Chapter 12
Olive Breeding

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Abstract The olive (*Olea europaea* L.) is, at the same time, one of the most ancient domesticated fruit trees and the most extensively cultivated fruit crop in the world, covering an area of about 7.5 million hectares. The recent diffusion of olive outside its traditional area of cultivation, the Mediterranean basin, together with a continuous trend in the modernisation of its industry, has greatly increased in recent years the demand for improved cultivars by olive growers. Hence, programmes of clonal selection and cross-breeding have been started in the main olive growing countries, aiming at selecting genotypes characterised by early bearing, resistance to pests and to abiotic stresses (such as frost and drought), limited alternate bearing, suitability to intensive culture and to mechanical harvesting, as well as high-quality productions, in terms of both organoleptic characteristics of fruits and oils, and high contents in substances useful for human health. This chapter reviews the recent advances in olive breeding, providing extended information on flower biology, main world cultivars, germplasm collection and preservation, propagation techniques, main characters for olive improvement and traditional breeding techniques (clonal selection, cross breeding and mutagenesis). In addition, information on the recent developments of olive biotechnology for the improvement and the safeguard of genetic resources (tissue culture, synthetic seed technology, genetic transformation and cryopreservation) is also reported.

12.1 Introduction

The olive (*O. europaea* L.) is one of the most ancient domesticated fruit trees and its products have been valued since ancient times. The oil extracted from the fruit mesocarp is a valuable and healthy food, but in ancient times its importance was also due to other uses, such as lamp fuel, wool treatment, medicine and cosmetic, soap production and the like. As a food, it is used for salads, for cooking and to preserve

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S.M. Jain, P.M. Priyadarshan (eds.), *Breeding Plantation Tree Crops: Tropical Species*,

Table 12.1 Average world production of olive (2000–2005) according to the International Olive Oil Council (http://www.internationaloliveoil.org/)

	Olive oil			Table olives	
	($\times 10^3$ t)	%		($\times 10^3$ t)	%
World	2739.3	100.0		1541.9	100.0
EU countries	2171.1	79.3		681.8	44.2
Other countries	568.2	20.7		860.1	55.8
Leading countries					
Spain	1051.3	38.3	Spain	497.9	32.2
Italy	683.1	24.9	Turkey	161.5	10.5
Greece	394.2	14.3	Egypt	136.7	8.9
Tunisia	142.8	5.2	Syria	134.2	8.7
Turkey	112.3	4.1	Greece	101.7	6.5

other foods. Table olives are also a typical component of the Mediterranean diet and are consumed after processing and pickling in different ways.

The olive originated in the Mediterranean area and the countries bordering the sea still produce 97% of the world oil production. The main producers are Spain, Italy and Greece for olive oils and Spain and Turkey for table olives (Table 12.1). In addition, olive culture is expanding to many other countries outside the Mediterranean basin, such as the United States (California), Australia, China, South Africa, as well as in other sub-tropical and warm temperate areas, usually in fairly arid regions and on well-drained soil. Today, olive is the most extensively cultivated fruit crop in the world, covering an area of 7.5 million of hectares (http://faostat.fao.org/).

The earliest signs of olive cultivation can be traced back to 4 B.C. and before (Zohary and Spiegel-Roy 1975; Loukas and Krimbas 1983; Zohary and Hopf 1994) to areas of the Eastern Mediterranean coasts and islands, although the ancestors of currently grown olive cultivars are still believed to have been domesticated in the mountainous territory, south of the Caucasus, covering today's western Iran, Eastern Turkey, Lebanon, northern Israel, Syria and northern Iraq. From the Eastern Mediterranean the olive moved westwards to Greece and the Aegean archipelago, although Crete and Cyprus probably belong to the oldest olive centre of diversity. In these areas, collectively considered a secondary centre of diversity, the olive grew in importance and underwent further selection by humans, in the period between 3 and 2 B.C. In Crete, in 16 B.C., there existed in Knossos a huge deposit of clay jars (still visible today in the excavation site) able to store five times the amount of oil the local population could consume in 1 year, thus indicating a well-developed trade in olive oil.

Around the beginning of 1 B.C., a second migration appears to have taken place, again westwards, to Sicily and Tunisia, an area regarded as olive's tertiary centre of diversity. From there, around 600 B.C., probably through Etruria (today's Tuscany), the crop is reported by the classical historians to have reached the Romans. Up to this point the olive had moved slowly westwards, first on the ships of Phoenician merchants and later on those of Greek colonists. These peoples had spread the crop in many other places of the Mediterranean Sea including Spain, France

and North Africa with varied results. But the conquest of the whole area by the Roman legions and its transformation into a vast, united empire, made trade and communications far more intense. The olive benefited from this situation, and the Romans spread its cultivation in new areas or favoured it in places where it stagnated, especially when Italy appeared unable to provide the required supply of olive oil. The crop achieved its maximum economic importance in the II–III centuries A.D., particularly in Northern Africa, but also in Spain, Dalmatia and the French Provence. With the fall of the Roman Empire information about the olive becomes scarce. Its cultivation dropped dramatically with the reduction in population and the abandonment of large areas that took place in the course of the early Middle Ages. This was not the case in the territories under Arab rule, where the crop remained important to the point that its cultivation was forbidden in Sicily, in order to protect production in North Africa, probably the main producer at the time (Acerbo 1937).

In Europe, olive oil acquired new importance only in XVI–XVII° centuries, when it became a significant trading commodity for Venetians who imported it from their Aegean possessions, such as Crete and Cyprus. Thus, slowly olive plantations began to spread in the Mediterranean areas where they can still be found today, with the exception of most of North Africa, where it was reintroduced on a large scale much more recently.

The arrival of the olive in the Western and Southern hemispheres is recent. Argentina, California, Australia and South Africa, where the enthusiasm for the crop of Mediterranean migrants had ensured its introduction across the last century, all proved to have suitable environments for olive commercial cultivation.

12.2 Botany of *Olea europaea* L.

12.2.1 Vegetative Structures

Olive is a long-living evergreen tree. In suitable environments millenary trees are not uncommon. The wood resists decay and the tree, if destroyed by adverse events, can easily regenerate from suckers (‘pollards’), which are abundantly produced by roots and by particular structures located around the collar, the ovules.

The cultivated olive is a medium-size tree, 4 to 8 metres high, according to cultivar and management. The trunk has a smooth surface in the young tree that soon becomes rough and twisted. The canopy, if unpruned, tends to form a dense globe. The shoots are slender and may carry either leaf buds or flower buds at leaf axils; mixed buds are rare but can occur. Seedlings have a long juvenile stage and the tree is traditionally propagated by cutting or grafting (see Section 12.4.1.). Juvenile structures, which at times are seen as suckers produced by the rootstock, have shorter internodes, smaller and darker leaves and can be thorny. The leaves are thick, persistent (may last 2–3 years), oppositely arranged. The upper surface is strongly cutinized and the lower surface, where stomata are, is covered by a thick felt of peltate hairs that gives it a silvery appearance. The root system, in

spite of the marked resistance of the tree to drought, is relatively shallow, although soil characteristics and management have a major influence on its distribution (Rapoport 1999).

12.2.2 Flower Biology

12.2.2.1 Inflorescence Formation and Flowering

Flower bud inflorescences are borne at leaf axils (hence a maximum of two per node). Usually flower buds are formed on the shoots developing the year before anthesis. The formation of flower bearing buds is a process requiring the passage of the meristematic apex of the bud, undifferentiated in its early stages of growth, to a structure carrying flowers. Flower differentiation takes place in winter (that is, in the Northern Hemisphere, in the period from late February to mid-March) although in some areas it may last longer. The timing and extent of flower differentiation seem to depend on the achievement of specific chilling requirements, as low winter temperatures influence not so much floral evocation as rather the expression of a flowering potential already determined in warmer periods. As a rule, floral differentiation occurs during the 40–60 days before anthesis and the process is completed as the inflorescence emerges and develops (Fabbri and Benelli 2000).

Inflorescence development begins in early spring, roughly one month after the onset of flower differentiation, usually starting on the south side of the tree (in the Northern Hemisphere). It is gradual, and the time between inflorescence emission and anthesis is usually around 4 weeks, up to 6 or more in warmer climates, in separate flushes. As a rule, the earlier the emission of inflorescences, the higher the expected production, as fruit set may take place in less dry conditions. However, environmental events may markedly alter the forecast. Flowers are usually borne on 1-year-old shoots. Only occasionally inflorescences develop on 2- or 3-year-old branches.

Two types of flowers are present each season: ‘perfect flowers’, containing stamens and pistils, and ‘staminate flowers’, containing aborted pistils and functional stamens. ‘Ovary abortion’ refers to absence of ovary or to small, imperfect, non-persistent ovary. All olive trees display ovary abortion, although at different extents, depending on cultivar, environment, year, inflorescence and type of shoot. Its incidence is variable, from less than 10–70% to more. In spite of that, production is usually not depressed as normal harvests require no more than 4% of fruit set. One-hundred percent abortion cannot exist in a commercial cultivar, with the exception of ‘Swan Hill’, an ornamental cultivar, selected by Hartmann in Australia (Hartmann 1967), which displays this character advantageous for olive trees utilised in urban forestry or as ornamentals.

12.2.2.2 Anthesis and Pollination

Full bloom occurs in full spring (e.g., May in warm areas such as California, Southern Italy, Greece and Spain; at higher altitudes and elevations full bloom is

delayed up to mid-June). Differences can be observed among cultivars, which are to be kept into account when selecting pollinators. Anthesis normally lasts 2–3 days on individual inflorescences and 5–6 days on the individual tree (up to 10–15 days if temperatures are relatively low). A flower is fully opened when both anthers and petals are separated. During the hottest part of the day anther dehiscence takes place and an abundant amount of pollen is shed.

The amount of pollen produced appears to be a varietal characteristic: for example, 'Leccino' and 'Frantoio' (two oil cultivars very common in Central Italy) produce small amounts of pollen, and larger quantities are produced by 'Ascolana', 'Manzanilla' and 'Pendolino'. More important is the pollen's ability to germinate: this characteristic appears to fluctuate (in vitro) between 12 and 60% (Zito and Spina 1956; Fernandez-Escobar et al. 1983).

Pollination is influenced by several factors, the most important being (Fabbri et al. 2004):

- temperature, which has the effect of enhancing tube growth, although, when too high, the stigma may get dry. For anther dehiscence the optimum is 30°C with 50% of Relative Humidity (RH). A good value of RH also enhances pollen germination;
- rain, which is always negative; indeed, it may determine pollen grain plasmolysis, dilute stigma secretions and hinder pollen transport;
- wind, which is fundamental for this anemophilous species. When too strong, the wind may transport masses of pollen away from the grove. Although olive pollen can be found as far as 12 km from the tree, the effective range is considered to be not larger than 30 m.

12.2.2.3 Sterility

Sterility may be due to factors different from those affecting ovary abortions, such as anomalies during meiosis producing imperfect gametophytes ('cytological sterility'), quite rare in olive, and incompatibility ('factorial sterility'). Incompatibility occurs when a perfect pollen grain fails to germinate on the stigma or germinates, but its tube growth is somehow impeded. Incompatibility may be between two cultivars ('inter-' or 'cross-incompatibility') or when a cultivar is genetically programmed not to be fertilised by its own pollen ('self-incompatibility').

