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Genetic improvement of olive (*Olea europaea* L.) by conventional and *in vitro* biotechnology methods

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Abstract

In olive (*Olea europaea* L.) traditional methods of genetic improvement have to now produced limited results. Intensification of olive growing requires appropriate new cultivars for fully mechanized groves, but among the large number of the traditional varieties very few are suitable. High-density and super high-density hedge row orchards require genotypes with reduced size, reduced apical dominance, a semi-erect growth habit, easy to propagate, resistant to abiotic and biotic stresses, with reliably high productivity and quality of both fruits and oil. Innovative strategies supported by molecular and biotechnological techniques are required to speed up novel hybridisation methods. Among traditional approaches the Gene Pool Method seems a reasonable option, but it requires availability of widely diverse germplasm from both cultivated and wild genotypes, supported by a detailed knowledge of their genetic relationships. The practice of "gene therapy" for the most important existing cultivars, combined with conventional methods, could accelerate achievement of the main goals, but efforts to overcome some technical and ideological obstacles are needed. The present review describes the benefits that olive and its products may obtain from genetic improvement using state of the art of conventional and unconventional methods, and includes progress made in the field of *in vitro* techniques. The uses of both traditional and modern technologies are discussed with recommendations.

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1. Introduction

Over 750 million olive trees are cultivated worldwide and 95% are located in Mediterranean Basin countries (between latitudes 30 and 45) characterized by hot dry summers and cool winters. The plants are sensitive to winter cold (-8 °C) but are able to tolerate drought and heat. Olive is cultivated both for oil extraction and table consumption with a world average production of 3-3.2 million tons per year. Europe produces 76.1% of all olive oil, followed by Asia with 12.2%, Africa with 10.7%, America 0.8%, and Oceania 0.1%. In Europe about 2.2 million tonnes of olive oil are produced by 1.9 million farmers which make up roughly one-third of all European Union (EU) farmers (Niaounakis and Halvadakis, 2006). Spain is the largest producer with an average 1 million tons per year, followed by Italy and Greece with 560 and 350 thousand tons respectively, and they together account for about 97% of EU olive oil production (FAOSTAT, 2013). Other countries that produce significant amounts of olive oil are Tunisia, Turkey, Syria, and Morocco. However, production drastically varies each year due to adverse climate conditions, particularly drought, and to pests and diseases, in addition to alternate bearing typical of the species.

In the traditional area of cultivation this species provides low anthropogenic pressures and agronomic inputs, being a biocenosis in equilibrium able to ensure the stability of the ecosystem with high homeostatic ability. This peculiarity has been held in high regard by the European Union which introduced the principle of "environmental conditionality". The concept of conditionality tends to strongly bring out the link between agriculture and territory, as a strategic factor to create favourable conditions for mutual enhancement as the principal benefit of rural areas. In this context, the application of environmental issues is a priority in order to promote agricultural production methods aimed at reducing environmental impacts, encouraging conservation of natural habitats, biodiversity of the agricultural landscape and exerting an ecological and hydro-geological defence, while at the same time characterizing the landscape. In other words the olive plays food, environmental and rural functions.

In the past olives were extensively cultivated under traditional regimes, but over recent decades a limited number of groves have been progressively established with more plants per hectare under irrigation. In most areas of the Mediterranean Basin olive groves are still characterized by old plants of great size, with low yield and alternate bearing, located in steeply sloping areas, and shaped according to the vase training system. These are not suitable for full mechanization of harvesting and pruning, and so do not guarantee profits to the land owners. For these reasons the search for new ways to exploit and promote its main product, the oil, is fundamental to maintain the groves which often host monumental plants, and are part of historical memory and culture - representing art under different form.

2 New approaches of cultivation and specific requirements from cultivars and rootstocks

Notwithstanding the objective of maintaining some of the traditional groves, there are means to establish new groves with high density plantations, thus fulfilling the aim to increase oil production to both meet the increasing demand for a good extra-virgin olive oil and to guarantee more income for farmers. The use of available cultivars, that are productive although oil quality is not excellent, might be a temporary solution in order to assure the increasing demand for extra-virgin oil, perhaps used to blend with oil from traditional groves to improve the quality sought under the current imperfect oil classification based on acidity and extraction methods.

A complete review derived from experience with super high-density cultivation has been reported by Rallo (2014). Previously Fontanazza et al., (1998a), and Moutier et al. (2010)

reported various methods to improve training systems for the new orchards based on tree architecture, tree training and use of low vigour genotypes.

Among more than one thousand known varieties, of which there are about 600 in Italy, very few are suitable for high density and super high-density cultivation systems that require 250-400 and 900-1200 plants per hectare respectively; most of the traditional cultivars are suitable for 250 plants/ha, that are normally used in new plantations of several countries. At the present time these modern groves are mainly realized with a few genotypes chosen among the traditional cultivars, such as Arbequina, Arbosana and Koroneiki. Other new varieties are also promising, such as the Italian FS17 (Favolosa) and Don Carlo (Fontanazza et al., 1998a), the Spanish Sikitita and Oliana (Bellini et al., 2008), and the Israeli Askal (Lavee et al., 2003). Varieties suitable for many of the required environments are not well known, because olive trees can change their general performance from one environment to another, including the production of secondary metabolites in the fruits which confer taste and health properties, or fatty acid composition, particularly oleic acid level. The reduction of this compound below a certain concentration compromises not only the quality but also marketability as extra-virgin olive oils.

Development through traditional cross-breeding of new cultivars suitable for mechanical pruning and harvesting is time consuming, whereas planting of vigorous traditional local cultivars grafted on dwarfing rootstocks appears a feasible strategy as an alternative to breeding of dwarf cultivars. In addition, this allows maintenance of the best local cultivars which are known for their oil quality, agronomic and healthy traits.

However, the availability of dwarfing rootstocks is very limited and their effectiveness cultivar-specific. A few accessions are currently under investigation, such as FS17 (Fontanazza et al., 1998b) and LD (Nardini et al., 2006; Rugini et al., 1996), together with some selections among traditional cultivars, seedlings and *in vitro* plantlets (both diploids and tetraploids) derived from mutagenesis (Rugini et al., 2011). However, in order to speed up rootstocks selection it would be advisable to identify them among available cultivars for which the phenotypic stability of the adult phase is known, rather than among seedlings which will require time for reliable selection. Consequently a large number of genotypes, of both cultivars and rootstocks, are required for genetic selection to fit in different environments, requirements of modern farming techniques, and criteria of oil and table fruit consumers. Research on new cultivars of table olives is still limited to a few breeding programmes (Lavee, 2013; Medina et al., 2012; Rallo, 2014a).

Variety renewal has been hampered by the extreme longevity of olive trees, the long period of juvenility of their offspring, and the diffidence of the public to accept genotypes obtained with advanced biotechnological approaches. Modern biotechnological techniques are suitable for olive improvement because they both allow direct correction of main defects, practicing a sort of “gene therapy” on the existing known superior cultivars, and can also support traditional breeding using the great genetic variability present in the species, to guide crossing of genotypes chosen among the olive populations of different sites.

In general cultivars to be employed for modern high-density and super high-density cultivation should possess a number of characteristics. Their form should be of reduced size, medium-low vigour, reduced apical dominance with a semi-erect habit, and a high number of lateral branches of small diameter (Rosati et al., 2013). They should also be resistant to abiotic and biotic stresses, provide consistent high production of high quality oil, and easy-to-propagate. Ideally, new cultivars should be self-fertile with high ability to bear in clusters (several fruit per inflorescence, as found for some species belonging to the genus *Olea*), early bearing with large fruits greater than two grams, with a progressive degradation during fruit ripening of phenolic and pectic compounds, with high flesh firmness, and progressive reduction of fruit detachment.

The oil should have a balanced composition of fatty acids, with high phenol content, including other compounds with health properties and characteristic flavour. An extra-virgin olive oil requires a high content of oleic acid (at least80% of the total fatty acids), a phenols content between 40 and 1500 mg/kg, and α-tocopherol between 50 and 750 mg/kg. Plants with these characteristics are necessary for all orchard types.

Rootstocks should be able to transmit to the scions a growth habit of dense and less vigorous shoots which are generally more efficient in flowering. The rootstocks should also provide stress resistance including drought, salt stress, heavy soils, and to root diseases.

Genetic improvement is now facilitated by combining traditional and modern *in vitro* technologies that have reached an advanced stage of development. Conventional methods include: a) clonal selection, b) *in vivo* induced mutation, c) crossing and selection after hybridization (bulk selection) and d) selection from programmed hybridization (*Gene Pool Method*). Unconventional methods include: a) selection of mutants from *in vitro* somaclonal variation, b) protoplast technology, c) selection of stable mutants from natural or induced *in vivo* mutagenesis, d) selection of haploids and polyploids, and finally e) transgenic plants for which a great number of valuable genes are available.

3 Clonal selection

This is a very useful technique for germplasm preservation, but very little success has been obtained in selecting olive genotypes suitable for cultivation. Although many selections have been made within populations of traditional cultivars; these have been labelled for sale by nurseries with little substantial improvement; but several with slightly improved characteristics (better fertility, more tolerance to pests and diseases, early ripening, larger fruits, dwarfing habit, grafting compatibility) have been described and reported in the literature (Fontanazza, 1987; Pannelli et al., 1993; Parlati et al., 1994, Loreti et al., 1994; Leitao et al., 1999, Sebastiani and Vitagliano, 1999, Serrano, 1990; Fiorino et al., 1999; Garcìa-Berenguer, 1978; Suarez et al., 1990; Tous et al., 1993; Tous et al., 1999; Abela and Serrano, 1984; Leitao et al., 1994; Martins et al., 1997; Serrano et al., 1997; Leitao et al., 1999; Serrano et al., 1999; Gregoriou, 1996; Gregoriou, 1999; Kafazi, 1987; Osmani, 1993; Delmas, 1986; Boulouha, 1986; Boulouha et al., 1992; Lansari and Bouchra, 1996; Khlif and Trigui, 1986, 1990; Trigui, 1996; Grati Kamoun et al., 2000; Arsel and Cirik, 1994; Krestnikov, 1981; Sholokhova, 1984; Ben-Ari G.,2014). Selection continues both in traditional and new olive growing countries where plants grow wild. Our experience in Nepal is that the flowering time between wild species (i.e. ssp *Cuspidata* and *europaea*) is staggered by about 2-3 weeks, reducing the probability of natural crossing.

4 Induced mutations

Poor results have been achieved by irradiation-induced mutations, but further exploration is warranted. It is quite easy to produce apparently dwarf mutants, but generally these are not stable, i.e. “Briscola”, a chimeric mutant of cv Ascolana Tenera, can be used only for ornamental purposes (Roselli and Donini, 1982). However an exception has been reported by Pannelli et al. (1990) and Rugini et al. (1996) with a mutant LD (Dwarf Leccino) that originated from irradiated Leccino cuttings. This mutant is stable and shows promising as a rootstock genotype able to reduce scion vigour in some cultivars. It is not suitable as a variety in its own right as it requires very late blooming pollinators, which is very rare among the known olive varieties. From the same experiment, which included cv Frantoio, several mixoploid mutants

were also produced. These plants allowed selection of triploids among seedlings, and tetraploid plants through *in vitro* culture of fragmented shoot tips (Rugini et al., 1996). These tetraploid plants were self-fertile. In addition, diploid mutants derived from self-incompatible "Leccino" were found to be self-compatible (unpublished data).

