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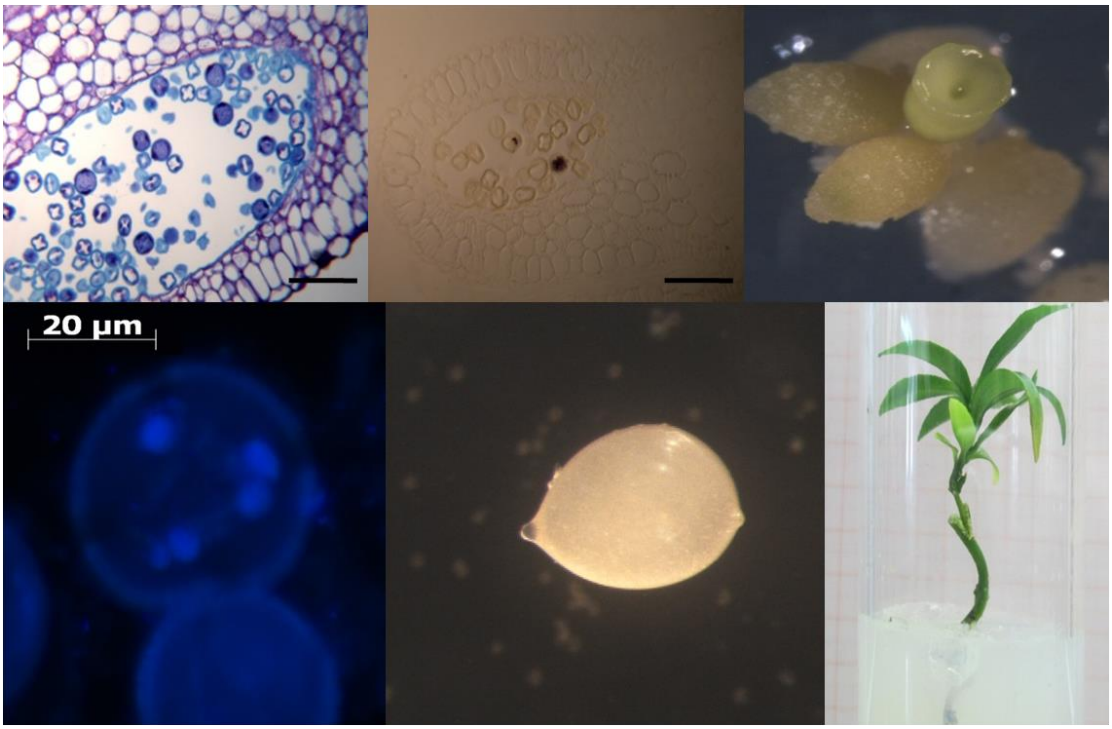
STUDY ON GAMETIC EMBRYOGENESIS *VIA IN VITRO* ANTHER AND ISOLATED MICROSPORE CULTURE IN FRUIT CROPS



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Abstract

Fruit breeding is mainly based on both conventional (hybridization, mutation and selection) or biotechnological methods (somatic hybridization, genetic transformation and haploid production). The genetic improvement through the conventional methods is limited by many factors such as fruit trees long juvenile period, high heterozygosity, large size and sexual incompatibility. Haploids and doubled haploids, obtained through gametic embryogenesis have a potential use in fruit crops genetic improvement. The change of the microspores fate from the normal gametopytic pathway towards the sporophytic induction is affected by numerous factors. Genotype, medium composition and stress were considered the most important factors required to switch the pollen embryogenic development.

During this doctoral course, researches have been carried out on gametic embryogenesis in different fruit crops via anther and isolated microspore culture. Particularly, for the first time, embryos were obtained by isolated microspore culture technique in *Citrus* spp. Morover, somatic embryogenesis callus and regeneration of plantlets were achieved via anther culture in blood sweet oranges, *C. sinensis* L. Osbeck, cvs. Moro, Tarocco Meli, Tarocco TDV and Tarocco S. Alfio, homozygous callus, embryos and plantlets were instead obtained from *Citrus clementina* Hort. ex Tan. cvs. Hernandina and Corsica. These results represent an advancement of plant breeding and propagation techniques in *Citrus* spp.

Research has been also carried out on *Olea europaea* L. gametic embryogenesis, multicellular structures have been obtained from anther and isolated microspore culture, as the first step towards haploid olive embryos production.

Furthermore, a preliminary experiment has been carried out on of several hazelnut (*Corylus avellana* L.) cultivars to obtain regeneration through anther and isolated microspore culture.

Key words: anther culture; *Citrus*; fruit trees breeding; hazelnut; homozygsity; isolated microspore culture; olive; pre-treatments; pollen embryogenesis; somatic embryogenesis.

FIRST PART

1- Introduction

Genetic improvement in fruit crops, carried out by conventional methods is time consuming and limited by many factors. Anther and isolated microspore culture are efficient biotechnological methods used to produce haploid and double haploid plants in fruit trees breeding. To switch microspore development towards the sporophytic embryogenesis pathway it is necessary to take into account many factors such as genotype, physiological state of donor plants, microspore developmental stage, medium composition, physical and chemical stress, pretreatment and environmental condition used for *in vitro* culture. Particularly, stress pre-treatments are considered to be an important factor in inducing embryogenesis in microspores and they have been found necessary for triggering pollen embryogenesis. In fact, commonly without stress, microspores follow their normal gametophytic pathway to form pollen grains (Touraev et al., 1997). Stress can be applied through the growing conditions, provided to the donor plants, as pretreatment to the floral buds or to the isolated microspores, or during the culture. Actually, all aspects of the *in vitro* culture protocol could be classified as stresses (Ferrie and Caswell 2010).

Investigations have been conducted to obtain regeneration through anther and isolated microspore culture techniques, studying and testing the response of microspores to different stress treatments applied before and during the culture.

Although isolated microspore culture technique is considered the most effective system for studying the microspores induction and embryogenesis, in many species anther culture has proven to be more effective than isolated microspore for producing haploids and doubled haploid. This could be due to the effect of anther wall tissues on embryogenesis.

Studies have been carried out through years 2011, 2012 and 2013 to improve both anther and isolated microspore culture techniques, investigating different factors affecting microspore embryogenesis.

Particularly, in 2011, the effect of two thermal shocks applied to the *in vitro* cultured anthers of six *Citrus* cultivars (four blood sweet oranges**:** *C. sinensis* L. Osbeck, cvs. Tarocco Meli, Tarocco TDV, Tarocco S. Alfio, Moro and two clementines: *Citrus clementina* Hort. ex Tan. cvs. Corsica and Hernandina) was investigated. Somatic

embryogenesis callus and regeneration of plantlets from blood sweet oranges *C. sinensis*L. Osbeck, cvs. Moro, Tarocco Meli, Tarocco TDV and Tarocco S. Alfio were achieved, homozygous callus, embryos and plantlets were obtained from *Citrus clementina* Hort. ex Tan. cvs. Hernandina and Corsica.These results represent advancement in plant breeding and propagation techniques in *Citrus spp*.

In the same year, anther culture of the following five olive cultivars, Biancolilla Napolitana, Cerasuola Sciacca, Galatina, Nocellara del Belice and Verdello, have been carried out. Moreover, in four olive cultivars (Biancolilla Napolitana, Nocellara del Belice, Tonda Iblea and Verdello) the isolated microspore culture was performed, observing their development (the number of microspores uninucleated with no development, binucleated, trinucleated, multinucleated and forming calli) by fluorescence microscope (Zeiss, Axiophot, Germany).

In the second year (2012), a six months training stage has been carried out at the Centro de Investigaciones Biológicas (CIB), of the CSIC (Consejo Superior de Investigaciones Cientìfica) in Madrid, Spain, on different cell biology methodologies, to monitor isolated microspore and anther cultures of different olive and *Citrus* spp cultivars have been studied. Particularly, including fixation and embedding techniques for *in vitro* samples to investigate structural preservation by microscopical analysis, learning different protocols (resin semi-thin sectioning and different staining methods for specific cellular components, microscopical analysis by various methods: phase contrast, bright field and epifluorescence; digital image acquisition in a CCD camera, and image processing by appropriate softwares; a short experience on spectral confocal laser scanning microscopes: Leica SP2 and SP5) have been focused.

In the same year, studies on previous cultures have been carried out, sub-culturing and monitoring by 4',6-diamidino-2-phenylindole (DAPI) staining and observations through afluorescence microscope (Zeiss, Axiophot, Germany) of the *Citrus* and olive cultures.

In 2013, anther and isolated microspore cultures have been performed in the following six different hazelnut (*Corylus avellana*) cultivars: Tonda Romana, Meraviglia De Bolwiller, Imperatrice Eugenia, Carrello, Nostrana and Gentile Romana.The anthers response of two thermal shocks and the microspores response to four thermal shocks and two media were observed.

In the same year (2013), the anther and microspore response to five different growth regulators combinations, added to the P induction medium (Germana` et al., 1997;

Germanà and Chiancone 2003) were studied in *C. sinensis* L. Osbeck, cvs.Moro and in *Citrus clementina* Hort. ex Tan, cvs.Nules and MAR.

For the first time, embryos were obtained from isolated microspore culture technique in *Citrus spp*.

In *Olea europaea* L., multicellular structures have been obtained from anther and isolated microspore culture, as first step towards haploid olive embryos production.

In hazelnut (*Corylus avellana* L.) for the first time, an experiment has been carried out to obtain regeneration through anther and isolated microspore cultured, testing different temperature stresses applied to the explants.

2-Status of art of research on gametic embryogenesis

2.1 Objectives of breeding programs in fruit trees

Plant breeding has a significant potential in increasing crop production to meet the needs of an ever-growing world population, improving food quality to ensure a long and healthy life and address the problems of global warming and environment pollution, together with the challenges of developing novel sources of biofuels (Germanà 2011a). A great effort is needed to develop and modify some aspects of current plant breeding to be more effective in improving fruit crops. Fruit tree breeding is based on conventional (hybridization, mutation and selection) or biotechnological methods (somatic hybridization, genetic transformation, haploid production, etc...). In fruit trees, as well as in most woody plants, genetic improvement by conventional methods is time-consuming and limited by many factors. Biotechnologies can provide powerful tools for plant breeding. The main goals of fruit breeding programs are to obtain new varieties with a shorter juvenile non-fruiting period, an increased yield, a longer ripening season, regular bearing, seedlessness and improved external and internal quality of the fruits. Another important aim in fruit tree improvement research is to make available new scions and rootstocks selected for resistance or tolerance to biotic and abiotic stresses (Germanà 1997, 2006).

2.2 The importance of haploids (Hs) and doubled haploids (DHs) in plant breeding

Haploid plants (Hs) are sporophytes with the gametic chromosome number (n instead of 2n). When spontaneous or induced chromosome duplication of a haploid occurs, the resulting plant is called doubled haploid (DH). The interest of breeders in haploids and doubled haploids mainly relies in the possibility to obtain homozygosity in woody plants, generally characterized by a long reproductive cycle, a high degree of heterozygosity, large size, and, sometimes, by self-incompatibility (Germanà 2006, 2011a).

Haploid plants arouse interest in the fields of genetic and developmental studies, as well as of plant breeding. The importance of haploids in plant breeding and genetic research was immediately recognized when the first natural haploid was discovered in *Datura stramonium* and in *Nicotiana* spp. by Bergner, as reported by Blakeslee and Belling (1924) and by Kostoff (1929).

Haploid technology is important for its potential use in mutation research, selection, genetic analysis, transformation and in the production of homozygous cultivars (Germanà 2010; 2011a). Moreover, haploid lines can be used as parents in protoplast fusion with diploid plants in order to obtain triploids, which are particularly important since they are seedless.

Moreover, DHs have featured strongly in basic and applied genetic studies of crop plants and have an important role in the development and exploitation of structured mutant populations for forward and reverse genetics (reviewed by Forster and Thomas 2005). DHs are also very useful for genome mapping, providing reliable information on the location of major genes.

The first DH crop plant released was the cv. Maris Haplona of rapeseed *Brassica napus* (Thompson 1972), followed by the cv. cultivar Mingo in barley *Hordeum vulgare* in 1980. Later, haploids were reported in a range of species.

