protocols (one-step, nested, multiplex) applicable to crude sap or TNA extracts. A well-performing single-step RT-PCR procedure for the detection of eight olive-infecting viruses (ArMV, CLRV,

SLRSV, CMV, OLV-1, OLV-2, OLYaV, and TNV) included in the Italian phytosanitary certification protocol, has recently been developed and validated through an interlaboratory ring test (Loconsole et al. 2010). Real-time PCR protocols are also being developed with encouraging results (Albanese et al. 2012).

Bumpy fruits. This disease was first observed in Italy in cultivar "Ascolana tenera" (Marte et al. 1986), then in Portugal in cv. "Negrinha de Freixo" (Henriques et al. 1992). Infected trees bear pear-shaped, puckered fruits with deformed kernels, show narrow and twisted leaves and bushy growth. The disease has been reproduced in healthy seedlings by grafting. The yield is affected and cuttings have a reduced rooting ability. The latter trait, however, was not confirmed for the Italian cultivar "Raggiola" whose cuttings rooted as well as those of apparently healthy "Frantoio" plants (Roschetti et al. 2009). The interest of this finding lies in the fact that "Raggiola" and "Frantoio" are apparently genetically identical but are retained as different cultivars due to some morphological of "Raggiola" (narrow leaves, small inflorescences), which are attributed to SLRSV infection (Ferretti et al. 2002). The putative agent of bumpy fruits, the aforementioned SLRSV, is a soil-borne (nematode-transmitted) unassigned member of the family *Secoviridae* (Sanfaçon et al. 2011) identified in 15 different Portuguese cultivars and in a number of others in eight different countries (Table 2), very few of which, however, show symptoms. Modifications of olive drupes resembling very much bumpy fruits were observed in Greece, but the presence of SLRV in symptomatic plants was not ascertained.

Leaf yellowing complex. The bright yellow discolorations of the foliage observed in several Italian regions and described under the name of "vein yellowing," "leaf yellowing," and "yellow mottling and decline" constitute the "Leaf yellowing complex." Three different filamentous viruses are associated with this complex: (i) a putative potexvirus, Olive vein yellowing-associated virus (OVYaV) (Faggioli and Barba 1995); (ii) Olive yellow mottling and decline-associated virus (OYMDaV), a virus belonging to an undetermined genus (Savino et al. 1996); (iii) Olive leaf yellowing-associated virus (OLYaV) a member of the family *Closteroviridae* (Sabanadzovic et al. 1990). The leaf yellowing condition which OYMDaV and OLYaV are associated with was reproduced in healthy seedlings by grafting, suggesting the systemic infection ability of the virus. OLYaV has been found in symptomatic or, more often, symptomless trees from 18 different countries (Table 2).

Other diseases. They include the following: (i) low vigor, leaf chlorosis, fasciation, and deformation of the shoots shown by several Portuguese cultivars infected by *Olive latent virus 1* (OLV-1) were suggested as being putatively induced by this virus (Felix et al. 2007); (ii) deformations of leaves and drupes go together with yellowing of the canopy were observed in Croatia in plants infected by *Cherry leafroll virus* (CLRV) which was retained as the putative agent of the disease (Luigi et al. 2011); (iii) "vein banding" and "vein clearing" are two additional disorders reported from Italy (Table 1). Beside the described symptoms (Triolo et al. 1996), there is no information on their origin and the role, if any, played by the viruses associated with them [*Tobacco mosaic virus* (TMV)] and *Olive semilatent virus* (OSLV), respectively.

3.2 *Xylella fastidiosa*

Xylella fastidiosa (*Xf*) is a xylem-restricted pathogenic bacterium native to the Americas, where it has been confined for long time. *Xf* is the agent of destructive diseases of many agriculturally relevant crops (e.g., blueberry, citrus, coffee, grapevine, several stone fruits) and of different shade trees (Hopkins and Purcell 2002). Unfortunately, *Xf* has no longer a geographical distribution limited to the Americas. Its presence in Taiwan is a potential threat to continental Asia, while the recent landing in Italy and France and the ascertained occurrence in Iran contribute

to a permanent modification to its geographic range (Almeida and Nanni 2015). The bacterium continues to spread into new areas, where it may settle in traditional hosts or move into new ones, eliciting destructive diseases.

The most common pathway leading to *Xf* epidemics is the introduction of exotic genotypes into environments that are ecologically adapted to the maintenance of the bacterium in the plant community. One of the most dramatic recent examples of a new *Xf*–host association is that with olive in Southern Italy. The unexpected arrival of the pathogen in the Salento peninsula has created relevant economic issues beyond the agricultural sector, considering the importance of the olive oil production chain in that region.

Xf infections to olive were first reported in 2014 (Krugner et al. 2014) in trees exhibiting leaf scorch and dieback symptoms in California (USA). The putative agent of this condition, whose pathogenicity is still under scrutiny, is a strain of *X. fastidiosa* subsp. *multiplex*., i.e., a bacterium taxonomically different from *X. fastidiosa* subsp. *pauca*. This bacterium is the major if not the unique agent of the olive quick decline syndrome (OQDS), the disease which is devastating the Salentinian olives (Saponari et al. 2013). OQDS is characterized by leaf scorching and scattered desiccation of twigs and small branches which, in the early stages of the infection, prevail on the upper part of the canopy. Leaf tips and margins turn dark yellow to brown, and desiccate. Symptoms become increasingly severe over time and extend to the rest of the crown, which acquires a blighted appearance. Desiccated leaves and mummified drupes remain attached to the shoots. Affected trees, especially those of the major local cultivars, "Cellina di Nardò" and "Ogliarola salentina," decline slowly and die, regardless of their age. Declining trees, especially the aged, century-old ones, very often exhibit a discolored sapwood, which is colonized by fungi of different genera (e.g., *Phaeoacremonium*, *Phaeomoniella*, *Pleumostomophora,* and *Neofusicoccum*) which are thought to act as disease aggravators (Nigro et al. 2013).

Interestingly, olive trees showing symptoms strikingly resembling those of the Apulian OQDS have been reported in Argentina (Haelterman et al. 2015) and Brazil (Coletta-Filho et al. 2016). In both cases, symptomatic plants are infected by *X. fastidiosa* strains genetically closely related to the subspecies *pauca*. Although belonging to the same subspecies occurring in Apulia, the Argentinean, and Brazilian *Xf* strains differ from the Salentinin isolate, known as CoDiRO, whose genome, a DNA molecule of *ca*. 2,500,000 bp in size, has been sequenced (Giampetruzzi et al. 2015) and found to be molecularly identical to a bacterial isolate from Costa Rica.

Although *X. fastidiosa* has not yet been proven to be the only agent causing OQDS as pointed out by Coletta-Filho et al. (2016), a convincingly strong correlation between symptoms in olive trees and the presence of this pathogen appears evident in three distant geographic regions of the world (Southern Italy, Argentina, and Brazil).

Accurate detection of the bacterium in olive trees has been achieved by serological and molecular assays (Loconsole et al. 2014; Yaseen et al. 2015). The bacterium was isolated in pure culture from symptomatic oleander (Cariddi et al. 2014), olives (Saponari et al. 2014), and a number of other naturally infected hosts (Saponari, unpublished). These cultures have been used for artificial inoculation assays of different olive cultivars and hosts. *X. fastidiosa* is exclusively transmitted by xylem-sap feeding insects belonging to the order Hemiptera, sub-order Cicadomorpha. While in the Americas there are numerous sharpshooters species (family Cicadellidae, subfamily Cicadellinae) and almost sixty have been identified as *X. fastidiosa* vectors, very few sharpshooter species are present in Europe. While there is no information about the vector that transmits the bacterium in Argentinean and Brazilian olive groves, search for the putative vector of *Xf* in southern Italy has identified *Philaenus spumarius* as the predominant vector species. Indeed, this spittlebug represents the most common and widespread species, and

the one that more than any other thrives on olive. Populations of hundreds of adults of *P. spumarius* colonize olive trees in spring-late summer, and a high number of individuals are *Xylella*-positive, up to nearly 100 % in summer (Cornara et al. 2014). Thus, *P. spumarius* has a tremendous inoculum potential that is discharged on olive trees, the species with which it entertains a preferential relationship.

As to the risks for Europe and the Mediterranean basin represented by the introduction of *X. fastidiosa* into Italy, and more recently in France, it has been predicted (Purcell 1997; Bosso et al. 2015) how widely the bacterium will spread in these regions, should nothing be done to confine it within its current boundaries. Likewise, a disease management strategy aimed at restraining bacterial dispersal by reducing the inoculum sources and by controlling vector's juveniles (mechanical weeding in late winter) and adults (a pesticide treatment in late spring when they move to olives) had been envisaged.

4 Some of the Major Olive Diseases Caused by Pathogenic Fungi

Verticillium dahliae. Verticillium wilt of olive (VWO), caused by the soil-borne fungus *Verticillium dahliae* Kleb, is one of the most important diseases affecting this woody crop. Loss from Verticillium wilt includes the death of trees and the reduction in fruit yield. The trees may be infected by two pathotypes of *V. dahliae,* classified as defoliating (D) and non-defoliating (ND) based on their aptitude to induce or not defoliation of green leaves, respectively. Severity of attacks depends upon virulence: The ND pathotype is relatively severe, and in infected plants symptoms may resolve completely. On the contrary, infections by the D pathotype can be lethal (Schnathorst and Sibbett 1971). Moreover, the pathogen can survive in the soil for long periods of time and chemical compounds are not effective (Wilhelm 1955). The use of resistant cultivars or rootstocks for grafting of VWO-susceptible varieties may represent a valuable tool to counteract the disease.

During the last 20 years, VWO attacks have considerably increased in many Mediterranean regions. Multiple factors such as (i) the use of infected propagation material or pathogen-infested soils, (ii) the abuse on fertilization and irrigation, (iii) the pathogen's dispersal efficacy and the endurance of its infective structures (microsclerotia), (iv) climatic factors and edaphic variables, (v) the genetic/pathogenic diversity of pathogen's populations (i.e., defoliating [D] and non-defoliating [ND] pathotypes), or (vi) changes in cultivation systems have contributed to boost the disease. This scenario makes necessary to implement an integrated disease management strategy for the effective control of VWO (López-Escudero and Mercado-Blanco 2011).

Spilocaea oleagina. Olive leaf spot (OLS), also called peacock spot disease or *Cycloconium* leaf spot, is caused by the fungus *S. oleagina*, Fries (syn. *Cycloconium oleaginum* Cast). This disease usually arises on the upper surface of the olive leaf and is associated with the fall of leaves and fruit as well as low quality of olive oil, causing considerable losses in many olive-growing areas worldwide (Viruega et al. 2013). Resistance of olive cultivars to *S. oleagina* attacks has been reported to be variable although the underlying mechanisms are not known (Mekuria et al. 2001). Some cultivars have been described as relatively tolerant (based on symptom severity), but not "immune" to the pathogen. The disease may be chemically controlled by the application of fungicides (Sistani et al. 2009), but treatments appear to be not always effective (Obanor et al. 2008). In addition, chemical fungicides may lead to the onset of resistant pathogen races (Vanneste et al. 2003) as well as disorder of the plant metabolism (Obanor et al. 2008).

5 The Interaction of Olive with Its Enemies

5.1 The Molecular Responses of the Olive Fruit to *Bactrocera oleae* Infestation

Despite the dominant importance of the OLF in the vast majority of olive cultivated areas (Malheiro et al. 2015a), research efforts to examine olive response to the fruit fly are scarce. Among others, two main factors may account for the limited information available. Firstly, the study of the interaction is hampered by the virtual impossibility to use the so-called controlled conditions. The cultivation of olive plants in confined environments, such as growth chambers or greenhouses, and the fruit fly rearing pose large structural and economic burdens. Besides being a perennial, slow-growing, shallow-rooted, biennial bearing tree, olives require a specialized workforce and large dedicated space. For instance, trees require some cold for proper fruit setting but they are sensitive to hard freezing. Moreover, although considerable progress has been made, mass rearing of the insect without negative effects on pest performance cannot be considered a routine approach (Rempoulakis et al. 2014; Sagri et al. 2014a). While the absence of controlled conditions likely reduces the reproducibility of the experiments, it is fair to add that not all the results in a confined environment may be necessarily significant in field conditions. On the other hand, the selection of appropriate uninfested drupes from undamaged trees in field conditions is essential to avoid false negatives, and it is a technical challenge that requires entomological expertise. Another factor to consider is the ample genetic variability of the cultivated olive. A very large number of cultivated varieties characterize this species (Belaj et al. 2002). Considering that different cultivars have different levels of tolerance to the fruit fly (Daane and Johnson 2010), it is expected that also molecular response may differ. It is also likely that the same cultivar may display different behavior in different environments, especially in response to different climates or soil types.

The interaction between *B. oleae* and olive drupes is a relatively complex process. Some traits of the plant affect the interaction even before the pest has been in physical contact with its host. The fruit fly can locate potential host plants at a distance and employs non-random behaviors (e.g., based on fruit dimension and phenological state) to increase the probability of landing on a suitable host. Moreover, before oviposition, the OLF evaluates the acceptability by fruit probing. Olive cultivars may have a very different constitutive tolerance to OLF, affected by a high number of factors. These include plant-based traits, pest population density, climate, and their interaction (Lo Scalzo et al. 1994; Scarpati et al. 1996; Massei and Hartley 2000; Burrack and Zalom 2008; Gonçalves et al. 2012). Unfortunately, cultivars that are considered very tolerant to OLF may suffer considerable attacks under intense infestation (Iannotta et al. 2007). The differential susceptibility to the OLF oviposition may involve a number of morphological, physiological, and phenological parameters (Neuenschwander et al. 1985; Kombargi et al. 1998; Rizzo et al. 2012), which make the study of constitutive defense challenging. Some attempts have been made to investigate the molecular reaction of the drupe to the fruit fly sting. Although limited to a small number of genes, it seems clear that inducible genes have a different response to the *B. oleae* puncture, to mechanical wounding, and to the feeding larvae (Corrado et al. 2012). This is expected considering that the larva actively takes away nutrients from the drupe, which is consistent with the higher magnitude of the olive response observed for this interaction.

The first effort to achieve a more comprehensive understanding of the molecular basis and related signaling pathways involved in olive interaction with *B. oleae* larvae was done by PCR-based suppression subtractive hybridization (SSH) (Corrado et al. 2012). The SSH method allows selective PCR amplification of cDNA fragments that differ between a control and

experimental transcriptome without any prior genomic knowledge. For this reason, this technique has been employed to investigate the plant response to biotic and abiotic stress (Ouyang et al. 2007; Estrada-Hernandez et al. 2009). The functional characterization of the subtracted library indicated a higher representation of ESTs involved in the plant response to stress. On the other hand, a noteworthy proportion of sequenced transcripts were similar to uncharacterized olive transcripts, suggesting that the olive response to *B. oleae* also involves a number of olive specific genes yet to be discovered. Considering that in total, less than half of the identified olive transcripts could be annotated, a critical barrier for working with the olive is the dependence gene ontology repertoires and genomic information that are primarily based on model species (Kültz et al. 2007). According to a similarity-based GO-analysis, various classes of genes are affected by the feeding larva. Differentially represented transcripts found a significant similarity with genes involved in the response to biotic stress, such as wounding and pathogen attack, as well as abiotic stress, such as high or low temperature, drought, and NaCl. The identified transcripts were also putatively involved in the production, signal transduction, or response to hormones and molecules involved in pest response (e.g., jasmonic acid and ROS). Transcripts putatively encoding for resistance-related traits such as proteinase inhibitors (i.e., trypsin inhibitors, a type of serine protease inhibitors) were highly overexpressed in two cultivars following larval feeding. Serine inhibitors in plants belong to a large multigene family and, in absence of a reference genome, it was difficult to ascertain the possible contribution or more than one member. Larval infestation maintained high levels of trypsin protease inhibitors in ripe fruits (Alagna et al. 2016). Approximately, a third of the functionally annotated transcripts were homologous to genes that were first described in plant–pathogen interaction. The concurrent presence of genes involved in different signaling pathways suggests that a clear dichotomy between responses to arthropods and pathogens is not present in olive, as also noted for the interaction between olive and *S. oleaginea* (Benitez et al. 2005). It is known that feeding tunnels, as well as stings, may represent an opportunity for "secondary" pathogens. In addition, *B. oleae* larvae require their natural complement of bacteria to growth in unripe olives (Ben-Yosef et al. 2015). The olive defense mechanisms should include also indirect defense that is the ability of the plant to attract natural enemies of the herbivores. OLF induces an ethylene burst and a quantitative and qualitative variation in the volatile organic compounds (VOCs) from fruits (Alagna et al. 2016). Moreover, it has been also proposed that volatiles emitted by olive leaves may interfere in olive fly females' host selection (Malheiro et al. 2016).

As previously mentioned, transcriptome sequencing in olive is not only able to describe differences in gene expression but, with limited genomics information, it is also useful to identify transcripts and biological processes involved in specific plant reactions. Among the various olive transcripts that have been linked to OLF infestation (Corrado et al. 2012), different research lines indicated that genes coding for beta-glucosidases are important element of the olive response (Koudounas et al. 2015). Beta-glucosidase activity promotes the formation of toxic glutaraldehyde-like structure from oleuropein. Oleuropein is a major secoiridoid compound in olive, whose presence has been linked to olive resistance to the fly (Lo Scalzo et al. 1994; Noce et al. 2014), as well as pest resistance in other members of the Oleaceae family (Konno et al. 1999).

Proteomics studies in olive have been mainly focused on fruit development and quality. A study of the effect of the *B. oleae* larvae on drupes indicated that the differentially expressed proteins are primarily involved in carbohydrate metabolism, redox processes, and defense responses, such as a beta-glucosidase (Corrado et al. 2012).

Current evidence indicates that olive tolerance to the OLF is the outcome of a complex response, in which both genetic and environmental variabilities play a role that is worth investigating. Plants make use of pre-existing

physical and chemical barriers to reduce their suitability. The phenological state at the time of the pest outbreak is also a factor that influences host selection, but the relative importance of constitutive and inducible traits to the olive tolerance to the OLF is a question that remains open. Inducible defense mechanisms are activated upon attack and they include both direct and indirect defense mechanisms. The physical interaction between the drupe and the OLF activates components of different signaling pathways and leads to the production of chemically different compounds that directly and indirectly should reduce pest performance.

The complexity of the olive-*B. oleae*-environment interactions requires interdisciplinary approaches in order to elucidate the importance of the genetic factors and of the molecular basis of induced resistance. Future research cannot understand olive defense without a more integrative experimental designs that will require the expertise of different scientific fields. Available information indicates that herbivore-induced response should affect a number of relevant biochemical and morphological features of the drupe. Therefore, the molecular investigation of the effects upon olive yield and quality represent an interesting challenge for the applied research in the field.

5.2 The Olive Responsive Transcriptome to a Vascular Soil-Borne Pathogen

5.2.1 *Verticillium dahliae*

Understanding the genetic and molecular mechanisms triggered in olive upon the infection by *V. dahliae* would be instrumental to design novel control tools to confront the disease. While our knowledge on plant–pathogen interactions has steadily increased over the years, particularly with the development of powerful "-omic" approaches, the information about the genetic and molecular bases underlying plant defense responses against vascular and/or root pathogens is until now scarce (Larroque et al. 2013; Yadeta and Thomma 2013). In the case of *V. dahliae*, plant tissue reactions so far reported can be structural, i.e., the formation of tyloses in the xylem, and/or biochemical, i.e., accumulation of phenolic compounds (Baídez et al. 2007; Markakis et al. 2010). They can be either constitutive (Mueller and Morgham 1993) or induced in response to pathogen infection (Daayf et al. 1997; Markakis et al. 2010). In olive, tolerance of cultivar "Frantoio" to *V. dahliae* has been suggested to be mostly mediated by biochemical mechanisms induced in the root tissues, rather than structural responses such as vascular plugging (Bubici and Cirulli 2012). Overall, these defense reactions seem to take place to a lower extent in susceptible varieties than in tolerant ones (Baídez et al. 2007; Markakis et al. 2010; Bubici and Cirulli 2012).

Our knowledge on the genetic basis underlying *V. dahliae*-olive interaction has been recently enhanced by using the SSH methodology (Diatchenko et al. 1996). The interaction under study was the VWO-tolerant cultivar "Frantoio" and the D, highly virulent pathotype of *V. dahliae* (Gómez-Lama Cabanás et al. 2015). cDNA libraries enriched in up-(FU) or down-regulated (FD) olive genes from above-ground tissues upon root infection by the pathogen were generated. Broad transcriptomics changes taking place in aerial organs were observed as consequence of root infection by *V. dahliae*. It is interesting to emphasize that many of the genes systemically induced or repressed related to defense against diverse stress agents. For instance, genes putatively coding for catalase, calmodulin-binding family protein, ET-responsive transcription factor rap2-12-like, acetone cyanohydrin lyase or thaumatin like-protein were identified as up-regulated genes, whereas defensin, chloroplastic 6-phosphogluconolactonase, or phosphatase 2c 25 were found as down-regulated. Some 2688 expressed sequence tags (EST) were sequenced and analyzed from FU and FD libraries, eventually generating 976 unigenes. A total of 585 transcripts corresponded to up-regulated genes while 381 were down-regulated. Sequence comparison revealed that 37 % of the ESTs matched

to coding sequences previously identified in genomes of woody plants but only 3.5 and 5.9 % of the unigenes found in FU and FD libraries, respectively, showed significant identity with olive genes (Gómez-Lama Cabanás et al. 2015). As also previously stated, this result underlines the scarcity of genetic/genomic information on olive. Bioinformatics analysis showed that colonization of "Frantoio" roots by the *V. dahliae* D pathotype induced (19.8 % of unigenes identified in FU) and repressed (25.7 % of unigenes identified in FD) a broad range of plant defense responses to stresses (i.e., phenylpropanoid biosynthesis or terpenoids, hormones biosynthesis, and salicylic acid [SA]-related proteins). Besides, genes coding for transcription factors (TFs) involved in a/biotic stress such as *GRAS1* and *WRKY*'s (i.e., *WRKY 33* and *20*) were systemically up-regulated, suggesting that these TFs may play important roles in defense against this vascular pathogen. Elongation factors (i.e., *EF-1α*) and genes coding for β-amylases were also found in both cDNA libraries. Recently, β-amylases have been reported as negative regulators in Arabidopsis partial resistance against *V. dahliae* (Gkizi et al. 2015). Nevertheless, the roles of EFs and β-amylases coding genes in the olive-*V. dahliae* interaction remain to be elucidated. Finally, it is interesting to emphasize that 4 % (FU library) and 19 % (FD library) of the identified unigenes are related to the photosynthesis processes. Bilgin et al. (2010) have suggested that down-regulation of photosynthesis-related genes is part of a defense response, particularly against biotic stress.

The study of Gómez-Lama Cabanás et al. (2015) has also analyzed, in "Frantoio" plants, the relative expression pattern along time of seven genes involved in defense responses to different stresses. The genes were: *ACO* (ACC oxidase), *CO-MT* (caffeoyl-O-methyltransferase), *ACL* (acetone cyanohydrin lyase), the TF *GRAS1*, *DRR2* (disease resistance response protein), *PR10* (pathogenesis-related protein), and *DEF* (plant defensin protein). *GRAS1* and *CO-MT* genes were validated indicating that the phenylpropanoid pathway and this TF are systemically induced when *V. dahliae* colonizes olive roots. Four of these genes (*GRAS1*, *DRR2*, *ACL,* and *ACO*) were further assessed on their early- and middle-term expression patterns in olive cultivars showing differential susceptibility to VWO ("Picual" susceptible; "Frantoio" and "Changlot Real" tolerant). Interestingly, similarities in the expression pattern were found depending on the VWO susceptibility/tolerance level of the cv. tested. Thus, *GRAS1* slightly increased its relative expression along time in tolerant cultivars, while it showed a trend to be repressed in "Picual" plants. The putative olive *DRR2* gene, identified in the FD library, showed a trend to be up-regulated in the susceptible cv. unlike the overall down-regulated over time observed in tolerant varieties (Gómez-Lama Cabanás et al. 2015). This transcriptomics approach has provided for the first time crucial information about an as yet poorly understood interaction between the most devastating olive vascular pathogen and a VWO-tolerant cultivar. Data will be useful in terms of both acquisition of fundamental knowledge and the potential development of novel control tools such as genetic markers to evaluate VWO susceptibility/tolerance degree of olive genotypes. While SSH has revealed to be a robust technique, the implementation of more powerful approaches such as RNAseq (Wang et al. 2009) will yield more comprehensive information to understand, among others, this biotic interaction, as already used to study transcriptomics changes taking place during cold acclimation of olive plants (Leyva-Pérez et al. 2015).

5.3 The Olive Responsive Transcriptome to a Foliar Pathogen, *Spilocaea oleagina*

A transcriptomics approach based on differential display was used to elucidate molecular responses during the interaction with *S. oleagina* (Benitez et al. 2005). The interaction of "Lechín de Sevilla," a variety considered to be resistant to "peacock spot" (Trapero and Blanco 2001), with *S. oleagina*, up-regulated 162 olive transcripts.

The relative expression pattern of 21 selected genes revealed differences in gene induction along time. Early induction of several genes involved in signaling, transcriptional control, oxidative stress, a/biotic stresses, as well as a number of genes with unknown function, was observed after infection. However, induction of genes involved in metabolism and cellular maintenance was delayed. In contrast, up-regulation of these 21 selected genes in the susceptible cv. "Picual" was delayed and/or reduced in response to *S. oleagina* inoculation. Basal expression of some genes in control plants of the resistant cv. was higher than in the susceptible one, indicating a constitutive activation of defense responses (Benitez et al. 2005). These results shed light on the underlying mechanisms of this plant–pathogen interaction, indicating that resistance to *S. oleagina* in olive relies on an active genotype-dependent defense response. This conclusion is based on the observation that constitutive expression of defense genes was lower in the susceptible variety than in the resistant one, the latter showing a faster and stronger induction of gene expression after pathogen inoculation.

6 The Interaction of Olive with a Biological Control Agent, *Pseudomonas fluorescens*

Bacterial endophytes are probably present in all plant species, providing benefits to the host plant and positively influencing its growth, fitness, and development (Hardoim et al. 2015). Endophytic bacteria constitute a yet-to-be-explored tool for agricultural biotechnology (Mercado-Blanco and Lugtenberg 2014). For instance, endophytic bacteria exerting biological control may activate control mechanisms once established within plant tissues, potentially setting off a long-term plant protection status (Rosenblueth and Martínez-Romero 2006; Reinhold-Hurek and Hurek 2011). However, many questions on how a plant–endophyte interaction is successfully established remain to be elucidated. To develop such a lifestyle means that endophytes must be adapted to the plant interior and that are able to overcome, elude, or modulate the plant immune response to be recognized by the host plant as beneficial organisms (Reinhold-Hurek and Hurek 2011; Mercado-Blanco 2015).

Pseudomonas fluorescens PICF7 is an indigenous inhabitant of olive roots (Martínez-García et al. 2015). It shows in vitro antagonism against *V. dahliae* (Mercado-Blanco et al. 2004) and is able to endophytically colonize olive roots under different experimental conditions (Prieto et al. 2011; Maldonado-González et al. 2015). It has been previously shown that PICF7 is an effective biological control agent (BCA) against VWO (Mercado-Blanco et al. 2004), and that effective suppression of the disease requires the establishment of the BCA at both the surface and the interior of olive roots, prior to colonization by *V. dahliae* (Prieto et al. 2009).

In the case of olive, our understanding of the transcriptomics changes occurring during the interaction with a beneficial, endophytic bacterium has been recently enhanced. In order to elucidate the genetic processes taking place in roots (Schilirò et al. 2012) and aerial tissues (Gómez-Lama Cabanás et al. 2014) during the colonization of olive roots by strain PICF7, SSH cDNA libraries of the VWO-susceptible cv. "Arbequina" inoculated with strain PICF7 were generated, enabling the identification of a broad set of up-regulated olive genes at both local and systemic levels (Fig. 2). Schilirò et al. (2012) demonstrated that colonization by PICF7 induced a broad set of defense responses in olive root tissues, including genes related to ISR (induced systemic resistance) and SAR (systemic acquired resistance). Computational analysis of 445 unigenes induced in olive roots upon PICF7 inoculation showed that more than 40 % of them were associated with plant defense and response to stresses. A high percentage of unigenes (43.8 %) represented sequences present in genomes of woody plants, although only 2.5 % corresponded to olive, similarly to what observed in the *V. dahliae*–olive interaction.

Relative expression of selected genes involved in plant hormones biosynthesis and responsive transcription (*ACL*; lipoxygenase,

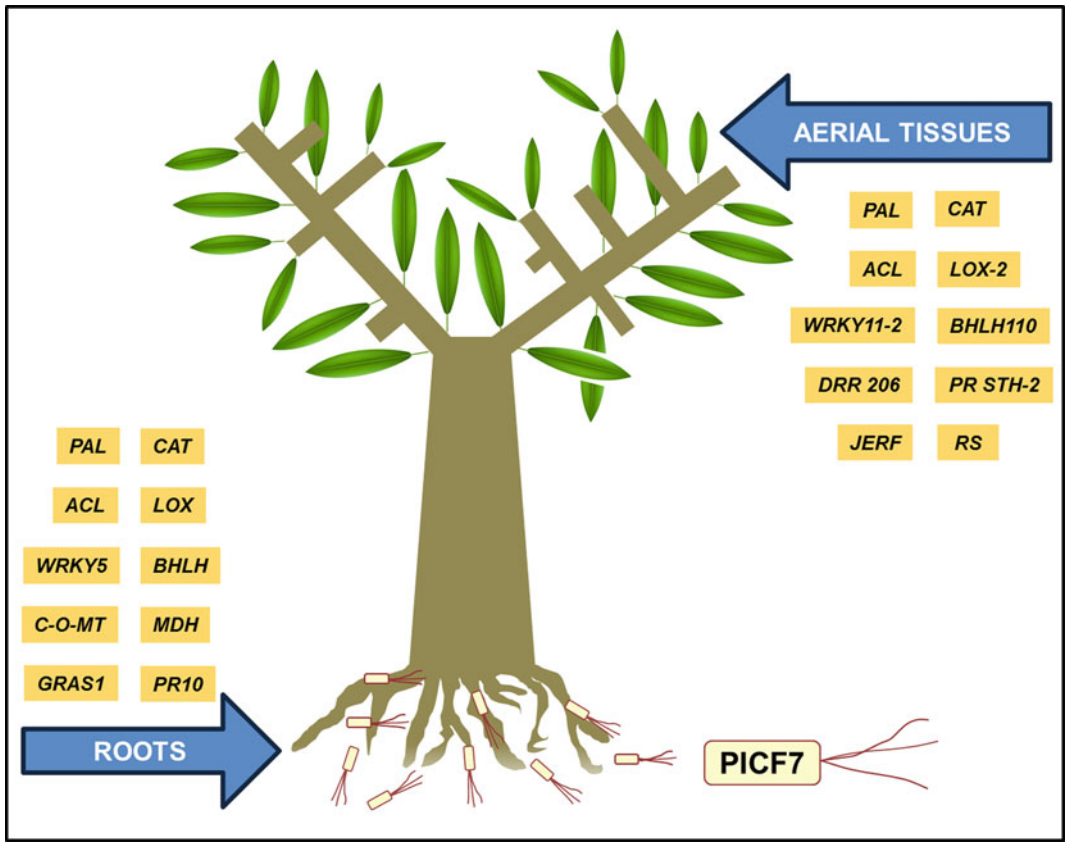

Fig. 2 A schematic representation of some genes involved in defense responses which are induced in olive roots and/or aerial tissues during the interaction of *Pseudomonas fluorescens* PICF7 (a beneficial root endophyte effective against *Verticillium dahliae*) with roots. *ACL* Acetone cyanohydrin lyase; *CAT* Catalase; *C-O-MT* Caffeoyl-O-methyltransferase; *BHLH110* and *BHLH* Basic helix-loop-helix transcription factors; *JERF* Transcription factor JERF; *LOX/LOX-2* Lipoxygenases; *MDH* Malate dehydrogenase; *PAL* Phenylalanine ammonia-lyase; *WRKY5* and *WRKY11-2* WRKY transcription factors; *GRAS1* transcription factor GRAS; *PR10* and *PR STH2* Pathogenesis-related proteins; *DRR 206* Disease resistance response protein; and *RS* Raffinose synthase. Based on Schilirò et al. (2012) and Gómez-Lama Cabanás et al. (2014)

LOX; malate dehydrogenase, and *WRKY5*, *bHLH*; *ARF2* [TFs]), phenylpropanoid biosynthesis (phenylalanine ammonia-lyase, *PAL*) and signal transduction defense response (*GRAS1*, a TF) validated the results from the generated SSH cDNA library. For instance, induction of *PAL* gene transcript suggested that this response pathway may be activated upon PICF7 colonization and may play some role in the biocontrol activity displayed by this bacterium. Up-regulation of *GRAS1* could indicate a modulation of olive defense network signaling after PICF7 treatment. Additionally, the ARF family of TFs regulates a broad range of plant responses to auxin (Tiwari et al. 2003). Up-regulation of *ARF2* and *WRKY5* may contribute to elicit a SAR response in olive as a consequence of PICF7 colonization. Moreover, a vast number of new candidate genes participating in this beneficial interaction, such as intrinsic membrane proteins, catalases (CATs), or purine permeases related to cellular communication, carbohydrates, and ZIP family iron transporters, were identified. Gómez-Lama Cabanás et al. (2014) aimed to elucidate whether similar systemic defense responses were also triggered in "Arbequina" aerial tissues upon

root inoculation by PICF7. In this case, sequencing of 1344 ESTs provided a set of 376 induced unigenes. Computational analysis revealed that many of them were potentially involved, as observed for roots tissues, in plant defense, and response to different stresses. Percentage of sequences homologous to woody plants was somewhat lower than that found in root tissues (34.6 %), while percentage of sequences showing significant identity with olive genes was higher (4.3 %). Interestingly, some genes involved in defense response were up-regulated in both tissues. This suggested that strain PICF7 could play an important role as a BCA against olive pathogens other than *V. dahliae* through a systemic defense mechanism (i.e., ISR). Among others, genes involved in plant hormones and phenylpropanoids biosynthesis (i.e., *PAL*, *ACL*, *ACO*, *LOX-2*), oxidative stress (*CAT*), and Ca^{2+} metabolism implicated in systemic defensive responses were induced in above-ground organs. In addition, expression of several TFs related to plant defense were also up-regulated, i.e., JERF, bHLH, and WRKY (Gómez-Lama Cabanás et al. 2014). In their study, five genes *ACO*, *ACL*, *LOX-2*, *CAT,* and *PAL*, all related to plant defense responses, were then selected for validation of the SSH library at different time points. *ACO* was moderately up-regulated in aerial tissues upon root colonization by PICF7. The putative olive *CAT*, also found as up-regulated gene in root tissues (Schilirò et al. 2012), was highly induced at middle-term after inoculation. Calmodulin (CaM) and other Ca^{2+}-related proteins were induced in aerial tissues as well, suggesting that the complex Ca^{2+}/CaM can decrease H_2O_2 levels in plants by activating CATs, supporting their possible role in plant defense responses as previously reported (Yang and Poovaiah 2002). Olive *LOX-2* was found to be up-regulated in the long term, and another *LOX* gene implicated in biosynthesis of JA was induced in olive roots (Schilirò et al. 2012). The same *ACL* found as up-regulated in roots (Schilirò et al. 2012) was also induced in aerial tissues. The induction of the *PAL* gene in both olive roots (Schilirò et al. 2012) and aerial tissues (Gómez-Lama Cabanás et al. 2014) upon PICF7 treatment indicates that this defense response pathway is activated after PICF7 colonization, and that this BCA seems to be recognized by the host plant, at least transiently (maximum relative expression at middle term), as a stress-inducing agent. Overall, middle-term up-regulation of defense-related genes in olive aerial tissues could be a response of the plant to defend against strain PICF7 colonization, whereas the subsequent decrease in gene expression could indicate that presence of this endophytic bacterium in roots is somehow recognized as "non-hostile." How the plant response is attenuated or overcome by PICF7 remains to be elucidated.