Rawashdeh (2003) reported the first attempts to measure the effect of radiation treatments on DNA level in olive trees, using RADP markers to detect genetic variation.

Oražem et al. (2013) investigated the effect of X-ray irradiation treatments of shoot cultures of cv Canino by detailed genetic analysis of induced mutation events comparing morphological measurements, studies of nuclear DNA content and molecular marker (SSR and AFLP) analyses. They concluded that X-ray irradiation of *in vitro* grown shoots provides an efficient system that generates a sufficient number of induced mutations, and that mutation events can be effectively scored by measurement of nuclear DNA content and AFLP profiling.

5 Crossing, hybrid selection and molecular markers.

In the absence of information on the genetic basis of target characteristics, the parents and their offspring are crossed with each other, which normally does not include self-fertilization due to self-incompatibility, and to avoid inbreeding depression. Cross breeding is very tedious because olive flowers are very small and fruit-set is very low (1-2%). Self-sterile mother genotypes are generally used to avoid emasculation, although some risk may occur due to partial self-compatibility observed in several varieties, which should be identified by molecular analysis. More research is necessary as the literature reports that a gametophytic self-incompatibility (GSI) system might exist in olive, even though cyto-histological observations and bio-molecular evidence support the presence in olive of a sporophytic self-incompatibility (SSI) system (Collani et al., 2012).

However, using conventional breeding, some genotypes with agronomical interesting traits have been selected and most are employed in high density groves. Hybrids have been particularly sought with tolerance to fungal diseases, high oil content and reduced size. New genotypes such as Barnea (Lavee et al., 1986), Masepo, Tamir and Askal (Lavee et al., 2003) have been released in Israel; the FS17, Don Carlo, Giulia and Tosca 07 in Italy (Fontanazza et al., 1998 a, b); and Sikitita in Spain (Bellini et al., 2008). Bellini et al. (2000) licensed three new cultivars after breeding and selection, and other promising breeding activities are under investigation from crossing of the cultivars Ascolana tenera, Carboncella, Dritta, Gentile di Chieti, Intossio and Leccino (Alfei et al., 2008). Further crossing are under development in many traditional olive countries, with the aim to improve the characteristic of local varieties and for obtaining new genotypes for table olives and dual-purpose cultivars (Trigui, 1996; Jardak, 2006; Laz, 2006; Arsel and Cirik, 1994; Yalcinkaya et al., 2000; Zafer Can and Isfendiyaroglu, 2006; Zeinanloo, 2006).

Furthermore, several other promising hybrids, derived from crossing cv Leccino with cv Bianca di Tirana, are under field evaluation at Tuscia University for self-fertility, dwarfing habit, and tolerance to peacock eye and olive fly.

Limited information on the hereditability of main traits has been reported. Nevertheless Zeinanloo et al. (2009) reported hereditability of fruit weight, percentage of dry matter, fruit width, fruit length and oil percentage in Iranian cultivars in order to find the best combination of parental genotypes. Moral et al., (2013) studied genetic effects on length of juvenile period (JP) and vigour (plant height and trunk diameter), finding that seedling vigour and JP significantly correlated with female parent and year of germination. Ben Sadok et al. (2013) investigated the genetic determinism of architectural traits in the F1 progeny from crossing two contrasting

olive genotypes, Olivière and Arbequina. They identified quantitative traits controlling growth, branching, first flowering and fruiting; the models were able to show a broad sense of heritability for traits including year of growth and branching order. Perez et al. (2014) reported that the major phenolic compounds (tyrosol or hydroxy-tyrosol molecules, lignans, flavonoids, and phenolic acids) of virgin olive oil in progeny derived from the cross of Picual x Arbequina cultivars, displayed a large degree of genetic variability, widely transgressing the parental levels, demonstrating a high degree of variability with just a single cross. The relationship between tissue size and cell number in the ovary, and tissue size in the fruit, was studied in eight cultivars with different fruit and ovary size by Rosati et al. (2012). They reported that tissue growth and partitioning in the fruit is largely determined by the characteristics of the ovary tissues at flowering, providing important information for plant breeding and crop management. Finally, it is important to pay attention to the choice of female parent since, in addition to the above mentioned traits, fruit shape, ripening, and oil accumulation are dominated by the female parent (Lavee et al., 2014).

Molecular markers have been used both in theoretical and applied research for applications such as resolution of the species origin, genetic characterization of the extensive olive germplasm, breeding programs, and traceability of oil. A description of the main genetic markers and the most relevant fields of application to olives were reviewed by Baldoni (2014) and Bracci et al. (2011). With respect to genomic research, knowledge regarding the olive tree is very limited when compared with other crops. The first sequences of *Olea europaea* were released in the last decade of the 20th century, as for other plant species, but research has since proceeded more slowly. Recently, many efforts have been made to fill this gap with improvement in the identification and annotation of genes prevalently based on identification of ESTs (expressed sequence tags), which are mainly related to allergens in olive pollen, and characteristics of fruits, olive oil and disease resistance (Merkuria et al., 2001). Significant progress in understanding the olive transcriptome was achieved by the identification of genes expressed during flower and fruit development. The transcriptome of fruits from two cultivars, Coratina and Tendellone, characterized by low and high phenolic content, was investigated using a 454 sequencing platform (Alagna et al., 2009). Bazakos et al. (2012) reported the identification of 209 and 36 differentially expressed transcripts in the salt-tolerant and salt-sensitive cultivar respectively.

Moreover, a large set of genes differentially expressed at different stages of fruit development in Leccino cultivar were identified using a SSH (Suppressive Subtractive Hybridization) approach (Galla et al., 2009). Finally, in recent times, the entire plastome of the Italian cultivar Frantoio has been published and new polymorphic plastid markers were identified (Mariotti et al., 2010). Research groups, mostly in Europe, have now started work on olive genome sequencing; these include the “Oleagen Genomics Project” in Spain, and the “Olea-Olive Genome Project” in Italy (http://genomes.cribi.unipd.it/olive/).

6 Selection from programmed hybridization (*Gene Pool Method*).

It has been suggested by Rugini et al. (2011a) that the Gene Pool Method (GPM), already adopted for herbaceous species, be introduced to olive breeding. This method uses information from phylogeny, domestication, and hybridisation affinity among cultivated, wild ecotypes, and other *Olea* species. The wild olive (*Oleaster*) is spread from the centre of origin (Minor Asia) to the Mediterranean basin. The large geographical area provided isolation that differentiated many different *oleaster* ecotypes, which differently affected olive domestication (Hannachi et al., 2008). The final result was the natural formation of different groups of domesticated olive according to environmental adaptation, morphology and performance

(Lavee and Zohary, 2011), although among this homogeneous autochthonous population it is often possible to find foreign cultivars introduced after domestication (Baldoni et al., 2000; Belaj et al., 2007; Muzzalupo et al., 2007). This diversity must be considered in any breeding program, as parents of widely separated origin produce offspring which may exhibit heterosis for many specific traits. The monumental ancient olive trees, which often cannot be identified among the local cultivars, represent a good source for parent choice, having survived local biotic and abiotic stresses for centuries.

Many important traits are polygenic, including tree vigour, productivity, resistance to biotic and abiotic stresses, and oil productivity. Before starting any breeding program the availability of specific genetic markers for such traits can be very valuable in the choice of parents.

The primary gene pool (GP1) of *O. europaea* ssp *europaea*, includes all the diploid and inter-fertile Mediterranean forms of *O. europaea* including the wild *oleaster,* for which the alleles for wildness have been discovered (Lumaret and Ouazani, 2001) distinguishing *olevaster* from cultivated varieties.

The secondary gene pool (GP2) of *O. europaea* ssp. *europaea* includes species having pre-zygotic incompatibility (overcome by *in vitro* pollination) with different ploidy level, such as *O. europaea* ssp *europaea* var *cerasiformis*, *O. europaea* ssp *europaea* var *maroccana, O. europaea* ssp *europaea* var *guanchica, O. europaea* ssp *europaea* var *laperrini*, *O. europaea* ssp *europaea* var *cuspidata.*

The tertiary gene pool (GP3) groups most of the *Ligustroides* species, such as *O. exasperata, O. capensis* ssp. *macrocarpa, O. capensis* spp. *capensis, O. woodiana, O. lancea, O. paniculata,* from which gene transfer to *O. europaea* ssp. *europaea* could occur only through *in vitro* culture of hybrid embryos (embryo rescue) as post-zygotic incompatibility occurs.

The quaternary gene-pool (GP4) of *O. europaea* ssp. *europaea,* is represented by highly incompatible genotypes for which gene mixing can only be overcome with genetic engineering.

Recourse to the use of related species with all known genetic technologies (conventional and unconventional methods) could be very useful in breeding programs. The oil quality of different *Olea* species have a similar composition to *Olea europaea* (Hannachi et al., 2009), and they possess important characters to be transferred such as disease resistance and fruit set. To realize such genetic improvement it is fundamental to collect and carefully evaluate the available agro-biodiversity across all the *Olea* taxa, and to undertake genetic mapping. To date, an international centralized service for conservation does not exist, but several institutions carry on recovery, propagation and maintenance of a large number of accessions, mainly local ones. Seventy-six collection centers are present in 24 countries (Rallo et al., 2005, Tous et al., 2005), but none of them are complete collections, although it would be very important to have large clonal collections replicated in different environments in order to observe their phenotypic behavior and to reduce risk of losing valuable germplasm. The International Olive Oil Council (IOOC) promoted a campaign aimed at retrieving and preserving accessions from distant locations and countries where preservation is not provided (Fabbri et al., 2004). These germplasm field banks (*ex situ*) are mainly located in traditional countries including Spain, Tunisia, Syria, Morocco, Algeria, Libia e Libano, Iran, Israel (Amiri, 2008; Aradhya and Preece, 2012; Biton et al., 2015) and also in Italy - in Calabria, Tuscany, and Sicily. These are very useful to characterize the varieties before use as Belaj et al. (2012) did for genotypes collected in the world's largest germplasm collection located in Spain, which provided a core collection based on molecular markers (AFLP, DArTs, SSRs, SNPs) and agronomic traits. But an international collaboration would substantially enhance this. Use of the "Gene Pool Method" should now be feasible with development

of required technologies, including *in vitro* techniques, material for gene transformation, pollination and fertilization *in vitro,* and embryo rescue to produce haploids, dihaploids, and polyploids, and to preserve genetic resources.

In addition to information about the hereditability of valuable characteristics, the availability of molecular markers assists definition of geographical sites of origin for *oleaster* and other *Olea spp.*; and may an interesting prospect is that these powerful markers may be used to reduce juvenility of offspring through the choice of appropriate cultivars as pollinators since this is genotype-dependent (Bellini, 1990; Fontanazza and Bartolozzi, 1998). Two strategies are currently used to shorten the long juvenile period. The first consists of forcing seedling growth in the greenhouse and nursery to increase growth in height, together with pruning the lateral shoots and, when it works well, grafting onto adult rootstocks. The second strategy is the identification and early selection of genotypes with a short juvenile period, which might be phenotypically recognizable at an early stage.