'Self-' and 'cross-incompatibility' mechanisms are both common in olive and have been the main reason for the large genetic variability typical of the species. This aspect has been extensively studied due to its recognised importance in the establishment of olive orchards (to assure inter-compatibility among cultivars and, hence, productivity) as well as when breeding programmes are designed. Notwithstanding, contradictory results have been often reported as the behaviour of some of the main important cultivars (e.g., 'Arbequina', 'Frantoio', 'Manzanilla de Sevilla' and 'Picual') resulted, according to different studies, either 'self-incompatible' or 'self-compatible' (Diaz et al. 2006a). More investigation seems necessary on this aspect particularly to ascertain the genetic control of the 'self-' and 'cross-compatibility' mechanism in olive.

12.2.2.4 Fruit and Seed Development

In olive, the average final fruit set (i.e., the ratio between number of fruits persisting until maturity and the initial number of flowers) is around 2%, although yearly fluctuations can be wide. Fruit drop is utilised by the plant to adapt production to its elaborating surface. Other factors may influence fruit drop, such as nutritional and water deficiencies, weather conditions during bloom, sterility, lack of pollinators and pests.

The olive fruit is a drupe, which means it is made of two main parts, that is, pericarp and seed. The pericarp is made of (i) the skin (exocarp), free of hairs and with stomata, (ii) the flesh (mesocarp), the tissue containing oil, and (iii) the pit (endocarp), a lignified shell enclosing the 'true seed'. The pit and the contained seed are the olive 'stone'. The seed consists in a seed coat and a thick endosperm that ensheath a large embryo, made of flat cotyledons and of short radicle and plumule. As a rule there is only one seed per fruit, rarely two. In some Spanish cultivars the occurrence of nucellar polyembryony has been reported. Fruit shape and size, pit size and surface morphology vary greatly among cultivars, and are the most reliable morphological features to distinguish between cultivars.

The embryo makes up for most of the seed volume. The seed coat, derived from the integuments that represented the main ovular tissues, is thin, leathery and rich in vascular ridges. Between seed coat and embryo is a layer of endosperm, rich in starch (King 1938). The embryo has two quite evident large cotyledons, the embryonic leaves. A short radicle, located at the lowest end of the embryonic axis, will give rise to the root system. Between the cotyledons is a small plumule from which the future epigeic system (i.e., the plant parts that will be exposed to the open atmosphere) will develop. The embryo is usually completely formed after five months from full bloom. No further morphological or anatomical changes appear to occur in the embryo, although dormancy is imposed on the seed later in the season. Seed growth means a gradual embryo enlargement, which at the end occupies most of the space inside the endocarp at the expense of the endosperm.

Usually pollination and fecundation are essential for fruit set and early seed development. The presence of a vital seed in a growing drupe is not essential for fruit development. Indeed, many apparently normal fruits have no seeds. The fruit can also develop without the presence of a fertilised ovule (parthenocarpy), but in this case the fruit remains distinctly smaller.

12.3 Genetic Resources

The latin scientific name of the cultivated olive is that given by Linnaeus in 1764, *O. europaea* L. The latin name of the genus is believed to derive from the Greek word *elaion* (oil), while the name of the species underlines its European (or, better, Mediterranean) distribution. The Mediterranean climate is characterised by mid-season rains, dry summers and winters, a short-lived cold season with occasional frosts. So typical is the olive of such climate that it is the species better suited to

define a climate as Mediterranean. Few other areas in the world possess similar climatic features and olive culture has been introduced recently in many of them.

The cultivated olive belongs to the family Oleaceae. The family, made up of more than 30 genera and over 500 species, is distributed in tropical and temperate regions of the world. The genera include some ornamental and agricultural plants, such as *Forsythia, Fraxinus, Osmanthus, Jasminum, Ligustrum* and *Syringa*. The fruits of the Family are drupes (*Olea* spp.), berries (*Jasminum* spp.), capsules (*Syringa* spp.) or samaras (*Fraxinus* spp.). *O. europaea* is the only species of the genus *Olea* that can be found in the Mediterranean basin and the cultivated olive originated from the wild form 'oleaster' that is still present in most coastal areas (Zohary and Hopf 1994; Fig. 12.1). All the other species (over 30) are distributed in subtropical to warm temperate areas of both the Northern and Southern Hemispheres (Bartolini and Petruccelli 2002).

12.3.1 Wild and Cultivated Species

Within the cultivated *Olea* two subspecies are distinguished: *O. europaea* L. subsp. *sativa* (Loudon) Arcangeli (= subsp. *europaea*), to which belong the numerous cultivated varieties, and *O. europaea* L. subsp. *oleaster* (Hoffm. & Link) Negodi (= subsp. *sylvestris*) (Miller) Hegi, to which belong the spontaneous forms commonly called 'oleasters' (Ciferri 1950; Zohary 1994). The relationship between the cultivated varieties and the wild forms has been the object of many hypotheses. The ancestor of cultivated olive is supposed to be the wild form, easily distinguished by thorny shoots, small and roundish leaves, small and elliptical fruits with a thin oily mesocarp (Rugini and Lavee 1992; Zohary 1994; Amane et al. 1999). The domestication of the oleaster goes back to 4,000–3,000 B.C. when mass selection of trees started, presumably choosing the trees whose fruits were larger and richer in oil.

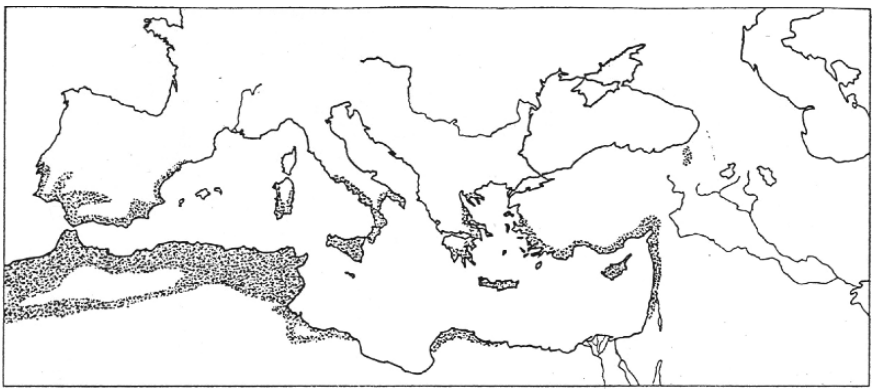

Fig. 12.1 Distribution of the wild olive (*O. europaea* subsp. *oleaster*) is the progenitor of the cultivated olive. This ancient form of the species is still present in several coastal areas of the Mediterranean basin (from Zohary and Spiegel-Roy 1975)

The selection was made possible by the easy vegetative propagation of the species with traditional techniques.

The botanical origin of the olive is not completely clear, and several *Olea* species have been considered as possible ancestors. A marked morphological similarity exists between spontaneous and cultivated forms of *O. europaea*, and species growing outside the Mediterranean basin – such as *O. chrysophylla* Lam. (= *O. africana* Miller), diffused in Africa and Asia, *O. excelsa* Ait. of the Canary Islands and *O. ferruginea* Royle (= *O. cuspidata* Wall.), native of Central Asia – have been indicated to be the ancient parents of the olive (Chevalier 1948). For Ciferri and Breviglieri (1942) and for Vavilov (1951) the ancestors could have been *O. Laperrini, O. chrysophylla* and *O. cuspidata*. Abundant data is being produced by molecular analysis of *Olea* genomes and more light is expected to be shed on the origin of the species (Ganino et al. 2006a).

Cultivated olives are nearly all diploid with $2n = 2x = 46$; occasional triploids and tetraploids have been reported as well as one case of polysomaty ($2n = 55$). The chromosome number of the species was first established by Breviglieri and Battaglia (1954) whose observations on chromosome morphology led them to assume that the species had originated by allopolyploidy, probably by parents whose haploid chromosome numbers were $n = 11$ and $n = 12$. These chromosomal numbers are present in several species of the Oleaceae Family. The hybrid is sterile and unstable, and its survival must have been granted by duplication of the genome. With reference to the centromere position, the chromosomes are of the median and sub median type. Three pairs of satellite chromosomes have been identified, smaller than in other species (Falistocco and Tosti 1996).

12.3.1.1 Characterization of the Cultivars by Morphological, Biochemical and Molecular Markers

Due to its early domestication and its large spread in the Mediterranean basin, the olive is particularly rich with cultivars, with a large number of synonyms and homonyms that make their description and classification particularly difficult. Cultivars are better defined as 'cultivar-populations' as they generally comprise clones that are separated by a number of minor characters (Morettini 1954a; Scaramuzzi and Roselli 1986; Roselli 1990). A detailed study has listed over 1,200 cultivars originating from 34 countries and preserved worldwide in 79 collections, with over 3,200 synonyms (Bartolini, Prevost, Messeri and Carignani 1998). More than 800 cultivars are for oil production, over 100 are table olives and the remaining are used for dual purpose.

Many botanists tried to describe olive cultivars without resulting in an accurate classification (e.g., Prevost and Mostardini 1999; Ganino et al. 2006a). At the onset of the nineteenth century, Simòn de Rojas Clemente (in Barranco and Rallo 1984) and Tavanti (1819) classified the olive cultivars depending on leaf, fruit and endocarp characteristics. Based on these three morphological characteristics, Ruby (1917) evaluated the differences among French cultivars. However, with the necessity to evaluate biological and ecological characteristics, in addition to the

morphological ones, Ciferri et al. (1942) made a morpho-ecological classification of olive. Using these numerous morphological and biological characters, a very detailed descriptor list was prepared for 70 olive cultivars grown in central Italy. Although the system had some limitations, the list soon became a fundamental reference for scientists. Afterwards, Barranco and Rallo (1984) prepared a pomological list (including characters of fruiting branches, leaves, inflorences, fruits and endocarps) to identify Andalusian olive cultivars. With the aim of making uniform the methodology of germplasm data collection, another descriptor list was prepared in 1985 under the supervision of Union Internationale pour la Protection des Obtentions Végétales, Geneva (UPOV). Other descriptor lists worthy of mention have been, in the following years, those of Leitão et al. (1986), Cimato et al. (1997) and Rallo et al. (2005).

The complexity of classification and the drawbacks of the morphological markers, which can be affected by the environment and the plant developmental stage, was the reason for the development of biochemical (isozymes and allozymes) and molecular (e.g., RFLP, RAPD, AFLP, SSR) markers for cultivar characterisation. A first attempt for the discrimination among olive cultivars by biochemical markers (i.e., the isozyme analysis of the olive pollen) dates back to the early 1980s (Pontikis et al. 1980). Following investigations demonstrated that biochemical markers, based on the detection of polymorphisms in enzyme protein composition, were suitable to characterise olive germplasm (Trujillo et al. 1990; Potes et al. 1999). A drawback in the use of pollen for varietal identification lay in the fact that sample collections were possible only at specific periods of the year (Trujillo et al. 1999). Analyses of phenolic content (Heimler et al. 1994) and of seed storage proteins (Durante et al. 1992) have been other attempts made in olive for the biochemical identification of cultivars. However, in time it was evident that these approaches were insufficient for a clear discrimination of plants. The reason was due to the limitations of isoenzymatic analyses, such as (i) only a small part of the structural genes is represented, (ii) nucleotide alterations are often undetectable because not all nucleotide substitutions result in a variation of amino acids, and thus at the protein level, and (iii) isozymes produced as a result of transcription and translation are regulated by several factors, including origin and physiological stage of tissues and environmental conditions, and thus they present scarce reproducibility.

