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Review

Applications of biotechnology in olive

Geraldo Magela de Almeida Cançado[1]*, Tesfahun Alemu Setotaw[1] and Juliano Lino Ferreira[2]

[1]Plant Biotechnology Laboratory, Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG), Caldas, MG, Zip Code 37.780-000, Brazil.
[2]Embrapa Pecuária Sul. Bagé, RS, Zip Code 96401-970, Brazil.

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Many scientific and technological fields make use of biotechnology. Among the most important applications of biotechnology in agriculture are large-scale commercial micropropagation, genetic transformation and the development of transgenic varieties, embryo rescue in plant breeding programs, genotyping based on DNA markers, studies of genetic evolution and diversity, and genome sequencing. These myriad applications of modern biotechnology and molecular biology are being applied to the olive tree, a crop cultivated for thousands of years in many places of the world and whose products are consumed globally. The selection and development of olive cultivars by conventional breeding methods is costly and time consuming, therefore, the application of biotechnology procedures and techniques, such as *in vitro* cultivation, molecular markers, and genomic and genetic transformation in this species may facilitate the improvement of important traits of this crop, such as biotic and abiotic resistance and tolerance, yield performance and oil quality. In this review, we present current applications of modern biotechnology and molecular biology in olive species.

Key words: *Olea europaea*, tissue culture, molecular markers, genetic transformation, genomic diversity.

INTRODUCTION

Olive (*Olea europaea* L.) is a perennial plant species that is widely cultivated and consumed worldwide, including in Brazil. The olive is of great importance in the international food market due to the health benefits and culinary qualities of its products. In addition, increasing scientific evidence of the beneficial properties of the regular consumption of olive oil has encouraged an increase in its consumption worldwide. For most plant species, including the olive tree, advances in field performance depend on knowledge obtained via modern biotechnology and molecular biology, which supports new and more accurate approaches in many research fields, including genetics, plant pathology, physiology and biochemistry, entomology and plant nutrition. The definition of biotechnology includes many concepts, but one widely used definition of biotechnology is the combination of techniques that permit the manipulation and use of microorganisms, plants and animals with the objective of developing processes and products of interest for people. Modern biotechnology has applications in diverse fields, and the products of biotechnology can be found in a variety of markets and goods, such as foods, clothing, cosmetics, pharmaceuticals and a number of industrial materials. These products are already part of the daily lives of common citizens, even if unconsciously so.

Biotechnology processes are essential tools for increasing the quality and productivity of agriculture in different countries worldwide because conventional methods alone are inadequate to continue to achieve significant gains. The technologies developed in the fields of biotechnology and molecular biology can be combined with traditional approaches, such as conventional breeding, for the potential development of new genotypes with superior traits more rapidly (Sartoretto et al., 2008) and aggressively. This practical application of biotechnology is already a reality for crops such as

*Corresponding author. E-mail: gcancado@gmail.com. Tel: +5503537351566.

soybeans, corn and cotton.

IN VITRO CULTURE OF OLIVE

Plant tissue culture or *in vitro* culture has been a very successful technique in plant biotechnology. *In vitro* culture has been intensively studied and improved over the last six decades, which is now regularly adopted as a routine process in several plant species to accelerate the efficient clonal propagation of various commercial crops. The contributions of *in vitro* culture include the multiplication and preservation of clones with superior genetic and phytosanitary quality, the development of new cultivars by using genetic transformation methods, germplasm conservation, which reduces space and cost, and the rescue of zygotic embryos with low viability rates (Souza et al., 2011). *In vitro* culture is also an indispensable tool for physiological and biochemical studies of plant species, including wild relatives whose potential remains unexploited, because it allows researchers to replicate environmental conditions more reproducibly.

In vitro culture is based on the totipotentiality of plant cells. The theory of totipotentiality was first proposed by Haberlandt in 1902 which states that each plant cell possesses the genetic potential to generate a new organism identical to the originating organism (George, 2008). Based on this concept, a number of studies have been performed to investigate the capacity of plant cells to regenerate whole plants using artificial nutrient medium. For the olive, explants are cultivated in *in vitro* culture in a highly controlled environment and are frequently used in experiments that require a high level of homogeneity. In addition, *in vitro culture* permits the use of organs, tissue, or even cells as the initial explant source. Although, plant tissue culture is widely used for research purposes and is well documented in the olive, its commercial application remains challenging and almost unexplored.

Despite low rates of success, the multiplication of the olive *in vitro* remains a major focus in many research labs due to its huge advantages as a large-scale cloning technique compared to conventional methods such as rooting of stem cuttings and grafting. However, the olive tree presents a high level of outcrossing, and therefore the practice of producing seedlings from seeds is avoided for commercial use and is used mainly with the aim of genetic breeding. The plants obtained from seeds produce genotypes different from that of the mother plant, and consequently, they may not exhibit the same agronomic qualities as the mother plant due to the genetic segregation. In addition, the occurrence of a lengthy juvenile phase in olive plants obtained from seed postpones the production of flowers and fruits, which, depending on the situation, can delay the first harvest by two to five years.

The rooting of stem cuttings and grafting are often employed in olive orchards for the production of olive plantlets, although these process present low efficiency and are influenced by environmental factors, genetic constitution, nutrition and the sanitary status of the plant material utilized. Given these limitations, the *in vitro* clone propagation of olive may assume an important role by reducing or even overcoming some of these limitations (Rugini and Caricato, 1995). Furthermore, the biotechnology associated with olive tissue culture encompasses a myriad of widely used techniques. In the following topics, the application of the biotechnology process in olive production will be presented in detail.

MICROPROPAGATION OF OLIVE

This practice is a routine process in tissue culture and transgenic plant development with high practical impact in agriculture. Micropropagation is used frequently in plant species that do not respond adequately to conventional propagation methods. The popularization of this technique and the reduction of its cost have stimulated its application in agriculture. Additional advantages of micropropagation include the continuous generation of plant material throughout the year, independent of seasonal issues, the small space requirement, large-scale cloning of elite genotypes from a few stock plants, and high phytosanitary quality.

However, the vegetative propagation of olive tree and other woody species by conventional methods has some drawbacks, mainly due to seasonal influences and the demand for large areas to produce buds for grafting and stem cuttings for plantlet rooting. The bottleneck in commercial cultivation of olive in Brazil is the absence of sufficient certified olive plantlets with adequate phytosanitary quality and genetic identity. In addition, the lack of knowledge in strategic areas of olive cultivation and production, such as propagation, genetic breeding, irrigation and fertilizer management, disease and pest control, and post-harvest and fruit/oil processing, is also an obstacle for the cultivation of this crop.

Cost is another consideration in the installation of olive micropropagation facilities. The experience accumulated from other species, such as eucalyptus, strawberry, banana, floriculture and potato, has demonstrated that the cost required for installing and maintaining this type of infrastructure will decrease and that the initial capital investments will be compensated for by the scaling-up of production after a few cycles of production. The critical step that should be considered in *in vitro* olive cultivation, whether for commercial or research purposes, is the development of adequate protocols. Similar to the rooting of stem cuttings, *in vitro* rooting is very dependent on the genetic background and is strongly influenced by the olive variety used. The influence of the genetic background on the response to the environment affects

the intricate metabolic processes involved in plant development.

Although, olive trees are not a deciduous species and do not exhibit characteristics such as a period of dormancy, a significant reduction in growth development occurs during the winter season, principally in regions with harsh winters. Olive explants exhibit variable responses to *in vitro* multiplication depending on the seasons in which they are collected.