To the present, breeding work has been based on cultivated genotypes, except in few cases where feral olive populations have been tested (Hannachi et al., 2009). Wild olive germplasm contains more variability than the cultivated varieties (Belaj et al., 2010; Baldoni and Belaj, 2009), and is adapted to several environments. In particular the *Olea europaea* subsp. *europaea* var. *sylvestris,* may be a very important source of resistance to abiotic stresses such as drought, salt, wind and low temperature (Aranda et al., 2011; Baldoni et al., 2006; Klepo et al., 2013; Meddad-Hamza et al., 2010; Mulas, 1999) and biotic stresses such as *Verticillum wilt* (Sesli et al., 2010), peacock spot (Ciccarese et al., 2002), and olive fly (Mkize et al., 2008).

Besnard et al., (2001) first reported hybrids between olives and wild relatives of the same genus. Subsequently, Caceres et al., 2014 have produced hybrids by reciprocal inter-subspecific crosses between *Olea europaea* ssp. *europaea* and *Olea europaea* ssp. *cuspidata.* The morphology of the offspring, for which their hybrid nature has been proved by AFLP and SSR molecular markers, resembled the female parent.

To reach targeted goals it will be necessary to find breeding materials with promising alleles for their transfer. This will require intensive research on olive genetic resources studying inheritance and identifying target genotypes. On the other hand, appropriate application to the gene pool of biotechnological procedures based on *in vitro* culture can directly help breeding programmes. These procedures are useful in inducing new genetic variation by mutation breeding, in transferring genes from the wild to cultivated genomes by hybridization and clonal selection, to induce somaclonal variation, to change the ploidy level and to obtain homozygous plants.

7 In vitro techniques and genetic transformation

Micro-propagation by axillary bud stimulation is now available for many olive cultivars, and it can contribute to genetic improvement by both conventional and unconventional methods through: 1) rapid propagation of new genotypes, 2) pathogen elimination from the mother plants or offspring, 3) embryo rescue, 4) germplasm preservation, 5) plant regeneration from cell tissues, and 6) synthetic seed production.

7.1 Rapid propagation of new genotypes

The establishment of axenic cultures is best carried out using vigorous nodal explants; meristems or shoot-tips from field-grown or greenhouse plants are generally unsuccessful due to rapid oxidation of tissues in the sterilising solution. Pre-soaking nodal explants in PPM®

(Plant Preservative Mixture), and adding it to the medium, is generally effective to obtain sterile explants. The rapid growth, tender and elongated shoots are obtained on Olive Medium (OM) (Rugini, 1984) with addition of a mixture of growth regulators (Zeatin, 6-Benzylaminopurine, Thidiazuron, Metatopolin, Giberellic acid), mannitol as carbon source, and for some cultivars Dikegulac is effective to reduce apical dominance (Mendoza de Gyves et al., 2008). Peixe et al. (2007) reported that zeatin was successfully replaced by coconut water in combination with 6-Benzylaminopurine or kinetin with the cultivar Galega. In some cultivars satisfactory shoot proliferation is obtained by reinvigoration of the first shoots sprouting from the initial nodal explants. This could be achieved by inducing sprouting of basal buds on the initial shoots by systematic elimination of apical buds during the first subcultures of the shoots, or by *in vitro* grafting of the shoots onto juvenile seedlings. Rooting of explants takes advantage of basal darkening (Rugini et al., 1987) or by placing the whole culture in the dark for one week. Putrescine at 160 mg/l promoted early and effective rooting by increasing total peroxidase activity at the shoot base, which is essential for root induction (Rugini et al., 1997). The acclimatization phase after root emergence can be accelerated by forcing cuticle formation, stomata functionality and shoot growth by adding liquid medium over the solid medium (double layer), with the liquid containing a high amount of mannitol or sucrose (4-5%), putrescine (160 mg/l), and Giberellic acid (40-50 mg/l). Where possible, it is preferable to root *ex-vitro*, a process successfully verified for some cultivars, including cv Chemlali (Yacoub-Bougdal et al., 2007) and Italian cvs Frantoio, Maurino and Coratina; continuous light during this phase is essential (Leva, 2011).

7.2 Pathogen elimination from the mother plants and offspring

According to the present European legislation, olive plants can be certified only if they are totally virus-free. Since most olive plants are affected by several viruses, the diffusion of pathogen-free plants could be further progressed by olive nurseries. Meristem culture is not possible with collection from *in vivo* growing plants, but is relatively easy if the meristems are excised from *in vitro* growing shoots and rigorously placed on top of a small cube (5 mm) of solid proliferation OM medium in Petri dishes or multiwell plates. Plants of three Italian cultivars obtained by this method and grown in the field since 8 years are still virus-free (Rugini and Bottalico, 2011).

7.3 Immature embryo culture

Immature embryos of several Italian and Iranian cultivars, rescued less than two months after fertilization, have been successfully germinated (Hossein and Hajnajari, 2006; Rugini, 1988). Rapid germination was also recently achieved with three-month old embryos (unpublished data); these turned green within two days and after 15 to 20 days, with two or three nodes, the seedlings were ready to be transplanted to the pots for the hardening phase. This technique allows reduction by at least 18 months the time traditionally required to overcome the long seed dormancy period.

7.4 Germplasm preservation

The current trend is to establish groves with a small number of cultivars, generally the most productive or most suited to expect environmental conditions, without much consideration for germplasm preservation.

It is essential to avoid loss of important genotypes within particular gene pools. The conservation of seeds has little sense for vegetative propagated plants with a high degree of heterozygosis, such as olive, so *in vitro* preservation by both slow growth and, in particular, cryo-preservation, seems to be a promising alternative to field conservation as outdoor plants may be subjected to serious risk of both abiotic and biotic stress.

7.4.1 Slow growth preservation

Experiments carried out using two cultivars (Leccino and Frantoio) cultured *in vitro* on solid medium under dark condition at +4°C, allowed shoots to be preserved for 8 months (Lambardi et al., 2000). Micheli et al., 2007 reported a simple method of encapsulation in alginate nutrient gel of "Moraiolo" nodes in plastic cuvettes. After storage at +4 °C, compared with a control at room temperature, both for 15 and 30 days, axillary buds successfully developed, indicating a possible use of this technique for germplasm exchange over long distances.

7.4.2 Cryo-preservation

Various organs and tissues of olives have been cryo-preserved including somatic embryos or embryogenic tissues, seeds with or without endocarp, and shoot tips (Benelli et al., 2013). This allows long-term conservation of olive germplasm by direct immersion of tissues into liquid nitrogen (LN) (–196°C), or by using a vitrification solution before LN immersion. In the first case, Martinez et al. (1999) obtained 30% survival of olive shoot tips of the cv Arbequina following the removal of up to 30% of their moisture content and re-warming at room temperature. Success was obtained with a procedure of vitrification and one-step freezing in liquid nitrogen of shoot tips excised from *in vitro* grown shoot cultures of the cv Frantoio. In addition, somatic embryogenic cultures of Canino cultivar proved to be a highly suitable material for cryo-conservation using the vitrification approach (Lambardi et al., 2000). In general, olive embryogenic lines appeared to be highly suitable materials for cryo-preservation (Benelli et al., 2001; Lynch et al., 2011; Sanchez-Romero et al., 2009; Shibli and Al-Juboory, 2000). Cryo-preservation of shoot tips also appears promising using the vitrification technique; Benelli et al. (2001) obtained satisfactory post-rewarming shoot-tip survival with cvs 'Canino' and 'Gentile di Larino' respectively, but with poor re-growth, while good re-growth (38%) was observed in cv Frantoio following two steps dehydration with PVS2 (50% PVS2 for 30 min, then 100% PVS2 for 1 h), direct immersion in LN, and post-rewarming culture on medium containing a high concentration of zeatin (46 μM) (Lynch et al., 2007). An alternative to embryo conservation is the encapsulation of apical and nodal buds from micropropagated shoot cultures, but this currently shows a low rate of conversion into plants (Micheli et al., 1998).

7.5 Plant regeneration from cell tissues

7.5.1 Shoot organogenesis

Shoot organogenesis has been obtained both from zygotic and mature tissues. The work was aimed at organogenesis from mature explants since zygotic material is not suitable for genetic improvement due to the high heterozygosity of olive. Petioles from leaves of *in vitro* growing shoots of several cultivars (Canino, Moraiolo, Dolce Agogia and Halkidikis) showed good potential organogenesis (Mencuccini and Rugini, 1993), but is still not efficient enough to regenerate plants from modified cells with any biotechnological technique (gene transfer,

somaclonal variation, or induced mutation by physical or chemical approaches). It is considered important to develop an effective procedure for somatic embryogenesis.

7.5.2 Somatic Embryogenesis

Somatic embryogenesis has been successfully achieved from zygotic embryos 60 to 75 days after fertilization (Leva et al., 1995; Rugini, 1988), however this capacity can be extended to a period of at least two months by storing whole detached fruitlets at 14-15°C (Rugini, 1995). Somatic embryogenesis has also been achieved from non-germinated mature embryos of both wild (Orinos and Mitrakos, 1991) and cultivated olive (Mitrakos et al., 1992; Shibli et al., 2001).

Somatic embryogenesis from mature tissues is still difficult to achieve, however, cyclic somatic embryogenesis has been obtained from two cultivars, Canino and Moraiolo, through the novel 'double regeneration system' (Rugini and Caricato, 1995). These embryos originated from very small leaflets of adventitious neo-formed buds from petiole tissues. Rejuvenation through adventitious shoot organogenesis does not seem to be essential for an embryogenic response for all cultivars. For example, this technique was not required in cv Dabbhia (Mazri et al., 2013) and in a genotype of *O. europaea* L. var. *sylvestris* (Capelo et al., 2010). The growth regulator thidiazuron (TDZ) seems to be a very important trigger for induction of somatic embryogenesis; in all experiments reported above it was effective. In addition the antibiotic cefotaxime seems to be an essential factor, since in all cases above reported it was always present in the medium. Secondary embryos differentiate mainly with unicellular origin from the epidermal surface (Lambardi et al., 1999), establishing a very efficient cycle of somatic embryogenesis. The long-term cyclic production of embryos direct from epidermal cells could be a great advantage in regenerating plants from transgenic cells because it avoids callus formation, which could be a wide source of undesirable genetic variability. The high production of somatic embryos should make it useful to employ for somaclonal variation under selection pressure but, in our experience, phenotypic variability was never observed after many years in field-trials with the cv Canino; this was confirmed by Lopes et al. (2009) in *Olea* spp., where genome integrity was maintained throughout the stages of embryogenesis. However, in a case only, a different vegetative behaviour (bushy and columnar phenotype) was reported in plants derived from somatic embryos of the same cotyledon in the cv Frangivento (Leva and Petruccelli, 2007). These conflicting results suggest consideration be given to potential for unwanted variation before using somatic embryogenesis for propagating true-to-type olive plants. Somatic embryogenesis can be applied for production of "synthetic seeds" which can also be useful for germplasm preservation (Lynch et al., 2007).

An efficient method of plant regeneration from cells is essential to support unconventional genetic improvement such as selection of mutants derived from somaclonal variation, both *in vitro* and *in vivo*, natural or induced, protoplast technology, haploids and polyploids, and production and selection of transgenic plants.

7.6 Haploids

Production of homozygous olive plants by self-fertilization seems to be dubious considering the frequent self-sterility and the long juvenile phase which characterize this species. Homozygous plants would be of great interest for isolation of mutants and recessive traits. Anther, ovary, pollen and ovule cultures should be explored with the aim of producing di-haploid plants by doubling the number of chromosomes in a short period of time (Germanà, 2006). Recently, promising results were obtained employing a new method of isolated microspore culture which

led to cell division and pro-embryo formation in the cvs Arbequina and Picual (Bueno et al., 2005). Various experiments have been carried out by using compatible pollen treated with physical agents (UV and X-rays), and treatments with toluidine blue at different time after pollination. The fruit recovered showed different classes of fruit size and shape. This embryo culture produced several plantlets which are under investigation.