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Physiological Responses to Abiotic Stresses

7

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Abstract

Olive (*Olea europaea* L.) trees are widespread in Mediterranean agroecosystems and are now extensively cultivated in different warm-temperate regions of the world such as North and South America, Australia, New Zealand, and South Africa, and even in the monsoon systems of China and India. In the Mediterranean area, the biological and agronomical success of this species is due to its adaptability to the Mediterranean climatic conditions: mild, wet winters with temperatures that drop below 10 °C but rarely below 0 °C and warm, dry summers. When weather conditions become more extreme (drought, high, or low temperatures) or soil conditions are not optimal for olive growth (salinity, low oxygen, nutrient deficiencies), the plant can be subjected to abiotic stresses, which may have negative effects on its physiology. The damages derived from stresses caused by environmental constrains are often not immediately recognized in olive orchards, since plants are largely grown in non-specialized planting systems that are managed with limited cultural practices. However, due to the renewed interest in extra-virgin olive oil for its beneficial health effects, olive cultivation has now been modified from traditional low-density and low-input to high-density and high-input growing systems. Information on the effect of abiotic stresses on trees under the new cultivation systems is scarce due to the wide differences in management practices, environmental conditions and the increase in the

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use of selected varieties. Under these new conditions, the abiotic factors and their related stresses might have a strong impact on both yield and quality. In this chapter, we focus on physiological responses of olive trees to drought, salinity, and temperature stress. The reader can refer to the existing literature for other abiotic stresses.

1 Drought Stress

In Mediterranean agroecosystems, drought is one of the main stress factors that limit olive growth and productivity, despite the morphological, anatomical, and physiological adaptive traits of this species. These traits confer olive trees a high resistance to water scarcity ensuring to regulate water loss, maintain water uptake at the beginning of drought stress, and preserving cell turgor while tolerating dehydration when stress increases (Connor and Fereres 2005). In plants, water is continuously absorbed and lost in large quantities. Transpiration is the essential process that enables the exchange of CO_2 and water vapor with the atmosphere. Transpiration is also an important mechanism for dissipating the heat input from sunlight (nearly half of the net heat input on the leaf). In olive, Bongi and Palliotti (1994) estimated that for each gram of fruit dry matter, approximately 315 g of water are absorbed by the roots, transported toward the stems and the leaves, and then lost into the atmosphere. To ensure this continuous and intense water flow, the maintenance of hydraulic functionality is very important. Recently, the most relevant physiological adaptations to water stresses in olive trees and the mechanisms adopted by the plant to maintain hydraulic functionality and xylem water potential above the safety threshold to avoid hydraulic conductance loss have been highlighted (Fernández 2014). The water stream from roots to leaves serves for the transport of minerals elements following uptake by roots. For these reasons and many others that are beyond the scope of this chapter, olive trees must precisely balance water uptake and loss, as slight imbalances in water flow might induce severe damage. Furthermore, crop performance in relation to water stress is a time-dependent process as stress intensity, duration, and timing during the olive production cycle change continuously and interact synergistically with many others stress factors, e.g., mineral nutrients availability, high temperature, salinity, and many others.

1.1 Anatomical and Morphological Features of Roots, Stems, and Leaves

The root system of the olive tree is well adapted to explore the top soil layers and take advantage from the scarce and intermittent rainfall events typical of Mediterranean climate (Fernández et al. 1991; Searles et al. 2009). Moreover, the presence of active roots in soil volumes close to the trunk increases plant efficiency in absorbing the water coming from rainfall and running down the stem (Gómez et al. 2001).

In olive stems, the xylem is diffuse-porous, with small-diameter vessels uniformly distributed in the annual growth ring, rich in fibers, and with scarce parenchymatic cells (Salleo et al. 1985; Rossi et al. 2013) (Fig. 1). The diameter of the xylem vessels in nodal and internodal portions of a 1-year-old stem is less than 40 μm, and as much as 90 % of the vessels have a diameter of less than 20 μm (Lo Gullo and Salleo 1990). The frequency of vessel diameter classes in rings of mature olive plants varies with water availability. Rossi et al. (2013) reported that irrigated trees have fewer vessels with a diameter <20 μm than rainfed trees: 4.3–8.3 % versus 8.3–15 % (as range variation in annual ring). Overall, the diameter classes higher than 20 μm were on average more abundant in irrigated trees than in rainfed ones. Small-diameter xylem vessels have a lower hydraulic

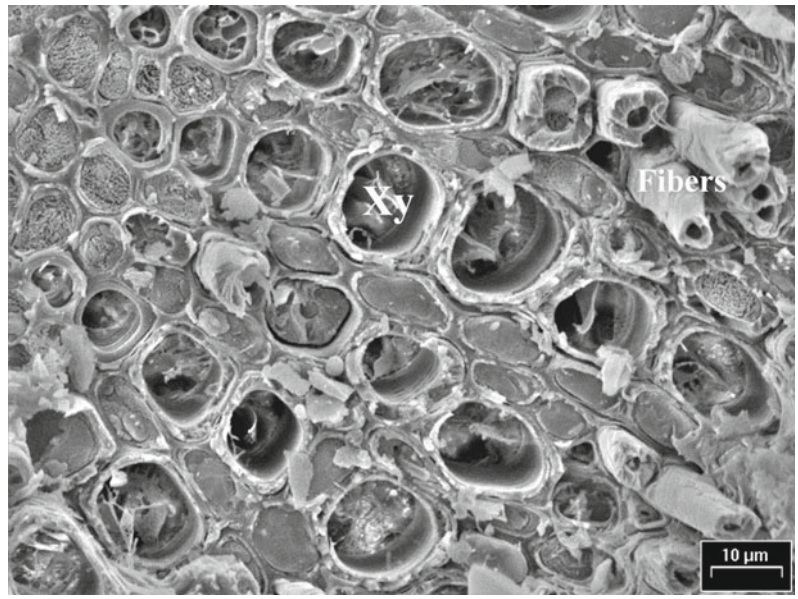

Fig. 1 Cryo-SEM image of frozen-hydrated current-year shoot of olive, freeze fractured transversally. Xy, xylem cell and fibers. *Photograph* A. Minnocci

conductivity, which can increase the plant resistance to water-deficit stress by reducing the probability of embolism occurring in the xylem. One of the causes of xylem embolism is cavitation that occurs when the tension in the vessel is greater than the vapor pressure of water; when this phenomenon happens, liquid water transforms into vapor water (Fernández 2014). A xylem vessel can also be blocked by air bubbles into the transpiration stream through the pit membrane, a phenomenon known as air-seeding. Due to olive anatomical features, it has been estimated that only 5 % of the xylem vessels of the stem undergo cavitation when the leaf water potential (ψ_{leaf}) approaches −3.5 MPa (Salleo and Nardini 1999). For that level of water stress, the percent loss of conductance in current-year shoots of 'Manzanilla' trees has been reported to be about 25 % (Torres-Ruiz et al. 2014).

In species adapted to xeric habitats, thick and tough leaves with a highest fiber-to-protein ratio are usually present (Larcher 1995). The evergreen and long-living (2–3 years) olive leaves have many sclerophylly traits, including a thick cuticle and stomata located on the abaxial side, where they are protected by a multilayered *indumentum* of peltate scales, or trichomes, (Marchi et al. 2005, 2007) (Fig. 2) that create microenvironmental conditions favorable to gas exchange under dry conditions (Besnard et al. 2009). Sclerophylly characteristics, such as higher density of leaf tissue, thickening of the cuticle and density of the trichomes, increase further in response to water-deficit stress (Bosabalidis and Kofidis 2002). Moreover, Bacelar et al. (2004) found extensive genotypic differences among cultivars: an enhanced sclerophylly was observed in 'Manzanilla,' for example, as the production of more parenchyma tissues and increased protective structures such as the upper cuticle and the upper and lower epidermis, while in 'Cobrançosa' water loss is prevented through high-density foliar tissue and thicker cuticle and trichome layers. Studies investigating which genes are responsible for these anatomical and morphological traits would be extremely useful in developing new varieties incorporating these characteristics.

1.2 Physiological and Molecular Features

The visible symptoms of drought stress appear late in field-grown trees, when growth and yield are already affected. Early senescence of leaves and leaf drop are evident symptoms of drought,

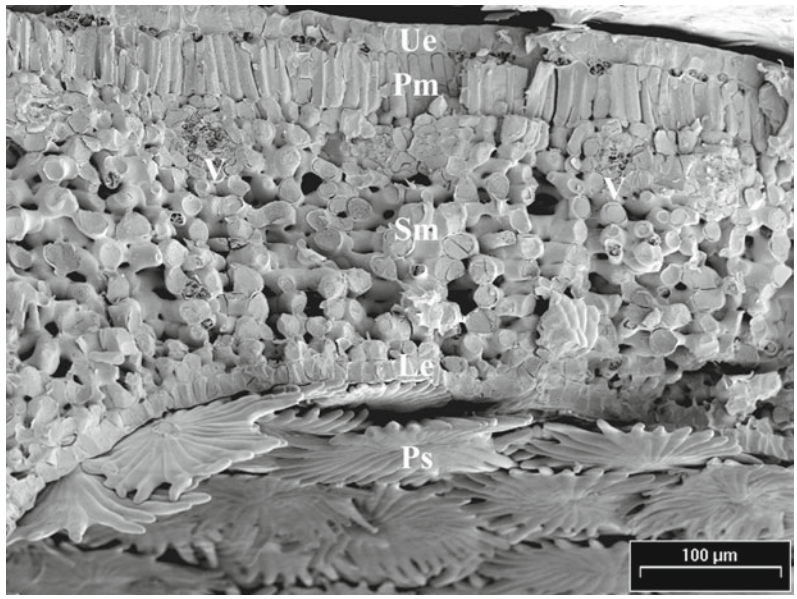

Fig. 2 Cryo-SEM image of frozen-hydrated young olive (cv. Leccino) leaf, freeze fractured transversally. At the *top* are the upper epidermis (Ue) and palisade mesophyll (Pm), in the *center* the spongy mesophyll (Sm) with veins (V) and below the *lower* epidermis (Le). On the abaxial surface are present several peltate scales, or trichomes (Ps, peltate scales). *Photograph* A. Minnocci

but are common to other types of stresses as well. Under controlled experimental conditions, the progression of water-deficit symptoms starts with leaf blade folding and changes in leaf angle and ends with leaf shedding. In the early stages, the visible symptoms are mainly localized in the youngest leaves and occur during the warmest period of the day. Loss of turgor and exposure of the trichomes-rich abaxial surface of the leaf (Fig. 3), obtained by lowering the insertion angle of the petiole of the leaf onto the stem, enable the reflection of the incoming solar radiation (Baldini et al. 1997). These mechanisms allow to decrease transpiration through a reduction in leaf energy balance, which limits temperature rise and photoinhibition in the leaf (Schwabe and Lionakis 1996). As water stress progresses, symptoms include turgor loss, chlorosis, bronzing, and blade folding appear on older leaves (Gucci et al. 2003), shoot wilting and shriveled fruits (Fig. 4). In addition, olive has the capacity to develop tolerance mechanisms via a series of physiological responses, which result in the maintenance of tissue water status within a physiological range that is still suitable for metabolic processes (Lo Gullo and Salleo 1988). These adaptations enable olive plants to establish a high water potential between leaves and roots, and consequently to extract water up to a soil water potential (ψ_{soil}) of −2.5/−3.5 MPa (Lo Gullo and Salleo 1988; Dichio et al. 2003, 2005) before wilting. In fact, olive plants tolerate low water potential (ψ_w) (down to −6 or even −8 MPa) and leaf dehydration (leaves lose almost 40 % of their water while retaining full rehydration capacity) (Rhizopoulou et al. 1991). In the early stages of water-deficit stress, when the water absorbed by the roots and transported through the xylem into the leaves is no longer sufficient to replenish losses through transpiration, both the ψ_w and the turgor potential (ψ_p) decrease rapidly (Lo Gullo and Salleo 1988). These changes immediately inhibit cell division and expansion in the growing organs, and water loss from the tissues determines a reduction in osmotic potential (ψ_π) caused by both passive (driven by dehydration) and active mechanisms generating an increase in the concentration of intracellular solutes. Indeed, olives have a high capacity for osmotic adjustment and active synthesis and

Fig. 3 Decrease of the angle between the leaf and the stem in current-year shoots of 'Manzanilla' olive trees when the available soil water decreases. **a** Well-irrigated tree. **b** water stressed tree. *Photograph* J.E. Fernández

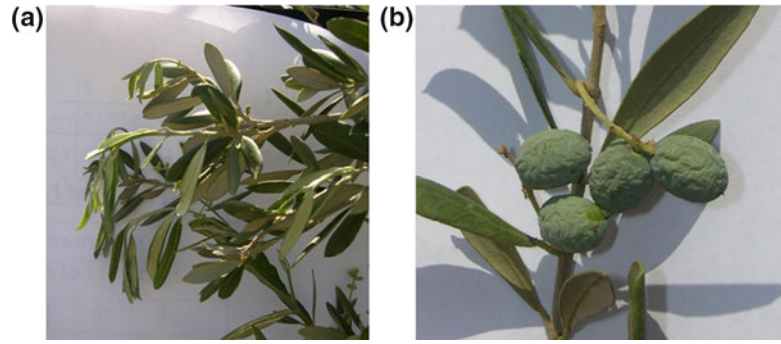

Fig. 4 **a** Shoot wilting and leaf curling in a severely stressed 'Manzanilla' olive tree; **b** shriveled fruits in a severely stressed 'Arbequina' olive tree. *Photograph* J.E. Fernández

accumulation of osmotically active and metabolically compatible solutes that can lower the osmotic potential of the cells. It has been suggested that active mechanisms of osmotic regulation in response to water-deficit stress, including accumulation of soluble carbohydrates, inorganic cations, and organic acids or amino acids reduce the cells' osmotic potential and enable plants to tolerate short- or long-term water-deficit stress (Ingram and Bartels 1996). Mannitol, glucose, and organic acids were shown to play an important role in the active osmotic adjustments in drought-stressed olive plants (Fig. 5). For severely stressed trees, Dichio et al. (2005) reported an osmotic adjustment ranging from 2.4 to 3.8 MPa and 'Coratina' plants subjected to different levels of water stress showed an active osmotic adjustment, mainly due to

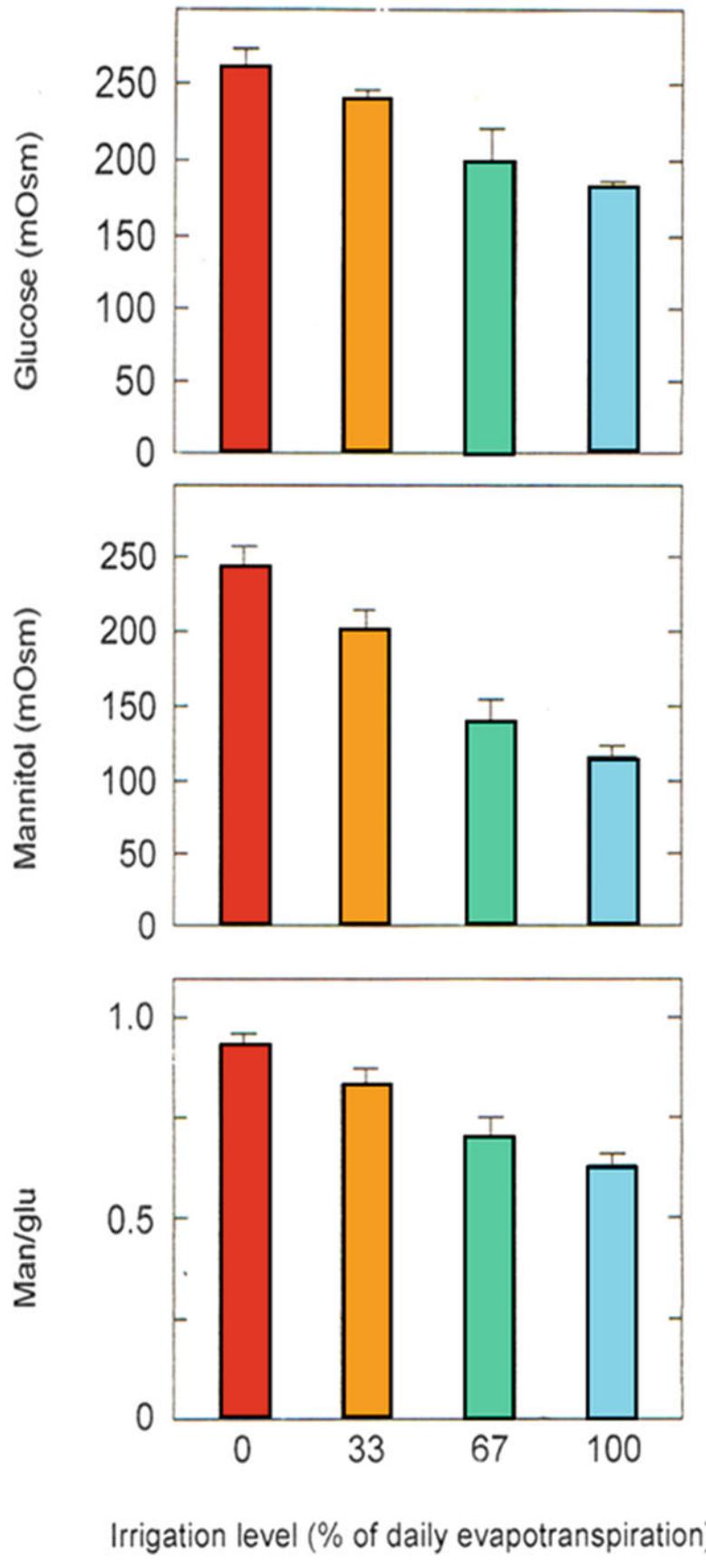

Fig. 5 Glucose concentration, mannitol concentration, and the mannitol-to-glucose ratio in leaves of olive trees subjected to different levels of irrigation

mannitol, of 0.45–0.8 MPa for leaves and 0.75–1.42 MPa for roots. Moreover, the maximum elastic modulus increased from 11.6 MPa (control plants) to 18.6 MPa (highly stressed plants) (Dichio et al. 2003). The osmotic adjustment enables cell turgor maintenance and contributes to the plant's ability to extract water at lower ψ_{soil}. In plants with pre-dawn ψ_{leaf} of −5.2 MPa, roots can reach osmotic adjustments varying from 1.67 MPa (roots of 4–5 mm) to 0.2 MPa (roots less than 1 mm diameter) (Xiloyannis et al. 1999). These osmotic changes are responsible for the increase in the root-to-soil ψ_w gradient and provide the cell turgor necessary to prevent the detachment of fine roots from soil particles.

Growth inhibition is accompanied by a slowdown in photosynthetic activity (Bongi and Palliotti 1994). Photosynthesis in leaves is determined by diffusional and non-diffusional limitations. Diffusional limitation is due to gas phase resistances (controlled by stomatal conductance—g_s, and mesophyll conductance—g_m) in CO_2 transport pathway from the atmosphere to the carboxylation sites of chloroplasts. Non-diffusional limitation includes all the biochemical processes responsible for carboxylation and overall for photosynthesis efficiency, which is ultimately influenced by photoinhibition. To prevent the loss of water by transpiration, stomatal pores close, causing a reduction in g_s and photosynthesis (Fernández et al. 1997; Giorio et al. 1999). Studies on the gas exchange of olive plants subjected to different levels of water deficit showed that the net CO_2 assimilation rate (A_n) and g_s reach their maximum values early in the morning in both well-watered and stressed plants, but decline more and faster in plants exposed to stressful conditions (Xiloyannis et al. 1999; Moriana et al. 2002). Jorba et al. (1985) found that A_n was reduced by 85 % when the relative water content (RWC) changed from 96 to 65 %; Larcher et al. (1981) observed a decrease in A_n at ψ_{leaf} lower than −1.3 MPa, and 50 % reduction at ψ_{leaf} lower than −2.2 MPa. However, olive plants still maintain a slight net assimilation rate (10 % of well-watered plants) at very low (−6.0 MPa) pre-dawn ψ_{leaf} (Xiloyannis et al. 1999) and A_n is still detectable at −7.0 MPa ψ_{leaf} (Dichio et al. 2005) and −8.0 MPa stem water potential (ψ_{stem}) (Moriana et al. 2002). Tognetti et al. (2004, 2005) described, after measuring seasonal leaf water relations and transpiration in irrigated and non-irrigated olive plants, how these variables respond to daily and seasonal variations in tree water status, soil moisture, and evaporative demand. In a recent work, Tugendhaft et al. (2016) found the newly bred 'Barnea' to have relatively high stomatal conductance and net photosynthesis but lower drought tolerance compared to cultivars such as 'Picual' and 'Souri,' common in rainfed agricultural system. The latter suggests a trade-off between higher yields under irrigated conditions and drought tolerance.

Unlike most other species, g_s in olive declines steadily as water-deficit stress increases, causing a reduction in photosynthesis for a wide range of ψ_{stem}. However, Moriana et al. (2002) found that A_n decreases much faster than g_s at $\psi_{stem} < -4$ MPa. Under moderate water deficit, in fact, the effect of drought stress on photosynthesis is mediated primarily by the reduction in g_s, while later in the season under higher stress, its effect also impact non-stomatal components, such as g_m or light-dependent inhibition associated with the primary photochemical reaction center, the photosystem II (PSII) (Angelopoulos et al. 1996). Notably, g_m also decreases in the summer mainly due to the changes in temperature and vapor pressure deficit of the air. Diaz-Espejo et al. (2007), for example, found a $g_m = 0.224$ mol m^{-2} s^{-1} at a leaf temperature of 29.6 °C, and a $g_m = 0.14$ mol m^{-2} s^{-1} at a leaf temperature of 40 °C. The value of A_n can also change when leaf develops under severe summer conditions, as reported by Bosabalidis and Kofidis (2002). They observed an increasing number of mesophyll cells, chloroplasts, and a higher CO_2-uptake cell surface. In general, even if the olive leaves become net exporters of assimilates at the beginning of their expansion cycle (Marchi et al. 2005, 2007), A_n increases until leaves approach their full expansion, since mesophyll thickness doubles from initial through final leaf developmental stage Marchi et al. (2007). Moreover, current-season leaves had greater A_n levels than

1-year-old leaves (Proietti et al. 2012). Anatomical studies proved that g_m is correlated with chloroplast exposed surface to leaf area ratio and mesophyll cell wall thickness (Tomás et al. 2013). Recently, both aquaporins and carbonic anhydrase have been found actively involved in the regulation of g_s and g_m in olive. Perez-Martin et al. (2014) measured, in a short-term water stress and recovery experiment, the evolution of leaf gas exchange, chlorophyll fluorescence, and plant water status and correlated these data with the gene expression of *OePIP1.1* and *OePIP2.1* aquaporins as well as stomatal carbonic anhydrase. Using structural equation modeling, the authors concluded that both *OePIP1.1* and *OePIP2.1* expression could explain most of the variations observed for g_s and g_m, while the stromal carbonic anhydrase had a small but significant effect on g_m in olive under drought conditions. These findings could greatly help molecular breeders in identifying new metabolic pathways in olive response to drought and speedup breeding.

The maintenance of the A_n activity during drought stress enables olive plants to continue the production of photoassimilates that can be transported and accumulated into the root system. This mechanism sustains root growth and increases the root-to-crown ratio in water stressed plants (Xiloyannis et al. 1999). Since root growth is fundamental for the exploration of new soil volumes, these adaptations result in better tolerance of the plants to drought conditions. When the daily minimum ψ_{stem} reaches values lower than −3.5 MPa, the olive leaf approaches the point of turgor loss. Under these conditions, g_s and transpiration are very low, while the relative water content of the leaf is approximately 75 % (Lo Gullo and Salleo 1988; Giorio et al. 1999). In this low transpiration phase, recovery of the water transpired by the leaf is not very effective because of the drop in hydraulic conductivity of the xylematic vessels. Torres-Ruiz et al. (2014) showed the percentage loss of hydraulic conductivity as a function of the xylem water potential (ψ_{xylem}) in current-year shoots of 'Manzanilla' olive trees; when ψ_{xylem} approached −5 MPa, the percentage loss of hydraulic conductivity reached 50 %. Despite, more negative water potentials can reduce further the olive hydraulic conductivity. The plant is able to withstand these low values with only minor seasonal xylem embolism (Salleo and Lo Gullo 1983; Torres-Ruiz et al. 2013). In general, the reduction in hydraulic conductivity due to cavitation is low (25–30 %) and seems to be caused mainly by the large-diameter vessels, which are most vulnerable to the cavitation process. The key variable determining vulnerability to embolism is not clear. It seems that the diameter of the intervessel pit membrane pore is more relevant than the diameter of the xylem vessel itself (Tyree and Sperry 1988). Under the pit area hypothesis (Wheeler et al. 2005), the ψ_{xylem} value corresponding to 50 % loss of hydraulic conductivity is determined by the largest pit pore in the total pit area of a vessel. Increasing evidence shows that water in the xylem vessels under tension contains a large number of bubbles of nanometric size. Jansen et al. (2009) observed how the origin and size of the bubbles correlated with the structure of the pit membrane, rather than with the pore diameter. When formed, these bubbles can explode, leading to embolism, or can shrink, causing nocturnal embolism repair (Brodersen et al. 2013). Olive is capable to maintain a very high water potential gradient between leaves and roots, from −6.5 MPa in the leaves and stems to −3.5 MPa in roots with diameters of less than 4 mm (Dichio et al. 1994). This characteristic allows the plant to take up water from drying soils even when the soil ψ_w is very low, preserving the tissues' rehydration capability (Rhizopoulou et al. 1991). The rewatering process for the drought-stressed olive plants is preceded by a period of inertia in leaf activity, such that the full recovery of the leaf's functions during the process of rehydration normally takes a few days. This effect is probably dependent on hormone balance and hydraulic conductivity of the xylem system. Short-term studies on water-use dynamics in olive after rewatering by heat-pulse measurements of the sap flux (Moreno et al. 1996) showed differences between irrigated and non-irrigated trees. Up to 3 days after irrigation, the irrigated plants

maintained a transpiration rate of 1.65 mm^3 mm^{-2} d^{-1} on a leaf basis. After this phase, the rate of water use declined and transpiration fell. The sap flow in the near-surface roots dropped concomitantly. In non-irrigated trees, irrigation raised the transpiration rate to 1.12 mm^3 mm^{-2} d^{-1} only and leaf water potential did not recover. Tognetti et al. (2004) also observed that water scarcity during the summer reduces gas exchange and ψ_{stem}, gradually increasing the hydraulic resistance. This effect is more evident in non-irrigated plants and correlate with changes in hydraulic properties at the root-soil interface. After water stress recovery, the non-irrigated plants showed physiological performance similar to that of their irrigated counterparts. Taken together, all these data show that even after rewatering olive behaves as a water saving species.

Water deficit produces a stomatal conductance response in the whole plant, and the phytohormone abscisic acid (ABA) could have a major role as an endogenous messenger in this root-to-shoot signaling mechanism. In fact, ABA can specifically target the guard cells and induce stomatal closure through ion channels activation and changes in gene expression that enforce more complex regulation (Christmann et al. 2006). Recent findings suggest that ABA has long-lasting effects on plant hydraulic properties via the activity of aquaporins (AQPs) (see below), which contributes to the maintenance of a favorable plant water status (Parent et al. 2009). In olive, Kitsaki and Drossopoulos (2005) reported a significant correlation between ABA and ψ_{leaf}. Guerfel et al. (2009) run an enzyme-linked immunosorbent assay to study differences in root and leaf ABA accumulation between 'Chemlali' and 'Chetoui' cultivars during water-deficit stress. The cultivar-specific differences in ABA accumulation observed during water stress reflected the degree of stress tolerance. The drought-tolerant 'Chemlali' accumulated lower levels of ABA in its leaves in response to water stress than the drought-sensitive 'Chetoui.'

The flow of water across cell membranes occurs not only directly across the lipid bilayer, but it is facilitated by the presence of specific proteins known as AQPs. AQPs are present in all living organisms and they may increase considerably the hydraulic conductivity of membranes (Preston et al. 1992). These proteins are tetramers containing six α-helices arranged in a right-handed bundle that crosses the membrane, with the N- and C-terminal residues located on the cytoplasmic surface of the membrane (Tyerman et al. 2002). Water stress causes significant modulations in the expression levels of genes related to AQPs (Baiges et al. 2002). Higher AQP expression can lead to an increase in water permeability when water is less available (Yamada et al. 1997) but, at the same time, a decrease in AQP expression can ensure better conservation of water during water stress (Smart et al. 2001). It is therefore highly likely that during water-deficit stress, these two mechanisms work simultaneously to maintain adequate water status in the plant tissues. In olive, Secchi et al. (2007) found putative AQPs in both the plasma membrane and the tonoplast and recovered three sequences (identified as *OePIP1.1*, *OePIP2.1,* and *OeTIP1.1*). They demonstrated that *OePIP2.1* and *OeTIP1.1* code for water-transport proteins and that the expression levels of *OePIP1.1* and *OePIP2.1* are high in roots and low in branches and leaves. Moreover, the expression levels of these genes during a period of water-deficit stress significantly decreased in all organs, suggesting their role in water-conservation mechanisms. As previously discussed, non-stomatal factors (e.g., g_m) have been reported to limit photosynthesis as much as g_s (Warren 2007). In this respect, both carbonic anhydrase (Price et al. 1994) and AQPs (Flexas et al. 2006) play an important role in CO_2 transport in cells. Therefore, the internal conductance to CO_2 is not only controlled by leaf anatomy and morphology (Marchi et al. 2008), but it is also influenced by these molecular and biochemical factors that help to explain the rapid modifications occurring in internal conductance to CO_2 under water stress (Perez-Martin et al. 2014).

Drought stress in Mediterranean climate is normally accompanied by high temperature and irradiation levels, which ultimately cause photoinhibition, photooxidation, and photorespiration (Osmond et al. 1997). All these mechanisms

facilitate the production of reactive oxygen species (ROS), which are well-known harmful molecules also acting as cellular signals in response to stress. Thus, accurate regulation of their levels in cells and tissues is essential (Noctor 2006). To control ROS homeostasis in cells, plants have developed non-enzymatic (α-tocopherol, β-carotene, phenolic compounds, ascorbate, and glutathione) and enzymatic (superoxide dismutase, catalase, peroxidase, among others) systems. In olive leaves, Corpas et al. (2006) studied the localization and expression levels of superoxide dismutase (SOD), an enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. SOD is present in different isoenzymatic forms (Mn-SOD, Fe-SOD, and CuZn-SOD). Transcript analysis showed that these forms represent 82, 17 and 0.8 % of total SOD, respectively. In the palisade cells, expression levels of Fe-SOD were higher, followed by those of Mn-SOD and CuZn-SOD. In the phloem, the most abundant isoform was Mn-SOD, which is also the only one present in the xylem. The activities of several antioxidant enzymes, including SOD, have been studied in 'Coratina' trees subjected to water-deficit and recovery under different irradiance conditions. The activity of most of these antioxidant enzymes was reduced during the period of recovery in both leaves and roots, while that of polyphenol oxidase increased (Sofo et al. 2004a). Light intensity plays a major role in determining the extent of these reductions. It is therefore possible to hypothesize higher ROS production during the water stress recovery phase under high irradiance conditions. Progressive increases in lipoxygenase activity and malondialdehyde content were observed in leaves and roots of olive trees exposed to increasing levels of drought stress (from −0.5 to −2.4 MPa; from −2.5 to −4.9 MPa, and from −5.0 to −6.3 MPa). These changes were associated with an increasing level of membrane lipid peroxidation (Sofo et al. 2004b). Increased levels of proline, an amino acid that has multiple functions in plants, were also found. Proline accumulation increases the osmotic potential of the cells, and high-proline contents are compatible with cellular functions. Proline can also function as a source of nitrogen, protecting membranes and proteins during water-deficit stress (Ain-Lhout et al. 2001), and as an electron acceptor to prevent ROS damage to the photosystem (Hare et al. 1998). Proline, therefore, is an important component of the olive response to water stress and might be a useful biological marker for drought resistance.