Many reviews have been make available, focusing on methods used for haploid and doubled haploid production as well as on the importance of Hs and DHs for plant breeding (Dunwell 2010; Zhang et al., 1990; Andersen 2005; etc.). Moreover, extensive efforts have been made to obtain haploids from several hundreds of species (Magoon and Khanna 1963; Maluszynski et al., 2003; Palmer et al., 2005a; Xu et al., 2007; Germanà 1997; 2006; 2011a,b).

The release of new fruit trees cultivars through DH technology is much more difficult and more time requiring (Germanà 2009). In many fruit trees, such as apple, papaya and peach, H and DHs have been obtained, but it takes considerable time to characterize them (Pooler and Scorza 1995; Yahata et al., 2005; Rimberia et al., 2007).

Regarding H and DHs production in *Citrus* and their relatives, anther culture is the most employed methods to recover haploids. Alternative methods can be: selection of seedlings (Esen and Soost 1972; Toolapong et al., 1996), *in situ* parthenogenesis (the production of an embryo from an egg cell without the participation of the male gamete) induced by irradiated pollen followed by *in vitro* culture of embryos (Ollitrault et al., 1996); *in situ* (Oiyama and Kobayashi, 1993) or *in vitro* (Germanà and Chiancone 2001) parthenogenesis induced by pollen from a triploid plant, followed by *in vitro* culture of embryos. Irradiation or triploidy do not hinder pollen germination, but prevent pollen fertilization, stimulating the development of haploid embryoids from ovules.

Although a lot of research has been carried out on gametic embryogenesis (Germanà 1997; 2011a,b), not much of it has been successful. In *Citrus*, the first haploid seedlings were obtained by the application of gamma rays in *Citrus natsudaidai* (Karasawa 1971) after that, many studies have been carried out on gametic embryogenesis to obtaining haploid and doubled haploid plants through anther and isolated microspore culture but not much of it has been succeeded. Only heterozygous plantlets have been obtained by anther culture in *C. aurantium* (Hidaka et al., 1982; Germanà unpublished), *C. sinensis* (Hidaka 1984b), *C. aurantifolia* (Chaturvedi and Sharma 1985), *C. madurensis* (Ling et al., 1988), *C. reticulata* (Germanà et al., 1994), *Poncirus trifoliata* (Deng et al., 1992) and *C. sinensis*. (Cardoso et al., unpublished). Haploid plantlets have been recovered from *Poncirus trifoliata* L. Raf. (Hidaka et al., 1979) and *C. madurensis* Lour. (Chen et al., 1980), one doubled haploid plantlet has been obtained from the hybrid No. 14 of *C. ichangensis* x *C. reticulata* (Deng et al., 1992); haploid plantlets and highly embryogenic haploid calli of *C. clementina* Hort. ex Tan. (Germanà et al. 1994; 2000a, Germanà and Chiancone 2003); haploid but albino embryoids of Mapo tangelo *C. deliciosa* x *C. paradisi* (Germanà and Reforgiato 1997); haploid and diploid calli, embryoids and leafy structures but no green plants of *C. limon* L. Burm. (Germanà et al., 1991), haploid embryoids of *Clausenae xcavata* (Froelicher and Ollitrault 2000) have been also achieved. Also regeneration of triploids by *in vitro* anther and isolated microspore culture are very important in *Citrus* spp because of their seedlessness (Kobayashi et al., 1997; Ollitrault el al., 1996).

In many fruit trees and other plant species, considerable researches have been carried out to obtain haploids and doubled haploids through gametic embryogenesis, for example in: *Olea europaea* (Bueno et al., 2004, 2005, 2006; Solis et al., 2008; Chiancone et al., 2013), *Prunus armeniaca* (Peixe et al., 2004; Germanà et al., 2010), *Eriobotrya japonica* (Germanà et al., 2006; Padoan et al., 2011), *Malus domestica* (Fei and Xue 1981; Xue and Niu 1984; Zarsky et al., 1986; Höfer and Hanke 1990; Zhang and Lespinasse 1990; Verdoodt et al., 1998; Höfer 1995, 2003), *Phoenix dactylifera* (Chaibi et al., 2002; Chen et al., 1980), *Prunus persica* L. (Michellon et al., 1974; Seirlis et al., 1979), *Musa balbisiana* (Assani et al., 2003), *Carica papaya* L. (Litz and Conover 1978; Tsay and Su 1985; Rimbeira et al., 2005), *Annona squamosa* L. (Nair et al., 1983), *Gingko biloba* (Laurain et al. 1993), *Feijoa sellowiana* Berg.(Canhoto and Cruz 1993), *Coffea arabica* (Ascanio and Asdrúbal 1994), *Pyrus pyrifolia* Nakai (Kadota and Niimi 2004), *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), *Quercus suber* (Bueno et al., 1997), *Clausena excavata* (Froelicher and Ollitrault 2000).

2.3 Microspore embryogenesis via anther culture technique

More than 50 years ago, Guha and Maheshwari (1964) discovered that anthers of *Datura spp.* containing immature pollen grains (microspores) and *in vitro* cultured at specific conditions, could develop into haploid embryos. After this discovery, many experiments have been carried out to set up efficient and replicable protocols focused on obtaining haploids through anther culture (Germanà 2011a, 2011b). Also isolated microspores at the right developmental stage, after suitable pre-treatments and at appropriate culture conditions, may change their usual gametophytic pathway towards pollen maturation, to develop haploid embryoids or embryogenic callus.

Through anther culture technique, it is possible to recover also diploid embryoids. They can originate from the microspores, later duplicating their chromosomal number, and so they are homozygous, or they may come from the somatic tissue of the anther, so they are heterozygous as the mother plant (Cardoso et al., unpublished). Those somatic calli and plants have high value in fruit trees breeding, since they can be used for protoplast fusion, genetic transformation or *in vitro* germplasm storage.

Anther culture technique has been employed to recover haploids in many fruit crops, among which citrus and stone fruits (Zhang et al., 1990). Regarding apple, for example, the induction of embryogenesis from cultured anthers is still low and highly genotype-dependent (Hofer 1995; De Witte et al., 1999). Haploid plantlets and highly embryogenic haploid calli of *Citrus* spp (Germanà et al., 1994; 2000a, Germanà and Chiancone 2002, 2003, Germanà et al., 2005), *Prunus armeniaca* (Germanà et al., 2010) *Eriobotrya japonica* (Germanà et al., 2006; Padoan et al., 2011) have been also obtained.

Generally in fruit tree anther culture technique, flower buds are collected from the trees cultivated in the field when the microspores are at around the uninucleated stage of development.

The stage of pollen development is commonly determined by staining one or more anthers per bud size by acetocarmine or by DAPI (4', 6-diamidino-2-phenylindole). After a pre-treatment and surface sterilization, the anthers are aseptically and carefully

dissected and placed into the medium. The sterilization is usually performed by immersion for 1-2 minutes in 70% (v/v) ethyl alcohol followed by immersion in sodium hypochlorite solution (about 0.5% active chlorine in water) for 10-15 minutes. Before removing the petals by a small forceps, the floral buds are washed two or three times with sterile distilled water.

Some morphological and chemical changes indicate the starting of the embryogenesis process. The microspore enlarges significantly, the nucleus is placed in the cell center, the cytoplasm clears and the large vacuole breaks apart into smaller fragments (Touraev et al., 2001). These rearrangements provide, in the induced microspores of many species, a star-like morphology, that is considered a transient early morphological marker of embryogenic commitment (Indrianto et al., 2001; Maraschin et al., 2005). After that, microspores symmetric division occurred, as the first morphological evidence of the embryogenic pathway (Zaki and Dicknson, 1991), then microspore continue its division patterns towards multicellular structures and embryos formation. Otherwise, during the gametophytic pathway, microspores divided and differentiated into generative and vegetative nuclei different in size and shape.

2.4 Microspore embryogenesis via isolated microspore technique

A decade later after the discovery by Guha and Maheshwari (1964), Nitsch (1974) cultured *Nicotiana* microspores naturally released from anther tissue (‘shed microspore’ culture). Only in 1982, Lichter mechanically isolated microspores from *Brassica* buds prior to culturing them, paving the way for further and numerous studies on microspore culture. In fact, many studies have been carried out to develop protocols for the different species and increasing the frequency of embryogenesis via isolated microspore culture.

While in anther culture, the whole immature anthers are *in vitro* cultured, in microspore culture, the immature male gametes are isolated before the culture, removing somatic anther tissue by different methods.

Although anther culture is often the method of choice for doubled haploid production in many crops, because of its higher efficiency and advantageous simplicity (Germanà 2011a), the technique of the isolated microspore culture provides a better way of investigating the cellular, physiological, biochemical, and molecular processes involved in pollen embryogenesis (Nitsch 1977; Reinert and Bajaj 1977), even if it requires better equipment and more skills than anther culture does. Moreover, isolated microspore

culture avoids the regeneration of diploid embryos and plants from somatic anther tissue (Ferrie and Caswell 2010; Germanà 2011a, 2011b).

Essentially, the steps involved in an isolated microspore culture protocol are: growing donor plants, harvesting floral organs, isolating microspores, culturing and inducing microspores, regenerating embryos, and doubling the chromosome number (Ferrie and Caswell 2010).

The differences between anther and isolated microspore culture could be due to the effect of anther wall tissues on embryogenesis, the anther wall may negatively impact the microspores, or may produce diploid somatic callus and subsequently embryos. Anther culture is extremely time-consuming more than isolated microspore culture; the isolated microspore culture system allows for better nutrient availability to the developing microspores and provides a superior method for tracking and studying microspore maturation and embryo development (Ferrie and Caswell 2010). In microspore culture, the immature male gametes are isolated prior to culture, removing somatic anther tissue by different methods. Although anther culture is often the method of choice for doubled haploid production in many crops, because of its higher efficiency and advantageous simplicity (Germanà 2010, 2011a), the technique of the isolated microspore culture provides a better way of investigating the cellular, physiological, biochemical, and molecular processes involved in pollen embryogenesis (Nitsch 1977; Renert and Bajaj 1977), even if it requires better equipment and more skills than it does in anther culture. Moreover, isolated microspore culture avoid the regeneration of diploid embryos and plants from somatic anther tissue.

Embryogenesis via isolated microspore is affected by numerous factors: genotype, pretreatment, microspore developmental stage, donor plant physiological state and growth condition, stress treatments, culture media, condition of incubation. As the first step in any isolated microspore protocol, identifying the correct flower bud and anther size with microspores in optimal developmental stage provided a very important effect in embryogenesis, and uni-nucleate to early binucleate stages are usually selected. DAPI (4', 6-diamidino-2-phenylindole) and acetocarmine are the stains most commonly used for determining the developmental stage of the microspore (Fan et al., 1987).

For many species, usually flower buds or anthers are pre-treated, placed in nutrient solution, media, water, or inducer chemicals and kept for up to several weeks prior to microspore isolation (Ferrie and Caswell 2010). Most temperature pretreatments are at 4

– 10°C, but short heat shock conditions of 33°C for 48 - 72 h can also be used (Liu et al., 2002).

The most effective technique used to isolate the microspores in many fruit trees crops involves mechanically crushing the surface-sterilized buds to release the microspores from the anthers by a sterilized blender, then filtering the microspores to remove somatic anther wall and bud tissue from the microspores. The microspores are subsequently separated by centrifugation. Percoll gradients (Fan et al., 1987; Joersbo Brunstedt, 1990), maltose density gradients (Kasha et al., 2001), sucrose gradients (Maraschin et al., 2003) and mannitol gradients have been used to separate the somatic tissues and to obtain clean isolated microspore cultures (Germanà et al., 2010; Chiancone et al., 2013)

High practical skills is required for obtaining a clean microspore culture, and somatic tissues like anther wall, filaments and somatic hairs must be separated from the microspores (Iqbal and Wijesekara, 2007). In *Compositae* species, *Helianthus annuus* L. (sunflower), multicellular somatic hairs were difficult to eliminate during microspore isolation: the hairs were highly reactive and produced calli, requiring the use of percoll gradients (Coumans and Zhong 1995) or Mannitol gradients (Germanà et al., 2010; Chiancone et al., 2013) to purify the microspore preparations.