7.7 Triploids and tetraploids

Tetraploid plants do not naturally exist in *Olea europaea* ssp *sativa*. Rugini et al. (1996) produced several stable tetraploid plants of the cultivars Leccino and Frantoio by *in vitro* shoot-tip fragmentation, starting from mixoploid plants generated by γ-ray irradiation of cuttings. The separation of tetraploids (*4n*) from diploid (*2n*) shoots has been carried out since two distinct groups appeared during the subcultures; shoots that were wide, long and thick corresponded to stable tetraploids, while the other group was diploid. In a field trial the *4n* plants maintained the same leaf morphology but showed a reduction of trunk and canopy size, flexible branches, absence of juvenility, and uniformly large flower and fruit size; some of the embryos from the large fruits were triploid. The *4n* "Leccino" acquired self-fertility while the *2n* mutants, although retaining auto-incompatibility acquired inter-fertility among other *2n* mutants and with self-sterile Leccino mother plants. This could be a benefit for genetic improvement since reciprocal crosses could reduce heterozygosity within the cultivar. Furthermore both *2n* and *4n* mutants, when used as rootstock, reduced the size of the cv Canino scion within 8 years observation. The *2n* mutants of Leccino were characterized by consistently extraordinary cropping and pollination capacities, while maintaining the same oil characteristics and tolerance to *Spilocea oleagina* as the Leccino mother plants. For these reasons this mutant could be a candidate for high density olive cultivation.

7.8 Genetic transformation and plant recovery

Gene transfer technology might be a useful alternative to speed up genetic improvement by correcting defects in important commercial cultivars (gene therapy). However, two important factors are essential: the availability of morphogenetic tissues of valuable cultivars and the availability of useful genes. With the aim of modifying canopy architecture, increasing rooting ability, and tolerance to abiotic and abiotic stresses, chimeric plants with *ri-TDNA* of *Agrobacterium rhizogenes*, and uniform transgenic plants with *rolABC* and *osmotin* genes, were produced and evaluated in a field trial over ten years (Rugini, 2015).
Several Italian olive cultivars have been rooted by simply infecting the basal part of *in vitro* proliferated shoots with *Agrobacterium rhizogenes* wild type, or strain NCPPB 1855 (Rugini, 1992; Rugini 1986). However, the roots are rarely transgenic, allowing us to speculate that the roots originate from non-transformed neighbour cells to transformed ones or, less likely, the roots were induced by unknown compounds present in the *Agrobacterium* exudates (Rugini et al., 2000). Strobel et al. (1988) carried out greenhouse experiments with some Tunisian olive plantlets using another strain of *A. rhizogenes* (strain 232) and, by infecting the root system after uniformly trimming to 4-5 cm, observed after sixty days a root mass greater than the controls, with transgenic roots emerging from the primary roots. They reported a beneficial effect both on vegetative growth and reproductive parameters, although the new roots appear poorly connected with the existing primary ones. These observations encourage use of these techniques in some agricultural practices, as well as for rooting of recalcitrant olive genotypes or modification of some vegetative or reproductive parameters. However more scientific information is needed because different responses are obtained according to the variety or

587 species. The nature of the transformation event can also change the outcome, as observed in
588 other woody fruit species such as cherry and plum rootstocks, simple infection to induce root
589 formation can affect plant morphology and reproduction.
590 *Agrobacterium tumefaciens* strain LBA 4404 harbouring pBin19 with *rolABC* of *A. rhizogenes*
591 and the gene *nptII* for kanamycin resistance, under control of a natural promoter, have been
592 used for olive transformation. Both immature zygotic embryos of the cv Moraiolo and somatic
593 embryos from mature tissues of cv Canino, were used. In the first experiment zygotic embryos
594 resistant to kanamycin were selected (Rugini and Fedeli, 1990), in the second experiment,
595 transgenic plants were selected following cyclic somatic embryogenesis of the cv Canino and
596 field trials were carried out (Rugini et al., 2008). The *rolABC* plants showed the typical hairy
597 root phenotype, with prolonged vegetative growth to late autumn and a long juvenile phase;
598 although originated from mature tissues, the reproductive phase was not observed because the
599 Italian Minister of Environmental did not renew approval of the field trial when the first period
600 expired. However, after 10 years, the plants still maintained the same phenotype and the
601 presence of the foreign genes as shown by Real Time PCR analysis (Miano et al., 2004).
602 Through *in vitro* testing the transgenic plants revealed high rooting ability, which were 50%
603 rooted in auxin-free medium, reaching up to 60% rooting with addition of only 160 mg/l of
604 putrescine, while non-transformed plants did not root at all. The same high rooting ability has
605 been exhibited by the semi-hardwood cuttings collected from field grown plants, although in
606 this case a low amount of auxin was beneficial.
607 Somatic embryos of olives were transformed with *Agrobacterium tumefaciens* as described for
608 *rolABC* gene transformation to produce plants over-expressing tobacco *osmotin* (Rugini et al.,
609 2000). This gene is present in the genome of all plant species now tested and codes for a protein
610 of the PR5 (Pathogen Related Protein) family. This is expressed under both abiotic (mainly
611 under drought conditions) and biotic stresses, resulting in effective control of fungal diseases by
612 inducing an oxidative burst when hyphae come into contact with plant tissues. Recently the
613 osmotin protein was found to be a homologue of the human adiponectine, involved in glucose
614 metabolism; it seems to be the basis of new therapies for the treatment of various diseases
615 including diabetes, cancer and some diseases of the central nervous system (Naseer et al.,
616 2014). Transformation has been realized using *A. tumefaciens*, *LBA4404*, with the *pKYLX71*
617 plasmid harbouring the tobacco *osmotin* gene under control of the *35S* promoter, and plants
618 tested in the field (Rugini et al., 2000). After ten-years of growth they showed similar
619 phenotype to non-transformed plants also derived from somatic embryos, but with narrower
620 leaf lamina and high amounts of protein around the vacuoles in cells of epidermal and
621 sub-epidermal tissues (D'Angeli et al., 2001). These plants were more tolerant to peacock spot
622 (*Spilocea oleagina* Hughes), but showed an unexplained particular attraction to the insect
623 *Otiorhynchus cribricollis* Gyllenhal (unpublished data). Furthermore, over-expression of
624 osmotin induced cold protection by affecting programmed cell death and cytoskeleton
625 organization (D'Angeli and Altamura, 2007); these plants also showed an extraordinary
626 drought resistance. In a field trial young *osmotin* plants suffered under ordinary irrigation with
627 initial slow growth followed by leaf drop, and after few months died; this contrasted with
628 non-irrigated plants which grew indistinguishable from non-transformed plants for ten years
629 (Rugini et al., 2000). Experiments were carried out during summer in pots using two-year-old
630 plants derived from three different transformation events which were compared with analogous
631 plants of Canino wt and the Canino *rolABC* grafted on to Canino wt, which demonstrated an
632 extraordinary drought resistance of the transgenic plants: after four weeks all the transformed
633 plants showed normal growth, while the others died in the absence of water supply. Similar to
634 *rolABC* plants described earlier, the *osmotin* plants displayed a high degree of juvenility.
635 Finally, with *in vitro* experiments, transgenic shoots showed normal growth when 4% PEG

(polyethylene glycol) was present in the medium; this was accompanied by a greater accumulation in the tissues of proline and enzymes specific to drought stress (unpublished data), while the Canino *wt* showed clear signs of damage on the leaves with reduced growth evident.

Few other transgenic trials have since been attempted with olive. Recently Torreblanca et al. (2010) attempted transformation using embryogenic cultures derived from radicles of mature seeds cv Picual with *Agrobacterium tumefaciens*, harbouring *pBINUbiGUSint* or *pGUSINT* binary plasmids. These vectors contained the *nos-nptII* and the *uidA* gene driven by the maize poly-ubiquitin *Ubi1* and *CaMV35S* promoter, respectively.

Other traits in important commercial olive trees may be improved by "gene therapy", including examples such as: a) production of completely self-fertile plants, b) increase in the content and quality of oil, c) production of plants with parthenocarpic fruits, d) increased tolerance to cold and salt stress, e) regulation of fruit ripening and f) increase pathogens and parasites resistance. However, there are currently many genes of different origin available, whereas the number of olive genes isolated and characterized is still rare.

7.9 Protoplast technology

Protoplast technology is useful for studies, including protoplast fusion to produce hybrids and cybrids from cross-incompatible genotypes, to produce triploids and polyploids, or to introduce foreign naked DNA into cells by liposome. Viable olive protoplasts were isolated and cultured, and in some cases micro-calli were obtained, but plant regeneration has not been reported yet (Cañas et al., 1987; Mencuccini, 1991; Perri et al., 1994; Rugini, 1986).

7.10 In vitro mutagenesis

To increase the frequency of somaclonal variation above the spontaneous level with *in vitro* cultures, various physical and chemical agents can be used which, at the same time, might induce and/or select mutated cells. This technique requires a very efficient regeneration method such as the cyclic somatic embryogenesis mentioned above.

Ozair et al. (2014) added the mutagen oryzalin to OM culture medium and recovered shoots of cv Moraiolo with highly significant differences in shoot length, fresh weight, dry weight, leaf area, number of nodes, and number and length of roots. The use of mutagenic agents represents a useful tool not only to obtain new varieties, but also to study gene function. Application of the mutagen to *in vitro* shoots of cv Canino, during the multiplication phase, did not modify the vegetative habit but, in the field, the fruits in some plants were about 80% smaller, and for others about 20% larger, compared to the mother plants (unpublished data).

The olive species is characterized by the ability to produce suckers at the root transition region. It therefore should be possible, after *in vitro* roots emerge, to apply physical or chemical mutagens to this zone corresponding to where the suckers will originate in the field. This procedure should overcome both the difficulties of shoot organogenesis and, at the same time, should avoid chimeric tissues formation in the regenerated suckers since, being an example of organogenesis, they would normally originate from a single cell.

8. Conclusions and remarks

The genetic improvement of olive trees by classic and advanced biotechnological strategies offers to enhance yield and quality of its products, and speed up development of new cultivars

and rootstocks suitable for a modern intensive mechanized olive industry. Classical methods have failed to meet the requirements of olive production and to keep up with other demands of the olive industry. The application of advanced technologies as support or alone is essential to expand olive cultivation in arid and semiarid areas worldwide, and in other inhospitable areas, and to address additional characteristics to better use as oil or fruit for table consumption. It is advisable to employ the wild olive (*oleaster*) in new breeding programmes to increase variability. It is mainly due to their continuous omission that natural and cross breeding selection among autochthonous cultivars is not enough. Moreover, it is essential to introduce genes from other compatible *Olea* species and to breed among genotypes selected from widely distant sites. Deep genomic information is fundamental to facilitate the choice of parents, according to each specific aim, and to support effective use of genetic engineering. More systematic work is needed to overcome the lack of basic information on olives. However, biotechnological methodologies are now available, together with tools for marker assisted breeding, and these provide sufficient foundation to start massive genetic improvement.