During the exponential multiplication of olive cultivated *in vitro*, a satisfactory rate of genetic homogeneity must be maintained. During the multiplication phase, the occurrence of somaclonal variation must be avoided. Somaclonal variation can originate from genetic or epigenetic events and might be induced at a high rate by the stress during the successive subculture of explants *in vitro*. The effects of somaclonal variation occur directly on the deoxyribonucleic acid (DNA) due to the breaking of chromatin, the unbalanced migration of the chromosome during mitosis, inversions, deletions, translocations, duplications and changes in the nucleotide sequence of the DNA (mutations), which are transmitted in a hereditary manner to the progeny. Other sources of variation are derived from changes in the methylation pattern of DNA and chromatin condensation, the formation of stable complexes with histone molecules and synthesis of non-codifying RNA (Interference RNA), which can result in the loss of gene function (gene silencing). These events are known as epigenetic effects and are also induced by environmental effects, such as the stress caused by *in vitro* culture. However, the epigenetic changes do not alter the nucleotide sequence of the DNA, and in plants, they are transferred asexually through vegetative propagation.

Rhizogenesis (rooting) is considered as a critical step during *in vitro* plant regeneration of olive because it directly influences the next step, the acclimatization of the seedlings in an external environment (Rocha et al., 2008). Seedlings with weak root systems have a reduced chance of survival because they are not able to adapt their root performance to balance the uptake of water with evapotranspiration without undergoing drying. There is a relation between the internal level of auxins and cytokinin, which is responsible for initiating the rooting process (George, 2008). Other variables that can affect the process of adventitious root formation in olive are an excessive osmolarity of the culture medium, low availability of reserve substances in the tissue, lack of adequate nutrients, contamination by pathogens, juvenility of the materials used, thickness and lignification of the cell wall. All of these factors, individually or in combination, can completely inhibit the process of rooting. Peixe et al. (2007) has obtained a high rate of *in vitro* rooting (87%) among seedlings of the olive cultivar Galega after 60 days of culture by applying two types of auxins. Some explants were inoculated in OM medium (olive medium) supplemented with 4.9 µM indole-3-butyric acid (AIB), while others were immersed in a sterile solution containing 14.7 µM AIB for 10 s before inoculation in OM medium without the addition of growth regulators, a technique known as “pulsing”. One of the possible advantages of “pulsing” is avoiding constant exposure of the explants to the effects of auxins; excessive exposure to this class of growth regulators can inhibit rooting and induce callogenesis.

CULTURE OF OLIVE ZYGOTIC EMBRYOS

Among the techniques already cited, rescue of zygotic embryos using *in vitro* culture medium has been used for several purposes, such as the recovery of embryos that cannot germinate due to physical and chemical barriers, the development of plants obtained from controlled hybridization, the creation of genetically modified plants, and regeneration through organogenesis. Embryo culture permits the reduction of the time necessary to obtain a new individual, good uniformity and a high percentage of germination of embryos of species such as olive (Figure 1, Souza et al., 2011). When associated with *in vitro* culture, the germination of embryos permits the establishment of axenic cultures, which can be used as an efficient model for research purposes.

Among the desirable factors related to the donor plant and its fruit, the most important for embryo culture is the physiological maturity of the fruit. Therefore, a healthy donor plant with good nutritional status is required to obtain adequate fruits for embryo isolation. The genetic background of the variety utilized is also important, although the genetic profiles of olive zygotic embryos are distinct from their progenitors due to genetic recombination and segregation. Among the external factors that influence the efficiency of this procedure, the most important are the modification and adjustment of the composition of the culture medium and the environmental conditions during the incubation of the embryos. Variations in these factors can result in high or low percentages of germination in addition to directly influencing the late growth of the seedling produced (Souza et al., 2011).

SOMATIC EMBRYOGENESIS IN OLIVE

The propagation of olive can be achieved by using seeds, grafting or rooting of cutting stems. However, these techniques present disadvantages, including low regeneration and development rates, high heterogeneity during rooting mainly due to genetic constitution, sensitivity to environmental effects and genetic segregation, in addition to a long juvenile phase when plants are obtained from seeds. All of these factors, individually or in combination, increase the expense and time associated with olive propagation.

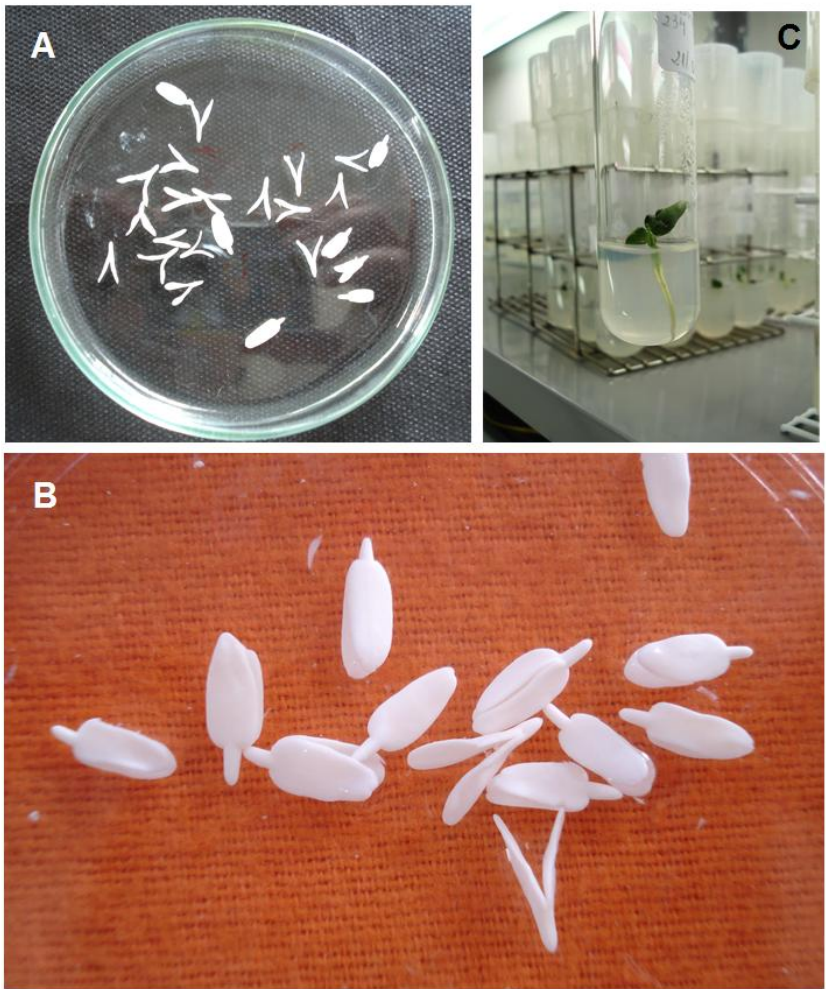

Figure 1. *In vitro* culture of olive embryos. A and B) Details of olive embryos isolated from physiologically mature seeds; C) seedlings of olive obtained from *in vitro* germination of isolated embryos. Source: Plant Biotechnology Laboratory (EPAMIG), Brazil.

One option is the use of cells, tissue and organs from elite olive genotypes to obtain clones on a large scale. Somatic embryogenesis is one of the most studied and applied techniques in plant tissue culture, mainly for research purposes rather than commercial multiplication. Basically, this approach consists of the proliferation of somatic cells with posterior organization into differentiated and bipolar structures similar to embryos, without the occurrence of fertilization (gamete fusion) (Williams and Maheswaran, 1986).

In theory, somatic embryogenesis in plants is divided into two major phases: induction and expression. When somatic cells are induced with chemicals or environmental stimulants, they can recover the ability of morphogenetic competence. After these structural changes, which are induced by external stimuli such as the composition of the culture medium, balance of growth regulators, and osmotic potential, among others inherent aspects of *in vitro* culture, cells differentiate into somatic embryos during the expression phase. In olive, the technique of somatic embryogenesis has great commercial potential as well as potential for use as an auxiliary model in areas of research, such as anatomy, physiology and genetics. Consequently, somatic embryogenesis is intensively studied (Rugini, 1998; Orinos and Mitrako, 1991; Rugini and Carcato, 1995) with the goal of solving its major bottlenecks.