A suitable culture medium containing all macro and micro-nutrients, vitamins, a carbohydrate source and all other required components depending on the genotype and culture, must be provided to the isolated microspores to obtaining embryogenesis (Germanà 1997; Germanà and Chiancone 2003). Antibiotics such as cefotaxime can also be added to the medium if contamination is a problem (Lantos et al., 2006).

Although isolated microspore culture is an efficient protocol to producing homozygous line for plant breeding programs, only a few reports of progress in fruit crops (Germanà 2006, 2011). Recently, many studies have been carried out to improve the haploidy protocols (Hofer 2005; Bueno et al., 2005, 2006; Ferrie and Caswell 2010; Germanà, 2009, 2010, 2011; Chiancone et al., 2013).

2.5 Factors affecting *in vitro* pollen embryogenesis

2.5.1 Genotype

Genotype, developmental stage, and culture conditions are the most important factors affecting the microspore developmental fate (Seguı`-Simarro and Nuez, 2008a,b). Because the induction rate of microspores depends on the genotype (Vasil 1980), it is

necessary to know the optimal conditions to turn the development of pollen towards the sporophytic pathway and to avoid embryo abortion (Germanà 2007). The effect of genotype on the success of the gametic embryogenesis is appeared clearly in *Citrus*, where homozygous regenerants have been produced in different cultivars of the one species *C. clementina*, although research on anther culture of numerous genotypes has been carried out (Germanà 1991, 1992, 1997, 2000a, 2003, 2005, 2006; Germanà and Chiancone 2003).

2.5.2 Donor plant

To obtain a successful microspores embryogenesis process, it is necessary to collect the flowers from healthy, fertilized, regularly irrigated and pest-free donor plants (Ferrie and Caswell 2010).

The donor plant's growth condition and physiological status can affected significantly the microspores embryogenesis and influence triggering it towards the sporophytic pathway (Wang et al., 2000). Diseases or stress applied to the donor plants can reduce the response to the pollen embryogenesis (Wang et al., 2000). Also the other environmental conditions around the donor plant (proper light, temperature, humidity, etc...) have a marked effect on the microspore embryogenesis. For example, donor plants of wheat and barley grown during October–December provided an excellent microspore responseg (Swapan and Datta 2005). In the rice crop, plants grown during the dry season provided the best microspore response under optimized conditions of a phytotron with controlled light, temperature, and humidity, which enable plants to maintain a healthy growth with disease and pest free status. In tobacco, Dunwell (1981) indicated that both photoperiod and light intensity affected the yield of microspore embryos and plantlets. The frequency of pollen grains is also increased by other conditions (short days, low temperatures) that are unfavorable for plant growth (Heberle-Bors and Reinert 1981; Heberle-Bors 1985).

Although the physiological state of the donor plant can dramatically affect the response of the anthers to the *in vitro* culture, this parameter has been investigated only in herbaceous plants, due to the difficulties involved with determining it in woody plants. In fact, the growth condition and physiological status of donor plants cannot be standardized in the anther culture of perennial plants, cultivated in open-air and affected by climatic (temperature, photoperiod and light intensity), cultural (pruning, irrigation, fertilization, etc.) and pedological conditions (especially during flower induction and

differentiation). This can explain why the response to the culture of woody plant anthers is very season dependent even when the same protocols are applied (Germanà 2010).

2.5.3 Pollen development stage

The induction rate of the pollen embryogenesis via anther and isolated microspore culture is strongly influenced by the pollen developmental stage. Microspores between the uninucleate and early binucleate stage (the stage around the first pollen mitosis) are the most responsive (Raghavan 1986; Pechan and Keller 1988; Smýkal 2000; Telmer et al., 1993; Touraev et al., 1996b; Gonzalez-Melendi et al., 1995 and 1996a,b). In *Nicotiana tabacum* Touraev et al. (1996a,b) reported that bicellular pollen stage is the best responding when starvation is used as pretreatment. When heat shock is applied instead, younger uninucleate microspore can be employed.

The stage of pollen development is commonly determined by staining one or more anthers per bud size by acetocarmine or by DAPI staining. Various researchers have observed in many genera that microspores at different developmental stages can be found within a single anther and between different anthers of the same flower bud (Hidaka et al., 1979, 198; Chen 1986).

Generally, the stage around the first pollen mitosis is the best responding, because after that the pollen grains start to accumulate storage reserves, they usually lose their embryogenic capacity and follow the gametophytic developmental pathway (Heberle-Bors 1985; Raghavan 1990). Without stress treatments, microspores follow their normal gametophytic pathway to form pollen grains (Touraev et al., 1997). After the stress treatment, the microspores are not able to follow their gametophytic pathway and they start to follow the sporophytic pathway and to form embryos (Zaki and Dickinson 1990; Sharma and Bhojwani 1985; Telmer et al., 1992).

2.5.4 Pre-treatment

Stress treatments are considered to induce embryogenesis in microspores and without stress, microspores follow their normal gametophytic pathway to form pollen grains (Touraev et al., 1996, 1997; Hu and Kasha 1999). Stress also plays an important role in the development of the embryos into green seedlings and consequently it improves the yield of green plants regeneration (Kasha et al., 2001; Li and Devaux 2003). It can be applied through the growing conditions applied to the donor plants, or as pretreatments to the floral buds or to the isolated anther or microspores, but also during the surface-

sterilization and with the culture conditions. In fact, all aspects of the microspore culture protocol could be classified as stresses (Ferrie and Caswell 2010).

Thermal shock are considered to be the most effective treatments to induce pollen embryogenic development. In several plants, such as *Citrus*, cold treatment is the most mainly used on pollen embryogenesis.

First studies on the effect of cold treatment on pollen gametic embryogenesis were done on *Datura innoxia* (Nitch and Norrel 1973) and later on tobacco (Duckan and Heberle-Bors 1976; Heberle-Bors and Reinert 1981; Rashid and Reinert 1983).

Cold pre-treatment applied to flowers has been reported to reprogram the gametophytic pathway in many species. In citrus anther culture, different cold pretreatment have been used starting form two hours (Starrantino 1986), overnight (Geraci and Starrantino 1990), four days (Germanà et al., 1991, 1994), one week up to three weeks (Deng et al., 1992; Germanà 1997, 2003, 2005, 2006). Also in other fruit crops and woody plant species, like olive *Olea europaea.* (Bueno et al., 2006; Chiancone et al., 2013), apple *Malus* sp. (Höfer 2003, 2004) oak *Quercus spp*. (Bueno et al., 2000, 2003; Ramirez et al., 2004), and poplar *Populus nigra* (Andersen 2003; Deutsch et al., 2004), cold pre-treatments have been used. In cereals and legumes, many studies have been carried out to discovering the effect of cold treatment on pollen embryogenesis, like in *Fabaceae* (Zagorska and Dimitrov 1995; Croser et al., 2004), in *Brassica* (Telmer et al., 1993; Custers et al., 1994; Binarova et al., 1997; Touraev et al., 1997; Indrianto et al., 1999; Smykal and Pechan 2000), in rye (Immonen and Anttila 2000; Immonen and Tenhola-Roininen 2003), in triticale (Ślusarkiewicz-Jarzina and Ponitka 1997; Marciniak et al., 1998; Gonzalez and Jouve 2000; Immonen and Robinson 2000; Tuvesson et al., 2003; Wędzony et al., 2003), in barley (Olsen 1987; Ziauddin et al., 1990; Scott and Lyne 1994; Salmenkallio-Marttila et al., 1995) and in wheat (Gustafson et al., 1995; Ingram et al. 2000; Redha et al., 2000).

In contrast, in *Solanum melongena* L., Rotino (1996) reported that high temperature showed a higher efficiency on the embryogenesis more than cold treatment. The same results have been obtained also in *Brassica oleracea* and in a number of related *Brassica* species (Duijs et al., 1992; Sato et al., 2002; Ferrie 2003) as well as in *Solanum chacoense* (Cappadocia et al., 1984).

In addition to the thermal treatments, there are various stress treatments applied to enhanc pollen embryogenesis. Cistué et al. (2006) reported that anthers pre-treated in 0.7 M mannitol for 5 days improved green plants regeneration. Labbani et al. (2007)

showed that a pre-treatment consisting in a combined mannitol 0.3 M and cold for 7 days had a strong effect on the number of embryos produced and on the regeneration of green plants. Heat shock combined with starvation (Touraev et al., 1996) or with cold and osmotic starvation combined with thermal treatments resulted useful to reprogram the gametophytic pathway (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; Davies 2003). Other protocols include treatments with oligosaccharides (Penhuizic et al., 2001), anti-microtubular drugs such as colchicine (Smykal and Pechan 2000), ethanol, gamma irradiation (Pechan and Keller 1989), and some other chemicals (Liu et al., 2002a, 2002b).

2.5.5 Microspore density

Microspore culture density is a very important factor affecting pollen embryogenesis. This parameter has been investigated and determined in woody plants and in many herbaceous plants. In fruit trees, Germanà (1996) resuspended citrus microspores using a density ranging from 1 x 10^3 to 10^4 microspore per ml, in olive Bueno (2006); Solis et al. (2008) adjusted the obtained microspore pellet to 1 x 10^5 microspore per ml. Germanà et al. (2010, 2013) and Chiancone et al. (2013) used 1 x 10^5 microspore per ml as a culture density for different olive, loquat and *Citrus* cultivars. Other isolated microspore culture densities have been studied and reported for different species: 10^4 microspores/ml, 2 x 10^4/ml, 3–4 x 10^4/ml, 8 x 10^4/ml and 10 x 10^4/ml (Ziauddin et al., 1990; Olsen 1991; Hoekstra et al., 1993; Gustafson et al., 1995; Prem et al., 2012).

2.5.6 Medium composition

The effect of medium composition on pollen embryogenesis have been widely investigated and the diverse genotypes show very different requirements. Usually, nutritional requirements of isolated microspore culture are much complicated than those of anther culture (Reinert and Bajaj 1977; Bajaj 1990).

Different carbon sources and plant growth regulators requirements are necessary to induce pollen derived plant formation (Germanà 1997). The most commonly used basal media for anther and isolated microspore culture are N6 medium (Chu 1978), modified MS medium (Murashige and Skoog 1962), N&N (Nitsch and Nitsch 1969). The nutrient medium not only provides nutrition to the microspores but also directs the pathway of embryo development.

Carbohydrate source is essential for embryo production in anther and isolated microspore culture because of their osmotic properties in the culture media, other than to be a energy source, to build structures and material.

Sucrose is the most common carbon source used in anther culture of citrus and their relatives, at 5% concentration (Hidaka et al., 1979, 1982; Geraci and Starrantino 1990; Froelicher and Ollitrault 2000). The influence of different carbohydrates has been tested on anther culture of *C. clementina* and *C. reticulata* (Germanà et al., 1994). Sucrose (at 5%) was found to be the best carbon source, in comparison to glucose in anther culture of two clementine and two mandarin cultivars (Germanà et al., 1994). In media used for pollen embryogenesis in many cereals, sucrose is added in the range of 2% to 3% and maltose in the ranges from 6% to 9% (Orshinsky et al., 1990; Otani and Shimada 1993; Karsai et al., 1994; Bishnoi et al., 2000; Barnabás, 2003; Pauk et al., 2003; Tuvesson et al., 2003; Wedsony 2003). Fructose and glucose have both been shown to be inhibitory to pollen embryogenesis in *Petunia* anther culture (Raquin 1989). Lactose at 18 g/l and galactose at 9 g/l are regularly used in clementine anther culture (Germanà 2003).

