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# Genetic improvement of olive

## Genetsko oplemenjivanje masline

**E. Rugini, Patricia Gutiérrez Pesce**

ABSTRACT AND CONCLUSIONS

In the last decade significant progress has been made in developing successful olive cloning techniques, although some difficulties still remain such as that with establishing sterile cultures in vitro and morphogenesis from mature tissue of cultivars. More work is required, although significant advances have also been made in shoot regeneration from petioles of in vitro grown shoots of several cultivars (Mencuccini and Rugini, 1993). However, the regeneration ability is still low for use in biotechnological applications. The novel strategy of the «double regeneration», developed to achieve somatic embryos in olive cv. Canino and Moraiolo may also be applicable in other cultivars. This technique can be generalised since at present it is essential for inducing and maintaining shoot morphogenic callus in other species such as cherry, apple, and pear (Gutiérrez Pesce et al., 1998; Rugini e Muganu 1998; Abdollahi et al., 2005).

Gene transfer techniques offer a more powerful strategy for genetic improvement in respect to traditional breeding methods. It allows the introduction into one genotype, one or a few pieces of genetic information without drastic modifications of the general characteristics of the plant. Transformation techniques have been developed, by using somatic embryogenesis, and transgenic plants, with some desirable agronomic traits, have already been generated in one cultivar. At present field trials, approved by the Italian Health Minister, are conducted on transgenic *rolABC*, and *osmotin* plants.

From transgenic olive plants, similar to kiwi transgenic plants with *rolABC* genes we expect plants with large root systems, compact vegetative habitus, smaller number of flowers per plant, and high rooting ability of cuttings. In plants over-expressing *osmotin* gene, we expect a higher tolerance to some fungi. Many genes have already been isolated from several species, which may be introduced in olive singly or associated with others. Transformation experiments with multiple genes (chitinase + *osmotin* + *PR1*) are in progress in our laboratory in order to increase fungal resistance.

Antibacterial genes (*thionin*, *cecropin*, attacin, etc.) against *Pseudomonas syringae* and genes for modifying the pattern of fruit ripening (ethylene, PG) are only a few

examples of the potential of genetic manipulation to improve olive. A high content of di-hydroxiphenols could confer the valuable bitter taste in the olive oil.

*Agrobacterium*-mediated gene transfer seems to be the most efficient method in olive.

The molecular techniques should not aim only to make clear the phylogenesis of the genus, but also to clone useful genes and promoters in olive. To facilitate this work, biotechnology, long-term breeding programs and biochemical research should be closely linked to achieve the objectives quickly.

Key words: genetic improvement, olive

## SAŽETAK I ZAKLJUČAK

U zadnjem desetljeću učinjen je znatan napredak u razvijanju uspješnih metoda kloniranja maslina iako će neke poteškoće ostati, kao utvrđivanje sterilnih kultura in vitro i morfogeneza iz zrelog tkiva kultivara. Potrebno je još raditi, makar je učinjen znatan napredak i u regeneraciji izbojaka iz petiola uzgojenih in vitro od nekoliko kultivara (Mencuccini i Rugini, 1993). Međutim, mogućnost regeneracije još je uvijek mala za biotehnološku primjenu. Nova strategija "dvostruke regeneracije", razvijena kako bi se dobili somatski embriji masline cv. Ganino i Moraiolo mogu se primijeniti i u drugim kultivarima. Ova se tehnika može generalizirati jer je danas bitna za induciranje i održavanje morfogenskog kalusa izbojka u drugim vrstama kao što su trešnja, jabuka i kruška (Gutierrez Pesce et al. 1998; Rugini e Muganu, 1998; Abdollahi et al., 2005).

Tehnike transfera gena pružaju snažniju strategiju za genetsko oplemenjivanje u odnosu na tradicionalne uzgojne metode. One omogućuju uvođenje u jedan genotip jedne ili više genetskih informacija bez drastičnih modifikacija općih značajki biljke. Razvijene su tehnike transformacije primjenom somatske embriogeneze i transgenske biljke određenih poželjnih agronomskih svojstava i već su proizvedene u jednom kultivaru. Upravo se provode pokusi na terenu, koje je odobrio talijanski Ministar zdravstva, na transgenskim *rolABC* i osmotinskim biljkama.

Od transgenskih biljaka masline, slično transgenskim biljkama kivija s genima *rolABC*, očekujemo biljke velikog sustava korijena, zbijenog vegetativnog habitusa, manjeg broja cvjetova po biljci i velike sposobnosti sadnica za ukorijenjenjem. U biljaka preizraženog osmotinskog gena očekujemo veću tolerantnost na neke gljive/gljivice. Izolirani su već mnogi geni iz nekoliko vrsta, što se mogu introducirati pojedinačno ili zajedno s drugima. Pokusi transformacije s mnogostrukim genima (chitinase + osmotin + PRL) su u tijeku u našem laboratoriju, kako bi se povećala otpornost na gljivice.

Antibakterijski geni (tionin, cecropin, atacin itd.) protiv Pseudomonas syringae i geni za modificiranje uzorka zriobe voća (etilen, PG) samo su neki primjeri potencijala genetske manipulacije za oplemenjivanje masline. Visok sadržaj dihidroksifenola

mogao bi prenijeti dragocjen gorki okus maslinovog ulja. Prijenos gena posredstvom agro-bakterija čini se najdjelotvornijom metodom u masline.

Cilj molekularnih tehnika ne bi trebao biti samo razjašnjenje filogeneze gena nego i kloniranje korisnih gena i stimulatora u maslini. Da bi se taj posao olakšao potrebno je usko povezati biotehnologiju, dugoročne uzgojne programe i biokemijska istraživanja radi brzog postizanja ciljeva.