The physiological, biochemical, and molecular machinery activated by water-deficit results in a high energy expenditure, which has adverse effects on vegetative growth and production in both current and following years. The effects of water scarcity on olive production largely depend on the biological phase during which water stress arises. The most relevant damages can be observed during the stages of flowering, beginning of pit hardening, and beginning of fruit ripening. At flowering, water deficiency can cause abnormalities in flower formation and a significant reduction in the number of flowers and, consequently, in the number of fruits per inflorescence. In the early stages of fruit development and ripening, limited water availability can increase fruit drop and significantly reduce fruit size at ripening (Inglese et al. 1996). Fruit growth under optimal ψ_{soil} is different from that of fruits of plants subjected to drought. A decrease in fruit fresh weight and volume has been observed in 'Frantoio' trees under water deficit. It has also been reported that drought stress mainly affects the mesocarp cell size, rather than the number of cells, while the oil content in mesocarp cells does not change (Costagli et al. 2003). Experiments with potted 'Leccino' plants showed a reduction in fruit fresh weight, volume, and cross-sectional area. The growth of the endocarp area was modified by water stress: 90 % of the final growth was achieved within 8 weeks from full bloom in irrigated plants, while under drought stress endocarp growth reached only 40 % of the expected growth (Rapoport et al. 2004). In field experiments with different olive cultivars, it has been shown that water supplies amounting to 33 % only of the crop evapotranspiration (ETc) during the dry season were enough to increase yield

significantly in comparison with non-irrigated plants. The higher production is determined by both a larger number of fruits per plant and a higher weight per fruit (d'Andria et al. 2000). Pulp-to-pit ratio of moderately stressed plants is equal or even slightly greater than in well-irrigated trees (Gucci et al. 2009). Experiments with 'Frantoio' and 'Leccino' trees irrigated from the beginning of pit hardening to early fruit veraison with 100, 66, or 33 % of ETc showed that differences in yield between treatments were mainly related to the mean fruit weight and that drought stress effects were more marked during flowering or early stages of fruit growth rather than late in the summer season.

Effects of water deficit on oil quality have been elucidated (Caruso et al. 2014; Gómez--del-Campo et al. 2014). In temperate environments, the oil from olive plants under dry-farming conditions do not differ from that of irrigated plants in terms of acidity and fatty acid composition, but they have a higher total polyphenol content at fruit ripening (Caruso et al. 2014; Patumi et al. 2002; d'Andria et al. 2004, 2009; Servili et al. 2007). Moreover, position in the canopy and water deficit determines significant interactions in oil quality, with some exclusive volatile organic compounds that are present only under certain conditions (Benelli et al. 2015). In arid climates, however, irrigation can lead to significant changes in fatty acid composition (Lavee and Schachtel 1999). Contrasting results have been obtained on the effect of irrigation on the oil percentage in the fruit. In general, both the oil percentage (expressed as % fresh weight) in the fruit and the percentage of oil extracted decrease with increasing level of water availability (Grattan et al. 2006). These effects can be caused by higher water content in the fruits of irrigated plants. In environments where annual rain is more than 450 mm $year^{-1}$, the percentage of oil in the fruit (calculated on a dry weight basis) does not correlate so closely with the level of drought stress (d'Andria et al. 2004). However, the quantity of olive oil per tree or per unit area decreases under conditions of water scarcity and this effect gets stronger as the climate becomes dryer (Grattan et al. 2006; Tognetti et al. 2006).

Olive fruit development is genetically programmed and largely influenced by environmental factors such as water availability that plays an important role in dry mass/oil accumulation as well as in many primary and secondary metabolism modifications. The number of identified and characterized genes involved in transcriptional networks and regulatory circuits related to olive fruit physiological and developmental processes is increasing and would constitute a relevant step in explaining how water-deficit stress affects olive oil quality and its health-related properties. In a recent study, Galla et al. (2009) identified and annotated many differentially expressed genes in different phases of fruit development (at 30 days after full bloom, pit hardening, and veraison) that might be involved in main processes of fruit growth, development, and ripening. Martinelli et al. (2011) showed data on genes involved in important pathways of secondary metabolism (flavonoids, polyphenols, terpenoids, and fatty acids) during fruit development in rainfed and fully irrigated olive plants.

2 Salinity Stress

High concentration of salts in the soil may be due to the geological origin of soils, the high evaporative demand of the environment, and the poor quality of irrigation water. In the last few decades, the increase in agricultural production and competition for water use with the civil and industrial sector have led to the use of poor quality water rich in ions that increase the risk of soil salinity. Sodium (Na^+) and chlorine (Cl^-) ions are usually responsible for salinization, whereas other ions like calcium (Ca^{2+}) and sulfate (SO_4^{2-}) are more rarely involved. In coastal areas of the Mediterranean basin, both the presence of numerous artesian wells and the high evapotranspiration during summer often cause

temporary infiltration of seawater into freshwater aquifers, exposing the nearest olive-growing areas to an increasing risk of salt stress.

Among fruit trees, olive is moderately resistant to salinity (FAO 1985; Rugini and Fedeli 1990; Gucci and Tattini 1997). Bernstein (1965), for example, measured a reduction in olive production of only 10 % when the electrical conductivity of a saturated-paste extract (EC_e) was 4–6 dS m^{-1}, or 6–8 dS m^{-1} when soils were rich in calcium. Maas and Hoffman (1977) established a mathematical relationship between production and EC_e. Olive production is reduced linearly as salinity is increased beyond an EC_e threshold of about 3–4 dS m^{-1}. In 'Arbequina' trees, the EC_e threshold for trunk growth is variable and decreases with age and exposure time, from 6.7 to 3.0 dS m^{-1} (Aragüés et al. 2005). In general, it is advisable to avoid water salinity exceeding 3–4 dS m^{-1} for irrigation, since olive performance decreases when water for irrigation has an EC greater than 5.5 dS m^{-1} (Freeman et al. 1994). Olive ability to tolerate high values of EC_e depends on the presence in the irrigation water of low percentages of NaCl or Na_2SO_4 that, according to some authors, is even more harmful (Bartolini et al. 1991). Young plants show significant reductions in growth when treated with NaCl solutions in the range of 40–100 mM, and concentrations greater than 100 mM threaten the plant survival. The maximum value of NaCl concentration that the olive tree can tolerate was estimated around 137 mM (Rugini and Fedeli 1990), while reductions in production were recorded above 30 mM NaCl (Gucci and Tattini 1997).

Numerous factors such as genotype, age of the plant, and agroenvironmental variables affect olive response to salinity (Chartzoulakis 2005). Tattini et al. (1992) highlighted great differences in salt tolerance between 'Frantoio' and 'Leccino,' while Chartzoulakis et al. (2002) showed that 'Kalamata' has a greater resistance to salinity than 'Mastoidis' and 'Amphissis.' Furthermore, Marin et al. (1995) analyzed the tolerance of 26 olive cultivars to 100 mM NaCl and reported a broad genotypic variability to salt stress, as indicated by wide changes (from 16 to 70 %) in shoot relative growth rate of salt-treated plants.

The visible symptoms of salt stress consist of foliar chlorosis and necrosis, desiccation of flowers, ovary abortion, desiccation of the apex of the leaves and, for the most severe conditions, necrosis of the stem tip, necrosis at root level, and abscission of leaves (Gucci et al. 2003). Other noticeable effects of salt stress are reduced growth, shortening of internodes, smaller leaves and a general reduction in leaf area, which is an early response mechanism common to many glycophyte species (Munns and Termaat 1986). Leaf abscission is a consequence of salinity stress, but also a defense mechanism useful to both reduce transpiration and eliminate toxic ions which normally accumulate in old leaves (Bongi and Loreto 1989; Loupassaki et al. 2002). The salt stress adversely affects not only the aerial part but also the root, although the effect is more pronounced on the former, such that the canopy to root ratio decreases when the stress intensity increases (Therios and Misopolinos 1988; Bongi and Loreto 1989; Gucci and Tattini 1997). Other effects caused by salinity are as follows: (a) reduced viability and germination of pollen, (b) low number of flowers per inflorescence, and (c) a reduction in the percentage of fruit set and fruit size (Gucci et al. 2003). Salinity decreases fruit weight but does not alter the oil content (Chartzoulakis 2005). In general, the saline stress modifies more the fruit production than that of oil (Gucci et al. 2003). The negative effect on production seems mostly due to a decrease in the crown volume.

Salinity in the root zone determines: (a) osmotic stress (osmotic effect), due to the decrease in ψ_{soil}; (b) modification of the homeostasis of ions in cells, by inhibiting the absorption of essential elements (e.g., K^+, Ca^{2+}, and NO^{3-}); and (c) cellular accumulation of potentially toxic concentrations of ions, such as Na^+ and Cl^- (specific ion effect) (Marschner 1995). These primary stresses rapidly induce a series of biochemical and molecular responses at the cellular

level involving second messengers such as Ca^{2+}, ROS, hormones, and transcription factors. The plant ability to exclude Na^+ and Cl^- from the cytoplasm and accumulate these ions in the vacuole is among the key component for the development of tolerance to salt stress. K^+ channels and some non-selective channels are considered responsible for the absorption of Na^+, while Na^+/H^+ antiports (Blumwald et al. 2000) regulate the outflow. Several attempts have been done to enhance salt tolerance in crops by traditional plant breeding approaches, as well as by biotechnological methods. Experiments with transgenic Arabidopsis plants overexpressing plasma membrane Na^+/H^+ antiporter (Shi et al. 2003) have shown an increase in salt tolerance in the transgenic plants, being that tolerance correlated with reduced Na^+ accumulation. In olive, Bracci et al. (2008) applied in vitro culture of microshoots, established from seed lines of free-pollinated 'Frantoio' and 'Moraiolo' trees, as a rapid evaluation tool for testing olive progenies tolerance to NaCl and tested progenies with different in vitro resistance.

In olive, salt tolerance is associated with the plant ability to exclude and retain Na^+ and Cl^- in the root (Gucci and Tattini 1997; Chartzoulakis 2005), limiting the translocation into the xylem, and the transport and accumulation in buds and leaves. Tolerant cultivars are able to provide a better control of the salt translocation to the aerial part (Tattini et al. 1995; Chartzoulakis 2005), by generating a gradient of Na^+ and Cl^- that decreases from the base to the apex of the shoot. In 'Leccino,' for example, it was observed at 120 mM NaCl a lower exclusion of Na^+ from the shoot than in 'Frantoio,' while the K^+/Na^+ ratio was higher in the aerial organs of 'Frantoio' than in those of 'Leccino' (Gucci and Tattini 1997; Gucci et al. 2003). Recently, Rossi et al. (2015) investigated the role of anatomical adjustment, namely the apoplastic barriers formation and tissues characteristics (Fig. 6), and ion localization in root of 'Leccino' and 'Frantoio' under salinity stress. Microscopic analyses showed that endodermis apoplastic barriers (Fig. 7) were formed closer to the root apex in 'Leccino' than in 'Frantoio,' and that Na^+ gradient from exodermis to stele tissues depend on genotypes and cell types. The apoplastic adjustments in roots seem to play a role, both in tolerant and sensitive olive cultivars, in reducing Na^+ fluxes in root tissues (from cortex to central cylinder) and consequently to the shoot. However, this mechanism alone is not sufficient to completely avoid salt translocation.

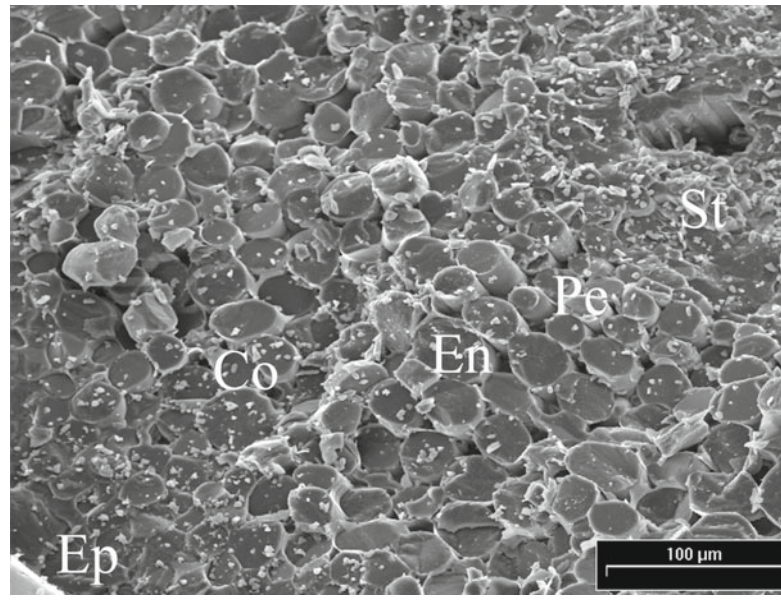

Fig. 6 Cryo-SEM image of frozen-hydrated olive (cv. Frantoio) root, freeze fractured transversally, showing the position of epidermis (Ep), cortex (Co), endodermis (En), pericycle (Pe), and stele (St). *Photograph* A. Minnocci

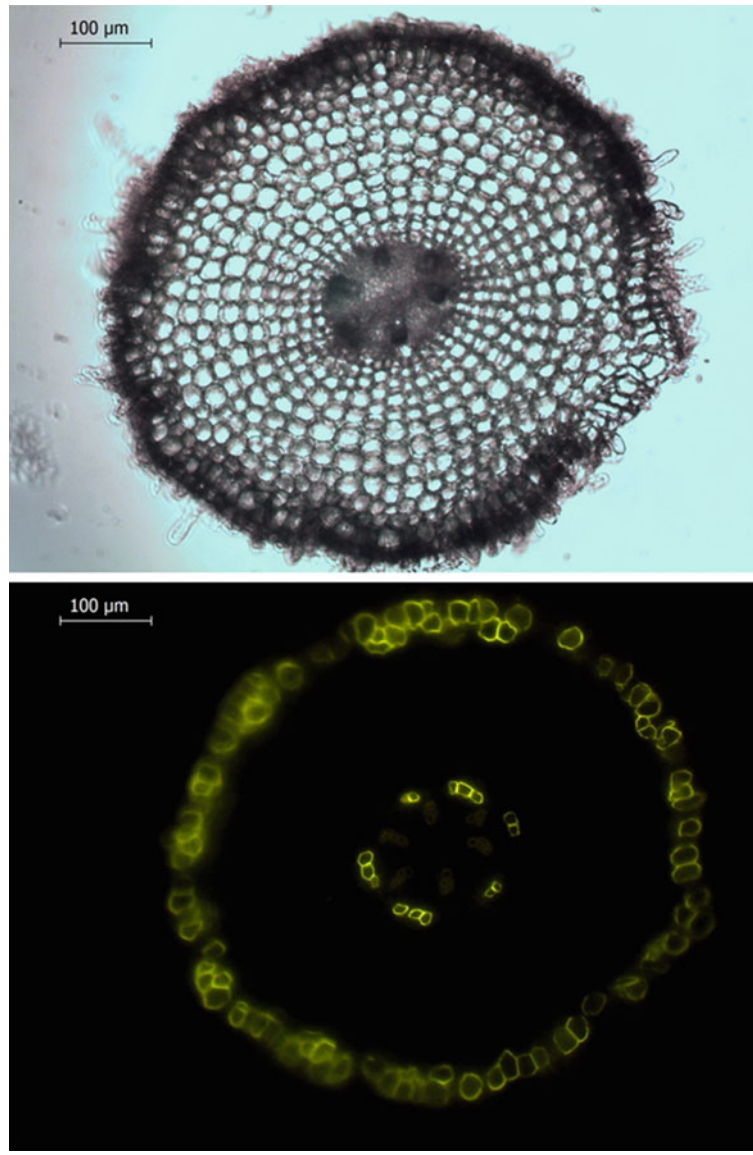

Fig. 7 Bright field and fluorescence (Fluorol yellow 088) images showing the exo- and endodermis suberin lamellae formation at the root tip of olive (cv. Frantoio) grown at NaCl 120 mM. *Photograph* L. Rossi

The accumulation of Na^+ and Cl^- can cause imbalances in essential mineral elements homeostasis. Calcium, for example, has an important role in reducing Na^+ toxicity (Melgar et al. 2006) and a high Ca^{2+}/Na^+ ratio is important to reduce the negative effects of Na^+ on depolarization and selectivity of the plasma membranes in olive (Rinaldelli and Mancuso 1996). During salinity stress, the concentration of K^+ is reduced in many glycophyte species (Greenway and Munns 1980) as olive. The genotype appears to have a minor role (Bartolini et al. 1991; Tattini et al. 1995; Chartzoulakis et al. 2002), and the strongest reduction in K^+ is observed in roots and old leaves. This mechanism could be used to maintain the ionic balance in tissues and an appropriate K^+/Na^+ ratio in the young and still growing organs (Chartzoulakis 2005). Tabatabaei (2006) studied the interactions of salinity with nitrogen in sensitive and tolerant cultivars demonstrating reductions in the activity of the nitrate reductase enzyme when plants were treated with 150 mM

NaCl. This effect was accompanied by a reduction in total nitrogen and nitrate absorption.

In addition to the ionic effects, salinity alters the plant's water relations. At the initial stages of salinity stress, the ψ_{soil} solution increases, roots have more difficulties to take up water (Therios and Misopolinos 1988), transpiration decreases and the cell water status is affected (Gucci et al. 1997). Significant reductions in pre-dawn ψ_w, ψ_π and in the relative water content (RWC) were observed in 'Frantoio' and 'Leccino' plants exposed to 100 and 200 mM NaCl (Gucci et al. 1997). The reduction of ψ_w occurs through the reduction of ψ_π, so that the ψ_p of plants exposed to salinity stress remains at values comparable to or higher than those measured in the control plants. Furthermore, the reduction in ψ_{leaf} is proportional to the salt concentration of the external solution, allowing olive to maintain a stable gradient between the concentration of the salt in the external medium and that of the root cells. The cell turgor effect is maintained provided the salt concentration does not become too high or the stress does not persist for long periods. To counterbalance the high osmotic pressure due to the accumulation of Na^+ and Cl^-, olive leaves accumulate glucose and mannitol (Tattini et al. 1996). The osmotic effect provided by glucose and mannitol has been estimated to be around 25–30 % of the total effect and therefore less than that generated by inorganic solutes, which represent the main component of the osmotic adjustment under salinity. Gucci et al. (1998) showed a differential partitioning of carbon in fully expanded leaves of 'Frantoio' at 100 mM NaCl and an increase in mannitol concentration in leaf mesophyll cells. This effect was due to a different partitioning of the assimilated carbon toward mannitol instead toward glucose and sucrose. In tissue culture experiments, it was observed that the addition of NaCl to suspension-cultured cells of olive enhanced the capacity of the polyol:H^+ symport system and the amount of mannitol transporter 1 (*OeMaT1*) transcripts, whereas it strongly repressed mannitol dehydrogenase activity 1 (*OeMTD1*). This mechanism provides intracellular accumulation of mannitol (Conde et al. 2007).

Olive leaves have a thick cuticle and tightly packed mesophyll cells, both limiting the movement of CO_2. These characteristics are more evident during salt stress (Bongi and Loreto 1989). Olive plants exposed to salinity stress show a reduction of the photosynthetic activity that depends on salt concentration and genotype (Bongi and Loreto 1989; Tattini et al. 1995; Chartzoulakis et al. 2002). In general, the main reductions were observed in cultivars that exhibited the highest values of photosynthesis and stomatal conductance, while no correlations were found between the accumulation of salt and reduction of photosynthesis both in young and old leaves (Loreto et al. 2003). The effects of salinity on gas exchange are controversial and can be related, to some extent, to water relations: At initial stages of stress, turgor is higher in 'Leccino' than in 'Frantoio' and this may explain why gas exchange parameters of the former cultivar remain high. Tattini et al. (1995, 1997) observed that A_n and g_s decreased more in tolerant 'Frantoio' than in sensitive 'Leccino' cultivar, while a good correlation was observed during the recovery phase. On the contrary, Chartzoulakis (2005) reported a good degree of correlation between the decrease of photosynthesis in young leaves and tolerance: 20 % for the tolerant 'Kalamata' and 62 % for the moderately sensitive 'Amphissis.' Stomatal limitations to photosynthesis seem to be prevalent during the early stages of salt stress. However, Loreto et al. (2003) showed that the low concentration of CO_2 at the chloroplast level is determined by both low g_s and g_m.

In general, olive tolerance to salinity is mainly due to mechanisms of exclusion or retention of salt at the roots, thus avoiding the accumulation of Na^+ and Cl^- in the aerial organs. Therefore, more attention should be given to the molecular machinery responsible for the perception, transfer, and adaptation to salt stress. Following a transcriptomic approach, Bazakos et al. (2015) investigated the molecular response of olive leaves and roots of the 'Kalamon' trees to salinity using next-generation sequencing technology. In this study, many differentially expressed genes that are related to salt tolerance response were

Fig. 8 Necrotic fruitlet (*arrow*) caused by high temperature stress. *Photograph* R. Gucci

identified. This approach will likely lead to identification of the genes responsible for salinity tolerance in olive and opens new possibility for breeders.

3 Suboptimal Temperatures

The olive tree is more resistant to high than low temperatures. However, climate change and consequent temperature rise are exposing olive trees to high temperature stress even in traditional areas of cultivation. Moreover, the recent and continuous expansion of olive cultivation areas has increased the likelihood of low temperature stress. The visible damage such as stem sunburn and leaf chlorosis occurs rarely and only when plants have been subjected simultaneously to high temperatures, severe water shortages and high light intensity. Branches and leaves exposed to sunlight can reach higher temperatures than those of the surrounding environment. Data for the overheating of the olive leaves during summer are not available in the literature. Leaves of Mediterranean species, such as *Quercus ilex*, undergo an increase of 4–8 °C respect the ambient temperature when exposed to an intense solar radiation (Larcher 2000) and similar changes have been measured in olive leaves of severely stressed trees (Gucci et al., unpublished data). To reduce the heat load, olive has developed a number of features: (a) small leaf size; (b) partially open stomata, even under severe water deficit; (c) reduced angle of insertion of the leaf petiole on the stem, as discussed in the drought stress section; and (d) a thick carpet of trichomes on the abaxial side that filter out UV light and reflect infrared radiation (Baldini et al.

1997; Liakoura et al. 1997). Photosynthesis is sensitive to high temperatures, due to the heat sensitivity of thylakoid membranes (Berry and Bjorkman 1980). In olive, measurements of chlorophyll fluorescence in response to high temperature showed a critical threshold of 46–47 °C. At 48–49 °C, the first necrosis on the leaves appear (Gucci et al. 2003). Heat tolerance depends on the genotype, although differences are not wide; varieties from northern areas show less tolerance to high temperatures than varieties originated in warmer climates (Mancuso and Azzarello 2002). Seasonal variations in heat tolerance have been observed in several species, including olive (Kappen 1981). For this species, during the summer is it possible to measure an increase in resistance of 3–4 °C when compared to the winter period; such variations may be correlated to the synthesis of specific proteins (HSP, heat-shock proteins) produced by plants in response to high temperatures stress (Nover et al. 1989). Flowering, fruit set, and fruitlet growth are quite sensitive to high temperature stress that can cause flower wilting and production of shotberries can be observed. Fruit growth is also sensitive to temperatures higher than 35 °C. Fruit shriveling, necrosis, and abscission are caused by the increase in tissue temperature due to high temperatures and water deficit during the first few weeks of fruitlet growth (Fig. 8). High temperatures during fruit development also decrease the oleic acid concentration in olive oil, and temperature effects on fatty acid composition have been indicated in the range 16–32 °C (García-Inza et al. 2014).

Low winter temperatures have caused extensive damage to olive plants at intervals of 25–40 years. In international scientific terminology, the low temperature stress can be divided into two categories: (a) stress caused by temperatures between 10–15 and 0 °C (chilling); (b) stress caused by temperatures below 0 °C (freezing). In olive, the chilling stress is not as easily visible as in other species such as citrus fruits that show symptoms such as chlorosis and necrosis. Still, it causes a slowdown of the metabolic processes. Optimal temperatures for olive growth range between 20 and 30 °C (Rinaldelli and Mancuso 1994), whereas the plant progressively slows the metabolism when temperatures drop below optimal values. Lower temperatures reduce respiration and enzymatic activity, uptake of water and nutrients, photosynthetic efficiency, and cellular processes determining growth inhibition (Mancuso 2000).

The highest damage to olive plant results from temperatures below 0 °C. The threshold temperature for freezing symptoms depends on several factors. Some of them are related to the plant, such as genotype, phenological stage, nutritional and health status, and age and type of organ. Others are related to air humidity, duration of the freezing temperatures, temperature drop rates, direction, and speed of wind. When freezing occurs, the visible symptoms consist in desiccation of the shoot tip, leaf drop, longitudinal cracks in the bark, and split of the sapwood. During the initial phase of freezing, the extracellular water freezes, and this process continues until all the liquid water is converted into ice. In this phase, if the cells are acclimated to freezing, the pressure exerted by extracellular ice crystals is not harmful for the wall and membranes (Stout et al. 1987). During the freezing process of the extracellular water, a vapor pressure difference between the apoplast and the simplast is generated and the ψ_w value determines the gradual release of water from the cell (exosmosis). The cell membrane permeability to water flow is critical to ensure the maintenance of a thermodynamic equilibrium that, if altered, initiates the intracellular freezing (Levitt 1980). During extracellular freezing, the intracellular solute concentration increases; lowering the intracellular cell solution freezing temperature, a phenomenon that would lead, in this phase, to the immediate death of the cell. The accumulation of intracellular compatible solutes helps to reduce the risk of intracellular freezing. Below a certain temperature, the formation of intracellular ice starts. This event requires the nucleation of the cytoplasmic solution, a process that can take place homogeneously or heterogeneously from the extracellular ice and that is strongly influenced by the state of acclimatization before freezing (Levitt 1980). The presence of a

supercooling mechanism that allows conservation of water in the liquid state at freezing temperatures was reported for olive by Fiorino and Mancuso (2000). This allows olive cells to lower the freezing temperatures of their cytoplasm down to −7 or even −18 °C depending on the solute accumulation, variety, and organ type. Recently, Arias et al. (2015) studied the role of apoplastic water, solute content, and cell wall rigidity in the freezing avoidance by supercooling mechanism.

Freezing temperatures show significant differences between varieties and olive organs, with a descending order of sensitivity from the fruits to roots, leaves, branches, and buds (Gucci et al. 2003). Attempts have been made to classify olive cultivars according to their tolerance to freezing, identifying also some variability for this character within the same variety (La Porta et al. 1994). Bartolozzi and Fontanazza (1999) used a variety of markers (visible symptoms, electrolyte-release analysis, and differential thermal analysis) to determine freezing tolerance in olive cultivars. Among all methods, electrolyte-release analysis was the best and allowed to identify that the 'Bouteillan' and 'Nostrale di Rigali' cultivars were the most tolerant and 'Borsciona' the less. Mancuso (2000) measured the electrical resistance of different organs (leaves, stems, buds, and roots) during freezing and proposed this system as a fast, simple and non-destructive methodology. Electrical resistance measures enable to estimate the values of LT_{50} (lethal temperature at which 50 % of damage occurs) for some cultivars: −12.0, −12.8, −15.6 and −18.3 °C, respectively, for 'Coratina,' 'Frantoio,' 'Leccino,' and 'Ascolana.'

Freezing tolerance occurs only in genetically competent species, through a mechanism known as acclimation. The acclimation to freezing is mediated by a complex series of molecular, biochemical, and physiological events that are activated by environmental stimuli such as temperature and photoperiod (Sakai and Larcher 1987). In the Northern Hemisphere, acclimation begins during autumn, when the temperatures start to become suboptimal (10–15 °C) for growth and the day length shortens. During acclimation, olive exposure to sublethal freezing (some degrees below zero) temperatures may induce an increase in frost resistance (Sebastiani et al. 2002). Since the olive tree lacks dormancy (unlike deciduous species), once acclimated to freezing the plant can easily lose acclimation when winter have mild temperatures. Field observations showed a partial loss of acclimation in about 6 days with average temperatures above 16 °C (Gucci et al. 2003). The main environmental factor for the acclimation of the root is soil temperature. However, olive roots have a poor acclimation to freezing. In drupes, Matteucci et al. (2011) investigated the relationship among development, cold response, expression of fatty acid desaturase (FAD) genes, and unsaturated fatty acid composition in genotypes differing in leaf cold tolerance. In all genotypes, cold sensitivity was high in the epi- and mesocarp cells before oil body formation, and decreased during oil biogenesis. Genotype-dependent differences were observed at the end of the oil production cycle. Results showed a direct relationship between FAD expression and lipid desaturation in the drupes of the cold-sensitive genotype, and an inverse relationship in those of the cold-resistant genotype, suggesting that drupe cold acclimation requires a fine FAD posttranscriptional regulation.

The physiological events that lead to acclimation are activated by perception and transduction of environmental stimuli. Specific gene families are activated, inducing both major modifications in metabolism and intracellular accumulation of solutes and changes in the lipid composition of the membranes. In olive, the study of biochemical markers during acclimation and freezing has not allowed to establish accurate correlations with the degree of tolerance. Transient changes in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) were observed in response to low temperatures, as signaling factor for cold acclimation (Knight and Knight 2000). Using olive protoplasts, D'Angeli et al. (2003) demonstrated transient increases of $[Ca^{2+}]_{cyt}$ in response to low temperatures. They are caused both by Ca^{2+} efflux from the organelles and Ca^{2+} influx through the plasma membrane. Afterward, D'Angeli and Altamura (2007) studied the

presence of osmotin in olive plants. This is a stress-responsive antifungal protein belonging to the pathogenesis-related (PR)-5 family that confers tolerance to both biotic and abiotic stresses. In control plants (non-transgenic), osmotin was present only after acclimation and in tissues showing a programmed cell death (PCD). In transgenic plants, osmotin was always present. Parallel measurements of $[Ca^{2+}]_{cyt}$ showed that changes occurred only in the control plants (non-transgenic and non-acclimatized). All together, these results show that the osmotin is positively correlated with the PCD induced by acclimation, block $[Ca^{2+}]_{cyt}$ changes, and also mediate the low temperature-induced cytoskeleton modifications.

Hashempour et al. (2014) studied the role of the antioxidant enzymes in freezing tolerance, by comparing cold acclimated and non-acclimated olive plants. Data showed that cold acclimation enhanced the activities of superoxide dismutase, peroxidase, ascorbate peroxidase, catalase, and polyphenol oxidase. Moreover, the lethal temperature for 50 % of the population (LT_{50}) correlated to peroxidase, catalase, and polyphenol oxidase activity, suggesting that these three enzymes could be used as selection criteria in screening tolerant olive cultivars.

The study of short- and long-term transcriptional changes in 'Leccino' leaves exposed to progressive freezing temperatures was undertaken by Guerra et al. (2015). Transcriptomic data identified the typical and conserved components of the molecular pathways leading to the plant cold response. This included changes in membrane composition-related genes, induction of coldregulated genes, transcription factors, and downregulation of photosynthesis-related genes. However, some specific features characterizing the cold response of the olive tree were identified, namely genes of the glutathione cycle, polyamine and flavonoid pathways (likely to support ROS scavenging), as well as genes of the raffinose and trehalose biosynthetic pathways that sustain osmolytes accumulation, and signaling pathway of ABA. Using suppression subtractive hybridization and sequencing, Bernardi et al. (2015) studied cold-sensitive and cold-tolerant 'Leccino' clones treated with decreasing temperatures (down to −10 °C). Several genes whose expression was differentially modulated in the two clones were found: *chloroplast ycf2 protein*, *rubredoxin family protein*, *bark storage protein*, *carbonic anhydrase*, and *chlorophyll a/b-binding protein*.

This growing body of information is a good starting point to understand the biochemical and molecular mechanisms that control the tolerance to freezing in olive and gives the opportunity to apply effective molecular breeding approaches.

4 Concluding Remarks

In recent decades, olive physiology and biochemistry during abiotic stresses have been extensively investigated. Field studies have highlighted wide genotypic variability and disclosed several of the physiological mechanisms involved in olive tolerance and resistance. Further cellular and molecular studies are likely to promote the understanding of resistance mechanisms in olive plants and to provide new breeding tools for stress resistances. There is a lack of research and data on production of olives in the new areas of olive growing, on the effects of spring high temperature, and rain on olive productivity. Moreover, investigation as the relationships between responses to various environmental stresses is highly required to cope with the changing climate, rising temperatures, increase in precipitation abnormalities and freezing temperatures, and salinization that will expose olive trees to concurrent environmental constraints. Last but not least, stress and new cultivars alone and together have many impacts on olive oil composition and quality.

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Metabolomics of Olive Fruit: A Focus on the Secondary Metabolites

8

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Abstract

Metabolomics studies are widely used in systems biology approaches with the aim to understand the metabolism and physiology of living organisms. Metabolomics of olive fruit can be defined as the application of metabolomics in the study of the multitude of molecules that characterize olive metabolism during the different fruit's phenological stages, in response to the crop management choices and environmental variables. The study of olive fruit metabolomics has increased in the recent years, because olive products (oil and table olive) are directly related to nutrition and human health. In this chapter, we will describe the recent trends and applications of metabolomics to the characterization of olive secondary metabolites including the genotypes, the agronomical, and the environmental variables that could modify their composition.