The supplement of other substances, such as growth regulators, casine, proline, glutamine, biotin, coconut water, polyamines (PAs), anti oxidant activated charcoal and antibiotics to the culture media has been found to increase the number of pollen-derived embryos in many species. The presence of growth regulators (auxins, cytokinines) is crucial for microspore-derived embryo production in the majority of plant species (Maheshwari et al., 1982). The type and the concentration of auxins seem to determine the pathway of microspore development (Ball et al., 1993). Many studies have been focused on growth regulators combinations on anther culture and isolated microspore culture in *Citrus spp* and many other fruit crops. (Starrantino 1986), studying the effect of different auxine and cytokinin combination on citrus anther culture. Germanà et al., (1991, 1994) obtained embryonic structures from *C. sinensis* and *C. paradisi*. and tested the effect of numerous combination of KIN, NAA and ZEA on *Citrus spp.* Founding that the best combination was 0.02 mg/l NAA + 0.5 mg/l ZEA + 0.5 mg/l Kin in anther culture medium of *C. clementina* and *C. reticulata*. Regarding cereals, the effect of growth regulators, such as IAA, NAA, BAA added to the induction media alone or in combination at various concentrations was tested in maize (Scott and Lyne 1994; Davies 2003; Caredda et al., 2000; Jacquard et al., 2003) and in barly (Scott and Lyne 1994; Davies 2003; Caredda et al., 2000; Jacquard et al., 2003). In anther culture of wheat, triticale and rye, 2,4-D and kinetin are used in the induction media and NAA

with kinetin in stimulate regeneration (Otani and Shimada 1993; Redha et al., 2000; Pauk et al., 2003).

Other addition, such as activated charcoal, was also tested in many experiments. In *Brassica* Prem et al. (2008) reported that, the microspores embryogenesis in *B. juncea* has increased when silver nitrate and activated charcoal were included in the culture medium. Other recent improvements include the addition of the anti-auxin to enhance microspore embryogenesis in *B. juncea* (Agarwal et al., 2006).

Antibiotics such as cefotaxime can also be added to the medium if contamination is a problem (Davis 2003; Lantos et al., 2006).

Alifatic polyamines (PAs), such as putrescine (PUT) and spermidine (SPE) are low molecular mass polycations present in all living organisms (Martin-Tanguy 2001; Bagni and Tassoni 2001). These growth regulators play a very important role in flower initiation, pollen tube growth, cell division (Bagni and Tassoni 2001). The addition of PAs to the medium stimulate gametic embryogenesis in *Citrus clementina* Hort. ex Tan (Chiancone et al., 2006), in some wheat cultivars (Rajyalakshmi et al., 1995) and in cucumber (Ashok and Kumar et al., 2004).

2.5.7 Characterization of regenerants: ploidy analysis

For identification of regenerants from anther and isolated microspore culture, flow cytometry has been used to analyze the ploidy level (Ollitrault et al., 1996; Froelicher and Ollitrault 2000; Germanà et al., 2005b). Other indirect methods have been used to estimate ploidy level, such as counting the chloroplast number in stomatal guard cells and determining plastid sizes (Qin and Rotino 1995). Not only haploids or doubled haploids have been obtained by gametic embryogenesis. Actually, non-haploid (diploid, triploid, tetraploid, pentaploid, and hexaploid) embryos and plantlets have been obtained via anther culture of different genotypes (D'Amato 1977; Dunwell 2010).

2.5.8 Characterization of regenerants: detection of homozygosity

Isozyme analyses, random amplified polymorphic DNA markers and microsatellites can be utilized to assess homozygosis (Germanà 2006). In *Citrus*, isozyme analyses have been employed to confirm the gametic origin of calluses and plantlet (Germanà et al., 1991, 1994, 2000a, 2000b; Ollitrault et al., 1996; Germanà and Reforgiato 1997; Germanà and Chiancone 2001). Isozyme techniques allow the distinction between androgenetic and somatic tissue when the enzyme is heterozygotic in the diploid

condition of the donor plant and the regenerant show lack of an allel (Germanà 2007). In addition, also RAPD markers, sequence characterized amplified region (SCAR), and simple sequence repeats (SSRs) have been utilized to assess homozygosis and todetermine the gametic origin of calluses and regenerated plantlets, irrespective of their ploidy (Germanà 2011a).

2.5.9 Plant recovery

Plantlet formation from pollen embryogenesis programs may occur either directly through embryogenesis of microspores or indirectly through organogenesis or embryogenesis of microspore-derived callus (Germanà 2007). In citrus, the highly embryogenic haploid callus is multiplied in 15 x 100 Petri dishes, with MS medium (Murashige and Skoog, 1962) containing MS Salts, MS Vitamins, 34 g/l sucrose, 0.5 g/l Ascorbic acid, 0.5 g/l mannitol, 1 mgl^{-1} GA_3, 0.02 mgl^{-1} NAA and 8.5 g/l agar. This procedure was repeated every month until plantlet regeneration (Germanà et al., 2005, 2006; Germanà and Chiancone 2003).

Although the *Citrus* embryos germinated vigorously, usually the homozygous plantlets grow slowly in soil, presumably due to the expression of harmful recessive genes expressed in homozygosity (inbreeding depression). These homozygous plantlets always died as a result of fungal infection (Germanà 2007).

To improve the survival of plantlets *in vitro* grafting of homozygous small shoots (2-3 mm) onto etiolated 20 day old Troyer citrange seedlings (cultured in MS micro grafting medium containing MS Salts , MS Vitamins, 30 g/l sucrose, 0.1 g/l Myoinositol and 6 g/l agar) can be executed.

After about 4 months of the micro grafting, when the grafted plantlets obtained were about 4-5 cm high, plantlets were washed with sterilized water and subsequently transferred to the greenhouse in pots containing sterilized peat moss, sand and soil in the ratio of 1:1:1 for the hardening phase (Germanà 2000b; 2007).

SECOND PART

Introduction

Haploids and doubled-haploid plants are of great interest for breeding and genomic studies. They can be obtained through the gametic embryogenesis, one of the most important example of cellular totipotency, that is the ability of the single plant cell to regenerate a whole plant.

It can be achieved starting from both the female gamete ("gynogenesis"), and from the male gamete with the commonly so-called "pollen embryogenesis".

The research conducted during the three years of doctorate was carried out to induce and to study gametic embryogenesis in some fruit crops (*Olea europaea*, *Citrus clementina* Hort. ex Tan., *Citrus sinensis* (Osbeck) L., and *Corylus avellana* L.), trough anther and isolated microspore culture.

1. *Citrus* (*Citrus* spp.)

1.1 Introduction

Citrus are the most important cultivated fruit species, ranked first among all fruit crops worldwide. A great amount of world *Citrus* fruit production (more than 126 million tons produced during 2013, FAOSTAT database) comes from the Mediterranean countries. About 80% of total global *Citrus* produced are orange and tangerine. All cultivated forms of *Citrus* and related genera (*Poncirus*, *Fortunella*, etc.) are diploid with a monoploid number of chromosomes (n=x=9) (Frost 1925). Triploid and tetraploid forms of *Citrus* also exist. The interest of breeders in haploids (Hs) or doubled haploids (haploid that doubled their chromosome number, DHs), lies in the possibility of shorting the time needed to produce complete homozygous lines (Germanà 2006, 2010). In *Citrus* anther culture, stress treatments have been found necessary for development and triggering embryogenesis (Starrantino 1986; Germanà et al., 1991, 1994, 1997, 2009, 2005). Stress treatments (such as chilling, high temperature, high humidity, water stress. etc.) applied to excised floral buds or to anthers before culture act as a trigger for inducing the sporophytic pathway, preventing the maturation of microspores (Touraev et al., 1997), although sometimes different results have been obtained.

The stress, triggering the microspore development from gametophytic to sporophytic pathway, seems to act by altering the polarity of the division at the first haploid mitosis involving reorganization of the cytoskeleton (Reynolds 1997), delaying and modifying pollen mitosis, blocking starch production or dissolving micro tubules (Nitsch 1977).

Many studies have been focused on influence of growth regulators on anther culture and isolated microspore culture in *Citrus spp* and in many other fruit crops (Starrantino 1986). Observing the effect of different auxine and cytokinin combination on *Citrus* spp anther culture, embryonic structures from *C. sinensis* and *C. paradisi* were obtained (Germanà et al., 1991, 1994).

"Pollen embryogenesis", can be induced through anther or isolated microspore *in vitro* culture. Mostly, anther culture is the method of choice for doubled haploid production in many crops, because of its simplicity. In *Citrus* and their relatives, haploid plantlets have been recovered from *Poncirus trifoliata* L. Raf. (Hidaka et al., 1979), one doubled haploid plantlet has been obtained from the hybrid No. 14 of *C. ichangensis* x *C. reticulata* (Deng et al., 1992). Moreover, homozygous plants with different ploidy and

highly homozygous embryogenic calli of *C. clementina* Hort. ex Tan. (Germanà et al., 1994; 2000b; 2005; Germanà and Chiancone 2003), haploid, but albino embryos of 'Mapo' tangelo (*C. deliciosa* x *C. paradisi*) (Germanà and Reforgiato 1997), haploid and diploid calli, embryos and leafy structures but no green plants of *C. limon* L (Germanà et al., 1991).

Numerous endogenous and exogenous factors affect embryogenic response of anthers in culture (Smykal 2000; Wang et al., 2000). Particularly, genotype, physiological state and growth conditions of donor plants, stage of gamete development, pre-treatment to flower buds and *in vitro* culture media and conditions of incubation, together with their interactions, are all factors that greatly affect the anther response to the *in vitro* culture (Germanà 2011a; 2011b). There is no single standard condition or protocol to obtain plant formation by anther culture, and it is possible that anthers not only of different species, but also of different cultivars within a species, show very diverse requirements to undertake an embryogenic route and development.

This study has been carried out through three years: 2011, 2012 and 2013, to obtain regeneration through anther and isolated microspore culture of several *Citrus* genotypes, and testing different treatments to the anthers and microspores in culture and achieving gametic or somatic embryogenesis, depending on the genotype.

1.2 Anther culture

1.2.1 Materials and methods

Plant material

The experiments were carried out through two years 2011 and 2012, studying the response to anther culture of *C. sinensis* L. Osbeck and *Citrus clementina* Hort. ex Tan.
In particular, flower buds of the following varieties of blood sweet oranges, *C. sinensis* L. Osbeck, were collected: Tarocco Meli, Tarocco TDV, Tarocco S. Alfio (collected in Lentini, Italy) (2011) and Moro (collected in Palermo, Italy) (2011-2012).
Regarding clementine, *Citrus clementina* Hort. ex Tan., anther culture was carried out with the following cultivars: Corsica and Hernandina (collected in Lentini, Italy) (2011).

Correlation of anther size and microspore gametophyte development

For both *in vitro* culture techniques: anther and isolated microspore culture, the stage of pollen development was tested to identify the vacuolated stage. Anthers from different cultivars and different buds sizes were squashed in a few drops of 4',6-diamidino-2-phenylindole (DAPI) staining solution and observed under fluorescence microscope (Zeiss, Axiophot, Germany).The vacuolated stage (Fig.1A) was previously determined to be the most responsive for *Citrus* gametic embryogenesis (Germanà 2007; 2009). For the experiments, only the flower buds of the same sizes as those with anthers containing microspores at the vacuolated stage (5.0-10 mm in length for oranges, 3.5–5.0 mm for clementine) (Fig. 1B and 1C) were selected for the culture.

Flower bud sterilization and in vitro culture

After a cold pretreatment (4°C for 8-15 days in the dark), flower bud surfaces were sterilized by immersion for 3 min in 70% (v/v) ethyl alcohol, followed by immersion for 20 min in 25% (v/v) commercial bleach (about 0.5% active chlorine in water). Finally, flower buds were rinsed three times for 5 minutes each with sterilized distilled water.
Petals were aseptically removed with small forceps and anthers were carefully dissected and placed in 6 cm diameter Petri dishes containing 10 ml of solid medium.

Medium composition

P induction medium (Germanà et al., 1996) was used as a basal media for all anther culture experiments in all 2011 experiments.

In the second experimental season 2012, N6 medium (Germanà and Chiancone, 2003) and P mediumwere both used as induction media (Table 1).

Experimental design and data collection

2011 Influence of two thermal shocks on anther culture of Citrus clementina Hort. ex Tan, cvs. Corsica and Hernandina, and of Citrus sinensis L***. Osbeck, cvs. Tarocco Meli, Tarocco Sant'Alfio, Tarocco TDV and Moro***

The effect of two thermal shocks on the response of anthers was studied, applying to the *in vitro* cultured explants (after the inoculation and before the culture, at the above reported conditions) the two following treatments:

1) **-20°C for 30 minutes (F).**
2) **37°C for 60 minutes (H).**
3) **No thermal shock (C).**

2012 Influence of two cold treatments and two culture media on anther culture of *Citrus sinensis L. Osbeck****, cv. Moro***

The effect on anther response of two cold treatments and two culture media was studied, culturing the anthers on two media, P and N6, and after that, storing the Petri dishes, for 30 or 60 minutes at -20°C.