Development of molecular markers revolutionised genetic analysis of plant genomes as they are free from environmental influence and have potential to identify variations at the DNA level. Restriction Fragment Length Polymorphism (RFLP) markers have been used to distinguish wild olives from cultivated varieties, confirming the Mediterranean basin as the site of olive domestication (Besnard et al. 2001; Besnard et al. 2002). They also permitted the analysis at molecular level of 95 plants obtained by the crossing of ‘Leccino’ with ‘Dolce Agogia’ (De la Rosa et al. 2003).

The Random Amplified Polymorphic DNA (RAPD) analysis, based on the Polymerase Chain Reaction (PCR) technique, does not contain the technical inconveniences of RFLP markers as only a very small amount of genomic DNA (25–100 ng) is sufficient, and the process is relatively fast and simple. This technique

has been applied successfully to identify olive cultivars from several countries (Fabbri et al. 1995; Cresti et al. 1996; Durante et al. 1999; Mekuria et al. 1999; Belaj et al. 2001; Ergulen et al. 2002; Wu et al. 2004; Ganino and Fabbri 2005). In spite of their wide use for olive cultivar characterisation, RAPDs are dominant markers and thus cannot differentiate homozygote from heterozygote, hence limiting their potential to be used directly as a selection tool for desirable traits in breeding programmes.

The combination of enzymatic digestion of DNAs and selective amplification of fragments has favoured the use of Amplified Fragment Length Polymorphism (AFLP) markers, which have high reproducibility and wide applications in cultivar identification, germplasm analysis and genetic mapping. AFLP markers have been used to determine genetic similarities and/or polymorphisms among the different forms of *Olea* (Angiolillo et al. 1999; Sanz-Cortéz et al. 2003; Montemurro et al. 2005; Owen et al. 2005; Grati Kamoun et al. 2006). AFLP markers have also been used jointly with RAPDs to study the presence of intra-cultivar variability (Belaj et al. 2004) as well as with SSRs (Bracci et al. 2006a; Montemurro et al. 2006)

The Microsatellites or Simple Sequence Repeats (SSRs) are the most recent and promising molecular approach to olive cultivar identification. The SSRs are codominant, easily reproducible, randomly and widely distributed in the genome, characteristics that make them very useful in plant breeding programmes. In a specific study on genetic diversity and relationships among 32 Italian and Spanish cultivars, SSRs showed the highest level of polymorphism and provided more information than AFLPs and RAPDs, although AFLPs was the technique revealing the highest number of bands per reaction (Belaj et al. 2003). In a few years, the application of SSRs to olive has been impressive and several groups have already given important contributions to discriminate among olive cultivars (Rallo et al. 2000; Sefc et al. 2000; Bandelj et al. 2002; Lopes et al. 2004; La Mantia et al. 2005; Bracci et al. 2006b; Trujillo et al. 2006; Diaz et al. 2006b; Ganino et al. 2006b). In addition, important information is also expected from the applications of the SSR technique to the mapping and breeding of olive genome (Cipriani et al. 2002; De la Rosa et al. 2004; Wu et al. 2004) also to construct genomic linkage maps of olive and to allow for early selection of progenies according to their growth and fruiting characteristics (‘marker-assisted selection’). The technique has been used as well to determine the varietal composition of olive oils through the analysis of DNA extracted from the oil (Breton et al. 2004; Testolin and Lain 2005). The major drawback of the analysis lies in the time and cost required for SSR isolation.

Other molecular markers such as Sequenced Characterised Amplified Region (SCARs) and Inter-Simple Sequence Repeats (ISSR) have been used to a lesser extent in olive (Hess et al. 2000; Gemas et al. 2004; Busconi et al. 2006; Essadki et al. 2006). However, a promising research is in progress on single nucleotide polymorphisms (SNPs) in a fragment of phytochrome A gene of olive, using high-resolution DNA melting analysis to simultaneously scan mutations and genotypes with unlabeled probes (Muleo et al. 2006).

12.3.1.2 Main World Cultivars

Spain, the olive leading country with the highest table olive and oil production, has 183 olive cultivars (Bartolini et al. 1998), among which 'Sevillana' and 'Manzanilla' are known as the most important table cultivars. The former has large and golden-yellow coloured fruits with a flesh/stone ratio of 7.5:1. A clone of 'Sevillana', the 'Spanish Sevillana', is also grown in Algeria. The cv Manzanilla, grown worldwide, has medium size, symmetrical and apple-shaped fruits with green skin including tiny whitish spots. In addition to table olive cultivars, Spain has many important cultivars that are mainly used for oil production. Among them, 'Picual' is grown on almost 1/3 of the whole planted surface in Spain, and is therefore one of the most cultivated varieties in the world. It has an elongated and nearly symmetrical fruit with a medium/high oil content (22–23%). 'Arbequina' is another important oil cultivar of Spain, which is not only grown in Catalonia (North Spain), but also widely used for new plantations in Argentina and Chile; its suitability to mechanical harvesting is increasing its importance worldwide. The small, generally round shaped fruits of 'Arbequina' contain 20–21% of oil under non-irrigated conditions. Besides 'Picual' and 'Arbequina', 'Hojiblanca', which is grown mainly in the Cordoba district of Andalusia, has a medium content (17%) of good quality oil (Barranco 1999) and can be also used as a table olive. Other important oil cultivars are 'Cornicabra' and 'Morisca'.

Italian olive culture is characterised by an extremely high number of cultivars due to the earliness of introduction of the species, the variety of environments that olive finds in the country and the political fragmentation of its territory in the past centuries. As many as 538 cultivars, with over 1,300 synonyms, are reported (Bartolini et al. 1998). 'Frantoio' is one of the major oil cultivar. It originated in Tuscany, but it is now largely present in other regions of the country, often with different names (synonyms). It adapts to the most varied climates and, for this reason (and also for the high quality of its oil), it has been adopted in emerging olive-growing countries, such as the United States, Australia, South Africa, Argentina and Chile. It has an elongated fruit with 17–20% of oil content. 'Leccino' is another important Italian oil cultivar, originated from central Italy. The cultivar has a good resistance to strong frosts, which in several areas of Central Italy are periodically the cause of high damage to olive trees. Hence the cultivar is largely used in breeding programmes with the aim to select cold-resistant olive genotypes. Its elliptic-shaped fruit has around 17% oil content. 'Pendolino' is another cultivar largely used in olive orchards of central Italy, mainly because it is considered a good pollinator. 'Ogliarola' is the name of a group of cultivars of southern Italy characterised by high productivity and oil content. Other important Italian oil cultivars are 'Coratina', 'Canino', 'Carolea', 'Moraiolo' and 'Biancolilla'. Although Italy is one of the most important producers of olive oil, it is not considered among the leading countries for table olive production. Nevertheless, it has large-fruited cultivars, producing high-quality table olives. Among them, the cv Nocellara del Belice, which is similar to the Spanish 'Manzanilla' and the Greek 'Amphisis', is considered to be the best Italian table olive cultivar. 'Ascolana tenera' is another very important Italian cultivar, today

diffused also in other countries such as Israel, Mexico, Argentina and California. Among the dual-purpose cultivars, ‘Itrana’, ‘Giarraffa’ and ‘Tonda Iblea’ are worthy of mention.

‘Koroneiki’ is the main oil variety of Greece and is planted in over 50% of the country’s olive area, particularly in the Peloponnesus, in Crete and in other islands. It has a small ovoid-shaped fruit with high oil content; it is resistant to drought, but not to low temperatures. ‘Mastoidis’, ‘Kalamata’ (‘Kalamon’) and ‘Chalkidiki’ are other important Greek varieties with high oil content and a good flesh/stone ratio. ‘Amphisis’ (‘Konservolia’) is the main black table olive cultivar, constituting 80–85% of Greek table olive production. This cultivar is mainly grown in central Greece and it has a round-to-oval shaped fruits with the colour gradually changing, at maturity, from deep green to black with white dots.

‘Picholine’ is grown in Southern France and, together with the cultivar ‘Lucques’, is the most used in the French table olive industry. It is considered as a dual-purpose cultivar. It is also cultivated in Italy, Israel, Morocco and, occasionally, other olive-growing countries. Its fruit has 15–18% oil content under non-irrigated conditions. The oil is rather light in colour and of very high quality.

Turkey, Syria, Morocco and Tunisia, which have relatively high productions of table olives, generally prefer to use dual-purpose cultivars. Among them, Turkey and Syria have a high table olive consumption. ‘Ayvalik’ is the most important Turkish oil cultivar while ‘Domat’ and ‘Gemlik’ are those mainly used as table olives, where the former is consumed as green table olive and the latter is for black table olive (Ergulen et al. 2002). ‘Massabi’, ‘Sourani’ and ‘Temprani’ are among the best Syrian dual-purpose cultivars.

Other important cultivars are ‘Picholine marocaine’ and ‘Zitoun’ (Morocco), ‘Chemlali’ and ‘Chitoui’ (Tunisia), ‘Sigoise’ (Algeria) and ‘Nabali’. The latter, in particular, is a dual-purpose cultivar largely diffused in the Middle East, having a high oil content (about 30%).

For a detailed list of world olive cultivars, the ‘Olive Germplasm: Cultivars and World-Wide Collections’ database (http://apps3.fao.org/wiews/olive/oliv.jsp) can be consulted. The database is the 2005 web edition of a previous report (Bartolini et al. 1998) and contains information on 1,208 cultivars. Essential information on the main characteristics of cultivars (e.g., productivity, oil content and extraction, rooting ability, tolerance to abiotic and biotic stresses, biochemical and molecular identification) is also reported.

12.4 Olive Propagation

12.4.1 Traditional Propagation Techniques

In the olive-growing countries, olive propagation is achieved by rooting of leafy stem or softwood cuttings, by grafting pieces of stem (scions) onto seedlings or clonal rootstocks or, today only occasionally, by regenerating whole plants from the

ovules, that is, characteristic tissue hyperplasia that appear as protuberances at the collar of old trees (Fabbri et al. 2004). Among these techniques, rooting of leafy stem cuttings under mist is by far the most common technique. By the mid-1950s, the technique spread, especially in countries like Spain, where grafting had never acquired importance, and grew to become the source of over 70% of propagation material, leaving only about 20% of the market to grafted plants (Fabbri 2006). In general, cuttings are obtained from one-year-old or younger shoots by dividing them into 10–15 cm pieces of 4–6 mm in diameter, with 4–6 nodes and with the 4–6 leaves at the distal nodes maintained on the cuttings. In order to stimulate rooting, before insertion in the rooting substrate, the cuttings are treated at their basal ends with a root-promoting agent, that is, an hydro-alcoholic solution or a talcum powder formulation containing auxins, mainly indole-3-butyric acid (IBA), in high concentration (generally, 2,000–5,000 ppm). The basal ends of treated cuttings are then inserted 3–4 cm in the rooting medium (e.g., perlite), inside a rooting bench covered with a transparent plastic film, and maintained under mist conditions for the period necessary to form multiple and well-developed adventitious roots.

