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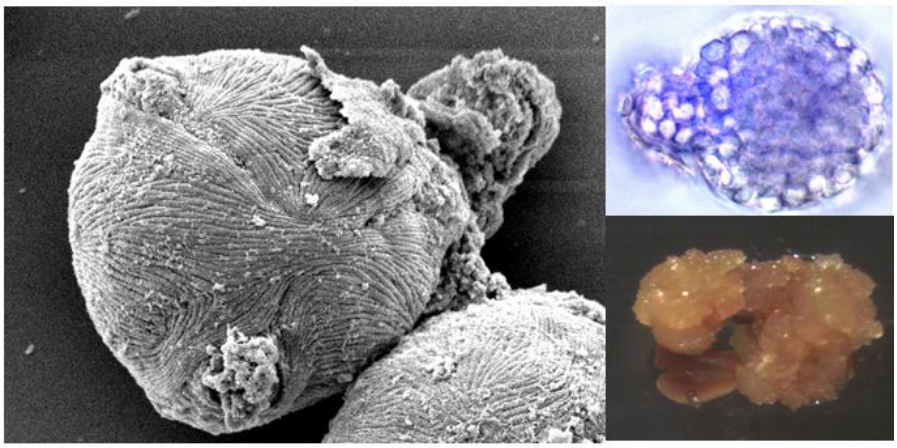
# Gametic embryogenesis in Mediterranean fruit crops

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# Contents

# Acronyms

| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| AMP | Aminoprophosmethyl |
| ANOVA | Analysis of variance |
| BA | Benzylaminopurine |
| DAPI | 4,6- Diamidino-2-phenylindole |
| DH | Doubled haploid |
| DNA | Deoxyribose nucleic acid |
| EMS | Ethyl methanesulfonate |
| ENU | Ethyl nitrosourea |
| FDA | Fluoresceine diacetate |
| GA3 | Gibberellic acid |
| IBA | Indole butyric acid |
| Kin. | Kinetin |
| LU | Late Uninucleate |
| MS | Murashige & Skoog's medium (1962) |
| NAA | Naphthalene acetic acid |
| PAs | polyamines |
| rpm | Rotations per minute |
| TDZ | thidiazuron (N-phenyl-1,2,3,-thi-diazol-5-ylurea) |
| UV | Ultra violet |
| Zea | Zeatin |

# Abstract

Haploids and doubled-haploid plants are of great interest for breeding and genomic studies, for investigation of the basic aspects of plant embryogenesis and can be also useful in conjugation with other biotechnological methods such as induced mutations, disease resistance, gene transfer and mutation breeding.

Haploids develop spontaneously in small number and are produced mainly through gametic embryogenesis. The gametic embryogenesis is one of the most important example of cellular totipotency, that means the ability of plant cells to regenerate a whole plant from a single cell. It can be obtained from both a female gamete ("gynogenesis"), and from a male gamete with the commonly so-called "pollen embryogenesis".

The present work was undertake for the induction and study of the gametic embryogenesis in some Mediterranean typical plants trough isolated microspore and anther culture.

*Eriobotrya japonica*, *Olea europaea and Prunus armeniaca* were the species selected for the pollen embryogenesis study.

Moreover, a genetic diversity evaluation of a Sicilia loquat germplasm collection was carried out in order to a biodiversity conservation and study the possible origin on different cultivars largely cultivated in Spain and in Italy.

# First part

# 1 Introduction

Fruit tree breeding goals are focused on increasing the external and internal quality of the product, the resistance or tolerance of the scions and of the rootstocks to biotic and abiotic stresses, always keeping in mind the necessity of a more sustainable agriculture. To achieve such improvements, conventional (hybridization, selection, mutation) or biotechnological methods are available. Embryo culture, regeneration from protoplasts, somatic hybridization, *in vitro* mutant selection, genetic transformation and haploid production can greatly help fruit crop breeding. Among the biotechnological methods, haploid and doubled haploid production, through gametic embryogenesis, allows the single-step development of complete homozygous line from the heterozygous parents, increasing the efficiency of perennial crop breeding programmes. In fact, there is no way to produce homozygous breeding lines in fruit trees through conventional methods that involve several generations of selfing.

Haploids (with a gametophytic set of chromosomes in the sporophyte) and doubled-haploid plants (DH, haploids which has undergone a doubling of chromosomes) are of great interest for breeding and genomic studies. In addition, haploid technology can be also useful to investigate the basic aspects of plant embryogenesis.

Haploids develop spontaneously in small number and are produced mainly through gametic embryogenesis. The gametic embryogenesis is one of the most important example of cellular totipotency, that means the ability of plant cells to regenerate a whole plant from a single cell (Reynolds, 1997). It can be obtained from both a female gamete ("gynogenesis"), and from a male gamete with the commonly so-called "pollen embryogenesis". There are several factors affecting embryogenesis of microspores, including the genotype, the physiological state of the donor plant, the stage of development of gametes, the pre-treatment (heat shock, starvation, use of chemical inducers, etc.) and the culture conditions (temperature, induction medium, etc.).

The study of introduction, development and establishment of haploid technology for the improvement of some selected fruits crops forms the main part of this work. In fact, this thesis has been carried out to study and to induce gametic embryogenesis by *in vitro* culture of anthers and isolated microspores in some fruit trees present in the Mediterranean area. In addition, a research has been carried out on the application of molecular markers (SSR and AFLP) for the genetic characterisation of a Sicilian loquat germplasm collection.

# 2 Status of art of gametic embryogenesis

Haploid technology includes the regeneration of haploid embryos from male or female gametes and the production of haploid (H) and doubled haploid (DH) plants from them. This technique is the most rapid route to achieve homozygosity as well as to produce pure lines. The first spontaneous haploid were observed in *Datura stramonium* L. by Blakeslee et al. (1922) and forty-two years later, Guha and Maheshwari (1964) reported a new finding. They observed numerous embryos from *in vitro* culture of *Datura innoxia* Mill anthers. Furthermore, they confirmed that the embryos and regenerated plants had originated from immature pollen grains with a haploid number of chromosomes (Guha and Maheshwari, 1966). This discovery demonstrated that the male gametophytic cell is totipotent, for what the immature pollen grains can be stimulated toward a sporophytic divisions, which afterwards lead to the production of embryos and complete plants. This process is named "pollen embryogenesis". DH plants are produced through spontaneous doubling of haploids, or can be obtained by the induction of chromosome doubling using colchicine treatment at various phases of the haploid production, such as microspores, embryos, plantlets or even plants (Jansen, 1974).

In a relatively short period, these discovery had a tremendous impact in stimulating further development of *in vitro* procedures for the obtaining of haploid and doubled haploid plants in various important agriculture crops, such as tobacco (Nakata and Tanaka, 1968; Nitsch and Nitsch, 1969), barley (Kasha and Kao, 1970; Clapham, 1973), brassica (Kameya and Hinata, 1970; Thomas and Wenzel, 1975), tomato (Sharp et al., 1972; Segui-Simarro and Nuez, 2007), and pepper (Wang et al., 1973; George and Narayanaswamy, 1973).

Furthermore, pollen embryogenesis became the most common method of choice to achieve haploidy in many crops when compared to the other available *in vitro* methods, i.e. gynogenesis and chromosome elimination (Bajaj, 1990; Ferrie et al., 1994, 1995; Palmer and Keller, 1999).

With the development of *in vitro* techniques, it soon became evident that H and DH plants can be produced by different methods other than the anther culture. For instance, pollen embryogenesis can also be induced via direct culture of isolated microspores (Reinert and Bajaj, 1977; Lichter, 1982) or by passively shed-microspores from anther culture in liquid medium (Ziauddin et al., 1990). In some species, for example in *Brassica napus*, direct culture of isolated microspores has proven to be more efficient

than anther culture for embryo production (Siebel and Pauls, 1989). The advantages of microspore culture over anther culture include a high number and more homogeneous population of microspores as starting material, the absence of growth-inhibiting substances leaking out from the degrading of anther tissue and the absence of competition for growth, such as from the connective tissue of anther (Nitsch, 1977).

Moreover, other advantages are that the isolated microspore culture can be exposed directly to treatments without interfering of the maternal tissue, and that the culture conditions can be more rigorously controlled. In some species, for instance in *Brassica oleracea*, some genotypes were responsive to microspore culture in spite of their failure to respond to anther culture (Takahata and Keller, 1991; Duijs et al., 1992). Microspore culture was effective in reducing the problem of albino formation in cereal anther culture (Heberle-Bors et al., 1996), and resulted in a high frequency of spontaneous chromosome doubling (Kasha and Maluszynski, 2003).

## 2.1 The importance of haploid and doubled haploids

Doubled haploids are plants produced by spontaneous or artificial doubling of the chromosomes of haploid plants. Such plants are valuable because the chromosomes that are created by induced/spontaneous doubling are exact copies of the chromosomes that were present in the haploid plant. Doubled haploids offer a major advantage by attainment of homozygosity in a single step, thus significantly increasing the breeding cycle along with speedy, efficiency and

### 2.1.1 Achievement of homozygosity

According to conventional breeding approaches, homozygosity may be achieved by repeated selfing and rigorous selection for several generations (Allard, 1960). Normally this exercise requires a lot of years for varietal development programs, and is most efficient in self-pollinated crops that do not show inbreeding depression. Doubled haploids greatly reduce the time required for obtaining homozygous plants if an efficient haploid reproduction protocol is available. Moreover, attaining homozygosity for recessive and quantitatively controlled traits is an even greater Mammoth task because of the involvement of many loci and masking of recessive alleles in heterozygous state (Baily and Comstock, 1976). Doubled haploids may be obtained in a single step thus fixing the genotypic combinations in a single generation (Rajhathy, 1976; Ferrie et al., 1995).

DH technologies also enhance the effectiveness of selection of desired recombinants, especially when quantitative traits are evaluated (Forster and Thomas, 2005). More than 280 new superior varieties produced with the use of various DH methods in many annual crops (http://www.scri.sari.ac.uk/assoc/COST851/, see also Forster and Thomas, 2005).

### 2.1.2 Haploidy and mutation

Doubled haploids offer the unique advantage of utilization of the haploid phase for selection. This fact assumes a greater importance for selection in case of induced mutations which are generally recessive in nature or controlled by recessive alleles (Maluszynski et al., 1995; Forster and Thomas, 2005).

Haploid cells are also ideal for mutation induction and selection, because they facilitate screening for both recessive and dominant mutants, and avoid chimeras, thus shortening the breeding time. The most interesting aspect of the mutagenic treatments applied to haploid cells is the immediate expression of recessive mutations and the possibility to obtain, by doubling chromosomes, complete homozygous diploids. The development of improved DH protocols now makes this a viable option for many species (Maluszynsky et al., 2003).

Mutations are immediately expressed in haploid and doubled haploid plants, hence these are very lucrative targets for mutation research. For example, herbicide resistance has been introduced using doubled haploid technique in conjugation with chemical mutagenesis (Swanson et al., 1988).

### 2.1.3 Haploidy and *in vitro* selection

Microspore cultures are one of the most excellent targets for *in vitro* selection for disease resistance, provided that the disease defence system is active at such an early stage of plant development (Downey and Rimmer, 1993). Gametoclonal variation, i.e. the morphological, biochemical and genetic variation observed among plants regenerated from cultured gametic cells (Evans et al. 1984; Morrison and Evans 1987), is another reason to use haploids and DHs in crop improvement. It results from both meiotic and mitotic division other than from the *in vitro* tissue culture “somaclonal variation”, (Larkin and Scowcroft 1981), and, because of their homozygousity, the gametoclones show the direct expression of both dominant and recessive mutations.

### 2.1.4 Haploidy and genetic transformation

Isolated microspores in culture form a good target for gene transfer systems such as PEG, electroporation, microinjection and biolistic methods. Also, microspore derived embyros have been used as recipient cell system for *Agrobacterium*-mediated gene transfer (Swanson and Erickson, 1989; Fukuoka, et al., 1998) .

### 2.1.5 Haploidy and genomic analysis

DHs are also very useful for genome mapping, providing reliable information on the location of major genes and QTLs for economically important traits (Khush and Virmani 1996).DNA based procedures such as RFLP/AFLP analysis are being increasingly employed in plant breeding, due to their enormous range of application. DNA markers provide unprecedented refinement in genetic analysis through the construction of nearly saturated genetic maps. This provides the breeder with a highly efficient marker aided selection tool.

In genetics, DHs may serve to recover recessives. Using DHs, linkage data can be obtained directly by sampling gametes as monoploids. DHs are ideal for the study of mutation frequencies and spectra. As DHs represent homozygous, immortal and true breeding lines, they can be repeatedly phenotyped and genotyped so phenotypic and genotypic information can be accumulated over years and across laboratories. In genomics, DHs are therefore ideal for studying complex traits that are quantitatively inherited which may require replicated trials over many years and locations for accurate phenotyping. DH populations are desirable genetic materials for genetic mapping including the construction of genetic linkage maps and gene tagging using genetic markers. They can be produced relatively rapidly, requiring 1–1.5 years to become established after the initial cross and they provide an ongoing population that can be used indefinitely for mapping. QTL analysis is facilitated by using DH mapping populations and the homozygosity of DHs enables accurate phenotyping by replicate trials at multiple sites (Forster and Thomas, 2005). In addition, in DH populations, dominant markers are as efficient as co-dominant markers because linkage statistics are estimated with equal efficiency. DHs can be also used to increase the expression level of a transgene.

## 2.2 Gametic embryogenesis in woody plants

A sporophytic (2n) and a gametophytic (n) generation constitute the living cycle of higher plants, where the gametes are products of meiosis and the sporophytic generation, usually diploid, develops by fertilization producing the zygote. The gametophyte size and lifespan have been progressively reduced during evolution, compared with those of the sporophytic generation. Heslop-Harrison (1979) termed this step of angiosperm life the "forgotten generation", because the influence of the gametophytic phase on the sporophyte is usually underestimated and gametes are only considered as vectors for the genome transmission to the next sporophytic generation.

Haploid and DHs are important in the fields of genetic and developmental studies, as well as for plant breeding. In fact, they have a potential use in mutation research, selection, genetic analysis, transformation and in the production of homozygous cultivars also required to utilize as parental lines for F1 hybrids (in exploiting heterosis).

The release of new fruit trees cultivars through DH technology is much more difficult and more time requiring, as several years are required for the *in vitro* regenerated plants to go through the juvenile period and begin flowering. Many haploids and DHs are under observations in many fruit trees such as citrus, apple, papaya and peach, but it takes considerable time to characterize them (Pooler and Scorza 1995; Germanà et al. 2000a, 2005; Höfer et al. 2002; Yahata et al. 2005; Vanwynsberhe et al. 2005; Rimberia et al. 2005).

Over the past 30 years, research in gametic embryogenesis contributed to increase the number of species in which haploid (Ferrie et al., 1995; Sopory and Munshi, 1996; Raghawan, 1997; Maluszynski et al., 2003) and doubled-haploid plants were obtained (Konzak et al., 1999, Zheng et al., 2001, Liu et al., 2002).

In woody plants, characterized by a long reproductive cycle, a long juvenile phase, a high degree of heterozygosity and, often, also by self-incompatibility, it is extremely difficult to obtain haploid using conventional methods. Instead, through gametic embryogenesis, haploids are obtained in a single step.

Some example of gametic embryogenesis induced form anthers or isolated microspores are: *Cassia fistula*, *Jacaranda acutifolia* and *Poinciana regia* (Bajaj and Dhanju, 1983), *Cocos nucifera* (Thanh-Tuyen and De Guzmán, 1983), *Quercus petraea* and *Fagus silvatica* (Jörgensen, 1988), *Aesculus carnea* (Radojevic et al., 1989), *Hevea brasiliensis* (Chen, 1990), *Populus* spp. (Baldursson et al., 1993), *Coffea arabica*

(Ascanio and Asdrúbal, 1994; Carneiro, 1993), *Quercus suber* (Bueno et al., 1997), *Clausena excavata* (Froelicher and Ollitrault, 2000), *Phoenix dactylifera* (Chaibi et al., 2002), *Citrus clementina* Hort. ex Tan. (Germanà et al., 1994; Germanà and Chiancone 2003), *Citrus limon* Burm (Germanà et al., 1991), Citrus madurensis Lour (Chen et al., 1980), Malus domestica (Fei and Xue 1981; Xue and Niu 1984; Zarsky et al. 1986; Zhang et al. 1987; Höfer and Hanke 1990; Verdoodt et al. 1998; Höfer 1995, 2003), Musa balbisiana (Assani et al.,2003), Pyrus pyrifolia Nakai (Kadota and Niimi, 2004), Poncirus trifoliata (L.). (Hidaka et al., 1979), C. deliciosa x C. paradisi (Germanà and Reforgiato, 1997), Prunus armeniaca (Peixe et al., 2004; Germanà et al., 2010), *Prunus persica* (L.) Batsch (Michellon et al., 1974; Seirlis et al., 1979; Hammerschlag, 1983), [*Musa balbisiana* (BB)] (Assani et al., 2003; Kerbellec, 1996), *Carica papaya* L. (Litz and Conover, 1978; Tsay and Su, 1985; Rimbeira et al., 2005) *Annona squamosa* L. (Nair et al., 1983), *Olea europaea* (Bueno et al., 2004, 2005, 2006), *Levisticum officinale* (Ferrier and Caswell, 2010), *Pimpinella anisum* (Ferrie et al. 2005), *Gingko biloba* (Laurain et al. 1993), *Saponaria vaccaria* (Kernan and Ferrie 2006), *Feijoa sellowiana Berg.* (Canhoto and Cruz 1993) and *Eriobotrya japonica* (Germanà et al., 2006; Li et al., 2008; Padoan et al., 2010).

Parthenogenesis and gynogenesis have been applied in: *Populus* spp. (Von Kopecky, 1960; Winton and Einspahr, 1968; Stettler and Bawa, 1971; Illies, 1974a,b), *Citrus clementina* Hort ex Tan. (Germanà and Chiancone, 2001), *Citrus reticulata* Blanco (Ollitrault et al., 1996; Froelicher et al., 2007; Aleza et al., 2009), *Malus domestica* (Zhang and Lespinasse, 1991; Höfer and Lespinasse, 1996), *Prunus avium* L. (Seirlis et al., 1979; Höfer and Hanke, 1990), *Morus alba* L. (Dennis Thomas et al., 1999), *Opuntia ficus-indica* (Gonzalez-Melendi et al. 2005b) *Pyrus communis* (Bouvier et al., 1993; Kadota et al., 2002), *Actinidia deliciosa* (Pandey et al., 1990; Fraser et al., 1991) and *Pseudotsuga menziesii* (Livingston, 1972).

## 2.3 Methods to obtain haploids

The methods for obtaining haploid have been well established in a range of economically important crop species and can be divided into four main groups (Jensen, 1986; Germanà, 2006):

### 2.3.1 Development of spontaneous haploids

Spontaneous haploid recovery has been reported in over 100 angiosperm species (Kasha, 1983). Particularly in fruit trees, spontaneous and low viable haploid plants have been recovered in apple, pear, peach, plum, apricot, etc., but with a frequency too low for practical application in fruit breeding (Zhang et al., 1990).

The origin of spontaneously occurring haploids can be trough parthenogenesis (i.e., the production of an embryo from an egg cell without the participation of the male gamete) or apogamy (the production of an embryo from a gametophytic cell other than the ovum).

### 2.3.2 Wide crossing

This method is based on the crossing between two genetically distant species (Jensen, 1977; Mujeeb-Kazi and Riera-Lizarazu, 1997). In some interspecific or intergeneric crosses, fertilization is followed by paternal chromosomes elimination from hybrid embryo. This technique is very useful in cereals and in these crosses where the endosperm is either not formed or poorly developed; therefore, such embryos do not mature in the caryopsis and embryo rescue and *in vitro* culture are necessary (Laurie et al. 1990; Mujeeb-Kazi and Riera-Lizarazu 1997). Intergeneric crosses are applied also in potato where, however, the mechanism does not involve chromatin elimination but parthogenetic induction of the egg cell.

### 2.3.3 Gynogenesis

Gynogenesis is usually selected as the alternative method in species where pollen embryogenesis fails (Sestilli and Ficcadeni, 1996). The basic protocol was developed in the late 1980s (Bossoutrot and Hosemans, 1985; Goska 1985; Doctrinal et al., 1989) and it was induced in certain genotypes of wheat (Zhu et al., 1981; Matzk, 1981; Comeau et al. 1992; Matzk et al.1995), barley (Gaj and Gaj 1996; Gaj, 1998) and rice (Zhou and Yang, 1981).

Gynogenesis induced from isolated ovules appeared to be successful, in recalcitrant plants to the anther culture, for sugar beet (Bossoutrot and Hosemans, 1985; Goska, 1985; Galatowitsch and Smith, 1990; Lux et al., 1990; Ragot and Steen, 1992; Gürel et al., 2000; Wremerth-Weich and Levall, 2003), red beet (Baransky, 1996) and fodder beet (Kikindonov, 2003).