In particular, the following theses were tested:

1) **P medium and no thermal shock (P C).**
2) **N6 medium and no thermal shock (N6 C).**
3) **P medium + 30 min at -20 °C (P F).**
4) **N6 medium + 30 min at -20 °C (N6 F).**
5) **P medium + 60 min at -20 °C (P F+).**
6) **N6 medium + 60 min at -20 °C (N6 F+).**

In all the experiments, per each treatment, 10 Petri dishes were prepared (600 anthers /treatment). Moreover, as control, 10 Petri dishes were incubated directly in the growth chamber, at the same conditions above mentioned (C). Sixty anthers were placed in

each Petri dish, sealed with parafilm, then incubated at 26±1°C, for thirty days in the dark, and then placed under cool white fluorescent lamps (Philips TLM 30W/84) with a photosynthetic photon flux density of 35 μmol m^{-2} s^{-1} and a photoperiod of 16 light hrs.

Every month, anthers in culture were observed and after 10 months, the number of anthers that did not develop (Fig. 1D), that were swollen (Fig. 1E) or producing non morphogenic calli or embryogenic calli and was registered per each Petri dish.

This time was chosen because it was previously observed that, after ten months, *Citrus* anthers did not undergo further significant development. These data were used to calculate the means.

The effects of the treatments and of the genotype on registered data were tested by analysis of variance. When values were not normally distributed, data were transformed by arcsin square root. To compare the means, the Games-Howell's test or the Tukey's test were used.

Germination medium and calli multiplication

The embryos and the embryogenic calli were transferred into the germination medium in 15 x 100 Petri dishes, with MS medium (Murashige and Skoog, 1962) containing MS Salts, MS Vitamins, 34 g/l sucrose, 0.5 g/l Ascorbic acid, 0.5 g/l mannitol, 1 mgl^{-1} GA_3, 0.02 mgl^{-1} NAA and 8.5 g/l agar. This procedure was repeated every month until plantlet regeneration.

In vitro grafting and plant recovery

Although the *Citrus* embryos germinated vigorously, usually the homozygous plantlets grow slowly in soil, presumably due to the expression of harmful recessive genes expressed in homozygosity (inbreeding depression). These homozygous plantlets always died as a result of fungal infection (Germanà 2007).

To improve the survival of plantlets *in vitro*, grafting of homozygous small shoots (2-3 mm) was carried out onto etiolated 20 day old Troyer citrange seedlings (Fig. 1F),obtained from seed germination in MS micro grafting medium containing MS Salts, MS Vitamins, 30 g/l sucrose, 0.1 g/l Myoinositol and 6 g/l agar.

After about 4 months of the micro grafting, when the grafted plantlets obtained were about 4-5 cm high, plantlets were washed with sterilized water and subsequently transferred to the greenhouse in pots containing sterilized peat moss, sand and soil in the ratio of 1:1:1 for the hardening phase.

Characterization of regenerants: ploidy analysis

Ploidy level of the obtained regenerants was evaluated by flow cytometry analysis. Tissue from calli, embryoids and plantlets was chopped with a razor blade in presence of 1 ml of nuclei extraction buffer (Partec) and a diploid control sample. The suspension was filtered through 30μm nylon filter and mixed with 4 ml of a DAPI stain solution. Relative DNA content of each sample was determined using a Partec Cell analyser PA II (Partec GmbH, Münster, Germany) and the reference control sample.In 2011, a total of 96 regenerants were analyzed: of these 30 were analyzed from blood sweet orange cultivars (9 from Moro, 12 from Tarocco S. Alfio, 3 from Tarocco TDV and 6 from Tarocco Meli) and 66 from clementine cultivars (51 from Hernandina e 15 from Corsica).In 2012, a total of 7 regenerants were analyzed from Moro blood sweet orange.

Characterization of regenerants: analysis of heterozygosity **(carried out by Prof. Testolin and Dr. Orietta Lain, Dipartimento di Scienze Agrarie e Ambientali, Univerisity of Udine. Italy)**

In order to analyse and characterize anther regenerants, samples were collected and Simple Sequence Repeats (SSRs) have been utilized to assess homozygosis or heterozygosis and to determine the origin (gametic or somatic) of calli and of regenerated plantlets, irrespectively of their ploidy. Total DNA was extracted from 0.2 g of plant material (young leaves or calli).

The followings regenerants were analysed: 3 of Moro, 3 of Tarocco Meli, 3 of Tarocco S. Alfio and 1 of Tarocco TDV, 1 of Corsica and 5 of Hernandina (2011), 10 of Moro (2012), by means of the Qiagen Plant DNA Mini Kit following the manufacturer's protocol. DNA concentration in samples was determined by fluorometer (Hoefer DyNA Quant 200) using the Hoechst H 33258 fluorescent dye and human DNA (50-250 ng/μL) as a standard.

Fourteen tri-nucleotide microsatellites isolated by Kijias et al. (1997) from *Citrus x limonia* (rangpur lime) x *Poncirus trifoliata* were screened in a preliminary test and four of them (TAA1, TAA15, TAA41, and CAC15) were selected for the analysis for their polymorphism, the easily scorable pattern, and the little stuttering.

PCRs were carried out on 8 μL volume containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.3 μM each primer (forward primer was

labelled with either FAM or HEX fluorescent dye), 20 ng genomic DNA and 0.3 U of Taq polymerase (Amersham Biosciences, USA) using a PT 100 termal cycler (MJ Research, USA). The PCR thermal profile was as follows: 94 °C for 5' for 1 cycle; 94°C for 60", 55 °C for 30", 72 °C for 60" for 32 cycles, 72 °C for 5'.

One μL of desalted PCR product was mixed with 2.75 μL of loading solution (70 % formamide, 1 mM EDTA), 0.25 μL ET-ROX dye (Et400-R size standard, Amersham Biosciences, USA), and 1.0 μL deionised H_2O, centrifuged at 900 rpm for 2', denatured at 95°C for 4', cooled in ice and electrophoresed on a MegaBACE 500 capillary sequencer (Amersham Biosciences, USA). Fragment profile was analysed using Genetic Profiler v2.0 (Amersham Biosciences, USA). Each PCR/EF run was repeated 4 to 6 times.

Cellular architecture

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 and after about ten 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) 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).

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.

1.2.2 Results and discussion

Anther culture, embryo and plantlet regeneration

After one week of culture, many anthers start already to swell and to produce calli. Anther-derived calli were non-morphogenic, or highly embryogenic (Fig. 2A). The route of regeneration was also, but more rarely, through direct embryogenesis (Fig. 2B), through organogenesis (Fig. 2C) or through secondary embryogenesis observed at the basis of an embryo (Fig. 2D). The embryogenic calli differentiate into a clump of embryos (Fig. 2E).

The embryogenic callus was multiplied (Fig. 2F), and, once the embryos appear, they were germinated in Petri dishes and afterward transferred to Magenta boxes (Sigma V8505) or to test tubes. Most of the obtained embryos were well-structured and they developed like zygotic embryos, through the globular, the heart, the torpedo and the cotyledonary stages.

The anthers collected at different times during the culture, squashed and DAPI stained, observed under fluorescence microscopy showed the presence of different features. Also, an asynchrony in the developmental response of the microspores was observed. In fact, bicellular pollen grains contained nuclei with different chromatin condensation pattern, such as the larger vegetative nucleus and the smaller generative one were also detected (Fig. 2G), denoting an asymmetrical division, typical of pollen maturation.

Additionally, in all genotypes, pollen grains with two similar nuclei (Fig. 2H), formed by a symmetrical division, together with multinucleate pollen grains (Fig. 2I), developed when the nucleus divides without cell division, were observed, indicating that these microspores switched their developmental programme towards proliferation. In some anthers, after six months in culture, large multicellular structures or proembryos were observed. In fact, association between the polarity of first pollen division and ability to embryogenesis induction from microspores has been showed (Twell and Howden 1998), and symmetric division of pollen nucleus is considered as first indication of onset of embryogenic program (Seguí-Simarro and Nuez 2008).

2011 Influence of two thermal shocks on anther culture of *Citrus clementina Hort. ex Tan****, cvs. Corsica and Hernandina, and of*** *Citrus sinensis* L.*Osbeck****, cvs.Tarocco Meli, Tarocco Sant'Alfio, Tarocco TDV and Moro.***

Results regarding the comparison among the responses of anther culture of two cultivars of *Citrus clementina* Hort. ex Tan: Hernandina and Corsica, and of four cultivars of *Citrus sinensis* (L.) Osbeck: Moro, Tarocco S Alfio, Tarocco TDV and TaroccoMeli, after ten months of culture, are reported in Table 2. All genotypes gave rise to a morphogenesis process from *in vitro* cultured anthers, even if with great genotype-dependent differences.

In fact, a strong genotype effect was observed for all parameters registered (not developed, swollen, with callus and with embryogenic callus or with embryos anthers). Particularly, significant differences were observed in the percentages of not developed, swollen anthers and anther with callus: Tarocco S. Alfio and Meli showed the lowest amount of the first type and the highest of the second one. Regarding the anthers with callus, the highest percentages were observed in clementine Corsica and in oranges Tarocco S. Alfio and Meli, although not always the calli produced were embryogenic. Moreover, regarding the percentages of anthers producing embryogenic calli, the genotype exhibiting more anthers with embryogenic calli (Hernandina 3.3 %), was not among that ones with the highest value of anthers with callus. The percentage of anthers producing embryogenic calli, the most interesting data, was higher in clementines compared to the blood oranges.

Furthermore, regarding the effect of the temperature treatments, it was observed a different behavior between sweet oranges and clementines, even if not supported by statistical analysis, since the cold treatment (-20°C) induced a noticeably greater production of embryogenic calli in anthers of Hernandina (5.6%) and of Corsica (3.7%), while it had not clear effect in the development of sweet orange explants. Among the blood oranges, it was observed that the cultivar 'Tarocco S. Alfio' showed the highest response of anthers with callus and with embryogenic callus, regardless of the temperature treatment tested, followed by the cultivar 'Tarocco Meli' (Fig. 3).

2012 Influence of two cold treatment and two culture media on anther culture of Citrus sinensis* L. *Osbeck, cv. Moro.

Results regarding this experiment are reported in table 3.

Statistical analysis carried out on data recorded after ten months in culture, did not show statistically differences for the parameters not developed anthers, while regarding the swollen anthers, the factor that had the main influence was the cold treatment, more than the culture medium. In fact, the treatment -20°C for 30 min gave the best results. On the contrary, for the parameter anther with callus, the main factor was the culture medium. Medium P induced a higher callus production than N6 medium. Moreover, even if no significative interaction was recorded, it is possible to conclude that the worst combination was medium N6 with cold treatment -20°C for 30 min.

A low percentage of anthers regenerated embryogenic calli; in particular, 0.7% and 0.1% has been detected, respectively, in anthers subjected to the following treatments: P control and P + -20°C for 30 min. Because it was not possible to observe regeneration in all theses, it was not possible to carry out the statistical analysis.

Particularly in recalcitrant genotypes, the use of a stress can be required to efficiently switch the gamete development towards the sporophytic pathway. In fact, it has been observed in many genotypes that treatments (both physical or chemical) applied before the culture to the excised flower buds, to the whole inflorescences or to the isolated anthers, work as a trigger to induce the sporophytic pathway, preventing the pollen maturation (gametophytic pathway) (Germanà 2011a; 2011b). Among these treatments, chilling, high temperature, high humidity, water stress, anaerobic treatment, centrifugation, sucrose and nitrogen starvation, ethanol, irradiation, microtubuli disruptive agents, electrostimulation, high medium pH, heavy metal treatment, etc. have been used in anther and in microspore culture. Shariatpanahi et al. (2006) classified them in three categories: widely used, neglected, and novel.

