Olive Breeding with Classical and Modern Approaches

10

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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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