Gynogenesis achieved by *in situ* pollination with irradiated pollen, has been successfully reported for fruit trees in *Malus domestica* (Zhang and Lespinasse 1991; Höfer and Lespinasse 1996), *Pyrus communis* (Sniezko and Visser 1987; Bouvier et al. 1993), *Actinidia deliciosa* (Pandey et al. 1990; Chalak and Legave 1997), *Citrus* (De Lange and Vincent, 1988; Oiyama and Kobayashi, 1993; Ollitrault et al., 1996; Germanà and Chiancone, 2001; Froelicher et al., 2007; Aleza et al., 2009 ) and *Theobroma cacao* (Falque et al., 1992). These methods rely on pollination with pollen irradiated by gamma rays from cobalt 60 followed by *in vitro* culture of immature seeds or embryos. The selection of an efficient radiation dose, the optimization of the pollination method, the seed collection time, the developmental stage and the cultural media and conditions are important factors affecting the success of this technique and the number of haploid embryos rescued through this technique.

### 2.3.4 Pollen embryogenesis

The pollen embryogenesis, often incorrectly called "androgenesis," is the most deeply studied and the most widely effective technology deployed in obtaining DHs. Two vital *in vitro* steps are required: (1) the induction of the sporophytic division, and (2) the regeneration of H/DH plants. The induction phase is often preceded by pre-treatments of plants, inflorescences/flowers or anthers. The regeneration phase is sometimes followed by an *in vitro* rooting phase. Finally, the plantlets obtained are acclimated to the ex vitro conditions. In most cases, the chromosome constitution of haploid plants needs to be doubled by treatment with chemicals. In some species, however, "spontaneous duplication" of chromosomes occurs during the culture at a sufficiently high rate for practical purposes.

The development of a microspore to a plant can be direct i.e. through formation of a pollen-derived androgenic embryo (often described in the literature as an "embryo-like structure"). In appropriate culture conditions, ELSs germinate directly into plants. Induced microspores can also form callus tissue that regenerate plants via indirect embryogenesis or organogenesis. Often all the developmental pathways co-exist in the same culture and their proportion depends on genotype and culture condition used.

Although the application of pollen embryogenesis is widespread, the cellular, biochemical and molecular bases for the transformation of microspores into pollen embryoids are poorly understood. However, some results and factors are clear. For example, it is well known that in fruit trees the capacity to regenerate from a male gamete

is genotype-dependent, and that the stage of microspore development is a critical factor for induction (Germanà 2006; 2009). Particularly, the fruit male gametes become competent to differentiate in a different way from the gametophytic pathway around the time of the first haploid mitosis (late uni-nucleate, vacuolate or early bi-cellular pollen stage).

Usually, physical, thermal (heat, cold) or chemical (water stress, starvation) stresses are necessary to enable competent microspores to follow the sporophytic pathway instead of the gametophytic one. *In vitro* anther or isolated microspore culture are the most effective and widely used methods of producing haploids and DHs.

Based on the technology used, the pollen embryogenesis can be archived thought anther culture or isolated microspore culture. For the latter, two alternative methods of microspore isolation are used. Flowers or inflorescences at the optimal developmental stage are isolated by hand or by blending and microspores are then separated from the debris by a series of washes, sieving and/or centrifugation steps (Zaki and Dickinson 1990; Hu and Kasha, 1997). Anthers can also be isolated manually from flowers and placed in liquid medium containing mannitol where the microspores are spontaneously released into the medium and then filtered or separated by centrifugation and re-suspended in the induction medium (Touraev et al. 1996 a,b; 1997). It the other hand, anthers from close flower buds are isolated and cultured intact on a solid or semi-liquid media.

### 2.3.5 Diploidization

Haploid plants are mostly sexually sterile. In the absence of homologous chromosome, meiosis is abnormal and, as a result, viable gametes are not formed. In order to obtain fertile homozygous diploids, the chromosomes of the haploids must be duplicated. In some plants, spontaneous duplication of the chromosome number occurs at high rate (>50%). This is especially true for the plants where pollen embryogenesis occurs via callusing, as in many cereals, including wheat, barley and rice. This spontaneous process is highly advantageous as it avoids the use of antimitotic agents. Where the frequency of spontaneous doubling of the chromosome exceeds 50%, there is no requirement for any special treatment to obtain fertile homozygous doubled haploids. In other cases, the pollen plants should be treated with 0,1 -0.4 % colchicine (Blakeslee, 1937), oryzalin and trifluralin (Bartels and Hilton, 1973; Strachan and Hess, 1983; Morejohn et al., 1987, Cleary and Hardham, 1988). or aminoprophosmethyl (APM) (Aya

et al.,1975) solution to diploidize the chromosome. Different methods have been followed to diploidize the haploid plants. Generally, the pollen-derived plants, with three to four leaves, are soaked in aqueous solution for 24-48 h and, after washing with distilled water, transferred to a potting mixture for hardening and further growth (Rao and Suprasana, 1996).

## 2.4 Factors influencing microspore embryogenesis

The ability to induce totipotency in anther or isolated microspore cultures is greatly influenced by several factors. These include genetic and external factors that may have strong implications on *in vitro* microspore development.

### 2.4.1 Genotype

The genotype of the donor plants have been reported to deeply effect the microspore embryogenic response. Genotypic variations in haploid embryo development have been observed in several crop species (Maheshwari et al., 1982). In *Brassica*, for example, genotypic variability for microspore embryogenesis response in isolated microspore culture has been reported in *Brassica campestris* (Ferrier et al., 1995; Burnett et al., 1992; Baillie et al., 1992; Guo and Pulli, 1996) and *Brassica juncea* (Thiagarajah and Stringam, 1993; Lionneton et al., 2001; Hiramatsu, et al., 1995).

In woody plants, effects of genotype were studied (Redenbaugh et al., 1981; Tsay and Su, 1985; Chen, 1986; Uddin et al., 1988; Milewska-Pawliczuk, 1990; Baldursson et al., 1993; Baldursson and Ahuja, 1996; Germanà et al., 1991; Germanà et al., 2006).

### 2.4.2 Donor plant's growth condition

The donor plant's growth condition has a marked effect on the physiological processes influencing also the microspore embryogenic ability. Proper light, temperature, humidity and nutrients are all necessary to develop healthy plants (Wang et al., 2000). Varying growth conditions of donor plants have been tested for their influence on microspore embryogenesis, ranging from plants grown under field conditions to plants grown under artificial or growth room conditions. Any kind of diseases or stress applied to the donor plants, can reduce the response to the pollen embryogenesis (Wang et al., 2000).

### 2.4.3 Microspore development stage

The microspore development stage is a key factor influencing the microspore's ability to turn totipotent. This is primarily due to the fact that microspores would only respond to embryo formation at a developmental stage when they are not committed to develop into pollen grains (Zaki and Dickinson 1990; Sharma and Bhojwani, 1985; Telmer et al., 1992). Moreover, the microspore development is asynchronous and microspores of different developmental stages may be observed in a developing anther. Therefore, selection of buds that have maximum proportion of embryogenic microspores is essential for efficient microspore-derived embryo yield. The microspore development may be divided into three basic stages viz. the tetrad stage (when the microspore mother cell splits into four haploid cells), the uninucleate stage (when the uninucleate microspore prepares for the nuclear division to form the vegetative and generative nuclei) and the bicellular stage (when the microspore contains a generative and a vegetative nucleus).

It has been established that there is an optimum development stage (embryogenic window) that corresponds to the late uninucleate to early bicellular stage of development, during which a large number of microspores could undergo embryogenesis (Raghavan, 1986; Pechan and Keller 1988; Smýkal, 2000; Pechan and Smykal, 2001; Telmer et al., 1993; Touraev et al., 1996b; Gonzalez-Melendi et al., 1995 and 1996; Satpute et al., 2005). Moreover, it has been proposed that non-embryogenic microspores produce inhibitory substances that suppress embryo development in the embryogenic microspores (Heberle-Bors, 1985; Kott et al., 1988). This may be because of the rupturing of non-embryogenic bicellular microspores thus reducing the embryogenic frequency and altering the morphology of embryos. Replacement of culture media after microspore isolation helps in reducing the autotoxins thus allowing normal embryo development. Similar influence of microspore developmental stage on microspore embryogenesis has been reported in olive (Bueno et al., 2003, 2005, 2006).

### 2.4.4 Pretreatment

Abiotic stresses play a very important role in androgenic induction, as first established in tobacco (Duckan and Heberle-Bors, 1976; Heberle-Bors and Reinert, 1981). Low or high temperature shocks are applied as pre-treatments or at the early stages of induction in most protocols developed for both, mono- and dicotyledonous plants.

At the beginning of the 1990s in cereal pollen embryogenesis, treatment of spikes with low temperature of 4–7°C for a period of 3 or 4 weeks was regarded as a

prerequisite for androgenic induction in barley (Olsen 1987; Ziauddin et al., 1990; Mordhorst and Lörz 1993; Scott and Lyne 1994; Evans and Batty 1994; Salmenkallio-Marttila et al., 1995). Cold pre-treatment of spikes for over 1 week was also applied to wheat (Gustafson et al., 1995; Ingram et al., 2000; Redha et al., 2000), triticale (Ślusarkiewicz-Jarzina and Ponitka 1997; Marciniak et al. 1998; Gonzalez and Jouve 2000; Immonen and Robinson 2000; Tuvesson et al. 2003; Wędzony 2003) and rye (Immonen and Anttila 2000; Immonen and Tenhola-Roininen 2003). Along with other pre-treatments, the cold pre-treatment favours the synchronization of the developmental process of the microspores (Hu and Kasha 1999). The role of temperature in induction of pollen embryogenesis was clearly shown in the case of *Brassica napus* microspore culture (Telmer et al. 1993; Custers et al. 1994; Binarova et al. 1997; Touraev et al. 1997; Indrianto et al. 1999; Smykal and Pechan 2000). At a temperature of 25°C or lower, microspores in suspension continue their development into pollen, while an 8 hour treatment with at a higher temperature of 32°C was enough to induce the androgenic process. There is evidence to suggest that prolonged stress treatment raises the proportion of albino plants in monocot cultures. By examination of various treatment lengths, Ohnoutková et al., (2000) and Zheng et al. (2001) demonstrated that the yield of green plants could be optimised for a range of starting materials including freshly collected anthers. The role of temperature in androgenic induction is now better understood as discussed in Touraev et al., (1997), where it is described as one of the main stress factors influencing microspore transition from gametophytic to sporophytic development.

Indeed, low temperature stress can be combined or replaced by other stress treatments, sometimes with better success than previously described protocols. Osmotic and starvation stress are nowadays frequently applied to cereals in combination with a relatively short, 3–5 day treatment with low temperature (Touraev et al. 1996a, b; Hoekstra et al. 1997; Hu and Kasha 1997; Caredda et al., 2000; Kasha et al., 2001; Li and Devaux 2001; Wojnarowiez et al. 2002; Jacquard et al., 2003; Cistué et al., 1995; Davies 2003). An improvement in androgenic efficiency in maize was achieved by the application of a cold pre-treatment (7°C) of tassels (Barnabás 2003a) or by pre-treatment at 14°C in a medium containing mannitol (Nageli et al., 1999; Obert et al., 2000; Zheng et al., 2003). Zheng et al., (2002; 2003) and Liu et al., (2002) used the combination of growth regulators and a short cold pre-treatment of spikes and/or starvation stress in mannitol with satisfactory results.

Novel methods were also published for wheat in which the cold pre-treatment of spikes was replaced with heat shock, 33°C for 48–72 hours (Touraev et al. 1996a, b; Liu et al. 2002). High temperatures were routinely used in androgenic induction of rapeseed microspores. Pechan et al. (1991) demonstrated that a treatment of 32°C for 8 hours provided a synchronous and irreversible switch in the development of embryogenic microspores of cv. Topas. It was confirmed later that, in certain culture conditions, temperature alone controls the developmental fate of rapeseed microspores and provides a perfect system to study various aspects of the pollen embryogenesis process (Hause et al., 1993; Binarova et al., 1993; Boutilier et al., 1994; Telmer et al., 1995; Custers et al., 1999, 2001). However, in some microspore suspensions heat shock could be replaced by colchicine treatment (Zhao et al., 1996a; b) and here a high number of the regenerated plants were doubled haploids, an important practical benefit. Pollen embryogenesis induction was proven to be successful in a number of related *Brassica* species when heat shock protocols were applied (Duijs et al. 1992; Barro and Martin 1999; Sato et al., 2002).

Various "stress treatments" have been applied widely. For example, application of low temperature pre-treatment was proven to favour pollen embryogenesis induction in trees: oak (*Quercus* sp.) (Bueno et al., 2000, 2003; Raminez et al., 2004), apple (*Malus* sp.) (Höfer 2003, 2004), citrus (*Citrus clementina*) (Germanà et al., 1994) and poplar (*Populus nigra*) (Andersen 2003; Deutsch et al., 2004). It also favours androgenic induction in some *Fabaceae* species (Zagorska and Dimitrov 1995; Kaltchuk-Santos et al., 1997; Croser et al., 2004; De Moraes et al., 2004). Comparative studies by Bayliss et al., (2004) showed that low temperature stress gives better results in comparison to high temperature when applied to *Lupinus* sp.

It can be concluded that in most present protocols to induce pollen embryogenesis, stress treatments of various kinds are widely applied, while in numerous other studies pretreatments and/or pre-cultures are omitted. In the latter case, one has to take into consideration that disinfection and isolation procedures are, in themselves, stress treatments. Thus, the way in which material is handled before the start of the induction culture may play an important role as a stress factor, is not completely controlled and not yet entirely understood.

### 2.4.5 Media composition

Improvements in the formulation of culture media have also contributed to the progress of pollen embryogenesis methods. The composition of basic salts and micro-elements is wide and varied. The most often modified components are: (1) the source of organic nitrogen, (2) carbohydrates, and (3) growth regulators. The first significant step towards a better efficiency in barley anther culture was achieved by lowering the ammonium nitrate content and increasing the glutamine level as a source of organic nitrogen (Olsen 1987). The role of various nitrogen sources was later studied in detail by Mordhorst and Lörz (1993). Most recent barley media recommend usage of glutamine at levels above 500 mg/l of medium (Li and Devaux 2001; Ritala et al., 2001; Kasha et al. 2003; Szarejko 2003). Raised level of glutamine or asparagine also became a component of media in DH production of triticale (Pauk et al. 2003b; Wędzony 2003) and rye (Immonen and Tenhola- Roininen 2003). Glutamine and serine at relatively high amounts are components of media used in *Brassica* ssp. (Da Silva Dias 2003; Ferrie 2003; Hansen 2003). These data suggest that amino acids serve as a better nitrogen source for developing ELSs compared to inorganic chemicals containing nitrate. Thus, it is recommended that organic nitrogen levels are raised in the induction medium until the induction rate and quality of ELSs are optimised.

Carbohydrates serve as a source of energy, building material and a component that regulates the osmotic properties of the culture media. The most spectacular success in protocols efficiency was achieved in barley, potato and wheat by the replacement of sucrose by maltose in numerous versions of induction media (Hunter 1987; Scott et al., 1995; Orshinsky et al., 1990; Mejza et al., 1993).

Sucrose and maltose are the main sugar components of the media reported the literature with few examples of other carbohydrates tested. An interesting experiment was performed by Ilic-Grubor et al. (1998) who successfully induced pollen embryogenesis in rapeseed cv. ‘Topas’ in a medium of low sucrose concentration, but with the addition of polyethylene glycol (PEG) as an osmoticum. Zhou et al. (1991) showed that the osmotic potential of media influences green plant regeneration. This opens up another issue: the role of osmotic pressure in the media, an important physiological parameter rarely reported in papers and not routinely measured in media preparation.

Numerous substances are active as growth regulators *in vitro*, many of them are synthetic analogous of plant hormones. The kind of substance, its dose and the

proportions in which several components are composed remain of substantial importance in regulating cell division and morphogenesis. Surprisingly, in many protocols for isolated microspore culture, growth regulators are omitted in the induction medium.

Early attempts to produce doubled haploids in maize by anther culture showed high genotypic dependence of the protocol efficiency. Delalononde and Coumans (1998) connected variability in androgenic response with the level of endogenous auxine. Abscisic acid (ABA) was applied to improve regeneration of induced embryos (Hansen 2000). Microspore suspensions are often cultured without the addition of growth regulators although the most successful media are conditioned with ovaries (Hansen and Svinset 1993; Bruins et al. 1996; Puolimatka et al. 1996; Hu and Kasha 1997; Zheng et al. 2002). Conditioning with an actively growing suspension culture was also successfully applied to induce *in vitro* development of isolated zygotes (Bakos et al. 2005). It can be presumed that the ovaries provide a source of active ingredients, phytohormones or other signalling molecules important for androgenic induction or ELS maturation. However, the data from detailed analysis of conditioned media have not yet been published.

Maize microspore culture was used recently as a model to study androgenic processes (Testillano et al. 2000, 2004; Borderies et al. 2004; Góralski et al. 2005; Mathys-Rochon 2005). Among others, the latter authors showed that arabinogalactan proteins added to the medium improved regeneration in low responsive genotypes. This discovery opens up new possibilities in improving the regeneration process, and may have beneficial effects for other species. It is probable, that other molecules that play regulatory role are secreted into the conditioned media however, to prove this hypothesis more detail studies of media during culture are required.

### 2.4.6 Microspore density

The density of isolated microspores in the culture media is another essential factor responsable for normal development of embryos. Microspore culture density ranging from 10,000 to 500,000 cells/ml has been reported for different species. Different density of microspores have been studied by several researchers to get the optimum embryo yield such as $10^4$ microspores/ml, 2 $10^4$/ml, 3–4 $10^4$/ml, 8 $10^4$/ml and 10 $10^4$/ml (Ziauddin et al., 1990; Olsen, 1991; Hoekstra et al., 1997; Gustafson et al., 1995).

# Second part

# 1 Apricot (*Prunus armeniaca* L.)

**First stages of microspore reprogramming to embryogenesis through anther culture in *Prunus armeniaca* L.**

## 1.1 Introduction

*Prunus armeniaca* L. is a worldwide known species with over 3.8 million tonnes of fruits produced in 2009 (FAOSTAT Database), very important particularly in the Mediterranean basin. Apricot is diploid with a monoploid number of chromosomes n = x = 8. Apricot has been primarily domesticated in China, but has a secondary centre of origin in Middle East (Watkins, 1976). It is a traditional fruit of North Africa and, in Algeria, it is considered a very profitable fruit crop for the local climate. Evidence of its cultivation in Maghreb can be traced as far as the XII century. Fruits are consumed fresh or dried and are considered as a staple food crop by the local populations (Panaud et al., 2002). Limited in vitro culture protocols for organ regeneration and propagation have been reported for Prunus species (Espinosa et al., 2006; Koubouris and Vasilakakis, 2006; Arbeloa et al., 2009; Canli and Tian, 2009; Petri and Scorza, 2010) Regarding research on pollen embryogenesis in apricot, Harn and Kim (1972) obtained callus formation from apricot anther culture, but its ploidy level was not determined. The formation of calli from cultured anthers of apricot 'Harcot', as well as the differentiation of nodular structures have been also reported by Peixe et al.(2004). The authors studied the influence of the temperature during the pre-treatment and evaluated, by flow cytometry, the plaid level of calluses that ranged from haploid to octoploid.

Induction of microspore embryogenesis *in vitro* is switched by a stress treatment, in many cases temperature stress. In different herbaceous and tree species, heat or cold stress has been reported to trigger pollen embryogenesis, the response being genotype dependent (reviewed in Maluszynski et al., 2003). We hypothesized that microspore reprogramming could be induced in apricot cultivars by cold stress through anther culture.

In the present study, we analysed whether a cold stress treatment followed by the *in vitro* culture in appropriate culture media was able to induce embryogenic responses in the microspores of several apricot cultivars; and whether the response was genotype dependent. The results showed the formation of multicellular pollen and proembryos in

different cultivars of P. *armeniaca* through the reported *in vitro* anther culture protocol, confirming the hypothesis.

The first stages of microspore reprogramming were analyzed also through structural and confocal microscopy observations, performed at different times of the culture, to monitor the main cellular changes of the apricot microspores during the anther culture.

## 1.2 Materials and methods

In this set of experiments the factors affecting microspore totipotency were critically examined. These included: genotypic variation for embryogenic response, bud size and microspore stage correlation, use of different media compositions for microspore culture, use of different treatments after isolation and use of growth regulators/ media additives.

### 1.2.1 Plant material and genotype selection

One year branches were harvested in February from a collection field, located in Lascari (Palermo, Italy) and, as inductive pre-treatment, they were subjected to 4 ◦C, in the dark, for two weeks. In order to evaluate the influence of genotype on the gametic embryogenesis induction from microspore, five cultivars of *P. armeniaca* L., and, more in details, three cultivars from Italy: Ninfa, Palumella, Portici, one from Canada: Orange Red and one from Tunisia: Sajeb, were tested.