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Genetic improvement of olive (*Olea europaea* L.) by conventional and *in vitro* biotechnology methods

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Abstract

In olive (*Olea europaea* L.) traditional methods of genetic improvement have to now produced limited results. Intensification of olive growing requires appropriate new cultivars for fully mechanized groves, but among the large number of the traditional varieties very few are suitable. High-density and super high-density hedge row orchards require genotypes with reduced size, reduced apical dominance, a semi-erect growth habit, easy to propagate, resistant to abiotic and biotic stresses, with reliably high productivity and quality of both fruits and oil. Innovative strategies supported by molecular and biotechnological techniques are required to speed up novel hybridisation methods. Among traditional approaches the Gene Pool Method seems a reasonable option, but it requires availability of widely diverse germplasm from both cultivated and wild genotypes, supported by a detailed knowledge of their genetic relationships. The practice of "gene therapy" for the most important existing cultivars, combined with conventional methods, could accelerate achievement of the main goals, but efforts to overcome some technical and ideological obstacles are needed. The present review describes the benefits that olive and its products may obtain from genetic improvement using state of the art of conventional and unconventional methods, and includes progress made in the field of *in vitro* techniques. The uses of both traditional and modern technologies are discussed with recommendations.

Keywords: *Olea europaea*, intensive cultivation, rootstocks, gene pool hybridization method, *in vitro* techniques, gene availability, genetic transformation

1. Introduction

Over 750 million olive trees are cultivated worldwide and 95% are located in Mediterranean Basin countries (between latitudes 30 and 45) characterized by hot dry summers and cool winters. The plants are sensitive to winter cold (-8 °C) but are able to tolerate drought and heat. Olive is cultivated both for oil extraction and table consumption with a world average production of 3-3.2 million tons per year. Europe produces 76.1% of all olive oil, followed by Asia with 12.2%, Africa with 10.7%, America 0.8%, and Oceania 0.1%. In Europe about 2.2 million tonnes of olive oil are produced by 1.9 million farmers which make up roughly one-third of all European Union (EU) farmers (Niaounakis and Halvadakis, 2006). Spain is the largest producer with an average 1 million tons per year, followed by Italy and Greece with 560 and 350 thousand tons respectively, and they together account for about 97% of EU olive oil production (FAOSTAT, 2013). Other countries that produce significant amounts of olive oil are Tunisia, Turkey, Syria, and Morocco. However, production drastically varies each year due to adverse climate conditions, particularly drought, and to pests and diseases, in addition to alternate bearing typical of the species.

In the traditional area of cultivation this species provides low anthropogenic pressures and agronomic inputs, being a biocenosis in equilibrium able to ensure the stability of the ecosystem with high homeostatic ability. This peculiarity has been held in high regard by the European Union which introduced the principle of "environmental conditionality". The concept of conditionality tends to strongly bring out the link between agriculture and territory, as a strategic factor to create favourable conditions for mutual enhancement as the principal benefit of rural areas. In this context, the application of environmental issues is a priority in order to promote agricultural production methods aimed at reducing environmental impacts, encouraging conservation of natural habitats, biodiversity of the agricultural landscape and exerting an ecological and hydro-geological defence, while at the same time characterizing the landscape. In other words the olive plays food, environmental and rural functions.

In the past olives were extensively cultivated under traditional regimes, but over recent decades a limited number of groves have been progressively established with more plants per hectare under irrigation. In most areas of the Mediterranean Basin olive groves are still characterized by old plants of great size, with low yield and alternate bearing, located in steeply sloping areas, and shaped according to the vase training system. These are not suitable for full mechanization of harvesting and pruning, and so do not guarantee profits to the land owners. For these reasons the search for new ways to exploit and promote its main product, the oil, is fundamental to maintain the groves which often host monumental plants, and are part of historical memory and culture - representing art under different form.

2 New approaches of cultivation and specific requirements from cultivars and rootstocks

Notwithstanding the objective of maintaining some of the traditional groves, there are means to establish new groves with high density plantations, thus fulfilling the aim to increase oil production to both meet the increasing demand for a good extra-virgin olive oil and to guarantee more income for farmers. The use of available cultivars, that are productive although oil quality is not excellent, might be a temporary solution in order to assure the increasing demand for extra-virgin oil, perhaps used to blend with oil from traditional groves to improve the quality sought under the current imperfect oil classification based on acidity and extraction methods.

A complete review derived from experience with super high-density cultivation has been reported by Rallo (2014). Previously Fontanazza et al., (1998a), and Moutier et al. (2010)

reported various methods to improve training systems for the new orchards based on tree architecture, tree training and use of low vigour genotypes.

Among more than one thousand known varieties, of which there are about 600 in Italy, very few are suitable for high density and super high-density cultivation systems that require 250-400 and 900-1200 plants per hectare respectively; **most of the traditional cultivars are suitable for 250 plants/ha, that are normally used in new plantations of several countries.** At the present time these modern groves are mainly realized with a few genotypes chosen among the traditional cultivars, such as Arbequina, Arbosana and Koroneiki. Other new varieties are also promising, such as the Italian FS17 (Favolosa) and Don Carlo (**Fontanazza et al., 1998a**), the Spanish Sikitita and Oliana (**Bellini et al., 2008**), and the Israeli Askal (**Lavee et al., 2003**). Varieties suitable for many of the required environments are not well known, because olive trees can change their general performance from one environment to another, including the production of secondary metabolites in the fruits which confer taste and health properties, or fatty acid composition, particularly oleic acid level. The reduction of this compound below a certain concentration compromises not only the quality but also marketability as extra-virgin olive oils.

Development through traditional cross-breeding of new cultivars suitable for mechanical pruning and harvesting is time consuming, whereas planting of vigorous traditional local cultivars grafted on dwarfing rootstocks appears a feasible strategy as an alternative to breeding of dwarf cultivars. In addition, this allows maintenance of the best local cultivars which are known for their oil quality, agronomic and healthy traits.

However, the availability of dwarfing rootstocks is very limited and their effectiveness cultivar-specific. A few accessions are currently under investigation, such as FS17 (Fontanazza et al., 1998b) and LD (Nardini et al., 2006; Rugini et al., 1996), together with some selections among traditional cultivars, seedlings and *in vitro* plantlets (both diploids and tetraploids) derived from mutagenesis (Rugini et al., 2011). However, in order to speed up rootstocks selection it would be advisable to identify them among available cultivars for which the phenotypic stability of the adult phase is known, rather than among seedlings which will require time for reliable selection. Consequently a large number of genotypes, of both cultivars and rootstocks, are required for genetic selection to fit in different environments, requirements of modern farming techniques, and criteria of oil and table fruit consumers. Research on new cultivars of table olives is still limited to a few breeding programmes (Lavee, 2013; Medina et al., 2012; Rallo, 2014a).

Variety renewal has been hampered by the extreme longevity of olive trees, the long period of juvenility of their offspring, and the diffidence of the public to accept genotypes obtained with advanced biotechnological approaches. Modern biotechnological techniques are suitable for olive improvement because they both allow direct correction of main defects, practicing a sort of "gene therapy" on the existing known superior cultivars, and can also support traditional breeding using the great genetic variability present in the species, to guide crossing of genotypes chosen among the olive populations of different sites.

In general cultivars to be employed for modern high-density and super high-density cultivation should possess a number of characteristics. Their form should be of reduced size, medium-low vigour, reduced apical dominance with a semi-erect habit, and a high number of lateral branches of small diameter (Rosati et al., 2013). They should also be resistant to abiotic and biotic stresses, provide consistent high production of high quality oil, and easy-to-propagate. Ideally, new cultivars should be self-fertile with high ability to bear in clusters (several fruit per inflorescence, as found for some species belonging to the genus *Olea*), early bearing with large fruits greater than two grams, with a progressive degradation during fruit ripening of phenolic and pectic compounds, with high flesh firmness, and progressive reduction of fruit detachment.

The oil should have a balanced composition of fatty acids, with high phenol content, including other compounds with health properties and characteristic flavour. An extra-virgin olive oil requires a high content of oleic acid (at least80% of the total fatty acids), a phenols content between 40 and 1500 mg/kg, and α-tocopherol between 50 and 750 mg/kg. Plants with these characteristics are necessary for all orchard types.

Rootstocks should be able to transmit to the scions a growth habit of dense and less vigorous shoots which are generally more efficient in flowering. The rootstocks should also provide stress resistance including drought, salt stress, heavy soils, and to root diseases.

Genetic improvement is now facilitated by combining traditional and modern *in vitro* technologies that have reached an advanced stage of development. Conventional methods include: a) clonal selection, b) *in vivo* induced mutation, c) crossing and selection after hybridization (bulk selection) and d) selection from programmed hybridization (*Gene Pool Method*). Unconventional methods include: a) selection of mutants from *in vitro* somaclonal variation, b) protoplast technology, c) selection of stable mutants from natural or induced *in vivo* mutagenesis, d) selection of haploids and polyploids, and finally e) transgenic plants for which a great number of valuable genes are available.

3 Clonal selection

This is a very useful technique for germplasm preservation, but very little success has been obtained in selecting olive genotypes suitable for cultivation. Although many selections have been made within **populations** of traditional cultivars; these have been labelled for sale by nurseries with little substantial improvement; but several with slightly improved characteristics (better fertility, more tolerance to pests and diseases, early ripening, larger fruits, dwarfing habit, grafting compatibility) have been described and reported in the literature (Fontanazza, 1987; Pannelli et al., 1993; Parlati et al., 1994, Loreti et al., 1994; Leitao et al., 1999, Sebastiani and Vitagliano, 1999, Serrano, 1990; Fiorino et al., 1999; Garcìa-Berenguer, 1978; Suarez et al., 1990; Tous et al., 1993; Tous et al., 1999; Abela and Serrano, 1984; Leitao et al., 1994; Martins et al., 1997; Serrano et al., 1997; Leitao et al., 1999; Serrano et al., 1999; Gregoriou, 1996; Gregoriou, 1999; Kafazi, 1987; Osmani, 1993; Delmas, 1986; Boulouha, 1986; Boulouha et al., 1992; Lansari and Bouchra, 1996; Khlif and Trigui, 1986, 1990; Trigui, 1996; Grati Kamoun et al., 2000; Arsel and Cirik, 1994; Krestnikov, 1981; Sholokhova, 1984; Ben-Ari G.,2014). Selection continues both in traditional and new olive growing countries where plants grow wild. Our experience in Nepal is that the flowering time between wild species (i.e. ssp *Cuspidata* and *europaea*) is staggered by about 2-3 weeks, reducing the probability of natural crossing.

4 Induced mutations

Poor results have been achieved by irradiation-induced mutations, but further exploration is warranted. It is quite easy to produce apparently dwarf mutants, but generally these are not stable, i.e. “Briscola”, a chimeric mutant of cv Ascolana Tenera, can be used only for ornamental purposes (Roselli and Donini, 1982). However an exception has been reported by Pannelli et al. (1990) and Rugini et al. (1996) with a mutant LD (Dwarf Leccino) that originated from irradiated Leccino cuttings. This mutant is stable and shows promising as a rootstock genotype able to reduce scion vigour in some cultivars. It is not suitable as a variety in its own right as it requires very late blooming pollinators, which is very rare among the known olive varieties. From the same experiment, which included cv Frantoio, several mixoploid mutants

were also produced. These plants allowed selection of triploids among seedlings, and tetraploid plants through *in vitro* culture of fragmented shoot tips (Rugini et al., 1996). These tetraploid plants were self-fertile. In addition, diploid mutants derived from self-incompatible "Leccino" were found to be self-compatible (unpublished data).
Rawashdeh (2003) reported the first attempts to measure the effect of radiation treatments on DNA level in olive trees, using RADP markers to detect genetic variation.
Oražem et al. (2013) investigated the effect of X-ray irradiation treatments of shoot cultures of cv Canino by detailed genetic analysis of induced mutation events comparing morphological measurements, studies of nuclear DNA content and molecular marker (SSR and AFLP) analyses. They concluded that X-ray irradiation of *in vitro* grown shoots provides an efficient system that generates a sufficient number of induced mutations, and that mutation events can be effectively scored by measurement of nuclear DNA content and AFLP profiling.