1 Introduction

Metabolomics is one of the 'omics' technologies that have characterized the molecular studies of this last decade, and it is aimed to the identification and quantitation of 'as-many-small-metabolites-as-possible' in a system (Cevallos-Cevallos et al. 2009). It has become an important tool in many research areas depicting, in a certain moment, the specific metabolites of cells, tissues, organs, and organisms' sample (Weckwerth and Fiehn 2002). Since metabolomics reproduces the physiological status of a living organism and gives a deeper insight into its cellular activity, it becomes a powerful tool for studying plant metabolism and physiology. In plants, metabolomics approaches have been applied for many purposes, such as in biomarkers identification, investigation of unknown metabolic pathways, and stress tolerance mechanisms, up to the food, since food direct impact on nutrition and human

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health. The reviews published since now show the broad impact and rapid growth of metabolomics in plant science (Hall et al. 2008; Wolfender et al. 2009; Allwood and Goodacre 2010; Kusano et al. 2011).

Metabolomic analyses can be classified as targeted or untargeted. Targeted apply to a specific group of metabolites that requires the identification and quantification of as many metabolites as possible within the group (Ramautar et al. 2006). These analyses are important for evaluating the fate of a specific group of molecules under well-defined conditions and generally require higher level of purification and a selective extraction of metabolites. Untargeted apply to the detection of as many groups of metabolites as possible. The aim is to obtain patterns or fingerprints without necessarily detecting or quantifying a specific compound (Monton and Soga 2007). Another classification is based on the specific objective of the analysis and data manipulation, so that metabolomic studies can be: (a) discriminative; (b) informative; and (c) predictive (Cevallos-Cevallos et al. 2009). Discriminative approaches are used to discover differences between samples without automatically producing statistical models or assessing pathways that may explain the differences (i.e., classify the oil samples by olive cultivar). Informative approaches are focused on the identification and quantification of targeted or untargeted metabolites aimed to obtain intrinsic information from the sample under study. Informative metabolomics is used to discover metabolic pathways, novel bioactive molecules, biomarkers, metabolite databases, and metabolites functionality (i.e., olive fruit response to stress and nutraceutical approaches). Predictive approaches used statistical modeling based on metabolite profile and abundance are aimed to predict a variable difficult to quantify (i.e., metabolite-based models for prediction of olive oil sensory quality). Discriminative, informative, and predictive metabolomics can also be used all together for quality, nutrition, and food components analysis (Wishart 2008) (Fig. 1).

Metabolomics approaches must adopt satisfactory sampling, homogenization, extraction, storage, and preparation methods to maintain the whole process unbiased. Since numerous techniques exist for metabolite detection, these should be case by case evaluated and for a detailed analysis of these aspect readers can refer to Weckwerth and Fiehn (2002).

The olive fruit is a drupe at its mass differed considerably among cultivars; (Rosati et al. 2009) measured a sixfold/fivefold difference in fruit dry weight (DW) between cultivars during a

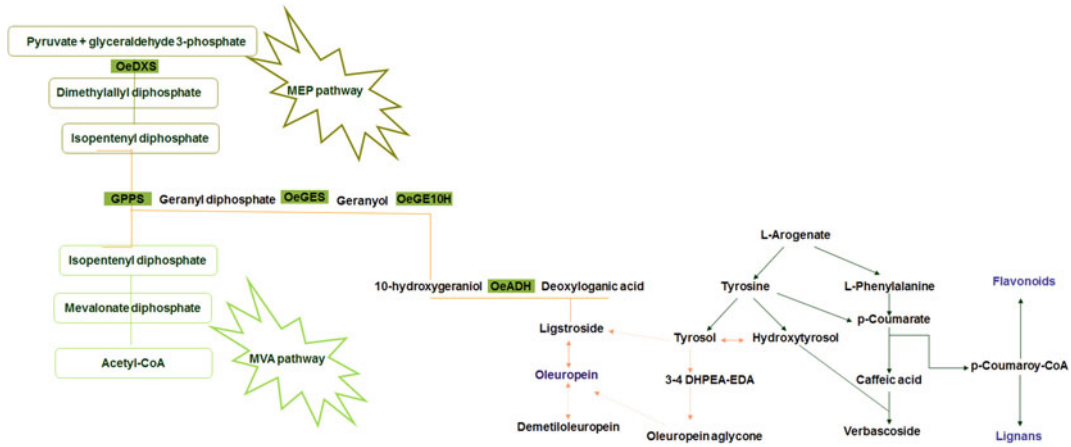


Fig. 1 Schematic representation depicting the putative biosynthetic pathways of main secondary compounds of olive fruits (Obied-Hassan et al. 2008; Alagna et al. 2012). *OeDXS* 1-deoxy-D-xylulose-5-P synthase; *GPP* Geranyl diphosphate synthase; *OeGES* Geraniol synthase; *OeGE10H* Geraniol 10-hydroxylase; *OeADH* Arogenate dehydrogenase; *MEP* Methyl-D-erythritol 4-phosphate; *MVA* Mevalonate. *Dotted arrows* indicate uncertain biosynthetic steps

three-year experimental period, with a maximum of 2.3 g_{DW} for the Nocellara del Belice and a minimum of 0.4 g_{DW} for Koroneiki. Olive drupe consists of three distinct anatomical parts: epicarp (or skin/peel, 1–3 % of the total fruit mass), mesocarp (or pulp/flesh, 70–90 % of the total fruit mass), and endocarp (or stone/pit, 9–27 % of the total fruit mass) that encloses the seed (2–3 % of the total fruit mass).

In olive fruit, the role of the epicarp cells is mainly that to protect the internal tissues and for this reason cells are covered by a cuticle, which consists of cutin (an insoluble polymer) and waxes (a complex mixture of aliphatic and cyclic lipids) that can be intra-cuticular or present on the fruit surface as three-dimensional epi-cuticular waxes (Lanza and Di Serio 2015). Further, fruit ripening is accompanied by a change in the skin color due to a modification of pigment concentration in the epidermal cells and color change from green to purple. This change is due to an accumulation of anthocyanins (Ryan et al. 2002) together with the degradation of chlorophylls and carotenoids (Minguez-Mosquera and Garrido-Fernandez 1989).

The mesocarp is soft and pulpy flesh and accumulates a wide range of molecules including water, proteins, lipids, carbohydrates, inorganic substances, and many secondary metabolites. Together with the skin, the mesocarp represent the edible portion of olives and contains a large proportion of water (70–75 % of the mesocarp weight) and oil (ranging from 14 to 15 % in green table olives to about 30 % in black, mature olives) (Bianchi, 2003). Other important molecules are oxalic, succinic, malic, and citric acids (1.2–2.1 % of dry flesh) that together with the free fatty acids represent the free organic acids fraction of olives. Regarding sugars, glucose and fructose prevail over saccharose and mannitol (3.5–6 % of the flesh), while protein content varies between 1.5 and 2.2 % of the fruit weight (Bianchi 2003). The stone is characteristic of a cultivar and has been used as morphological descriptors in pomology. The enclosed seed comprise 2–3 % of the weight and is composed by 30 % water, 27 % oil, 27 % carbohydrates, and 10 % protein (Connor and Fereres 2005), whereas the woody shell contains not more than 1 % of oil and is largely made of lignocellulosic material with hemicellulose (21–28 %), cellulose (30–34 %), and lignin (21–25 %) as main components (Rodríguez et al. 2008).

Since a description of all of them is still difficult, due to the limited numbers of publications using informative metabolomics approaches (Martinelli et al. 2012, 2013); in this chapter, we will briefly discuss the main olive fruit metabolites' classes and then focus on those belonging to the secondary metabolites, namely the phenolic compounds. For these molecules, we will examine in detail the role of genotypes, agronomical, and environmental variables in modifying their composition in olive fruit.

2 Lipids and Sugars in Olive Fruit

Lipids in the olive drupe are mainly located in the mesocarp. The glyceridic fraction represents almost 98 % of the olive components and it includes triglycerides, fatty acids, phospholipids, and waxes. In particular, at the fruit ripening the triglycerides characterize almost entirely the saponifiable fraction for 98–99 % of total fats, while the diglycerides and monoglycerides are present in small amounts with a range of 1–1.5 % and less than 1 % of total fats, respectively (Inglese et al. 2011). The biosynthesis of mono, di, and triglycerides (TAGs) is carried out with the primary metabolism, which occurs in different successive stages: first, the fatty acids are biosynthesized and then these are assembled to glycerol through predominantly the glycerol 3-phosphate or Kennedy pathway (Sánchez and Harwood 2002). This biosynthetic pathway involves in a series of four reactions that yield TAGs as end products.

The fatty acid composition of olive fruit is characterized by: saturated fatty acids (palmitic (C16:0) and stearic (C18:0) acids); monounsaturated fatty acids (palmitoleic (C16:1) and oleic (C18:1) acids); and polyunsaturated fatty acids (linoleic (C18:2) and linolenic (C18:3) acids) (Table 1) (Montedoro et al. 2003). The last two

Table 1 Main fatty acids of olive oil

Compound	Concentrations (%)
Myristic acid	0.0–0.1
Palmitic acid	7.5–20
Palmitoleic acid	0.3–3.5
Margaric acid	0.0–0.4
Heptadecanoic acid	0.0–0.4
Stearic acid	1.0–4.0
Oleic acid	47.0–84.0
Linoleic acid	3.0–21
Linolenic acid	0.2–1.5
Arachic acid	0.1–0.7
Eicosenoic acid	0.0–0.4
Behenic acid	0.0–0.2
Lignoceric acid	0.0–0.4

are essential fatty acids and, therefore, they should be taken with diet are not synthesized by humans. During the fruit growth, the amount of all the fatty acids increases, the most noticeable is that of oleic acid that at the end of ripening represents 70–80 % of the total, followed by the palmitic acid (10–15 %), linoleic acid (5–10 %), and the stearic acid (2–3 %) (Inglese et al. 2011). The intermediate key for the de novo fatty acid biosynthesis is the acetyl-CoA that comes mainly from the pyruvate produced in the sugar metabolism (Sánchez and Harwood 2002). Fatty acids are synthesized in the stroma of plastid from malonyl-ACP (acyl carrier protein), which is formed by the malonyl-CoA in turn by acetyl-CoA carboxylase. The pathway involves a series of reactions (seven cyclical repetitions) building the fatty acid molecule from malonyl-CoA where, in each cycle, the end product of the previous cycle is added, determining the growth in length of the acyl chain, by two carbon atoms, until the palmitoyl-ACP (C16:0-ACP) formation (Sánchez and Harwood 2002). The product 'palmitic acid' in the biosynthesis of fatty acids can be subsequently modified through reactions catalyzed by specific enzymatic systems which lead to its elongation to stearic acid and, in some cases, the introduction of double bonds, taking place the oleic acid formation. In particular, the oleic acid, the most prevalent constituent of olive fatty acids, is produced by desaturation of stearoyl-ACP, a reaction catalyzed by the stearoyl-ACP Δ9-desaturase (Harwood 1996). Traditionally, the health-promoting effects of virgin olive oil (VOO) have been ascribed to its high amount of oleic acid exerting high efficiency in the modulation of gastrointestinal and metabolic functions and of extrinsic cardiovascular risk factors.

Phospholipids, being the major components of biological membranes both in plant tissues and in animal, constitute the most important class of polar lipids. In VOO the their content ranges between VOO 21 and 124 mg kg^{-1} of oil (Koidis and Boskou 2006). The waxes represent a further class of minor saponifiable fraction of olive fruit. They form the external hydrophobic layer produced by plants as a barrier against the biotic and abiotic environmental stresses. The waxes are characterized by homologous series of very-long-chain aliphatics, i.e., fatty acids, aldehydes, alcohols, ketones, alkanes, and alkyl esters (Bianchi 2003).

The olive fruit as well as VOO are the richest vegetable source of squalene ranged from 110 to 839 mg 100 g^{-1} (Beltrán et al. 2015). This compound is a polyunsaturated triterpene comprising of six isoprene units and plays a key role as intermediate metabolite in cholesterol and others sterols (Boskou 2009). Cultivar, ripening

stage of the olive fruit, and agroclimatic conditions as well as extraction technology strategies of VOO are the main agricultural and technological factors affecting the squalene content (Wiesman 2009; Beltrán et al. 2015). A recent study carried out by Fernández-Cuesta et al. (2013) on the evolution of squalene content in the fruit flesh of four Spanish cultivars shows a significant increase during the fruit ripeness. However, in a previous study, other authors reported the maximum accumulation of squalene during the early stage of fruit growth and a remarkable decrease in full fruit maturity, hypothesizing that its reduction is only due to the involvement of squalene in the biosynthesis of sterols and terpenoids through via the acetate/mevalonate pathway (Sakouhi et al. 2001). The mevalonate pathway seems to promote also the secoiridoid synthesis in olive fruit (Obied-Hassan et al. 2008). The sterol composition and content of olive fruits and related oils typically between 1000 and 2000 mg kg^{-1} depend on several agronomical and technological factors (Fernández-Cuesta et al. 2013). Particular attention has been paid to the composition of the sterols of olive oil as very useful parameter for detecting adulterations or to check authenticity, since it can be considered as a fingerprint. The sterolic fraction is characterized mainly by β-sitosterol achieving 75–90 % of the sterols and to a lesser extent by Δ5-avenasterol (5 and 20 % of total sterol). During the olive development, a significant change of the sterol profile can occur. Thus, a decrease in β-sitosterol content and simultaneously increase in Δ5-avenasterol have been observed (Aparicio and Luna 2002; Vekiari et al. 2010; Fernández-Cuesta et al. 2013). Fernández-Cuesta et al. (2013) suggested that the different levels of accumulation of both sterols is accountable to variable enzymatic activities according with fruit development stages, not only due to a change in the flesh/stone ratio during ripening, as explained by Aparicio and Luna (2002).

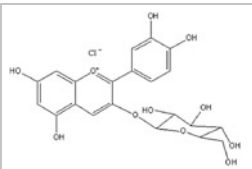
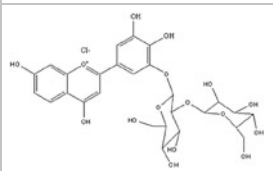
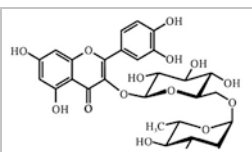
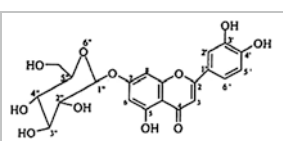
Sugars are essential molecules for the synthesis of lipids in plant cells. Two sources of carbohydrates for growth and lipid biosynthesis in olive fruit have been identified in experiments done with heterotrophic and autotrophic olives (Sánchez, 1995; Sánchez and Harwood 2002): (a) sugars from mature leaves translocated by the phloem (the mayor one); (b) sugars synthesized by fruits photosynthetic activity (the minor one). Analysis of phloem exudates showed that the photoassimilates translocated from olive leaves were oligosaccharides of the raffinose family (mainly stachyose) with sucrose, and mannitol also being translocated through the phloem (Flora and Madore 1993; Gucci et al. 1998). Existing information on phloem unloading and assimilates transport suggest both symplastic and apoplastic mechanisms, although the apoplastic pathway is supposed to prevail during fruit ripening (Ruan and Patrick 1995; Zhang et al. 2006). The hydrolysis of stachyose and raffinose by extracellular α-galactosidase produce galactose and sucrose that is hydrolyzed to glucose and fructose by cell-wall-bound invertases. Galactose, fructose, and glucose are then transported into olive mesocarp cells, together with mannitol, the characteristic sugar soluble components of olive tissues (Marsilio et al. 2001; Conde et al. 2007). During ripening, when storage lipids accumulate into the mesocarp cells, glucose concentration falls steadily while the mannitol content increases. This behavior, very likely, reflects the function of mannitol as a reserve carbohydrate and osmoprotectant, and Marsilio et al. (2001) suggested that the relative amount of mannitol in the fruit could be used as an indicator of the cultivar potential for oil biosynthesis.

Additional information on lipids and sugars biosynthesis and transport in olive can be found in Conde et al. (2008).

3 Phenolic Compounds in Olive Fruit

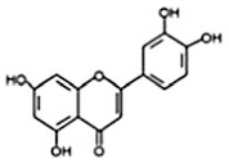
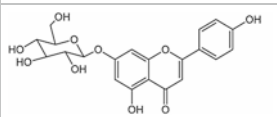
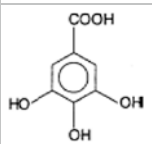
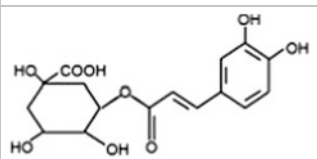
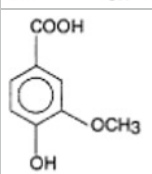
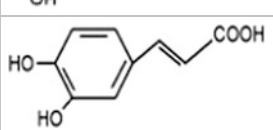
Members of Oleaceae, including olive, contains many compounds, such as, simple phenolic, carotenoids, tocopherols, chlorophylls, and anthocyanins that are common to many other species, and a group of complex phenolics that are specific of this family. This latter category is represented by phenolic oleosides or secoiridoids that are typical of the Oleaceae and few other

Table 2 The main phenolic compounds of olive fruit. The Chemical structures were drawn based on available literature data (Robards et al. 1999; Servili and Montedoro 2002; Obied-Hassan et al. 2007)

Class	Compound	Concentrations (mg kg^{-1} FW)			Chemical structure	Reference
		Mean	Upper quartile	Lower quartile		
Flavonoids	*Anthocyanins*					
	Cyanidin 3-O-glucoside	273.5	881.7	52.3		Romani et al. (1999)
	Cyanidin 3-O-rutinoside	1021.9	3205.7	248.3		Romani et al. (1999)
	Flavonols					
	Quercetin-3-O-rutinoside	180.8	273.0	111.2		Romani et al. (1999)
	Flavones					
	Luteolin 7-O-glucoside	61.9	129.1	4.8		Romani et al. (1999)

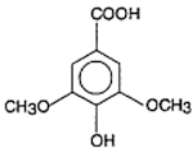
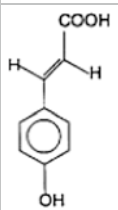
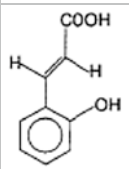
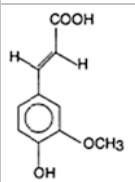
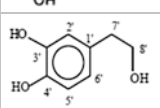
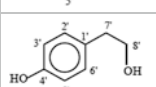
(continued)

Table 2 (continued)

Class	Compound	Concentrations (mg kg^{-1} FW)			Chemical structure	Reference
		Mean	Upper quartile	Lower quartile		
	Luteolin	20.9	47.9	1.0		Romani et al. (1999)
	Apigenin 7-O-glucoside	24.2	40.6	6.2		Romani et al. (1999)
Phenolic acids	Gallic acid	1.0	3.4	0.0		Dağdelen et al. (2013)
	Chlorogenic acid	2.3	8.8	0.0		Dağdelen et al. (2013)
	Vanillic acid	9.8	31.0	2.6		Dağdelen et al. (2013)
	Caffeic acid	0.5	1.9	0.0		Dağdelen et al. (2013)

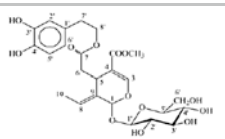
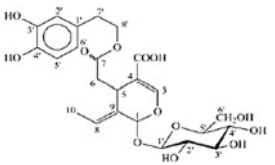
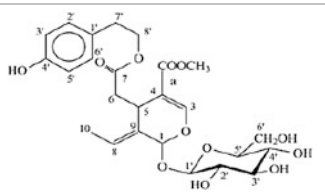
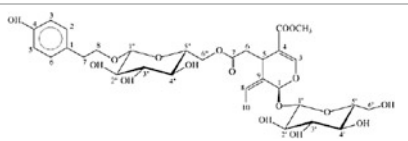
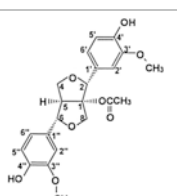
(continued)

Table 2 (continued)

Class	Compound	Concentrations (mg kg^{-1} FW)			Chemical structure	Reference
		Mean	Upper quartile	Lower quartile		
	Syringic acid	1.1	3.3	0.0		Dağdelen et al. (2013)
	p-Coumaric acid	1.7	8.1	0.0		Dağdelen et al. (2013)
	o-Coumaric acid	2.7	11.7	0.0		Dağdelen et al. (2013)
	Ferulic acid	0.3	1.6	0.0		Dağdelen et al. (2013)
Phenolic alcohols	(3,4-Dihydroxyphenyl) ethanol (3,4-DHPEA)	454.6	967.3	224.4		Unpublished data
	(p-Hydroxyphenyl) ethanol (p-HPEA)	267.7	465.1	112.7		Unpublished data

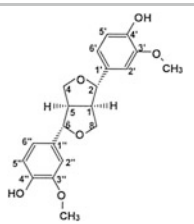
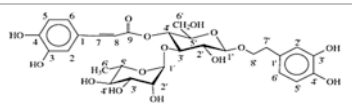
(continued)

Table 2 (continued)

Class	Compound	Concentrations (mg kg^{-1} FW)			Chemical structure	Reference
		Mean	Upper quartile	Lower quartile		
Secoiridoids	Oleuropein	25241.0	43695.0	2088.3		Unpublished data
	Demethyloleuropein	8395.9	13764.7	647.3		Unpublished data
	Ligstroside	2211.4	3167.2	1443.6		Unpublished data
	Nüzhenide	1758.3	1905.0	1525.0		Unpublished data
Lignans	(+)-1-acetoxypinoresinol	800.5	1132.0	513.6		Unpublished data

(continued)

Table 2 (continued)

Class	Compound	Concentrations (mg kg^{-1} FW)			Chemical structure	Reference
		Mean	Upper quartile	Lower quartile		
	(+)-pinoresinol	271.4	347.1	183.2		Unpublished data
Hydroxycinamic acid derivatives	Verbascoside	8926.6	28978.0	1508.1		Unpublished data

dicotyledonous families (Ryan et al. 2002). Overall, the concentrations of phenolic compounds in olive drupes range between 1 and 3 % of the pulp fresh weight (FW) and the leading classes of phenols present are: (1) phenolic acids; (2) phenolic alcohols; (3) flavonoids; and (4) secoiridoids (Table 2). Several phenolic acids with the basic chemical structure of C6-C1 (benzoic acids) and C6-C3 (cinnamic acid) have been found in olive fruits (Obied-Hassan et al. 2012). Beside phenolic alcohols, (3,4-dihydroxy phenyl)ethanol (3,4-DHPEA) and (p-hydroxy phenyl)ethanol (p-HPEA) are the most abundant. The flavonoids group is represented by the flavonol glycosides (luteolin-7-glucoside and rutin), anthocyanins, cyanidin, and delphinidin glycosides. The secoiridoids group is characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure and oleuropein, demethyloleuropein, ligstroside, and nüzhenide are the most abundant secoiridoids glucoside (Servili and Montedoro 2002). Regarding this last class of molecules, oleuropein was observed for the first time long time ago by Bourquelot and Vintilesco (1908), and subsequently Panizzi et al. (1960) discovered its chemical structure. Demethyloleuropein was isolated and characterized by Ragazzi et al. (1973) and ligstroside by Kubo and Matsumoto (1984). Regarding the hydroxycinnamic acid derivatives, verbascoside chemical structure was discovered by Andary et al. (1982) in *Orobanche rapum-genista* and confirmed by Servili et al. (1999a) in olive. Oleuropein, demethyloleuropein, and verbascoside were found in peel, pulp, and seed, but they are more abundant in the pulp, whereas nüzhenide was found only in the seed (Servili et al. 1999b).

Several factors affect the metabolomics profile of the phenolic compounds in olive fruits, such as cultivar, growing site, climatic conditions, alternate bearing, and ripening stage. Since this plurality of factors, finding a general objective criterion to describe how phenolic molecules change in fruit is a difficult task. For that reason, the main aspects analyzed were as follows: (a) genotypes and phenological stages and (b) agronomical and environmental variables.

3.1 Genotypes and Phenological Stages

During ripening, the profile of phenolic compounds in olive fruit of different cultivars undergo wide modifications that strongly influence sensorial attributes, shelf-life, and the nutritional value of olive and olive oil. Since the last century, Romani et al. (1999) studying 'Frantoio', 'Rossellino', 'Ciliegino', 'Cuoricino', and 'Grossolana' fruits showed that oleuropein and its aglycon were present in large amount (concentration range 1136–2406 and 1312–1991 mg kg^{-1}, respectively) in slow-ripening cultivars ('Ciliegino', 'Cuoricino', and 'Grossolana'), whereas in the precocious-ripening cultivar Rossellino, these compounds were present at lower concentrations (36 and 24 mg kg^{-1}, respectively). Since olives of the different cultivars were harvested at the same time, these changes were likely due to the grade of ripening that was higher for 'Rossellino'. Regarding hydroxytyrosol, the trend was opposite and the highest value was observed for 'Rossellino' (4133 mg kg^{-1}).

Gomez-Rico et al. (2009) studied the effect of both the cultivar and the degree of ripening on the olive fruit biophenolic for six Spanish cultivars: 'Arbequina', 'Cornicabra', 'Morisca', 'Picolimón', 'Picudo', and 'Picual'. They confirmed that oleuropein was the main phenolic and found significant differences in the cultivars studied and that this oleoside decreased significantly from unripe to ripe fruit. During ripening, oleuropein concentration decreased from 2230 to 60 mg kg^{-1} FW in 'Arbequina' and from 11,600 to 6340 mg kg^{-1} FW in 'Cornicabra' variety. Regarding other biophenols such as demethyloleuropein, it was found exclusively in 'Arbequina' and its content ranged between 984 and 1985 mg kg^{-1} FW, becoming its major phenolic compound at black stage. Demethyloleuropein is most likely a degradation product of the oleuropein (Amiot et al. 1986; Servili et al. 1999b), and this data were in agreement with Amiot et al. (1989) who found demethyloleuropein only in two of eleven French cultivars and with Esti et al. (1998) who found this compound only in two out

of the eight Italian cultivars studied. Simple phenol like hydroxytyrosol increased during ripening in 'Arbequina': from 219 mg kg^{-1} FW at green stage to 349 mg kg^{-1} FW at black stage. In the other cultivars, the changes were not statistically significant. Verbascoside was the main hydroxycinnamic derivative and steadily increased from green to black stage in many varieties (from 665 to 1231 mg kg^{-1} FW in Arbequina to 64 to 173 mg kg^{-1} FW in Picual); while in others like 'Cornicabra' and 'Picudo', it was not detected. Regarding anthocyanin, the most abundant was cyanidin 3-O-rutinoside that ranged between 1058 and 3236 mg kg^{-1} FW at black stage, except for 'Picudo' where it was absent. In literature, it is well known the case of 'Leucocarpa', a natural mutant producing small fruits, with low oil content and an ivory-white color at ripening instead of the usual purple black, with a very low or null amount of anthocyanins since their synthesis is blocked (Lavee 1986). In 'Leucocarpa', total anthocyanins are in the range of 5–12 mg kg^{-1} FW at 155–180 days after full bloom, whereas in 'Leccino' they reached 297 mg kg^{-1} FW at 155 days after full bloom (Cirilli et al. 2016). This difference between olive genotypes that produce very low amount of anthocyanins could help in the identification of genes controlling anthocyanin biosynthesis and accumulation in olive fruits.

More recently, Alagna et al. (2012) measured the concentration of oleuropein, demethyloleuropein, 3-4 DHPEA-EDA, ligstroside, tyrosol, hydroxytyrosol, verbascoside, and lignans in the developing olive fruits of 12 cultivars. Again, these compounds proved to vary significantly among cultivars and showed some degree of specificity. At 45 days after flowering (DAF), the total phenolic content in all cultivars ranged between 50 and 350 mg kg^{-1} DW, with higher values in 'Coratina' and 'Rosciola' and lower values in 'Tendellone' and 'Dolce d'Andria'. In general, total phenolic content concentration decreased during fruit development and maturation. In 'Coratina', as an example, the total phenolic content dropped from almost 350 mg kg^{-1} DW at 45 DAF to less than 150 mg kg^{-1} DW at 165 DAF. In 'Dolce d'Andria' the initial total phenolic content was very low (less than 50 mg kg^{-1} DW at 45 DAF), the phenolic concentration in fruit at 165 DAF were almost negligible. Regarding oleuropein, demethyloleuropein, 3-4DHPEA-EDA, ligstroside, tyrosol, hydroxytyrosol, verbascoside, and lignans, the variability was also very large between cultivars and showed peculiar trends at different fruit phenological stages. In olive drupe, oleuropein was the most abundant biophenol and decreased gradually after fruit set when the initial total phenolic content was very high. On the contrary, when the initial total phenolic concentration in the drupe was low, the most abundant biophenolic was 3-4 DHPEA-EDA. Other biophenols, such as demethyloleuropein, ligstroside, and lignans undergo an increase in concentration during ripening, even if this trend differ significantly between cultivars having high and low biophenols content in fruit. Using a deep metabolomic analysis targeted to these secondary metabolites, Alagna et al. (2012) also studied if contrasting phenotypes for phenolic metabolism have transcriptional differences that could explain this behavior. Following this approach, 27 transcripts that could be putatively involved in the synthesis of the major olive fruit compounds (secoiridoids, phenolics, terpenes, and sterols) were identified. In particular, the sequences encoding for putative 1-deoxy-D-xylulose-5-P synthase (OeDXS), geraniol synthase (OeGES), geraniol 10-hydroxylase (OeGE10H), and arogenate dehydrogenase (OeADH) were most abundant at 45 days after flowering (DAF), suggesting that these genes and their corresponding enzymes might play a role in regulating secoiridoid accumulation during fruit development.

3.2 Agronomical and Environmental Variables

The large variability of olive phenolic concentration is affected by the interaction between cultivar and growing environment (Inglese et al. 2011).

Beside the numerous environmental factors and agronomic practices, water availability and irrigation have been shown to strongly affect the phenolic composition of olives (Ripa et al. 2008; Inglese et al. 2011; Di Vaio et al. 2013).

The environmental effect includes water availability, which is partly controllable with an irrigation regime. The relationships between the water availability during fruit ripening and phenolic profile have been widely elucidated. Generally, an increase in water availability in the soil implies a reduction of concentration of the total phenolic compounds in olive flesh and therefore in corresponding VOO (Servili et al. 2007; Inglese et al. 2011; Caruso et al. 2014). However, the irrigation regimes seem to exert a different effect on specific phenol compounds (Martinelli et al. 2012). The different irrigation volumes (full, deficit, and complementary) determined changes in the phenolic fraction of cv. 'Frantoio', adversely affecting the secoiridoid compounds, which are directly responsible for the oxidation stability of VOO, healthy effects, and sensorial properties (Caruso et al. 2014). While the lignans concentrations ((+)-1-acetoxypinoresinol and (+)-1-pinoresinol) are generally unaffected by the irrigation regime, which is in agreement with finding by Servili et al. (2007). This decrease in hydrophilic phenols can be explained by a less enzymatic activity of L-phenylalanine ammonya-lyase (PAL) responsible for their synthesis (Tovar et al. 2002; Servili et al. 2007). As the PAL activity is greater under stress condition (Tovar et al. 2002).

In contrast to this observation, other studies carried out by Dabbou et al. (2010, 2011) showed an opposite impact of irrigation regimes on phenolic amount of Arbequina and Koroneiki cultivars under Tunisian growing conditions. These results indicated that the irrigation regime could ameliorate the quality of Arbequina oils in term of phenolic content.

According to the available literature, the effect of temperatures on phenolic composition is still controversial. In a study carried out by Farinelli et al. (2011), in dry summers and autumns an increase in phenol content has been observed. Whereas the total amount of them decreased when the higher the degree days accumulated from fruit set to harvest (Ripa et al. 2008).

Furthermore, the light exposure enhances the final fruit size, the oil content, and phenolic compounds, secoiridoids in particular, except for the lignans. Low light levels significantly slowed down fruit maturation, whereas conditions of water deficit accelerated the maturation process.

The role of orchard management (i.e., N-fertilization) on phenolic concentration in olive fruits and oil is scarcely studied (Fernandez-Escobar et al. 2006; Ninfali et al. 2008; Inglese et al. 2011; Rosati et al. 2014). Several authors discussed the negative relationship between the leaf nitrogen status and the secondary plant metabolites amount, phenols in particular. The adverse effects of nitrogen accumulation in leaves on olive phenolic fraction imply a biosynthesis inhibition of phenols or its precursors is duo to protein/phenol competition sharing a common precursor: the phenylalanine (Jones and Hartley 1999; Fernandez-Escobar et al. 2006; Erel et al. 2013). With nitrogen over-fertilization of olive trees, the phenylalanine preferentially flows into protein synthesis rather than toward the synthesis of phenols via PAL explaining the decrease in phenolic compounds in olive fruit (Jones and Hartley 1999).

4 Volatiles and Lipoxygenase Pathway

The amount of prevalent volatile compounds is low in intact and healthy cell tissues of olive fruit. However, they are rapidly originated during VOO extraction as a result of the breakdown of fruit integrity produced by enzymatic activities included in the lipoxygenase (LOX) pathway (Kalua et al. 2007). Among them, C6 and C5 compounds, in particular C6 linear unsaturated and saturated aldehydes and alcohols and their corresponding esters are the most important compounds of the volatile fraction, from either a quantitative or a qualitative point of view (Tatticchi et al. 2014). The levels and the activities of enzymes involved in the LOX pathway, which are genetically fixed, play a noticeable impact on

development of volatile compounds in VOO. The LOX are a pool of endogenous enzymes that use lipids as substrates and give arise a cascade series of events that eventually lead to volatile compounds formation responsible for 'green', 'fruity', 'almond' etc. sensory notes (Angerosa 2002, 2004; Kalua et al. 2007; Servili et al. 2009). Particular emphasis was paid to the biogenesis of them.