The use of somatic embryogenesis to regenerate olive *in vitro* has been reported by various research groups. Shibli et al. (2001) obtained satisfactory results after carefully studying the influence of various factors during the formation of somatic embryos, such as the explant source, the presence of osmotic agents in the medium and the balance of growth regulators. Lopes et al. (2009) verified the genetic integrity of two species of *Olea* using molecular markers at the different steps of somatic

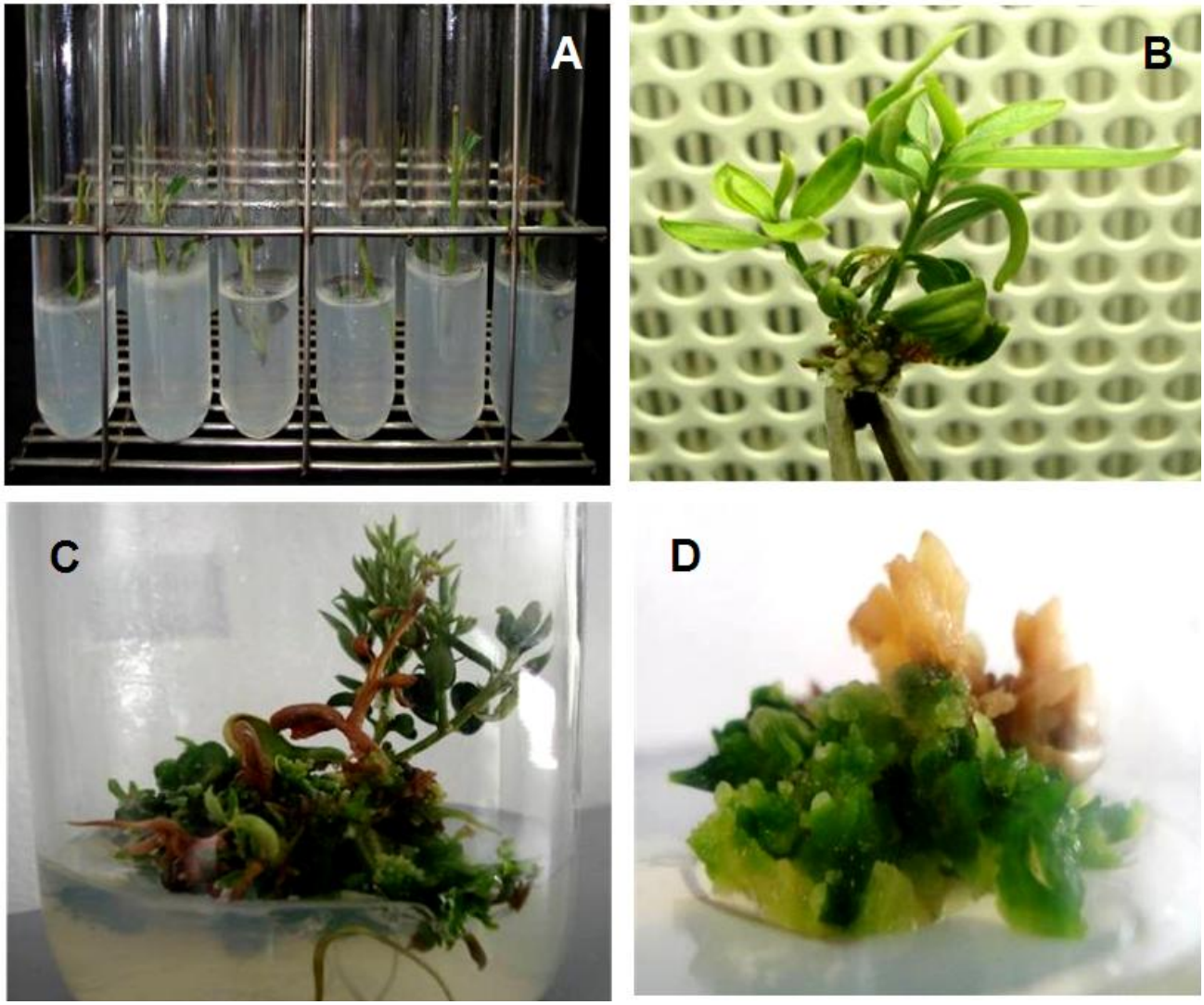

Figure 2. *In vitro* establishment of olive: **A)** nodal segment of olive cultivars cultured *in vitro*; **B)** development of new buds from nodal segments; **C)** formation of callus with regenerative competence and posterior development of plantlets *in vitro*; and **D)** detail of the beginning of the formation of embryonic structure from olive callus. Source: Plant Biotechnology Laboratory (EPAMIG), Brazil.

embryogenesis. These authors did not detect any somaclonal variation, indicating that this method is a reliable way of multiplying olive while maintaining genetic integrity. Pérez-Barranco et al. (2009) used the embryogenesis callus of olive as source material for genetic transformation. In the Plant Biotechnology Laboratory of EPAMIG, experiments are being conducted to optimize protocols for the generation of somatic embryos of several olive varieties. Molecular markers such as SSR and Inter Simple Sequence Repeat (ISSR) are being applied to monitor the occurrence of genetic variation during somatic embryogenesis.

Thus, the results indicate that olive tissues from nodal segments, zygotic embryos and cotyledons might be used as sources of explant that exhibit high levels of competency for the proliferation of buds. Alternatively, olive plantlets already established *in vitro* can be used as a source of tissue for somatic embryogenesis (Figure 2). Thus, the identification of more responsive olive genotypes for regeneration and *in vitro* cultivation are crucial because genotype-dependent responses in this species are well documented.

The application of somatic embryogenesis extends all of the previously cited advantages of micropropagation by presenting an opportunity for the generation of a large number of plantlets of good quality and at low cost, after routine optimization. Somatic embryogenesis, when compared to other methods, requires small spaces and limited production time, thus assuming the status of a routinely adopted, facile technique.

Despite the advantages of somatic embryogenesis, it presents some limitations, such as the fusion of embryos or the occurrence of abnormal morphogenesis during cell division and the formation of genetic chimeras, in addition to the occurrence of somaclonal variation discussed earlier. However, these events can be efficiently monitored and controlled by evaluating the olive plantlets with molecular tools.

GENETIC TRANSFORMATION OF OLIVE

Conventional genetic breeding was long and is the only option for the development of cultivars with superior agronomic traits. However, the traditional method of genetic recombination, in which new individuals are crossed and selected, suffers serious drawbacks as a consequence of a reduction in variability in highly improved populations, making it more difficult to identify the sources of genes involved in the response to biotic and abiotic stress in the same species. In addition, incompatible crossing between genetically distinct progenitors limits or even prevents gene transference among different species. Others drawbacks, mainly for the genetic breeding of perennial crops such as olive, are the long time and large areas required for the evaluation and selection of promising genotypes.

Thus, the development of genetic transformation methods creates new opportunities to improve plants in a more controlled way because it allows the direct introduction of selected genes obtained from any organism, including microorganisms, plants and animals, into the genome of the target plant (Perani et al., 1986). This possibility permits the plant breeders to overcome the sexual barriers between species, genera, families and even phyla, permitting the unrestricted exchange of genetic material among organisms. This technique is also very valuable to avoid the undesirable effects of genetic drag, which occurs when genes of interest are genetically linked to genes controlling undesirable traits. Genetic drag is very frequent when plant breeders use wild genotypes as a source of genetic variability for conventional hybridization. The elimination of these unwanted genes takes several years to be accomplished, delaying the recovery of the original genetic background. Consequently, recombinant DNA technology is used to improve cultivars while avoiding the need for slow backcrossing processes to maintain the original phenotype.