Ključne riječi: genetsko oplemenjivanje, maslina

## 1. INTRODUCTION

Olive crops occupy the world production area of 10 million hectares of which 60% as main cultivation. In some countries like Italy and Greece many olive plantations are obsolete, so the substitution with new olive plants is necessary. Among countries traditionally olive producers, Spain has for some time been renewing olive plantations by using few cultivars. New plantations are grown in countries not traditionally olive growing like Australia, China, South Africa and South America with the cultivar of the Mediterranean Basin derived from selections carried out among a wide clonal population. Almost non-existent in fact are the cultivars derived from programmed crossings because of the long juvenile phase of the progeny derived from seeds. Nevertheless, most of them are not suitable for the modern techniques of agriculture, because they do not present a correct balance between the production and the excessive vegetative growth, creating problems during harvest. Furthermore, most of them are not resistant to the olive fly and to the eye of peacock.

For modern oliviculture it is necessary to adopt new cultivars derived either from traditional or modern technologies for genetic improvement, or from the selection among the numerous genotypes in the Mediterranean Basin which are estimated to be around 4000 between cultivars and accessions (Bartolini et al., 1998). This variability could be greatly reduced when the problem of synonymy and homonymy due to environment and inner pathogen is solved. There is an urgent need for a genetic certification of the propagation material, using new biotechnological techniques that could also contribute to safeguard genotypes with peculiar features, by genetic identification, eventual protection from viruses, and their preservation with nonconventional technologies. Besides, it appears important to begin a genetic improvement programme with conventional and nonconventional technologies.

## 2. OBJECTIVES OF THE GENETIC IMPROVEMENT OF OLIVE

The genetic improvement programmes are directed to solve agronomic and commercial problems such as: a) the production of auto-fertile plants, b) the regulation of fruit ripening and increase of oil content and quality, c) the production of plants with parthenocarpic fruits, d) increase of cold and salt tolerance, e) modification of the vegetative habitus, and g) resistance to biotic stress.

At present a lot of genes of different origin are available (Owen, 1995), while the number of characterized and isolated genes of olive species is still limited.

### *2.1 Regulation of fruit ripening and increase of the oil content and quality*

A lot of cultivars have good agronomic characteristics, but they present early ripening, high water content of the fruits, and early dropping. This pattern may be corrected by generating transgenic plants with a reduced ethylene biosynthesis, by using an antisense gene. At present, two molecular strategies can be used to modify oil composition and content:

- alteration of the major fatty acid level by suppressing or expressing a specific key enzyme in lipid biosynthesis.
- creation of an unusual fatty acid.

By anti-sense suppression or co-suppression of oleate desaturase it is possible to increase oleic acid (C18:1) by more than threefold (from 24% to 80%) in the oil of transgenic soybean (Humann, 1997). The same strategy was adopted to increase stearate (C18:0) by up to 30% both in canola and soybean oils. Unusual fatty acids can be produced in one plant by transferring a gene encoding the specific biosynthetic enzyme. An example can be seen in canola which naturally does not produce laurate (C12:0), while a new transgenic genotype does contain laurate. The oil content of some nut crops used for cosmetics, such as almond, could be increased or their composition could be modified by these techniques.

### *2.2 Parthenocarpy*

A major limiting factor for olive productivity is the alternate bearing, but often the lack of pollination causes this problem in areas with less intensive olive cultivation. Blossom synchronization between cultivars is often variable

during the years. This problem is more important in areas where the cultivation intensity is low and the environmental conditions are unfavourable. The introduction of the parthenocarpic traits in these species may allow fruit development and subsequently boost the yield. Olive shows some tendency to natural parthenocarpy, under adverse environmental conditions, although the fruits remain very small. The introduction of parthenocarpy Arabidopsis gene, already successful in several herbaceous species, including aubergine (Rotino et al., 1997) may overcome the pollination problem.

*2.3 Cold and winter chilling tolerance*

Olive plants with greater cold resistance are recommended. In other species, transgenic plants with gene encoding the antifreeze protein have been generated (Hightower et al., 1991) and the over expression of SOD (Super Oxide Dismutase) gene has also been demonstrated to repair frost damaged cells (Van Camp et al., 1993; McKerzie et al., 1993). Over expression of *Arabidopsis CBF1* gene enhances freezing tolerance by inducing genes associated with cold acclimatisation (Jaglo-Ottosen et al., 1993), and could also be useful in olive.

*2.4 Alteration of the vegetative and reproductive habitus*

Genetic improvement is oriented to dwarfness and changes in canopy architecture producing dwarf and semi-dwarf plants with shorter and numerous shoots, that are more suitable for high-density orchards. Moreover, developing plants with an extensive root system and/or with reduced water consumption could be an advantage drought season and in areas with not enough available water. Some genes of the TL-DNA of *Agrobacterium rhizogenes* that affect plant growth and development have already been introduced in olive (Rugini et al., 1999), in kiwi (Rugini et al., 1991), in cherry (Gutiérrez Pesce et al., 1998; Rugini and Gutierrez-Pesce, 1999) and peach (Hammerschlag and Smigocki, 1998). In all these species, the reduction of plant size and apical dominance is confirmed and the current field trials will show their agronomic value. In addition, the reduction of flower numbers observed in kiwi (Rugini et al., 1997) may contribute to solving the waste of energy and to reducing the alternate bearing.

Another possibility offered by biotechnology for modifying the growth and reproductive behaviour, is the modification of plant receptors in order to change the light perception. The transformation with phytochrome genes

(*phyA*, and/or *phyB*, sense or antisense), which, together with other photoreceptors, control plant development (circadian rhythms, apical dominance, blossoms, growth and fruit ripening, photosynthesis products distribution, development of photosynthetic systems, transpiration control and hormone synthesis) (Vince-Prue and Canham, 1983; Tucker, 1976; Muleo and Thomas, 1993, 1997; Baraldi et al., 1992), may contribute to developing plants with high agronomic value and suitable for very high density planting.

### *2.5 Pests and diseases resistance*

The challenge of using molecular biology in this area is to generate broad resistance mechanisms that have been difficult to achieve with classical plant breeding approaches. For enhancing protection from fungi, in addition to osmotin gene or an association of other genes, as already introduced in olive, other genes may be introduced after first testing the gene product against the main fungi of olive. *Stilbene syntase* gene (Hain et al., 1993), ribosome-inactivation protein gene (Longmann et al., 1992), *glucose oxidase* gene (Wu et al., 1995) and genes encoding hydrolytic enzymes such as chitinase and glucanase, which degrade fungal cell wall component (Broglie et al., 1991; Yoshikaw et al. 1993), may contribute to protecting olive. Particular attention is given to Polygalacturonase-Inhibiting Proteins (PGIPs) that specifically inhibit the activity of endo-polygalacturonases released by fungi on invasion of the plant cell wall.