Several studies were focused on the enzymatic mechanism involved in LOX pathway. Ab initio, the production of 9- and 13-hydroperoxides of linoleic (LA) and linolenic (LnA) acids mediated by lipoxygenase (LOX) represents the first step of the pathway. Therefore, very specific hydroperoxide lyases (HPL) catalyzed the cleavage of 13-hydroperoxides and results in C6 aldehydes, whose unsaturated ones can isomerize from cis-3 to the more stable trans-2 form. The C6 aldehydes were reduced to corresponding alcohols by alcohol dehydrogenase (ADH), which can produce esters because of the catalytic activity of alcohol acetyl transferases (AAT). When the substrate is LnA, a further branch of the LOX pathway is activated. LOX would catalyze the formation of stabilized 1,3-pentene radicals that can dimerize resulting in C10 hydrocarbons (known as pentene dimers) or can react with a hydroxy radical, producing C5 alcohols, which can be enzymatically oxidated to corresponding C5 carbonyl compounds (Aparicio et al. 1996; Aparicio and Morales 1998, Angerosa et al. 2004).

The outcomes of several studies suggested that the profile of volatile compounds is strongly modified by behavior of LOX pathway enzymes that depended on cultivar, and numerous agronomic and processing conditions. In this way, the LOX activities seem to be cultivar-dependent (Angerosa et al. 2004). Furthermore, the advanced fruit ripening reduces the LOX activities decreasing the quantity of the volatile compounds responsible for the positive VOO sensory attributes (Angerosa et al. 2004). Some studies on the relationships between the water availability during the fruit growth and volatile compounds were carried out by several authors (Servili et al. 2007; Dabbou et al. 2010; Inglese et al. 2011; Benelli et al. 2015; Taticchi et al. 2014). In particular, as mentioned previously, the water stress conditions stimulate the synthesis of phenolic compounds in olive fruits and therefore their increase in corresponding oils, on the contrary, they exert the negative effects on activation of the LOX (Servili et al. 2007). The finding by Servili et al. (2007) showed that in cv. Leccino grown under three different water conditions (full, deficit, and stress irrigation) the several volatile compounds such as hexanal, (E)-2-hexanal, and other LOX derivative compounds were positively correlated with the irrigation rate.

However, during the storage of olive, a reduction of the volatile compounds responsible for the positive perception was observed probably due to the inhibition of the LOX pathway. The presence of molds, yeasts, and bacterial contaminations on olive surface, together with their corresponding metabolism is due to development of the off-flavor in VOO (Angerosa et al. 2004), whereas sugar fermentation leads to acetic acid and ethyl acetate production, which are ascribed to be responsible for the 'vinegar' defect.

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Genome Sequencing, Transcriptomics, and Proteomics

9

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Abstract

This review encompasses the current status of major areas of progress in olive tree genome sequencing, including insights into genome function derived from large-scale gene expressing profiling, and studies on genomic architecture of repetitive sequences, smaller RNA, and proteomics. Olive tree genomics, as well as other omics, is progressing owing to recent developments in next-generation sequencing (NGS) technologies. Biological insights, therefore, are not only resulted from the sequencing initiative, since from genetic mapping, gene expression profiling, gene discovery research, and proteomics over nearly last seven years a large amount of information has been provided by different laboratories. The availability of high-quality genome assembly provides olive biologists with valuable new tools to improve and develop new varieties more efficiently, enabling the implementation of marker-assisted selection and genomic selection, and contributing to the comprehension of the

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molecular determinants of key traits peculiar to the species of olive tree and giving important clues concerning the evolution of its complex genome.

1 Introduction

Unraveling the information through studies of omics can be equated to the discovery of the whole experience, which has been biologically accumulated during the evolutionary history of an organism, and it is the baseline which characterizes the set of adaptive events switched on in response to environmental factors and crop management choices that occur during the developmental stages of olive tree. The olive tree-cultivated form (*Olea europaea* L. subsp. *Europa*ea var. europaea) and the wild form (*Olea europaea* subsp. *Europaea* var. *sylvestris*) are mainly grown in the Mediterranean basin and Near Eastern, where the majority of a large number of olive cultivars estimated in more than 1200 (Bartolini et al. 1994) are also conserved. Olive is a diploid species ($2n = 2x = 46$) and the genome size ranges between 2.90 pg/2C and 3.07 pg/2C, with 1C = 1400–1500 Mbp (Loureiro et al. 2007).

Olive species is becoming one of the most economically important evergreen fruit crops in all Mediterranean climate types around the world. Despite its global importance and its metabolic peculiarities, available information on genomic and transcriotomic sequences for olive are still scarces, recently, an increasing number of expressed gene functions are being described. Besides, the Olive Genome Project (OLEA) (http://www.oleagenome.org) and the International Olive (*O. europaea*) Genome Consortium (IOGC) (http://olivegenome.karatekin.edu.tr) are expected to provide high-resolution information for functional studies and for discovery of new molecular markers. A striking feature coming from the analysis of studies conducted until now on olive genome indicates the presence of a greater number of repeated elements and among them the tandem repeat sequences (excluding rDNA) accounted for 31.16 % of the reads, the LTR-REs (*Gypsy* plus *Copia* elements) accounted for 38.84 % of the reads matching the whole genome set of assembled sequences (WGSAS), while low percentages of the presence were accounted for DNA transposons and non-LTR-REs. In this chapter, more accurate details are reported. From these studies, the peculiarity of genome evolution in this species has been evidenced with a very large fraction of the genome produced by tandem repeats amplification and LTR-RES. The role of this very large fraction of genome still remains unknown, but it can negatively affect the assembly of genome since olive is a highly heterozygous species.

The occurrence of a large and highly variable germplasm for this species, and for the related species, will allow to explore genetic variability concerning this genome fraction, possibly enabling to clarify the mechanisms by which such sequences have been produced and maintained during evolution and their function. The acquired knowledge will identify the relevant differences in the control of gene expression of the same sets of genes that exist among different genotypes. Following the genome assembly, the considerable task of annotating the genome remains. This includes predicting key features such as polymorphisms, GC content, repeated sequences, and genes. A suite of bioinformatics tools is available for predicting protein-coding genes and repeated sequences, based on sequence homology with other sequenced genomes and alignment of RNA sequences onto the assembly pseudo-chromosomes.

High-density genetic marker screen technology has been developed for olive, including single-nucleotide polymorphism (SNP) arrays (Kaya et al. 2013) and genotyping-by-sequencing (GBS) (İpek et al. 2016; Marchese et al. 2016). These technologies will be helpful

for developing high-density genetic maps, fine mapping of major loci, genome-wide association studies (GWAS), genomic selection, and accelerate plant breeding (He et al. 2014). The data of structural and functional genomics, together with those from proteomics, metabolomics, mapping and genotyping, will be extremely useful for linking genotype to phenotype and pull out under-exploited natural diversity that is present in the *Olea* complex and in olive germplasm, enabling olive tree scientists to develop an understanding of the genetic regulatory mechanisms of key traits of high-quality production, synthesis of functional compounds, and those involved in plant–environment interactions and improved yield, and will provide fascinating opportunity in olive breeding programs, reducing the length and number of breeding cycles, labor, and cost (van Nocker and Gardiner 2014).

2 Genome Sequencing and Assembly

Two independent projects are focused on sequencing the olive genome. The Italian OLEA project is focused on the Leccino variety (Muleo et al. 2012), while the IOGC International Consortium has sequenced and assembled the genome of wild olive tree (*O. europaea*, var. *sylvestris*) with a coverage of 246X (Unver et al. 2016).

The *O. europaea* var. *sylvestris* genome was assembled with SOAPdenovo that produced a draft genome of 1.48 Gb, with the quality of genome assembled (N50) of 228 kb into twenty-three linkage groups that were anchored 50 % of the sequences, as resulted from the association with a newly constructed genetic map. Moreover, about 50 % of the total genome assembly was composed of repetitive DNA. The number of predicted gene models is 60,214, and 36,381 of them were anchored to chromosomes. Phylogenetic studies have highlighted that the genome underwent whole genome duplication event, before speciation from sesame. The olive genome with the last species shares a high degree of synteny for a large number of blocks.

The first draft of the olive genome sequence has been recently released by researchers of another independent project focused on sequencing the genome of almost 1200-year-old olive tree of Spanish cv. Farga (Cruz et al. 2016). The authors have assembled sequence data of 155,000 fosmid clones and 543 GB of raw DNA sequence from whole genome shotgun (WGS) that were generated by a combination of illumina sequencers run on short-insert paired-end (PE) libraries. Half of the 13,038 scaffolds (N50) were larger than 443.1 kb, and the final genome assembly of scaffolds indicated a total length of 1.31 Gb (95 % of 1.38 Gb estimated genome size), and the C-value with a median at 1.59 pg. These results confirm the existence of notable variation in the repetitive fraction of the genome for the species. The pipeline CEGMA estimated a genome completeness of about 98.79 %, and the heterozygous ratio identified by kmer individuals' analysis was 0.054. The number of gene-coding sequences with 56.339 predicted unique proteins generated from genome annotation was also supported by RNA sequencing from leaf, root, and fruit tissues at various stages. The higher number of proteins compared to closely related *Erynthranthe guttata* (24,373 predicted proteins) is consistent with the putative event of genome duplication in *O. europaea*. In this species, the chromosomal number is almost the double ($2n = 46$) than that found in *Sesamum indicum* ($2n = 26$) by Zhang and co-workers (2013), and *E. guttata* ($2n = 28$) by Fishman and co-workers (2014).

The olive genome of the cultivated variety Leccino is being sequenced, by the Olive Genome Project (OLEA) (http://www.oleagenome.org), using a combination of NGS Technologies and a combination of assembly approaches. The WGS approach to assemble the genome is being pursued using Illumina and 454-sequencing with a combination of long single reads, paired-end reads, and mate pairs until a coverage of at least 40 genome equivalents is reached. The assembly is being performed using Abyss and CLC assemblers. A bacterial artificial

chromosome (BAC) pooling approach is being used to sequence random pools of 384 BACs using Illumina paired-end reads. A BAC coverage of approximately 3–4 genome equivalents is going to be sequenced, with each BAC pool sequenced at least at a 50X coverage. The advantages of the BAC approach are of two types: on the one hand, each BAC pool is much smaller in size than in the total genome size, reducing the assembly complexity. On the other hand, within each BAC pool we should not face the problem posed by sequence heterozygosity among maternally and paternally derived genomes that strongly affects WGS approaches and that is particularly challenging in the olive genome. The advantage of the WGS approach is the much more complete and homogeneous coverage of the entire genome. The two assemblies produced, the WGS and the pooled BAC assembly, will therefore be combined using a proprietary algorithm (GAM) to produce a consensus assembly. The consensus assembly will finally be anchored to the genetic map through the use of high-throughput genotyping technologies.

As of today, all the data needed for the WGS component have been produced. Gbp of Illumina sequence data was approximately produced, corresponding to a nominal coverage of 60X of the genome of cv Leccino. The Illumina sequences were obtained from two paired-end libraries with 500–600-bp inserts that were sequenced on the Illumina Genome Analyzer IIx producing 150-bp reads for a total coverage of 43X (65 Gbp) and from one paired-end library with 1000-bp inserts that was sequenced on the Illumina HiSeq 2000 system producing 100-bp reads for the remaining 17X coverage (25 Gbp). Finally, two mate-pair libraries with 3-kbp inserts were constructed and sequenced on the HiSeq 2000 to produce 100-bp reads and to reach a coverage of 4 genome equivalents (6 Gbp).

Eighteen Gbp of Roche-454 sequence data was approximately produced, corresponding to 12X coverage approximately. Twelve Gbp was obtained as long single reads of which approximately one-third was 400-bp-long reads (FLX TITANIUM technology) and two-thirds were 700-bp-long reads (FLX XL PLUS technology). Additionally, 6.2 Gbp of sequence data was obtained as paired-end reads from three libraries with 3-kbp inserts (3.8 Gbp) and 10 libraries with 8-kbp inserts (4.4 Gbp).

The 454 single reds and the Illumina paired-end reads are being used in a traditional WGS assembly. The Illumina mate-pair and the 454 paired-end sequences (i.e., all those sequences that have been obtained from inserts of larger size) will be utilized in order to scaffold into larger assemblies than the contigs obtained from the assembly of reads from the shorter inserts, with the aim to try to overcome the assembly problems posed by the occurrence of repetitive elements. Since many of the transposable elements in plant genomes are larger than 3 kbp, the larger inserts are going to be of crucial importance.

A number of assemblies were performed to test different strategies and to obtain a first rough draft of the olive genome. We tested assemblies both using the Illumina data only, as well as using Illumina and 454 data. All datasets have been initially filtered for low-quality sequences and for chloroplast DNA contamination and then were subject to assembly using the CLCBio assembler. When only the Illumina data were used (53X coverage after filtering), we produced an assembly of total size of 1.1 Gbp and N50 size of 1.7 kbp. The scaffolding using the mate-pair and paired-end information on the same assembly using the SSPACE tool increased the N50 size to 2.3 kbp. The addition of an initial set of 454 data (3.5 genome equivalents after filtering, single reads only) increased the total assembly size to 1.5 Gbp and the N50 size of contigs and scaffolds to 2.8 and 3.7 kbp, respectively. Finally, the addition of the remaining 454 sequences from the large insert libraries (3- and 8-kbp inserts) greatly improved the assembly, increasing considerably the N50 size of the scaffolds. The restriction to scaffolds of minimum 500 bp long the final assembly is 1.4 Gb long, and the N50 size of scaffolds is increased up to 10 kbp. However, due to the problems posed by the high levels of sequence heterozygosity present in the genome of cultivar Leccino, we consider the sequencing of the BAC

pools a necessary component of our strategy in order to obtain a satisfactory assembly.

A large insert library (>100 kbp) of BAC clones was obtained from cultivar Leccino. 43,008 BAC clones were pooled into 112 plates of 384 BAC clones each. Eleven pools were initially sequenced with both Illumina Hiseq 2000 and Illumina Miseq, 100-bp and 250-bp paired-end, respectively, for a total of 60 Gbp. Reads were de novo assembled, and a total of 350 Mbp with N50 ranging in the 11 pools from 10 kbp to 21 kbp was produced, and some BACs were fully reconstructed (>100 kbp).

In order to evaluate the level of polymorphism in the *Olea* genome, we aligned the reads produced within the WGS approach on the assembled BAC pools. For each of the 11 pools, on single-copy regions, we detected SNPs. An high-frequency of SNP was found, detecting one SNP every 30 to 40 bp, proving a very high level of heterozygosity in this species. The degree of heterozygous in olive is comparable to that of the most heterozygous species, classifying it among complex genomes, such as Ciona savignyi (Small et al. 2007), Branchiostoma floridae (Putnam et al. 2008), and Strongylocentrotus purpuratus (Sea Urchin Genome Sequencing Consortium 2006). Further resequencing of different varieties is in progress and might reveal an even higher level of polymorphism within the *Olea* genome.

The International Consortium IOGC has used SOAPdenon method to assembly the genome, which generated a draft genome of 1.48 Gb. The dimension of the assembled genome resulted to be near to the estimated dimension of ~1.46 Gb. The researchers were able to anchor 50 % of sequences into 23 linkage groups, by using a constructed genetic map; the sequences have included 572 Mb. About 50 % of the total genome assembly was found to be composed of repetitive DNA. Transposable elements and interspersed repeats occupied 47 % of the genome. Phylogenetic and synteny analysis, and whole genome duplication analyses highlighted that the olive genome underwent duplication, before the event of speciation from sesame.

3 Analysis of the Repetitive Component and Olive Genome Composition

Some of the biggest technical challenges in sequencing eukaryotic genomes are caused by repetitive DNA (Faino and Thomma 2014): that is, sequences that are similar or identical to sequences elsewhere in the genome. An initial assembly of olive Illumina and 454 reads using RepeatExplorer (Novák et al. 2010) clearly showed five major clusters corresponding to five repeat families containing tandem repeats (Barghini et al. 2014). The repeat unit of four of these families (Oe80, Oe86, Oe178, and Oe218) were already identified as tandem repeats, isolated from genomic libraries, and, in some instances, localized by cytological hybridization on olive chromosomes (Katsiotis et al. 1998; Minelli et al. 2000; Lorite et al. 2001; Contento et al. 2002). The remaining family (Oe179) and a sixth minor family (Oe51) were also identified as tandem repeats. Besides clusters of tandem repeats, a number of minor clusters related mostly to *Gypsy* and *Copia* long-terminal-repeat (LTR) retrotransposons (REs) were identified (Fig. 1).

Then, a de novo assembly procedure was used to produce a large set of genomic sequences from Illumina and 454 reads (Barghini et al. 2014). The resulting whole genome set of assembled sequences (WGSAS) was composed of 210,068 sequences. Because of the relatively low genome coverage of the sequencing, most of the contigs that were obtained by both methods do not represent specific genomic loci; instead, they are probably composed of reads derived from multiple copies of repetitive elements, thus representing consensus sequences of genomic repeats (Novák et al. 2010). Although the exact form of this consensus does not necessarily occur in the genome, this representation of repetitive elements has been shown to be sufficiently accurate to enable amplification of the whole-length repetitive elements using PCR (Swaminathan et al. 2007). Moreover, the comparison with an available sequence library obtained by Sanger sequencing indicated a good correspondence

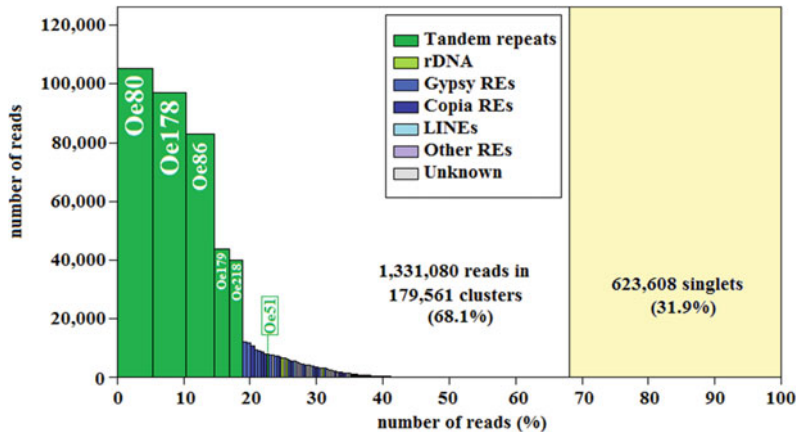

Fig. 1 Repeat abundance based on one genome equivalent of Illumina reads clustered using RepeatExplorer (Novák et al. 2010). Each bar in the histograms shows the individual size (height) of each cluster and the size relative to the total (width). The composition of each cluster is indicated by color, and single-copy, unclustered sequences are reflected to the right of the vertical bar. For the most redundant clusters, the annotation is reported within the bar (Barghini et al. 2014)

between virtual and real sequences (Barghini et al. 2014).

Assuming that Illumina sequence reads were sampled without bias for particular sequence types, mapping Illumina reads onto the WGSAS provided a method for estimating the redundancy of any genomic sequence in the dataset (Swaminathan et al. 2007; Tenaillon et al. 2011; Natali et al. 2013). All contigs with estimated redundancy higher than 100X (83,324 sequences) were selected and annotated to produce a collection of olive repeated sequences, hereafter called OLEAREP (Barghini et al. 2014).

The frequency distribution of different sequence types in OLEAREP is reported in Fig. 2, in which the dataset was further subdivided into two fractions, according to their average coverage, highly repeated (HR, average coverage >16,200), and medium repeated (MR, average coverage ranging between 16.2 and 16,200). Concerning the HR fraction, tandem repeats were the largest component, accounting around 2/3 of these contigs (Fig. 2). LTR-REs were also represented in the HR fraction, with *Gypsy* REs being more abundant in this fraction than *Copia* ones. Other classes of repeats (DNA transposons, rDNA, and putative genes) accounted only for minimal portions of HR set.

By converse, the MR fraction was mainly composed of LTR-REs (66.1 %), with *Gypsy* and *Copia* REs showing similar percentages (Fig. 2). Non-LTR-REs were poorly represented, as frequently observed in plant genomes. Putative DNA transposons accounted for 9.65 % of the MR fraction. All types of plant DNA transposons were found. Putative hAT and Mutator elements were by far the most redundant in this class, followed by putative Helitrons and CACTA elements. Tandem repeats were much less represented in this genome fraction than in HR.

Olive genome composition was estimated by counting the number of reads that mapped to each sequence. The percentage of HR sequences in the *Olea* genome was very high, amounting to 38.62 % at least. MR sequences accounted at least for 34.16 % of the genome, and low- or single-copy sequences represented only 16.92 % of the olive genome.

Olive genome composition was estimated also in terms of repeat types. The frequencies of each repeat type are reported in Table 1. Tandem repeat sequences (excluding rDNA) accounted for 31.16 % of the reads matching the WGSAS. LTR-REs amounted to 38.84 %, with *Gypsy* elements prevailing over *Copia* ones. DNA

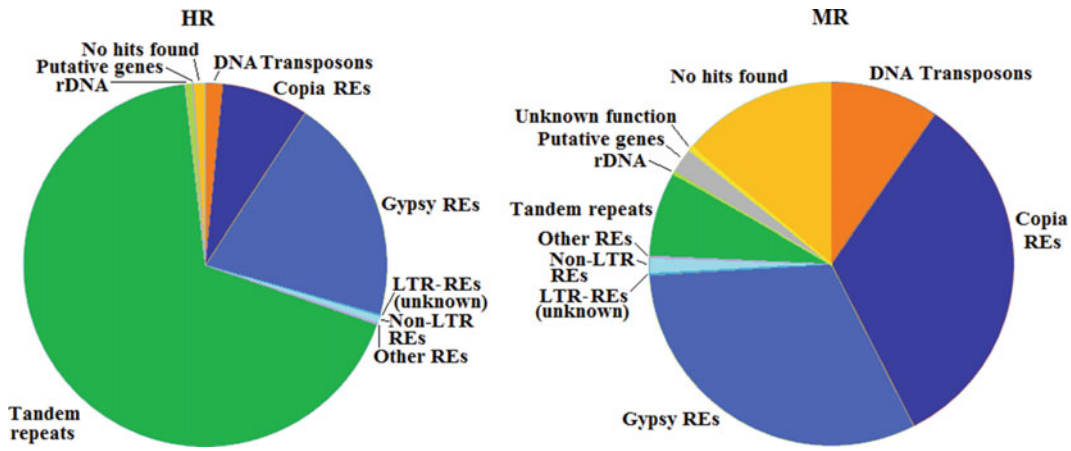

Fig. 2 Sequence composition of the OLEAREP database (HR and MR sequences) (Barghini et al. 2014)

transposons and non-LTR-REs showed low percentages.

On the whole, the OLEAREP database gives a precise characterization of the repetitive component of the *O. europaea* genome. It includes all already known olive repetitive sequences but also new, unknown sequences with high redundancy, which might represent new repeats to be still identified and characterized.

3.1 Analysis of Tandem Repeats

The large fraction of genome formed by tandem repeats is a peculiar feature of the olive genome. In many studies on genome assembly, tandem repeats are preliminarily removed, representing a negligible fraction of the genome and facilitating the assembly procedure (see e.g., for the sunflower genome, Staton et al. 2012). Until today, the largest fraction of tandem repeats found in a plant genome was estimated at around 23 % in the genome of cucumber (Huang et al. 2009).

Olive tandem repeats belong to six major families, defined according to their sequence and length. The first three families (Oe80, Oe178, and Oe86) correspond to the OeTaq80, OeTaq178, and OeGEM86 families described by Bitonti et al. (1999) and by Minelli and co-workers (2000) and account for about 72 % of tandem repeats. The fourth family (Oe179) was for the first time identified in this survey: It represents 12.6 % of the tandem repeats and the most common repeat unit is 179 bp in length; within this family, a number of repeats were truncated, with a variable length. In some cases, truncated elements were also arranged in repeat arrays, suggesting that the truncation has occurred while Oe179 was still replicating, with the truncated units that have continued their amplification.

The fifth family is Oe218, already described by Katsiotis and co-workers (1998), and accounting for 12.3 % of tandem repeats. The sixth major family was observed for the first time in this survey, representing only 2.2 % of the tandem repeats; the repeat unit is 51 bp.

Oe80, Oe178, and Oe218 constitute GC-rich, heavy satellites, having a GC content of 45.4, 43.2, and 41.8 %, respectively. By converse, Oe51 has a GC content of 33.5 %, constituting a light satellite. The GC contents of Oe86 and Oe179 (36.0 for each type) are similar to the mean GC content. All repeat families are present in multiple distinct contigs, indicating that distinct subtypes and higher-order structures of these sequences are present in the olive genome.

A distance tree, constructed using 100 sequences for each of the six repeat types, showed low sequence similarity among major tandem repeat families, suggesting an independent origin from each other (Fig. 3).

Table 1 Percentage distribution of repeat classes in the olive genome

Sequence type	Order	Superfamily	Number of contigs	Number of matched reads	Percentage
Retrotransposons	Unclassified		42	34,017	0.025
(Class I)	LTR	*Copia*	54,110	24,725,640	17.821
		Gypsy	47,920	28,884,342	20.819
		Retrovirus	101	74,960	0.054
		Endogenous retrovirus	4	6314	0.005
		Solo-LTR	52	18,355	0.013
		Unknown	189	174,016	0.125
	LINE	L1	2384	1,739,119	1.253
		RTE	453	123,845	0.089
		Unknown	38	20,591	0.015
	SINE	tRNA	268	64,093	0.046
	Total				40.265
DNA transposons (Class II)	Unclassified		67	32,668	0.024
	Subclass I	Tc1-Mariner	217	74,711	0.054
		hAT	7187	2,784,674	2.007
		Mutator	5790	3,335,678	2.404
		PiggyBac	1	34	0.000
		PIF-Harbinger	754	250,771	0.181
		CACTA	1212	496,957	0.358
		Crypton	7	2054	0.001
	Subclass II	Helitron	1297	672,682	0.485
	Total				5.514
Tandem repeats			11,260	43,233,770	31.161
rDNA			356	1,932,081	1.393
Unknown			308	179,225	0.129
No hits found			74,292	14,584,090	10.512
Total reads excluding organellar ones		138,741,954			

The measurement of the nucleotide diversity (the number of nucleotide substitutions per site), of each tandem repeat family, has shown that Oe218 is the most variable, followed by Oe178, and Oe80. Minor variations were observed within the families Oe179, Oe86, and Oe51. Actually, it is known that tandem repeats are characterized by large instability, depending on the repeat unit length, on the purity (i.e., similarity) of repeats, on the base composition, on external factors such as biotic and abiotic stresses (Gemayel et al. 2012). Moreover, the mutation rate in tandem repeats is estimated between 10^{-3} and 10^{-6} per cellular generation (Verstrepen et al. 2005). Such a high mutation rate should be related to the hypermethylation of these sequences (Hu et al. 2012).

It is hypothesized that the tandem repeats have a role in the genome. Beside their structural role in participating in centromeres and telomeres (Gemayel et al. 2012), tandem repeats can accumulate and generate intercalary heterochromatic regions. For example, in maize, tandem repeats form chromosomal knobs that reduce

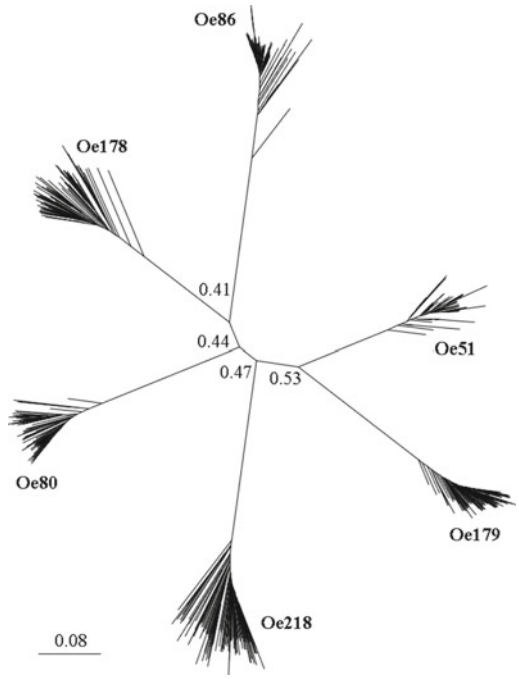

Fig. 3 Distance tree of olive tandem repeats (100 sequences per family); bootstrap values higher than 0.4 are shown. *Bar* represents the nucleotide distance (Barghini et al. 2014)

recombination rate in adjacent regions (Ghaffari et al. 2013).

In conclusion, our findings on olive genome evidenced the peculiarity of genome evolution in this species, with a very large fraction of the genome produced by tandem repeats amplification. The occurrence of a large and highly variable germplasm for this species will allow to explore genetic variability concerning this genome fraction, possibly enabling to clarify the mechanisms by which such sequences have been produced and maintained during evolution and their function.

3.2 Analysis of LTR Retrotransposons

Olive retrotransposon fragments were isolated and sequenced (Stergiou et al. 2002; Natali et al. 2007). However, the identification and accurate characterization of LTR-REs require the availability of sequences that span element length. In the frame of the project aimed to sequence the olive genome, a number of BAC clones were sequenced. These sequences were scored to identify full-length LTR-REs, searching for structural features and sequence similarities, i.e., the occurrence of two relatively intact LTRs, of identified polypurine tracts and primer-binding-sites, and of flanking tandem-site-duplications, and allowing the first characterization of intact elements in olive.

A set of 254 putative full-length REs was isolated (Barghini et al. 2015). The majority of isolated full-length REs belonged to the *Copia* superfamily (166), followed by the *Gypsy* superfamily (81, of which 36 contained an integrase chromodomain). Only seven REs remained unclassified.

In angiosperms, *Gypsy* and *Copia* superfamilies are differently represented in the genomes. Different ratios between *Gypsy* and *Copia* RE frequencies were reported ranging from 5:1 in papaya to 1:2 in grapevine (Vitte et al. 2014). Analysis of the whole olive genome showed a ratio of 1.17:1 (Barghini et al. 2014). The isolated olive full-length REs showed on the contrary a prevalence of *Copia* over *Gypsy* elements, indicating that the number of *Gypsy* families is lower than that of *Copia*, but *Gypsy* REs are more abundant than *Copia* REs (Barghini et al. 2015).

The relatively low frequency of REs in the olive genome could be related to a low rate of retrotransposition, but also to RE loss (Ma et al. 2004). RE DNA removal is driven in plants by a number of mechanisms, including DNA rearrangements and unequal homologous recombination; solo-LTRs are the main products of such processes (Ma and Bennetzen 2004).

Analyzing the relative redundancy of LTRs and inter-LTR regions in one and the same full-length RE was performed for evaluating the occurrence of solo-LTRs related (i.e, belonging to the same family) to that RE. Solo-LTRs related to the isolated full-length REs were rare: only 16 out of 254 REs showed a ratio between the number of mapped reads per Kb of LTR and inter-LTR >2.5. These ratios were especially high for two *Gypsy* and two *Copia* elements, indicating the occurrence of a large number of

solo-LTRs for RE families that are related to these full-length elements (Barghini et al. 2015).

Concerning the amplification of REs, the identification of sister LTRs allowed us to date the insertion of REs in the olive genome, using the method established by San Miguel and co-workers (1998) in maize. Intact retroelements have a built-in molecular clock that is useful for estimating their insertion times, based on sister LTR divergence. In fact, when an RE inserts into the genome, its LTRs are usually 100 % identical (Kumar and Bennetzen 1999). Mutations then occur within the two LTRs, and as more time passes since the insertion, the larger the genetic distance between LTRs becomes. Hence, the RE insertion time can be estimated using a nucleotide substitution rate suitable for such elements (Ma and Bennetzen 2004).

Using a substitution rate per year of 3.6×10^{-9}, calculated comparing orthologous genes of olive and ash trees, the putative insertion times were calculated for each full-length LTR-RE. The putative mean age of analyzed LTR-REs was 17.94 MY (Barghini et al. 2015). Analysis of sister LTR similarity indicates that, in olive, both *Gypsy* and *Copia* REs have been active in the same period. Nearly all the identified full-length elements appear to be mobilized in a time-span of 40 MY (Fig. 4).

The mean insertion date of olive *Copia* full-length REs is lower than that of *Gypsy*. The insertion date profiles indicate that, during the last 40 MY, *Copia* and *Gypsy* REs have both been active, but with different time-courses. For example, only one isolated *Gypsy* full-length RE inserted between 1 and 8 MY ago. Moreover, the percentage of *Gypsy* REs inserted between 10 and 25 MY ago; hence, presumably, their retrotransposition activity is by far larger than that of *Copia* elements.

In contrast to other species, such as maize (Brunner et al. 2005) and sunflower (Buti et al. 2011) in which the retrotransposon burst is very recent and probably still occurring, in the olive genome the insertion of new REs for both *Gypsy* and *Copia* REs appears to be decreasing in frequency in the last 8 MY. A similar time-course of the RE amplification wave was reported in the genome of a gymnosperm, the Norway spruce (Nystedt et al. 2013). The observation of a considerable number of elements that have inserted more than 10 MY ago represents a clear

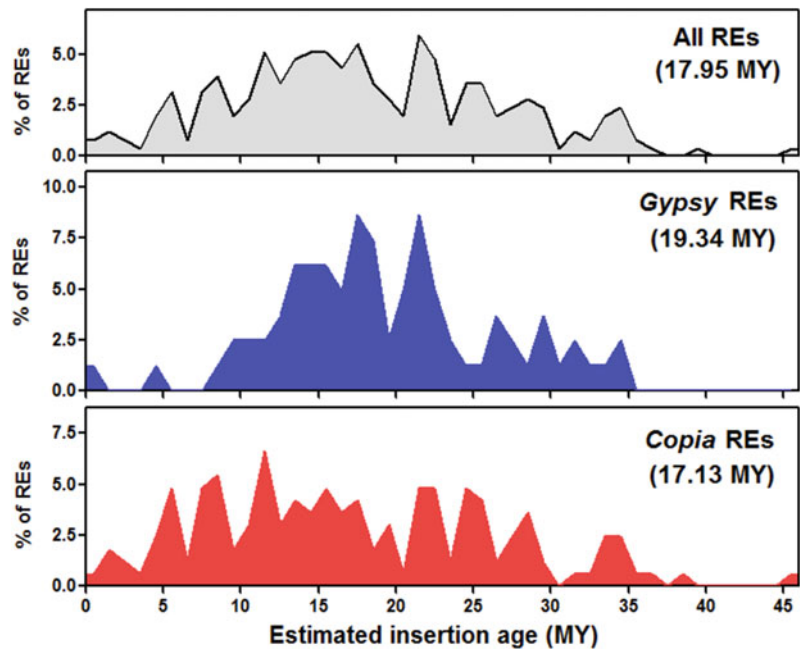

Fig. 4 Distributions of full-length REs of *Olea europaea*, according to their estimated insertion ages (MY). Mean insertion dates are reported in parentheses (Barghini et al. 2015)

distinctive feature of the olive genome in comparison with other angiosperm genomes analyzed so far.