### 1.2.2 Bud size and microspore stage correlation

After the cold inductive pre-treatment, the branches were forced under 25°C day/night temperature and 16-h photoperiod until the flower buds reached the phenological stage B described by Fleckinger (1964) in which buds swell get longer and petals appear (Fig. 1.1b). Particularly, at this phase, inside the anthers there are mostly microspores at the vacuolated stage, that is the most suitable for gametic embryogenesis induction (Peixe et al., 2004). To check the pollen developmental stage, one anther per each flower size was selected and the microspores were stained and then observed under a fluorescent microscope. Only flower buds of the same size of the ones containing vacuolate microspores (Fig. 1.1d and e) were selected for anther culture.

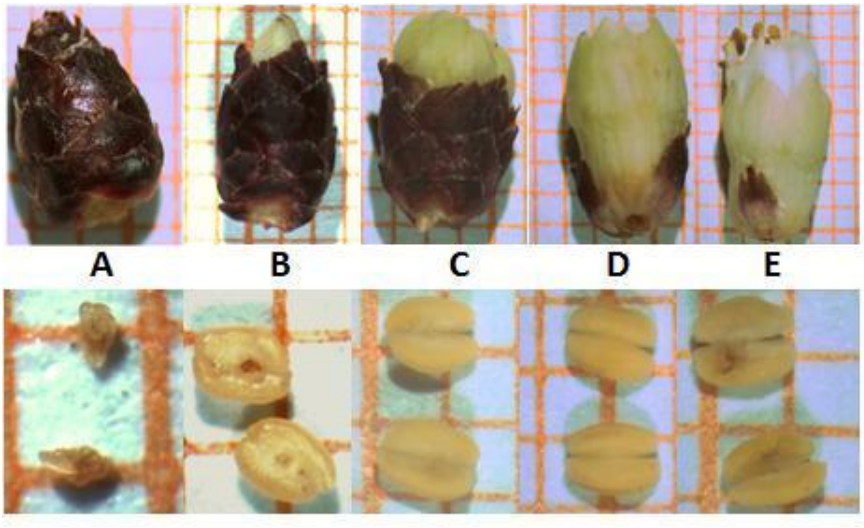


**Fig. 1.1 Correlation between phenological stages of flower buds and anther development**

### 1.2.3 Flower bud sterilization and anther culture

After evaluation, flower buds of the size containing a higher percentage of late uninucleate microspore, were removed from the brunch and, after pre-treatment, were surface sterilizated. The sterilization is usually performed by immersion for 5 minutes in 70% (v/v) ethyl alcohol followed by immersion in sodium hypochlorite solution (about 0.5% active chlorine in water) for 20 minutes. Then, the floral buds were washed three times with sterile distilled water and processed.

So, after sterilization, about 50 anthers were placed in each Petri dish, containing 10 ml of solid medium. 10 Petri dishes were prepared per each genotype and per each medium (500 anthers per each genotype and per each medium). Petri dishes were sealed with parafilm, incubated at 26±1°C, in the dark for the first 30 days and then placed under cool white fluorescent lamps (Philips TLM 30W/84, France) with a photosynthetic photon flux density of 35 µmol $m^{-2}$ $s^{-1}$ and a photoperiod of 16 light hours.

### 1.2.4 Media composition

Anthers of all genotypes were cultured on medium P, reported by Germanà and Chiancone (2003) and anthers of three selected genotypes (Ninfa, Palumella, Sajeb), were placed also on the medium NN4, (N6 of Chu (1978), added with 4% sucrose, 4.52µM 2,4-dichlorophenoxyacetic acid (2,4-D); 2.85µM indole-3-acetic acid (IAA) and 4.56µM zeatin), resulted the best one in the experiments carried out by Peixe et al. (2004) (Table 1.1). Calli obtained from anther culture were transferred to a regeneration medium (RM) containing half basal MS medium (Murashige and Skoog, 1962) added with 4% sucrose and 5.70 µM IAA, 4.55 µM zeatin and 4.55 µM thidiazuron (N-phenyl-1,2,3,-thi-diazol-

5-ylurea: TDZ), as reported by Peixe et al. (2004). 10 Petri dishes were prepared per each genotype and per each medium (500 anthers per each genotype and per each medium). Petri dishes were sealed with parafilm, incubated at 26±1°C, in the dark for the first 30 days and then placed under cool white fluorescent lamps (Philips TLM 30W/84, France) with a photosynthetic photon flux density of 35 μmolm−2 s−1 and a photoperiod of 16 light hours.

**Table 1.1 Media composition**

| Components | Medium | | |
|---|---|---|---|
| | P | NN4 | RM |
| | Per liter | | |
| N6 Chu Salts | 1X | 1X | |
| MS Salts | 1X | | ½ X |
| MS Vitamins | 1X | | ½ X |
| Sucrose | | 40 g | 40g |
| Galactose | 18 g | | |
| Lactose | 36 g | | |
| Ascorbic Acid | 500 mg | | |
| Myoinositol | 5 g | | |
| Biotin | 0,5 mg | | |
| Thiamine | 5 mg | | |
| Pyridoxine | 5 mg | | |
| 2,4-D | 0,5 mg | 1 mg | |
| IAA | | 0,5 mg | 1 mg |
| Kinetin | 0,5 mg | | |
| Zeatin | 0,5 mg | 1 mg | 1 mg |
| GA3 | 0,5 mg | | |
| TDZ | 0,5 mg | | 1 mg |
| BAP | 1 mg | | |
| Coconut water | 50 ml | | |
| Casein | 500 mg | | |
| Serine | 100 mg | | |
| Glicine | 2 mg | | |
| Glutamine | 800 mg | | |
| Malt extract | 500 mg | | |
| Agar | 8,5 g | 8,5 g | 8,5 g |
| pH | 5,8 | 5,8 | 5,8 |

### 1.2.5 Statistical analysis

The number of anthers not developed, swollen, or with calli was observed after two months of culture in each Petri dish. These values were used to calculate means. Differences among cultivars were tested by analysis of variance at $P < 0.01$ level. Differences among means were tested by Tukey's multiple comparison test.

### 1.2.6 Fixation and processing for light microscopy

In order to characterize the main changes in the cellular architecture of microspores in culture, as well as to identify the developmental stages of the formation of microspore-derived multicellular structures, a microscopical analysis was carried out.

Samples, at different times: just collected from the tree, after one week, two weeks and two months of culture were selected, processed, and resin embedded. Particularly, anthers were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.3, overnight at 4 ◦C. After washing in PBS, samples were dehydrated through an acetone series, infiltrated and embedded in Technovit 8100 acrylic resin (Kulzer, Germany), at 4 ◦C, as previously described (Solís et al., 2008), except for the time of infiltration which was increased in 48 h for apricot anthers, to optimize the penetration of the resin into the tissue. Semithin sections were stained with different methods and analyzed under light and confocal laser scanning microscopy (Leica CLSM).

### 1.2.7 Toluidine blue and iodide–potassium-iodide ($I_2KI$) staining cytochemistry for starch

Toluidine blue staining was used to observe the cellular organization under light microscopy. Iodide-based cytochemistry was performed at specific stages for localization of starch granules. Staining solutions (0.075% toluidine blue in water; 2 g of KI and 0.2 g of I in 100 ml of water) were applied on Technovit sections for 10–15 min. After rinsing and drying, preparations were mounted in Eukitt and observed under bright field in a Leitz Laborlux 12 microscope equipped with a DP10 Olympus digital camera.

### 1.2.8 DAPI staining for nuclei observation on resin sections and confocal analysis

DAPI staining was analyzed under confocal microscopy for the nuclei examination. 1µg/ml 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) solution in PBS was applied for 15 min to semithin Technovit sections, which after rinsing were mounted in

Mowiol and observed in a confocal laser scanning microscope Leica CLSM TCS SP2 under UV irradiation, DAPI fluorescence optical sections and their projections were captured.

## 1.3 Results and discussion

### 1.3.1 Bud size and microspore stage correlation

An analysis to correlate the phenological stage of flower buds, anther size and pollen developmental stage was firstly carried out in order to establish a convenient selection criterion of the buds. Several flower bud stages containing anthers of different sizes were determined according to the total length and their sepal-petal lengths ratio. DAPI staining for DNA was performed on anthers to reveal the developmental stage of the microspores contained in each bud/anther type. Selected stages showed young and late vacuolated microspores (Fig. 1.2d, e, g and h) inside buds which exhibited the petals appearing with a length lesser than the sepals (Fig. 1.2a and b), and bicellular pollen (Fig. 1.2f and i) and mature pollen inside the longer buds, with the length of petals higher than the sepals (Fig. 1.2c). These results permitted us to select the buds at the most appropriate stage for the *in vitro* culture, there are those containing vacuolated microspores (Fig. 3.2a and b), according to most reports on pollen embryogenesis (Ramírez et al., 2003; Peixe et al., 2004; Germanà et al., 2006; Solís et al., 2008).

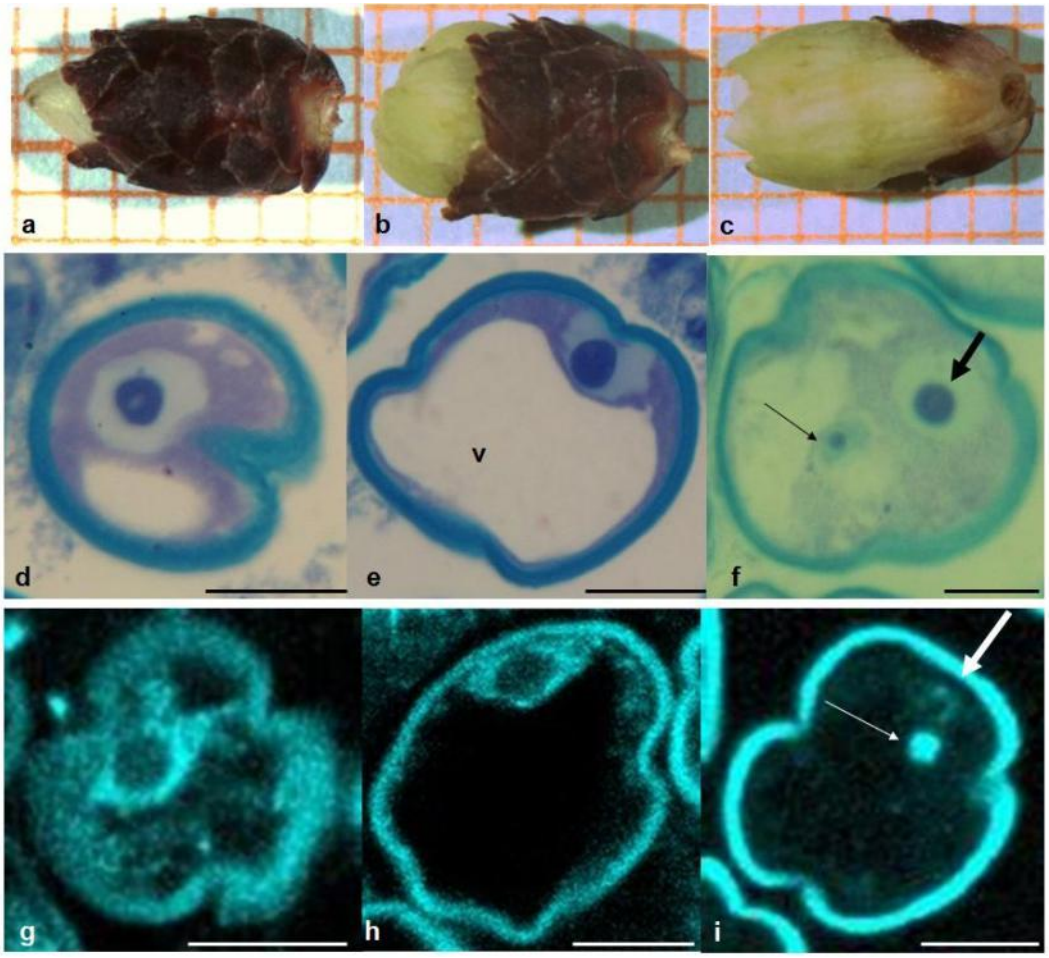


**Fig. 1.2 Correlation between phenological stages of flower buds and pollen development. Sequential stages of floral development in apricot (a–c) and microspore development in vivo (d–i). Toluidine blue staining (d–f) and DAPI staining for DNA under confocal microscope (g–i). (d and g) Young vacuolated microspore; (e and h) late vacuolated microspore; (f and i) bicellular pollen. Thin arrows: generative cell; thick arrow: vegetative cell. Bars represent: 10µm.**

### 1.3.2 Anther culture

Different features have been described in anther cultures as signs of initiation of a morphogenic response and of a change of the developmental pathway. After one week of culture, while the non- responsive anthers decreased in volume and turgenscence (Fig. 1.3b), in other anthers, the swelling, that is the first anatomical change accompanying the morphogenic response (Germanà et al., 2006), was observed (Fig. 1.3c) and keep swelling for more than four weeks; then, some of the swollen anthers started to produce callus (Fig. 1.3d and e). Different types of callus were observed: in few cases, calli were white, transparent and friable (Fig. 1.3d), but in most cases, calli were yellowish (Fig. 1.3e), with some globular structures (Fig. 1.3f). DAPI staining performed after two months of culture, on anthers with globular structures, showed some multinucleated

structures (Fig. 1.3g), indicating that the microspores were induced to follow the sporophytic pathway. Different responses were observed among anthers cultured on medium P (Table 1.2).

**Table 1.2 Response of five apricot cultivars to the induction medium P.**

| Cultivar | Not developed anthers | | Swollen anthers | | Anther with callus |
|---|---|---|---|---|---|
| | % | | % | | % |
| Ninfa | 57.9 | ab | 41.4 | bc | 0.8 |
| Orange red | 70.5 | a | 28.3 | c | 1.2 |
| Palumella | 49.4 | bc | 49.8 | abc | 0.8 |
| Portici | 59.2 | ab | 39.8 | c | 1.0 |
| Sajeb | 41.8 | c | 56.3 | a | 1.8 |

Values within each column followed by different letters are significantly different at P≤0.01 (Tukey's test)

Statistical analysis showed that the best results were obtained from the cultivar Sajeb, Palumella and Ninfa; in fact, these cultivars showed the lowest percentage of not developed anthers (41.8%, 49,4% and 57,9% respectively) and the highest percentages of swollen anthers (56.3%, 49,8% and 41,4% respectively). No statistically significant differences were observed among cultivars regarding callus production (Table 1.2). These three cultivars were selected for the culture in the medium NN4, which was previously reported for anther culture of another cultivar of apricot (Peixe et al., 2004). The cultivar Ninfa gave the best results, showing the highest percentage of anthers producing callus (12.2%) (Table 1.3), a percentage much higher than that one registered in the medium P (0.8%) (Table 1.2). Moreover, there was a statistically significant interaction between the two factors (genotype and medium). The best medium for apricot anther culture was the NN4; in fact, for Ninfa as well as for Sajeb, the lowest percentage of not developed anthers (respectively 40.5% and 33.9%) and the statistical highest percentage of anthers with callus (12.2% and 6.2%) were observed in this medium (Table 1.3). Probably, P medium, set up for citrus anther culture and characterized by a higher number of components (amino acid, coconut water, ascorbic acid, etc.) and by a lower concentration of growth regulators is less suitable for apricot pollen embryogenesis. The quantitative study also showed that the best response was found in Ninfa cultivar since it displayed a notably higher percentage of anthers with callus (12.2%), in comparison with the other cultivars.

**Table 1.3 Effect of the two induction media on the in vitro anther development.**

| Cultivar | Medium | Not developed anthers % | Swollen anthers % | Anthers with callus % |
|---|---|---|---|---|
| Ninfa | P | 57,88 | 41,37 | 0,75 |
| | NN | 40,47 | 47,31 | 12,22 |
| Palumella | P | 49,37 | 49,84 | 0,80 |
| | NN | 53,09 | 45,12 | 1,79 |
| Sajeb | P | 41,84 | 56,35 | 1,81 |
| | NN | 33,92 | 59,78 | 6,30 |
| Cultivar[a] | | P≤0.001 | P≤0.001 | P≤0.001 |
| Medium[a] | | P≤0.001 | P = 0.339 | P≤0.001 |
| Cultivar × medium[a] | | P≤0.001 | P = 0.024 | P≤0.001 |

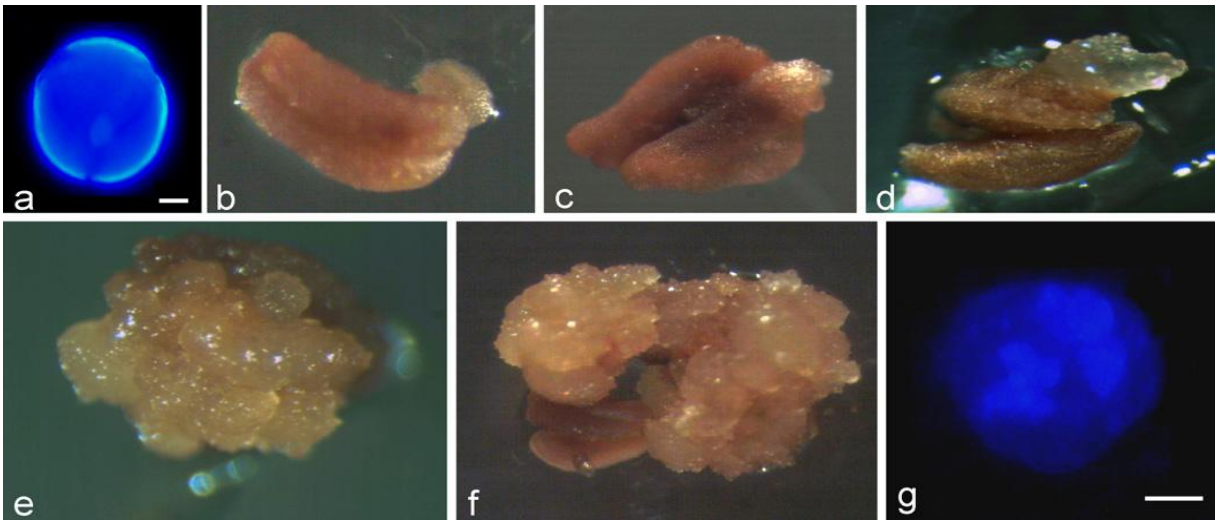


**Fig. 1.3 Progression of the in vitro anther culture in apricot. (a) Vacuolate microspore at the initiation of in vitro culture, DAPI staining; (b) not developed anther after one month in culture, cv. Sajeb; (c) swollen anther after one month in culture, cv. Sajeb; (d) anther with white callus; (e) anther with yellow callus; (f) anther with globular structures, cv Ninfa; (g) multinucleated microspore.**

### 1.3.3 Cellular architecture analysis during pollen embryogenesis

The progression of the *in vitro* anther cultures was monitored and the changes in the cellular organization of the microspores were analyzed in comparison with the gametophytic development. At the beginning of the culture (Fig. 1.4a), the microspores exhibited the typical architecture of the vacuolated microspores observed during the gametophytic development (Fig. 1.4e and h) and after one week in culture, microspores developed in culture. After embryogenic inductive conditions, exhibited differential features, showing two nuclei with similar size and organization (Fig. 1.4b and c), in

contrast with the two different nuclei of the bicellular pollen developed *in vivo* (compare Fig. 1.4f with 1.3b). These two-cell structures indicated that the microspore *in vitro* underwent a symmetrical division and switched from their gametophytic developmental pathway towards proliferation and were the result of the first embryogenic division of the microspore which still exhibited large cytoplasmic vacuoles (Fig. 1.4b). At later stages, cytoplasmic vacuoles progressively disappeared and multicellular structures were observed with dense cytoplasms, still surrounded by exine, the pollen wall (Fig. 1.4d) The iodide-based cytochemistry revealed the presence of starch granules in the multicellular structures (data not shown), as it was reported in the microspore-derived multicellular proembryos of other woody species like *Citrus* ssp. Ramírez et al., 2003) and *Eryobotrya japonica* Lindl. (Germanà et al., 2006). At later stages, after two months in culture, the responsive anthers were broken and showed callus and globular structures (Fig. 1.4e). The microscopical analysis of sections of these broken anthers revealed remnants of the somatic tissues of the anther and rounded undifferentiated cells with large vacuoles and different sizes, typical callus cells (Fig. 1.4f), which appeared grouped and dispersed inside and out of the anther. Interestingly, at the interior of the broken anthers rounded multicellular masses, similar to multicellular proembryos, were found (Fig. 1.4e and f). They were formed by cells with a typical architecture of proliferative cells with large nuclei, dense cytoplasms and thin walls (Fig. 1.4f and g); the globular proembryos appeared as individual structures limited by a layer of cells completely different, with elongated shape and cytoplasmic inclusions (Fig. 1.4f and g). The presence of specific layers of cells and cell wall components surrounding multicellular organogenic masses and proembryos has been reported in other organogenic *in vitro* systems (Fortes et al., 2002; Bárány et al., 2010). It has been suggested that these peripheral components were specific of the periphery of cellular masses with organogenic competence (Fortes et al., 2002). These proembryos would be originated by the subsequent proliferation of the multicellular pollen grains after the exine breakdown, as it has been found in other anther cultures for embryogenesis induction of woody species (Ramírez et al., 2003, 2004). The iodide-based cytochemistry for starch revealed very small starch grains in the cytoplasm of the proembryo cells, whereas the cells surrounding the proembryos and the rest of the somatic cells displayed very large starch inclusions (Fig. 1.4h).