Several cultivars, mainly used as table olives, are very hard to root or do not root at all. In addition, the de-novo formed root apparatus is often poorly functional. Grafting is the only viable technique for clonal propagation of such cultivars. In comparison with cutting propagation, the production of grafted trees is a more complex operation that requires long practice and, as a consequence, is usually restricted to specialised nurseries where skilled labour is present. In olive, grafting is performed by inserting a small portion of a stem (scion) onto a clonal or a seed rootstock. Clonal rootstocks (i.e., rootstocks reproduced by cutting propagation) are used in Spain where they are obtained from specific olive cultivars (e.g., 'Verdal', 'Lechin de Sevilla', 'Oblonga' and 'Gordal').

The method of grafting scions on seedlings is still used in Italy and in some 'new' olive-growing countries, such as Argentina, where it has allowed a rapid diffusion of olive cultivation. In this technique, a short piece of shoot, mainly just one node, is grafted onto a rootstock that is developed from a seed. The main advantage of rootstock production by seed propagation lies mostly in the possibility of a cheap production of large numbers of high-quality virus-free rootstocks, even in nurseries having little skill and equipment. On the other hand, one drawback is that the seedlings are not homogeneous in terms of vigour and root development, hence influencing growth characteristics of grafted plants that can differ quite markedly. A proper handling of olive seeds (from fruit collection up to seed germination and seedling development) can greatly improve the characteristics of rootstocks, which in turn perform much better during grafting and contribute to produce well-developed grafted trees. In this sense, the use of high-quality seed (i.e., seeds of known provenance, clean and free from disease and insects containing viable embryos and showing high germinability) is of prime importance for rootstock production.

Several other techniques of propagation, based mainly on the traditions of ancient olive growers, have been developed in the time: (i) the use of rooted suckers or large cuttings from old branches (named 'estacas' and 'garrotes' in Spain), (ii) the

grafting on suckers or wild olive trees and (iii) the grafted-cuttings method (Fabbri et al. 2004). Few of them still maintain a certain importance in traditional areas of olive cultivation.

12.4.2 In Vitro Propagation (Micropropagation and Micrografting)

Micropropagation represents the most important advancement in plant propagation in the last 100 years. For a large number of species, a consistent improvement in the sanitary and qualitative characteristics of propagated plants was obtained after effective in vitro propagation protocols were developed. In plant breeding, micropropagation has become an important tool to reproduce large numbers of selected plants easily and in a shorter time, if compared to traditional propagation techniques. However, unlike the majority of fruit species, at the beginning of the 1990s only a few olive cultivars could be efficiently propagated in vitro by micropropagation (Rugini and Fedeli 1990). Moreover, at that time, micropropagation was often initiated using explants from embryos and seedlings (e.g., Bao et al. 1980; García-Berenguer and Durán González 1990; Cañas et al. 1992), but this approach is of minor interest when reproducing selected cultivars or clones. When using explants from adult trees, several problems hindered the development of effective protocols of micropropagation, among which (i) the heavy oxidation of tissues when explants (nodal segments and buds) are collected from in-field or greenhouse plants, (ii) the difficulty of getting sterile shoots when nodal explants were used and (iii) the laboriousness of establishing shoot cultures with some cultivars. Over the last decade, many advances have been made towards the solution of these problems and the optimisation of the various steps involved in olive micropropagation so that complete protocols (from the introduction in vitro of explants to the acclimatation of rooted plants) are today available for several cultivars from different Mediterranean countries (Lambardi and Rugini 2003; Giorgio et al. 2006).

12.4.2.1 Initiation and In Vitro Establishment of Shoot Cultures

The initiation of olive micropropagation using buds or nodal segments from adult field-grown plants is difficult and time consuming, mainly because of high contaminations and the rapid oxidation of tissues after plating. The same explants collected from potted stock plants, grown in greenhouse, are instead the ideal material to introduce in vitro the olive, particularly when tender apical twigs and nodal segments are excised from vigorous shoots soon after sprouting (Rugini and Fedeli 1990). Tissue disinfection before its introduction in vitro is a fundamental step in olive micropropagation, and the present tendency is to avoid the use of ethyl alcohol that causes tissue dehydration. Hence a treatment of 10–20 min with calcium or sodium hypochloride, at different concentrations, is the most common approach for explant disinfection (Mencuccini 1995).

In the micropropagation of olive, the development of a specific olive medium (OM) for shoot proliferation marked an important step towards the improvement

of the technique. The medium was formulated on the basis of the analysis of the main mineral elements of shoot apices from field plants during their rapid growth (Rugini 1984). The major differences between MS (Murashige and Skoog 1962) and OM medium formulations are: (i) the OM medium is richer in Ca, Mg, S, P, B, Cu and Zn, (ii) it has a slightly different Ca/N ratio (1:11) and (iii) it also contains glutamine as a nitrogen source. Unlike other fruit species, mannitol (one of the major carbohydrates of olive metabolism) has repeatedly proved to be the best carbon source in the shoot proliferation medium.

Olive is characterised by a strong apical dominance. As a consequence, shoot proliferation is achieved mainly by means of uni- or binodal segmentation of elongated shoots (Fig. 12.5, *top left*), instead of axillary bud proliferation – the typical approach with the majority of fruit species. Zeatin (a natural cytokinin) plays a major role in the regulation of this phenomenon. According to the olive cultivar, its concentration in the proliferation medium can range from 0.5 up to 10 mg/l.

Recently, micropropagation of olive in Temporary Immersion System (TIS) showed to be promising to limit the expression of shoot apical dominance and to increase proliferation rates (Lambardi et al. 2006a).

12.4.2.2 Shoot Rooting and Acclimatation

Great advances have been made in rooting of micropropagated shoots over the last decade so that even cultivars 'recalcitrant' to cutting propagation (such as several table olive cultivars) can now be satisfactorily rooted in vitro (Lambardi and Rugini 2003). The common approach in olive is to root elongated shoots when still in vitro by means of a simple transfer of single shoots to an auxin-containing medium. 1-Naphthaleneacetic acid (NAA) and IBA, at concentrations ranging from 1 to 4 mg/l, are generally used to root olive shoots. Over time, alternative or additional procedures to the traditional subculturing of shoots in a gelled auxin-containing medium have been proposed: (i) the 'pulse' treatment of shoots, that is, dipping for a short time the basal part of microcuttings in a highly concentrated auxin solution (e.g., Bartolini et al. 1990; Rugini and Fedeli 1990), (ii) the basal etiolation of shoots, performed by black painting of the outside of the jars and by covering the agarized rooting medium with sterile black polycarbonate granules (Rugini et al. 1993) and (iii) the addition of polyamines to the rooting medium (Rugini et al. 1997). However, these methods, although effective in enhancing in vitro adventitious rooting, never found practical application in olive micropropagation protocols.

Acclimation is another critical point in olive micropropagation due to the drastic change of climatic conditions (humidity, light intensity and asepsis) that characterises the passage from the in vitro to the in vivo environment. This problem is accentuated by the particular histology of leaves from in vitro culture, which makes them even more prone to desiccation, as well as poorly functional in the acquisition of autotrophic conditions. As a consequence, just after the exit from the in vitro conditions, olive plantlets are potted in small pots filled with appropriate compost substrates (e.g., peat moss, perlite and polystyrene granules, 2:2:1; Rugini and

Fedeli 1990) and acclimatated under a transparent plastic film or in fog conditions. Following acclimatation, a one-year hardening period is required before their final plantation in orchard (Mencuccini 1995).

To date, few reports have dealt with the genetic and agronomic characteristics of micropropagated olive trees after in-field plantation. However, no evidence has been produced as concerns the loss of the genetic fidelity of in vitro propagated trees to the donor plant (Garcia-Fèrriz et al. 2002; Leva et al. 2002). When transferred on to field, micropropagated plants have usually given satisfactory results with reference to overall growth and onset of flowering; moreover, the occasional appearance of juvenile traits is transitory (Briccoli Bati et al. 2002; Leva et al. 2002). Not all cultivars, though, respond equally well to micropropagation in terms of productivity (Briccoli Bati et al. 2006).

12.4.2.3 Micrografting of Olive

In addition to micropropagation, the micrografting technique has been recently proposed for the olive. Cycles of shoot micrografting on in vitro-grown seedling rootstocks, for instance, proved to be successful in inducing rejuvenation of mature olive trees (Revilla et al. 1996; Farahani et al. 2006). Moreover, Troncoso et al. (1999) cleft-micrografted uninodal explants (from in vitro-grown ‘Cañivano’ seedlings) on in vitro ‘Arbequina’ seedlings, prepared with a cut just under the basal pair of leaves, obtaining 67% of plantlet survival and hardening in vivo. These results suggest that micrografting should be further explored as an additional approach to olive multiplication.

12.5 Breeding Objectives

The recent diffusion of olive outside its traditional areas of growth, together with a continuous trend in the modernisation of its cultivation, has greatly increased in recent years the demand by olive growers of improved cultivars (e.g., more suitable to mechanisation and utilisation in intensive orchards). The large genetic variability of olive, as expressed by the high number of cultivar populations, could offer great opportunities for a marked improvement of olive characteristics. Notwithstanding, the genetic improvement of olive is still far from being comparable to that of other temperate fruit species. For a long time, old farmers, particularly in countries with ancient olive traditions, have considered the olive an ‘easy’ tree, which did not require the particular attentions (in terms of culture management and care) that were reserved for other fruit species, such as, for instance, the grape. Hence the onset of advanced studies for the improvement of olive culture is a relatively recent story. Indeed, an important impulse towards new research and development in olive culture and oil production came after 1974 from the FAO, which was the promoter of international projects based on modern scientific approaches. Soon the necessity of an effort to improve plant material was evident as traditional cultivars showed to be not always adequate to support the modernisation and the intensification of olive

orchards. In concomitance, although the Mediterranean basin is the area which still has 95% of the olive orchards of the world, over the last 30 years the production and the consumption of olive oil have greatly increased, particularly outside this elective area of cultivation, interesting countries of different Continents, such as Japan, China, South Africa, USA, Argentina and Australia. Remarkable increases in olive cultivation and oil production (up to 10 fold) have been observed in some of these countries, such as Australia. Hence, the volume of olive oil consumed annually worldwide is expected to soon exceed three million tonnes. Such volume of olive oil requires active farming programmes and selected olive trees for both new orchards and replacement in old olive groves. Moreover, as the olive industry moves from traditional manual methods to mechanised operations, planting stock will need to be developed to meet future challenges. As a consequence, selection is directed to genotypes that are early bearing, resistant to pests and to abiotic stresses (such as frost and drought), with a limited alternate bearing, suitable for intensive culture and mechanical harvesting and characterised by high-quality productions in terms of both organoleptic characteristics of fruits, and high content in substances useful for human health.