4 Olive Chloroplast Genome

The chloroplast genome of the olive is already available from two independent groups (Mariotti et al. 2010; Besnard et al. 2011), with 155,889 bp in length. This genome has an organization and gene order conserved among numerous Angiosperm species and do not contain any of the inversions, gene duplications, insertions, inverted repeat expansions and gene/intron losses that have been found in the chloroplast genomes of the genera *Jasminum* and *Menodora*, from the same family as *Olea* (Mariotti et al. 2010). Forty polymorphisms have been identified in the plastome sequence, leading to a low number of chlorotypes distinguishing the olive cultivars.

5 Transcriptomics and Proteomics in Olive

Over the past several years, in olive, classical breeding programs have been focused on selecting for traits as short juvenile period, plant architecture suited for mechanical harvest, or oil characteristics, including fatty acid composition, phenolic, and volatile compounds to suit new markets. However, a better understanding of the genomic organization and the development of suitable molecular tools are mandatory steps to improve the efficiency of such breeding programmes. Nonetheless, transcriptomic data are already available for some of the olive genes involved in specific traits, such as fruit ripening, growth, and juvenile-phase transition. In the following paragraphs, we will focus on the gene expression studies performed so far by using high-throughput transcriptome sequencing technologies. Such transcriptomic approaches allowed to select a number of candidate genes that affect olive biology.

5.1 Flower and Fruit Development

In olive, floral biology has important practical implications, in addition to its scientific relevance, given that flower features and bloom affect fruits and yield. Furthermore, fruit development is the result of genetically programmed processes and environmental cues. High-throughput transcriptomics represent a key step for understanding the regulatory networks underlying plant reproduction and fruit growth and ripening.

Olive is a wind-pollinated, andromonoecious species whose cluster inflorescences are paniculate and whose flower position on the inflorescence may affect its development and fertility (Ben et al. 2013). Complex molecular and cellular processes are required for the development of reproductive tissues and the sculpting of the final form of the different organs (Irish 2010). A single tree may produce as many as 500,000 flowers, but only a small percentage of them (1–2 %) may set fruits due to several factors, such as wind pollination syndrome, flower development defects (i.e., ovary abortion), plant reproductive barriers (i.e, self-incompatibility and male sterility), and competition for maternal nutritional resources (Lavee et al. 1999; Rosati et al. 2011). The physiological changes that occur throughout flower development in olive have been investigated in the cultivar (cv.) Leccino at pre-anthesis and anthesis stages (Alagna et al. 2016). Analysis of the transcriptomic data generated by 454 sequencing (Table 2) revealed that among the flower transcripts, a large number of genes involved in the modification and degradation of proteins represented the substantial changes that occur during the development of flower verticils, including reproductive organs. Transcripts involved in cell-wall remodeling and polyamine biosynthesis were found upregulated

at anthesis, while phenylpropanoid and flavonoid biosynthesis-related transcripts were downregulated during flower development. Finally, several genes involved in carbohydrates, lipid metabolism, transport, and cellular component organization were found more expressed at later stages of flower development (Alagna et al. 2016).

Some olive cv are characterized by a high number of male flowers, due to a high rate of ovary abortion and pistil desiccation (Reale et al. 2009; Rosati et al. 2011; Rapoport et al. 2012). The incidence of pistil abortion is influenced by nutritional or stress conditions (Bouranis et al. 1999; Fernández-Escobar et al. 2008), and it has been proposed that starch and sucrose metabolism might have a role in this process (Reale et al. 2009). In fact, flowers need carbohydrates to complete their differentiation, and changes in starch synthesis, degradation, and mobilization might affect the correct balance of nutrients in flower organs with consequences on the regular development of the pistils and ovary. Male sterility may also occur in olive (Cavallotti et al. 2003).

Transcripts potentially involved in the ovary abortion process were investigated by comparing cv. Leccino (low-ovary aborted flowers) and cv. Dolce Agogia (high-ovary aborted flowers) (Alagna et al. 2016). The analysis of the transcriptomic data (Table 2) identified several olive homologs of the genes involved in starch and sucrose metabolism, polyamine biosynthesis, cell-wall metabolism, programmed cell death (PCD), regulation of flavonoid biosynthesis, and MYB and MADS transcription factors (Alagna et al. 2016).

Self-incompatibility and interincompatibility represent the most important reproductive barriers in olive. In self-incompatible plants, the main recognition step is accomplished by the interaction between female and male determinants, which are usually encoded at a single polymorphic locus (*S*-locus) (Iwano and Takayama 2012). Subsequently, apposite cell signaling and cell–cell communication are fundamental for pollen acceptance and growth, requiring intricate intercellular communication between male and female cells. Pollen tube growth occurs directionally through the stigma and style to enter the ovary and is influenced by chemotropic agents, as well as a variety of lipids, ions, proteins, and metabolites that are produced by the pistil (Chapman and Goring 2010). A representative transcriptome of pollen and pistils has been just released (Carmona et al. 2015) (Table 2); cDNA libraries from pollen and pistil at different maturing and developing stages (with leaf and root as vegetative control) were 454 sequenced to provide a reproductive transcriptome and a user-friendly database (http://reprolive.eez.csic.es).

Candidate genes potentially involved in pollen–pistil interactions were identified by comparing the transcriptomes of cv. Frantoio (self-compatible) and cv. Leccino (self-incompatible) at anthesis (when the pollen grains reach the stigma upon self-pollination and potentially trigger the incompatibility reaction) (Alagna et al. 2016) (Table 2). The authors found more than 26 % of the upregulated genes in the self-incompatible cv. were involved in cell-wall degradation, whereas the upregulated genes in self-compatible cv. were mainly related to catabolic metabolism. Noteworthy, a significant number of altered transcripts belonged to hydrolase family (Alagna et al. 2016). It has been proposed that these enzymes may be involved in the remodeling of the pollen tube cell wall during its growth along the stylar transmitting tissues (Mollet et al. 2000), and in olive, they might also have a specific role in the self-incompatible interactions.

Fruit development and ripening takes place in about 4–5 months and includes the following phases: fruit set after fertilization, seed development, pit hardening, mesocarp development, and ripening. During the ripening process, fruit tissues undergo physiological and biochemical changes that include cell division and expansion, oil accumulation, metabolite storage, softening, phenol degradation, color change (due to anthocyanin accumulation in outer mesocarp cells). Several candidate genes putatively involved in olive fruit development were identified by comparative large-scale transcriptome analysis performed on fruits of cv. Coratina (high phenolic content) and cv. Tendellone (oleuropein-lacking

Table 2 An overview of the main set of transcriptome data generated from different cultivars, plant organs, and adaptive responses to stresses

Biological process	Publication	Cultivar	Tissue	Sequencing technology	Reads
Flower and fruit development	Alagna et al. (2016)	Leccino; Dolce Agogia; Frantoio	Flower (pre-anthesis, anthesis)	Pyrosequencing	465,000
	Carmona et al. (2015)		Pollen (mature, 1–5 h germination); pistil (stages 2–3–4); leaf (mature); root (mature, radicle)	Sanger	1549
				Pyrosequencing	2,077,309
	Alagna et al. 2009	Coratina; Tendellone	Fruit (45–135 days after flowering)	Pyrosequencing	261,485
	Iaria et al. (2016)	Leucocarpa; Cassanese	Fruit (100–130 days after flowering)	Illumina	103,359
	Muñoz-Merida et al. (2013)	Lechin de Sevilla; Picual; Arbequina; Picual*x*Arbequina	Fruit mesocarp (green, turning, purple); shoot (juvenile, adult, dormant); root (juvenile, adult); seed (green fruits); leaf (young)	Sanger	38,183
				Pyrosequencing	1,742,850
Fruit abscission	Gil-Amado and Gomez-Jimenez (2012)	Picual	Fruit AZ (54–217 days post-anthesis)	Pyrosequencing	443,811
	Parra et al. (2013)	Picual	Fruit pericarp and AZ tissues (217 days post-anthesis)	Pyrosequencing	397,457
Abiotic stress responses	Bazakos et al. (2015)	Kalamon	Leaf; root	Pyrosequencing	291,958
	Leyva-Perez et al. (2015)	Picual	Leaf	Illumina	149,638,888
	Guerra et al. (2015)	Leccino			75,645,229
miRNA	Donaire et al. (2011)	Picual; Arbequina	Shoot (juvenile, adult)	Pyrosequencing	169,699
	Yanik et al. (2013)	Ayvalik	Fruit (ripe, unripe); leaf ("on-year" and "off-year")	Illumina	92,823,293

natural variant), at two developmental stages: 45 and 135 days after flowering (DAFs) (Alagna et al. 2009) (Table 2). About 25 % of the annotated enzyme-coding transcripts were involved in biosynthesis of lipids and fruit metabolites. Transcript fluctuations were consistent with the physiological status of the fruit. The higher expression of transcripts related to the

biosynthesis of structural proteins at 45 DAF may be correlated with the intense and rapid cell divisions during fruit growth, while the higher expression of transcripts putatively associated with fatty acid biosynthesis and with the assembly of storage triacylglycerols at 135 DAF is in agreement with fatty acid accumulation pattern in olive fruits, starting at about 90 DAF until the end of fruit maturation (Conde et al. 2008). Among genotype-specific transcripts, several ones putatively involved in the biosynthesis of steroids with nutritional and health benefits were reported exclusively in cv. Coratina (Alagna et al. 2009).

To investigate whether these changes at the mRNA level correspond to variations at the protein level, a comparison between transcript and protein profiles was performed in Bianco and co-authors (Bianco et al. 2013). So far, this paper is the only one in the literature that monitors the proteome variations associated with olive fruit development by using comparative proteomics based on 2-DE coupled to MALDI-TOF mass spectrometry, providing new and important insights into fruit metabolism and oil accumulation process (Bianco et al. 2013).

Interestingly, comparison between transcriptomic and proteomic datasets revealed that most of the proteins and their putative transcripts associated with fatty acids biosynthesis and metabolism (enoyl ACP reductase and lipoxygenase), as well as transcripts and proteins linked to cell cycle, biosynthesis of structural proteins involved in cell expansion showed a similar increased pattern during drupe development (Bianco et al. 2013). Transcript and protein profile comparison also revealed some divergent patterns, indicative of possible post- transcriptional events in RuBisCO large subunit-binding protein subunit alpha and proteins associated with detoxification and oxidation-reduction processes (Bianco et al. 2013).

Transcripts involved in flavonoid and anthocyanin metabolism during drupe development were identified by comparing different Illumina RNA-seq libraries generated from drupes of cv. Leucocarpa (characterized by a switch-off in skin color at full ripeness) and cv. Cassanese (as control), sampled at 100 and 130 DAF (Iaria et al. 2016) (Table 2). The cv. Leucocarpa was characterized by a broad downregulation of chalcone synthase, dihydroflavonol 4-reductase, and anthocyanidin synthase transcripts compared to cv. Cassanese. Moreover, several members of MYB, MYC, and WD transcription factors related to the regulatory complexes that control anthocyanin structural genes at the transcriptional level were identified as differentially expressed (Iaria et al. 2016).

Oil synthesis starts after pit hardening, reaching a plateau after 75–90 days, while the phenolic fraction is maximum at fruit set and decreases rapidly along fruit development. To get information about genes involved in determining oil content and composition, mesocarp and seed transcriptomes from fruits of different cv (Picual and Arbequina with different characteristics regarding fruit and oil organoleptic properties) were investigated (Muñoz-Merida et al. 2013) (Table 2). To date, this paper is the largest contribution to transcript information in *Olea* (about 2 M reads) (Table 2). The assembly has rendered over 81,020 unigenes that have been functionally annotated. Interestingly, numerous transcripts are involved in lipid metabolic/biosynthetic process or lipid fatty acid metabolic/biosynthetic process and then associated with oil characteristics and production (Muñoz-Merida et al. 2013).

5.2 Fruit Abscission

Abscission and senescence are key physiological events that occur during the growth and development of fruits in higher plants. These bear commercial implications both for the plant yield and the harvest. In agricultural research, the manipulation of genes governing these phenomena is crucial to develop varieties that can produce fruits with longer shelf-life as well as crops that tolerate greater environmental stress. After fruit ripening, many fruit tree species undergo massive natural fruit abscission. Abscission occurs in an anatomically distinct layer of cells known as the abscission zone (AZ) (Gonzalez-Carranza and Roberts 2012) located between the pedicel and fruit, and the patterns of mature

fruit abscission differ between cultivars (Gomez-Jimenez et al. 2010). Olive fruit has several AZs in the pedicel, but only one AZ at a time is selectively activated per specific developmental stage (Parra-Lobato and Gomez-Jimenez 2011). Probably, the induction of abscission depends on a complex interplay of plant hormone concentrations in addition to factors that alter the responsiveness and sensitivity of the tissues (Gonzalez-Carranza and Roberts 2012). To identify differences in transcript abundance related to the mature fruit abscission in olive, 454 pyrosequencing technology was used in cv. Picual comparing AZ transcripts at two different stages: pre-abscission vs. abscission (Gil-Amado and Gomez-Jimenez 2013) (Table 2). The authors identified 70 transcription factor genes induced during mature fruit abscission in AZ. Among them, the classes that are well represented included bZIP proteins, MYB proteins, and homeobox domain proteins (Gil-Amado and Gomez-Jimenez 2013). To significantly expand the olive transcript catalog, 454 pyrosequencing technology was also used to sequence two cDNA samples from fruit pericarp and AZ, which were collected from ripe fruits, when abscission occurs (Parra et al. 2013) (Table 2). Functional categorization of the differentially expressed genes showed that AZ tissues have an apparently higher response to external stimuli than that of ripe fruit, revealing a higher expression of genes involved in auxin-signaling, lignin, aromatic amino acid, isoprenoid, amino acid dephosphorylation-transport, and photosynthesis pathways (Parra et al. 2013). By contrast, fruit-enriched transcripts are involved in ATP synthesis coupled with proton transport, glycolysis, and cell-wall organization. Regarding the cross-talk between fruit and AZ, several transcription factors were identified, especially MADS-box, ZF, homeobox domain proteins, bHLH, and bZIP families (Parra et al. 2013). This represents the first effort to elucidate the molecular bases related to the mature fruit abscission in olive, as a model to study fleshy fruit abscission. In fact, most studies identifying transcriptional regulators during organ abscission have used *Arabidopsis* (Nath et al. 2007), while regarding fruit abscission transcriptomic data are available only in apple (Botton et al. 2011).

5.3 Abiotic Stress Responses

Abiotic stresses such as salinity, drought, and cold cause a plethora of responses at the morphological, physiological, biochemical, and molecular levels which reduce yield and plant productivity. All three abiotic stresses cause a primary loss of cell water and as a result a decrease of cell osmotic potential (Duque et al. 2013). Plants have evolved highly complex mechanisms to respond and tolerate such stresses which are partly coordinated by intricate gene regulatory networks.

Although olive is a tree species well adapted to xerothermic conditions and, therefore, to environments of high temperature and long drought; the rapid expansion of olive cultivation increases the need for use of low-quality saline water for irrigation. Such water causes salt stress which negatively affects shoots growth and fruit productivity. In olive, there are salt-tolerant and salt-sensitive genotypes which differ in their ability to exclude toxic ions and to control the net salt import to the shoot.

The molecular basis of this tolerance was investigated by comparative transcriptome analysis of two olive cultivars using microarrays (Bazakos et al. 2012). Despite the limited number of probe sets, transcriptional regulatory networks were constructed for both, cv. Kalamon and cv. Chondrolia Chalkidikis, while several hierarchically clustered interacting transcription factor regulators such as JERF and bZIP were identified (Bazakos et al. 2012). The higher complexity of the cv. Kalamon transcription factor network compared to the cv. Chondrolia Chalkidikis network might be indicative of a more coordinated effort to adapt to salinity. Moreover, the comparison of the interactions among transcription factors in olive with those reported for *Arabidopsis* indicates similarities in the response of a tree species with *Arabidopsis* at

the transcriptional level under salinity stress (Bazakos et al. 2012).

A 454 pyrosequencing approach was also employed to characterize the transcriptome of leaves and roots of cv Kalamon in response to salinity (Bazakos et al. 2015) (Table 2). In roots, 24 differentially expressed clusters were identified comprising 9 down- and 15 upregulated genes, while 14 down- and 56 upregulated clusters of differentially expressed genes were identified in leaves (Bazakos et al. 2015). In addition, 433 unique transcripts encoding transcription factors were determined while the most abundant among them appeared to be senescence-associated as well as NAC domain family transcription factors which are known to be involved in salt stress responses (Bazakos et al. 2015). Transcripts implicated in salt tolerance, such as glutathione reductase, superoxide dismutase, and proline dehydrogenase, were also identified in the leaf transcriptome exposed to salinity (Bazakos et al. 2015).

In another report, transcriptome analysis of olive leaves of cv Picual during cold acclimation conditions resulted in the identification of 6309 differentially expressed transcripts (Leyva-Perez et al. 2015) (Table 2). Among them, the early response genes comprised of C-repeat binding factor transcription factors, fatty acid desaturases, wax synthesis and oligosaccharide metabolism (Leyva-Perez et al. 2015).

A RNA-Seq analysis was performed to identify in olive genes associated to cold stress response, studying short- and long-term transcriptional changes occurring in leaves of cv. Leccino exposed to a progressive lowering of temperatures until −4 °C (Guerra et al. 2015). The Illumina (Illumina Genome Analyzer IIx) sequencing approach generated 93, 927,355 pair-end reads for a total of 27.24 Gb, reduced to 75, 645,229 pair-end high-quality reads and 20.33 Gb after the trimming and filtering process. A total number of 85,752 contigs resulted and 44,332 leaf transcripts has been de novo assembled. Among them, 5464 differentially expressed genes (DEGs) were identified. Most of the typical components of the known and conserved molecular repertoire of the plant cold response have been found into the set of transcriptomic data, as transcriptions factors of cold signaling, induction of coldregulated genes (*cor*), genes involved in changes of membrane composition, and downregulation of photosynthesis-related genes (Guerra et al. 2015). Specific cold response genes of olive tree leaves, induced during cold acclimation, were identified, including genes of the glutathione cycle, polyamine and flavonoid pathways, likely to support reactive oxygen species (ROS) scavenging, as well as genes of the raffinose and trehalose carbohydrate biosynthetic pathways to sustain the accumulation of osmolytes. Moreover, genes involved in the signaling pathway of abscisic acid (ABA), synthesis of callose and lignins, indicated changes in composition of cell wall, were also strongly present (Guerra et al. 2015). The RNA-Seq data about CBF-like transcript has been confirmed trough expression profile studied by RT-PCR trials conducted in Leccino and in seven other cultivars differing for cold tolerance (Guerra et al. 2015).

The high-throughput transcriptome analyses of olive trees under abiotic stress resulted in the identification of a large number of genes involved in adaptation and tolerance, but future functional characterization will determine their physiological significance in these conditions.

5.4 Small RNAs

The microRNAs (miRNAs) are noncoding small RNA found in diverse eukaryotes, negatively regulating specific target messenger RNA (Reinhart et al. 2002). The plant miRNAs range in size from 20 to 24 bases (Dugas and Bartel 2004). They act as key regulators controlling the gene expression in a multitude of developmental and physiological processes (Pulido and Laufs 2010; Sunkar et al. 2012). So far, their involvement in developmental regulation and flowering processes has been extensively studied in a wide variety of herbaceous plant species (http://www.mirbase.org/) while the list of miRNAs from woody plants is scarce and restricted to conifers, poplar, grapevine, and citrus (Lu et al. 2008;

Morin et al. 2008; Song et al. 2009; Pantaleo et al. 2010). Recently, the first inventory of miRNAs in olive was reported (Donaire et al. 2011) (Table 2). Two distinct miRNA cDNA libraries were prepared from juvenile and adult shoots from the progeny of a genetic cross between the cv. Picual and cv. Arbequina and sequenced by deep pyrosequencing (Table 2). The vast majority of sequences (80 %) were singletons suggesting that the miRNA libraries were far from saturated and that, consequently, olive contained a large and diverse miRNA population. A hallmark signature of the olive miRNA population is the vast presence of the 24-nt species at a higher level with respect to many other plant species. Donaire and colleagues suggest an active role of heterochromatin silencing in the maintenance and integrity of the olive large genome (Donaire et al. 2011). Currently, miRNAs from about 24 broadly conserved families have been identified from eudicots to basal plants and deposited in the public miRNA database miRBase (Griffiths-Jones et al. 2008). In the olive miRNA dataset were identified 18 out of the 24 known miRNA families (Donaire et al. 2011).

Regulation of miRNA has a significant impact on the olive tree alternate bearing (Yanik et al. 2013). Alternate bearing is a common phenomenon among crop plants, defined as the tendency of certain fruit trees to produce a high-yield crop one year ("on-year"), followed by a low yield or even no crop the following year ("off-year"). Thus, this phenomenon may severely affect the olive fruit yield. Several miRNAs related to the alternate bearing were identified in a study performed in Yanik et al. 2013. In this work, six miRNA libraries were constructed from fruits (ripe and unripe) and leaves ("on-year" and "off-year" in July and in November, respectively) (Table 2). About 15,587,819 reads from each library were generated with the high-throughput Illumina sequencing system (Yanik et al. 2013). Predicted targets of miRNA were categorized into 108 process ontology groups with significant abundance. Among those, several alternate bearing-associated processes were found, such as development, hormone-mediated signaling, and organ morphogenesis.

Deeper sequencing or even alternative sequencing platforms could give better resolution in the olive small RNA population, therefore unraveling more miRNA.

6 Conclusions

With the availability of highly assembled genome sequence, the research activities will be focused on the challenge to translate the decoded genome into new tools that can be implemented by olive biologists and tree breeders for variety improvement. High-quality genome assembly greatly facilitates this task by enabling the complete inventory of DNA variation in olive species, including copy number variations, single-nucleotide mutations (insertions and deletions), epigenetic variations, such as DNA methylation, smaller RNA, and mobile elements. The next-generation sequencing (NGS) technology (Metzker 2010), which enables the rapid generation of a massive amount of sequencing data with a limited low cost, gives the opportunity to sequence whole genome and DNA variant identification of cultivated varieties and wild species. The capacity of sequencing large DNA fragments of several kbps, of increasing the proportion of the assembly anchored to genetic maps, assembling larger haplotyped scaffolds by new molecular and bioinformatics procedures will improve the current genome assembly in the near future.

The transcriptomic approaches discussed clearly demonstrate that the catalog of olive transcripts has been significantly expanded in recent years (Table 2), providing several answers to the various biological questions affecting the olive biology. Large datasets of transcriptomic sequences including miRNAs, mainly generated by pyrosequencing, have recently been reported for several tissues (Fig. 5a, b). However, genomic information in olive is well behind other species of woody plants, such as grape (Velasco et al. 2007) or poplar (Tuskan et al. 2006) whose complete genome sequences are already available. The lack of a complete and annotated

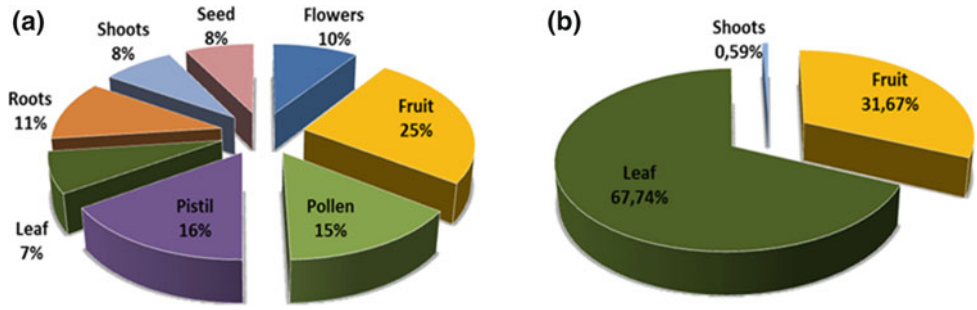

Fig. 5 **a** Pie-sectioned representation of transcriptomic reads generated for olive tissues (Illumina reads of leaf are not included). **b** Pie-sectioned representation of miRNA reads generated for olive tissues

genome makes impossible a deep and detailed interpretation of data already available in olive.

Recently, the advancements on sequencing technology have been impressive. The newer generation of sequencing methods based on single-molecule sequencing and in situ sequencing (to read nucleic acid composition directly in fixed cells and tissues), allows to obtain a great number of reads of several Mb in length, no GC bias, and high read accuracy at lower costs (Buermans and den Dunnen 2014). By applying these emerging sequencing technologies, the amount of genomic information will become accessible in a shorter time, allowing the easier sequencing and resequencing of the olive genome. All this will make easier to identify new gene functions and new molecular markers involved in the expression of fundamental agronomic and productive traits affecting olive biology, opening the possibility of developing molecular tools to the level currently available for other model plant species.

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Olive Breeding with Classical and Modern Approaches

10

Eddo Rugini and Ciro De Pace

Abstract

Olive breeding aims to the adoption of a fast-track breeding methodology to rapidly identify and select *ortets* within the available gene pool or in progenies from planned mating design for the development of new varieties that meet the current objectives of the olive industry. Basic information is needed on the breeding objectives, the genetic basis of the desired traits, the selection criteria to be adopted, and the genetic diversity available for trait enhancement and new varieties needed by the current and future olive farmers. The available genetic diversity is not yet well organized according to the gene pool concept that greatly facilitates the choice of breeding materials and breeding procedure to adopt. In addition, despite recent significant efforts, the progress of knowledge on single-locus traits and QTLs is still limited, placing the efficiency of olive breeding at a crossroad. To overcome this important limiting factor, the current selection activities could be merged with the biotechnological advancements to formulate a faster trait-enhancement procedure based on cloning and genotyping of immature embryos from planned mating designs. Developments in DNA sequencing will now allow a cost-efficient increase of genomic resources for driving the rapid acquisition of information on genes for important economical and agronomical olive traits. The in vitro germination of immature zygotic embryos, zygotic embryo cloning, and application of modern genomic resources will set the stage for an accelerated olive breeding procedure.

1 Introduction

The olive industry in the Mediterranean Basin has ancient roots and contributes about 80 % to the world olive production.

The olive orchards in the Mediterranean area were established for extensive cultivation under

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the rainfed condition, where plant density was low to maintain the crop with large plants for several decades. Those plants, often located in steeply sloping areas, are characterized by low crop capacity and alternate bearing. In addition, a vase shape is given to the tree foliage which is not suitable for harvesting and pruning mechanization, and the income for the land owners is normally very low according to the current international market oil prize.

In the last thirty years, the taste and health properties of olive oil have been appreciated in several parts of the world including Japan, USA, Australia, China, South America, and South Africa. Farmers in those countries decided then to introduce the olive crop adopting higher plant density and drip irrigation. In those areas, it is also possible to produce easily organic olive oil for the absence of the major pests and diseases present in the traditional olive growing countries. In addition, farmers are not obligated, as in Europe, to maintain the traditional olive cropping system for its landscape functions and may devote efforts to increase the quality of the extraction of olive oil and increase their income.

The traditional areas of olive cultivation in the European Union is now managed under the measure of 'environmental conditionality' to increase the link between agriculture and territory, and create favorable conditions for the mutual benefit of farmers in rural areas and consumers.

The measure aims to the promotion of agricultural production methods that reduce the environmental impacts and encourage the conservation of natural habitats and biodiversity of the agricultural landscape exerting also an ecological and hydrogeological defense of the territory. In those areas, hundreds of varieties are present in small farmers' fields although a few dozen is in cultivation in large farms. Some of those such as the Spanish varieties 'Arbequina,' 'Arbosana,' 'Sikitita,' and 'Oliana' (Bellini et al. 2008), the Greek var. 'Koroneiki,' the Italian varieties 'FS17' ('Favolosa') and 'Don Carlo' (Fontanazza et al. 1998), and the Israelian var. 'Askal' (Lavee et al. 2003) are suitable for modern olive cropping system. Those systems are intensive and super-intensive, with 250–400 and 900–1200 plants per hectare, respectively, with the canopy suitable for mechanical pruning and harvesting.

Very few varieties, such as 'Leccino,' maintain stable cropping performance and oil quality in different environments. Therefore, a breeding activity leading to new clonal varieties with possibly larger adaptation, good agronomic and stress tolerance performance, environmental stability of oil quality, and with canopy of reduced size suitable for mechanical pruning and harvesting, obtainable also with grafting on dwarfing rootstocks, are the variety traits that farmers like to find for converting their current crop area to olive plantation.

The renewal of varieties has been hampered by the extreme longevity of olive trees, the long period of juvenility of their offspring, the deference that man had for this plant, and recently also the diffidence of the public to accept genotypes obtained with advanced biotechnological approaches.

In this chapter, the genetic basis of olive traits (Sect. 2), the selection criteria (Sect. 3), the available genetic diversity (Sect. 4), and the conventional and biotechnological approaches (Sect. 5) are considered to evidence the main efforts carried out to breed new olive varieties.

2 Approaches for Detecting the Genetic Basis of Traits to Be Enhanced and to Measure the Available Genetic Variability for Breeding

The genetic complexity of any given species is accounted by the size of its nuclear DNA. Olive is a diploid species having 46 chromosomes ($2n = 2x = 46$) and the nuclear DNA content of olive varieties was determined for the first time by Rugini et al. (1996). Feulgen cytophotometric analyses indicated a mean (2C) nuclear DNA content of var. 'Frantoio' and var. 'Leccino' of 2.26 and 2.20 pg of DNA per haploid nucleus, respectively (Rugini et al. 1996; Bitonti et al. 1999; Loureiro et al. 2007).

The genome sizes of the wild species *Olea europaea africana, Opuntia ficus-indica,* and *Olea ferruginea* were much lower (1.6–1.85 pg) than in the cultivated olive varieties (1.95–2.35 pg) (Bitonti et al. 1999).

Besnard et al. (2008) found a 2C value = 7.88 ± 0.19 pg in ssp. *maroccana* and 5.52 ± 0.28 pg in ssp. *cerasiformis*; the populations of the other four subspecies displayed a 2C value ranging from 2.93 to 3.75 pg. The estimated 1C genome size (Mbp) ranged from 1450 to 1558 Mbp (Dolezel et al. 2003).

Based on the flow cytometry and genetic analyses, strong evidence for polyploidy was obtained in ssp. *cerasiformis* (tetraploid) and ssp. *maroccana* (hexaploid), whereas the other subspecies appeared to be diploids (Besnard et al. 2008).

A methodology for isolating triploid and tetraploid olive genotypes was developed by Rugini et al. (1996). The polyploids were isolated from two mixoploid somatic mutants obtained earlier by treating 'Frantoio' and 'Leccino' plantlets with gamma irradiation.

The variation in the nuclear DNA content of the mixoploid mutants was closely correlated with the variation in their pollen size, crop capacity, and the production of large fruit. The mixoploid mutants produced a mixture of normal drupes and some abnormally large ones, almost twice normal size. Triploid genotypes with 69 chromosomes were isolated by germinating the seeds of these large fruits, collected from both the mixoploid mutants. Tetraploid plantlets, with 92 chromosomes, were obtained from 'Frantoio' and 'Leccino' by selecting in vitro, during several proliferation phases of the mixoploid shoots, those shoots with ovate leaf shape which occurred among the shoots with normal lanceolate or intermediate leaf shape. The shoots with normal lanceolate or intermediate leaf shape were diploid.

Usually, traits with discrete phenotypic classes express Mendelian inheritance. However, recognition of traits with Mendelian inheritance in olive has been problematic although several fruit traits express discrete phenotypic classes (Bartolini et al. 2006). Many other fruit traits (i.e., dry matter in fruit flesh) display continuous variation and quantitative genetic inheritance. Narrow sense heritability (h^2_N) for fruit traits ranged between 0.17 (flesh and stone weight ratio) and 0.36 (percentage of dry matter in fruit flesh) (Zeinanloo et al. 2009), while broad sense heritability was high (>0.81) for all the studied fruit traits. The narrow sense heritability for fruit size components (fruit width, $h^2_N = 0.22$; fruit length, $h^2_N = 0.25$) was low compared to the broad sense heritability for similar traits estimated by Padula et al. (2008). A large set of genes involved in olive flower development has been identified by Alagna et al. (2016).

3 The Breeding Objectives and Selection Criteria

The primary olive breeding objectives include: shortening the unproductive period (juvenility), flowering earliness and flowers on moderate density clusters with abundant pollen load and tendency to anemophily in order to set fruits in dense detachable bunches, and reduce dependence on pollinators for bearing fruits. The olive tree should also be easy to propagate and resistant to abiotic and biotic stresses, and provide a high and constant crop of fruits every year (Rallo 2014a). Early bearing, high cropping, resistance to chief diseases (particularly Verticillium wilt caused by *Verticillium dahliae* and 'olive quick decline syndrome' (OQDS) caused by *Xylella fastidiosa* bacterial strain), industrial suitability, and high capacity to differentiate flowers in twigs older than one year should be pursued.