Stress seems to act in different ways: altering the polarity of the division at the first haploid mitosis, involving reorganization of the cytoskeleton (Nitsch and Norreel 1973, Reynolds 1997), delaying and modifying pollen mitosis (two equal-size vegetative-type nuclei instead of one vegetative and one generative) and blocking starch production or dissolving microtubules (Nitsch 1977). Others profound cytoplasmic and nuclear rearrangements, and gene expression occurs before and along induction of microspore embryogenesis (Seguí-Simarro and Nuez 2008).

The temperature shock isdefinitely the most effective and the easier to apply treatment to induce pollen embryogenesis development. However, the optimum level and duration of the shock, change depending on the genotype (Dunwell et al., 1983). Actually, in this study different results have been produced by the same thermal shock. Thermal shock, as other stress treatments, is frequently associated with biosynthesis of heat-shock proteins (HSPs), but actual studies showed that HSPs have an indirect effect on role of microspore embryogenesis (Seguí-Simarro and Nuez, 2007, Seguí-Simarro 2010), that is directly related to stress tolerance (Seguí-Simarro and Nuez, 2008a, 2008b).

Characterization of regenerants: ploidy analysis

The ploidy analysis by flow cytometry revealed that 30 regenerants (embryos and plantlets) obtained through 2011, all sweet orange anther culture regenerants were diploid (2n), the same results were obtained from the 7 regenerants of Moro blood sweet orange in 2012 anther culture experiment, regardless of the cultivar or temperature treatment used (Fig. 4A). On the contrary, all 66 analyzed regenerants from the anther culture of clementine were triploids (Fig. 4B).

Previous investigations on the ploidy of 94 regenerants obtained through anther culture of clementine, carried out by flow cytometry analysis, already showed that about 82% of them were tri-haploids, rather than haploids or doubled-haploids, as expected (Germanà et al 2005a; Germanà 2007; 2009). Non-haploid (diploid, triploid, tetraploid, pentaploid, hexaploid) embryos and plantlets have been obtained from anther culture of other genotypes (D'Amato 1977). In fruit crops, triploids have been often regenerated through anther culture in apple (Hofer 1994; Hofer et al 2002), *Pyrus pyrifolia* Nakai (Kadota and Niimi 2004) and *Carica papaya* L. (Rimbeira et al 2006).

Triploids are important for fruit breeding, due to the seedlessness of their fruits and for this reason anther culture can be used to produce triploid plants that may be of great commercial importance when seedlessness is required by consumers, such as for *Citrus* or table grape. Triploids, traditionally produced by 2x x 4x and 4x x 2x crosses, can also be obtained in *Citrus* through *in vitro* culture of endosperm or by fusion between haploid and diploid protoplasts (Kobayashi et al 1997; Ollitrault et al 2000). In addition, also anther culture can be a reliable technique to recover new triploid varieties in clementine.

Characterization of regenerants: analysis of heterozygosity

Because of the spontaneous chromosome doubling occurring in the haploid calli and embryos, ploidy level analysis cannot identify pollen-derived plants. In fact, when *in vitro* anther or ovary are cultured, diploid plants, such the ones obtained in this experiment, can be either homozygous spontaneously doubled haploids or heterozygous diploids derived from somatic tissue.

To discriminate between these two kinds of regenerants, microsatellite DNA loci that were heterozygous in the parental genotypes at a preliminary screening were used.

Three out of four SSRs selected, that is TAA1, TAA15, and TAA1, were heterozygous in the orange clones of either 'Tarocco' and 'Moro' cultivars, while CAC15 was apparently homozygous displaying a single allele, that has been considered at homozygous state; the SSRs selected were all heterozygous in the clementine cultivars 'Hernandina' and 'Corsica'.

The allele size ranges were compatible with data reported in the literature (Kijias et al. 1995) with small differences in absolute size in base pairs depending on the size standard, polymer in the capillary and machine as known from the literature (Testolin and Cipriani 2010).

All regenerants obtained from the orange varieties 'Tarocco' and 'Moro' through 2011 and 2012 anther culture experiments showed allelic patterns identical to those of the original mother plants from which regenerants were obtained and, therefore, from a genetic point of view they were heterozygous of somatic origin.

All regenerants of clementines 'Hernandina' and 'Corsica' analysed, showed homozygous allelic pattern, displaying either allele of the mother plant. Results were absolutely consistent at all SSR analysed and should indicate a gametic origin of regenerants (Fig. 5).

Interestingly,it was observed that all 'Tarocco' and 'Moro' orange clones showed the same allelic profiles at all SSR analysed. In spite of the small number of SSR analysed that does not allow to draw any ultimate conclusion, the identical profile at four loci could indicate a common somatic origin of the cultivars and clones of study, a common occurrence in many *Citrus* species as observed by several authors (Fang and Roose 1997).

Microsatellites have been previously employed to characterize regenerants obtained from *Citrus* anther culture (Germanà and Chiancone, 2003; Germanà et al., 2005). Biochemical markers can also be employed to demonstrate the androgenetic or origin of

regenerants from *Citrus* anther culture (Deng et al., 1992; Germanà et al., 1991; 1994; 2000a, b; Germanà and Reforgiato, 1997). In fact, in previous research, isozyme techniques allowed to distinguish between gametic and somatic tissue (Germanà et al., 1991; 1994; 2000a, b; Germanà and Reforgiato, 1997).

Cellular architecture

In 2011, anther culture experiments, the cellular changes and organization promoted by the treatments were used to study the relation between structural development and triggering of the sequent differentiation events towards the embryogenic pathway.

Anthers from all different *Citrus* cultivars were directly excised from the trees (time zero) then fixed and processed as well as the anthers taken from the different treatments after about ten months of culture. Results illustrated that, most of anthers collected at time zero mainly contained vacuolated microspores at a later developmental stage and rarely bi-cellular pollen with a large nucleus. In oranges cvs., the structural analysis of anthers development under the long-term anther culture condition showed differences between the different treatments in structural features, anthers wall somatic tissues, the covering layers of the pollen sac, the microspores development stages and in anthers inter-cellular communication respect to non-cultured anthers (time zero). Generally the results demonstrated less anthers wall damage and tissues decay after one year of culture from the control treatment. Meanwhile at the high temperature treatment (34 ºC 1h) has been recorded the highest percentage of somatic tissues decay). No structures differences were significantly affected by the different treatments in clementine cvs. Hernandina and Corsica.

Cell to cell communication is a very important factor controlling growth, development and triggering the cell differentiation. The inter-cellular communication controls the selective exchange of proteins, transportation of salts, sugars and other different nutrients substances (Kim and Zambryski, 2005; Kobayash et al, 2007; Barany *et al*., 2005a,b, 2010a,b).

Results illustrated that, in some microspores, nucleus divided producing two cells equivalent in size and structural features, in all the different treatments and cultivars. The symmetric division of the microspores occurring during the normal gametophyte development is the first morphological evidence of the embryogenic pathway (Zaki and Dicknson, 1991).

Different microspore division patterns have been recorded. Some multicellular structures were obtained especially from clementine. In most of long-term woody plant anther cultures, many microspores died or stopped their development or turned into what it called Pollen-like structure. Otherwise it may appear as unusual clear microspores. In orange cv. Moro, the percentage of stopped microspores was higher in the control treatment comparing with the other treatments. In other cultivars, the percentage of stopped and died microspores appeared to be independent.
Starch storage deposition at time zero, and after one year of anther culture were investigated. Results showed that, starch accumulation and deposition were changed temporally and spatially during the anther culture depending on the genotype and treatment.

In orange, cv. Tarocco, the starch distribution after culture was higher in the somatic anther wall tissues comparing with the percentage of starch accumulation in microspores. No starch was detected by the iodide based staining (I_2KI) in many microspores after culture and before it (time zero). In clementine cultivars, starch deposition appear to be independently localized at these experimental conditions. These results, particularly in orange cvs., indicated that, an higher percentage of starch cytochemistry in somatic anther tissues than in microspores after culture could have an important role in blood sweet oranges embryogenesis.
Carbohydrates are energy source for the cell carbon framework and biosynthetic processing. They also act as osmotic agents and contribute to the maintenance of plasma membrane integrity (Pareddy and Greyson 1989; Tremblay and Tremblay 1991). Embryo development stages are influenced by the metabolic levels of sugars that act as developmental signals (Rook and Bevan 2003). The high and practically constant amount of carbohydrates in every embryogenic stagein *Acca sellowiana* suggests the importance of sugars in the embryo development (Iraqui and Tremblay 2001). found that carbohydrate ratio could act as triggers influencing division and differentiation in the early development during somatic and zygotic embryogenesis of *Acca sellowiana.*
In *Eriobotrya japonica* (Germanà et al. 2006) observed a high starch accumulation in the bicellular pollen grain as an early microspore embryogenic pathway after about 6 months of anther culture. Starch accumulation and deposition has been observed and suggested to be associated with polarity establishment and cell differentiation (Raghavan 2000; Testillano et al., 2000; Indrianto et al., 2001).

In this study, the results showed that starch accumulation was significantly higher in somatic anthers tissues than in the microspores after about one year of oranges anther culture. The role of starch storage and deposition in gametic embryogenesis remains to be clarified (Fig. 6).

Tables

Table 1.Response to anther culture of two cultivars of *Citrus clementina*, Hernandina and Corsica, and of four cultivars of *Citrus sinensis*, Moro, Tarocco S Alfio, Tarocco TDV, Tarocco Meli, after ten months of culture.

Cultivar	Not developed anthers (%)		Swollen anthers (%)		Anthers with callus (%)		Anthers with embyoids or embryogenic callus (%)	
Hernandina	58.5	a	27.5	b	10.6	b	3.3	a
Corsica	49.9	a	31.4	b	16.2	a	2.4	a
Moro	60.4	a	29.3	b	10.0	b	0.3	b
Tarocco S. Alfio	12.8	b	65.0	a	21.4	a	0.8	b
Tarocco TDV	47.9	a	42.8	b	9.1	b	0.2	b
Tarocco Meli	19.1	b	63.9	a	16.8	a	0.3	b

Values within each column followed by different letters are significantly different at $p \leq 0.05$ (Games-Howell for not developed, swollen anthers and anthers with embyoids or embryogenic callus; Tukey's test for anthers with callus)

Table 2. Influence of two different types of medium and of thermal shocks on the anther response recorded after 10 months of *in vitro* anther culture cv. Moro and the interaction between the different factors

Medium	Thermal shock	Not developed anthers (%)	Swollen anthers (%)	Anthers with callus (%)
P	**Control**	40.3	51.4	7.7
P	**-20 ˚C 30 min**	36.1	59.8	3.9
P	**-20 ˚C 60 min**	30.6	62.7	6.8
N6	**Control**	55.4	40.7	3.9
N6	**-20 ˚C 30 min**	38.1	60.7	1.2
N6	**-20 ˚C 60 min**	41.4	57.3	1.3
Medium		p = 0,101	p = 0,307	p = 0,007
Thermal shock		p = 0,289	p = <0,001	p = 0,218
Medium x Thermal shock		p = 0,430	p = 0,607	p = 0,608

Two-way ANOVA, Tukey's test at p≤0.05.