12.5.1 In-field Collections of Olive Cultivars

The collection, characterisation and preservation of olive cultivars can be considered the first fundamental steps against the risks of genetic erosion and towards the exploitation of genetic resources for breeding programmes. The renewal of old groves in the main olive-producing countries, the use of a limited number of cultivars more suitable for the new intensive and mechanised orchards and the diffusion of new cultivars, already available or which are going to be released by ongoing breeding programmes, are all factors producing a progressive abandonment of autochthonous and 'local' cultivars and, as a consequence, a real risk of erosion of olive genetic resources. In addition, a patrimony still exists of genetic resources to be characterised in traditional olive-growing countries outside Europe (e.g., in Tunisia, Morocco, Syria and Turkey) as well as a 'new-emerging' genetic variability in other countries (e.g., Argentina, California and Australia) due to the common use of seed propagation to produce rootstocks.

Because of that, in recent years various public institutions, both at the national and international level, have promoted a thorough conservation campaign with the goal to retrieve and preserve accessions from distant locations and countries where conservation is not provided. The most important olive international Institution, the International Olive Oil Council (IOOC), for instance, has been the promoter in 1995 of the European project RESGEN ('Conservation, characterization, collection and utilization of the olive genetic resources') aimed at the collection, characterisation and conservation of olive genetic resources as well as at the introduction of germplasm from different countries in national in-field collections. The project, financially supported by the European Union, was initially developed only for EU members (Spain, Greece, Italy, Portugal and

France), but soon the interest generated in other olive-growing countries opened the door to the participation of nine more IOOC Members, that is, Algeria, Syria, Morocco, Tunisia, Cyprus, Egypt, Israel, Slovenia and Croatia (Essid 2006). As a main result of RESGEN, more than 1,400 accessions have already been collected in national repositories, 500 of which are autochthonous cultivars (see http://www.internationaloliveoil.org/resgen/index.html). In these clonal orchards, the trees are maintained, agronomically evaluated, morphologically and molecularly described, and are the source of propagation material. In addition, an action aimed at the identification and the survey of forests of the Mediterranean basin, containing rare wild and feral forms of olive trees, is also ongoing (Ouazzani and Lumaret 2006).

Important collections are today present in all the main olive-growing countries. The most important is in Spain where the collection of the Olive World Germplasm Bank (OWGB) of Cordoba accounted in 2005 for more than 400 accessions from 20 olive-growing countries, about half of which are already registered and authenticated by means of morphological descriptors and/or molecular markers. The collection is continuously implemented with new accessions from Spain and other countries (Caballero et al. 2006). In Tunisia, a germplasm bank was established in 1990 by the National Conservatory of Boughrara-Sfax, and contains at present about 120 accessions comprising autochthonous varieties, local forms and foreign cultivars (Trigui et al. 2006). In Italy, in addition to various national and international clonal collections established by public institutions, a descriptor-list of Italian cultivars (from Tuscany) has been realised and is available for consultation in internet (Iannì et al. 1995, http://www.ivalsa.cnr.it/archivio%20fruit/olivo/indice.htm).

12.5.2 Main Characters for Olive Improvement

In the last two decades, various olive-growing countries (Spain, Italy, Israel and Greece) have been the promoters of programmes for olive improvement based mainly on the direct observation, clonal selection and cross-breeding of plants from local cultivars, exhibiting interesting phenotypic characteristics. Two main reasons can explain this traditional approach to the improvement of olive genetic resources: (i) local cultivars are the result of a ‘natural’ selection, that is, the process of adaptation of a plant to a specific environment in order to optimise its growth and functions. In terms of productivity (quantity and quality of olive products), the process was driven by the hand and the expertise of old olive growers. Local cultivar populations are the result of this long-lasting process, and they present satisfactory characteristics of productivity and adaptability to the climatic and pedological conditions of the specific area of cultivation. The main drawback lies in the fact that, when moved to a different environment, out of their area of origin, these cultivars often show great difficulties of adaptation with a consequent decrease of their yield performance; (ii) in olive, almost nil is the information available concerning the genetic control of characters and their hereditability.

Olive improvement is therefore at present focussing its attention on the evaluation of adaptability of the main cultivars to different areas of cultivation, through the establishment of clonal collections where genetic resources from different countries are not only preserved, but also comparatively evaluated. Specific programmes of clonal selection and cross breeding are ongoing, mainly at national levels, including quite a high number of agronomic and production characters. In addition, due to the maintenance of some traditional peculiarities in each olive-growing country, minor characters that are of interest in one country can be almost neglected in others. For instance, the characters 'germinability of seeds' and 'seedling morphology' are considered important only in Italy for the improvement of olive rootstocks as this country still has an economically important nursery production of plants grafted onto seed rootstocks (Fabbri et al. 2004). A list of the characters at present considered of major importance in olive breeding is reported in Table 12.2.

In recent years, several olive breeders concentrated their attention to the 'vigour and form' of the plants in order to select compact genotypes suitable for the new intensive orchards. Vigour and form are characters strictly linked in olive, and some methods were proposed in the past for a precocious characterisation of low-vigour genotypes, among which are the content in abscisic acid in shoots and leaves (Yadava and Dayton 1972) and stoma density in leaves (Bartolini et al. 1979). However, Del Rio et al. (2002) reported that the characterisation of young plants with reference to their high or low vigour cannot start before they are at least 6-years old. Initially the breeding activity concerning dwarf plants produced interesting results in terms of new cultivars suitable for ornamental purpose (Hartmann 1967; Roselli and Donini 1982). Then the interest moved to the selection of compact cultivars (such as one selection from the cv Frantoio, the 'Fs-17'; Fontanazza et al. 1990) as well as dwarfing rootstocks (Buffa et al. 2006) with the aim to promote high-planting density and mechanisation of pruning and harvesting operations.

As regards fruiting and fruit characteristics, plenty of efforts have been put in the selection of cultivars with high or low oil content and, more recently, of dual-purpose cultivars. Investigations in this field had already hypothesized a genetic control of the oil content in fruits, a character showing high variability among the different cultivars (Fontanazza and Bartolozzi 1998). Unlike oil cultivars, requirements for table olives are low oil content to favour preservation and high reducing sugar contents to ensure a good lactic fermentation. A cultivar with these characteristics, 'Kadesh', was selected by Lavee (1978) and became fairly popular in Israel and Argentina.

Another character worthy of mention for its economical consequences is alternate bearing. This is a widespread phenomenon in olive cultivars, with negative effects on fruiting, vegetative growth and, as a consequence, on tree management (Lavee 2006). To limit the expression of alternate bearing, particular attention is required during fertilisation and pruning of olive trees, which in turn cause an increase of costs for skilled hand labour. Significant differences have been observed in the leaf protein content in fruiting and non-fruiting trees as well as in its quantitative change during the growing season (Lavee and Avidan 1994), supporting the hypothesis of specific gene activation or repression. Research in this topic is moving towards the characterisation of genes involved in flower bud induction.

Table 12.2 Examples of studies on characters important for olive improvement and relative cultivar selection

Character	Aim of investigation	Selected Cultivar	Reference
Tree vigour and form	Selection of dwarf forms, suitable for ornamental scope	'Swan Hill'	Hartmann (1967)
		'Briscola'	Roselli and Donini (1982)
	Selection of dwarfing rootstocks	'Fs-17'	Fontanazza et al. (1990)
			Buffa et al. (2006)
	Studies to find a correlation between physiological and morphological characters of young trees and their vigour		Bartolini et al. (1979)
			Del Rio et al. (2002)
Fruiting and fruit characteristics	Studies on alternate bearing		Lavee and Avidan (1994) and Lavee (2006)
	Short juvenility (early flowering) of trees		Leitão (1990), Bellini (1993) and Santos Antunes et al. (1999)
	Oil yield		Fontanazza and Bartolozzi (1998)
	Selection of cultivars with high-oil content	'Barnea'	Lavee et al. (1986)
	Selection of cultivars with low-oil content (table olives)	'Kadesh'	Lavee (1978)
	Selection of dual purpose (table olives and oil) cultivars	'Arno', 'Tevere', 'Basento'	Bellini et al. (2002)
	Selection of clones of 'Chemlali Sfax' for quality and regularity of production		Grati Kamoun et al. (2002)
	Hereditability of fruit characters in table olives		Fanizza (1982)
	Hereditability of fruit characters (size, weight and colour) in progenies from oil cultivars		Bellini (1993) and Parlati et al. (1994)
	Genetic variability of fruit histological characteristics		Mulas (1994)

Table 12.2 (continued)

Character	Aim of investigation	Selected Cultivar	Reference
Adventitious rooting	Improvement of rooting efficiency by means of *Agrobacterium rhizogenes* transformation		Rugini and Mariotti (1992), Rugini (1992), and Rugini et al. (2000)
	Progeny evaluation as regards rooting potential of cuttings		Voyiatzi et al. (2002)
	Classification of olive cultivars with reference to high, medium and low rooting ability		Fabbri et al. (2004)
Resistance/susceptibility to pests	Selection of cultivars resistant to *Spilocaea oleagina*	'Maalot'	Lavee et al. (1999)
	Selection of cultivars resistant to *Verticillum* wilt	'Oblonga'	Hartmann et al. (1971)
	Selection of cultivars resistant to *Cycloconium*		Lavee (1990)
	Classification of cultivars as resistant, tolerant and susceptible to the main olive phytopathologies		Bellini et al. (2003)
Tolerance to cold and frost conditions	Variability among Italian cultivars to frosts		Antognozzi et al. (1994, La Porta et al. (1994) and Bartolozzi and Fontanazza (1999)
	Studies on correlations between morphological/physiological characters and cold tolerance		Roselli et al. (1989), Roselli and Venora (1990) and Bartolini et al. (1999)
Salt tolerance	Studies on physiological and anatomical characters of salt tolerant plants		Gucci and Tattini (1997), Cantos et al. (2002)
	Different salt tolerance of olive cultivars		Therios and Misopolinos (1988), Tattini et al. (1994), Benlloch et al. (1994), Marin et al. (1995) and Chartzoulakis et al. (2006)
	Selection in vitro of salt tolerant genotypes		Fodale et al. (2006)

The improvement of adventitious rooting ability of olive cultivars is a character of high interest for the nurserymen. The character is under genetic control, as proved by the very high variability of rooting potential (both natural and after auxin treatments) when cuttings are collected from different cultivars (Fabbri et al. 2004). Here, promising advances have been made using a biotechnological approach (see Section 12.7.4).