Features that facilitate mechanical harvesting of the fruits need to be considered when selecting progenies for the *ortet* of future clonal varieties (Rallo 2014b). Plant traits such as low vigor with a compact growth habit, mild-force required for fruit removal, natural fruit abscission, resistance to bruising, and low competition between growing shoots and inflorescences during fruit-set period should be addressed as selection criteria.

The oil should have a high content of oleic acid (around 70–80 % of the total fatty acids), a phenols content between 40 and 1500 mg/kg,

and α-tocopherol between 50 and 750 mg/kg, including other compounds with health properties and characteristic flavor (see Servili et al.'s chapter in this book).

The specific traits for producing varieties of high-quality table olives are related to fruit set, fruit drop, fruit size, pit size, yield per tree, and flesh to pit ratio (Lavee 2008; Rallo 2014a; Medina et al. 2012). In some countries fruit size and quality can be enhanced by thinning with naphthaleneacetic acid (NAA) application to minimize alternate bearing behavior in olives, thereby increasing economic returns in the 'on' year (Barone et al. 2014).

The proper scion/rootstock combination needs also to be part of the breeding objectives because the rootstock genotype affects the ability to transmit to the scions some important growth features such as dense and less vigorous shoots, which are generally more efficient in flower differentiation and modification of phenological phases. In addition, rootstock may provide tolerance to stresses, including those due to drought and salt stress, heavy soils, and to root diseases.

Further breeding objectives stem from the current vision of orchard typology, which points to high-density and super-high-density cultivation.

3.1 Fast-Track Breeding Programs to Overcome Juvenility

The multi-year period between seed germination and the first flowering, called the juvenile period (JP), has been the main obstacle in cross-breeding programs for both fruit (Janick and Moore 1996) and olive (Moral et al. 2013) crops. This period may last up to 15–20 years in trees growing under natural conditions (Rugini and Fedeli 1990; Bellini et al. 2002a, b).

An accelerated breeding approach may be achieved by controlling and inducing a flowering gene or/and silencing a floral repressor to shorten the juvenile phase in olive as it has been achieved in other fruit crops (Flachowsky et al. 2007, 2011; Wenzel et al. 2013).

3.2 Flowering and Fruit Traits and Genetic Diversity for the Reproductive System

Very few varieties are self-fertile and the majority of olive varieties are self-incompatible or show some level of self-incompatibility (SI) and need to be fertilized by other varieties for successful fruit set (Seifi et al. 2011; Fabbri et al. 2004; Conner and Fereres 2005; Diaz et al. 2006). Cytoplasmic male sterility also occurs in some olive varieties. It was identified in var. 'Cerasòla' and was attributed to a duplication event at the *cox3* locus (Cavallotti et al. 2003).

Regular bearing over the years is desired but rarely obtained by varietal selection. It would occur when a very delicate balance between fully vegetative and reproductive branches is attained, quantitatively and qualitatively, by a proper pruning intervention for smoothing the interaction between sources of carbohydrate (leaves), the number of florigenic buds, and climatic factors.

As an outcrossed wind-pollinated species, some olive varieties are male sterile but the majority of olive varieties are self-incompatible (Besnard et al. 2000). Varieties such as 'Lucques,' 'Olivière' (France), and 'Farga' (Spain) are considered male sterile (Villemur et al. 1984; Besnard et al. 2000; Serrano et al. 2010; Breton et al. 2014).

Besnard et al. (2000) identified three different male-sterile phenotypes in olive. In the cross 'Olivière' (male sterile) × 'Arbequina,' the male-sterile trait was maternally inherited and affected all progenies. The male sterility (ms 2) displayed by 'Olivière' plus six other varieties and three oleasters was strictly associated with the CCK chlorotype and the MCK mitotype. Oleaster*s* carrying that cytotype showed the presence of restorer alleles. The male-sterile phenotypes displayed by 'Lucques' (ms 1) and 'Tanche' (ms 3) were associated with the ME1 mitotype but it has not been demonstrated that it is a type of cytoplasmic male sterility.

SI is one of the most important systems adopted by many flowering plants to prevent inbreeding and maintain diversity within the species. Most olive varieties are not strictly self-incompatible nevertheless they require foreign pollen to enhance fruit yield and, consequently, orchards should contain pollinisers to ensure fruit set on the main variety. Sexual compatibility and floral biology of several olive varieties have been studied (Seifi et al. 2011; Koubouris et al. 2014; Selak et al. 2014; Marchese et al. 2016).

The system of incompatibility of olive is still undefined (Alagna et al. 2016), but the first evidence of the occurrence of a sporophytic self-incompatibility (SSI) system (Iwano and Takayama 2012) has been recently provided (Collani et al. 2010, 2012; Breton and Berville 2012; Breton et al. 2014).

Cytohistological and biomolecular analyses conducted in putative self-compatible 'Frantoio' and self-incompatible 'Leccino' varieties led Collani et al. (2012) to identify some transcripts of the main genes known to play a crucial role as female determinants of the SSI system typical of Brassicaceae.

Due to the extensive occurrence of SI and male sterility, olive growers need to plant more than one variety in their orchards to ensure sufficient cross-pollination (Martin et al. 2005; Mookerjee et al. 2005).

Five *Olea* species in Malesia (*O. borneensis*, *O. brachiata*, *O. decussata*, *O. dentata*, *O. javanica*) express dioecy (Kiew 1979) while *Olea paniculata* has hermaphrodite flowers.

3.3 Oil Quality

Improvement of oil quality is a difficult task because, in addition to the genetic factors, the growing environment and time of harvest of the fruits play important roles in shaping the oil characteristics, flavor, and salutistic properties.

In fact, Alruqaie et al. (2013) assessed that the differences in fatty acids content among different varieties are due to genetic, environmental, and field location features. Perez et al. (2014) reported the genetic variability of the major phenolic compounds (tyrosol or hydroxytyrosol, lignans, flavonoids, and phenolic acids) of virgin olive oil. A progeny derived from the cross of 'Picual' x 'Arbequina' varieties displayed a large degree of variability, widely transgressing the parenal levels, demonstrating a high degree of variability within just a single cross.

3.4 Choice of the Rootstock for Shaping Branch Architecture

Canopy-architecture traits in modern fruit crop orchards are mainly molded by the rootstock genotypes, which allow also the cultivation of the scion varieties in unsuited soils or environments.

The tree architecture should be dwarfing, with the initial fast growth of flexible and numerous twigs to facilitate mechanical pruning and harvesting (Rugini et al. 2003; Rosati et al. 2013) (see Rallo et al.'s chapter in this book).

A dwarfing rootstock is necessary when the self-rooted scion is not sufficient to get the desirable plant size (Rugini et al. 2016b) and when the cultivar does not express multi-resistance to various biotic stress factors (soil pathogens) and multi-tolerance to abiotic stresses.

Ben Sadok et al. (2013) investigated the genetic determinism of architectural traits in the F_1 progeny derived from crossing of two contrasting genotypes, 'Olivière' and 'Arbequina.' They dissected the tree architecture into quantitative traits related to growth , branching and first flowering and fruiting. In addition, they designed tree architcture models that included the year of growth, branching order, and genotype effects, and estimated broad sense heritability for those traits.

A large number of scion-variety versus rootstock-variety combinations need to be tested for fitting adequately the different environments where olive trees could be grown. However, the trend in rootstock breeding is to incorporate more traits into the list of the ideotype features, compared to the current genetic features of the rootstocks. Additional trait enhancements are sought

for improving propagation ability and grafting compatibility, resistance to replant in problematic soils due to new disease complexes, which may involve fungi, bacteria, and nematodes, rooting depth, mineral nutrient uptake, fruit bearing precocity and quality, and level of dwarfing. Seedling rootstocks were used in the past to propagate difficult-to-root olive cultivars, such as 'Gordal Sevillana' (Hartmann and Whisler 1970; Troncoso et al. 1990), or to make the plants more stable in the windy environment for the presence of taproot in the seedling rootstocks. However, the emission of new roots from the scion guarantees the plant size uniformity in the field, excluding the effect of the rootstock on the tree crown size.

Clonal rootstocks selected among traditional varieties or from shoots obtained by in vitro micropropagation of diploid and tetraploid meristems derived from mutagenesis of fruiting varieties (Rugini et al. 1996) are currently investigated (Rugini et al. 2016b). For example, the 'FS17' (Fontanazza et al. 1998) and 'LD' ('Leccino Dwarf') (Rugini et al. 1996; Nardini et al. 2006) clones and the 'LM3-2*n*' and 'LM3-4*n*' plants selected in vitro by shoot-tip fragmentation of mutagenized apical meristems from var. 'Leccino' (Rugini et al. 2016b) are being tested as rootstocks with dwarfing ability when grafted with scions from 'Leccino' itself and 'Canino.' The other mutants such as 'Leccino Compact' and 'LD' (Rugini et al. 1996) reduced the total leaf area, the hydraulic conductance, and the xylem conduit diameter (Buffa et al. 2006; Fabbri et al. 2006; Pannelli 2006; Nardini et al. 2006; Trifilò et al. 2007; Di Vaio et al. 2012).

3.5 Response to Biotic and Abiotic Challenges

The olive tree and its products can be damaged by many diseases and pests. The most dangerous bacteria are *Pseudomonas savastanoi*, which produces tubercules on the branches and stems, and *X. fastidiosa* subsp. *pauca* (*Xfp*) strain CoDiRO, recently reported in olive trees, causing the OQDS (Martelli et al. 2016). One hundred and 24 fungal species (obligate parasites, primary, or secondary invaders) are pathogenic to the olive (Chliyeh et al. 2014) but the most dangerous are *Spilocaea oleagina* that causes injury on the leaves and fruits and *Verticillium dahliae* which is harmful to the root apparatus and to the growth of the olive plants. Among insects, the most aggressive are the olive fruit fly (*Bactrocera olea* Gmelin), the olive moth (*Prays oleae* Bernard), and black scale (*Saissetia oleae* Olivier) (see Corrado et al.'s and Sebastiani et al.'s chapters in this book).

The olive tree should be resistant to the mentioned diseases and pests. However, the general picture that stems from the analysis of the olive host response to the causal agent (virus, bacteria, fungi, and insects) of biotic stresses is of a complex intricacy of gene interactions. The connections involve coding sequences for transcription factors, enzymatic and stress-related proteins, and metabolic components either inhibiting the pathogen or parasite larvae or attracting parasite enemies (see Corrado et al.'s chapter in this book).

Indeed, the main tool to elucidate the molecular basis and related signaling pathways involved in olive genome interaction with biotic agent stressor has been the PCR-based suppression subtractive hybridization (SSH) (Diatchenko et al. 1996), which reveals a large amount of the expressed genes in response to the susceptible host compared to the response in the tolerant or resistant host. In fact, it is based on the selective PCR amplification of cDNA fragments that differ between the transcriptome of the biotic stress tolerant or resistant olive host variety and that of the susceptible host variety, without any prior genomic knowledge (Estrada-Hernandez et al. 2009; Ouyang et al. 2007). In all instances, several dozens of over- or under-expressed genes have been detected studying the response of olive drupe to *B. oleae* larvae (Corrado et al. 2012), the olive response to the highly virulent V937I pathotype of *V. dahliae* (Gómez-Lama Cabanás 2015) and to *X. fastidiosa* ssp *pauca* strain CoDiRO (Giampetruzzi et al. 2016). In this last study, it was assessed that 659 and 447 genes were differentially regulated in var.

'Leccino' and var. 'Ogliarola Salentina,' respectively, upon *Xfp* infection. Upregulation of genes encoding receptor-like kinases (RLK) and receptor-like proteins (RLP) is the predominant response of var. 'Leccino,' which is missing in var. 'Ogliarola Salentina.' These data suggest that *Xfp* determines a lower pathogen concentration in var. 'Leccino' and indicates that this cultivar may harbor structural genes and/or regulatory elements which counteract *Xfp* infection.

Crosses between the *Xfp*-tolerant var. 'Leccino' and other valuable varieties should be programmed to get new *Xfp*-tolerant varieties with drupes having high oil quality and quantity.

In natural wild olive populations, the greatest adaptive response to abiotic stresses is expected when populations are large, have high genetic variability, natural selection is strong, and there is an ecological opportunity for the establishment of better-adapted genotypes (Alberto et al. 2013). Hints on the genetic basis of resistance or tolerance to abiotic stresses are obtained by comparative transcriptome analysis of olive varieties expressing a divergent response to environmental challenges such as the olive response to NaCl stress or by exposing seedlings at different NaCl treatments in terms of concentration or duration of exposure (Bazakos et al. 2012).

Saline stress may occur when low quality (i.e., drainage water) or salty water (3–8 g/l NaCl) is used for olive irrigation. Saline water negatively affects olive shoot growth, causes morphological changes in leaves, and affects fruit productivity (Chartzoulakis 2005).

There are salt-tolerant and salt-sensitive varieties. Tolerant varieties such as 'Frantoio' (Italy), 'Kalamata,' 'Megaritiki' (Greece), 'Picual' and 'Lechin de Sevilla' (Spain), and 'Chemlali' (Tunisia) have greater ability to exclude toxic ions and control the net salt import to the shoots (Chartzoulakis 2005).

The olive tree is able to tolerate the low availability of water in soil by means of morphological, physiological, and biochemical adaptations acquired in response to periods of water shortage often lasting throughout the spring-summer period (Connor and Fereres 2005; Sofo et al. 2008). The extremely drought-resistant ssp. *laperrinei* could act as a genetic resource to improve its domestic counterparts in case of most severe drought occur in the Mediterranean countries as a consequence of climate changes (Besnard et al. 2012). Also ssp. *cuspidata* is a valuable genetic resource to improve drought tolerance in cultivated olive (see Sebastiani et al.'s chapter in this book).

4 The Genetic Diversity Available for the Trait Improvement

The olive industry faces global economy and dynamic transformations due to decreasing labor availability, increasing environmental concerns, the cost of energy, climate change and epidemics of new and invasive insects, and bacterial and other diseases. The generally reactive response, rather than proactive actions against the new challenges, hampers the release in due time of the new cultivars endowed with the proper traits to mitigate the negative impact of the stresses. The inability to have a rapid varietal turn over in olive and other fruit tree crops is mainly due to the length of the juvenile phase, which in conventional breeding methods based on phenotypic selection widen the breeding cycle. However, the successful search for optimal growth conditions for seedlings from seeds of selected trees allowed breeders to reduce the length of the juvenility phase and shortening the breeding cycle.

Recent advancements in botany, physiology, biotechnologies, genetics, genomics, gene transfer, and gene editing provide the tools to discover recombination hotspots on the genome and promote rapid trait inheritance assessment, genetic linkage mapping, heterosis in hybrids and hybrid clones, chromosome engineering, mutagenesis and polyploidy induction, molecular genetics, nucleotide sequence editing in genes, tissue culture, and genetic transformation. These achievements allowed the transition from conventional breeding techniques based largely on phenotype to molecular marker-assisted breeding approaches, cisgenesis, or targeted nucleotide alteration in genes, providing new alleles with large phenotypic effects and reducing the duration and

number of breeding cycles for new cultivar release.

There are several excellent reference books that describe the theory and compare methods of traditional and unconventional approaches in crop breeding. Because the breeding methods share several procedural stages, here we provide a general overview of the steps for the breeding process to release new olive varieties.

The olive breeding processes are based on the exploration of the germplasm available especially those entries composing the primary gene pool (GP1) of the olive.

When the genetic variation in the GP1 is narrow and new genetic combinations are desired, the second step is the increase of genetic diversity by hybridization of selected heterozygous parental plants from the same or different GP categories or by induction of new genetic variation through in vitro culture, targeted mutagenesis, gene editing, and acceptable genetic transformation (i.e., cisgenesis) methodologies.

Once enough genetic diversity is available for the target trait and associated molecular markers have been identified, then the third step is the screening and selection of the plants possessing the desired allelic combinations for the sought phenotype.

Finally, the last step is the multi-year and multi-location testing of the yielding ability of the promising plant genotypes, which is concluded by cultivar development through variety registration and certification of the commercial planting material.

Based upon the Harlan and de Wet (1971)'s gene pool concept, the primary gene pool or GP1 of olive includes the cultivated crop taxa (*O. europaea* ssp. *europaea* var. *europaea*) and the wild forms (*O. europaea* ssp. *europaea* var. *sylvestris*) that cross easily with the crop. Alleles for wildness distinguishing oleaster from cultivated varieties have been discovered (Lumaret and Ouazzani 2001). Belaj et al. (chapter in this book) provide a thorough description of the germplasm resources available in the GP1.

The wild ssp. *cuspidata* (Wall. ex G. Don) Cif. widespread in the northern to southern Africa, Arabia, India to China, and eastern Australia (i.e., in the coastal and subcoastal districts of eastern New South Wales) is a candidate of the GP2 of cultivated olive. The ssp. *cuspidata* was known also as *O.* ssp. *africana*, *Olea chrysophylla,* and *O. ferruginea* Royle; the attributed common names were 'Brown,' 'African,' or 'Indian' olive. Many ssp. *cuspidata* ecotypes exist throughout southern Africa and beyond, growing from the coastal and subtropical forest regions to semidesert highlands adapted to frost, drought, or high humidity and tolerates temperatures ranging from about −5 to 40 °C (Costa 2014). The high adaptability of ssp. *cuspidata* is found in Australia where, from its introduction for horticultural purposes, it became an invasive and potentially dangerous plant (Cáceres et al. 2015). The drupe of ssp. *cuspidata* from Kenya shows less oleic acid than var. *europaea* (Hannachi et al. 2009).

The tertiary gene pool (GP3) must include most of the *Ligustroides* species, such *as O. exasperata*, *O. capensis* ssp. *macrocarpa*, *O. capensis* spp. *capensis*, *O. woodiana*, *O. lancea*, and *O. paniculata*, from which the gene transfer to *O. europaea* ssp. *europaea* is expected to occur by hybridization and in vitro culture of the resulting hybrid embryos, because of post-zygotic incompatibility effects.

The quaternary gene pool (GP4) of *O. europaea* ssp. *europaea* is represented by highly sexual incompatible genotypes for which gene transfer can occur only with genetic engineering tools.

5 The Breeding Methods

5.1 Clonal Selection

Traditionally the olive varieties of many Mediterranean regions are the outcome of selections from the local wild populations and the field performance of plants expressing novel fruit traits was found in oleaster populations. In some cases, the olive clones domesticated in other regions were probably transferred to other regions by cloning the best variants found in the originally domesticated trees (Lavee 2013).

Genuine oleaster populations contain more variability than the cultivated olives (Baldoni and Belaj 2009; Belaj et al. 2010) and are adapted to several environments. Oleasters may be a very important source of resistance to abiotic stresses such as drought, salt, wind and low temperature (Mulas 1999; Baldoni et al. 2006; Meddad-Hamza et al. 2010; Aranda et al. 2011; Klepo et al. 2013), biotic stresses, such as Verticillium wilt (Sesli et al. 2010), peacock spot (Ciccarese et al. 2002), olive fly (Mkize et al. 2008), and OQDS caused by *Xfp*. Most of the traditional varieties composed of aged trees are selections from the wild or only 1–2 generations away from the oleaster gene pool and the genetic diversity of those trees is wide.

The old landraces that make the bulk of locally adapted olive trees in small farms as well as the large olive plantations with aged trees have been established also by clonal selection and clonal propagation (Oz et al. 2008). Many selections have been obtained from traditional varieties (i.e., 'Moraiolo,' 'Canino,' 'Manzanillo,' 'Chimlali,' 'Picual,' and 'Souri'). However, further clonal selection in those materials gave poor results as the genetic composition of those selections is basically unchanged or only slightly modified from that of the original variety (Lavee 2013). They were labeled with letters and numbers, but few of them expressed further improved characteristics (better fertility, more tolerance to pests and diseases, early ripening, larger fruits, and dwarfing habit) (Berenguer 1978; Khlif and Trigui 1986, 1990; Fontanazza 1987; Garcia Berenguer 1988; Suarez et al. 1990; Pannelli et al. 1993; Parlati et al. 1994; Tous et al. 1999; Lavee et al. 2008). On the other hand, a high level of genetic variability was detected within the 'Biancolilla,' 'Giarraffa,' and 'Moresca' Sicilian local varieties (Caruso et al. 2014) and within the local varieties in Sardinia (Marra et al. 2014) due to somatic mutations and polyclonal propagation of feral forms within the local varieties.

Twenty-three clones have been identified in the olive var. 'Zutica' growing on the Montenegrin coast since 2000 years. The clones clustered into two main groups of 8 and 13, respectively, differing for fruit size and oil content in the fruit (Lazovic et al. 2014).

To overcome the limits of clonal selection within current varieties and local populations in small-holder fields affected by genetic uniformity, the reinvention of domestication within wild var. *sylvestris* germplasm has been proposed (Lavee 2013).

Feral olive populations have also been tested (Sedgley 2000; Hannachi et al. 2009) as sources of materials for cloning new varieties (Guerin et al. 2002).

Cloning of hybrids between olive and wild relatives has also been proposed to broaden the genetic diversity available for selection of new varieties (Besnard et al. 2001). Biton et al. (2012) suggested the use of partially inbred plants belonging to other *O. europaea.* ssp as parents in olive cross-breeding programs in order to exploit heterosis and select vigorous hybrid clones. Cloning of inter-ssp hybrids such as those from var. *europea* (female) x ssp. *cuspidata* (male) could provide new and interesting genotypes to test as new varieties since the hybrid offspring resemble the female parent but contain male-specific alleles as confirmed by amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) molecular markers (Caceres et al. 2015).

5.2 Exploiting Genetic Diversity by Intercrossings Within the Primary Gene Pool

5.2.1 Selection Within Progenies from Open Pollination Among Members of the Primary Gene Pool

In other instances, the progenies for selecting promising genotypes are produced by open pollination of plants of a given accession. In this case, the members of the progeny are half-sibs (HS) and are easily obtained because only the collecting and planting the seeds from a given mother plant is required. Using HS progenies, it has been possible to ascertain, for example, that

the length of the JP in the seedling significantly varied according to the mother plants that provided the seeds (Moral et al. 2013). The evaluated mother plants were classified into three groups that differed in the length of the JP of their progeny: short ('Arbequina' and 'UCI 7–34'); medium ('Lechín de Sevilla,' 'Manzanilla de Sevilla,' 'Picual,' 'UCI 11–28' and 'Zaity'); and long JP ('Frantoio,' 'Memecik,' and 'UCI 10–30'). The height of the seedling at planting was taken as a measure of its vigor and was significantly correlated with the length of the JP for all progenies except for those of 'Lechín de Sevilla,' 'Memecik,' and 'UCI 10–30' because most of their seedlings did not flower during the 14 years of the study.

Two open-pollinated progenies (o.p.), originated from a wild olive ('Alga05') and the main Spanish olive var. 'Picual,' revealed great seedling polymorphism for SSR markers and high levels of morpho-agronomic and genetic diversity (Klepo et al. 2013). As expected, for most of the morpho-agronomical traits, 'Picual' o.p. progeny showed superior values in comparison with the wild o.p. progeny. However, 'Alga05' wild olive progeny was more vigorous, with shorter JP and more abundant flowering than 'Picual' o.p. For both progenies, principal component analysis showed a strong association between different agro-morphological traits (fruit *vs* stone dimensions in the wild olive progeny, and fruit trait *vs* oil content in the 'Picual' progeny) which could facilitate the selection of the most appropriate traits and increase the efficiency of olive breeding programs.

5.2.2 Programmed Hybridization Using Homozygous Genotypes from Haploid in Vitro Culture

The analyses of progenies obtained by close inbreeding in olive varieties would be of great interest for isolation of clones expressing recessive traits. However, the breeding system of olive varieties is based on intercrossing due to the high proportion of varieties expressing SI. Therefore, with the exception of few well-known case of self-fertility in olive, and the lack of knowledge on the coefficient of inbreeding of each variety due to coancestry, the production of homozygous olive plants by close inbreeding is impractical. Therefore, alternative methods should be applied to get homozygous plants. The most promising are anther, pollen, ovary, and ovule cultures to produce, in a short period of time, dihaploid (DH) plants by doubling the number of chromosomes of the regenerated haploid shoots (Germanà 2006). Bueno et al. (2005) were able to induce cell division and proembryos formation in the culture of isolated microspore of the var. 'Arbequina' and var. 'Picual.'

Recently, experiments to obtain seeds with haploid embryos after pollination with compatible pollen treated with physical agents (UV-rays, X-rays, and toluidine blue) are expected to produce plants with a broad diversity for fruit size and shape. The embryo culture produced several plantlets, which are now evaluated for chromosome number using root-tip cytological preparations (Rugini personal communication).

Before the induction and identification of haploids become a routine biotechnological tool in olive breeding, it will be necessary to gain information on what factors influence haploid induction, the molecular basis of microspore embryogenesis, and the genetics underpinnings the ability of an olive cultivar to provide easy haploid induction.

The integration of genomic resources with DH technology will provide new opportunities for improving the selection methods, maximizing selection gains, and accelerate variety development through marker-aided olive breeding programs.

5.2.3 Planned Mating Designs

Controlled pollination experiments have been carried out in olive by either self- or cross-pollination and the results on seed setting have been compared with those from open pollination (Farinelli et al. 2004; Ibtissem et al. 2014). Farinelli et al. (2004) were mainly interested in studying the effect of pollination on the characteristics of seeds derived from self-, cross-, and open pollination of the varieties 'Carolea' and 'Kalamon.' The progenies from controlled crossing using pollen from the varieties

'Arbequina,' 'Carolea,' 'Frantoio,' 'Kalamon,' 'Leccino,' 'Maurino,' 'Moraiolo,' 'Nostrale di Rigali' and 'Orbetana' were used. The percentage of aborted seeds varied according to the pollinizer and mother plant. For example, seeds from crossing 'Carolea' to 'Dolce Agogia' determined the lowest percentages of aborted seeds (6.9 %), while 'Kalamon' pollinated with 'Dolce Agogia' gave the highest percentage (22 %) of aborted seeds. Pollen from 'Nostrale di Rigali' did not affect bi-seeding when it was used in crossing to 'Carolea' while it affected bi-seeding in over 20 % of the seeds obtained by crossing to 'Kalamon.'

Hybridization is mainly used to transfer from the donor accessions or the wild parental sub-gene-pool, some genes absent in the receiving variety (e.g., genes for resistance to insects or better adaptation). During the first generation after crossing, all the parental genes are reshuffled by both genetic recombination and a random assortment of the member of each chromosome pair. To select new genotypes possessing the desired combination of alleles at different loci, it is necessary to 'screen' a large progeny to retain those that phenotypically and genetically possess the right combination of morphological and molecular features.

Classic breeding programs by crossing and selection in the progenies have been carried out in Greece (Pritsa et al. 2003), Israel (Lavee et al. 1999, 2003, 2014), Italy (Fontanazza et al. 1998; Bellini et al. 2002a), Turkey (Arsel and Cirik 1994), Tunisia (Trigui 1996), and Spain (Rallo 1995). However, very few olive varieties have been obtained by the classical breeding program based on controlled crossing and selection (Lavee 1978, 1990; Brooks and Olmo 1997; Fontanazza et al. 1998; Lavee et al. 1986, 1999, 2003, 2004; Bellini et al. 2002b).

When hybridization has been used in olive breeding to take advantage of the qualified genetic diversity stemming from controlled crossings, the 'good x good' criterion has been used for choosing the parents to be included in the biparental cross-scheme. Applying that criterion, it was possible to: (a) avoid the appearance of many undesired phenotypes in the segregating progeny, and (b) increase the chance of finding plants with enhanced phenotypes directly in the segregating progeny, and (c) use them as *ortet* for clonal selection of new genotypes for variety registration. This strategy has been used in several olive breeding programs and some new clones have been selected (Lavee 1989; Fontanazza and Bartolozzi 1998; Bellini et al. 2008). The success of the biparental cross-breeding program will depend on the hereditability of the traits to be improved. This information rarely is available by comparison with other similar breeding programs and should be evaluated a posteriori from the material being studied. In this case, several 'good' accessions should be identified and many pairwise cross-combinations among them should be planned to increase the chance to find the desired *ortet* in the progeny. One 'good' x 'poor' cross should be performed for calibrating the progress that selection will allow in the 'good' x 'good' progenies.

Leon et al. (2007) described a breeding methodology based on the 'good x good'-controlled crossing and a growth-forcing step of the seedlings in the greenhouse to decrease the length of the juvenile phase of the progeny and speed up the selection process of promising genotypes.

Leon et al. (2007), in 1992 and 1993, selfed and crossed 'Arbequina,' 'Picual' (both 'good' early bearings), and 'Frantoio' ('poor' late bearing) olive varieties in the nine possible combinations to obtain progenies for selecting new early-bearing olive varieties. The seedlings of the progenies were subjected to a forcing growth protocol both in the greenhouse and in the field (Santos-Antunes et al. 2005).

Genotypes of the seedling progeny, which produced flowers and consequently fruits during the first years after field planting, were identified. After the field evaluation for three harvest seasons, 15 genotypes ('*ortet*') were selected from the initial population mainly on the basis of their early crop (short JP), high oil content, and, for some of them, for presenting outstanding agronomic values (León et al. 2004, 2005).

The seven-year-old '*ortet*' plants of those 15 selected genotypes and the three parental

varieties were vegetatively propagated by semi-hardwood stem cuttings. The one-year-old '*ramets*' of the 15 selected '*ortet*' and the propagated parental trees were planted in an open field at 6 × 5 m spacing in a randomized block design with 16 replications and one tree per elementary plot. Trees were trained as a single-trunk vase, with three-to-four main branches, and minimal pruning was carried out to allow early bearing. Standard cultural practices were followed, including irrigation supply by in-line drips to avoid water stress of plants.

Plants were systematically evaluated for earliness of bearing, vigor, crop, and yield efficiency in the 4 years after planting (from 2001 to 2005). Plant height and trunk diameter prior the beginning of each growing season were recorded. In the last year (February 2005), canopy height and width (measured east–west and north–south) were also recorded, and canopy surface and volume were calculated from these measurements (Del Río et al. 2005). 'Arbequina,' 'UC-I 7–34,' 'UC-I 9–67,' and 'UC-I 5–44' showed the highest earliness of bearing, with more than 80 % of trees bearing fruit two years after planting. These four genotypes and 'Picual' showed 100 % of fruiting trees in the 3rd year after planting. 'Frantoio' and other five additional genotypes showed 100 % of fruiting trees only at the 4th year after planting.

The breeding procedure and the adopted criteria for selecting early-bearing genotypes were effective for the identification of several new genotypes to become future varieties with short juvenile phase, early-bearing and high yield (both in fruit and in oil). Some of the early-bearing genotypes also presented low vigor and could fit in the high-density mechanically harvested orchard.

The length of the described procedure might be shortened if the immature zygotic embryo germination technology and the in vitro cloning of the zygotic embryo (see Sect. 5.5.2) are included in the process.

5.3 Adjustment of the Breeding Scheme for Scion Variety and Rootstock Selection

The first step for selecting plants to be used as scion varieties or rootstock is the massive germination of seeds for producing a large population of seedlings needed for screening. After the seeds have been extracted from mesocarp and washed in sodium hydroxide solution, they are stored in dry and ventilated environment at room temperature or maintained at a low temperature in the humid substrate, to overcome the dormancy; then, after 9 months (in August) they are placed to germinate in the greenhouse. When seedlings begin to grow, they may be screened for pest or disease resistance or for molecular markers in linkage disequilibrium with those traits. The individual seedlings may be cloned by cutting to provide multiple plants to be grafted for testing different varieties or testing as fruiting varieties. The process of producing multiple plants takes several years. However, to reduce of about two years the length of the process leading to the selection of seedlings, in vitro germination technique can be used (Sect. 5.5.2). When the seedlings have several nodes, uninodal explants can be rooted to get multiple plants, which are screened in vitro for molecular markers linked to genes for pest or disease resistance, and then the selected seedlings are hardened in pots for grafting.

Rootstock breeding and their development will steadily become important relative to scion breeding as an increasing number of useful characteristics and features of the scion varieties will be determined by rootstock traits such as resistance to soilborne pests and diseases. Rootstocks that deliver specific novel or rare functions to the scion are particularly interesting for their broader utility for adapting many scion varieties to just one rootstock type. For this purpose, a large number of genotypes, of both scions and rootstocks, are required for genetic selection to fit in different environments and meet the

requirements of modern farming techniques for both olive oil and table olive production (Medina et al. 2012; Lavee 2013; Rallo 2014a).

To simplify and to abbreviate the time of rootstock selection, it is advisable also to try to select them among the numerous olive varieties, for which are already known some useful characters. In addition being these varieties already in the adult phase, the scions will not be affected by the juvenility conditions of the seedling rootstocks if the grafting is carried out before overcoming the juvenile phase.

5.4 The Induced Genetic Diversity in Vivo and in Vitro

Mutagens can be applied to pollen, buds of unrooted cuttings, and potted plants. Subsequently, stable mutants can be recovered, both in vivo by grafting and in vitro by shoot-tip fragmentation or by shoot regeneration via organogenesis, which normally take place from a single cell. When shoot regeneration is difficult to achieve, it would be advisable to apply physical or chemical mutagens at the basal part of in vitro rooted shoots, just before transplanting them to pots. Once in the field, the natural capacity of plants to differentiate suckers in that zone might allow regeneration of mutated suckers. Both physical and chemical mutagens have been successfully used in olive, both in vivo and in vitro.