Figures

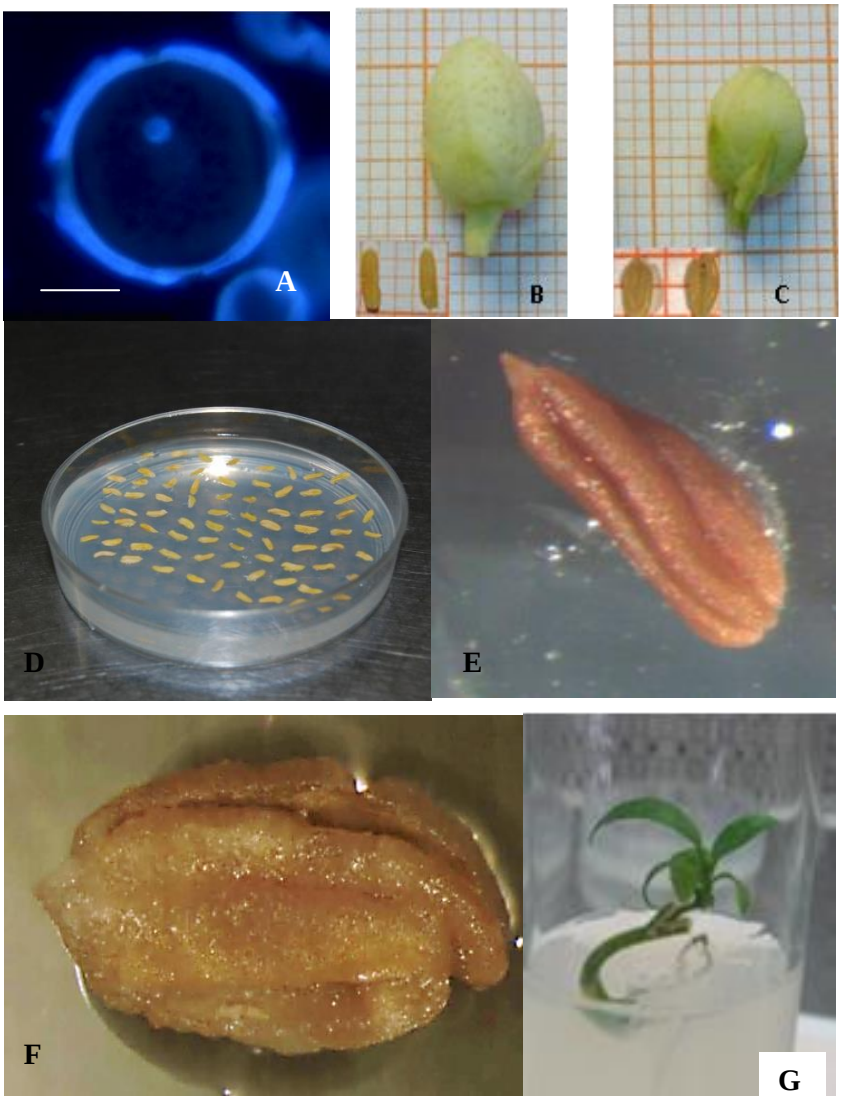


Fig. 1. Gametic and somatic embryogenesis in *C. sinensis* L. Osbeck and *Citrus clementina* Hort. ex Tan. **A**: Vacuolated microspore of *Citrus sinensis* cv Moro. **B**: Flower bud and anthers of *C. sinensis* L. Osbeck, cv Moro, with vacuolated microspores. **C:** Flower bud and anthers of *Citrus clementina* Hort. ex Tan, cv. Corsica, with vacuolated microspores. **D**: Petri dish with Moro anthers in culture; **E**: Not developed anther; **F**: Swollen anther; **G:***In vitro* grafting of small shoot apexes (2-3 mm) of homozygous clementine regenerant, onto 20 day old, etiolated 'Troyer' citrange seedling.

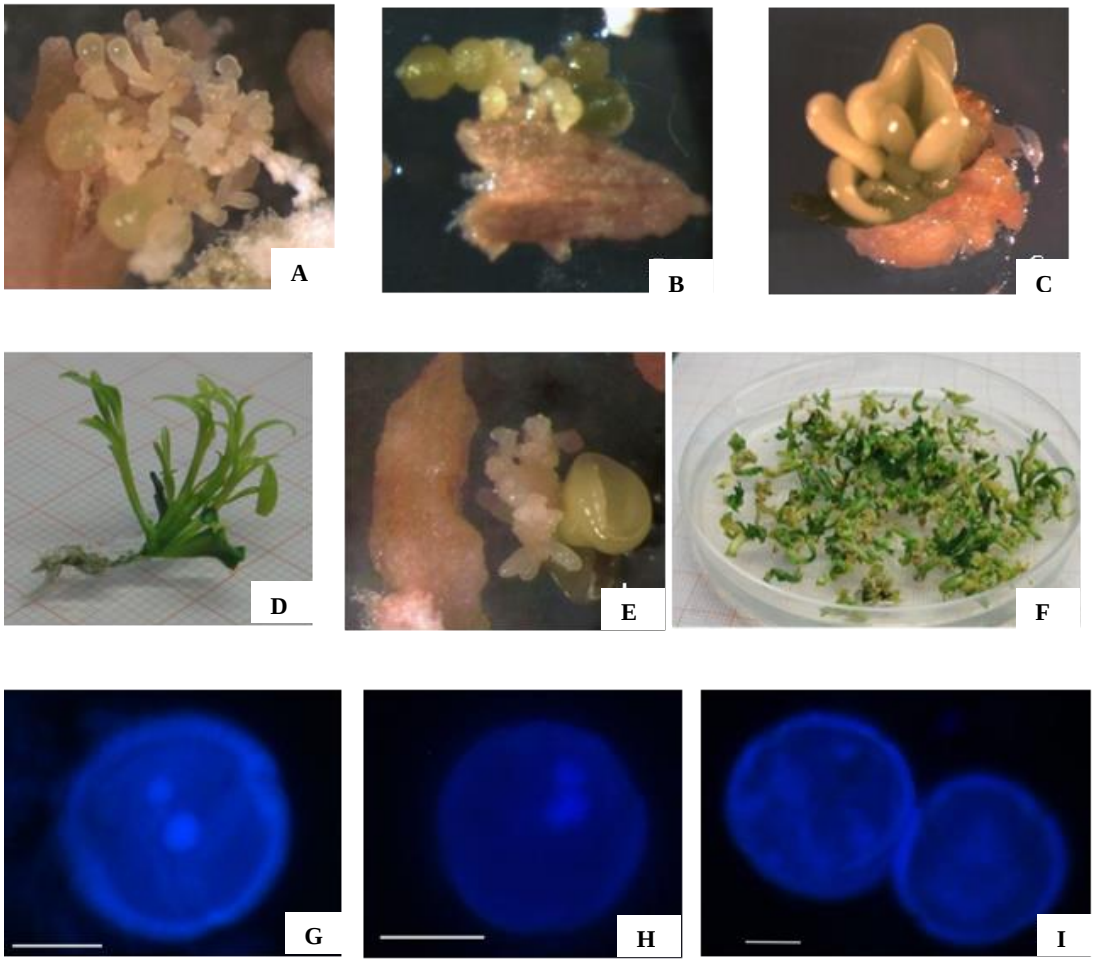


Fig. 2. **A**: Highly embryogenic callus of *C. sinensis* L. Osbeck, cv Moro. **B**: Embryogenic callus and embryos emerging from an anther of *C. clementina* Hort. ex Tan, cv. Hernandina. **C**: Direct embryogenesis from a *C. clementina* Hort. ex Tan, cv. Corsica. **D:** Organogensis in anther culture of *C. sinensis* L. Osbeck, cv Tarocco S. Alfio. **E**: Secondary embryogenesis observed at the basis of an embryo in Hernandina anther culture. **F**: Multiplication of highly embryogenic callus of Tarocco Meli. **G**: Bicellular pollen grain, containing nuclei with different chromatin condensation pattern. **H**: Pollen grain with two similar nuclei, formed by a symmetrical division. **I**: Multinucleated pollen grains of Corsica clementine. (Bars in G, H: 20 μm, in I: 10 μm).

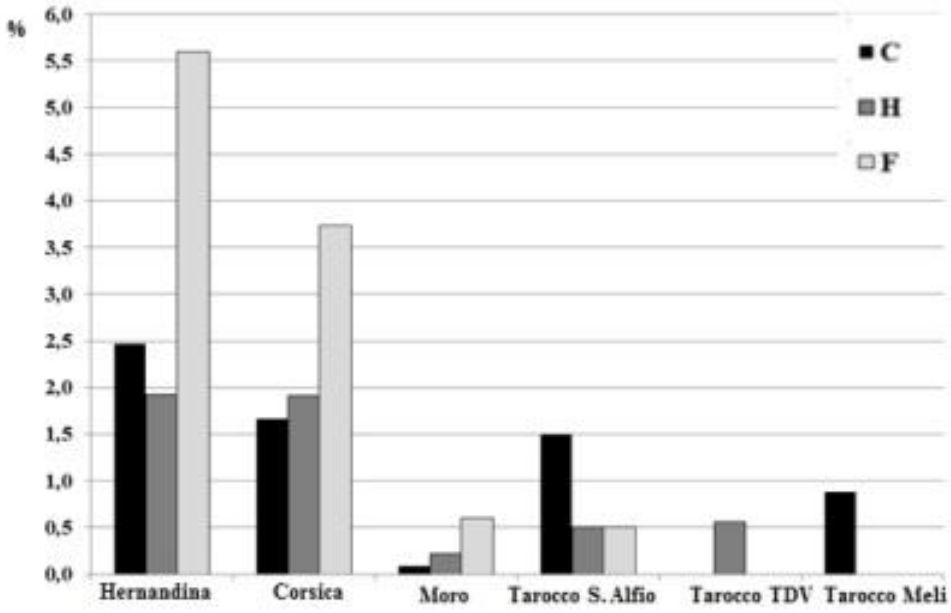
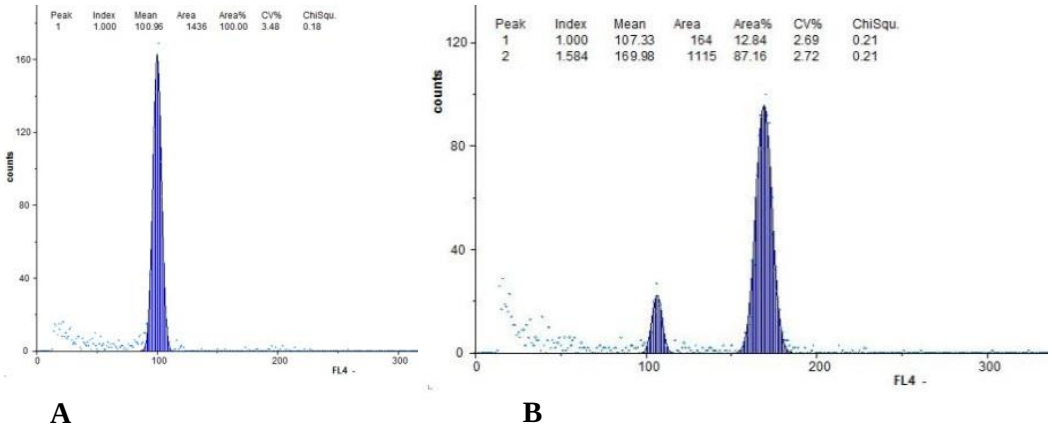


Fig. 3. Effect of the temperature treatments on the response of *C. sinensis* L. Osbeck and *Citrus clementina* Hort. ex Tan anthers with embyoids or embryogenic callus F: -20°C for 30 minutes; H: 37°C for 60 minutes; C: no temperature treatment.



A **B**

Fig. 4 Characterization of anther culture regenerants.**A**: Cytofluorimetric analysis: histograms of fluorescence intensity of nuclei from diploid leaf tissue of *C. sinensis* L. Osbeck and of a diploid regenerant. **B**: Cytofluorimetric analysis: histograms of fluorescence intensity of nuclei from diploid leaf tissue of *Citrus clementina* Hort. ex Tan mother plant (peak 1) and triploid embryo of *Citrus clementina* Hort. ex Tan, *cv* Corsica anther culture regenerant (peak 2).