Up-to-date, specific knowledge on the genetic control of the mechanisms of resistance/susceptibility of cultivars to biotic stresses is still lacking. Notwithstanding, generic information on different levels of resistance of cultivars to the main pests affecting the olive tree is available (Bellini et al. 2003), and on these bases important results have been obtained in time with the clonal selection of genotypes resistant to the peacock leaf spot (*Spilocaea oleagina*; Lavee et al. 1999) and the *Verticillium* wilt (*Verticillium dahliae*; Hartmann et al. 1971). It is still difficult, on the other hand, for the selection of genotypes resistant to the olive fruit fly (*Bactrocera oleae*) and to the olive moth (*Prays oleae*), among the most dangerous insects in olive because of their severe damage to fruits and, in the case of olive moth, to flowers and leaves. Indeed, up to now, it has not been possible to find cultivars showing clear evidence of resistance or tolerance to these pests, which are common in several olive-growing areas where they are often the cause of great losses of product and marked decrease of oil quality.

Also the studies concerning tolerance or susceptibility of olive cultivars to abiotic stresses (mainly frost and salt) are of great interest and economical importance. Frost is one of the main problems in olive-growing areas where winter temperatures fall frequently to 10°C or more below zero. This condition is typical, for instance, of central Italy and, together with early and late frosts (in spring and fall), has repeatedly been in the past the cause of great losses of olive groves due to the death of the epigeic part of the trees. Hence, particularly in Italy, research has moved towards the characterisation of cultivars in terms of tolerance to low temperatures. Information is today available on the fair tolerance to winter frosts of some important cultivars, such as ‘Leccino’, ‘Ogliarola’, ‘Itrana’, ‘Tanche’ and ‘Moresca’; on the contrary, ‘Frantoio’ and ‘Moraiolo’ are highly susceptible (e.g., Antognozzi et al. 1994; La Porta et al. 1994; Bartolozzi and Fontanazza 1999). Some authors also report of an intra-cultivar variability of the cv Leccino to low-temperature tolerance (La Porta et al. 1994; Bartolini et al. 1999).

It has been repeatedly reported that the osmotic stress has several consequences on the vegetative growth of trees, particularly of the epigeic part. When growing in soils with high salt concentrations, the shoots have short internodes, small and thick leaves, and fruits of smaller size. The tree shows a general appearance of stunted growth and its productivity (both in terms of quality and quantity) can be negatively affected (Cresti et al. 1994). Several studies focussed attention on the evaluation of salt tolerance of different cultivars, assuming that, in general, ‘susceptible’ cultivars do not tolerate over 20 mM NaCl in the soil circulating solution, while ‘tolerant’ cultivars can resist up to 100 mM (Gucci et al. 1995). However, among the morphological symptoms that can be taken into consideration as ‘markers’ of salt tolerance/susceptibility, the quantification of growth reduction of trees

under osmotic conditions seems to be the most promising. Based on that, some of the main world cultivars are considered 'tolerant', such as the Spanish 'Arbequina', 'Lechin de Sevilla' and 'Picual' (Benlloch et al. 1994), the Italian 'Frantoio' (Tattini et al. 1994), the Tunisian 'Chemlali' (Ben Ahmed et al. 2006) and the Greek 'Megaritiki' (Therios and Misopolinos 1988), 'Kerkiras' and 'Kalamata' (Chartzoulakis et al. 2006).

12.6 Breeding Techniques

12.6.1 Clonal Selection

Due to the occurrence of self-incompatibility in olive germplasm, the cultivar populations of olive have a high degree of heterozygosity and the genetic variability is consequently high. This means that the potential for improvement is relevant, and by no doubt this feature of the olive has made possible the agronomical evolution of the crop in the Millennia, also because olive suitability to agamic propagation enabled farmers to preserve the selected types by vegetative propagation. As a result, the main producing countries of the Mediterranean basin possess hundreds of major and minor cultivars that represent an unfathomed variability which might yield the characters most useful for modern olive industry.

Clonal selection was proposed in the early 1960s for olive improvement in order to avoid the problems and the long time required for the development of programmes of cross-breeding and selection. Today, though considered a slow breeding technique, it still remains a valuable instrument that has been employed also recently in a number of olive producing countries, making it possible the improvement of the standard of numerous cultivars, as well as the increase of their homogeneity in terms of agronomic and productive characters. An additional positive aspect of clonal selection is the sanitary control that usually accompanies the procedure: the lines emerging from the clonal selection procedure are, as a rule, virus-free and more tolerant to pathogens, thus contributing to a general improvement of the industry.

In the last 50 years, in spite of the large amount of resources needed to pursue this kind of genetic improvement, all olive producing countries have promoted clonal selection programmes with often encouraging results (Lavee 1990). Selections were particularly initiated in regions with large-scale autochthonous olive populations and continued under different growing conditions, climates and levels of intensification (Lavee and Avidan 2002). Clones of standard cultivars have already been selected or are under evaluation in many countries, such as:

Spain, where the first studies were aimed at the improvement of cvs Picual, Manzanillo and Hojiblanca for characters of productivity and for the reduction of alternate bearing (García-Berenguer 1978). Later on, attention of breeders moved mainly to 'Manzanilla' (Suárez, Lopez-Rivares et al. 1990) and 'Arbequina' (Tous et al. 1993), and both these works resulted in the first selection of many interesting clones. One of them, the 'Arbequina' clone 'I-18', has had commercial diffusion for

its improved characteristics of productivity and the upright form of growth, which makes it suitable for mechanical harvesting;

Italy, working initially for the improvement of the cvs Frantoio, Moraiolo and Leccino, in order to improve their characters of productivity and winter-hardiness (Morettini 1961; Bartolini et al. 1995). An interesting clone of unknown origin, the 'I-77', was selected by Fontanazza (1993), having interesting characteristics of low vigour, self-fertility and early onset of bearing. At present, clonal selection is more active in the south of Italy and promising clones have already been obtained for the cvs Carolea (Parlati et al. 1995), Tonda Dolce (Mulè et al. 1992) and Nocellara del Belice (Mulè et al. 1994).

Portugal, where, as a result of a long-lasting clonal and sanitary selection programme, concerning 10 cultivars from the southern part of the country, 27 clones were selected, established in the field and are now under evaluation, mainly for characters of earliness of flowering and fructification (Serrano et al. 1999);

Tunisia, where the international restrictions on the local olive oil due to its fatty composition (i.e., oil rich in saturated fatty acids and poor in oleic acid) have imposed an improvement of local cultivars. In particular, an important programme of clonal selection is in progress with the cv Chemlali Sfax, aimed at the selection of genotypes more productive and able to give the quality of oil that can meet the international market criteria (Grati Kamoun et al. 2002).

Programmes of clonal selection have also been started in other countries, such as in France (e.g., with the cvs Picholine, Tanche and Lucques), Morocco (with the cv Picholine Marocaine), Turkey (mainly with the cvs Memecik, Ayvalik and Gemlik), Israel (with the cvs Souri and Nabali) and Cyprus (with the cv Local) (Bellini et al. 2003).

12.6.2 Cross Breeding

Cross breeding in the olive is usually more difficult and time consuming than in other fruit tree species. A main reason for this difficulty is the relatively scarce knowledge of the hereditary behaviour of the most important bioagronomic traits. Then additional objective obstacles are: (i) the high heterozygosity of the species (Rugini and Pannelli 1993) and its high degree of polymorphism; (ii) the self- and cross-incompatibility characteristics typical of the species, a condition which limits or makes harder the attempts at self-crossing or inter-crossing the cultivars; (iii) the long-lasting juvenile phase of plants, which is usually in the 10-year range or more. This, in turn, means that a minimum time for the release of a new cultivar is 20 years or more; (iv) the difficulty at emasculating the flowers, with the consequence that the nature of the pollen and of the parent tree can at times be uncertain. Also because, even in the case of self-incompatibility, a minimum amount of self-fertilisation can occur; and (v) the low fruit set rate (Rugini and Lavee 1992), and the even lower number of mature fruits containing vital seeds.

In addition, the linked heredity of various olive characters has to be taken into careful consideration in breeding activities, as in the classical example of the inverse

correlation between the oil content and the fruit size, which can be regarded as 'negative' for oil cultivars and 'positive' for the table olive ones.

As a consequence of all these problems, the olive has not received in recent decades the attention scientists gave to other fruit crops as concerns genetic improvement using cross-breeding approaches. This fact is clearly evidenced by the very limited number of new cultivars and rootstocks that have been released in the last 30 years (see Table 12.2). However, a promising change in this trend has been observed, as advances in the breeding technique (e.g., the in vitro embryo culture allowing the germination of naked embryos and the methods to shorten the juvenile period of plants) enhanced in recent years the onset of cross-breeding programmes in several olive-growing countries. Some of these programmes have already produced the first promising results (Fig. 12.2). As for the clonal selection, the majority of these programmes are today aimed at improving the autochthonous cultivars, and breeding activities are mainly based on selection within the F1 progenies, which display a marked variability even when self-pollination is adopted. Further improvement of cultivars for specific characters requires the utilisation of

Fig. 12.2 'Basento', a new olive cultivar released in the frame of a programme started in Italy in 1971. It is a dual-purpose olive cultivar obtained by cross-breeding ('Picholine′ × 'Manzanilla′) with semi-compact habit and high fertility. The characteristics of the fruit are good size, a very high flesh/stone ratio (> 19), medium-low oil content (11%) and very good organoleptic quality (Bellini et al. 2002)

second (F2) and third (F3) generation progenies. Hence the inclusion of characters from more than two parents and back crosses to amplify or reduce specific characters are presently a common strategy for olive genetic improvement.

12.6.3 Mutagenesis

Studies on induced mutation were first carried out in Italy by Morettini (1954b), in an attempt to find a solution to the problems related to the long juvenile phase of olive plants in breeding programmes. Later, advances were due to the studies of Donini, who applied X-rays (1975) and γ-rays (1976) to induce genetic alterations. Roselli and Donini (1982) were the first to patent a new cultivar, the cv Briscola, obtained by irradiation of self-rooted plantlets of 'Ascolana Tenera' (Fig. 12.3). Shoots of 'Briscola' have short internodes, a character that confers the plant a general dwarf form, resulting very interesting for ornamental purposes (Fig. 12.4).