Conceptually, genetic transformation is the controlled and stable introgression of genes directly into the genome of a target organism (Torres et al., 2000). Therefore, genetically modified plants are plants that express one or few exogenous genes, which may or may not exist in the original genome and which are obtained by genetic engineering techniques (Gander and Marcellino, 1997; Torres et al., 2000). Transgenic plants are frequently used as models in studies of gene expression and regulation; the role of genes in metabolism, biochemical and morphological pathways; and the response to diseases and pests, among many other uses. The recent adoption of transgenic plants by farmers for crops of commercial importance has promoted an agricultural revolution. The adoption of transgenic cultivars in commercial farms increases each year, and for crops such maize, cotton and soybean, the cultivated area of transgenic varieties is already greater than that of conventional varieties (James, 2009).

The *in vitro* culture of plant tissue is considered an essential requirement for the genetic transformation of the majority of plant species, once cultivated *in vitro*, it is possible to regenerate complete plants from one unique genetically modified cell altered by the introgression of a stable external gene (Pasquali and Zanettini, 2007; Sartoretto et al., 2008). However, the unavailability of an efficient technique for genetic transformation in olive has limited the widespread adoption of this technology in this species, principally due to the recalcitrant nature of olive for *in vitro* regeneration.

The cultivar Picual is the most popular in Spain for olive oil production and is the principal target for genetic transformation in this species. Pérez-Barranco et al. (2007) recently reported advanced biotechnological approaches for genetic improvement in olive. These authors developed an efficient system for the regeneration of young plantlets using somatic embryogenesis in root segments of mature zygotic embryos. According to these authors, this is the first step toward developing an efficient protocol for the genetic transformation of the Picual cultivar.

In addition to an efficient system for DNA integration, the successful transformation of plants requires an efficient selection of positive events by permitting the regeneration of transgenic cells and inhibiting the proliferation of escapes, which are tissues that were not transformed but were able to survive even in the presence of the selective agent. According to Pérez-Barranco et al. (2009), in olive, these parameters of genetic transformation still require intensive optimization to make this species more responsive and allow the adoption of genetic transformation as a routine procedure. The high rate of generation of escapes operationally prevents any attempt at transgenic event production on a large scale, either for scientific objectives or for commercial purposes, because it considerably increases the cost associated with the genotypic and phenotypic analysis of events.

There are different methods of transformation, and the choice of method depends on the species to be transformed, the type of explants to be used (callus, zygotic embryo, leaf tissue, protoplast, etc.), the regeneration capacity of the explant and the availability of the materials (Cançado et al., 2009). Plant genetic transformation techniques are classified into two

categories: direct and indirect genetic transformation. The direct genetic transfer of DNA is based on physical and chemical methods, such as biolistics (also known as particle bombardment), electroporation and PEG (polyethyleneglycol), while indirect genetic transformation of DNA uses the bacterium *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a vehicle of transformation (Chilton et al., 1982).

Results for the genetic transformation of olive have been limited. Lambardi et al. (1999) tested different devices and bombardment conditions for the transient transformation of somatic embryos of the olive cultivar Canino, without success. Recently, Pérez-Barranco et al. (2007) obtained transient expression of the *gus* gene in plants derived from olive embryonic cells by particle bombardment. Torreblanca et al. (2010) obtained a transformation frequency of 20 to 45% with the *A. tumefaciens* system and observed stable expression of the *npt*-II (neomycin phosphotransferase) gene, which confers antibiotic resistance in leaves of genetically transformed olive. The genetic transformation of the olive cultivar Canino with the *rol* ABC genes (plant oncogenes of *A. rhizogenes*) meditated by *A. tumefaciens* (Rugini et al. 2000; Rugini and Gutierrez-Pesce, 2006) has also been reported. This gene is responsible for the modification of vegetative growth patterns and stature of the plant. D'Angeli and Altamura (2007) also transformed olive with a gene that encodes the synthesis of osmotina, a compound involved in increasing resistance to abiotic stress. Mencuccini et al. (1999) used *A. tumefaciens* to introduce the *rol* ABC genes in the cultivar Dolce Agogia, using leaf petioles as the explant for transformation.

In addition to putative restrictions due to environmental effects (gene flow) and allergenicity, incorporating genetic transformation in olive breeding provides the attractive advantage of speed; the establishment of a new cultivar using traditional methods may take decades. The risks involved with this technology can be efficiently reduced or even avoided by the careful application of safety practices, thus potentially mitigating possible harmful effects to the environment and consumers.

MOLECULAR MARKERS FOR THE GENETIC STUDY OF OLIVE

The cultivation of olive trees for thousands of years and the commercial cultivation of a few genotypes all over the world has resulted in a decrease in genetic diversity into this species. In addition, the occurrence of synonymous (different names for the same varieties) and homonymous (different varieties with the same name) varieties are frequent in this species and complicate the standardization of variety names. Therefore, the accurate identification of accessions is one of the basic requirements for the correct management and preservation of olive germplasm. The identification and characterization of olive accessions are also critical for commercial applications because the quality and quantity of olive products are influenced by the properties of the varieties. By contrast, *O. europaea* is highly variable with respect to oil content, fruit size, and resistance/tolerance to biotic and abiotic stress (Bartolini et al., 1998). In the specific case of Brazilian olive germplasm, the absence of knowledge about genetic diversity is a barrier to its full utilization in breeding programs and the integration of other methods of germplasm conservation (for example, *in vitro* cultivation).

However, molecular markers are frequently used in diverse applications for the genetic characterization of olive trees, such as genotyping, pedigree analysis, identification of quantitative trait loci (QTLs), marker-assisted selection (MAS), parental selection and heterotic grouping, genetic mapping, formation of nuclear collections, identification of synonyms and homonyms, and tracking of gene insertion in transgenic olives, in addition to other uses.

IDENTIFICATION OF OLIVE CULTIVARS USING DNA FINGERPRINTING

For successful olive cultivation, the use of genetically certified plants is essential because quality and productivity are inherent to the genetic potential of each variety. There is high genetic diversity among available commercial varieties of olive, and in general, this high diversity results in significant alterations in characteristics such as oil content, fruit size, seed size and resistance/tolerance to biotic/abiotic stresses. Consequently, the accurate identification of olive varieties is fundamental for the optimum use of this crop. The use of molecular markers in association with morphological characterization is already being employed to identify olive varieties more accurately. In addition, outcrossing fertilization among cultivars makes this species a good model for molecular marker studies (Souza et al., 2012).

The identification of olive accessions or varieties is traditionally realized through the analysis and comparison of morphological and agronomics traits, most of which are only available during the stages of flowering and fruiting. Because olive requires three to five years to begin fruiting, the use of this method does not allow the early identification of mistakes or segregating plants. In addition, the phenotype of plants can be significantly altered by environmental effects, such as nutritional and sanitary status (Hannachi et al., 2008), making it more difficult to identify specific varieties. Another drawback is the excessive number of olive varieties available, more than two thousand, which makes the morphological differences among many of them very small and difficult to distinguish. To overcome this limitation, the use of molecular markers is becoming routine for the precise identification of olive cultivars.

In addition, molecular markers can be applied in early stages of development, even in embryos and plantlets. It is not a destructive method and requires a very small amount of plant tissue. Therefore, the use of molecular markers has become a very attractive technique. Nevertheless, the most important advantage of molecular markers is the high level of genetic identification, which is directly related to the nucleotide sequence of the DNA, eliminating any chances of external interference caused by environmental variation.