5.4.1 Induced Variation In Vivo by Physical Mutagens

Gamma rays have been used to induce mutations affecting plant architecture and phenological phases. Donini and Roselli (1972) recovered 'Briscola' as a chimeric mutant from irradiated cuttings of the var. 'Ascolana Tenera'; the mutant produce low and yearly variable fruit yield and often rise shoots with long internodes, whereby it is used only for the ornamental purpose. Other mutants have been produced as a result of irradiation of cuttings of var. 'Leccino' and var. 'Frantoio.' Only one mutant resulted stable, subsequently named LD, whereas most of the other mutants were chimeric, prevalently mixoploids. Using the shoot tip in vitro fragmentation technique (Rugini et al. 1996), stable diploid and tetraploid shoots were obtained from the mixoploid mutants. The 4*n* plants exhibited less growth, larger, and thicker leaves compared to the 2*n* plants. The stable 4*n* genotype from 'Leccino' acquired self-fertility and the 4*n* mutant from 'Frantoio' maintained the parental self-fertility. The 'LM3-*2n*' mutant from 'Leccino,' during nine years of observation, expressed constant and abundant fruit yield and its oil quality was similar to that of the 'Leccino' parent. In addition, it acquired the capacity to be inter-compatible with other diploid mutants from 'Leccino' and with the 'Leccino' parent. A fast rising of inbreeding is expected in the progenies from repeated backcrosses of 'LM3-*2n*' to 'Leccino.' When both the *4n* and *2n* 'Leccino' mutants were used as rootstocks, they proved to be very effective in reducing the scion size of the high-vigor 'Canino' variety (Rugini et al. 2016b), similar to the dwarfing ability expressed by the 'LD' mutant previously tested as a rootstock (Pannelli et al. 1992; Rugini et al. 1996; Nardini et al. 2006).

Oražem et al. (2013) combining morphological measurements, nuclear DNA content, and molecular marker (SSR and AFLP) analyses, evidenced that the physical X-ray irradiation of in vitro grown olive shoots of var. 'Canino' provided an efficient system for generating useful mutants. Those mutants were effectively differentiated by AFLP profiling.

5.4.2 Induced Variation In Vitro by Chemical Mutagens

The application of the chemical mutagen oryzalin to in vitro shoots of var. 'Canino' produced mutants that upon transplanting in the field exhibited a vegetative habit similar to that of the original variety. However, one mutant produced a few flowers and very small berries (about 80 % smaller than those of the original mother plant) and another mutant expressed normal flower density and fruits that were slightly larger than the original variety (Rugini personal communication).

The oryzalin mutagen was also used by Ozair et al. (2014) to induce genetic variation in

explants from var. 'Moraiolo.' When this chemical was used in the olive medium (OM) at the concentration of 300 mg/l, the new sprouted shoots displayed a significant increase in stem length, fresh and dry leaf weight, leaf area, the number of nodes, and number and length of roots compared to the shoots from the control (untreated) explants.

5.5 Nonconventional Methods and Breeding Innovations Introduced by Genomics and Biotechnologies

5.5.1 The Genetic Diversity Disclosed Using Genomic Resources

Molecular Markers

Several decades ago, the genetic diversity within the available olive germplasm for breeding was known for several morphological traits (Barranco and Rallo 1984), although they were influenced by environmental conditions. Subsequently, isozymes were used to evaluate varietal diversity (Trujillo et al. 1995) and DNA molecular markers were developed for the revision of the *Olea* taxonomy, the characterization of the olive germplasm, and the traceability of its oil (Bracci et al. 2011; Baldoni 2014; Baldoni et al.'s chapter in this book).

By the end of the last century, the first DNA-based marker introduced for a better genetic knowledge of olive was the random amplified polymorphic DNA (RAPD). Molecular markers have been used for estimating genetic distances among wild, feral, and cultivated olives from the Mediterranean Basin (Fabbri et al. 1995; Besnard and Bervillé 2000; Belaj et al. 2000, 2001) and for tracing the spread of olive in Macaronesia (Hess et al. 2000).

Multilocus molecular markers such as AFLPs were then adopted to gain insights on the distribution of genetic diversity at several sites in the nuclear genome. However, they are dominant and much of the information on the genetic structure of the progenies (average inbreeding at each locus, the rate of decay in linkage disequilibrium, etc.) remains undetected. AFLPs have been analyzed in wild, feral, and cultivated olives to study relationships between them (Angiolillo et al. 1999).

Subsequently, SSR markers have been used for effectively fingerprinting olive germplasm in several countries (Baldoni et al.'s chapter in this book) such as Argentina (Torres et al. 2014a, b), Turkey (Işik et al. 2011), Tunisia (Abdelhamid et al. 2013), Palestine (Obaid et al. 2014), Israel (Biton et al. 2012, 2015), Spain (Trujillo et al. 2014), and Italy (Bracci et al. 2009; Caruso et al. 2014; Marra et al. 2014). Eleven SSR loci have been sufficient to characterize 211 olive cultivars of an olive collection cultivated in six regions of southern Italy (Muzzalupo et al. 2009).

The SSR markers revealed the relationships between 23 accessions from Liguria Region (Italy) and 40 accessions from the olive germplasm of other Mediterranean origins. No cases of genetic identities were found between Ligurian and Mediterranean accessions revealing the uniqueness of olive varieties from the Italian region of Liguria (Bracci et al. 2009). Comparison of the SSR patterns among cultivated olives in Southern Italy evidenced that many local varieties in Sicily and Calabria contain a large proportion of feral forms (Caruso et al. 2014; Marra et al. 2014).

Single nucleotide polymorphism (SNP) markers are now in the olive breeding pipeline. They are codominant and multilocus genetic markers spread all over the genome and are cost-effective in terms of cost per marker. They can be discovered in expressed sequence tag (EST) libraries representing genes encoding proteins involved in the phenotypic expression of several traits such as fruit characteristics related to phenolic content in 'Coratina' and 'Tendellone' varieties (Alagna et al. 2009), and the flower and fruit development in var. 'Leccino' (Galla et al. 2009).

Next-generation sequencing (NGS) technologies are being used for genotyping SNPs. The NGS-based genotyping methods, known as genotyping by sequencing (GBS), enable the simultaneous detection of thousands of SNPs throughout the genome in mapping populations

or in a collection of clones (Baird et al. 2008; Elshire et al. 2011).

Linkage Mapping and QTL Identification

The first linkage mapping experiments in olive were performed on numerous F_1 cross-progenies using a relatively low number of markers (De la Rosa et al. 2003; Wu et al. 2004; El Aabidine et al. 2010; Khadari et al. 2010; Dominguez-Garcia et al. 2012; Ben Sadok et al. 2013). Recently, Ipek et al. (2016) constructed a high-density genetic linkage map for the olive genome using 5736 SNPs markers. Up to date, it has been impossible to deliver sound QTLs for MAS breeding, excepting for a few preliminary data (Ben Sadok et al. 2013; Atienza et al. 2014; Ates 2016; González-Plaza et al. 2016) (see Baldoni et al.'s chapter in this book).

Olive Genome Sequencing and de Novo Assemblies

The first data on the sequencing of the olive genome (Barghini et al. 2014; Muleo et al. 2012; Unver et al. 2016; Cruz et al. 2016; Muleo et al.'s chapter in this book) are available.

Unver et al. (2016) and Cruz et al. (2016) have reported the sequenced and assembled reads of the genome of the wild olive tree (*O. europaea* var. *sylvestris*) with coverage of 246X. A de novo sequencing methodology was used to assemble the genome, which resulted in a draft genome of 1.48 Gb with scaffold N50 of 228 kb, which is near to the previous estimates by flow cytometry and k-mer analyses ($\sim$1.46 Gb). They assembled 42,843 scaffolds (>1 kb) with about 80 % of the total assembly (1.16 Gb) and anchored 50 % of the sequences into 23 linkage groups, which included 572 Mb. About 50 % of the total genome assembly was found to be composed of repetitive DNA. Transposable elements and interspersed repeats occupied 47 % of the genome. Protein-encoding gene models were constructed and a total of 60,214 protein-encoding gene models were predicted for total assembly, of which 36,381 were anchored into chromosomes. The developed genomic resources (http://denovo.cnag.cat/genomes/olive/) will serve as a crucial source to facilitate more effective olive breeding programs.

Olive Breeding Assisted by the Targeted Use of Genomic Resources

With the new genomic resources developed through olive genome sequencing, the genome-wide marker genotyping in olive will become an integral part of any research that requires quantification and characterization of genetic diversity.

The most advanced genome-based breeding procedure is focused on the analysis of genetic variants by NGS and genomic selection (GS) which uses genome-wide markers to predict the breeding value of individuals to be selected. The genomic resources needed are prepared from (a) gene expression analysis, (b) GBS, and genome-wide association studies (GWAS) for the simultaneous characterization of hundreds of individuals plants for SNPs, candidate genes for specific traits, association of SNPs to QTLs, and variant discovery for a panel of relevant genes (Thomson 2014; Pandey et al. 2016).

QTL mapping, GWAS research, and GS studies will surely become prominent genomic approaches that will increase the selection efficiency of the desired genotypes in olive segregating populations.

5.5.2 In Vitro Techniques for Supporting Conventional and Unconventional Methods of Genetic Improvement

In Vitro Micropropagation

In olive, successful micropropagation has been reported for several varieties using axillary bud stimulation, organogenesis, and somatic embryogenesis techniques (Rugini et al. 2016a, b); in many cases, the resulting in vitro plants have been transplanted in the field. The axillary bud stimulation is currently used to produce plants on a large scale by commercial laboratories. Original OM (Rugini 1984) or modified OM by adding different

growth substances is currently used (Cozza et al. 1997; Mencuccini 2003; Saida et al. 2005) not only for *Olea* species (Grigoriadou et al. 2002) but also for other genera, such as *Fraxinus ornus* (Arrillaga et al. 1992). The micropropagated materials can be used to screen for resistance to biotic and abiotic stresses and for genetic improvement activity (Rugini et al. 2000; Bartolozzi et al. 2001) and to test the pathogen virulence and host interaction with parasites.

In Vitro Micropropagation by Axillary Bud Stimulation

In vitro micropropagation by axillary bud stimulation is available for many genotypes for commercial uses. This technique is essential to support conventional and unconventional genetic improvement, i.e., rapid propagation of new genotypes, pathogen elimination, immature embryo germination, germplasm preservation, plant regeneration from cell tissues to use for synthetic seed constitution and for genetic transformation or somaclonal variation induction and for protoplast technology.

The establishment of axenic cultures for axillary bud stimulation in olive is tricky, because the meristems or shoot tips from field-grown or greenhouse plants undergo rapid oxidation; therefore, nodal explants of vigorous twigs, with problems of contamination, are normally used as starting material. Recently in order to control internal infections, nano-silver particles (L-2000, NANO CID®, Iran) added to the media seem to be beneficial (Rostami and Shahsavar 2009). The rapid growth of tender and elongated shoots is accomplished on OM (Rugini 1984) with the addition of a mixture of growth regulators (Zeatin, BAP, TDZ, Metatopolin, and GA3) and mannitol as carbon source. Few varieties, such as the var. 'Maurino,' can be propagated also in a modified Murashige–Skoog (MS) medium (Leva et al. 1994). The rooting is improved if the entire explant or its basal part is placed in a dark environment for one week (Rugini et al. 1987). Putrescine at 160 mg/L generally promotes early and high percentage rooting by increasing total peroxidase activity, at the base of the shoot, essential for root induction (Rugini et al. 1997). When possible the in vivo rooting is preferred. Some varieties, such as 'Chimlali,' root easily in normal greenhouse conditions (Yakoub-Bougdal et al. 2007) while for others, such as 'Frantoio,' 'Maurino,' and 'Coratina,' continuous exposure to light during rooting phase resulted essential for root differentiation and emission (Leva 2011). Other details on olive micropropagation are reported by Rugini et al. (2016a, b).

This technique coupled with GA_3 treatment allows the flowering induction of in vitro growing shoots derived from micropropagation of seedlings from several African varieties (Chaari-Rkhis et al. 2006).

Pathogen Elimination from the Mother Plants or from Offspring

According to the present European legislation, olive plants can be certified only if they are virus free. Since most of the olive plants are affected by viruses, the pathogen-free plants could be a further progress in olive nursery activity. The meristem culture in olive is not possible if the explants are collected from in vivo grown plants, but it becomes relatively easy if the explants are collected from in vitro grown shoots. Meristem explants from in vitro grown shoots containing a virus will easily grow if placed on a small cube (5 mm) of solid OM into Petri dishes or multiwell plates. After ten years in field trials, the plants of three varieties, obtained by this method of virus eradication, are still virus free (Rugini and Bottalico 2011).

Immature Embryo Germination to Accelerate Breeding Programs

Germination of immature embryos sampled less than 3 months after fertilization, has been successfully achieved in several Italian (Rugini 1988) and Iranian (Hossein and Hajnajari 2006) varieties. This biotechnological innovation was further developed offering the possibility to accomplish early cloning from a single seedling and produce early-bearing '*ortet*' and/or '*ramets*' for the early identification of promising genotypes within the progenies from a planned mating design. The procedure starts by collecting the

fruits in August, when the embryo is still in the developing stage and before embryo-dormancy is triggered. Rapid germination of the immature embryo is achieved by using a solid specific medium (Rugini et al. *in litteris*) in glass test tubes. The embryo turns green within two days in a test tube and after 15–20 days form one or two nodes. At this stage, it can be transplanted to jiffy pots or, alternatively, in a layer of liquid OM plus 5–10 mg/l of GA_3 added to the solid medium in order to quickly stimulate the epicotyl to elongate up to 3–5 new nodes. Then the shoot is sectioned into 3–5 uni-nodal micro-cuttings which are transferred to jiffy pots after dipping them in 100 mg/l IBA for ten seconds. The juvenility status of the micro-cuttings allows 100 % of rooting within two weeks. The 3–5 potted plantlets of each embryo-ramet are grown in a greenhouse under continuous light to force rapid growth and early flowering, which, for some genotypes, occur after 2–3 years from the mating design. The only flowered ramets are transplanted in the field where the selection stage is started for the fruit traits (Fig. 1).

Germplasm Preservation

The current trend to establish olive groves with a reduced number of varieties (those which are the most productive or most suited for the environmental conditions) implies a reduction of the olive germplasm in the farmer's fields. To avoid the loss of important genotypes, it is necessary to implement germplasm conservation procedures such as in vitro preservation of olive genetic resources. In vitro slow growth and cryopreservation in liquid nitrogen of somatic tissues represent a promising alternative to seed storage or to field conservation of trees, where plants are subjected to serious risks due to abiotic and biotic stresses.

Slow-Growth Preservation

Lambardi et al. (2000) preserved the shoots of the varieties 'Leccino' and 'Frantoio' in vitro on solid medium under a dark condition at +4 °C for 8 months. Micheli et al. (2007) reported successful development of axillary buds of nodes of var. 'Moraiolo' encapsulated in alginate nutrient gel in plastic cuvettes, after storage at +4 °C for 15 and 30 days, indicating a possible use of this technique for germplasm exchange over long distances.

Cryopreservation

Various organs and tissues of olives including somatic embryos and embryogenic tissues, seeds with or without endocarp, and shoot tips have been preserved under liquid nitrogen (−196 °C) (Benelli et al. 2013). This technique allows long-term conservation of olive germplasm by immersion of tissues into liquid nitrogen directly or by using a vitrification solution before immersion in it. Martinez et al. (1999) after removal of up to 30 % of the moisture content from the shoot tip of var. 'Arbequina' followed by their immersion in liquid nitrogen, obtained 30 % survival after rewarming the shoot tips at room temperature. Lambardi et al. (2000) following the procedure of vitrification and one-step freezing in liquid nitrogen of shoot tips of the var. 'Frantoio' excised from in vitro grown shoots, achieved satisfactory results. Subsequently, Benelli et al. (2001) obtained satisfactory post-rewarming shoot-tip survival with var. 'Canino' and var. 'Gentile di Larino,' but with poor regrowth. Good regrowth of 38 % was reached in shoot tips of var. 'Frantoio' following a two-step dehydration with PVS2 (vitrification solution; Sakai et al 1990) (50 % PVS2 for 30 min and then 100 % PVS2 for 1 h), direct immersion of shoot tips in liquid nitrogen, and culture the tawed shoot tip on medium containing a high concentration of zeatin (46 μM) (Lynch et al. 2007). Although vitrification technique of shoot tips appears promising, the olive embryogenic lines seemed to be highly suitable materials for cryopreservation (Shibli and Al-Juboory 2000; Benelli et al. 2001; Sánchez-Romero et al. 2009; Lynch et al. 2011). In the absence of embryos to be cryopreserved, the encapsulation of both apical and nodal buds from micropropagated shoot could be adopted (Micheli et al. 1998), although a low rate of conversion into shoots was achieved.

DATE		TRADITIONAL MATURE SEED GERMINATION+ FORCING	IN *VITRO* IMMATURE EMBRYOS GERMINATION + FORCING
1st year	June 5	Starting mating design 	
	August 30		Germination in test tube in a specific solid medium 
	September 20		Add a layer of 2-3 ml liquid (OM medium+GA3) at first epycotylnode to stimulate new nodes to collect uni-nodal explants for rooting 
	October 20		Cloning each genotype by rooting in vivo the uni-nodal explants by dipping in 100 mg/l auxin water solution 
	November	Seeds from mature fruits, drying or storing at +4-5°C about one year to overcome the dormancy 	Forcing growth for early fruit bearing of 3-4 ramets under continous light in greenhouse until blooming 
2nd year	August	Place seeds to germinate	
	December	Starting seed germination	
3rd and 4th year: blooming of the earliest genotypes		1 plant per each genotype in 4 years	3-4 plants ('ramet') per each genotype are rised in 3 years

Fig. 1 Timetable and comparison of the embryo germination in conventional seed stratification and immature embryo germination in vitro

5.5.3 Plant Regeneration from in Vitro Cultured Tissues

Efficient methods of plant regeneration from tissue explants are essential to support conventional and unconventional genetic improvement, especially in olive and other high heterozygous tree crop species. Shoot organogenesis, which normally derives from a single cell, could be useful to isolate solid and stable mutant plants from chimeric tissues, which otherwise are difficult to obtain using traditional methods such as grafting small putative mutated twigs.

Shoot Organogenesis

Regeneration by organogenesis has been attained from both zygotic and mature tissues of some fruit crop varieties. In olive, considering its high heterozygosity, the research was addressed to organogenesis of mature explants of important varieties. Petioles from leaves of in vitro grown shoots of 'Canino,' 'Moraiolo,' 'Dolce Agogia,' and 'Halkidikis' varieties showed a good organogenesis potential (Mencuccini and Rugini 1993). However, the number of regenerated shoots was not enough for regenerating plants from modified cells by gene transfer, somaclonal variation, or induced mutation by physical or chemical approaches, but it seems to be an important step to accomplish somatic embryogenesis.

Somatic Embryogenesis and Constitution of Synthetic Seeds

The somatic embryogenesis has been successfully achieved by using tissues from either the zygotic embryo or somatic mature organs of plants. The most competent tissues for somatic embryogenesis are those from zygotic embryos harvested 60 to 75 days after fertilization (Rugini 1988; Leva et al. 1995). However, the temporal 'window of competence' can be extended for at least two months by storing the whole detached young fruits at 14–15 °C before dissecting the cotyledonary tissues (Rugini 1995). Somatic embryogenesis has been achieved also from non-germinated mature embryos of both wild (Orinos and Mitrakos 1991) and cultivated olive (Mitrakos et al. 1992; Shibli et al. 2001).

The somatic embryogenesis from mature tissues is still difficult to be accomplished, although cyclic somatic embryogenesis has been obtained from two varieties, 'Canino' and 'Moraiolo,' through a novel technique consisting of 'double regeneration system' (Rugini and Caricato 1995). The novel technique takes the advantage of using neo-formed organogenetic buds at the base of the petiole. The very small leaflets of those buds seem to be the most competent tissues to differentiate somatic embryos. Recently, multi-cotyledonary embryoids were obtained from petioles of the 'Picual' variety (Toufik et al. 2014), without double regeneration. Similar results were obtained by Capelo et al. (2010) and Mazri et al. (2013) with one wild genotype (var. *sylvestris*) and var. 'Dahbia,' respectively. Thidiazuron (TDZ) and cefotaxime seem to be two very important components of the growth medium to induce somatic embryogenesis (Rugini et al. 2016a).

Usually, secondary embryos are differentiated from cells of the epidermal surface of the somatic embryo, although their unicellular origin is not still clear (Lambardi et al. 1999). The unicellular origin of new somatic embryos is of great advantage in regenerating plants from transgenic explants because it avoids the formation of chimeric plantlets. Establishing a very efficient long-term cyclic somatic embryogenesis is an extremely useful approach to elude the onset of somaclonal variation during in vitro germplasm conservation.

In our experience, evident phenotypic variation was never observed in the field-grown plants of var. 'Canino' derived from few cycles of somatic embryogenesis. On the other hand, plants derived from long-term (more than 3 years in culture) cyclic somatic embryo culture displayed narrow leaves and reduced growth (Rugini personal communication) compared to the plant morphology of the original parental plant. This variation could be due to regeneration of embryos from calluses that sporadically can be formed in aged tissue cultures. A different vegetative behavior (bushy and columnar phenotype) has been reported by Leva and Petruccelli (2007) for plants derived from somatic embryos

after many cycles of regeneration from an original cotyledonary explant of one seed of 'Frangivento' olive variety. This suggested that genetic variation widens during the regeneration cycles. However, Lopes et al. (2009) observed a genome integrity throughout the stage of embryogenesis in the *Olea* spp.

These conflicting results suggest that for getting true-to-type olive plants it is necessary to pay attention to the genetic stability of the somatic embryos derived from cyclic somatic embryo cultures. However, at present it seems to be unlikely to use this technique for mass propagation, because the conversion to plants normally is too low and the rejuvenation of the derived plants may be high, delaying flowering. The evaluation of plants in the field at full maturity is advisable in order to discriminate the epigenetic variation, often due to juvenility of somatic embryo acquired in vitro, from the variation due to genetic mutations. Finally, somatic embryogenesis could be applied for the production of 'synthetic seeds' or 'encapsulated embryos,' useful also for germplasm preservation (Lynch et al. 2007).

Protoplast Technology

Protoplast technology is useful for several studies including protoplast fusion in an attempt to produce (a) somatic hybrids from cross-incompatible genotypes, (b) triploid and polyploid plants from protoplasts with different nuclear polyploidy levels, or (c) genetic transformation by introducing foreign naked DNA into cells by liposome carriers. Viable olive protoplasts from hypocotyls, cotyledons, and leaves of micropropagated shoots were isolated and cultured, and in some cases also microcalli have been obtained. However, plant regeneration has not been attempted yet (Rugini 1986; Cañas et al. 1987; Mencuccini 1991; Perri et al. 1994), although at present time morphogenetic tissues can be produced by using recent protocols of 'double regeneration system' on tissues from somatic embryos (Rugini et al. 2016a).

5.5.4 Genetic Transformation and Plant Recovery

'Plant gene therapy' aimed to correct the defects of the most important commercial varieties could be an important strategy to reduce the time and cost of the genetic improvement. However, two important factors are essential: the availability of morphogenetic tissues of valuable cultivars and the availability of useful genes. Transgenic plants with the *rolABC* and *osmotin* genes have been achieved with the aim to modify canopy architecture and to increase rooting ability and to improve tolerance to abiotic and abiotic stresses, respectively. Those plants have been tested in the field before flowering and then the trial was interrupted by the Italian Minister of Environment, who did not renew the authorization to continue (Rugini 2015). Subsequently, other attempts demonstrated the potentiality of this technology in improving some characters in the olive tree, and other research projects are underway to improve the technologies of gene transfer (Torreblanca et al 2010; Titouh et al. 2014).

Improvement of Rooting Ability with Agrobacterium Rhizogenes

The wild type strain of *Agrobacterium rhizogenes*, NCPPB 1855, has been used in vitro to induce rooting or to strengthen the root system of olive varieties. Nearly 100 % of the transgenic micro-cuttings, even those from varieties difficult to root, produced roots (Rugini 1986, 1992). Rarely the roots resulted transgenic, probably because they had originated from untransformed cells near the transformed ones or, to a less extent, the root induction derived by either unknown compounds present in the *Agrobacterium* exudates or by a transient expression of *Ri*T-DNA (Rugini et al. 2000). In greenhouse experiments, Strobel et al. (1988) increased the root mass, by infecting the primary root system (uniformly trimmed to 4–5 cm in length) with *A. rhizogenes* strain 232. The increase of root mass resulted beneficial in both vegetative and

reproductive growth parameters, although the new roots appeared poorly connected with the existing primary roots. However, more scientific information is needed to explain the many different responses that could be obtained by transforming different plant varieties or species with the same *A. rhizogenes* vector. The effect on the transformation event cannot be completely effective because a simple infection to induce root formation in cherry and plum varieties affected also the morphology and reproduction of the plants (Rugini 2015; Rugini et al. 2016a).

Modification of Canopy Architecture and Rooting Ability with Rol Genes

Several works in gene transformation of olive somatic tissues were carried out using *Agrobacterium tumefaciens* strain LBA 4404 harboring pBin19 with *rolABC* from *A. rhizogenes* and the gene *npt*II for kanamycin resistance, under the control of a natural promoter. In the first transformation, attempt of zygotic embryos of the var. 'Moraiolo' resistant to kanamycin was selected by Rugini and Fedeli (1990). Subsequently, transgenic plants were obtained from transformation of somatic embryos of the var. 'Canino.' The derived transgenic plants were cloned in vitro and transplanted to field in 1998 (Rugini et al. 2008). *RolABC* plants showed the typical hairy root phenotype and prolonged vegetative growth up to late autumn. The plants, although originated from mature tissues, expressed a long juvenile phase. However, after 10 years, the plants still maintained the initial phenotype and a correct transcription of the transgene, as shown by real-time PCR analysis (Miano et al. 2004). Transgenic plants revealed, in both in vitro and greenhouse tests, high sensitivity to auxin. Fifty percent of the in vitro explants rooted in the auxin-free medium while rooting was up to 60 % in medium containing only 160 mg/l of putrescine; the untransformed explants did not root at all. Similar results were obtained by the semi-hardwood cuttings collected from transgenic field-grown plants.

Genetic Transformation to Improve Tolerance to Biotic and Abiotic Stresses

Transgenic plants overexpressing the *osmotin* gene in the field have been obtained by genetic transformation of olive explants with *A. tumefaciens* LBA4404 harboring the pKYLX71 plasmid, containing the tobacco *osmotin* gene under the control of *35S* promoter (Rugini et al. 2000). The osmotin gene is present in all genomes of the plant species tested so far, and codes for a protein, belonging to the pathogen-related protein (PR5) family. In plants, this gene is normally expressed under both biotic and abiotic stresses, particularly under drought condition and fungal diseases. In addition, the osmotin protein proved to be a homolog of the mammalian hormone adiponectin, which is involved in glucose metabolism. Recent studies revealed that osmotin determines its therapeutic efficacy in different animal diseases modulating adiponectin receptor 1 and may become the basis of new therapeutic strategies for the treatment of various diseases including diabetes, cancer (Naseer et al. 2014), and central nervous system disorders including Alzheimer's disease (Shah et al. 2016). After ten years in the field, the osmotin-expressing transgenic plants showed a substantially similar phenotype to the untransformed plants derived by somatic embryogenesis. The few differences observed relate to the narrower leaf lamina and the high amount of osmotin around cell vacuoles of epidermal and subepidermal tissues of transgenic plants (D'Angeli et al. 2001). In addition, transgenic plants were more tolerant to *Spilocaea oleagina* but showed a particular and unexplained attractiveness for insects, such as *Otiorrhynchus cribricollis* Gyllenhal and *Lichtensia viburnii* Sign. Furthermore, overexpression of osmotin induced cold protection (D'Angeli and Altamura 2007) and an extraordinary drought resistance (Rugini 2015) by affecting programmed cell death and cytoskeleton organization. In the field trial, the young osmotin plants showed an evident suffering under ordinary irrigation supply, with initial slow growth, leaf drop,

root system rot, and plant death (Rugini et al. 2000), whereas the unirrigated plants were healthy until the end of the trial. In the experiments carried out in pots, during summer time, the 2-year-old osmotin-transgenic plants, derived from 3 different transformation events, demonstrated extraordinary drought resistance in comparison with analogous plants of 'Canino' and the '*rolABC* transgenic Canino' grafted on 'Canino' as rootstock (Rugini 2015). The drought resistance was confirmed in vitro under treatment with 2 % and 4 % polyethylene glycol (PEG): the osmotin-transgenic plants evidenced a greater tissue accumulation of proline and of other drought-stress-specific enzymes (Silvestri et al. submitted). Further olive-transformation experiments were carried out by Torreblanca et al. (2010) that attempted transformation using somatic embryos derived from radicles of mature seeds of var. 'Picual.' They used *A. tumefaciens* harboring pBINUbiGUSint or pGUSINT binary plasmids contained the *nos-npt*II and the *uidA* gene driven by the maize poly-ubiquitin *Ubi1* and *CaMV35S* promoter, respectively.

Using genetic transformation, many important traits with commercial significance may be improved in olive trees, including the production of completely self-fertile plants, the increase of fruit oil content and quality, the production of parthenocarpic fruits, the increased tolerance to cold and salt stress, the regulation of fruit ripening, and the increase of resistance and tolerance to pathogens and parasites.

6 Future Research Challenges and Potential Solutions Through Collaborative Research

The points raised in the previous paragraphs evidenced several aspects to be considered in olive tree breeding. Firstly, the olive clonal varieties share several similarities to other fruit tree crops because the olive trees are highly heterozygous, long-lived perennials with late sexual maturity, and a lengthy juvenile phase; genetic diversity among olive groves and adaptation to rapid climate changes is an insurance policy against alternate bearing and environmental challenges. Secondly, most olive varieties have narrow regional adaptation, so the number of varieties used for planting must be higher than those in most annual crops. Thirdly, olive trees serve as keystone species under climate change, so managing against loss of olive groves translates into more sustainable agricultural system management in Mediterranean environments. Fourthly, the residual wild olive (oleaster) populations should be preserved and become the target of a new domestication wave with some population-level improvement in adjacent agricultural areas providing the ecotonal features for gene flow between genuine oleasters and improved populations for maintaining the genetic diversity for new progeny haplotypes amenable to GS.

Over the last decades, top-down approaches from whole-plant phenotypes to the molecular genomic level have been developed to identify phenotype-to-gene associations for traits such as fruit yield. That approach progressively substituted those based on finding Mendelian genes only for traits exhibiting discrete phenotypic classes. In long-lived and slow-growth perennial species such as olive trees, a bottom-up approach from gene-to phenotype is now being developed for breeding new varieties. This approach lies in finding genome-wide marker data that effectively select for multi-genic quantitative traits early in the breeding cycle. Marker alleles identified by GBS, WGAS, and candidate genes discovered by gene expression profiling, genetic variant analysis, and Eco-TILLING (Wang et al. 2012) in full-sib and multi-parental intercrossed progenies promise the identification of natural mutations with large genetic effects for a trait phenotype.

Advancement in the sequence of other olive genomes such as the chloroplast (cp) genome will increase the efficiency of phylogeographic studies in *Olea* gene pools. The cp-genome has already been used as a versatile tool for *Olea* phylogenetics (Besnard et al. 2013). However, its resolution power can greatly be increased at lower taxonomic levels using specific DNA barcode (Mariotti et al. 2010). Selection of a suitable locus displaying adequate species-level

divergence (Kuang et al. 2011; Dong et al. 2012; Besnard et al. 2013) might enhance the ability to distinguish closely related plants at the species and population levels (Mariotti et al. 2010; Li et al. 2015).

The necessary genomic technologies to support conventional and unconventional genetic improvement have now been developed for olive. They allow us to get the necessary results for speeding up the breeding procedures and there are no more excuses for not immediately addressing the genetic improvement of this important species. The in vitro culture and cloning of immature embryos is an important insertion in the olive breeding procedure that significantly reduces the time to get replicated seedling genotypes which can be obtained in less than one year.

Therefore, the proper integration of genome-wide markers, WGAS, QTL mapping, and GS to predict the breeding value of *ortet* from in vitro immature embryo culture, and cloning of the olive *ortet* will allow the rapid multiplication and use of *ramets* for large-scale field evaluations, selection, and new olive variety release.

In addition, the shoot or somatic embryo regeneration from in vitro culture of tissues of adult and valuable varieties will allow an easy recovery of genetically stable plantlets from cells modified by several biotechniques (gene transfer, gene editing, or mutagenesis). A novel method to rescue mutants in varieties that are recalcitrant to in vitro regeneration is now available and is based on (a) gene modifications in cells close to the root system of in vitro plantlets, (b) transplant of the plantlets in the field, and (c) selection of mutant suckers spontaneously grown from putative genetically modified cells in the crown area of the plantlet.

7 Conclusions

Nowadays, olive breeding aims to the adoption of genomic resources to speed up the breeding methodology for rapid identification and selection of *ortets* within the available gene pool or in progenies from planned mating designs.

The available gene pools are not well characterized for the presence, inheritance, and efficiency of gene transfer to mitigate defects of the available olive varieties.

Despite recent significant efforts, the development of knowledge on single-locus traits and QTLs has stalled, leaving the efficiency of olive breeding at a crossroad.

No single-step breeding methodology is available to achieve the olive breeding goals in less than 6–7 years due to: (a) conventional management of the genetic resources to produce, evaluate, cloning, and selection of new genotypes, and (b) the delayed development of genomic resources for olive-wide genomics-associated studies.

To overcome these critical limiting factors, the current selection activities based on a 14-year breeding procedure to identify the genotypes for new olive varieties need to be accelerated by the integration of genome-wide markers, GS, and biotechnological advancements for in vitro embryo germination and cloning of the seedling genotypes.

Fortunately, developments in DNA sequencing started in 2016 will allow cost-efficient preparation of genomic resources from sequencing projects and will drive the acquisition of information on genes for important economical and agronomical olive traits and set the stage for an accelerated olive breeding procedure.

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