By irradiation of 'Frantoio' and 'Leccino' self-rooted plants, also Pannelli et al. (1990) obtained two vigorous but compact mutants (one for each cultivar) as well as one dwarf 'Leccino' mutant. The plants showed several morphological and physiological differences from the mother plants, such as shorter internodes, larger and thicker leaves, higher assimilation rates and water stress tolerance. Moreover, in vivo and in vitro selection allowed the isolation of tri- and tetraploid plants obtained

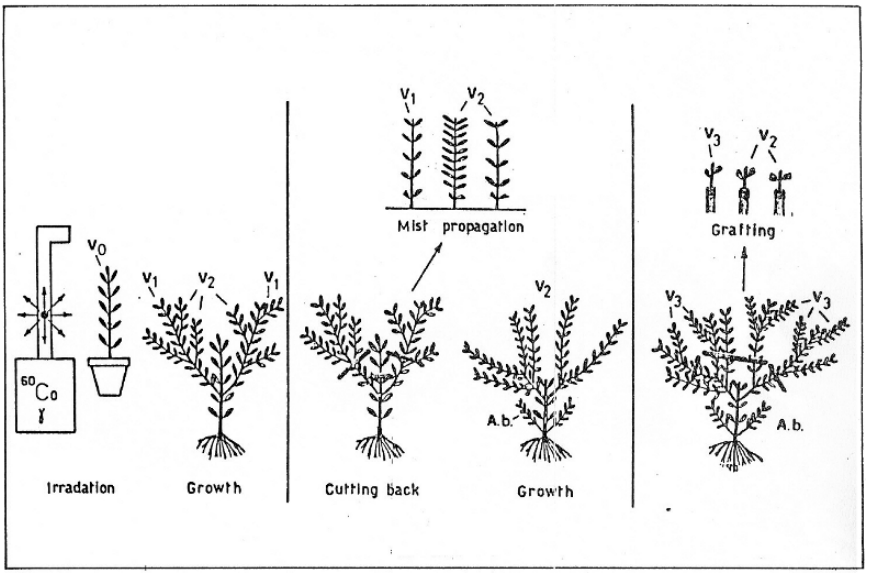

Fig. 12.3 Scheme proposed by Scaramuzzi and Roselli (1986) for the isolation of somatic mutations in olive trees, originated following the exposure of self-rooted plants to γ-rays. As the mutants always assumed chimeric forms, some cycles of cutting and grafting propagation were necessary to select wholly mutated shoots

Fig. 12.4 'Briscola' is an ornamental form of olive, which was obtained by induced mutagenesis, according to the scheme of Fig. 12.3. The cultivar is characterised by short internodes (*top*) and slow growth, which confer a general dwarf form (*bottom*) and make the tree particularly attractive for ornamental purposes (photos courtesy of G. Roselli and the 'SPO', Società Pesciatina di Orticoltura of Pescia, Italy)

by axillary bud stimulation of mixoploid mutants. In this way, plants were selected with a prevalence of tetraploid cells, which, by virtue of a thicker cell wall, showed to be more resistant to the peacock eye (Rugini et al. 1996). This study is still in progress to test the use of triploids and tetraploids plants as rootstocks (Rugini et al. 2006).

12.7 Biotechnological Approaches to Olive Improvement

In the last two decades, procedures of in vitro regeneration other than traditional micropropagation have been developed in olive, such as somatic embryogenesis and organogenesis from callus culture and the synthetic seed technology. The main

reason was to explore non-conventional methods of plant propagation, germplasm conservation and genetic improvement. Somatic embryogenesis, in particular, has been largely investigated because (i) it can be employed for mass micropropagation of plants, also overcoming difficulties faced in rooting numerous olive cultivars, (ii) dihaploid homozygous plants can be obtained from reproductive organs, such as anthers, pollen or ovules (Perri et al. 1994; Rugini et al. 1995), (iii) it can be used for the production of synthetic seeds (see Section 12.7.3), (iv) it can produce new variability via somaclonal variation and genetic transformation using either *Agrobacterium* or microprojectile bombardment techniques (see Section 12.7.4) and (v) it can be used in cryopreservation, providing an additional and powerful tool for the safe preservation of olive germplasm (see Section 12.7.5).

12.7.1 Somatic Embryogenesis

12.7.1.1 Induction of Embryogenic Lines from Zygotic Embryo and Seedling Explants

In olive, somatic embryogenesis has been induced mainly from juvenile explants, that is, immature (Rugini 1988; Leva et al. 1995) or mature zygotic embryos (Orinos and Mitrakos 1991; Mitrakos et al. 1992) with or without callus interposition. These studies showed that the maturation degree of the original explant has particular importance for the induction of somatic embryogenesis, either when entire zygotic embryos or excised cotyledonary explants are used. In the former system, somatic embryogenesis was reported only when zygotic embryos were harvested 75 days after full bloom and cultured in half-strength MS medium containing 0.5–2.5 μM BA (Rugini 1988). The existence of a 'window' of embryogenic competence during zygotic embryo development was also reported by Leva et al. (1995). They observed that only cotyledonary explants from immature embryos (cvs Picholine, Frangivento and Frantoio), harvested between 60 and 90 days after anthesis, were competent for embryogenic callus induction following their culture in SH (Schenk and Hildebrandt 1972) medium containing various combinations of NAA and isopentenyladenine (2iP). When cotyledons came from earlier (30 days) or later (130 days) collections, no evidence of somatic embryogenesis was observed. Differently, embryo-like structures could be observed in calli, originated from cotyledonary explants, when 'Chalkidikis' zygotic embryos were harvested 126 days after full bloom (Pritsa and Voyiatzis 1999).

A dissimilar morphogenetic expression of calli from different zygotic embryo tissues has also been evidenced in olive. Indeed, when mature embryos of the cv Koroneiki were used as the source of explants (Mitrakos et al. 1992), both rhizogenesis and somatic embryogenesis were high from radicle calli and low from distal-cotyledon calli, while only high rhizogenesis was promoted in calli from proximal cotyledon segments. It is notable that the high level of somatic embryogenesis (up to 40%) was obtained in radicle calli that, after 14–21 days in induction medium, were subcultured on OM medium without exogenous growth regulators. The high

embryogenic potential of root callus was confirmed in trials with seedlings of the cvs S. Agostino (Rugini et al. 1995) and Nabali (Shibli et al. 2001).

12.7.1.2 Induction of Embryogenic Lines from Mature Tissue Explants

Induction of embryogenic callus lines from mature tissues is by far the most useful technique for application to transformation studies of trees. However, in olive this approach was proved to be very difficult as, up to now, only one report is available where an effective embryogenic line was obtained from mature tissues (leaf petioles) excised from the cvs Canino and Moraiolo (Rugini and Caricato 1995). The regeneration system is described better as a 'secondary somatic embryogenesis' due to the fact that, once the embryogenic line was established, cycles of secondary somatic embryos were obtained directly from the epidermal tissue of primary somatic embryos (Fig. 12.5, *top right*). This way, the embryogenic line can be maintained for years by monthly subculturing. Histological observations showed that, in the embryogenic masses, together with a majority of perfect somatic embryos, sev-

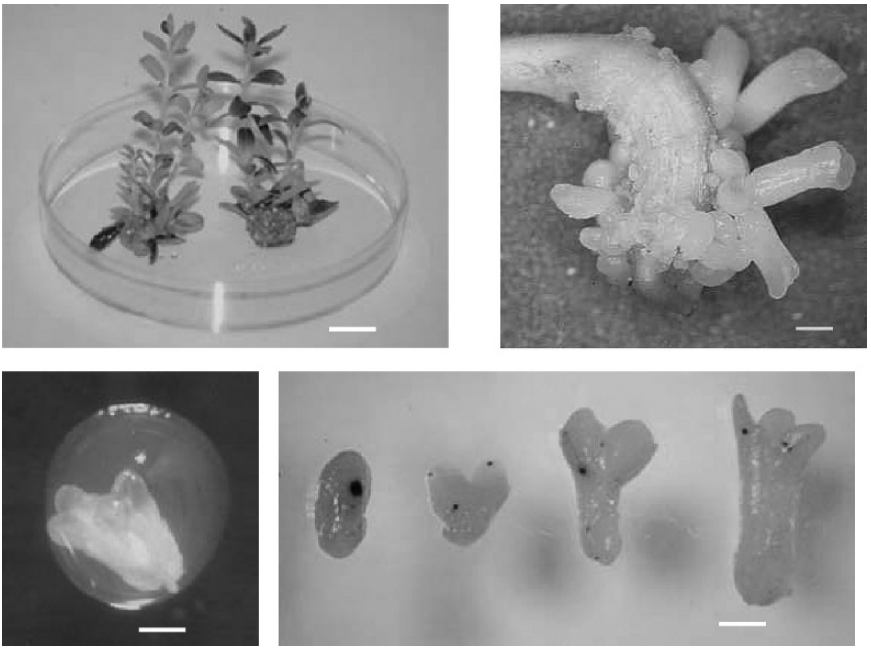

Fig. 12.5 Application of biotechnologies to the propagation and the genetic improvement of olive. *Top left*, Micropropagated shoots of the cv Frantoio just before to be cut at their base and transferred to the rooting medium (bar, 1 cm). *Top right*, Somatic embryogenesis in olive: many secondary somatic embryos, formed at the radicle end of a primary somatic embryo, with no evidence of interposed callus (bar, 1 mm). *Bottom left*, A synthetic seed of olive, containing a somatic embryo (bar, 1 mm). *Bottom right*, GUS gene expression in somatic embryos of olive at different stages of development, following their microprojectile bombardment (bar, 1 mm) (photos from Lambardi and Rugini 2003; *bottom right*, original photo of M. Lambardi)

eral other forms are generated, such as fused embryos, teratomic leaves, claviform structures and embryos with fused cotyledons (Benelli et al. 2001a).

12.7.2 Shoot Organogenesis

Since adventitious shoots and roots were obtained for the first time from seedling explants (Gilad and Lavee 1974), few reports have dealt with olive regeneration by shoot organogenesis. Direct shoot regeneration was induced on olive hypocotyls in White medium (White 1939), supplemented with 0.05 μM NAA and 2.5 μM BA (Bao et al. 1980) as well as from cotyledons of mature seeds (Rugini 1986). High shoot organogenesis was obtained from callus, previously induced on cotyledon segments excised from 'Tanche' and 'Picual' embryos (Cañas and Benbadis 1988). Initial callus proliferation was produced on OM medium containing high auxin/cytokinin ratio. Shoot organogenesis was then stimulated when the calli were transferred onto a 2iP-containing medium. Maximum shoot regeneration was observed in calli induced from cotyledon segments proximal to the embryo axes rather than the distal ones, suggesting that a gradient of regeneration potential existed from the proximal to the distal region of olive cotyledons. Rooting of adventitious shoots was obtained by transferring them to an IBA- or NAA-containing OM medium.

Mencuccini and Rugini (1993) obtained adventitious buds in petioles from in vitro-grown shoots of olive (cvs Moraiolo, Dolce Agogia and Chalkidikis). Interesting findings of this study concerned the efficiency of shoot organogenesis, which were heavily dependent on: (i) the cultivar ('Moraiolo' being the best), (ii) the position of the petiole along the shoot (apical nodes being better than basal ones), (iii) the medium/hormone combination and (iv) the culture in dark condition. Following rooting, the regenerated plantlets did not show morphological differences in comparison with the micropropagated donor plantlets.

12.7.3 Synthetic Seed Technology

Synthetic or artificial seeds (also named 'synseeds') are a recent evolution of tissue culture aimed not only at improving conventional micropropagation, but also at the easy storage of plant germplasm. Not only has the technology been mainly developed with ornamental species (Lambardi et al. 2006b), but also explants from fruit species can be successfully used for the production of synseeds (Standardi and Piccioni 1998). The synthetic seeds can be defined as 'artificially encapsulated somatic embryos, shoots, or other tissues which can be used for sowing under *in vitro* or *ex vitro* conditions' (Aitken-Christie et al. 1995). Synthetic seeds have also been recently tested for olive (Fig. 12.5, *bottom left*) with promising results. Micheli et al. (2002) used both microcuttings (apical and axillary buds) and somatic embryos of olive to produce synthetic seeds. The explants were first immersed in sodium

alginate solution (2.5%), after which drops of the solution (each drop containing one explant) were released into a complexing solution, that is, a water solution containing 100 mM of $CaCl_2$. The hardened beads were then washed from the solution and stored; after 'sowing' on an appropriate medium, they germinated successfully and converted into plantlets. Moreover, rooting and conversion to plantlets of 'Moraiolo' nodal segments are improved by dipping synthetic seeds in a sucrose- and IBA-containing solution before sowing (Micheli et al. 2006).