Genotypes with less susceptibility to *Pseudomonas syringae* pv *savastanoi* should be tried. Although, it is not so easy to introduce bacterial resistance into plants, because the product of the gene should act in the intercellular space, several species with enhanced resistance to bacteria have been obtained by introducing genes encoding bactericidal polypeptide. Thionin (Carmona et al., 1993) and its synthetic analogue MB39 (Mills et al., 1994), attacin E (Norelli et al., 1994) and cecropine (Huang et al., 1997) seem to enhance resistance to bacteria in other species. Particular attention should be paid to the human lysozyme, which confers resistance to both fungi and to bacterium *P. syringae* in tobacco plants (Nakaijma et, al. 1997).

In the battle against insect attack, the *Bt* gene from *Bacillus thuringensis* (Vaeck et al., 1987) has successfully been introduced in other species with encouraging results, and could be an alternative way to prevent olive fly damage. In addition, gene or genes isolation from resistant genotypes, such as «Bianca of Tirana» should be attempted.

## 3. OVERVIEW OF THE APPLICATION OF BIOTECHNOLOGIES FOR IN VITRO MULTIPLICATION AND CONSERVATION OF THE BIODIVERSITY

The biotechnologies could contribute, in significant manner, to the genetic improvement of the olive. It is indispensable to use in vitro technologies. This technique, after an initial difficulty for the axenic stabilization of the explants and the successive growth, today has satisfactory results.

In the olive, it is possible to produce plants from either zygotic and mature tissues from different cultivars under the three micropropagation techniques: a) by axillary buds stimulation, b) by organogenesis, and c) by somatic embryogenesis.

*Fig. 1. Olive tree cv Moraiolo during the proliferation phase. The apical dominance is strong, in spite of recent researches on the employment of hormones-like substances that showed the capacity to produce different shoots from a bud in some cultivars*

*Sl. l. Stablo masline cv. Moraiolo u fazi proliferacije. Apikalna dominacija je jaka, no najnovija su istraživanja o upotrebi tvari poput hormona pokazala da su one sposobne proizvesti izbojke iz pupa u nekih kultivara*

### *3.1 Micropropagation by axillary bud stimulation*

Protocols for in vitro propagation of the olive cultivars were reported many years ago (Rugini, 1984) and they are actually a commercial reality in the nursery production of Italy for more the 50 olive cultivars by using mature material. This technique allows high quality production and rapid growth of the plants, which are pathogen free on the surface and in the vascular system (Fig. 2). Rooting ouurs in high percentage (Fig. 3), even in cultivars considered recalcitrants when propagated from stem cuttings like 'Nocellara etnea' (Briccoli Bati and Lombardo, 1995). The rate of growth is superior to plants derived from stem cuttings, they produce flowers and behave in the same manner as plants derived from suckers or somatic embryos. Only micropropagated plants from mature material, from suckers or from somatic embryos (even if produced in vitro from adult material), spend the period of over 2 years under field conditions in respect to those derived from mature

shoots. The genetic stability of the in vitro plants was tested by PCR-RAPD analysis (Leva et al., 2000; Garcia-Fèrriz et al., 2000) and with agronomic and morphological observations (Rugini et al, 1996; Leva et al., 2000; Garcia-Fèrriz et al., 2000; Briccoli Bati et al., 2000). No genotypic and/or phenotypic variations were observed in different cultivas: Moraiolo, Canino, Nocellara, Carolea, Maurino, Frantoio, Leccino, Dolce Agogia, Empeltre, Arbequina e Picual and some North African cultivars.

*Fig. 2. Proliferation phase in a commercial laboratory, where beyond 50 cultivar are produced in vitro, some of which are of commercial interest*

*Sl. 2. Faza proliferacije u komercijalnom laboratoriju gdje se proizvodi in vitro više od 50 kultivara od kojih su neki tržišno zanimljivi*

*Fig. 3. Rooting phase in one olive cultivar. Up to now all the cv tested differentiate roots in high percentage, near 100%, including difficult-to-root cultivars by stem cuttings*

*Sl. 3. Faza ukorjenjivanja u jednom kultivaru masline. Do sada svi se testirani kultivari razlikuju u korijenu u visokom postotku, gotovo 100%, uključujući kultivare koji se teško ukorjenjuju iz sadnica stabljike*

*3.2 Micropropagation by organogenesis*

Organogenesis is the process in which the undifferentiated cells of any tissue differentiate an organ (buds or shoots, roots, flowers etc), directly from the cells of the tissue (direct organogenesis) or from the cells of a callus formed from any tissue (indirect organogenesis). The differentiated shoots will be induced to produced roots like those derived from pre-formed buds. Generally an only cell is involved in the regenerative process. This type of regeneration would facilitate the isolation of stable genotypes from chimeric tissues. The organogenesis was obtained in olive tree from zygotic tissues and from mature tissues of a cultivar, directly or through callus formation. A high frequency of organogenesis was obtained from cotyledonary fragments close to

the embryos of mature seeds (Rugini, 1986; Caňas and Banbadis, 1988) and sections of hypocotyls (Bao et al., 1980). Nevertheless, petioles of leaves derived from in vitro proliferated shoots are also able to regenerate buds, even though in low frequency, not more than 40%, in the function of a cultivar, of the position of the leaf on the shoot, of the substrate, as well as of the quality of the shoots during the phase of proliferation (Mencuccini and Rugini, 1992).

Commonly the organogenesis can not be considered a method of multiplication because from zygotic tissues it is not possible to reproduce the features of the mother plant due to the high heterozygosis of the species and because the efficiency of regeneration of a mature cultivar is too low. The use of this technology is essential as the first step in obtaining somatic embryogenesis.