5 Crossing, hybrid selection and molecular markers.

In the absence of information on the genetic basis of target characteristics, the parents and their offspring are crossed with each other, which normally does not include self-fertilization due to self-incompatibility, and to avoid inbreeding depression. Cross breeding is very tedious because olive flowers are very small and fruit-set is very low (1-2%). Self-sterile mother genotypes are generally used to avoid emasculation, although some risk may occur due to partial self-compatibility observed in several varieties, which should be identified by molecular analysis. More research is necessary as the literature reports that a gametophytic self-incompatibility (GSI) system might exist in olive, even though cyto-histological observations and bio-molecular evidence support the presence in olive of a sporophytic self-incompatibility (SSI) system (Collani et al., 2012).
However, using conventional breeding, some genotypes with agronomical interesting traits have been selected and most are employed in high density groves. Hybrids have been particularly sought with tolerance to fungal diseases, high oil content and reduced size. New genotypes such as Barnea (**Lavee et al., 1986**), Masepo, Tamir and Askal (**Lavee et al., 2003**) have been released in Israel; the FS17, Don Carlo, Giulia and Tosca 07 in Italy (**Fontanazza et al., 1998 a, b**); and Sikitita in Spain (Bellini et al., 2008).
Bellini et al. (2000) licensed three new cultivars after breeding and selection, and other promising breeding activities are under investigation from crossing of the cultivars Ascolana tenera, Carboncella, Dritta, Gentile di Chieti, Intossio and Leccino (Alfei et al., 2008). Further crossing are under development in many traditional olive countries, with the aim to improve the characteristic of local varieties and for obtaining new genotypes for table olives and dual-purpose cultivars (Trigui, 1996; Jardak, 2006; Laz, 2006; Arsel and Cirik, 1994; Yalcinkaya et al., 2000; Zafer Can and Isfendiyaroglu, 2006; Zeinanloo, 2006).
Furthermore, several other promising hybrids, derived from crossing cv Leccino with cv Bianca di Tirana, are under field evaluation at Tuscia University for self-fertility, dwarfing habit, and tolerance to peacock eye and olive fly.
Limited information on the hereditability of main traits has been reported. Nevertheless Zeinanloo et al. (2009) reported hereditability of fruit weight, percentage of dry matter, fruit width, fruit length and oil percentage in Iranian cultivars in order to find the best combination of parental genotypes. Moral et al., (2013) studied genetic effects on length of juvenile period (JP) and vigour (plant height and trunk diameter), finding that seedling vigour and JP significantly correlated with female parent and year of germination. Ben Sadok et al. (2013) investigated the genetic determinism of architectural traits in the F1 progeny from crossing two contrasting

olive genotypes, Olivière and Arbequina. They identified quantitative traits controlling growth, branching, first flowering and fruiting; the models were able to show a broad sense of heritability for traits including year of growth and branching order. Perez et al. (2014) reported that the major phenolic compounds (tyrosol or hydroxy-tyrosol molecules, lignans, flavonoids, and phenolic acids) of virgin olive oil in progeny derived from the cross of Picual x Arbequina cultivars, displayed a large degree of genetic variability, widely transgressing the parental levels, demonstrating a high degree of variability with just a single cross. The relationship between tissue size and cell number in the ovary, and tissue size in the fruit, was studied in eight cultivars with different fruit and ovary size by Rosati et al. (2012). They reported that tissue growth and partitioning in the fruit is largely determined by the characteristics of the ovary tissues at flowering, providing important information for plant breeding and crop management. Finally, it is important to pay attention to the choice of female parent since, in addition to the above mentioned traits, fruit shape, ripening, and oil accumulation are dominated by the female parent (Lavee et al., 2014).

Molecular markers have been used both in theoretical and applied research for applications such as resolution of the species origin, genetic characterization of the extensive olive germplasm, breeding programs, and traceability of oil. A description of the main genetic markers and the most relevant fields of application to olives were reviewed by Baldoni (2014) and Bracci et al. (2011). With respect to genomic research, knowledge regarding the olive tree is very limited when compared with other crops. The first sequences of *Olea europaea* were released in the last decade of the 20th century, as for other plant species, but research has since proceeded more slowly. Recently, many efforts have been made to fill this gap with improvement in the identification and annotation of genes prevalently based on identification of ESTs (expressed sequence tags), which are mainly related to allergens in olive pollen, and characteristics of fruits, olive oil and disease resistance (Merkuria et al., 2001). Significant progress in understanding the olive transcriptome was achieved by the identification of genes expressed during flower and fruit development. The transcriptome of fruits from two cultivars, Coratina and Tendellone, characterized by low and high phenolic content, was investigated using a 454 sequencing platform (Alagna et al., 2009). Bazakos et al. (2012) reported the identification of 209 and 36 differentially expressed transcripts in the salt-tolerant and salt-sensitive cultivar respectively.

Moreover, a large set of genes differentially expressed at different stages of fruit development in Leccino cultivar were identified using a SSH (Suppressive Subtractive Hybridization) approach (Galla et al., 2009). Finally, in recent times, the entire plastome of the Italian cultivar Frantoio has been published and new polymorphic plastid markers were identified (Mariotti et al., 2010). Research groups, mostly in Europe, have now started work on olive genome sequencing; these include the “Oleagen Genomics Project” in Spain, and the “Olea-Olive Genome Project” in Italy (http://genomes.cribi.unipd.it/olive/).

6 Selection from programmed hybridization (*Gene Pool Method*).

It has been suggested by Rugini et al. (2011a) that the Gene Pool Method (GPM), already adopted for herbaceous species, be introduced to olive breeding. This method uses information from phylogeny, domestication, and hybridisation affinity among cultivated, wild ecotypes, and other *Olea* species. The wild olive (*Oleaster*) is spread from the centre of origin (Minor Asia) to the Mediterranean basin. The large geographical area provided isolation that differentiated many different *oleaster* ecotypes, which differently affected olive domestication (Hannachi et al., 2008). The final result was the natural formation of different groups of domesticated olive according to environmental adaptation, morphology and performance

(Lavee and Zohary, 2011), although among this homogeneous autochthonous population it is often possible to find foreign cultivars introduced after domestication (Baldoni et al., 2000; Belaj et al., 2007; Muzzalupo et al., 2007). This diversity must be considered in any breeding program, as parents of widely separated origin produce offspring which may exhibit heterosis for many specific traits. The monumental ancient olive trees, which often cannot be identified among the local cultivars, represent a good source for parent choice, having survived local biotic and abiotic stresses for centuries.

Many important traits are polygenic, including tree vigour, productivity, resistance to biotic and abiotic stresses, and oil productivity. Before starting any breeding program the availability of specific genetic markers for such traits can be very valuable in the choice of parents.

The primary gene pool (GP1) of *O. europaea* ssp *europaea*, includes all the diploid and inter-fertile Mediterranean forms of *O. europaea* including the wild *oleaster,* for which the alleles for wildness have been discovered (Lumaret and Ouazani, 2001) distinguishing *olevaster* from cultivated varieties.

The secondary gene pool (GP2) of *O. europaea* ssp. *europaea* includes species having pre-zygotic incompatibility (overcome by *in vitro* pollination) with different ploidy level, such as *O. europaea* ssp *europaea* var *cerasiformis*, *O. europaea* ssp *europaea* var *maroccana, O. europaea* ssp *europaea* var *guanchica, O. europaea* ssp *europaea* var *laperrini*, *O. europaea* ssp *europaea* var *cuspidata.*

The tertiary gene pool (GP3) groups most of the *Ligustroides* species, such as *O. exasperata, O. capensis* ssp. *macrocarpa, O. capensis* spp. *capensis, O. woodiana, O. lancea, O. paniculata,* from which gene transfer to *O. europaea* ssp. *europaea* could occur only through *in vitro* culture of hybrid embryos (embryo rescue) as post-zygotic incompatibility occurs.

The quaternary gene-pool (GP4) of *O. europaea* ssp. *europaea,* is represented by highly incompatible genotypes for which gene mixing can only be overcome with genetic engineering.

Recourse to the use of related species with all known genetic technologies (conventional and unconventional methods) could be very useful in breeding programs. The oil quality of different *Olea* species have a similar composition to *Olea europaea* (Hannachi et al., 2009), and they possess important characters to be transferred such as disease resistance and fruit set. To realize such genetic improvement it is fundamental to collect and carefully evaluate the available agro-biodiversity across all the *Olea* taxa, and to undertake genetic mapping. To date, an international centralized service for conservation does not exist, but several institutions carry on recovery, propagation and maintenance of a large number of accessions, mainly local ones. Seventy-six collection centers are present in 24 countries (Rallo et al., 2005, Tous et al., 2005), but none of them are complete collections, although it would be very important to have large clonal collections replicated in different environments in order to observe their phenotypic behavior and to reduce risk of losing valuable germplasm. The International Olive Oil Council (IOOC) promoted a campaign aimed at retrieving and preserving accessions from distant locations and countries where preservation is not provided (Fabbri et al., 2004). These germplasm field banks (*ex situ*) are mainly located in traditional countries including Spain, Tunisia, Syria, Morocco, Algeria, Libia e Libano, Iran, Israel (Amiri, 2008; Aradhya and Preece, 2012; Biton et al., 2015) and also in Italy - in Calabria, Tuscany, and Sicily. These are very useful to characterize the varieties before use as Belaj et al. (2012) did for genotypes collected in the world's largest germplasm collection located in Spain, which provided a core collection based on molecular markers (AFLP, DArTs, SSRs, SNPs) and agronomic traits. But an international collaboration would substantially enhance this. Use of the "Gene Pool Method" should now be feasible with development

of required technologies, including *in vitro* techniques, material for gene transformation, pollination and fertilization *in vitro,* and embryo rescue to produce haploids, dihaploids, and polyploids, and to preserve genetic resources.

In addition to information about the hereditability of valuable characteristics, the availability of molecular markers assists definition of geographical sites of origin for *oleaster* and other *Olea spp.*; and may an interesting prospect is that these powerful markers may be used to reduce juvenility of offspring through the choice of appropriate cultivars as pollinators since this is genotype-dependent (Bellini, 1990; Fontanazza and Bartolozzi, 1998). Two strategies are currently used to shorten the long juvenile period. The first consists of forcing seedling growth in the greenhouse and nursery to increase growth in height, together with pruning the lateral shoots and, when it works well, grafting onto adult rootstocks. The second strategy is the identification and early selection of genotypes with a short juvenile period, which might be phenotypically recognizable at an early stage.