Molecular markers based on the polymerase chain reaction (PCR), such as random amplification of polymorphic DNA (RAPD), ISSR and microsatellite (SSR), have been used frequently with the objective of identifying olive plants. These techniques require modest laboratory infrastructure and are relatively low-cost when compared with other techniques. However, the cost of the amplified fragment length polymorphism (AFLP) technique is average compared with the other techniques, although it does not requires the very complex infrastructure that is frequently used for genetic study in olive trees.

The development of markers based on the sequencing of double-stranded DNA, such as single-nucleotide polymorphism (SNPs), has also been shown to be efficient for the identification of varieties or accessions of olive (Reale et al., 2006; Hakim et al., 2010). The application of this class of molecular markers in olive studies has been limited, but this will change in coming years with the adoption of new high-throughput DNA sequencing technologies (Sousa et al., 2009).

Currently, microsatellite molecular markers are some of the most applied in olive variety identification studies. Microsatellite molecular markers exhibit high reproducibility, enabling information exchange, data bank assembly and result standardization among different laboratories and research groups.

Many research studies have shown that a few microsatellite markers are sufficient to distinguish more than 100 olive genotypes (Muzzalupo et al., 2008; Sarri et al., 2006). These authors suggested that it is essential to select the correct combination of microsatellite markers to optimize the detection of genetic polymorphisms among the varieties to be analyzed.

Recently, Baldoni et al. (2009) examined 37 SSR markers in 21 varieties of olive. Analyses were performed independently in four distinct laboratories with the aim of standardizing the identification methodology among the laboratories. Each SSR marker was evaluated based on its reproducibility, discriminating power and the number of alleles amplified per sampled *locus*.

The pre-selected markers were used to identify 77 varieties of olive from different geographic origins. The result of this study identified a group of 11 standard microsatellite markers that can be used to identify olive varieties and generate a universal data bank of allelic profiles for this species.

GENETIC DIVERSITY STUDIES IN OLIVE

Genetic diversity is the variation among individuals in terms of the composition of genetic information or hereditary units (genes and its alleles) contained in all living species. This diversity involves not only different species but also individual populations of the same species that share similar characteristics. Genetic diversity is fundamental for maintaining the heterogeneity of species, which increases the effectiveness of their response to environmental changes.

The results that follow pertain to the genetic characterization of 60 olive accessions maintained in a germplasm bank in Brazil. The aim of the study was the measurement of genetic diversity using microsatellite markers and the detection of putative synonymous and homonymous varieties, as well as the assembly of all accessions into heterotic groups. This last resource is very useful for plant breeders because it maximizes crossing capacity by indicating the best parental combinations to be hybridized (Val et al., 2012).

Accessions from different regions of the world and varieties developed by the Brazilian olive breeding program were evaluated (Val et al., 2012). Thirteen SSR markers previously indicated as polymorphic for olive species (Chafari et al., 2008; Carriero et al., 2002; Cipriani et al., 2002) were used in this study. The data analysis permitted the identification of four main groups (Figure 3). Each predominant color on the graph represents each of the four groups identified by genetic analysis of the 60 accessions.

The principal coordinate analysis (Figure 4) also indicates the genetic dispersion of these olive accessions into four main groups. Based on the knowledge of the dispersion of the accession, an olive breeder can select a genotype combination with greater accuracy and precision, increasing the genetic gain by prioritizing the crossing of more divergent parents, improving the use of the available genetic variability, and, consequently, the probability of obtaining superior genotypes.

Because the knowledge produced from the molecular marker data is free from environmental interference or other difficult-to-measure factors, they allow the estimation of genetic variability with far more precision than that obtained by using phenotypic traits. Thus, the relative extent of the genetic potential of each olive accession is easily determined, but this information still must be confronted and correlated with agronomic traits, even if the molecular markers are very accurate.

EVALUATION OF CROSS-POLLINATION AND SELF-INCOMPATIBILITY IN OLIVE

Plants are classified as autogamous, allogamous or mixed based on the sexual reproduction system. This information is essential for genetic breeding because

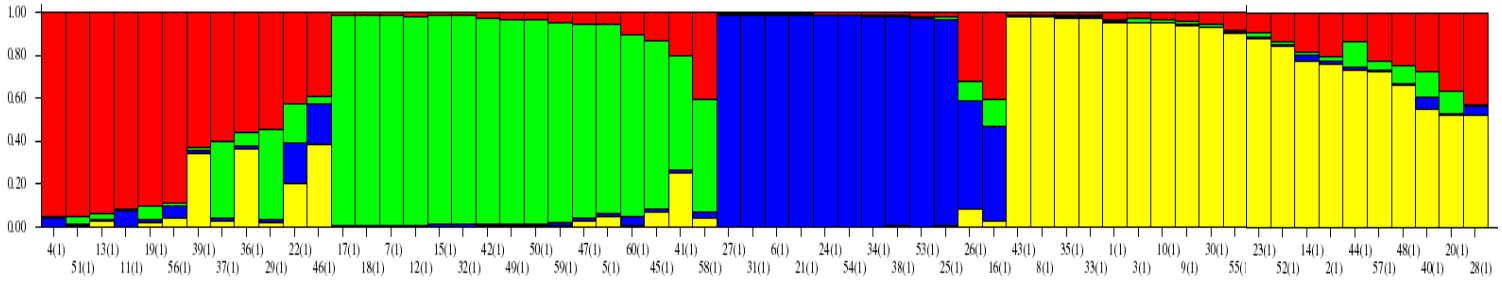

Figure 3. Bayesian analysis of 60 accessions of olive from the germplasm bank of EPAMIG, as produced by STRUCTURE version 2.3.1 indicating the existence of four distinct groups. Source: Val et al. (2012).

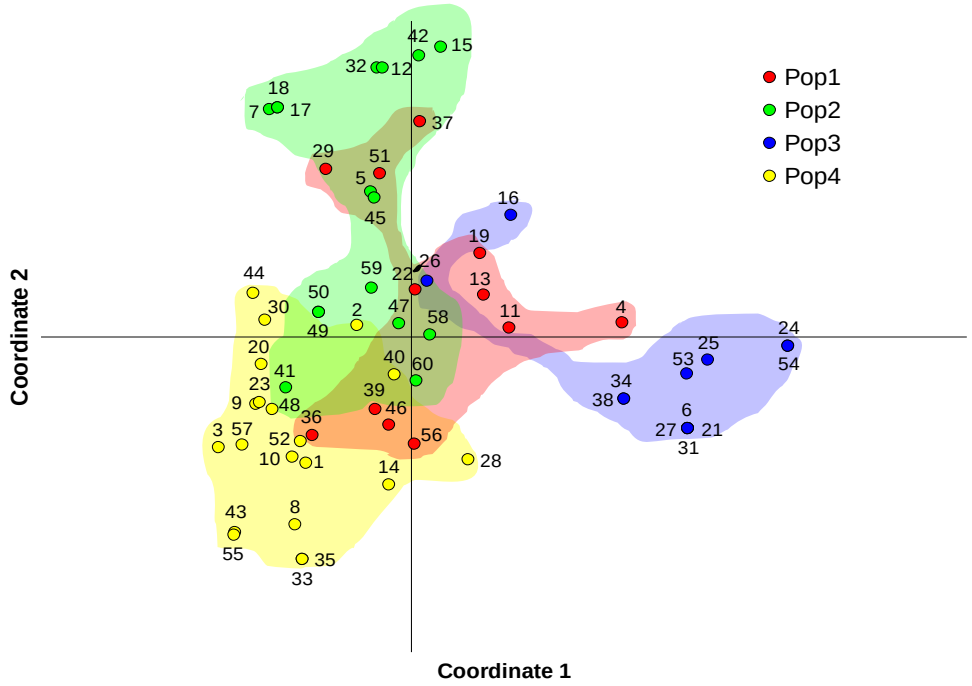

Figure 4. The assembly of 60 accessions of olive from the germplasm bank of EPAMIG, generated by Software GenAlex version 6 and indicating the existence of four overlapping populations (pop). Source: Val et al. (2012).

breeding methods are specific for each goal and differ depending on the reproduction of each species. However, the correct identification of the mode of sexual reproduction and knowledge of compatibility among cultivars are fundamental for the management of orchards because these factors directly alter the efficiency of fertilization and yield, among other outcomes.