*3.3 Micropropagation by somatic embryogenesis*

Similar to organogenesis, the somatic embryogenesis is a regeneration process where more than one cell is involved in embryo formation.

In the olive, somatic embryogenesis has been successful by achieved from several tissues of both zygotic and maternal origin: immature and mature zygotic embryos, seedling tissues and leaf petioles of cultivars. Two to two and a half months after fertilisation, is the best period to collect fruits to extract zygotic embryos, because during this period they show the highest somatic embryogenesis potential. However, as well as the date of embryo collection, other factors such as growth regulator quality and light should be taken into consideration, because they are critical in embryo formation (Rugini, 1988; Leva et al., 1995). The capacity of the immature zygotic embryos to form somatic embryos may be extended for at least two months by storing at 14 to 15 °C, the fruitlets collected 60-75 days after full bloom. Under these conditions, although the small embryos continue to develop, they do not, however, lose their embryogenic capacity, contrary to the corresponding embryos collected from fruits left on the plants (Rugini, 1995).

The first report on induction of somatic embryogenesis in the olive was achieved by wounding the roots still attached to the seedling; the callus produced in the wounded zone produced embryos which converted into plantlets (Rugini and Tarini, 1986). Callus from segments of non-germinated mature embryos (Orinos and Mitrakos, 1991; Mitrakos et al., 1992) could produce somatic embryogenesis. Calli derived from rootlets give the highest somatic embryogenesis. The difference in the amount of embryogenesis depends on the origin of the explant (proximal, medium and distal part of the

embryo) and on the last of the period of permanence of explants on callus induction medium.

Somatic embryogenesis from these tissues is still difficult to achieve. However, with two cultivars, «Canino» and «Moraiolo», it has been done using a strategy named «double regeneration system» (Rugini and Caricato, 1995; Rugini and Muganu, 1998). It consists in regenerating first adventitious buds from leaf petioles of in vitro growing shoots, then subculturing the small leaflets in a proper medium until pro-embryo masses appear. From the primary embryos it is easy to obtain the somatic embryogenic cycle, from the epidermis or from the under epidermal layer (Lambardi et al., 1999). When the embryos have been obtained, the cycle of somatic embryogenesis can be kept indefinitely, also in hormone free medium, either from normal embryos or from teratomic masses which can be of different morphology: fuse, claviform, and fused cotyledons (Rugini and Caricato 1995; Benelli et al., 2001; Rugini et al., 2005). The abnormal structures do not convert into plants, but conversion of normal embryos is often very difficult. The embryos differ mainly from epidermal surface with mainly unicellular origin (Lambardi et al., 1999). The continuous production of long-term cycles of embryos from epidermal cells could be a great advantage in regenerating plants from transgenic cells, because it avoids the callus formation which could be a wide source of undesirable genetic variability (Fig. 4). Plants derived from somatic embryos produce flowers 2-3 years later than micropropagated plants by axillary bud stimulation.

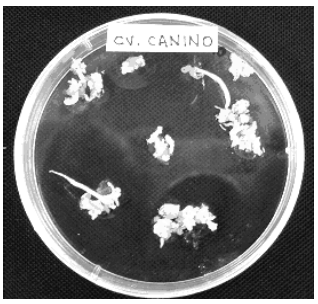


*Fig. 4. Somatic embriogenesis in olive tree, cv Canine obtained through the "double regeneration system" (regeneration of adventitious bud from leaves petioles of in vitro cultivars).*

*Sl. 4. Somatska embriogeneza u stablu masline cv. Canine, dobivena "sustavom dvostruke regeneracije" (regeneracije slučajno nastalog pupa iz peteljki listova kultivara in vitro)*

### *3.4 Production of pathogens free plants*

In olive propagation, almost always in asexual way, not only fungal and bacterial diseases but also viral diseases contribute to their spread. The diffusion of pathogen free olive material of commercial scale could be an

innovative field of work. The protocols for olive multiplication in vitro are available and can be applied to a wide number of olive cultivars (Fig. 5). From the in vitro grown shoots it is possible to take the meristem which is well known to be virus free. By this technology in collaboration with the Department of Plants Protection of the University of Bari – Italy, it was possible to clean 2 cultivars (Rugini, comm pers.) Up to now, the thermotherapy in vivo and in vitro applied to other species, has not given good results in the olive.

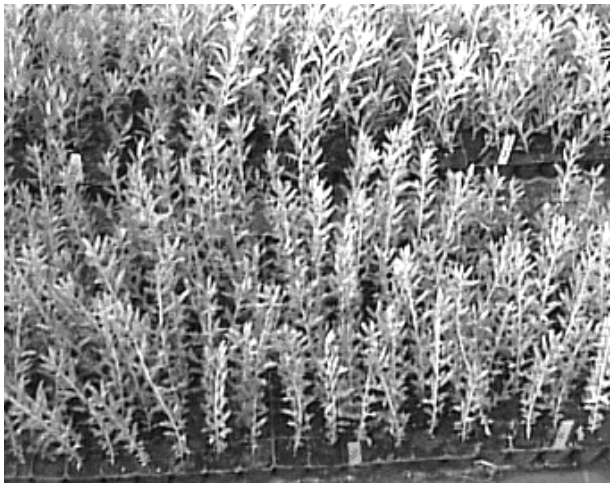

*Fig. 5. Plantlets of the cv Canine after the acclimatisation phase. They are characterized by a fast growth*

*Sl. 5. Biljčice cv. Canine poslije faze aklimatizacije. Odlikuju se brzim rastom*

### *3.5 Micrografting*

The olive presents difficulty to develop shoots from meristem on a common mineral substrate if it is not taken from in vitro grown shoots during the multiplication phase. Micrografting could represent a valid method of support to this technology in the case in which not all the genotypes are able to develop or to rejuvenate recalcitrant genotypes in the conditions in vitro in order to improve their adaptation to in vitro conditions. Revilla et al., (1996) succeeded to rejuvenate the cv Arbequina after a cycle of grafting on derived seed plants grown in vitro. Troncoso et al., (1999), developed a protocol of micrografting

under in vitro conditions among derived seed plants and obtained 67% of acclimatized plants. These results suggest that micrografting should be further explored as a simple and effective method for olive multiplication.