To the present, breeding work has been based on cultivated genotypes, except in few cases where feral olive populations have been tested (Hannachi et al., 2009). Wild olive germplasm contains more variability than the cultivated varieties (Belaj et al., 2010; Baldoni and Belaj, 2009), and is adapted to several environments. In particular the *Olea europaea* subsp. *europaea* var. *sylvestris,* may be a very important source of resistance to abiotic stresses such as drought, salt, wind and low temperature (Aranda et al., 2011; Baldoni et al., 2006; Klepo et al., 2013; Meddad-Hamza et al., 2010; Mulas, 1999) and biotic stresses such as *Verticillum wilt* (Sesli et al., 2010), peacock spot (Ciccarese et al., 2002), and olive fly (Mkize et al., 2008).

Besnard et al., (2001) first reported hybrids between olives and wild relatives of the same genus. Subsequently, Caceres et al., 2014 have produced hybrids by reciprocal inter-subspecific crosses between *Olea europaea* ssp. *europaea* and *Olea europaea* ssp. *cuspidata.* The morphology of the offspring, for which their hybrid nature has been proved by AFLP and SSR molecular markers, resembled the female parent.

To reach targeted goals it will be necessary to find breeding materials with promising alleles for their transfer. This will require intensive research on olive genetic resources studying inheritance and identifying target genotypes. On the other hand, appropriate application to the gene pool of biotechnological procedures based on *in vitro* culture can directly help breeding programmes. These procedures are useful in inducing new genetic variation by mutation breeding, in transferring genes from the wild to cultivated genomes by hybridization and clonal selection, to induce somaclonal variation, to change the ploidy level and to obtain homozygous plants.

7 In vitro techniques and genetic transformation

Micro-propagation by axillary bud stimulation is now available for many olive cultivars, and it can contribute to genetic improvement by both conventional and unconventional methods through: 1) rapid propagation of new genotypes, 2) pathogen elimination from the mother plants or offspring, 3) embryo rescue, 4) germplasm preservation, 5) plant regeneration from cell tissues, and 6) synthetic seed production.

7.1 Rapid propagation of new genotypes

The establishment of axenic cultures is best carried out using vigorous nodal explants; meristems or shoot-tips from field-grown or greenhouse plants are generally unsuccessful due to rapid oxidation of tissues in the sterilising solution. Pre-soaking nodal explants in PPM®

(Plant Preservative Mixture), and adding it to the medium, is generally effective to obtain sterile explants. The rapid growth, tender and elongated shoots are obtained on Olive Medium (OM) (Rugini, 1984) with addition of a mixture of growth regulators (Zeatin, 6-Benzylaminopurine, Thidiazuron, Metatopolin, Giberellic acid), mannitol as carbon source, and for some cultivars Dikegulac is effective to reduce apical dominance (Mendoza de Gyves et al., 2008). Peixe et al. (2007) reported that zeatin was successfully replaced by coconut water in combination with 6-Benzylaminopurine or kinetin with the cultivar Galega. In some cultivars satisfactory shoot proliferation is obtained by reinvigoration of the first shoots sprouting from the initial nodal explants. This could be achieved by inducing sprouting of basal buds on the initial shoots by systematic elimination of apical buds during the first subcultures of the shoots, or by *in vitro* grafting of the shoots onto juvenile seedlings. Rooting of explants takes advantage of basal darkening (Rugini et al., 1987) or by placing the whole culture in the dark for one week. Putrescine at 160 mg/l promoted early and effective rooting by increasing total peroxidase activity at the shoot base, which is essential for root induction (Rugini et al., 1997). The acclimatization phase after root emergence can be accelerated by forcing cuticle formation, stomata functionality and shoot growth by adding liquid medium over the solid medium (double layer), with the liquid containing a high amount of mannitol or sucrose (4-5%), putrescine (160 mg/l), and Giberellic acid (40-50 mg/l). Where possible, it is preferable to root *ex-vitro*, a process successfully verified for some cultivars, including cv Chemlali (Yacoub-Bougdal et al., 2007) and Italian cvs Frantoio, Maurino and Coratina; continuous light during this phase is essential (Leva, 2011).

7.2 Pathogen elimination from the mother plants and offspring

According to the present European legislation, olive plants can be certified only if they are totally virus-free. Since most olive plants are affected by several viruses, the diffusion of pathogen-free plants could be further progressed by olive nurseries. Meristem culture is not possible with collection from *in vivo* growing plants, but is relatively easy if the meristems are excised from *in vitro* growing shoots and rigorously placed on top of a small cube (5 mm) of solid proliferation OM medium in Petri dishes or multiwell plates. Plants of three Italian cultivars obtained by this method and grown in the field since 8 years are still virus-free (Rugini and Bottalico, 2011).

7.3 Immature embryo culture

Immature embryos of several Italian and Iranian cultivars, rescued less than two months after fertilization, have been successfully germinated (Hossein and Hajnajari, 2006; Rugini, 1988). Rapid germination was also recently achieved with three-month old embryos (unpublished data); these turned green within two days and after 15 to 20 days, with two or three nodes, the seedlings were ready to be transplanted to the pots for the hardening phase. This technique allows reduction by at least 18 months the time traditionally required to overcome the long seed dormancy period.

7.4 Germplasm preservation

The current trend is to establish groves with a small number of cultivars, generally the most productive or most suited to expect environmental conditions, without much consideration for germplasm preservation.

It is essential to avoid loss of important genotypes within particular gene pools. The conservation of seeds has little sense for vegetative propagated plants with a high degree of heterozygosis, such as olive, so *in vitro* preservation by both slow growth and, in particular, cryo-preservation, seems to be a promising alternative to field conservation as outdoor plants may be subjected to serious risk of both abiotic and biotic stress.

7.4.1 Slow growth preservation

Experiments carried out using two cultivars (Leccino and Frantoio) cultured *in vitro* on solid medium under dark condition at +4°C, allowed shoots to be preserved for 8 months (Lambardi et al., 2000). Micheli et al., 2007 reported a simple method of encapsulation in alginate nutrient gel of "Moraiolo" nodes in plastic cuvettes. After storage at +4 °C, compared with a control at room temperature, both for 15 and 30 days, axillary buds successfully developed, indicating a possible use of this technique for germplasm exchange over long distances.

7.4.2 Cryo-preservation

Various organs and tissues of olives have been cryo-preserved including somatic embryos or embryogenic tissues, seeds with or without endocarp, and shoot tips (Benelli et al., 2013). This allows long-term conservation of olive germplasm by direct immersion of tissues into liquid nitrogen (LN) (–196°C), or by using a vitrification solution before LN immersion. In the first case, Martinez et al. (1999) obtained 30% survival of olive shoot tips of the cv Arbequina following the removal of up to 30% of their moisture content and re-warming at room temperature. Success was obtained with a procedure of vitrification and one-step freezing in liquid nitrogen of shoot tips excised from *in vitro* grown shoot cultures of the cv Frantoio. In addition, somatic embryogenic cultures of Canino cultivar proved to be a highly suitable material for cryo-conservation using the vitrification approach (Lambardi et al., 2000). In general, olive embryogenic lines appeared to be highly suitable materials for cryo-preservation (Benelli et al., 2001; Lynch et al., 2011; Sanchez-Romero et al., 2009; Shibli and Al-Juboory, 2000). Cryo-preservation of shoot tips also appears promising using the vitrification technique; Benelli et al. (2001) obtained satisfactory post-rewarming shoot-tip survival with cvs 'Canino' and 'Gentile di Larino' respectively, but with poor re-growth, while good re-growth (38%) was observed in cv Frantoio following two steps dehydration with PVS2 (50% PVS2 for 30 min, then 100% PVS2 for 1 h), direct immersion in LN, and post-rewarming culture on medium containing a high concentration of zeatin (46 μM) (Lynch et al., 2007). An alternative to embryo conservation is the encapsulation of apical and nodal buds from micropropagated shoot cultures, but this currently shows a low rate of conversion into plants (Micheli et al., 1998).

7.5 Plant regeneration from cell tissues

7.5.1 Shoot organogenesis

Shoot organogenesis has been obtained both from zygotic and mature tissues. The work was aimed at organogenesis from mature explants since zygotic material is not suitable for genetic improvement due to the high heterozygosity of olive. Petioles from leaves of *in vitro* growing shoots of several cultivars (Canino, Moraiolo, Dolce Agogia and Halkidikis) showed good potential organogenesis (Mencuccini and Rugini, 1993), but is still not efficient enough to regenerate plants from modified cells with any biotechnological technique (gene transfer,

somaclonal variation, or induced mutation by physical or chemical approaches). It is considered important to develop an effective procedure for somatic embryogenesis.

7.5.2 Somatic Embryogenesis

Somatic embryogenesis has been successfully achieved from zygotic embryos 60 to 75 days after fertilization (Leva et al., 1995; Rugini, 1988), however this capacity can be extended to a period of at least two months by storing whole detached fruitlets at 14-15°C (Rugini, 1995). Somatic embryogenesis has also been achieved from non-germinated mature embryos of both wild (Orinos and Mitrakos, 1991) and cultivated olive (Mitrakos et al., 1992; Shibli et al., 2001).

Somatic embryogenesis from mature tissues is still difficult to achieve, however, cyclic somatic embryogenesis has been obtained from two cultivars, Canino and Moraiolo, through the novel 'double regeneration system' (Rugini and Caricato, 1995). These embryos originated from very small leaflets of adventitious neo-formed buds from petiole tissues. Rejuvenation through adventitious shoot organogenesis does not seem to be essential for an embryogenic response for all cultivars. For example, this technique was not required in cv Dabbhia (Mazri et al., 2013) and in a genotype of *O. europaea* L. var. *sylvestris* (Capelo et al., 2010). The growth regulator thidiazuron (TDZ) seems to be a very important trigger for induction of somatic embryogenesis; in all experiments reported above it was effective. In addition the antibiotic cefotaxime seems to be an essential factor, since in all cases above reported it was always present in the medium. Secondary embryos differentiate mainly with unicellular origin from the epidermal surface (Lambardi et al., 1999), establishing a very efficient cycle of somatic embryogenesis. The long-term cyclic production of embryos direct from epidermal cells could be a great advantage in regenerating plants from transgenic cells because it avoids callus formation, which could be a wide source of undesirable genetic variability. The high production of somatic embryos should make it useful to employ for somaclonal variation under selection pressure but, in our experience, phenotypic variability was never observed after many years in field-trials with the cv Canino; this was confirmed by Lopes et al. (2009) in *Olea* spp., where genome integrity was maintained throughout the stages of embryogenesis. However, in a case only, a different vegetative behaviour (bushy and columnar phenotype) was reported in plants derived from somatic embryos of the same cotyledon in the cv Frangivento (Leva and Petruccelli, 2007). These conflicting results suggest consideration be given to potential for unwanted variation before using somatic embryogenesis for propagating true-to-type olive plants. Somatic embryogenesis can be applied for production of "synthetic seeds" which can also be useful for germplasm preservation (Lynch et al., 2007).

An efficient method of plant regeneration from cells is essential to support unconventional genetic improvement such as selection of mutants derived from somaclonal variation, both *in vitro* and *in vivo*, natural or induced, protoplast technology, haploids and polyploids, and production and selection of transgenic plants.