Olive is considered allogamous, although some studies indicate that this condition varies depending on the cultivar and environment. According to Besnard (2007), olive is a diploid species (2n = 46) whose pollination occur principally by wind and whose seeds are dispersed by birds, animals and man. Cuevas and Polito (2004) classified olive as andromonoic (presenting perfect flowers, hermaphrodites, and male flowers in the same inflorescence), but according to Doveri et al. (2006), even if olive presents only hermaphrodite flowers, some cultivars are considered sterile males while others present only staminate flowers. Cuevas and Polito (2004) detected self-incompatibility in olive after observing that pollen tubes are not able to grow through the pistil and reach the ovule, preventing fertilization during self-pollination of olive genotypes. Meanwhile, the pollen tubes resulting from cross-pollination grew up through the pistil, reached the ovule and accomplished fertilization.

Studies of the compatibility of olive cultivars via pollen exchange have yielded inconsistent results, most likely due to differences in the environments in which the studies were conducted (Mookerjee et al., 2005); factors such as temperature, nutrition state, wind, and relative humidity can affect the rate of pollination.

Therefore, molecular markers can be a very useful tool to study fertilization in olive and to understand the self-incompatibility and cross-pollination of olive genotypes by tracking pollen donors and the flow of alleles in olive populations. Olive breeders who use uncontrolled cross-pollination for progeny generation frequently use molecular marker techniques to assist in the identification of putative male genitors. Mookerjee et al. (2005) used eight microsatellite markers to track paternity in olive plants, demonstrating the efficiency of this methodology to determine the frequency of pollen donors in seeds of olive trees growing in a mixed orchard. Among the 13 olive varieties evaluated, self-fertilization was rarely observed. Conversely, Souza et al. (2012) used microsatellite and ISSR markers to demonstrate a high rate of allogamy in two olive cultivars, Ascolano USA and MGS GRAP541, growing in a field among sixty olive genotypes. These same authors concluded that the number of pollen donors ranged from 4 to 5 in Ascolano USA and from 6 to 8 in MGS GRAP541.

Diaz et al. (2006) studied self-compatibility in olive by testing seeds obtained from a stock plant of the cultivars Picual and Arbequina. In their study, they used four microsatellite markers and concluded that these cultivars are self-incompatible. Rallo et al. (2000) also used microsatellite markers to confirm Mendelian segregation in a population of olive obtained by crossing the cultivars Leccino and Golce Agogia. Guerin and Sedgley (2007) used microsatellite markers to study cross-pollination and self-pollination in olive cultivars in two distinct locations. The results obtained confirmed the occurrence of self-incompatibility and cross-incompatibility in the cultivars studied.

Therefore, the application of molecular markers for the identification of olive cultivars, the evaluation of compatibility among genotypes, the tracking of paternity, and the optimization and selection of pollen donors can assist olive breeding programs. In addition to olive breeding, molecular markers have also proven to be a powerful tool for increasing field yield, given the peculiarities of the sexual reproduction of this species.

GENETIC MAPPING AND THE OLIVE GENOME

Genetic linkage maps and recombination information based on data generated by molecular markers are essential tools for genetic research and breeding of many plant species. These maps assist the analysis and selection of complex characteristics and are useful in studies of specific genes involved in the control and expression of polygenic traits (Tanksley, 1993). Given the long juvenile phase of olive, the use of marker-assisted selection (MAS) associated with QTL in breeding programs can accelerate the development and delivery of new cultivars. Because the selection can be started in young seedlings by identifying plants possessing molecular markers associated with important QTL, breeders are able to reduce the number of candidate genotypes evaluated until the adult and productive stage. Consequently, the use of molecular markers permits a reduction of the time and cost involved with the maintenance of plants in the field.

Wu et al. (2004) produced a genetic linkage map for olive varieties using molecular markers, such as microsatellite, RAPD, SSR and Sequenced Characterized Amplified Region (SCAR), and mapped 23 and 27 linkage groups in the olive varieties Kalamata and Frantoio, respectively. According to these authors, the genetic linkage group identified can be the reference point for understanding the structure, evolution and function of the olive genome. In addition, gene mapping using molecular markers can permit the association of morphological and agronomic traits, improving the selection of genitors and reducing the time necessary for the development of a new cultivar.

With the advent of high-throughput DNA sequencing and resequencing, the time and cost involved in this approach has been significantly reduced (Sousa et al., 2009). It is already possible to have detailed knowledge of the genome of an organism after a few weeks of sequencing. With the information generated by the

complete sequence of the genome, it is possible to identify the combination of genes responsible for the phenotype of an organism, as well as the factors that control their expression. A new branch of genome science studies the conservation among genomes and the order and number of genes in chromosomes of different organisms, providing researchers a new perspective on the progress of evolution and inferring with high accuracy the degree of relationship among species and the putative moment of genetic divergence. For plant breeding, the convenient applications of this knowledge are enormously important, particularly for the production of genetically modified plants. As reported above, the use of transgenic plants is already a reality for global agriculture, including Brazil, which is second in the world in cultivated area (James, 2009).

Although the complete genome of olive is still not available, Cattonaro et al. (2008) have used preliminary and incomplete data to suggest that the olive genome seems to be highly different from other species with a large genome size. According to these authors, preliminary evaluations indicate that a low percentage of transposable elements and a large number of repetitive sequences appear to be responsible for the large genome size of olive (1,400 to 1,500 Mbp). According to Bracci et al. (2011), several groups are working on the sequence of the olive genome, including the OLEAGEN genomics project. In addition, functional genomes have been described, mainly with the Expressed Sequence Tags (EST) approach, such that many sequences are now available. The central focus has primarily been genes that are differentially expressed in olive fruit and pollen allergen genes (Bracci et al. 2011).

Recently, Mariotti et al. (2010) published the sequence of the chloroplast genome of the Frantoio cultivar, which yielded some interesting results. First, the gene order and the genome organization are similar to those of other angiosperm species. Second, no inversion, gene duplication, insertions, inverted repeat expansion, or intron losses were identified in this organelle genome. Finally, some interesting polymorphisms were detected in the sequence alignments of six cultivated olives.

TRACEABILITY OF OLIVE OIL BY USING MOLECULAR TOOLS

Olive oil is the most prized product from olive trees. Extra-virgin and virgin olive oils are obtained mechanically by crushing fresh olive fruits (Brasil, 2005). The characteristics of olive oil are influenced by different environmental factors, such as plant nutrition, plant sanity, and plant age, but the intrinsic characteristics of the olive cultivar are considered the most determinant factors for olive oil quality. Varietal oil is considered superior to oils derived from unidentified plants or a mix of several cultivars. However, the traceability of the product from the field through industrial processing to its distribution in markets and customers is an expensive practice. Therefore, reliable, fast and low-cost methods can facilitate the monitoring of the authenticity and identification of the cultivars involved in the production of olive oil. Molecular markers meet these criteria, even if the sample has been subjected to some transformation and processing. Therefore, the use of this technique for the purpose of oil traceability increases the value of the product.