A lower amount or absence of intracellular starch granules have been described in young microspore-derived proembryos of other species, associated with cell proliferation

(Bárány et al., 2005; Solís et al., 2008), the increase in the starch accumulation being related with differentiation events (Bárány et al., 2005).

The evolution of the *in vitro* system described here, from two-cell and multicellular pollen grains to large multicellular proembryos indicated that the reprogramming of the microspore and the first steps of the embryogenic pathway have been achieved by the protocol assayed here in different varieties of P. *armeniaca*.

## 1.4 Conclusions

Breeding of perennial plants is usually cumbersome and time-consuming due to their long reproductive cycle, high degree of heterozygosity and complex reproductive biology and, so, the potential of gamete biotechnology can represent, particularly for them, a great advantage in comparison with the conventional methods. In the present paper, a new anther culture system has been developed for microspore embryogenesis in apricot. The evolution of the *in vitro* system has been characterized from two-cell, to multicellular pollen grains and to large multicellular proembryos, indicating that the reprogramming of the microspores and the first stages of the embryogenic pathway have been achieved. These findings constitute a crucial step in the design of protocols for the regeneration of microspore-derived embryos and DH plants, very important for future potential applications in breeding programmes of this fruit tree of economic interest.

Many are the factors affecting the success of the pollen embryogenesis (microspore developmental stage, anther or floral bud pre-treatments, medium composition, culture conditions, etc.) and, for this reason, a deeper knowledge and understanding of this process and how the factors drive morphogenic competence and development, will enable the effective deployment of gametic embryogenesis and haploid technology in the improvement of apricot and generally of fruit trees. Further efforts are necessary to obtain regeneration of microspore-derived embryos and plantlets.

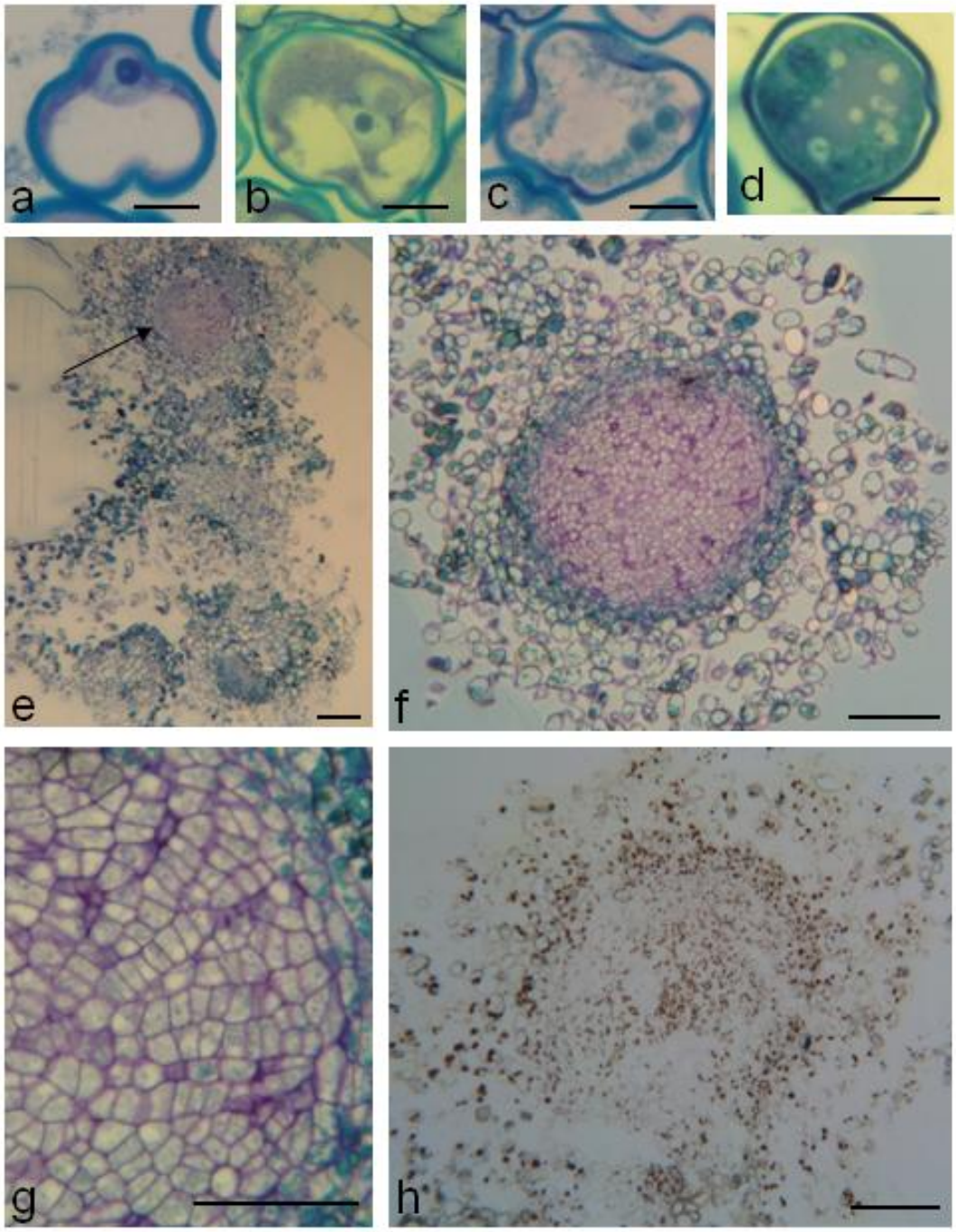


**Fig.1.4 Cellular monitoring during in vitro anther culture. Microspore development (a–d) and proembryos formation (e–h). Toluidine blue staining (a, b, d–g), DAPI staining for DNA under confocal microscope (c), and iodide-staining for starch (h). (a) Vacuolate microspore at the beginning of the culture; (b and c) microspore-derived two-cell structures after one week of culture; (d) multicellular pollen grain with dense cytoplasms after two weeks of culture; (e) panoramic view of an anther after 2 months in culture showing a rounded multicellular mass or proembryo (arrow); (f) proembryo surrounded by callus cells; (g) detail of proembryo cells; (h) starch cytochemistry, the proembryo cells (in the centre) show small and scarce starch granules whereas the surrounding cells exhibit larger and more abundant starch inclusions. Bars represent in (a–d): 10µm, in (e, f and h): 100µm, in (g): 50µm.**

# 2 Olive (*Olea europaea* L.)

**First stage of pollen embryogenesis in isolated microspore and anther culture of Olive**

## 2.1 Introduction

The Olive (*Olea europaea* L.) is a typical Mediterranean woody plants suitable for regions with a temperate climate, characterized by hot and dry summers (Lavee, 1992). The leaves are opposite and the flowers, present in racemes and emerging from the axils of the leaves, produce large quantities of pollen. Olive trees grow very slowly and rarely they reach more than 15 m in height, but they may have a lifespan of hundreds of years. The spread of this species in the Mediterranean basin has followed the human movement and the trades that have always characterized this geographical area. From a botanical point of view, the olive is the only edible fruit species, among approximately 600 species of the family *Oleaceae*.

Olive breeding conducted using traditional methods has always encountered problems due to various factors, such as self-incompatibility, long reproductive cycles, prolonged juvenile phase, a marked tendency to allogamy and high levels of heterozygosity. Like it has been demonstrated in other crops, biotechnological methods can improve the efficiency and increase the speed of breeding.

Gametic embryogenesis is a widely-used method to generate genetic variability, obtaining microspore-derived embryos and doubled-haploid plants, with many applications for plant breeding (Chupeau et al., 1998).

The pollen embryogenesis allows to obtain homozygous from heterozygous parents lines at all loci. It involves the reprogramming of the immature pollen (the microspore) toward a different development pathway that leads to the phenomenon of proliferation and differentiation with the formation of embryos and regeneration of haploid and double-haploid plants (Barany et al., 2005). The reprogramming of microspores, the induction of embryogenic divisions and the formation of multicellular proembryo constitute an important first step towards the production of haploid plants from pollen structures (Bueno et al., 2005, 2006; Solis et al., 2006, 2008). Isolated microspore culture of two olive cultivars (Arbequina and Picual) have been carried out resulting in induction

of sporophytic division, multi-nucleate microspores and multi-cellular structures (Bueno et al. 2004).

To induce gametic embryogenesis, exogenous polyamines (PAs) in culture medium seem to induce *in vitro* organogenesis and somatic and gametic embryogenesis in many species (Mengoli et al., 1989; Faure et al., 1991; Kevers et al., 2002; Rajesh et al., 2003; Bertoldi et al., 2004). Moreover, PAs in the culture medium increased the number of gynogenic embryos in *Allium cepa* L. (Martinez et al., 2000) and androgenic embryos in potato (Tiainen, 1992), in some Indian wheat cultivars (Rajyalakshmi et al., 1995), in *Citrus clementina* (Chiancone et al., 2006) and in cucumber (Ashok Kumar et al., 2004).

In the present research, the cellular changes induced by stress treatments, genotype and by the different culture media, anther and microspore culture were carried out.

## 2.2 Material and methods

### 2.2.1 Plant material

Experiments were conduced in two years (2009 and 2010).

In 2009, the following olive cultivars Minuta, Verdello, Caltabellotta, Nocellara Messinese, Nocellara del Belice, Moresca, Galatina, Cavaliere and Verdello, collected in Scillato, Palermo were used for isolated microspore culture, while for anther culture, a Sicilian cultivar Tonda Iblea and two cultivars from Tunisia, Zlamati and Chemlali were used.

In 2010, the cultivars Passulunara, Verdello, Caltabellotta, Nocellara del Belice, Moresca, Galatina and Cerasuola Sciacca, collected in Scillato, Palermo, and Moraiolo, collected in Perugia were used for microspore isolated culture, while for anther culture Verdello, Nocellara del Belice, Moresca, Galatina and Cerasuola Sciacca were used.

Olive branches were collected in May and subjected to 4°C, in the dark, for two weeks.

### 2.2.2 Bud size and microspore stage correlation

The relationship between the bud size and the microspore development stage was studied before the culture. In 2009, flower buds of cultivars Minuta, Verdello, Caltabellotta, Nocellara Messinese, Nocellara del Belice, Moresca, Galatina, Cavaliere and Tonda Iblea were collected randomly from the plant and were graded into three bud size range and then they were measured.

In order to study the correlation between the microspore develop and the flower bud size, anthers from two bud sizes were fixed in 200 µl alcohol: acetic acid (3:1) for 10 min, transferred and macerated in 200 µl Tris-Triton solution [Tris HCl (50 mM) + Triton X100 (1%)] to release the microspores. The macerated preparation, stained with 10 µl of 4,6- diamidino-2-phenylindole (DAPI) solution (0.001 µM DAPI in Tris-Triton solution) was observed under UV florescence microscope (Zeiss, Axiophot, Germany).

### 2.2.3 Anther culture

**Anther culture protocol**

Flower buds sterilized as previously described for apricot, were aseptically opened to collect anthers. About 50 anthers were placed in each Petri dish, containing 10 ml of solid medium. Then, all Petri dishes were put in the dark at 26±1°C for the first 30 days and then placed under cool white fluorescent lamps (Philips TLM 30W/84, France) with a photosynthetic photon flux density of 35 µmol $m^{-2}$ $s^{-1}$ and a photoperiod of 16 light hours.

**Media used for anther culture**

The media composition used in anthers culture is reported in Table 2.1. In 2009, the anthers were culture in Spe0, Spe1 and Spe4 media, while in 2010, the only medium used was the N6.

**Experimental design**

In 2009, the influence on gametic embryogenesis of three concentration of sperimidine (0g/l; 1g/l; 4g/l) was studied. The anthers of three cultivars (Verdello, Zlamati and Chemlali ) were cultured 10 Petri dishes with 50 anthers for each vessel were prepared per each genotype and media. After 8 months of culture, per each Petri dish, the number undeveloped anthers, of swollen anthers and of anthers with callus were recorded (Table 2.4). Statistical analysis was not possible because some Petri dishes were contaminated and data were not sufficient.

In 2010, the influence of two thermal shock on gametic embryogenesis was studied. The anthers of five cultivars (Verdello, Nocellara del Belice, Moresca, Galatina and Cerasuola Sciacca) were cultured. 30 Petri dishes with 50 anthers were prepared. Then, 10 Petri dishes were put in oven at 34°C for 1 hour, 10 freezer at -20°C for 30 minutes and others 10 were putted directly in dark.

Data on anther development were collected after seven months (Table 2.5) and differences among cultivars and treatments were tested by analysis of variance (two-way Anova) at $P < 0.01$ level.

## 2.2.4 Isolated microspore culture

**Isolation protocol**

The sterilized selected flower buds (Fig. 2.1a) were opened with sterile tweezers and about 800 anthers were put in Petri dish containing 6 ml of sterile 0.4 M mannitol solution at 4°C. The anthers were placed in a blender (sterile and cooled, Fig. 2.1b) and 14 ml of 0.4 M mannitol was added. The blender was used two times for 10 seconds at a minimum speed (Fig. 2.1c). The blender content was filtrated through 100 µm sterile filter placed in a plastic beaker also sterile. The remaining anthers were crashed again in the blender for 2 times for ten seconds. The content was poured and the filtrate was transferred into a Falcon tube (50 ml).

The tubes containing the suspended microspores were centrifuged at 2500 rpm for 10 minutes at 4 º C (Fig. 2.1e). The obtained microspore pellet was re-suspended in 30 ml of mannitol and centrifugated The procedure is repeated for at least two or three times to obtain a clean pellet.

After the last washing, microspores were counted immediately using a Burker camera, using 20 µl of the suspension and the density were measured. For the experiments, a density of 100,000 microspores per ml of medium ($10^5$ mcp/ml) was used. A volume of 1.0 ml was dispensed into each Petri dishes. All Petri dishes were put in the dark for the first 30 days and then placed under cool white fluorescent lamps (Philips TLM 30W/84, France) with a photosynthetic photon flux density of 35 µmol $m^{-2}$ $s^{-1}$ and a photoperiod of 16 light hours.

**Fig. 2.1 Microspore isolation protocol: (a) sterilization of flower buds with 70%ethanol solution; (b) blender; c) washing with mannitol solution; (d) re-suspending of microspore pellet; (e) Prime R centrifuge (Biofuge, Heraeus).**

**Media used for isolated microspore culture**

The media composition used for microspore culture is reported in Table 2.1. In 2009, the microspores were culture on P media while, in 2010, on N6 media.

**Experimental design**

In 2009, microspore viability in five cultivars (Minuta, Caltabellotta, Nocellara Messinese, Nocellara del Belice and Tonda Iblea) was evaluated by FDA in four moments: before the isolation, immediately after isolation and after treatment (32°C for 1 hour and -20°C for 30 minutes. Table 2.2). Statistical analysis was performed with one-way ANOVA, followed by Tukey's test ($P<0.01$).

In 2010, N6 media was used for isolated microspore culture of Moraiolo, Nocellara del Belice, Cerasuola Sciacca, Verdello and Galatina. The influence of two thermal shock was studied putting 5 Petri dishes at 34°C for 1 hour, 5 at -20°C for 30 minutes and 5 were put in the dark for the first 30 days at 26±1°C. Differences among cultivars and

treatments were tested by analysis of variance (two-way Anova) followed by Tukey's test, at $P < 0.01$ level.

. The size increasing was assumed as a signal of the switch of the development to the gametic embryogenesis. With this proposal, the microspore diameter was measured before and after three months of culture. 100 microspores per cultivar and per treatment were and measured.

### 2.2.5 Cellular architecture

DAPI staining were used to follow the development of microspores in culture (both for anthers and isolated microspores) as reported before. FDA staining (fluorescein diacetate solution (1.25 mg/ml)) was used to study the viability of the microspores.

Anthers, microspores and microspore derived structures developed in culture were processed for microscopy analysis; at specific time points: just after flower buds collection and every month for eight months. Samples were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.3, overnight at 4°C and than stored at 4°C in 0.1% FDA. After fixation, isolated microspores were gently centrifuged and the pellets containing the cells were embedded in 15 % gelatine for further dehydration and resin embedding. After washing in PBS, anthers and microspores in gelatine were dehydrated through an acetone series. Then, the samples were infiltrated and embedded in Techhnovit 8100 resin (Kulzer, Germany) at 4°C. Technovit semi-thin sections (1 µm) were stained with toluidine blue and observed under bright field of light microscope for structural analysis.

**Table 2.1 Media composition**

| Components | Media | | | | |
|---|---|---|---|---|---|
| | P | N6 | Spe0 | Spe1 | Spe4 |
| | Per litre | | | | |
| N6 Chu Salts | | 1X | | | |
| MS Salts | 1X | | 1X | 1X | 1X |
| MS Vitamins | 1X | 1X | 1X | 1X | 1X |
| Galactose | 18 g | 9 g | 9 g | 9 g | 9 g |
| Lactose | 36 g | 18 g | 18 g | 18 g | 18 g |
| Ascorbic Acid | 500 mg | 500 mg | 500 mg | 500 mg | 500 mg |
| Myoinositol | 5 g | | | | |
| Biotin | 500 mg | 500 mg | 500 mg | 500 mg | 500 mg |
| Thiamine | 5 mg | | | | |
| Pyridoxine | 5 mg | | | | |
| 2,4-D | 0.5 mg | 0.02 mg | 0.02 mg | 0.02 mg | 0.02 mg |
| IAA | | 0.02 mg | 0.02 mg | 0.02 mg | 0.02 mg |
| Kinetin | 0.5 mg | 1 mg | 1 mg | 1 mg | 1 mg |
| Zeatin | 0.5 mg | 0.5 mg | 0.5 mg | 0.5 mg | 0.5 mg |
| GA3 | 0.5 mg | 0.5 mg | 0.5 mg | 0.5 mg | 0.5 mg |
| TDZ | 0.5 mg | 0.1 mg | 0.1 mg | 0.1 mg | 0.1 mg |
| BAP | 1 mg | 0.5 mg | 0.5 mg | 0.5 mg | 0.5 mg |
| Coconut water | 50 ml | 50 ml | 50 ml | 50 ml | 50 ml |
| Casein | 500 mg | 500 mg | 500 mg | 500 mg | 500 mg |
| Serine | 100 mg | | | | |
| Glicine | 2 mg | | | | |
| Glutamine | 800 mg | 200 mg | 200 mg | 200 mg | 200 mg |
| Malt extract | 500 mg | | 500 mg | 500 mg | 500 mg |
| Spermidine | | | | 1g | 4g |
| pH | 5.8 | 5.8 | 5.8 | 5.8 | 5.8 |

## 2.3 Results and discussion

### 2.3.1 Bud size and microspore stage correlation

The appropriate microspore developmental stage is crucial in pollen embryogenesis. In this study, the developmental stages from tetrad (Fig. 2.3a) to mature pollen (Fig 2.3c) were observed in different anther and flower sizes.

In all the cultivars observed, unopened floral buds measuring approximately 2 mm long (Fig. 2.3b), with yellow-greenish anthers, were identified as those with higher proportion of microspores from the late uninucleate to the bicellular pollen stage. These microspores are at the optimal stage for the embryogenesis induction.

### 2.3.2 Anther culture

Results obtained from the experiment conduced in 2009 are extremely argumentative. In fact, the response of the three cultivars (two Tunisian and one Italian) is really different for all the treatments studied.

The most important results are reported for anther with callus. Each cultivar showed a different behaviour: not very big difference among media were observed for the cultivar Chemlali, while for the Zlemati, the highest percentage of anthers with callus was obtained in the spe0 medium. Instead, for Verdello the best results (30%) were reported in spe4.

**Table2.2 Effect of three induction media on the in vitro anther development after 8 months culture (year 2009)**

| Cultivar | Media | Not developed anthers % | Swollen anthers % | Anthers with callus % |
|---|---|---|---|---|
| Chemlali | spe0 | 35% | 44% | 21% |
| | spe1 | 28% | 53% | 20% |
| | spe4 | 37% | 46% | 18% |
| Zlemati | spe0 | 34% | 33% | 33% |
| | spe1 | 32% | 41% | 28% |
| | spe4 | 53% | 39% | 9% |
| Verdello | spe0 | 59% | 34% | 7% |
| | spe1 | 59% | 27% | 14% |
| | spe4 | 32% | 38% | 30% |

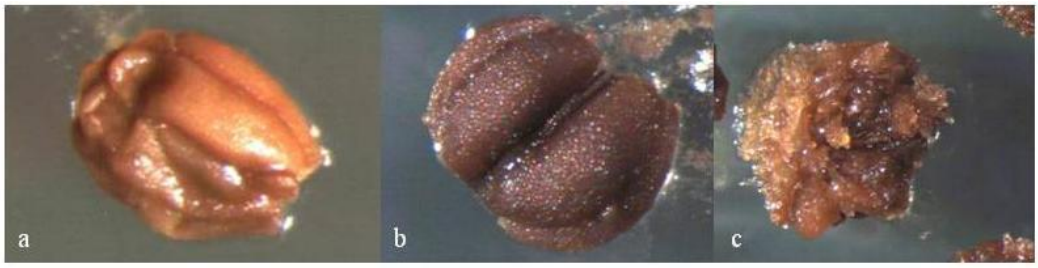


**Fig. 2.2 Progression of the in vitro anther culture in olive (a) not developed anther after one month in culture, cv. Galatina; (b) swollen anther after one month in culture, cv. Galatina; (c) anther with white callus after two months; cv. Verdello;**

Statistical analysis conduced on experiments of 2010 did not show any significant interaction between the two factors (cultivar and treatment). Different statistical significative were found among cultivar, for the swollen anthers and anthers with callus parameters (Table 2.3).