### *3.6 Synthetic seeds production*

Somatic embryogenesis could be used for the production of "synthetic seeds", that is, somatic embryos or apical buds encapsulated in a substrate of protection and/or nutrition, like sodium alginate to be used for micropropagation or for germoplasm conservation. Micheli et al., (1998) obtained good results with the cv Moraiolo after encapsulation in an alginate matrix, containing a nutritive medium, apical and nodal buds from micropropagated shoot cultures, they maintained up to 49% viability and grew satisfactorily when stored at 4°C for no more than 45 days. Lambardi and his collaborators tried with success to encapsulate somatic embryos of the cv Canino. Nevertheless, the somatic embryos to the present state present a low rate of conversion into plants.

### *3.7 Germplasm conservation*

Germoplasm conservation is also necessary for olive species in order not to lose interesting genotypes as a result of the novel tendency to cultivate few cultivars (more productive and more adaptable to most environmental conditions) in the new olive plantations. The conservation of seeds without doubt the simplest and less expensive technology, has had little sense in plants commonly propagated vegetatively which have a high degree of heterozygosis like olive species. For this reason preservation of the plant or its parts is indispensable.

At present, field conservation is the unique option for the olive species, even though there are varied risks of biotic and abiotic nature. In alternative to the clonal olive trees, the ex-situ conservation, that exploits the cultivation in vitro, seems to be promising by the slow growth conservation and the cryo-conservation. It offers enormous benefits: i) high speed of multiplication, ii) requires very limited space, and iii) possibility to maintain pathogen free plants.

#### 3.7.1 Slow growth conservation

It preserves the shoot under in vitro conditions in an ideal substrate able to slow down their growth at low temperature (about +4°C). Experiments

conducted on two cultivars (Leccino and Frantoio), preserved in refrigerator on substrate OM without hormones, either in the darkness or at 8 hours of light photoperiod at 20 μmol m-2 s-1, showed a good capacity of conservation for 8 months, but only in darkness (Lambardi et al., 2000).

Gardi et al. (2001) observed a different behaviour between among: cv Frantoio could be conserved at 6° C for 5 months showing 100% survival and growth after conservation, while for stored shoot cultures of 'Ascolana tenera' and 'Moraiolo' at 6°C and in the dark the maximum growth potential only up to 2 months.

### 3.7.2 Cryo-conservation

Promising results have been recently obtained with the application of cryogenic techniques to the long-term conservation of olive germplasm by applying the direct immersion into liquid nitrogen (–196°C) or by using the vitrification solution. 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, direct and rewarming at room temperature, while Lambardi et al. (2000) applied a procedure of vitrification and one-step freezing in liquid nitrogen to shoot tips excised from *in vitro*-grown shoot cultures of the cv Frantoio.

Alternatively to shoot tips, embryogenic cultures of olive proved to be a highly suitable material for cryopreservation using the vitrification approach, 40% of the cryopreserved embryogenic samples survived and soon started to proliferate new embryos after a pre-treatment with the vitrification solution and frozen in liquid (Lambardi et al., 2000).

## 4. EVALUATION OF THE GENETIC VARIABILITY IN OLIVE

Until very recent times the genetic variability of the cultivated olive trees was described only morphologically and agronomically. The evaluation of plants with genetic alterations is relatively simple if it is associated with phenotypic changes, but unfortunately from the observation of the phenotype it is not possible to verify the genetic changes nor the biochemical appearance not visible by instrumental examinations.

The recent development of the molecular biology allows evaluation of the genetic variability in olive trees in an objective and precise manner across

technologies that emphasize eventual genetic changes is of biochemical type (isoenzymes and allozymes), and molecular (RFLPs, RAPDs ISSR, AFLPs, SNIPs, micro satellites). Nevertheless, it is important to note that none of the methods used at present can be absolutely certain that genetic variations do not ouur during the cultivation in vitro or the germoplasm conservation.

### *4.1 Isoenzymes*

The isoenzymes represent only a small portion of the genome, they are submitted to the selective pressure and the number of loci under study is limited. They are used for cultivar identification and to determine the genetic relations between them.

The first attempts at the use of biochemical markers for understanding the domestication process in the olive started in the 80s by using isoenzymes extracted from pollen or from leaves, and they were able to distinguish the polimorfism between the cultivars (Pontikis et al., 1980; Loukas and Krimbas, 1983; Trujillo et al., 1990; Ouazzani et al., 1993). The isoenzymes, being the product of the gene expression, are regulated by different factors such as environmental conditions, tissue of origin, and the development of the plant. These factors gives back the isoenzymes few reproducible, giving back difficulties for their interpretation. However, this is a very polymorfic and co-dominating marker. In the olive, they isolated some genes codifying proteins involved in some essential biochemical processes. The first gene isolated was the Stearoil-ACP (δ9) Desaturasi (Baldoni et al., 1996), the key enzyme in the trial of insaturation of the fatty acids of the membranes and of the lipids of accumulation. It determines the formation of the double connection that transforms the stearic acid in to oleic acid,the main constituent of the olive oil.

Subsequently, other genes were isolated, like the RBCL (*Ribulose Biphosfate Carboxilase*) (Oxelman et al., 1997), the gene for NADH dehydrogenase of the chloroplast (Oxelman et al., 1999) and the gene of the crytochrome B5 (Martsinkvskaya et al., 1999). Different codifying genes for a family of proteins that induce allergic symptoms in the man were isolated in different research. These proteins difer in length but all of them are expressed during the formation of the pollen (Lambardero et al., 1994; Villalba et al., 1994; Batanero et al., 1997; Asturias et al., 1997; Ledesma et al., 1998).

This technology based on the electrophoretic discrimination of different molecular shapes of some enzymatic proteins facilitates and speeds up the work of the breeders in the evaluation of the germoplasm present for the

genetic improvement of the species and for the certification of the vegetable material propagated. They are carried out on a number limited genotypes that they have had common origin (Ouazzani et al., 1993; 1996).