The use of olive cultivars with low suitability for oil production reduces the quality of the olive oil. Moreover, the practice of adulterating olive oil by adding other, lower-cost vegetable oils is a frequent practice in some areas. For mixtures, chemical analysis is sufficient to confirm the authenticity of the product. However, when the objective is to identify olive oil of different cultivars, it is necessary to investigate the presence of genomic DNA from varietal contaminants.

In this case, molecular markers can be used because they are able to detect genetically distinct samples with high accuracy. Different markers are used to track the identity of olive oils to certify the quality of the final product. Alba et al. (2009) used microsatellite markers to evaluate the quality and origin of Italian olive oils produced from 7 certified olive cultivars. They compared the DNA from leaves against the olive oil from the fruits of the same plant. Doveri et al. (2006) used microsatellite markers, SCAR and SNPs to verify the presence of DNA of the pollen from the donor plant in olive oil from the cultivar Leccino. Pasqualone et al. (2007) also evaluated the efficiency of microsatellite markers for the identification of Italian olive oil using different mixtures of samples from olive oils produced from 5 different olive cultivars. In Italy, Muzzalupo et al. (2007) used microsatellite markers to determine the monovarietal origin from fruits with and without seeds and under different conditions of DNA extraction and amplification. These authors compared the results of the DNA obtained from oil samples with the results of the DNA from leaves of the same olive cultivar.

Despite the importance and applicability of molecular marker techniques in the olive processing industry, some studies have reported bottlenecks that could reduce their efficiency. These bottlenecks include the high-level degradation of genomic DNA during the process of oil production, which reduces the efficiency of DNA amplification and identification. Consequently, the availability of one efficient method for DNA extraction from olive oil is crucial for the incorporation of molecular marker techniques for routine quality analysis. International standardization of sampling and analysis methods, as well as the creation of a network of reference laboratories, are also required to validate results. Furthermore, results published by Doveri et al. (2006) confirmed that, in addition to maternal DNA, olive oil also contains alleles donated by several pollen

donors. In this context, olive oil DNA profiles should be interpreted carefully.

CONCLUSIONS

The aim of this review was to present the state of the art of the applications of biotechnology and molecular biology to the olive tree, as the consumption of olive products is growing due to their health benefits and excellent gastronomic and organoleptic qualities. For that reason, the commercial cultivation of olive is being encouraged by agroindustry and organized sectors of agriculture, such as cooperatives and farmer associations, stimulating the introduction and fast expansion of olive orchards in several areas of the world. In Brazil, practically all olive oil and other olive products that are domestically consumed are still being imported from other countries, but this scenario has changed rapidly in recent years.

To stimulate an increase in the sustainability and economic efficiency of olive production in countries in which this crop is not traditionally cultivated, such as Brazil, it is necessary to develop local varieties that are better adapted to specific environments. In addition, economic and environmental efficiency will also be improved by delivering technologies that are suited to each environment and present innovative solutions not only for olive breeding but also for soil and irrigation management, pruning and harvesting methods, plantlet production, and post-harvest and fruit processing, among other issues associated with the cultivation of this crop.

Therefore, modern biotechnologies, such as *in vitro* micropropagation, cloning via somatic embryogenesis, molecular marker-assisted selection, functional genomics and genetic transformation, are good examples of how adopting correct technological approaches can benefit the commercial, environmental and social aspects of the growth of plants such as olive trees.

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REFERENCES

Alba V, Sabetta W, Blanco A, Pasqualone A, Montemurro C (2009). Microsatellite markers to identify specific alleles in DNA extracted from monovarietal virgin olive oils. Eur. Food Res. Technol. 229(3):375-382.

Baldoni L, Cultrera NG, Mariotti R, Ricciolini C, Arcioni S, Vendarmin GG, Buonamici A, Porceddu A, Sarri V, Ojeda AM, Trujillo I, Rallo L, Belaj A, Perri E, Salimonti A, Muzzalupo I, Casagrande A, Testolin R, Lain O, Messina R, Testolin R (2009). A consensus list of microsatellite markers for olive genotyping. Mol. Breed. 24(3):213-231.

Bartolini G, Prevost G, Messeri C, Carignani C (1998). Olive Germplasm: Cultivars and World-wide Collections. FAO, Rome, Italy.

Besnard G, Henry P, Wille L, Cooke D, Chapuis E (2007). The origin of the invasive olives (*Olea europaea* L., Oleaceae). Heredity 99(6):608-619.

Bracci T, Busconi M, Fogher C, Sebastiani L (2011). Molecular studies in olive (*Olea europaea* L.): overview on DNA markers applications and recent advances in genome analysis. Plant Cell Rep. 30(4):449-462.

Brasil (2005). Resolução RDC Nº 270, de 22 de Setembro de 2005. Anvisa, Brasília, DF, Brazil. Available at: http://www.puntofocal.gov.ar/notific_otros_miembros/bra171a1_t.pdf.

Cançado GMA, Brasileiro ACM, Ribeiro AAP, Fernandes MCN, Sant'ana GC, Soares BDF, Rocha HS, Freitas GF (2009). Transgenic plants. Inf. Agropec. 30(253):14-23.

Carriero F, Fontanazza G, Cellini F, Giorio G (2002). Identification of simple sequence repeats (SSRs) in olive (*Olea europaea* L.). Theor. Appl. Genet. 104(2-3):301-307.

Cattonaro F, Felice N, Stefan A, Vendramin G, Baldoni L, Porceddu E, Porceddu A, Pe ME, Morgante M (2008). Toward olive genome sequencing: First insights into the genome organization. In: Proceedings of the 52nd Italian Society of Agricultural Genetics Annual Congress, Padova, Italy. pp. D. 78.

Chafari J, Meziane AE, Moukhli A, Boulouha B, Modafar CE, Khadari B (2008). Menara gardens: a Moroccan olive germplasm collection identified by a SSR locus-based genetic study. Genet. Resour. Crop Evol. 55(6):893-900.

Chilton MD, Tepfer DA, Petit A, David C, Casse-Delbart F, Tempé J (1982). *Agrobacterium rhizogenes* inserts T-DNA into the genomes of host plant root cells. Nature 295:432-434.

Cipriani G, Marrazzo MT, Marconi R, Cimato A, Testolin R (2002). Microsatellite markers isolated in olive are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars (*Olea europaea* L.). Theor. Appl. Genet. 104(2-3):223-228.

Cuevas J, Polito VS (2004). The role of staminate flowers in the breeding system of *Olea europaea* (Oleaceae): an andromonoecious, wind-pollinated taxon. Ann. Bot. 93(5):547-553.

D'Angeli S, Altamura MM (2007). Osmotin induces cold protection in olive trees by affecting programmed cell death and cytoskeleton organization. Planta 225(5):1147-1163.

Diaz A, Martin A, Rallo P, Barranco D, De la Rosa R (2006). Self-incompatibility of 'Arbequina' and 'Picual' olive assessed by SSR markers. J. Am. Soc. Hortic. Sci. 131(2):250-255.

Doveri S, O'Sullivan DM, Lee D (2006). Non-concordance between genetic profiles of olive oil and fruit: a cautionary note to the use of DNA markers for provenance testing. J. Agric. Food Chem. 54(24):9221-9226.

Gander ES, Marcellino LH (1997). Transgenic plants. Biotecnologia Cienc. Desenvolv. 1:34-37.

George EF (2008). Plant propagation by tissue culture. 3. ed. Exegetics, Edington, United Kingdom.

Guerin J, Sedgley M (2007). Cross-pollination in olive cultivars. Rural Industries Research and Development Corporation, Kingston, Australia.

Hakim IR, Kammoun NG, Makhloufi E, Rebai A (2010). Discovery and potential of SNP markers in characterization of Tunisian olive germplasm. Diversity 2(1):17-27.