Particularly, about the anther with callus, the best results were obtained from anthers treated at -20°C for 30 minutes for all the cultivars except for Galatina were the highest percentage of anther with callus was recorded in the control. Moreover, the percentage of anthers with callus of Galatina was the highest among the treatments and cultivars tested.

In conclusion, the results obtained in this experiment are encouraging because a very high percentage of anthers produced callus. Moreover, comparing the experiment conduced in the two years of research, the percentage of anthers with callus obtained after the cold treatment is two times higher than the one obtained after the culture on medium containing spermidine.

The results reported in this study on gametic embryogenesis on olive agree with these one reported by Bueno et al. (2006) that obtained the best results after treating microspores at low temperature. (3°C for 96 hours).

Due to the preliminaries and encouraging results obtained, more investigation has to be done, with the main goal of regenerating from the callus obtained.

**Table 2.3 Effect of three stress treatment on *in vitro* anther development; N6 media after 7 months culture; year 2010**

| Cultivar | Treatment | Not developed anthers % | Swollen anthers % | Anthers with callus % |
|---|---|---|---|---|
| Verdello | NT | 24,25 | 28,86 | 46,88 |
| | HT | 18,28 | 20,09 | 60,42 |
| | CT | 12,22 | 21,06 | 65,75 |
| Galatina | NT | 7,97 | 24,88 | 67,15 |
| | HT | 13,99 | 25,16 | 60,24 |
| | CT | 13,85 | 21,36 | 64,78 |
| Moresca | NT | 19,34 | 53,11 | 27,02 |
| | HT | 18,31 | 52,29 | 29,40 |
| | CT | 18,68 | 34,70 | 46,27 |
| Nocella del Belice | NT | 26,36 | 45,59 | 28,06 |
| | HT | 28,59 | 41,10 | 30,31 |
| | CT | 33,86 | 55,20 | 39,05 |
| Cerasuola Sciacca | NT | 19,16 | 45,99 | 34,85 |
| | HT | 20,66 | 42,62 | 36,71 |
| | CT | 16,00 | 36,38 | 47,62 |
| Cultivar[a] | | $P$=0.010 | $P$≤0.001 | $P$≤0.001 |
| Treatment[a] | | $P$=0.957 | $P$=0.159 | $P$=0.060 |
| Cultivar × treatment[a] | | $P$=0.645 | $P$=0.122 | $P$=0.898 |

*P* values for main factors and interactions of two-way ANOVA (Tukey's test).
**NT**: control; **HT**: 34°C/1h; **CT**: -20°C/30';

### 2.3.3 Microspore culture

The viability of microspores before isolation was high, and so the conservation of branches at 4°C was successful. Immediately after isolation, viability was 50–77%, while viability after heat-shock was 39–68% and after cold-shock was 26–32% (Table 2.4; Fig. 2.3 d, e).

The microspore viability was real high after before isolation and after the pretreatment at 4°C for 1 week, varying from 89% for Minuta and 74% for Nocellara del Belice. The isolation procedure has a genotype dependent influence on microspore viability. In fact, while for Nocellara del Belice, Nocellara Messinese and Tonda Iblea the viability decreases only for 10 points, for Moraiola and Caltabellotta, the viability decrease markedly (respectively 30 and 24 points).

The statistical analysis carried out on the effect of the thermal shock showed that only the cold shock negatively influence the microspore viability almost for every cultivar, excepted for Tonda Iblea (Table 2.4)

**Table 2.4 Percentage of microspore viability observed in five olive cultivars (2009).**

| | Viability | | | |
|---|---|---|---|---|
| | Before isolation | After Isolation Control | Heat shock 32°C 1h | Cold shock -20°C 30’ |
| Minuta | 89% | 50% a | 42% a | 33% b |
| Caltabellotta | 78% | 54% a | 44% a | 26% b |
| Nocellara del Belice | 74% | 65% a | 52% a | 32% b |
| Nocellara Messinese | 84% | 77% a | 68% a | 37% b |
| Tonda Iblea | 78% | 62% | 39% | 35% |

a Values within each column followed by different letters are significantly different at P<0.05 (SNK’s test)

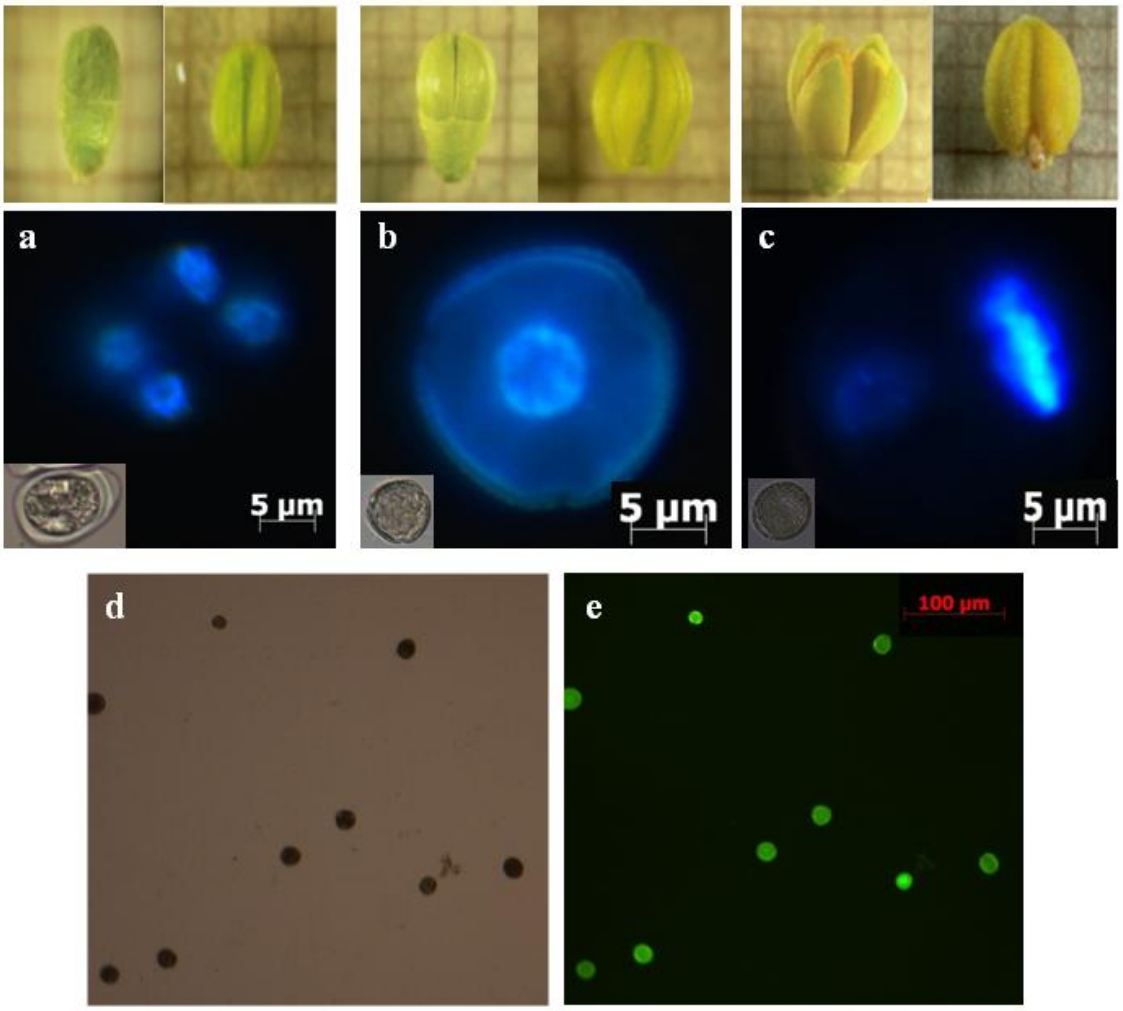


**Fig. 2.3 Correlation between phenological stages of flower buds, anther and microspore development (cv. Nocellara Messinese) (a-c) DAPI stained; microspore observed under phase contrast (d); FDA stained microspores under UV lamp (e).**

In 2010, after isolation, microspore measured around 8-10 µm. It has been observed that the cold shock at -20ºC in darkness for 30 minutes caused “enlarging” of about 16% (to 12µm) of the microspores after 1 day of the culture. After three months, the diameter average on the same treatment, was higher in microspore subjected to cold treatment than

the others. In fact, the diameter increased more than 2 times and the enlarged microspores continued embryogenic development (Fig. 2.4). Heat shock treatments produced also enlargement microspores and, in the cultivar Cerasuola Sciacca, microspores became bigger than the ones from the cold treatment. In the control conditions (no stress applied) microspores increase their diameter lass than other shock.

In the present study, both thermal shocks induced an increasing of the microspore size, and heat treatment resulted stimulating the gametic embryogenesis pathway.

No statistical differences were detected in the size of heat treatment and not treated microspores, while the cold one seems to significativily increase the microspore size, respect to the other treatment (Table 2.5). Except for Cerasuola Sciacca and Galatina, no differences were detected between the control and the two thermal shocks, meaning that the microspore size increasing is genotype dependent. In general, the good influence of cold treatment can be explained with the resumption of mitosis when the microspores are cultured at room temperatures, as reported by Zheng (2003).

**Table 2.5 The effect of cold and heat-treatments on increasing diameter of microspores after 3 months of culture.**

| Cultivars | Control µm | Heat Treatment µm | Cold Treatment µm |
|---|---|---|---|
| Moraiolo | 17.1 a | 16.3 a | 25.9 b |
| Nocella del Belice | 16.5 a | 23.1 a | 25.8 b |
| Cerasuola Sciacca | 12.9 a | 24.3 a | 22.4 b |
| Verdello | 13.5 a | 17.6 a | 20.0 b |
| Galatina | 15.6 a | 20.2 a | 25.1 b |

a Values within each column followed by different letters are significantly different at P<0.05 (SNK's test)

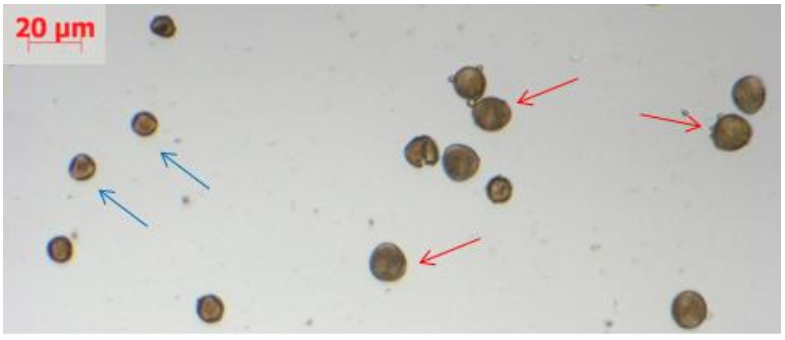


**Fig.2.4 Not developed (blue arrows, on left) and enlarged alived microspores (red arrows, on right) in culture (2010)**

Under the same culture conditions, microspores showed the indirect pollen embryogenesis. In fact, after 2-3 months of culture, the responding microspores produced brown and white callus. Pintos et al. (2007) observed that thermal treatment caused a very low frequency of "swollen" microspores and that these microspores continued to the pollen embryogenic development.

After 3 months in culture, microspore-derived calli, larger than 0.5 mm in diameter, were observed, and transferred to N6 solid medium in order to proliferate calli and to obtain regeneration. Calli was observed, in 2009 and 2010, in different genotypes, for example in Verdello (Fig. 2.5a) and in Galatina (Fig. 2.5b) in 2009, and in Cerasuola Sciacca (Fig. 2.5c) and Nocellara del Belice (Fig. 2.5d)

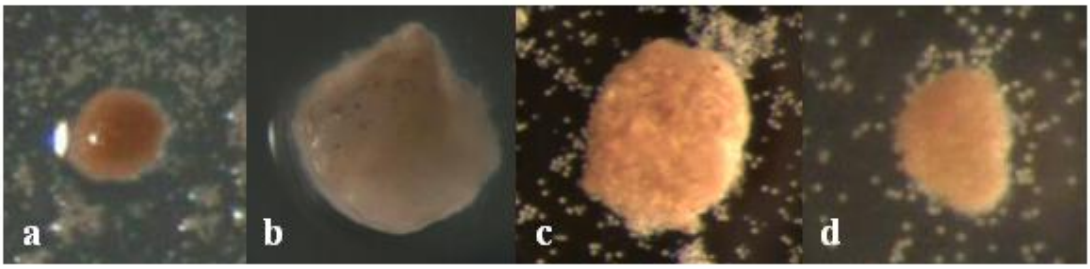


**Fig. 2.5 Calli obtained in microspore culture in 2009 (a,b) and 2010 (c,d); (a) cv. Verdello; (b) cv. Galatina; (c) cv. Cerasuola Sciacca; (d) cv. Nocellara del Belice**

### 2.3.4 Cellular architecture analysis

The progression of the *in vitro* microspore and anther cultures was monitored and the changes in the cellular organization of the microspores were analyzed. At the beginning of the culture, the microspores exhibited the typical architecture of the vacuolated microspores during the gametophytic development (Fig. 2.6a ).

After two weeks of culture, in microspore culture, pollen grains with two similar nuclei (Fig. 2.6b) formed by a symmetrical division were observed, indicating a high asynchrony in the embryogenic response of the microspores. These two-cell structures are the first step of the sporophytic pathway. In fact, after two or three months, several bi and tri nucleated microspores derived from symmetrical division of the nucleus were observed. Moreover, after three – four months, it was possible to observe many multinuclear and multicellular structures.

The anther cultures were fixed at different times in historesin and were monitored using toluidine blue staining. At the beginning of the culture, the microspores exhibited the

typical architecture of the vacuolated microspores during the gametophytic development (Fig. 2.6). After one week in culture, microspores developed in culture, after embryogenesis inductive conditions, exhibited differential features, showing two nuclei with similar size and organization

The results presented here suggested that the vacuolated microspore, very abundant in most of the anthers at the beginning of culture, was a responsive stage for induction in olive, as reported in many other species of herbaceous (González-Melendi et al. 1995a, b; Barany et al. 2005; Custers et al. 1994) and woody species (Ramírez et al. 2003, 2004).

## 2.4 Conclusion

This study reports the preliminary results on anther culture and isolated microspores of some cultivars of *Olea europaea* L. In the series of experiments, it was possible to optimize the protocol for isolation and culture of microspores with the selection of flower bud sizes and anthers containing uninucleate microspores.

In anther culture, the presence of spermidine on culture media was required for a better response of anther to the production of callus, while a cold or a heat treatment increasing the percentage of swollen and with callus anthers.

In the isolated microspore culture, it has been observed that a cold treatment at 4°C for two weeks combined with a thermal shock positively influenced the response of microspores.

Cytological analysis, realized on fixed and DAPI stained anthers and microspores allowed the observer the different steps of production of callus, of multicellular structures and of some proembryos.

These preliminary results recorded, for the first time in the olive cultivars, the first pollen embryogenesis stages from anther and isolated microspore culture.

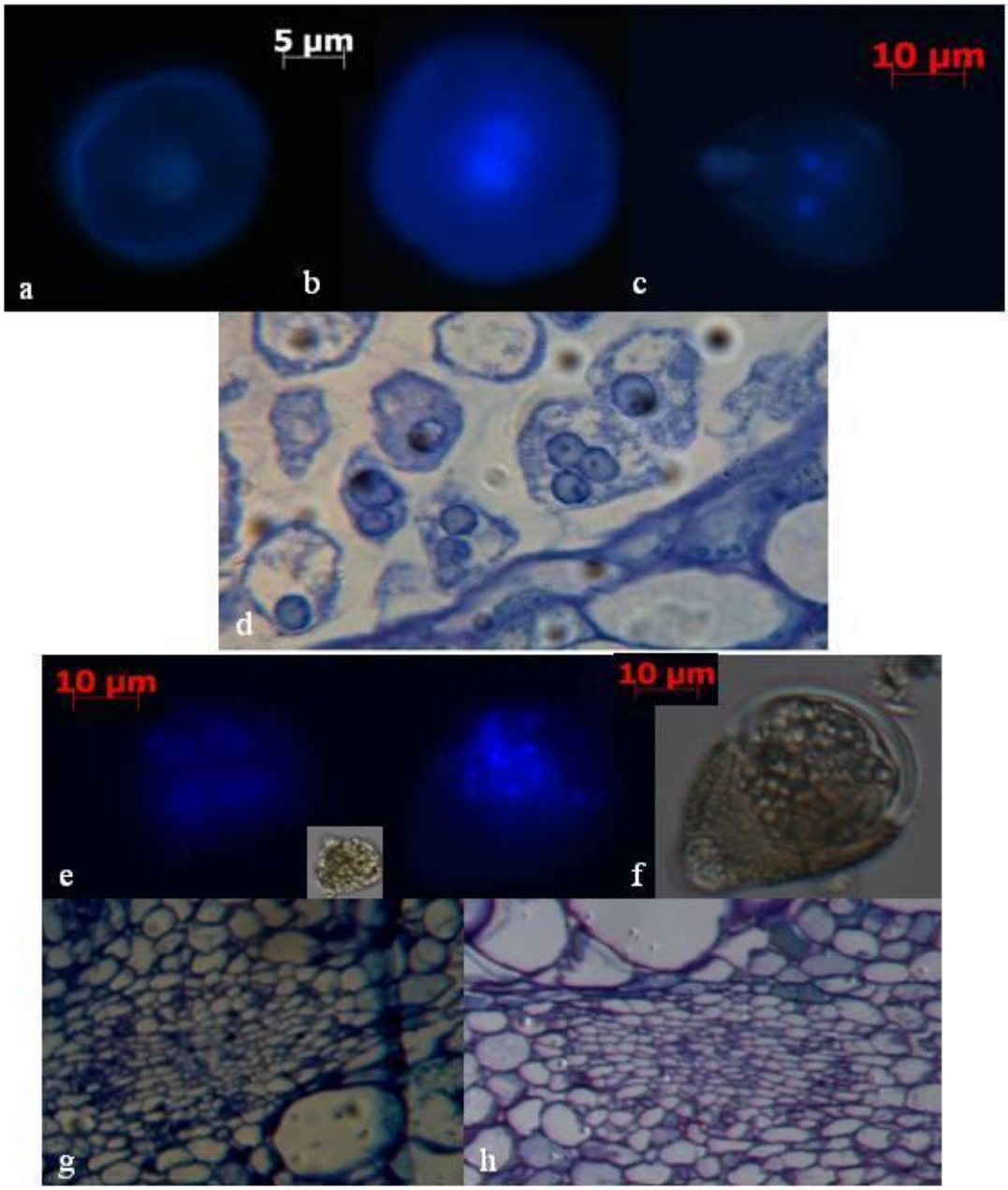


**Fig. 2.6 (a) Vacuolate microspore at the initiation of *in vitro* culture, DAPI staining; (b) microspore-derived two-cell structures after two weeks of culture; (c) tri-nucleated microspore after 1 month; (d) Anther section of cultivar Verdello. Uni, bi and trinucleated microspores (Toluidine blue staining); (e) tetra-nucleated structure after two months; (f) multinucleated like embryo structure; DAPI staining, microspore culture, cv. Nocellara del Belice. (g) proembryo structure surrounded by callus cells; (h) detail of proembryo structure derived after four months; cv. Tonda Iblea, anther culture; Toluidine blue staining.**

# 3 Loquat (*Eriobotrya japonica* Thunb.) Lindl.

**First Stages of Microspore Reprogramming to Embryogenesis Through Isolated Microspore Culture in *Eriobotrya japonica* (Thunb.) Lindl.**

## 3.1 Introduction

*Eriobotrya japonica* (Thunb.) Lindl., popularly known as 'loquat', is a subtropical evergreen fruit tree that was introduced from China to Japan in ancient times (XII cent.) and, later, to Europe (XVIII cent.). This crop showed a successful adaptation to the Mediterranean climate and now it was widely grown in regular orchards. Currently, Spain accounts for more than 50% of the total European production and some other countries, such as Italy and Israel, are also commercial producers (Gisbert et al., 2009). Loquats are consumed largely as fresh fruit, although small amounts are used in the preparation of jams, jellies, syrups, and pies (Lin et al., 1999).