Data banks are being created containing information on sequences isolated from RAPD fragments that do not correspond with other sequences and fragments found of DNA characterizing young and adult tissues (Bogani et al., 1994).

### *4.2 Molecular markers for identification of varieties*

Recently, olive DNA analyses have been conducted to find molecular markers to clarify the confusion created by so many synonyms and homonyms existing for olive cultivars, and for screening for biodiversity.

The first random amplified polymorphic DNA (RAPD) analysis was carried out in eleven cultivars by Bogani et al., (1994), using five arbitrary primers of ten nucleotides. Primers with a high G+C content were able to amplify many DNA fragments compared to primers with low G+C content. Authors also reported tables of similarity in which genetic diversity appeared between cultivars. Forty decamer oligonucleotides were used by Fabbri et al., (1995) to distinguish between 17 different cultivars. Only seventeen primers produced reproducible polymorphic markers among all cultivars with 47 total polymorphic DNA fragments. Estimated values of similarity were used to generate dendrograms by the UPGMA (Unweighted Pair Group Method using Arithmetic Averages) cluster analysis in which all cultivars were separated from each other, but no apparent relationship between olive cultivars and their geographic origin was shown (Fabbri et al., 1995). Many other researchers used RAPD analyses to look for polymorphism among both cultivars of *Oleaeuropaea* and diverse species of *Olea* (Roselli et al., 1995; 2002; Cresti et al., 1996; Vergari et al., 1996; Perri et al., 1997; Cavalieri et al., 1998; Wiesman et al., 1998); in all cases they used agarose gel to separate PCR products.

An increased number of fragments per primer, used in RAPD-PCR reaction, was found by Mekuria et al. (1999) separating amplification products by polyacrylamide gel electrophoresis, under denaturing conditions. A high degree of genetic similarity was found in cvs Manzanillo, Kalamata, Nevadillo, Picual, Correggiolo, and for small genetic variations the authors suggested that somatic mutations had occurred. In other cultivars analysed, a high degree of heterogeneity was found and the authors suggested that misnaming occurred on several occasions. Recently AFLP (Amplified Fragment Length Polymorphism),

has been used for marker fingerprinting, and has produced a sufficient number of polymorphic fragments to separate different species of *Olea* and to group genotypes within the cultivar of *Olea europaea* (Angiolillo et al., 1999). By this technique, they grouped genotypes coming from different geographical origin, using 5 AFLP-primers, which produced about 290 polymorphic fragments. One cluster was composed of olive cultivars, wild olive and North-West African species with a similarity level of 0.56. *Olea* species from East Africa and Asia grouped separately while species of the Indian Ocean and Australia showed the highest diversity. Examining olive genome in this way can help to identify the tools for distinguishing genotypes.

Two important works have been reported recently on repetitive DNA sequences, which are spread in all the genomes, sometimes called micro satellites (Katsiotis et al., 1998; Bitonti et al., 1999). The repetitive DNA sequences are 80 bp long and 81 bp long respectively, the latter is a family of repetitive sequence which is replicated in tandem from 2 to 4 times.

Up to now a lot of work has been carried out with molecular markers aiming at discriminating between the different genotypes, nonetheless, it is still not possible to distinguish clones of the same cultivar. Specific markers need to be found for providing molecular assistance in selection.

## 5. THE BIOTECHNOLOGIES APPLIED TO THE GENETIC IMPROVEMENT OF OLIVE

The new genetic improvement methods for the olive, such as genetic transformation, somaclonal variation with or without selective pressure or with mutagens, the manipulation of protoplasts, the haploids culture, can be applied only with an efficient and repeatable technology of regeneration.

The biotechnologies, besides allowing the preservation of the genetic pool of a species, can be employed to increase their variability by applying different exogenous treatments and continuing the regeneration process in order to produce new genotypes. The most used technologies are: i) simple regeneration from callus with or without selective agents (somaclonal variation), ii) changes in ploidy level (haploids and polyploids), iii) somatic hybridation, and iv) genetic transformation. These technologies are applicable with success only if efficient methods of regeneration from woven or better from callus are used.

*5.1 Somaclonal variation*

Up to now, few researches have been reported on inducing somaclonal variation. In order to achieve this objective it is indispensable to have a very efficient method of regeneration and possibly from mature material of affirmed cultivar aimed to improve certain characteristics. Few cultivars, including cv Canino, are able to regenerate from somatic embryos. In order to increase the genetic variation in a species, it is a common technique to grow the embryos or the embryogenic masses to ionising radiations, fungal filtered cultures, or filtered toxins of some pathogens or to other selective pressures that induce changes or allow the selection of changing material.

*5.2. Changes in ploidy level (haploids and polyploids)*

In the olive, to use homozygotic genotypes in the genetic improvement programme is of unquestionable importance. Considering the prevailing auto sterility and the juvenile phase that characterizes this species, the achievement of an homozygosis by auto-impollination seems very improbable, while the cultivation of macro or micro spores could help the achievement of such objective. Nevertheless, up to now, the regeneration of plants from these tissues is not possible (Perri et al., 1994; Rugini et al., 1996). To the contrary, the production of polyploids is more easily attained by using chemical products (colchicine, orizaline, etc.) or by physical means (ionizing radiations). Triploid and tetraploid plants have been produced from the mixoploid cultivar of Leccino and Frantoio, obtained by irradiation with gamma rays (Fig. 6). The tetraploid plants are recognizable because they present larger leaves. The triploids have been selected from seeds germination of the bigger fruits (arisen from diploid ovocell and haploid pollen) collected from mixoploid and tetraploid plants and later chromosomes count. Both types of plants are tested like rootstocks (Rugini et al., 1996).