7.6 Haploids

Production of homozygous olive plants by self-fertilization seems to be dubious considering the frequent self-sterility and the long juvenile phase which characterize this species. Homozygous plants would be of great interest for isolation of mutants and recessive traits. Anther, ovary, pollen and ovule cultures should be explored with the aim of producing di-haploid plants by doubling the number of chromosomes in a short period of time (Germanà, 2006). Recently, promising results were obtained employing a new method of isolated microspore culture which

led to cell division and pro-embryo formation in the cvs Arbequina and Picual (Bueno et al., 2005). Various experiments have been carried out by using compatible pollen treated with physical agents (UV and X-rays), and treatments with toluidine blue at different time after pollination. The fruit recovered showed different classes of fruit size and shape. This embryo culture produced several plantlets which are under investigation.

7.7 Triploids and tetraploids

Tetraploid plants do not naturally exist in *Olea europaea* ssp *sativa*. Rugini et al. (1996) produced several stable tetraploid plants of the cultivars Leccino and Frantoio by *in vitro* shoot-tip fragmentation, starting from mixoploid plants generated by γ-ray irradiation of cuttings. The separation of tetraploids (*4n*) from diploid (*2n*) shoots has been carried out since two distinct groups appeared during the subcultures; shoots that were wide, long and thick corresponded to stable tetraploids, while the other group was diploid. In a field trial the *4n* plants maintained the same leaf morphology but showed a reduction of trunk and canopy size, flexible branches, absence of juvenility, and uniformly large flower and fruit size; some of the embryos from the large fruits were triploid. The *4n* "Leccino" acquired self-fertility while the *2n* mutants, although retaining auto-incompatibility acquired inter-fertility among other *2n* mutants and with self-sterile Leccino mother plants. This could be a benefit for genetic improvement since reciprocal crosses could reduce heterozygosity within the cultivar. Furthermore both *2n* and *4n* mutants, when used as rootstock, reduced the size of the cv Canino scion within 8 years observation. The *2n* mutants of Leccino were characterized by consistently extraordinary cropping and pollination capacities, while maintaining the same oil characteristics and tolerance to *Spilocea oleagina* as the Leccino mother plants. For these reasons this mutant could be a candidate for high density olive cultivation.

7.8 Genetic transformation and plant recovery

Gene transfer technology might be a useful alternative to speed up genetic improvement by correcting defects in important commercial cultivars (gene therapy). However, two important factors are essential: the availability of morphogenetic tissues of valuable cultivars and the availability of useful genes. With the aim of modifying canopy architecture, increasing rooting ability, and tolerance to abiotic and abiotic stresses, chimeric plants with *ri-TDNA* of *Agrobacterium rhizogenes*, and uniform transgenic plants with *rolABC* and *osmotin* genes, were produced and evaluated in a field trial over ten years (Rugini, 2015).
Several Italian olive cultivars have been rooted by simply infecting the basal part of *in vitro* proliferated shoots with *Agrobacterium rhizogenes* wild type, or strain NCPPB 1855 (Rugini, 1992; Rugini 1986). However, the roots are rarely transgenic, allowing us to speculate that the roots originate from non-transformed neighbour cells to transformed ones or, less likely, the roots were induced by unknown compounds present in the *Agrobacterium* exudates (Rugini et al., 2000). Strobel et al. (1988) carried out greenhouse experiments with some Tunisian olive plantlets using another strain of *A. rhizogenes* (strain 232) and, by infecting the root system after uniformly trimming to 4-5 cm, observed after sixty days a root mass greater than the controls, with transgenic roots emerging from the primary roots. They reported a beneficial effect both on vegetative growth and reproductive parameters, although the new roots appear poorly connected with the existing primary ones. These observations encourage use of these techniques in some agricultural practices, as well as for rooting of recalcitrant olive genotypes or modification of some vegetative or reproductive parameters. However more scientific information is needed because different responses are obtained according to the variety or

587 species. The nature of the transformation event can also change the outcome, as observed in
588 other woody fruit species such as cherry and plum rootstocks, simple infection to induce root
589 formation can affect plant morphology and reproduction.
590 *Agrobacterium tumefaciens* strain LBA 4404 harbouring pBin19 with *rolABC* of *A. rhizogenes*
591 and the gene *nptII* for kanamycin resistance, under control of a natural promoter, have been
592 used for olive transformation. Both immature zygotic embryos of the cv Moraiolo and somatic
593 embryos from mature tissues of cv Canino, were used. In the first experiment zygotic embryos
594 resistant to kanamycin were selected (Rugini and Fedeli, 1990), in the second experiment,
595 transgenic plants were selected following cyclic somatic embryogenesis of the cv Canino and
596 field trials were carried out (Rugini et al., 2008). The *rolABC* plants showed the typical hairy
597 root phenotype, with prolonged vegetative growth to late autumn and a long juvenile phase;
598 although originated from mature tissues, the reproductive phase was not observed because the
599 Italian Minister of Environmental did not renew approval of the field trial when the first period
600 expired. However, after 10 years, the plants still maintained the same phenotype and the
601 presence of the foreign genes as shown by Real Time PCR analysis (Miano et al., 2004).
602 Through *in vitro* testing the transgenic plants revealed high rooting ability, which were 50%
603 rooted in auxin-free medium, reaching up to 60% rooting with addition of only 160 mg/l of
604 putrescine, while non-transformed plants did not root at all. The same high rooting ability has
605 been exhibited by the semi-hardwood cuttings collected from field grown plants, although in
606 this case a low amount of auxin was beneficial.
607 Somatic embryos of olives were transformed with *Agrobacterium tumefaciens* as described for
608 *rolABC* gene transformation to produce plants over-expressing tobacco *osmotin* (Rugini et al.,
609 2000). This gene is present in the genome of all plant species now tested and codes for a protein
610 of the PR5 (Pathogen Related Protein) family. This is expressed under both abiotic (mainly
611 under drought conditions) and biotic stresses, resulting in effective control of fungal diseases by
612 inducing an oxidative burst when hyphae come into contact with plant tissues. Recently the
613 osmotin protein was found to be a homologue of the human adiponectine, involved in glucose
614 metabolism; it seems to be the basis of new therapies for the treatment of various diseases
615 including diabetes, cancer and some diseases of the central nervous system (Naseer et al.,
616 2014). Transformation has been realized using *A. tumefaciens*, *LBA4404*, with the *pKYLX71*
617 plasmid harbouring the tobacco *osmotin* gene under control of the *35S* promoter, and plants
618 tested in the field (Rugini et al., 2000). After ten-years of growth they showed similar
619 phenotype to non-transformed plants also derived from somatic embryos, but with narrower
620 leaf lamina and high amounts of protein around the vacuoles in cells of epidermal and
621 sub-epidermal tissues (D'Angeli et al., 2001). These plants were more tolerant to peacock spot
622 (*Spilocea oleagina* Hughes), but showed an unexplained particular attraction to the insect
623 *Otiorhynchus cribricollis* Gyllenhal (unpublished data). Furthermore, over-expression of
624 osmotin induced cold protection by affecting programmed cell death and cytoskeleton
625 organization (D'Angeli and Altamura, 2007); these plants also showed an extraordinary
626 drought resistance. In a field trial young *osmotin* plants suffered under ordinary irrigation with
627 initial slow growth followed by leaf drop, and after few months died; this contrasted with
628 non-irrigated plants which grew indistinguishable from non-transformed plants for ten years
629 (Rugini et al., 2000). Experiments were carried out during summer in pots using two-year-old
630 plants derived from three different transformation events which were compared with analogous
631 plants of Canino wt and the Canino *rolABC* grafted on to Canino wt, which demonstrated an
632 extraordinary drought resistance of the transgenic plants: after four weeks all the transformed
633 plants showed normal growth, while the others died in the absence of water supply. Similar to
634 *rolABC* plants described earlier, the *osmotin* plants displayed a high degree of juvenility.
635 Finally, with *in vitro* experiments, transgenic shoots showed normal growth when 4% PEG

(polyethylene glycol) was present in the medium; this was accompanied by a greater accumulation in the tissues of proline and enzymes specific to drought stress (unpublished data), while the Canino *wt* showed clear signs of damage on the leaves with reduced growth evident.

Few other transgenic trials have since been attempted with olive. Recently Torreblanca et al. (2010) attempted transformation using embryogenic cultures derived from radicles of mature seeds cv Picual with *Agrobacterium tumefaciens*, harbouring *pBINUbiGUSint* or *pGUSINT* binary plasmids. These vectors contained the *nos-nptII* and the *uidA* gene driven by the maize poly-ubiquitin *Ubi1* and *CaMV35S* promoter, respectively.

Other traits in important commercial olive trees may be improved by "gene therapy", including examples such as: a) production of completely self-fertile plants, b) increase in the content and quality of oil, c) production of plants with parthenocarpic fruits, d) increased tolerance to cold and salt stress, e) regulation of fruit ripening and f) increase pathogens and parasites resistance. However, there are currently many genes of different origin available, whereas the number of olive genes isolated and characterized is still rare.

7.9 Protoplast technology

Protoplast technology is useful for studies, including protoplast fusion to produce hybrids and cybrids from cross-incompatible genotypes, to produce triploids and polyploids, or to introduce foreign naked DNA into cells by liposome. Viable olive protoplasts were isolated and cultured, and in some cases micro-calli were obtained, but plant regeneration has not been reported yet (Cañas et al., 1987; Mencuccini, 1991; Perri et al., 1994; Rugini, 1986).

7.10 In vitro mutagenesis

To increase the frequency of somaclonal variation above the spontaneous level with *in vitro* cultures, various physical and chemical agents can be used which, at the same time, might induce and/or select mutated cells. This technique requires a very efficient regeneration method such as the cyclic somatic embryogenesis mentioned above.

Ozair et al. (2014) added the mutagen oryzalin to OM culture medium and recovered shoots of cv Moraiolo with highly significant differences in shoot length, fresh weight, dry weight, leaf area, number of nodes, and number and length of roots. The use of mutagenic agents represents a useful tool not only to obtain new varieties, but also to study gene function. Application of the mutagen to *in vitro* shoots of cv Canino, during the multiplication phase, did not modify the vegetative habit but, in the field, the fruits in some plants were about 80% smaller, and for others about 20% larger, compared to the mother plants (unpublished data).

The olive species is characterized by the ability to produce suckers at the root transition region. It therefore should be possible, after *in vitro* roots emerge, to apply physical or chemical mutagens to this zone corresponding to where the suckers will originate in the field. This procedure should overcome both the difficulties of shoot organogenesis and, at the same time, should avoid chimeric tissues formation in the regenerated suckers since, being an example of organogenesis, they would normally originate from a single cell.

8. Conclusions and remarks

The genetic improvement of olive trees by classic and advanced biotechnological strategies offers to enhance yield and quality of its products, and speed up development of new cultivars

and rootstocks suitable for a modern intensive mechanized olive industry. Classical methods have failed to meet the requirements of olive production and to keep up with other demands of the olive industry. The application of advanced technologies as support or alone is essential to expand olive cultivation in arid and semiarid areas worldwide, and in other inhospitable areas, and to address additional characteristics to better use as oil or fruit for table consumption. It is advisable to employ the wild olive (*oleaster*) in new breeding programmes to increase variability. It is mainly due to their continuous omission that natural and cross breeding selection among autochthonous cultivars is not enough. Moreover, it is essential to introduce genes from other compatible *Olea* species and to breed among genotypes selected from widely distant sites. Deep genomic information is fundamental to facilitate the choice of parents, according to each specific aim, and to support effective use of genetic engineering. More systematic work is needed to overcome the lack of basic information on olives. However, biotechnological methodologies are now available, together with tools for marker assisted breeding, and these provide sufficient foundation to start massive genetic improvement.

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