*Maloideae*, including loquat, are functional diploids ($2n = 2x = 34$) for which an alloploid origin has been suggested (Lu and Lin, 1995). The prevalent heterozygosity and the absence of pure lines make selection and the genetic studies rather difficult to conduct in loquat. The perspective of raising haploid plants through *in vitro* isolated microspore culture, thus offers scores of foreseeable advantages, like shortening of the breeding period, production of homozygous diploid lines in a single step, through chromosome doubling, and isolation of valuable recessive traits at sporophytic level, that otherwise, remain accumulated and unexpressed in natural heterozygous diploid population (Srivastava and Chaturvedi, 2008). There have been reports on the establishment of plant regeneration system and haploid production through embryogenesis from anther culture of loquat (Germanà et al., 2006, Li et al., 2008). Therefore, experiments were undertaken to develop a reproducible method for regenerating doubled haploid (DH) plants of loquat, using, for the first time, isolated microspore culture. As part of this study, the results on first stages of microspore reprogramming to embryogenesis, through isolated microspore culture in loquat are reported.

## 3.2 Material and methods

### 3.2.1 Plant material

Terminal racemose inflorescences were collected in October-November 2009, from about 16-year-old trees of *Eriobtrya japonica*, cv. Algerie, BRT 20, Claudia, La Mantia, Nespolone di Trabia, Peluche, Sanfilippara, Tanaka and Virticchiara, grown at a collection field in Palermo, Italy. The inflorescences were kept in a plastic culture jars with approximately 50 ml of distilled water, to maintain high humidity and at 4°C in dark for about one week to provide a cold pre-treatment.

### 3.2.2 Bud size and microspore stage correlation

The relationship between the bud size and the microspore development stage was studied before initiation of microspore culture experiments. Flower buds were collected randomly from the plant and were graded into four bud sizes ranging and measured. In order to study the effect of the growth stage of the donor plant, the buds were collected and anthers from two buds were fixed in 200 µL alcohol: acetic acid (3:1) for 10 min, transferred and macerated in 200 µL Tris-Triton solution [Tris HCl (50 mM) + Triton X100 (1%)] to release the microspores. The macerated preparation, stained with 10 µL of 4,6- Diamidino-2-phenylindole (DAPI) solution (0.001 µM DAPI in Tris-Triton solution) was observed under UV florescence microscope (Zeiss, Axiophot, Germany). The cell counts were taken for different stages of microspores (as described by Telmer et al. 1992) at 20X magnification. The percentage of late uninucleate (LU) microspores were calculated by dividing the number of LU microspores by the total number of microspores observed under three different fields for each slide.

### 3.2.3 Microspore isolation and culture

Selected unopened flower buds were surface-sterilized by immersion in 70% (v/v) ethanol for 5 min, than in sodium hypochlorite solution (0.5% v/v) for 20 min, and finally, rinsed three times with sterile distilled water. Anthers from about 80 flower buds were collected with 10 ml of isolation medium containing Brewbaker and Kwack (1963) salts, mannitol at 7% and 500 mg/L ascorbic acid. Microspores were isolated as describe before in olive and, after last centrifugation, the pellet was re-suspended in the induction medium.

The induction medium composition was the following: N6 Chu (1978) medium added with 5% (w/v) sucrose (unless otherwise mentioned), 500 mg/L of ascorbic acid, 1.0 mg/L

of 2,4-D, 1.0 mg/L of indole-3-acetic acid (IAA). To study the influence of cytokinin to the gametic embryogenesis in loquat, isolated microspore were cultured on seven media: four containing alternatively 1.0 mg/L of 6-Benzylaminopurine (BA), Kinetin (KI), Zeatin (ZEA) or Thidiazuron (TDZ) and the others with three concentrations of TDZ 0.25, 0.5 and 1.0 mg/L. Moreover, the influence of the carbon source was studied adding to the induction medium containing 1.0 mg/L of ZEA, instead of the sucrose, the maltose at the same concentration (5%). Microspores were plated at a density of $10^6$microspores/ml (1 ml volume in 35 × 10 mm diameter sterile Petri plate) in various culture media and incubated at 25°C in the dark (Table 3.1).

**Table 3.1: Composition of the eight different media.**

| | Medium | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Components | M1 | M2 | M3 | M4 | M5 | M6 | M7 | M8 |
| N6 Chu Salts | x | x | x | x | x | x | x | x |
| MS Vitamins | x | x | x | x | x | x | x | x |
| Sucrose | 5% | | 5% | 5% | 5% | 5% | 5% | 5% |
| Maltose | | 5% | | | | | | |
| 2,4-D | 1mg/l | 1mg/l | 1mg/l | 1mg/l | 1mg/l | 1mg/l | 1mg/l | 1mg/l |
| Ascorbic Acid | 500mg/l | 500mg/l | 500mg/l | 500mg/l | 500mg/l | 500mg/l | 500mg/l | 500mg/l |
| BA | | | 1mg/l | | | | | |
| Zeatin | 1mg/l | 1mg/l | | | | | | |
| TDZ | | | | | 0mg/l | 0,25mg/l | 0,5mg/l | 1mg/l |
| Kinetin | | | | 1mg/l | | | | |
| pH | 5,8 | 5,8 | 5,8 | 5,8 | 5,8 | 5,8 | 5,8 | 5,8 |

### 3.2.4 Fixation and processing for light microscopy observation

Microspores and microspore derived structures developed in the culture were processed for microscopy analysis at specific time points. Samples were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.3, overnight at 4°C. After fixation, they were gently centrifuged and the pellets containing the cells were embedded in 15 % gelatine for further dehydration and resin embedding. After washing in PBS, samples were dehydrated through an acetone series. Then, the samples were infiltrated and embedded in Techhnovit 8100 resin (Kulzer, Germany) at 4°C. Technovit semi-thin

sections (1 µm) were stained with toluidine blue and observed under bright field of light microscope for structural analysis.

To determine the viability, microspores were stained with fluorescein diacetate (FDA) solution (1.25 mg/ml) and observed under a fluorescent microscope (Zeiss, Axiophot, Germany).

### 3.2.5 Fixation and processing for scanning electron microscope observation

Microspores cultured on various culture media and various development stage were fixed in 4% paraformaldehyde as described before and stored at 4°C .

Then, samples were washed in distilled water three-times (each for 15 min.) and dehydrated in a graded acetone series (10%-15 min., 30% -15 min, 50% -15 min., 70%-15 min., 95%-15 min) and after samples were washed three times 100% acetone each 15 min., and stored overnight in cold 100% acetone.

Samples were mounted on SEM stubs by using doubled-sided tape, sputter coated with gold and examined in a Phillips FEI Phillips scanning electron microscope.

## 3.3 Results and discussion

### 3.3.1 Flower bud size and microspore developmental stage

The present study focused on determining the flower bud size in nine cultivars of loquat at four typical microspore developmental stages i.e., tetrad, mid uninucleate, late uninucleate and bicellular stages using DAPI staining. In tetrad phase (Fig. 3.1a), the microspore cells were encapsulated by callose walls and arranged almost tetrahedrally. The microspores released from the tetrads were irregular and became sub-round soon, with less evident nucleus (Fig. 3.1b). The subsequent stage was the late uninucleate, with evident nuclei (Fig. 3.1c). Afterwards, in the microspores one mitotic division which produced one bigger vegetative nucleus and one smaller generative one was observed (Fig. 3.1d).

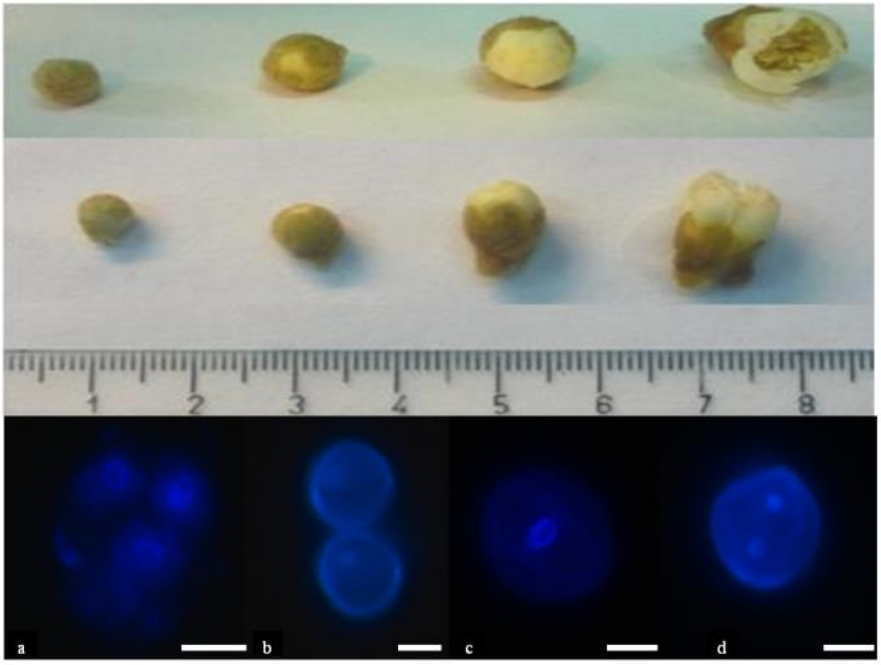


**Fig. 3.1: Cv Algerie microspores with tetrad (a), vacuolated uni (b,c), bi nucleate (d) development stages stained by DAPI observed under fluorescence microscopy under UV excitation and size of flower buds; bars represent 10µm**

The correlation studies suggested that flower bud size with uninucleate microspore development varied among loquat cultivars tested. Bud length around 6 mm (Virticchiara), 7 mm (Algerie, BRT 20, Nespolone di Trabia, Sanfilippara), 8 mm (Peluche, Claudia) and 9 mm (La Mantia, Tanaka) corresponded to microspores at the uninucleate stage of development (Table3.2). The correlation between sequential flower bud lengths and pollen developmental stages has previously been well established, constituting a useful criterion for handling and selection of specific stages for the microspore culture also in olive (Solis et al., 2008). Therefore, those flower buds with uninucleate development stage were selected as the most responsive developmental stage for the culture in loquat.

**Tab. 3.2: Flower bud size and microspore developmental stage**

| Genotype | Very Young Flower bud | | Young Flower bud | | Flower bud (about to open) | | Flower after anthesis | |
|---|---|---|---|---|---|---|---|---|
| | Flower size (mm) | Nuclear state | Flower size (mm) | Nuclear state | Flower size (mm) | Nuclear state | Flower size (mm) | Nuclear state |
| Virticchiara | 4.0- 4.9 | Tetrad | 5.0-5.9 | Uninucleate | 6.0-7.9 | Late Uninucleate | >8.0 | Bicellular |
| Peluche | 4.0-5.9 | Uninucleate | 6.0-6.9 | Late Uninulceate | 7.0-8.9 | Bicellular | >9.0 | Bicellular |
| Alghérie | 4.0-6.9 | Uninucleate | 7.0-7.9 | Bicellular | 8.0- 8.9 | Bicellular | >9.0 | Bicellular |
| Lamantia | 5.0-6.9 | Tetrad | 7.0-7.9 | Uninucleate | 8.0-8.9 | Late Uninulceate | >9.0 | Bicellular |
| Tanaka | 4.0-5.9 | Tetrad | 6.0-6.9 | Uninucleate | 7.0-8.9 | Late uninucletae | >9.0 | Bicellular |
| Claudia | 4.0-6.9 | Uninucleate | 7.0-8.9 | Late Uninucleate | 9.0- 9.9 | Bicellular | >10.0 | Bicellular |
| NDT | 4.0-5.9 | Tetrad | 6.0-6.9 | Uninucleate | 7.0-8.9 | Bicellular | >9.0 | Bicellular |
| Sanfillippara | 4.0-5.9 | Tetrad | 6.0-6.9 | Uninucleate | 7.0-8.9 | Bicellular | >9.0 | Bicellular |
| BRT | 4.0-5.9 | Uninucleate | 6.0-6.9 | Late Uninucleate | 7.0-8.9 | Bicellular | >9.0 | Bicellular |

Tests performed by FDA staining after the pre-treatment, revealed viability between 70-90% (Fig. 3.2). After 7 weeks of incubation, the greatest viability (50% in Algerie) was observed in culture medium supplemented with sucrose and BA.

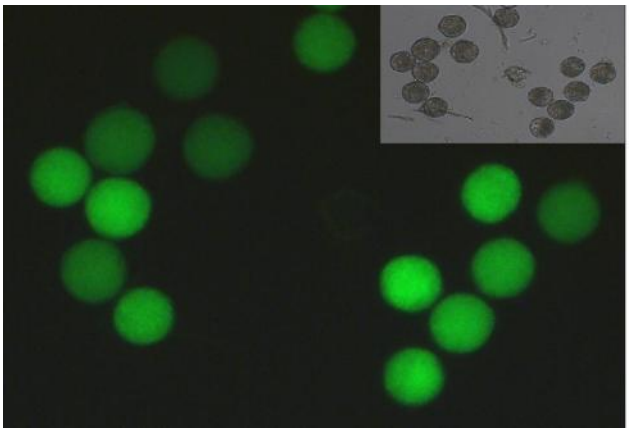

**Fig. 3.2: Microspores stained by FDA**

## 3.3.2 Microspore development in culture

Microspores of loquat isolated from flower buds of inflorescences exhibited a good competence to undergo embryogenesis after one week of cold treatment. Treatments of anthers or whole flowers with low temperature, for various lengths of time, prior to culture have been already applied in loquat (Germanà et al., 2006, Li et al., 2008). Cold treatment

is easy to practice and has been used to induce embryogenesis from isolated anthers as it seems to disrupt the cytoskeleton in microspores in the initial phase (Ferrie et al., 1995).

To study the pattern of cell division that takes place during early embryo formation from microspores, samples were taken from cultures at regular intervals and observed with DAPI staining to visualize the nuclei. Fluorescence microscopy analysis revealed structures with two (Fig. 3.3a) or three nuclei (Fig. 3.3b,c), still surrounded by the exine, as revealed by corresponding differential contrast image (DIC) of the same structure. The two nuclei of these microspores were similar in size, shape and chromatic condensation, suggesting that they were formed by a symmetrical division. It is widely accepted that the first morphological evidence of the embryogenic pathway is the symmetric division of the microspore, opposed to the asymmetric one, characteristic of the gametophytic pathway (Zaki and Dickinson, 1991) (Fig. 3.3c). Microspores with three nuclei also exhibited nuclei with similar size, shape and organization, which could indicate that the second division occurred asynchronically in the two previous nuclei, one of them dividing first, as previously reported in olive (Solis et al., 2008).

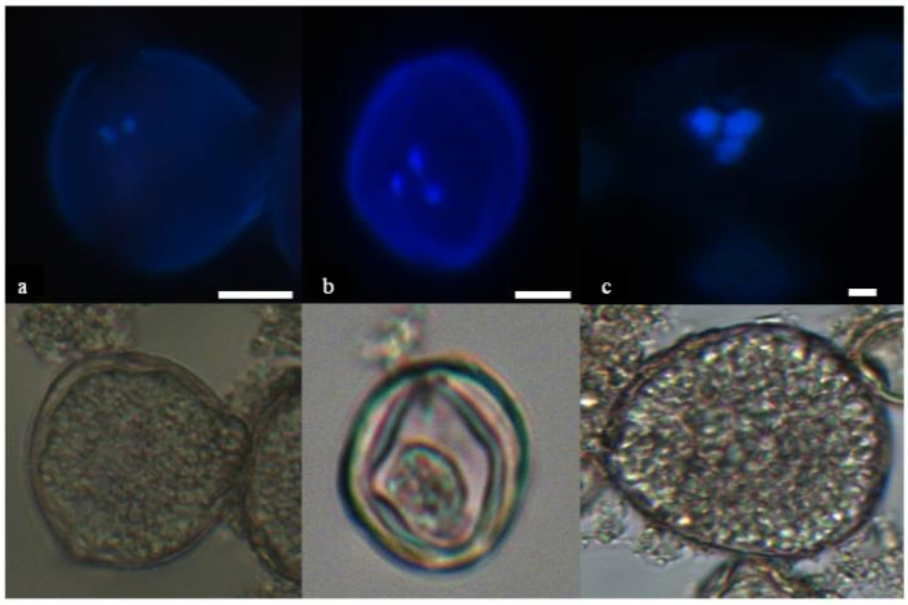


**Fig. 3.3: Microspores of cv Algerie; nuclei stained by DAPI; bars represent 10µm**

Multicellular and multinucleate pollen-derived structures, globular and irregular, were observed during the 3rd and the 4th month of culture, indicating a high asynchrony in the microspore embryogenic response (Fig 3.4). This fact illustrates that the reprogramming of the loquat microspores was started by the reactivation of proliferative activity, that is the initial event of the switch to embryogenesis.

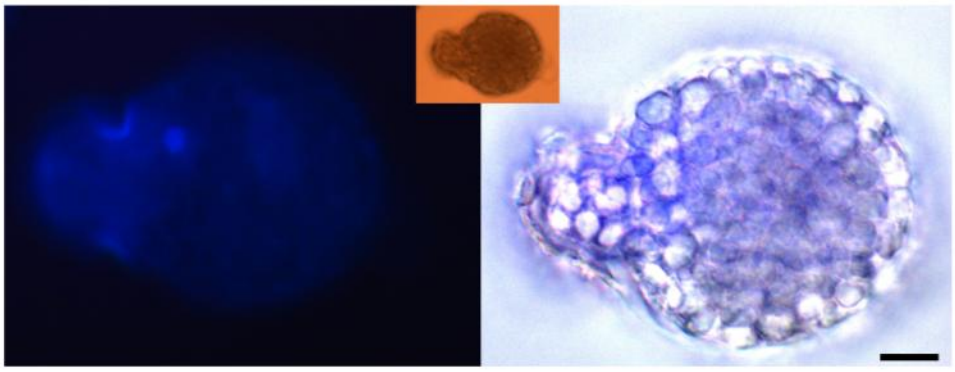

**Fig. 3.4 Multinucleated pollen-derived pro-embryo of cv Tanaka after 1 year; bars represent 10µm**

The observations performed on microspores in culture showed that embryogenic microspores were larger in size than non-embryogenic ones. In fact, microspores were about 15-17 µm at the beginning of culture and grew to 22-30 µm after 4 weeks.

After two months in culture, microspores show a response to the culture in term of dimension: in fact, in all the cultivars tested, microspores increased the size if compared with no responding (table 3.3). No statistically significant differences were observed among cultivars regarding size increasing, but Nespolone di Trabia, Sanfilippara and Virticchiara showed the biggest microspore, respectively 36.6µm, 38.0µm and 37.5µm in three different culturing media, containing the same carbon source (sucrose) but different kinds of cytokinins (zeatin, kinetin and TDZ).

The results obtained show as the response of loquat microspore to *in vitro* culture is highly medium-dependent and also genotype-dependent, as previously observed in other genotypes.

**Tab. 3.3 Diameter measure of microspores in culture observed after 2 months**

| media<br>size | M1<br>µm | M2<br>µm | M3<br>µm | M4<br>µm | M5<br>µm | M6<br>µm | M7<br>µm | M8<br>µm |
|---|---|---|---|---|---|---|---|---|
| Algerie | 27.9 | 22.1 | 24.5 | 25.6 | 21.2 | 25.2 | 28.5 | 28.8 |
| BRT 20 | 29.1 | 27.1 | 28.3 | 29.3 | 24.9 | 26.6 | 28.5 | 28.8 |
| Claudia | 34.5 | 31.3 | 34.4 | 26.5 | 30.4 | 27.9 | 27.9 | 27.1 |
| La Mantia | 20.7 | 22.4 | 26.5 | 28.3 | 22.3 | 26.0 | 26.5 | 28.3 |
| Nespolone.di Trabia | 33.2 | 22.1 | 23.4 | 36.6 | 20.6 | 31.2 | 28.0 | 28.2 |
| Peluche | 31.1 | 23.3 | 29.5 | 31.6 | 21.3 | 22.3 | 27.0 | 27.1 |
| Sanfilippara | 29.3 | 22.1 | 27.1 | 27.5 | 22.1 | 25.5 | 38.0 | 38.8 |
| Tanaka | 27.5 | 21.3 | 29.4 | 28.1 | 21.1 | 23.1 | 29.8 | 28.8 |
| Virticchiara | 37.5 | 29.5 | 30.6 | 34.9 | 30.9 | 30.6 | 31.6 | 35.6 |

Under the same culture conditions, microspores showed both direct and indirect types of morphogenesis. After 4 weeks of culture, the responding microspores produced yellowish/white callus (Fig. 3.5). Among the nine cultivars tested, five cultivars, La Mantia, Sanfilippara, Peluche, Algerie and Tanaka, produced callus from microspores. The highest response of callus was detected in Sanfilippara, followed by La Mantia, Peluche, Tanaka and Algerie in the decreasing order. As previously observed in loquat and other species, such as *Citrus*, the loquat microspore embryogenesis is genotype-dependent, meaning that different results have been obtained from different cultivars of the same species under the same cultural conditions (Germanà, 1997; Germanà et al., 2006).

The callus growth was observed up to 4 months indicating that the potential for microspore development lasts for a considerable period of time. Further experiments are in progress to transfer microspore derived calli to media devoid of growth regulators, for achieving the differentiation into multicellular structures, proembryos and plantlets.