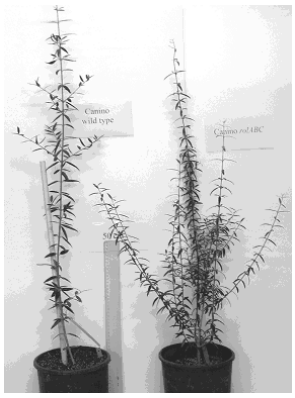


*Fig. 6. Transgenic plants for rolABC in pot. They were created with the objective to produce dwarf plant or rootstocks able to reduce the growth of the grafted cultivar*
*Sl. 6. Transgene biljke za rolABC u loncu. Stvorene su radi proizvodnje patuljaste biljke ili podloga sposobnih da smanje rast cijepljenog kultivara*

### *5.3 Somatic hybridization*

The somatic hybridation is useful for the production of somatic hybrids and for the production of cytoplasmatic hybrids (cibrids). In the olive, even though vital protoplast has been produced able to regenerate the cellular wall and subsequently microcalli (Rugini, 1986; Mencuccini, 1991; Perri et al., 1994), it is still not possible to regenerate complete plants from these cells. In case the regeneration succeeds, besides the production of hybrids plants could be obtained with high degree of variability as in regeneration from protoplasts.

### *5.4 Alien gene transfer*

The low efficiency of both conventional and some older unconventional genetic improvement techniques (protoplast technique and somaclonal variation) seem to indicate that genetic transformation is the most promising technique to speed up the development of new superior cultivars.

In order to apply this technique an efficient *in vitro* regeneration method and the availability of suitable genes are crucial conditions. The success of producing transgenic plants depends on the number of transformed cells and their ability to differentiate shoots and embryos. Common explants used in the olive for the transformation experiments are the leaf petioles, zygotic tissues and somatic embryos from in vitro grown cultivars because they are able to regenerate plants. Several procedures are currently used to accomplish gene transfer into plant cells: a) Microbe-vectored systems (*Agrobacterium*-mediated and virus-mediated), b) Artificial delivery system (DNA uptake by diffusion, membrane diffusion, electroporation, laser microbeam, microinjection and microprojectiles), and c) Natural delivery system (pollen tube pathway and floral tillers).

In the olive, both *Agrobacterium* and microprojectiles coated with DNA methods are currently used.

#### 5.4.1 Agrobacterium mediated gene transfer

Most of the reports on transformation and isolation of olive transgenic plants have involved *Agrobacterium*-mediated gene transfer. This involves the transfer of a segment of the Ti or Ri-plasmid known as T-DNA into the nuclear genome of the plant (Chilton et al., 1982). It is known that the efficiency of transformation depends on several factors that include: bacterium strains, plant cell competence, transgenic cell selection, and plant regeneration methods. The

ability of *Agrobacterium* to transform cells efficiently in the target tissue is essential. It is well known that the bacterial strains strongly influence the frequency of transformation. Most of the transformation experiments with *Agrobacterium* have been carried out with *rol* genes of the T-DNA of *A. rhizogenes* cloned in *A. tumefaciens*. The only *A. tumefaciens* strain used for transformation experiments up to now is LBA4404 containing the plasmid pBIN19 (Bevan, 1984). *Rol* ABC genes isolated from suitable *A. rhizogenes* wild type strains, containing plasmids p1855 and pBR322, have been transferred into *A. tumefaciens* to transform olive explants. Efficient selection of the transformed cells, which is usually carried out with the selective antibiotic kanamycin at a concentration varying according to the explant used, ranged from 50 to 100 μg/ml. The critical kanamycin concentration and the time to apply the selective pressure must be previously determined for each explant because the different tissues have different sensitivity to kanamycin. It is advisable to apply selection 3-4 weeks after co-cultivation with *Agrobacterium* to allow the formation of transgenic cell colonies in large numbers, which can survive the selection pressure better increasing the probability to differentiate organs. The lack of an efficient regeneration system from transformed cells is the major obstacle in mature tissues of olive cultivars, but not from zygotic tissues. However, an efficient protocol for regeneration via cyclic somatic embryogenesis has been developed in one cultivar making it easier to transform olive (Rugini et al., 1999; 2002). Another important factor is the stability of the inserted DNA in the plant genome in order to avoid transient expression of the foreign genes or the formation of chimeric plants. In addition, it is important to consider other factors that could affect transformation efficiency such as: length of the co-cultivation period, use of wounding agents during co-cultivation of the explants with the *Agrobacterium*, such as carborundum granules (Mariotti et al., 1991); and stimulating factors of bacterium virulence such as acetosyringone. The proper antibiotic agent to control *Agrobacterium* growth is fundamental. In olive, cefotaxime (200 mg/l) seems to be the appropriate antibiotic since it improves in some way the recovery of plantlets from somatic embryos (Rugini and Caricato, 1995).

### 5.4.2 Artificial delivery system

Biolistics has emerged recently as a simple and efficient technique for stable genetic transformation of recalcitrant species, and for studying gene expression and regulation. Most recently, people have used microprojectiles-

mediated DNA delivery to obtain transient expression in various tissues of woody species, such as embryogenic masses, zygotic and somatic embryos, needles, pollen and shoots buds (Ellis et al., 1991; Goldfarb et al., 1991; Duchesne et al., 1993; Charest et al., 1993; Hay et al., 1994). However, the successful genetic transformation requires the optimization of several factors, including *in vitro* regeneration systems, the methodologies for DNA delivery, and high expression of the introduced gene in the plant tissue. The only DNA delivery system being used in olive up to now is DNA delivery by microprojectile bombardment (Lambardi et al., 1998).

## 6. GENETIC TRANSFORMATION IN OLIVE

Gene transfer to the plant genome, modifications or substitutions of more efficient promoters or suppressed expression of undesirable genes are the alternative techniques to the conventional genetic improvement for increasing genetic variability . The first attempts of genetic transformation were reported in the 80's (Rugini and Fedeli, 1990) by using immature zygotic embryos from the cultivar Moraiolo.

Later, direct gene transfer or Agrobacterium mediated have been used successfully in an olive cultivar due to the efficient method of somatic embryogenesis.