Hannachi H, Breton C, Msallem M, Ben el Hadj S, El Gazzah M, Berville A (2008). Differences between native and introduced olive cultivars as revealed by morphology of drupes, oil composition and SSR polymorphisms: a case study in Tunisia. Sci. Hortic. 116(3):280-290.

James C (2009). Global Status of Commercialized Biotech/GM Crops: 2009. *ISAAA Brief* N°. 41. ISAAA, Ithaca, NY, USA.

Lambardi M, Benelli C, Amorosi S, Branca C, Caricato G, Rugini E (1999). Microprojectile-DNA delivery in somatic embryos of olive (*Olea europaea* L.). Acta Hortic. 474:505-509.

Lopes T, Capelo A, Brito G, Loureiro J, Santos C (2009). Genetic variability analyses of the somatic embryogenesis induction process in *Olea* spp. using nuclear microsatellites. Trees 23(1):29-36.

Mariotti R, Cultrera NGM, Díez CM, Baldoni L, Rubini A (2010). Identification of new polymorphic regions and differentiation of

cultivated olives (*Olea europaea* L.) through plastome sequence comparison. BMC Plant Biol. 10:211.
Mencuccini M, Micheli M, Angiolillo A, Baldoni L (1999). Genetic transformation of olive (*Olea europaea* L.) using *Agrobacterium tumefaciens*. Acta Hortic. 474:515-520.
Mookerjee S, Guerin J, Collins G, Ford C, Sedgley M (2005). Paternity analysis using microsatellite markers to identify pollen donors in an olive grove. Theor. Appl. Genet. 111(6):1174-1182.
Muzzalupo I, Lombardo N, Salimonti A, Perri E (2008). Molecular characterization of Italian olive cultivars by microsatellite markers. Adv. Hortic. Sci. 22(2):142-148.
Muzzalupo I, Pellgrino M, Perri E (2007). Detection of DNA in virgin olive oils extracted from destoned fruits. Eur. Food Res. Technol. 224(4):469-475.
Orinos T, Mitrakos K (1991). Rhizogenesis and somatic embryogenesis in calli from wild olive (*Olea europaea* var sylvestris (Miller) Lehr) mature zygotic embryos. Plant Cell Tissue Organ Cult. 17:183-187.
Pasquali G, Zanettini MHB (2007). Transgênese florestal. In: Borém, A. (ed). Biotecnologia florestal. Editora UFV, Viçosa, Brazil. pp. 317-334.
Pasqualone A, Montemurro C, Summo C, Sabetta W, Caponio F, Blanco A (2007). Effectiveness of microsatellite DNA markers in checking the identity of protected designation of origin extra virgin olive oil. J. Agric. Food Chem. 55(10):3857-3862.
Peixe A, Raposo A, Lourenço R, Cardoso H, Macedo E (2007). Coconut water and BAP successfully replaced zeatin in olive (*Olea europaea* L.) micropropagation. Sci. Hortic. 113(1):1-7.
Perani L, Radke S, Wilke –Douglas M, Bossert M (1986). Gene transfer methods for crop improvement: introduction of foreign DNA into plants. Physiol. Plant. 68(3):566-570.
Pérez-Barranco G, Mercado JA, Pliego-Alfaro F, Sánchez-Romero C (2007). Genetic transformation of olive somatic embryos through biolistic. Acta Hortic. 738:473-477.
Pérez-Barranco G, Torreblanca R, Padilla IMG, Sánchez-Romero C, Pliego-Alfaro F, Mercado JA (2009). Studies on genetic transformation of olive (*Olea europaea* L.) somatic embryos: I. Evaluation of different aminoglycoside antibiotics for nptII selection; II. Transient transformation via particle bombardment. Plant Cell Tissue Organ Cult. 97(3):243-251.
Rallo P, Dorado G, Martin A (2000). Application of microsatellite markers in olive breeding. Acta Hortic. 586:60-62.
Reale S, Doveri S, Díaz A, Angiolillo A, Lucentini L, Pilla F, Martín A, Donini P, Lee D (2006). SNP-based markers for discriminating olive (*Olea europaea* L.) cultivars. Genome 49(9):1193-1205.
Rocha MAC, Costa MAPC, Silva SA, Ledo CAS, Moreira MJS, Bastos LP (2008). *In vitro* rooting and acclimatation of genotypes of jenipapeiro (*Genipa americana* L.). Rev. Bras. Frutic. 30(3):769-774.
Rugini E (1988). Somatic embryogenesis and plant regeneration in olive (*Olea europaea* L.). Plant Cell Tissue Organ Cult. 14(3):207-214.
Rugini E, Caricato G (1995). Somatic embryogenesis and plant recovery from mature tissues of olive cultivars (*Olea europaea* L.) 'Canino' and 'Moraiolo'. Plant Cell Rep. 14(4):257-260.
Rugini E, Rita B, Rosario M (2000). Olive (*Olea europaea* var. sativa) transformation. In: Jain SM, Minocha SC (eds). Molecular Biology of Woody Plants. vol 2. Kluwer Academic Publishers, Dordrecht, Netherlands. pp. 245-279.
Rugini E, Gutiérrez-Pesce P (2006) Genetic improvement of olive. Pomologia Croatica 12:43-74.
Sarri V, Baldoni L, Porceddu A, Cultrera NGM, Contento A, Frediani M, Belaj A, Trujillo I, Cionini PG (2006). Microsatellite markers are powerful tools for discriminating among olive cultivars and assigning them to geographically defined populations. Genome 49(12):1606-1615.
Sartoretto ML, Saldanha CW, Corder MPM (2008). Genetic transformation: strategies for forest species breeding. Cienc. Rural 38(3):861-871.
Shibli RA, Shatnawi M, Abu E, Al-Juboory KH (2001). Somatic embryogenesis and plant recovery from callus of 'Nabali' olive (*Olea europaea* L.). Sci. Hortic. 88(3):243-256.
Sousa SM, Carneiro AA, Carneiro NP (2009). High throughput unveiling of genome sequences. Inf. Agropec. 30(253):76-84.
Souza RAV, Braga FT, Vieira-Neto J, Mendonça EAF, Azevedo PH, Cançado GMA (2011). Viability and germination of olive embryos subjected to different conditions of fruit storage. Pesq. Agropec. Bras. 46(3):309-314.
Souza RAV, Ferreira JL, Braga FT, Azevedo PH, Sant'Ana GC, Ribeiro AP, Borém L, Cançado GMA (2012). Outcrossing rate in olive assessed by microsatellite and inter simple sequence repeat (ISSR) markers. Afr. J. Biotechnol. 11(53):11580-11584.
Tanksley SD (1993). Mapping polygenes. Annu. Rev. Genet. 27:205-233.
Torreblanca R, Cerezo S, Palomo-Ríos E, Mercado JA, Pliego-Alfaro F (2010). Development of a high throughput system for genetic transformation of olive (*Olea europaea* L.) plants. Plant Cell Tissue Organ Cult. 103(1):61-69.
Torres AC, Ferreira AT, Sá FG, Buso JA, Caldas LS, Nascimento AS, Brígido MM, Romano E (2000). Glossário de biotecnologia vegetal. Editora Embrapa-CNPH, Brasília, DF, Brazil.
Val ADB, Ferreira JL, Vieira-Neto J, Pasqual M, Oliveira AF, Borém A, Cançado GMA (2012). Genetic diversity of Brazilian and introduced olive germplasms based on microsatellite markers. Genet. Mol. Res. 11(1):556-571.
Williams ES, Maheswaran B (1986). Somatic embryogenesis: factors influencing coordinated behavior of cells as an embryogenic group. Ann. Bot. 57(4):443-462.
Wu S, Collins G, Sedgley MA (2004). Molecular linkage map of olive (*Olea europaea* L.) based on RAPD, microsatellites and SCAR markers. Genome 47(1):26-35.