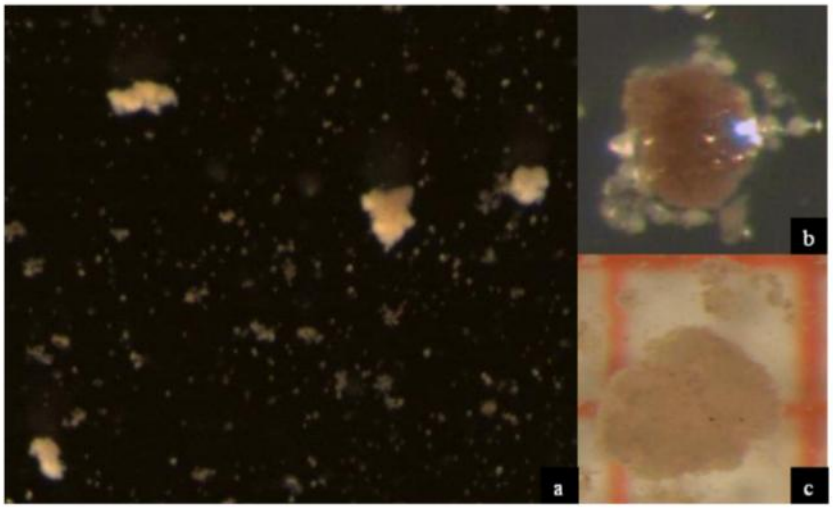


**Fig. 3.5 Callus of cv La Mantia in liquid medium after four weeks (a). Callus of cv Sanfilippara transferred in solid medium (b,c).**

### 3.3.3 Changes in cellular organization

Samples of the in vitro culture fixed and processed for further microscopical analysis, revealed that microspore-derived structures developed in the cultures, showing differential features. The selection of the processing method is a key point for structural studies, specially in the case of woody species (Ramírez et al. 2001, 2004). Formaldehyde fixation, low temperature processing and Lowicryl resin embedding have shown to be convenient for structural analysis of anther culture development in several trees, such as *Quercus*

(Ramírez et al. 2001, 2004), *Citrus* (Ramírez et al. 2003), and in this work for the first time, in *Erybotria*.

In toluidine blue stained sections and in SEM pictures, embryogenic microspores displayed rounded shapes and larger sizes than non-responsive microspores. Some of the microspore-derived structures showed ellipsoid shape and the exine, the special pollen wall, appeared broken in defined points and the cell contents grew out of this wall, forming a microspore-derived structure similar to a proembryo (Fig. 3.6). The presence of microspores and microspore derived-structures with different features not typical of the gametophytic pathway suggested that there were changes in the cellular organization, characteristics of the switch of developmental program towards embryogenesis (Ramirez et al., 2003), and similar to those found in loquat anther cultures (Germanà et al., 2006).

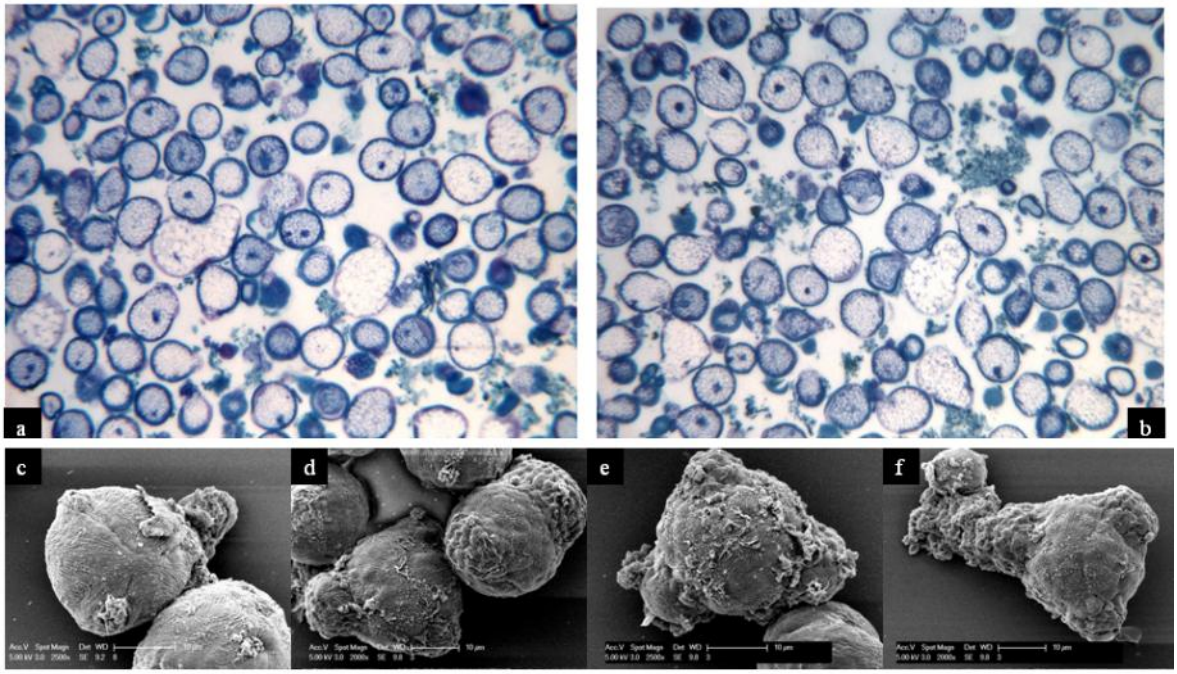


**Fig. 3.6 Cellular monitoring during *in vitro* microspore culture. Toluidine blue staining (a,b). Microspores on scanning electron microscope (c-f).**

Basic studies on the process of microspore embryogenesis have been mainly pursued in model species, like rape seed, tobacco and barley, but reports are very limited on other economically interesting crops and trees (Solis et al., 2008, Germanà et al., 2006). The present study reports for the first time the formation of multicellular microspores and main changes in cellular organization during the first stages of microspore embryogenesis in isolated microspore culture of loquat. This knowledge in a tree of economic interest like

loquat, could guide future strategies to induce gametic embryogenesis and regeneration of homozygous plants to improve and speed the breeding.

# 4 Genetic diversity evaluation of a Sicilian loquat germplasm collection by molecular markers.

## 4.1 Introduction

Loquat [*Eriobotrya japonica* (Thunb.)Lindl.] belongs to the *Rosaceae* family and is one of the few subtropical representatives. The Dadu River Valley, in southwestern China, is probably the center of origin of the genus *Eriotobrya* (Zhang et al., 1990). Loquat was introduced from China to Japan in ancient times (XII cent) and to Europe much more later (s. XVIII, Lin et al., 1999). The crop showed a successful adaptation to the Mediterranean climate and since the beginning of the twentieth century was grown in regular orchards.

Loquat has formed various ecological types in different zones over the course of its cultivation and acclimatization. Diversity within the species comes from seed propagation or bud spots which occur very often within the genus, although some of the mutations are not stable. Cultivation of the species has led to a large number of cultivars, due to different selection pressures applied by growers.

Now, several local loquat cultivars are currently threatened to disappear, because they are replaced by more profitable crops or because only some suitable cultivars were convenient with the market demand. Thus, severe genetic erosion concerns were rising and to preserve these genetic resources, threatened cultivars should be submitted to reliable conservation and selection programs.

In previous works, genetic diversity on loquat cultivars was assessed in different ways, including phenological observation and molecular markers derived from *Rosaceae* species (Zhang et al., 1990; Badenes et al., 2000; Martinez-Calvo et al., 2006; Soriano et al., 2005, Llacer et al., 2008; Gisbert et al., 2009 a,b). In particular, AFLP and microsatellites, in the last years, have become the markers of choice for multiple applications, proving to be particularly useful for integrating mapping results and assessing marker-gene association (Silfverberg-Dilworth et al., 2006). In *Rosaceae*, SSR and AFLPs markers have been shown to be extremely valuable for comparing maps from different genere exploiting their high transferability (Yamamoto et al., 2004). The development of genetic maps, based on molecular markers, will be useful tool to employ marker-assisted selection (MAS) within an improvement program.

The largest collection of loquat germplasm, more than 250 cultivars, is located in Fuzhou, China (Badenes et al, 2008). The second important germplasm collection is maintained at the National Institute of Fruit Tree Science in Japan (http://www.fruit.affrc.go.jp). The European loquat germplasm collection is held at the Instituto Valenciano de Investigaciones Agrarias (IVIA), in Spain. This collection was built from surveys made in the Mediterranean countries under the framework program GENRES, section of underutilized fruits (Badenes et al, 2004).

In this work, in order to evaluate the genetic diversity presents of a Sicilian loquat germplasm collection, 16 SSRs derived from Gisbert et al. (2008) and AFLPs loci originated from four different combinations of selective combinations of primers were used.

# 4.2 Material and methods

## 4.2.1 Plant material

Young leaves were collected in October-November 2009, from about 16-year-old trees of *Eriobtrya japonica* from a germplasm collection in Palermo (N 38° 4.657' E 013° 22.437'), Italy. Six shoot leaves with new leaves were collected and stored in a paper bags and kept at 8°C. Leaves were then separated from branches and quickly frozen at -80 °C until use.

For the genetic diversity evaluation, 20 cultivars (1 from Japan, 2 from USA, 13 from Italy and 4 from Spain) of loquat from different origins were used (Table 4.1).

**Table 4.1 Loquat accessions evaluated in the germplasm study, country and origin**

| Country | Name of cultivar | Origin |
|---|---|---|
| Japan | Tanaka[a,d] | A seedling originated in Japan. Introduced in USA in 1902 |
| Italy | 2L | Unknown |
| | 7L | Unknown |
| | BRT 20 [a] | Local seedling selected by Prof. Calabrese |
| | BRT 44 [a] | Local seedling selected by Prof. Calabrese |
| | Claudia [d] | Unknown |
| | Fiore [a] | Local seedling selected by Prof. Calabrese |
| | La Scala [a] | Unknown |
| | La Mantia [a] | Probably Algerie mutation. |
| | Marcenò | Unknown |
| | Marchetto[c] | Unknown |
| | Nespolone di Trabia[c] | Unknown |
| | Sanfilippara[a] | Unknown |
| | Virticchiara[a] | Unknown |
| Spain | Algerie (Altea)[b] | Unknown. Seedling selected in Algeria. Introduced in Spain in the 60´s |
| | El Buenet[a] | Unknown. Local cultivar (Alicante province) |
| | Bueno [a] | Unknown. |
| | Peluches[b] | Probably Algerie mutation. Local cultivar (Alicante province) |
| USA | Early Red | California |
| | Golden Nugged [d] | California |

[a] Calabrese et al., (2000)
[b] Martı´nez-Calvo et al. (2000)
[c] Martı´nez-Calvo et al. (2006)
[d] Martı´nez-Calvo et al. (2008)
[e] Morton (1987)

### 4.2.2 DNA Isolation

The solutions used for DNA isolation and processing are resumed in Table 4.4.

The isolation of high quality DNA from loquat leaves is particularly difficult because of their coriaceous nature and their content of phenolic compounds. DNA was extracted from 200 mg of young leaves following the method of Doyle and Doyle (1987) with some

modifications. Whole leaves were quickly washed in distilled water, frozen in liquid nitrogen and ground with a mortar and pestle, until a fine powder was obtained. The powder was transferred to a plastic tube (50ml) and after sublimation of the dry ice, 20 ml of lysis buffer at 65°C were added to each tube (Table 4.4). The suspensions were then incubated at 65°C during 45 min, with occasional mixing.

The lysed homogenate was extracted twice with an equal volume of chloroform isoamyl alcohol solution and centrifuged at 13000 rpm at 4°C for 15 min. Chloroform isoamyl alcohol binds to protein and lipids of cell membrane, dissolving them and making them precipitate. The aqueous phase was transferred to a new tube. DNA was precipitated at -20°C for at least 1 hour with 1 volume (20 ml) of cold isopropanol (Table 4.4). After centrifugation at 13000 rpm for 15 minutes, the pellet DNA was washed with 1 volume of absolute ethanol and centrifuged at 10000 rpm for 7 minutes. The pellet was dried under vacuum for one hour and then suspended in 200 µl ml TE buffer, pH 8.0. DNase-free RNase A (final concentration 0.2 mg/ml) was added and incubated at 37°C for 30 minutes (Table 4.4).

DNA quantification was performed with agarose gel (1%) in 0.5 X TBE buffer under constant voltage of 120V and by comparison with lambda DNA (Promega, Madison, WI) with 260nm wavelength by UV-spectrophotometer and stored at -20 °C.

### 4.2.3 Microsatellites (SSR) markers

A total of 16 SSR polymorphic microsatellite loci developed for *E. japonica* by Gisbert et al. (2008) have been tested (Table 4.2). Each polymerase chain reaction was performed with three primers: the specific forward primer of each microsatellite with M13(-21) tail at its 5′ end, the sequence-specific reverse primer, and the universal fluorescent-labeled M13(-21) primer (Schuelke 2000).

SSR amplifications were performed in a GeneAmp®PCR System 9700 thermal cycler (Perkin-Elmer Corp., Freemont, CA) in a final volume of 20 µl, containing 1× PCR, 1.5 mM MgCl2, 0.1 mM of each dNTP, 0.8 µM of each primer, 20 ng of genomic DNA and 1 U of Taq polymerase (Invitrogen), using the following temperature profile: 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, the optimized annealing temperature for 60 s and 72 °C for 1min and 30 s, finishing with 72 °C for 7min, as described by Gisbert et al. (2009a,b).

Allele lengths were determined using an ABI Prism 3130 Genetic Analyzer with the aid of GeneMapper software, version 4.0 (Applied Biosystems).

**Table 4.2: The 16 primers pairs used for the identification of genotypes and variability parameter values estimated for each SSR**

| Acronym | Repeat | No allele | Range (bp) | He | Ho | F | PD |
|---|---|---|---|---|---|---|---|
| *ssrEJ005* | $(AG)_{12}$ | 2 | 214-216 | 0.51 | 0.20 | 0.61 | 0.64 |
| *ssr*EJ012 | $(AG)_{19}$ | 3 | 224-239-242 | 0.53 | 0.40 | 0.25 | 0.57 |
| *ssr*EJ037 | $(AG)_{19}$ | 2 | 233-248 | 0.32 | 0.30 | 0.07 | 0.50 |
| *ssr*EJ039 | $(GA)_{12}$ | 2 | 236-242 | 0.48 | 0.35 | 0.27 | 0.48 |
| *ssr*EJ042 | $(GA)_{28}$ | 3 | 202-205-214 | 0.58 | 0.50 | 0.13 | 0.56 |
| *ssr*EJ049 | $(GA)_{27}$ | 2 | 214-291 | 0.10 | 0.10 | -0.03 | 0.35 |
| *ssr*EJ056 | $(AG)_{8}$ | 2 | 291-304 | 0.32 | 0.10 | 0.69 | 0.35 |
| *ssr*EJ075a | $(GA)_{11}$ | 2 | 222-228 | 0.36 | 0.35 | 0.02 | 0.47 |
| *ssr*EJ086 | $(GA)_{15}$ | 3 | 231-238-245 | 0.48 | 0.60 | -0.26 | 0.43 |
| *ssr*EJ088 | $(GA)_{15}$ | 3 | 215-231-245 | 0.59 | 0.75 | -0.26 | 0.46 |
| *ssr*EJ095b | $(GA)_{23}$(CT GTGT)$_3$ | 2 | 239-243 | 0.36 | 0.45 | -0.26 | 0.50 |
| *ssr*EJ104 | $(GA)_{21}$ | 3 | 162-165-168 | 0.41 | 0.40 | 0.02 | 0.86 |
| *ssr*EJ271 | $(AG)_{24}$ | 5 | 192-194-197-231-247 | 0.68 | 0.65 | 0.04 | 0.81 |
| *ssr*EJ282 | $(AG)_{12}$AA( AG)$_{11}$ | 4 | 154-162-185-187 | 0.64 | 0.55 | 0.14 | 0.72 |
| *ssr*EJ324 | $(AG)_{23}$ | 4 | 233-242-246-258 | 0.57 | 0.65 | -0.14 | 0.55 |
| *ssr*EJ329 | $(AG)_{19}$ | 3 | 154-157-175 | 0.34 | 0.40 | -0.018 | 0.47 |

### 4.2.4 AFLPs markers

A 250 ng sample of genomic DNA was digested (65°C for 3.5 hours) with 7 units of Tru9I restriction enzyme (recognition sequence: T/TAA; Roche Diagnostics GmbH, Germany) in M buffer (100 mM Tris-HCl, 500 mM NaCI, 100 mM MgCl2, 10 mM Dithioerythritol, pH 7.5), in a total volume of 15 µl, following by second digestion (37°C for 3.5 hours) with 5 units of EcoRI restriction enzyme (recognition sequence: CTGCA/G; Roche Diagnostics GmbH, Germany) in H buffer (500 mM Tris-HCl, 1 M NaCI, 100 mM MgCl2, 10 mM Dithioerythritol, pH 7.5).

The total sample obtained was incubated with T4 DNA ligase (T4 DNA Ligase; Roche Diagnostics GmbH, Germany) with Mse and Pst adapter in 1×ligation buffer (50 mM Tris-HCl, 10 mM dithioerythritol, bovine serum albumin, 500 µg/ml, pH 7.6) and the mixture of the resulting products was diluted 1:5 with double distilled water.

In order to reduce of 1/4 the number of fragments, a pre-selective PCR was performed using 10 µM of each MSE-C and ECO-A primes in a total volume of 25 µl, containing 1×

PCR buffer, 1.0 ml of dNTPs (10 mM), and 0.8 units of Taq polymerase (Roche), using the following temperature profile: 94 °C for 2 min, then 20 cycles of 94 °C for 20 s, 56°C for 30 s and 72°C for 25 s finishing with 72 °C for 30 minutes.

After pre-amplification, DNA was diluted again 1:10 in TE buffer. The selective amplification was performed on 20 µl aliquots using four additional selective combinations of primers (Table 4.3). DNA fragments were separated in an ABI Prism 3130 genetic analyzer (Applied Biosystems).

**Table 4.3 Restriction endonuclease used for the AFLP analysis**

| MseI | Pst I | Bands |
|---|---|---|
| Mse – CAG | Pst – AGG | 39 |
| Mse – CAG | Pst – ACA | 35 |
| Mse – CC | Pst – ACG | 30 |
| Mse – CTA | Pst – AGC | 29 |
| | Total: | 133 |

## 4.2.5 Loading and electrophoresis on fragment analyser

3 µl of PCR products were diluted with 20µl of milliQ water and 3ul of this solution were added with 12µl of formamide and 0.3 µl GeneScan™ 600 LIZ® Size Standard (Applied Biosystems, LLC), put to 95°C for 3 minutes, and then quickly on ice.

The sample tubes were placed in the 48-well sample tray; the software was set up and allele lengths were determined using an ABI Prism 3130 Genetic Analyzer with the aid of GeneMapper software, version 4.0 (Applied Biosystems) and resulting fragments were scored as binary traits (1 = present, 0 = absent) using GeneMapper v.4 software (Applied Biosystems) and data derived were analysed with Phylip 3.68, Genetix 4.05 and Hypertree software.

**Table 4.4 composition of the solutions used for DNA isolation and processing**

| |
|---|
| **CTAB 2%:** 1,4M NaCl: 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 0.14 M β-mercaptoethanol, CTAB 2%(w/w). |
| **Lysis buffer**: CTAB 2% with β-mercaptoethanol (0,2%) |
| **Chloroform-isoamyl solution**: CTAB 2% with chloroform 24:1; (v/v) |
| **CTAB buffer**: 100 mM Tris HCl pH 8.0, 20 mM EDTA |
| **RNase** A: 20 mg/mL (SIGMA). |
| **TE**: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. |
| **TBE** 5X: 54 g Tris, 27.5 g boric acid, 4.65 g EDTA per litre. |
| **M buffer** (100 mM Tris-HCl, 500 mM NaCI, 100 mM MgCl2, 10 mM Dithioerythritol, pH 7.5 |

### 4.2.6 Analysis of molecular data

Pairwise genetic similarities were estimated with the Dice (Sorensen) similarity coefficient Sij =2a/(2a1b1c), where a is the number of bands shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. The resulting genetic similarity matrices were used to generate a principal coordinates analysis (PCoA). Calculations were performed using the NTSYSpc2.0 software package (Applied Biostatistics Inc.).

Bootstrapped distance matrices were calculated using the Phyltools 1.32 software (Buntjer 1997) and used to test the stability of the neighbour joining tree constructed with the Phylip 3.6 package (Felsenstein 2005).

The following parameters were estimated, when possible, from the microsatellite marker data (Table 4.3): number of alleles per locus, expected heterozygosity ($He = 1-\Sigma p_i^2$, where $p_i$ is the frequency of the $i^{th}$ allele) (Nei, 1973), observed heterozygosity (*Ho*, calculated as the number of heterozygous genotypes divided by the total number of genotypes), fixation index ($F = 1-Ho/He$) (Wright, 1965) and the power of discrimination ($PD = 1-\Sigma g_i^2$, where $g_i$ is the frequency of the $i^{th}$ genotype) (Kloosterman et al, 1993). Putative alleles were named in alphabetical order starting with the largest size.