### *6.1. Obtaining chimeric plants (transgenic root system only)*

*Agrobacterium rhizogenes* grown in YMB liquid medium, are used to increase rooting of in vitro grown shoots (Rugini, 1984; Rugini and Fedeli, 1990; Rugini, 1992; Rugini and Mariotti, 1992). Shoots were inoculated in the middle of the stem by puncture or at base by a longitudinal wounding with a scalpel, stimulating rooting both from the inoculation point and at the basal part of shoots. The results support the hypothesis that the partial integration of T-DNA has a possible inductive role on the non-transgenic neighbour cells or perhaps more probably some unknown substances present in the bacterial secretions. The root system originating from treated shoots with *A. rhizogenes* also had more secondary roots during the first months. There are no news on the modification of the aerial part of the plant; however, in plum a drastic height reduction was observed when plants were transfered to the field (Rugini, personal comm.).

*6.2 Modifications of vegetative and reproductive habit and increased rooting efficiency*

After the development of the efficient protocol of a cyclic somatic embryogenesis in the cv Canino and Moraiolo (Rugini and Caricato, 1995), cv Dolce Agogia (Mencuccini et al., 1997), the *rolABC* genes of *Agrobacerium rhizogenes* and the marker gene *nptII* encoding resistance to the antibiotic kanamycin were transferred by using *A. tumefaciens* strain LBA 4404 (Rugini et al., 1999). The transgenic plants were transferred to the experimental field in 1999 under the authorization of the Minister of Health for further evaluation (Rugini et al., 2000). Transgenic plants showed increased juvenility which not allowing field evaluation. Transgenic plants for *rolABC* showed short internodes and increased rooting ability in respect to the nontransgenic controls. Furthermore, they grew slowly and presented many lateral buds giving them a bushy aspect (Fig. 6). Grafting combinations were carried out with a non transgenic cultivar which are under evaluation (Fig. 7).

*Fig. 7. Transgenic plants for rolABC and osmotin gene grown at the Università della Tuscia under the authorization of the Ministry of the Environment. They were created with the objective to produce dwarf plant or rootstocks able to reduce the growth of the grafted cultivar.*

*Sl. 7. Transgenske biljke za rolABC i osmotinski gen uzgojeni na Sveučilištu Tuscia pod ovlaštenjem Ministarstva okoliša. Stvoreni su radi proizvodnje patuljaste biljke ili podloga sposobnih da smanje rast cijepljenog kultivara.*

Regarding rooting ability, tests were done only with transgenic explants for *rolABC*. In vitro rooting test were done with uninodal explants of a selected somaclone subcultured in vitro and were compared with nontransgenic shoots coming from somatic embryos. In the first case, 50% of rooted explants were obtained in auxine free medium, and 60% of rooting in the medium containing 160 mg/l of putrescine, while in control clones 0% and 15% were obtained. When 0.1 and 0.5 mg/l auxine were added to the medium, the transgenic clones showed 100% of rooting efficiency, while control clones only 70-80%. These results show the increased rooting ability of the transgenic clones with *rolABC* in the presence of auxine.

### *6.3 Increasing resistance to biotic stress*

Transgenic plants with *osmotin* gene were obtained from somatic embryos from petioles of cv Canino with the same procedure as for the production of transgenic plants for *rolABC* of Canino (Rugini et al., 1999), by using *A. tumefaciens* strain LBA 4404 containing the *osmotin* gene in the pKYLX71 under 35S promoter (kindly supplied by Prof R. Bressan, Purdue University, USA). Osmotin is a gene present in all the plants tested up-to-date and belongs to the PR family (proteins for pathogenesis) is expressed under stress conditions, including pathogen attack. The osmotin plantlets grown in the greenhouse had similar phenotypic characteristic to untransformed ones. Several plants were used for field trials and showed to be more resistant to *Spilocaea oleagina* (D'Angeli et al., 2001) The resistant clones were grafted on nontransgenic rootstocks in order to evaluate their level of resistance to *S. oleagina*.

### *6.4 Genetic transformation with GUS and nptII reporter gene*

The use of these genes permits the gene transfer technique to improve and finding a more efficient promoter for the alien genes. Microprojectiles have recently been used to transform secondary somatic embryos derived from cv Canino. Transient gene expression was tested 3 days after the microprojectile-DNA delivery and the GUS gene was detected on bombarded somatic embryos using β-glucuronidase histological assay. Working with the Particle Inflow Gun (PIG) device, highest levels of transient GUS gene expression were observed when large-size somatic embryos (>5 mm length) were bombarded with the pZ085 and the pCGUδ0 plasmids at a pressure of 580-kPa. The use of different

particles (tungsten or gold) and apparatus for bombardment (PIG or PDS-1000/hE) did not affect substantially gene expression (Lambardi et al. 1999).

*Agrobacterium*-mediated system was used to transform the cvs Dolce Agogia and Moraiolo with the *gus* gene (Mencuccini et al., 1999). No transformed shoots have been recovered; only 7% of shoot regenerated from cv Dolce Agogia and 4% from Moraiolo. The GUS activity was assayed by histochemical analysis in somatic tissues three days after the *Agrobacterium* infection. The blue dye of the GUS activity was localized in leaf petiole basal sections and in small sectors of the callus.

## 7. MOLECULAR ANALYSIS OF THE TRANSGENES

Transformation and stability of the integrated DNA can be confirmed by a Southern blot analysis (Southern, 1975) of the transformed tissue or by PCR (Polymerase Chain Reaction) (Saiki, 1990). The PCR will reveal the presence of internal regions of the inserted T-DNA, whereas the border fragments are revealed by a variation of this technique referred to as inverse PCR (Ochman et al., 1990).

DNA from somatic embryos of olive cv Canino, were extracted in order to verify the stable insertion of *rolA*, *rolB*, *rolC* from the Ri TL-DNA of *A. rhizogenes* and the *osmotine-nptII* gene. PCR reactions were carried out by using specific primers for each gene. Southern analysis of the *EcoRI* digested genomic DNA confirmed that embryos were transgenics for the Eco 15 fragment of *A. rhizogenes* (Rugini et al., 1999).

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**Adrese autora – Authors addresses:**
Eddo Rugini
Patricia Gutiérrez Pesce
Università degli Studi della Tuscia,
Dipartimento di Produzione Vegetale, sezione di Ortofloroarboricoltura
01100 Viterbo

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