12.7.4 Genetic Transformation of Olive

The application to olive of genetic transformation studies dates back to 1984, when Rugini simply inoculated *A. rhizogenes* to the middle (by puncture) or the base (by longitudinal wounding) of in vitro-grown shoots of 'Dolce Agogia', with the aim of increasing its rooting potential by means of the creation of chimeric plants (Rugini 1986). A few years later, it was reported that the culturing of *A. rhizogenes*-inoculated shoots in putrescine-containing media dramatically increased rooting rates and basal callus formation (Rugini 1992). In spite of these first promising results, eventually olive was not intensively involved in genetic transformation studies mainly due to the difficulty of obtaining efficient morphogenetic lines from mature tissue that, once transformed, can guarantee high rates of regenerative events. In addition, the major olive-growing countries were subjected to the EU *moratorium* against 'Genetically Modified Organisms' (GMOs) and this condition is still a major obstacle to get significant advances in this research area. Notwithstanding, scientists are convinced that the genetic transformation of olive can be an important alternative to traditional breeding able to speed up the development of new genotypes improved for specific characters. For an exhaustive review of this topic, the reader is addressed to Rugini et al. (2000).

12.7.4.1 Transformation Techniques

Evidence has been produced showing the possibility to insert foreign genes into olive cells through both indirect (via *Agrobacterium*) and direct (by means of the biolistic technique) DNA transfer. *Agrobacterium*-mediated transformation was mainly used with *rol* genes of *A. rhizogenes*, in order to increase the potential of olive to produce adventitious roots. With this aim, suitable wild types of *A. rhizogenes* were used to isolate *rol* ABC genes. Then these genes, contained in p1855 and pBR322 plasmids, were cloned in LBA4404 strain of *A. tumefaciens* to transform olive tissues (Rugini et al., 2000). Selectable markers, allowing to distinguish transformed tissues by preventing the growth of the untransformed ones, play a crucial role for the development of transformation procedures. In olive, efficient selection of transformed cells was achieved by using the antibiotic kanamycin (50–100 μg/ml, depending on the type of explant) after 3–4 weeks of co-cultivation of explants with *Agrobacterium*, in order to increase the number of transgenic cell colonies (Rugini et al. 2000).

Besides the *Agrobacterium*-mediated technique, olive has been tested also for direct gene transfer by means of the microprojectile-DNA delivery system. The technique is based on the direct release into cells of specific genes (inserted into plasmid vectors, in turn adsorbed on the surface of gold or tungsten particles) by means of particular devices working at high helium pressures. In olive, the technique was applied to study transient gene expression of somatic embryos (cv Canino), following the optimisation of delivery parameters such as the pressure of bombardment, the type of particles (tungsten or gold) and the kind of particle delivery device, that is, the Particle Inflow Gun (PIG) or the Particle Delivery System (PDS)-1000/He (Lambardi et al. 1999). In this study, different plasmid vectors (the pZ085, containing the 35S promoter fused to the GUS gene, and the pCGUδ0, containing the sunflower ubiquitin promoter fused to the GUS gene with ubiquitin intron; Binet et al. 1991) were used to bombard somatic embryos at different stages of development. GUS gene expression could be detected in somatic embryos that were bombarded with gold particles (Fig. 12.5, *bottom right*), provided that appropriate delivery pressures were optimised for both devices. More recently, the biolistic system has been used also for the transformation of embryogenic cultures from 'Picual' juvenile material, using three different plasmids, that is, the pGUSINT (containing the 35S promoter), the pJGUS5 (in which the 35S promoter is coupled to an enhancer of expression) and the pCGUδI (Pliego-Alfero et al. 2005).

As mentioned, transient gene expression has been tested in olive following microprojectile DNA-delivery on somatic embryos of 'Canino' (Lambardi et al. 1999) and 'Picual' (Pliego-Alfero et al. 2005). It is interesting to note that, with the application of the β-glucuronidase histological assay, both reports evidenced the highest GUS gene expression when the pCGUδ0 plasmid, containing the ubiquitin promoter, was used for transient transformation.

12.7.4.2 First Attempts to Transfer Specific Genes in Olive

Rol genes of *A. rhizogenes* have been largely investigated with the aim to increase the potential of adventitious rooting of olive cultivars. In the abovementioned pioneer study of Rugini (1984), although the roots emerging from inoculated shoots were rarely transformed, an increased rooting ability with more secondary roots was observed, possibly demonstrating the inductive role of partial integration of T-DNA on the non-transformed neighbour cells (Rugini and Mariotti 1992). Afterwards, transformation procedures have been developed to transfer *rol* ABC genes to zygotic immature embryos of 'Moraiolo' (Rugini and Fedeli 1990) and to leaf petioles of 'Dolce Agogia' and 'Moraiolo' (Mencuccini et al. 1999) with promising results. The same gene construct (i.e., LBA 4404 strain of *A. tumefaciens*, encompassing the *rol* ABC genes in pBIN19 plasmid and the *nptII* gene encoding resistance to the kanamycin) was used in these studies. Selected plantlets, originated from transformed embryogenic calli, showed short internodes and high root potential. The transgenic plants are now under evaluation in experimental fields in Italy (Rugini et al. 2006).

Another interesting research line was developed to increase fungal disease resistance in olive. With this aim, somatic embryos of the cv Canino were transformed with the *A. tumefaciens* strain LBA 4404, containing the *osmotin* gene under the control of the 35S promoter. The somatic embryos, after selection for transgenicity, originated osmotin plant clones with no sign of phenotypic alterations (Rugini et al. 2000), but with promising characteristics of resistance to the peacock disease (Rugini et al. 2006).

It is expected that, in the near future, olive genetic transformation will be oriented mainly to induce changes in the tree morphology, for example, to produce dwarf and semi-dwarf plants with a large and well-developed root apparatus, characteristics that will make them more suitable for plantation in high-density orchards. To carry out this work, a large availability of olive genes will be necessary. To date, about 400 sequences of olive have been deposited in GeneBank, that is, 109 nuclear sequences, 90 ribosomal, 136 cpDNA +mtDNA, 26 EST, 44 SRAP Markers and 16 retrotrasposoms (Rugini et al. 2006).

12.7.5 Cryopreservation of Olive Germplasm

As described in Section 12.5.1, numerous programmes are today ongoing aimed at the preservation of the large genetic variability of olive through the establishment of in-field collections. However, olive germplasm kept this way is costly and is vulnerable to losses due to diseases, pests, extreme environmental conditions and economic pressures. Hence, some research groups are at present involved in the exploitation of tissue culture technology as a possible alternative approach to the preservation of olive germplasm. Among the various methods available (see, e.g., Lambardi and De Carlo 2002), plant cryopreservation (i.e., the storage of explants at the ultra-low temperature of liquid nitrogen) seems to be the most promising for the long-term conservation of olive germplasm. Shoot tips of the cv Arbequina, for instance, showed 30% survival after recovering from the storage in liquid nitrogen, provided that they were previously desiccated to 30% of their original moisture content (Martinez et al. 1999). Lambardi et al. (2002) applied a procedure of 'vitrification and one-step freezing in liquid nitrogen' to shoot tips excised from in vitro-grown shoot cultures of the cv Frantoio. Following the recovery of explants from liquid nitrogen and their plating in a regrowth medium, 15% survival rate was achieved but only from shoot tips that had been obtained from apical buds. With a similar procedure, promising results have been recently obtained with the cryopreservation of shoot tips from the Italian cvs Gentile di Larino and Ascolana Tenesa (Nisi et al. 2006). Unlike the 'vitrification' technique, the application of the 'encapsulation-dehydration' procedure was not effective in the protection of either 'Frantoio' (Benelli et al. 2001b) or 'Arbequina' (Martinez et al. 1999) explants during ultra-rapid freezing.

Alternatively to shoot tips, embryogenic cultures of olive proved to be a suitable material for cryopreservation using the 'vitrification' approach (Lambardi

et al. 2002). The technique was applied to portions of embryogenic masses of the cv Canino, containing somatic embryo primordia at different stages of development. After their recovery from the storage at −196°C, almost 40% of the cryopreserved embryogenic samples survived and promptly recovered to proliferate. Moreover, the recovered embryogenic cultures showed enhanced proliferative and morphogenetic activity, and the embryo primordia that were present in the embryogenic masses before cryopreservation greened when transferred into the light and developed rapidly to the cotyledonary stage. It must be emphasized that, due to the possibility of somaclonal variation occurrence during long-term culture of dedifferentiated cells, somatic embryos cannot be considered the best material for germplasm conservation. However, as evidenced in Section 12.7.4, the embryogenic cultures are a very important tool for genetic transformation studies and the possibility to store them in liquid nitrogen prevents the decline of embryogenic potential due to repeated subculturing.

12.8 Conclusions

The demand for olive oil is increasing in the world, not only for its gastronomic importance but also for its recognised value for human health, making it the 'king' of the typical Mediterranean diet. Hence a concomitant increase of the world production of high-quality olive oil is not only desirable and expected in the near future, but also to induce a positive effect in the price of extra-virgin olive oil, which is still often much higher than alternative vegetable fats, such as those from peanut, soybean, maize and sunflower. The economical aspect has particular importance for the Mediterranean countries where the olive is often among the most important cultivated fruit species. Hence the major olive-growing countries are presently deeply involved in an important work of transformation of the old groves (made of large and ancient trees) into modern, intensive and mechanisable orchards. It is obvious that a fundamental contribution is expected by the presently ongoing breeding programmes, as well as from the further development of strategies for the characterisation, the propagation and the conservation of genetic resources, all actions requiring the continuous support of international organisations and national institutions. This broad-spectrum activity is a requirement for the production of new cultivars which must drive the olive towards the 'new era' of intensive and mechanised orchards. The consequent improvement of yield and quality of olive productions in turn will make more remunerative the activity for the olive growers and, at the same time, will produce a beneficial effect on the market price of olive products.

An important contribution is also expected from the application of biotechnologies to olive, in terms of both the production of high-quality plants for new orchards and the creation of improved cultivars by genetic transformation having reduced size, superior rooting ability, resistance to abiotic and biotic stresses. In addition, the characterisation of cultivars by molecular markers (using RFLP, RAPD, AFLP and SSR techniques) as well as the safe conservation of genetic resources by non-conventional (cryopreservation) approach will also give their contribution to olive

improvement. To speed up this work, traditional breeding and biotechnological approach have to move in synergism, to drive the modernisation of olive culture in the 3rd millennium.

Acknowledgment This contribution was realised in the frame of a Bilateral Project (2006–2007) between the CNR of Italy (n° 2C) and the TUBITAK of Turkey (n° TBAG-U/120-104T260).

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