Genetic similarities were estimated using the Dice´s coefficient (Dice, 1945). The similarity matrix obtained was processed through the unweighted pair-group method (UPGMA) cluster analysis using the NTSYS program (Exeter Software, Setauket, N.Y.) (Rohlf, 1993) and finally depicted in a dendrogram. The goodness of fit of the cluster analysis was measured by the cophenetic correlation index. Nei distances (Nei and Li 1979) were calculated for 1000 bootstrapped data matrices using Phyltools 1.32 software (Buntjer 1997). Dice´s similarity values were also used to represent graphically genetic relationships among accessions by principal coordinates analysis (PCoA) (Gower, 1966) using the NTSYS software package.

## 4.3 Results

### 4.3.1 Polymorphism detected by SSRs

Twenty loquat cultivars were analyzed with 16 polymorphic SSRs. 45 alleles were clearly differentiated using the capillary electrophoresis sequencer and no discrepancies were found in the banding pattern of the duplicate analysis of each DNA sample. Allele sizes differed by two or more nucleotides, ranging from 154 to 304. Size differences detected between alleles at a locus ranged from 2 to 55 bp and was observed an average of 2.8 alleles per locus. Rare alleles (3) were also observed in the analysis.

UPGMA cluster analysis of the similarity matrix obtained considering the 45 SSR alleles resulted in a dendrogram of genetic relationship that grouped cultivars in general agreement with their geographic origins and pedigrees (Fig. 4.1). The cophenetic correlation value for this cluster analysis (r = 0.85) indicates a quite good fit, being statistically significant at the 1% level according to Lapointe and Legendre (1992).

### 4.3.2 Polymorphism detected by AFLP

Four AFLP primers combination were tested with loquat DNA, but only four selected primer combination colud produce clear and polymorphic bands. The AFLP analysis of 20 accessions with the this selected primer combinations generated 133 DNA fragments, of which 9 were monomorphic, yielding a polymorphism rate of 93.3%. The number of bands per primer pair ranged from 30 to 39.

## 4.4 Discussion

Information on the origin and relationship among cultivars is of great interest both for germplasm preservation and for breeding programs. Data analysis showed a very low degree of variability among the cultivars of loquat, as confirmed by Soriano et al. (2005), a high numbers of the average SSR alleles detected per locus (2.8), an expected heterozygosity mean of 0.45 and a power of discrimination mean of 0.54. These results demonstrate that SSRs are a more suitable tool for loquat cultivar identification than RAPD, which yielded 1.3 (Vilanova et al., 2001), and 1.9 (Badenes el al., 2004) alleles per primer on average.

There was enough variability found to distinguish all genotypes. However, some cultivars showed very similar marker pattern and this close genetic relationship agrees with

their putative origin. In particular, from the dendrogram, it can observed that Marcenò and Marchetto, La Scala and Sanfilippara, 7L and Tanaka, Peluche and Algerie are closed related(Fig.4.1). It means that genetically Marcenò derived Marchetto, La Scala could derive from Sanfilippara, 7L could derive from Tanaka and Peluche could derived from Algerie. In addition, Bueno and El Buenet are very close to Peluche, and in fact are very related to the Algerie group in terms of genetic distance (0.025) determinate by AFLP markers, that supports the hypothesis of common origin.

The fixation index mean (0.07) shows an excess of heterozygotes (Roughgargen 1979; Murray 1996; Perez de la Vega and Garcia, 2000), indicating that the behaviour of the collection studied as a whole is far from a Hardy-Weinberg equilibrium. This fact can be explained by genetic drift, such as mutation, outcrossing and overdominant selection (Soriano et al., 2005). In fact, although loquat is normally shelf pollinated, some cultivars need to be cross-pollinated by insects (Rodriguez 1983). Furthermore, most of the new selected genotypes originated by spontaneous mutation from known cultivars, suggesting that this phenomenon is quite common in loquat (Llacer et al., 2002; Martinez-Calvo et al., 2000).

The boostrapped tree derived from AFLPs placed La Mantia and 7L clearly separate from others cultivar, indicating these two cultivars are closely related which is in agreement with their origin: in fact, 7L is a seedling selection of La Mantia (Fig. 4.2).

In order to avoid the problem called "long branch attraction", that gives the impression that others accessions are more closed than reality, in the AFLP boostrapped tree La Mantia and 7L were eliminated. (Fig. 4.3)

These analysis grouped them in four main clusters that generally agree with their origin and pedigrees. The first cluster included Peluche and Bueno Spanish cultivars. The second included accessions probably originated from seeds or mutation "sports" from Golden Nugged (Virticchiara, Fiore, El Buenet and Marcenò), that originated from California and ancestrally form Japan, in according with Vilanova et al. (2001) that indicate a common origin of Virticchiara and Golden Nugged. The third cluster include typical Italian cultivars and the aspect and colour of the fruits (orange-red) suggests that were originated from the same ancestor, maybe from Early Red. The fourth one include accessions probably originated by spontaneous mutation from Algerie cultivar, suggesting that this phenomenon is quite common in loquat.

Zhang et al. (1990) observed an higher genetic diversity in Chinese than European accessions, suggesting China as centre of origin of the species. Later, Mogi cultivar was

introduced in Japan in 1840 and so loquat was diffused with the two main routes from Japan to the Mediterranean basin at the end of the 18$^{th}$ century, and to Florida from Europe and to California from Japan 100 years later (Lin et al., 1999). The plant material introduced could be different in each case, suggesting distinct origins for the two clusters.

This study showed that SSRs and AFLP molecular marker are suitable for loquat cultivar identification. However, for an in-depth study of genetic diversity within loquat species, it would be desirable to analyze a larger number of accession from different geographical area of Italy and of the world. But, just now it is clear that the Italian cultivars were originated from three different ancestrals of common origin, that afterwards originated Algerie, Golden Nugged and Early Red; more over, Italian and some Spanish cultivars could be due to one of the paths of the loquat to Spain from China was through Italy.

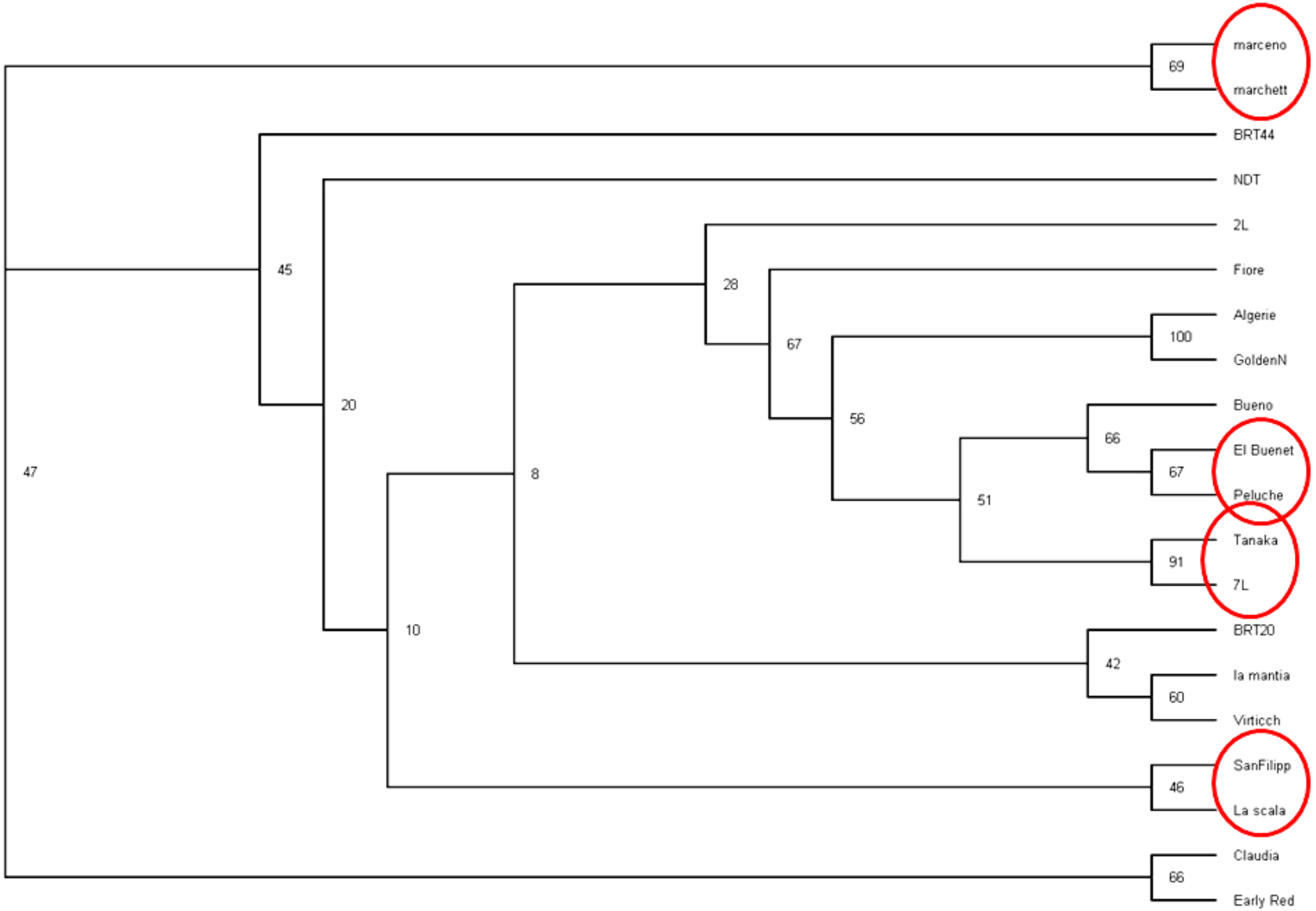


**Fig. 4.1: SSR Dendrogram of the 20 loquat cultivars included in this study generated by the unweighted pair-group method (UPGMA) cluster analysis from the similarity matrix obtained using Dice´s genetic distance.**

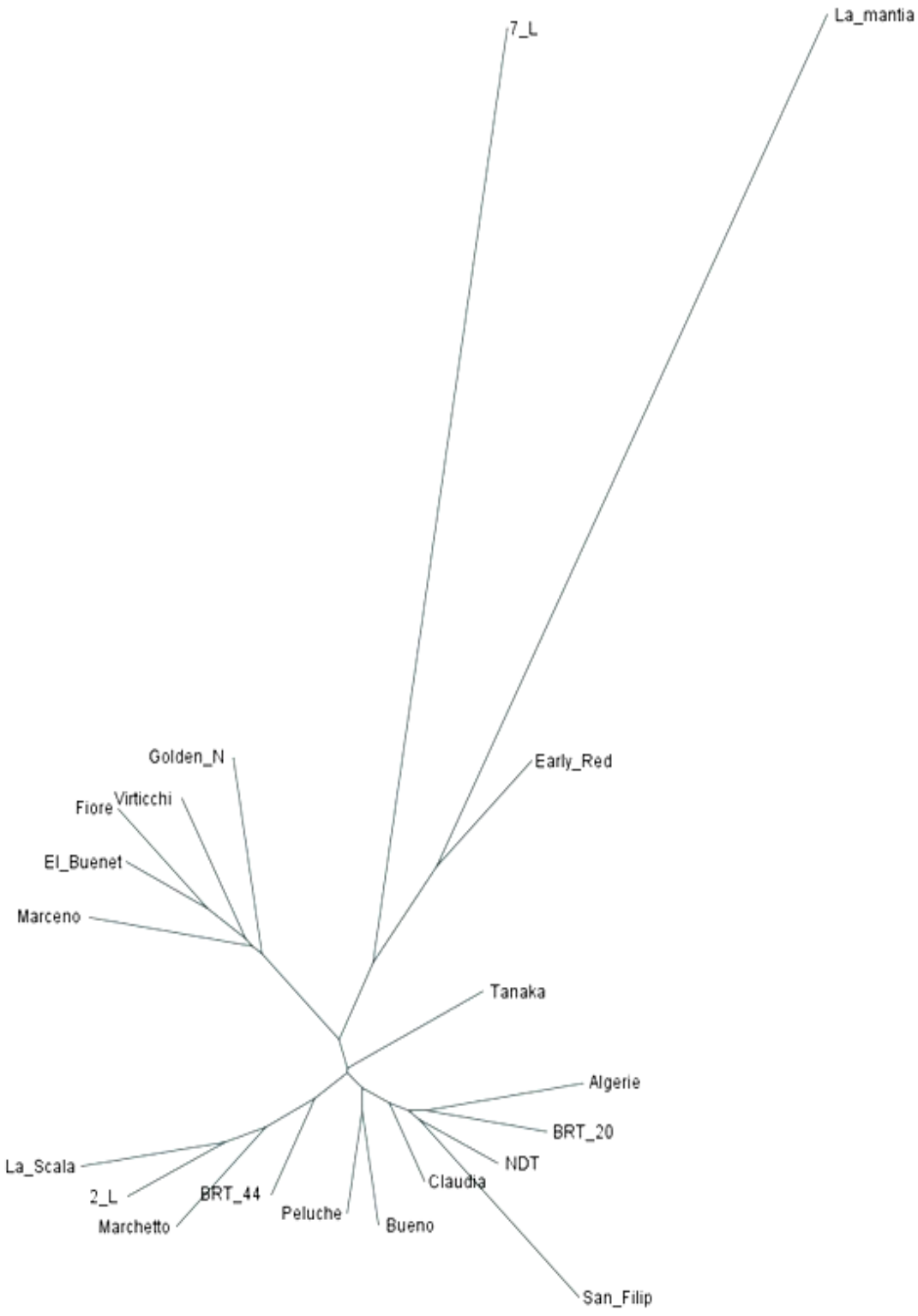


**Fig. 4.2 Boostrapped tree derived from AFLPs**

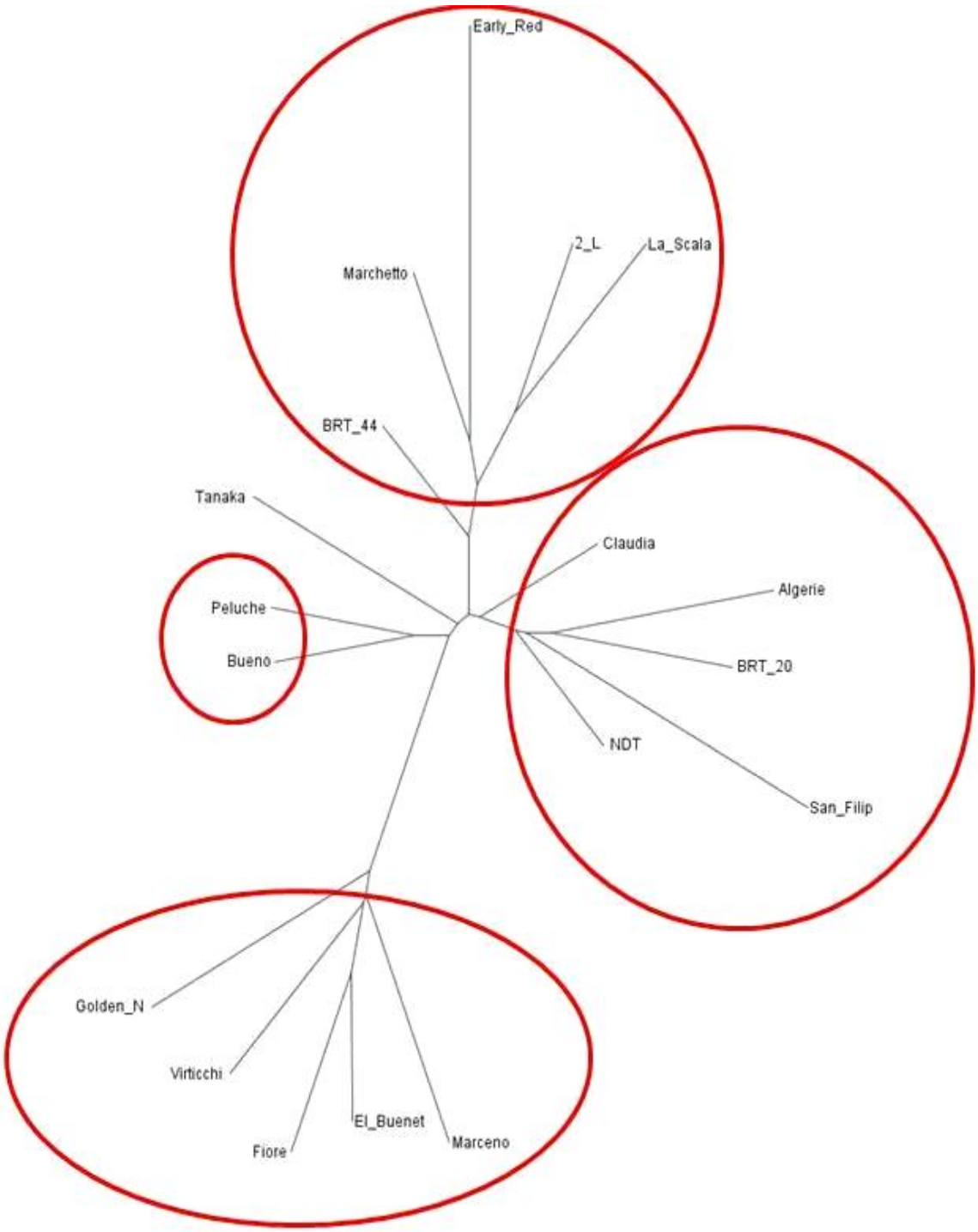


**Fig. 4.3: AFLP boostrapped tree without accessions La Mantia and 7L**

# 5 Conclusions

The present study was carried out during 2008 -2010, and the objective of the study was the development of an efficient protocol for pollen embryogenesis induction using anther/ microspore culture, stress treatment and different media condition, in some tipical Mediterranean fruit crops.

Increasing the number of genotype responding and following the development of the embryogenesis is of the fundamental importance for a better understanding of factors affecting the successful of methods.

The factors that affect haploid totipotency in anther and microspore cultures were studied. For anther culture these included optimum explant (microspore or anther), cold pre-treatment or high temperature incubation, varying sugar concentrations, various combinations of growth regulators in culture medium, culture of anthers from various bud sizes and different donor plant growth conditions. The isolated microspore culture experiments focused on the influence of donor plant growth environment, microspore development stage, culture media composition and incubation conditions on microspore embryogenesis.

For the first time, from isolated microspore culture in *Eriobotrya japonica* Lindl and from anther culture in some genotype of *Olea europaea* and in *Prunus armeniaca*, multicellular structure and proembryo Cold temperature pretreatment of flower bud 4 ± 1°C for 1-2 weeks was essential for triggering pollen totipotency. The correlation analysis between the bud size and microspore development stage were analyzed and revealed that late uninucleate microspores, collected from plants grown under normal field conditions, were observed to be most responsive to microspore embryogenesis. Genotype dependence for microspore totipotency was observed and a significant effect of genotype wise bud size selection was also established.

Results obtained, even if preliminary, have big importance for following study and for the potentialities of the haploid and doubled haploid production.

Moreover, the genetic diversity evaluation of a Sicilia loquat germplasm collection by molecular markers was carried out: this kind of analysis should be made in all genetic improvement programs in order to evaluate the genetic diversity available.

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# List of papers and works

Germanà, M., Chiancone, B., Padoan, D., Barany, I., Risueno, MC., Testillano, P.S. 2008. Induction of pollen embryogenesis in seven cultivars of *Prunus armeniaca* L. Acta Hort. Proceedings of the XIV International Symposium on Apricot Breeding and Culture; ISHS Acta Horticulturae 862 (Matera, Italy).

Padoan D; Chiancone B; Germanà M.A. 2009. Occurrence of spontaneous triploids from small seeds of *Hernandina Clementine*. 2nd International Citrus Biotechnology Symposium, Catania, Italy.

Chiancone B., Macaluso L., Padoan D., Germanà M.A. 2009 *In Vitro* plant regeneration from epicotyls segments of *carrizo citrange*. 2nd International Citrus Biotechnology Symposium, Catania, Italy.

Germanà M. A., Chiancone B., Padoan D., Bárány I., Risueno M.C. , Testillano P. S., 2010 First stages of microspore reprogramming to embryogenesis through anther culture in *Prunus armeniaca* L. Environ. Exp. Bot. 2010 DOI: 10.1016/j.envexpbot.2010.11.011

**Oral presentation**

First Stages of Microspore Reprogramming to Embryogenesis Through Isolated Microspore Culture in *Eriobotrya japonica* (Thunb.) Lindl. 3th International Symposium on Loquat 03th -06th May 2010 Antakya/HATAY (Turkey)

**Poster presentation**

Occurrence of spontaneous triploids from small seeds of *Hernandina clementine; In Vitro* plant regeneration from epicotyls segments of *Carrizo Citrange.* 2nd International Citrus Biotechnology Symposium, 30th Nov–2nd Dec 2009 Catania (Italy)

First stages of microspore reprogramming to embryogenesis through anther culture in *Prunus armeniaca* L." 14th International Symposium on Apricot Breeding and Culture; 16th - 20th June 2008 Matera (Italy).