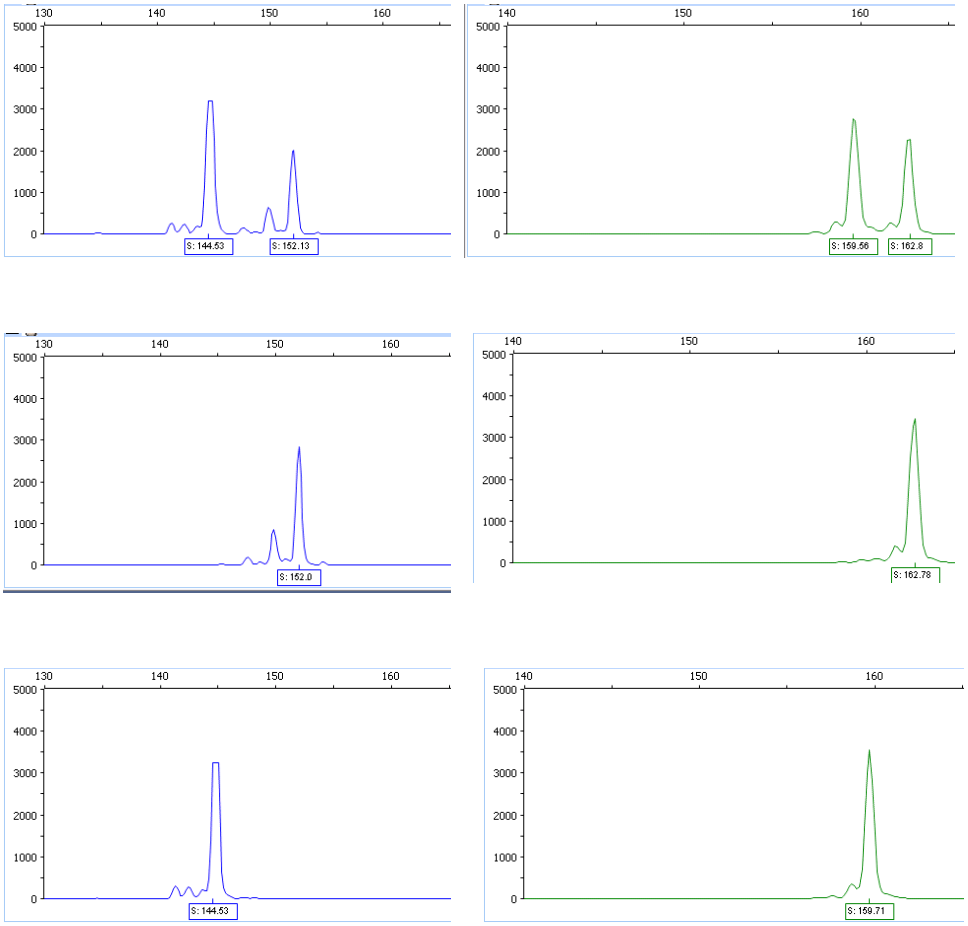


Fig. 5 Characterization of anther culture regenerants. Microsatellite analysis: Pherograms of the microsatellite markers TAA41 (left) and TAA1 (right) profiles of the mother plant (top) and two regenerants of Hernandina clementine (*C. clementina* Hort. ex Tan). While mother plant is heterozygous carrying two alleles, regenerants display either allele of their mother plant. The presence of alternative alleles of the mother plant has been considered as a proof of the gametic origin of regenerants

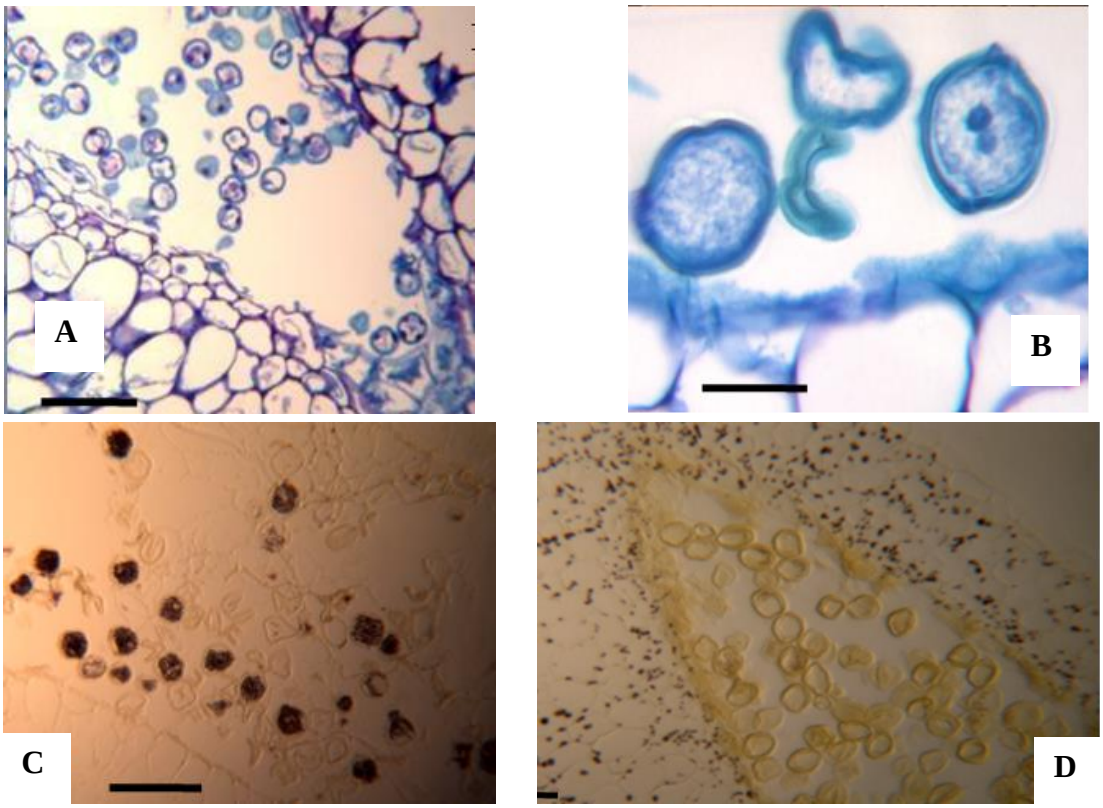


Fig. 6 Cellular architecture in orange, cv. Moro semithin technovit sections. **A-B:**, Toluidin blue staining for general structure. **A:** time zero vacuolated microspores. **B:** microspore producing two nuclei equivalent in size and structural features. **C-D**: Effect of long-term anther culture condition on structural features. I_2KI cytochemistry for starch. **C:** at time zero**,** numerous starch grains in the microspores as dark deposits. **D:** starch accumulation significantly higher in somatic anthers tissues than in the microspores after about one year of oranges anther culture **C:** (C-control. Bars represent in A,C, D: 50μm, in B: 10μm.

1.3 Isolated microspore culture

1.3.1 Material and methods

Plant material

The experiments were carried out in 2013**,** studied the response to gametic embryogenesis of one cultivar of *C. sinensis*L. Osbeck, Moro and two cultivars of *Citrus clementina* Hort. ex Tan., Monreal Rosso (MAR) and Nules, collected in Palermo (Italy).

To individuate the right flower bud and anther size to start the isolated microspore culture, the same protocol used for anther culture was followed.

Microspore isolation protocol

1- The flower buds sterilized as above reported, containing microspores at the late vacuolated stage,were opened with sterilized forceps and about 1000 anthers placed in Petri dish containing 6 ml of sterilized 0.4 M mannitol solution + 0.5 g/l Ascorbic acid at 4°C.

2- Anthers suspended in 6 ml0.4 M mannitol solution were placed in a sterilized blender and 14 ml of 0.4 M mannitol was added to complete the volume to20 ml.

3- The blender was used five times for 5 seconds each, at a minimum speed. Then, the blender content was filtered through 100 µm sterilized filter and placed in a sterile magenta box®. The remaining anthers were crashed again if needed.

4- The magenta box content was transferred into a Falcon tube (50 ml).

5- The Falcon tube (50 ml containing the suspended microspores) was centrifuged at 8500 rpm for 10 minutes at 4 º C. 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.

6- The clean pellet was re-suspended in 20 ml of mannitol and 20 µl of the suspension was used to measure the density by a Burker camera. The density prepared for all experiments was 100,000 microspores per ml of medium. A volume of 1.0 ml was placed into each Petri dish.

7- Finally all Petri dishes were put in the dark at 26±1˚C for the first 30 days, then placed under cool white fluorescent lamp (Philips TLM 30W/84, France) with a photosynthetic photon flux density of 35 μmol m^{-1} s^{-1} and photoperiod of 16 light hours.

Experimental design and data collection

In 2013, microspores response was evaluated to five different media obtained modifying the P medium (Germanà et al., 1996; Germanà and Chiancone, 2003) in the following way:

1) **P medium as control treatment (PC).**
2) **0.53 mgl^{-1} meta- Topolin+ 0.5 mgl^{-1} Zeatin (PMT/BA).**
3) **0.55 mgl^{-1} meta- Topolin+ 0.5 mgl^{-1} Benzylaminopurine (PMT/ZEA).**
4) **5.3mgl^{-1} meta- Topolin+ 0.5 mgl^{-1} Zeatin (PMT/BA10).**
5) **5.5 mgl^{-1} meta- Topolin+ 0.5 mgl^{-1} Benzylaminopurine (PMT/ZEA10).**

For each of the five media (Table 4), eight replicates were prepared (40 Petri dishes/cultivar).

After seven months of culture, 400 microspores per treatment were observed (four replicates with around 100 microspores per each) bya fluorescence microscope (Zeiss, Axiophot, Germany).Microspore developmental stages were determined by putting microspores in a few drops of DAPI solution (1 mg ml^{-1}) and observed.

For all the media tested, different structural features have been observed and registered: microspores uninucleated with no development (Fig. 7A), binucleated with two asymmetrical nuclei (normal gametophytic pathway: one vegetative and one generative nucleus) (Fig. 7B), binucleated with two equal-size vegetative-type nuclei that had just started their sporophytic pathway (Fig. 7C), trinucleated (Fig. 7D), tetranucleated, multinucleated (Fig. 7E and 7F), and, for the first time, calli (Fig. 7G) and embryos at different stages have been obtained (Fig. 7H).

These values were used to calculate means, differences among media were tested by one-way ANOVA performed to isolate the influence of the media composition. Differences among means were tested by Tukey's multiple comparison test at ($p \leq 0.05$).

1.3.2 Results and discussion

Effect of meta-Topolin on in vitro isolated microspore culture response of *Citrus sinensis* (L.) *Osbeck****, cv. Moro****.*

The statistical analysis performed after seven months of isolated microspore culture of *C. sinenesis* L. Osbeck, cv. Moro (Table 5), revealed that in the percentages of uninucleated, binucleated microspores no statistically differences have been obtained. The highest significant value of trinucleated microspores were observed in medium P control (13%), While the lowest in the media PMT/ZEA (6.8%) and PMT/BA (8.2%). On the contrary, the highest percentages of multinucleated microspores were observed with the same two media treatments PMT/ZEA (26.2%) and PMT/BA (28.3%). Although no statistical differences were obtained from both uninucleated and binucleated microspores, Medium P seems to induce the highest percentage of binucleated microspores (with the first sporophytic symmetrical nucleus division) and the statistically highest percentage of trinucleated microspores; in the medium P, it was observed the lowest percentage of multinucleated (21.9%). Possibly to go further in the gametic embryogenesis pathway, the support of the lowest concentration of Meta-Topolin was needed.

According to the percentage of microspores producing calli, the best results were observed in the medium PMT/ZEA10 (12.8%), meanwhile, the worse result was observed in the medium PMT/BA10 (2.7%).

In the number of microspores formed embryos, no statistical differences were recorded among the different media treatment even if the highest values have been recorded from both PMT/ZEA10 and PMT/BA10 media treatment.

Effect of meta-Topolin on in vitro isolated microspore culture response of Citrus clementina Hort. ex Tan, cv. Monreal Rosso and Nules

A strong genotype-medium interaction has been observed for the parameters: uninucleated, binucleated microspores and calli. In fact, each cultivar responded differently depending on the tested medium (Table 6). For the trinucleated and the multinucleated microspores, the medium composition was the main factor affecting their response (Graph 1). The sporophytic development of isolated microspores in culture has been, also, confirmed by the production of calli and pollen-derived embryos at different developmental stages. Finally, the cv. Monreal Rosso produced a statistically higher number of pollen-derived embryos per Petri dish, independently on the used medium (Graph 2).

Tables

Components	Media					
	P	**PMT/BA**	**PMT/ZEA**	**PMT/BA10**	**PMT/ZEA10**	**N6**
	Per litre					
N6 Chu Salts	1X	1X	1X	1X	1X	1X
N&N Vitamins	1X	1X	1X	1X	1X	1X
Galactose	18 g	18 g	18 g	18 g	18 g	9 g
Lactose	36 g	36 g	36 g	36 g	36 g	18 g
Ascorbic Acid	500 mg	500 mg	500 mg	500 mg	500 mg	500 mg
Myoinositol	5 g	5 g	5 g	5 g	5 g	-
Biotin	500 mg	500 mg	500 mg	500 mg	500 mg	500 mg
Thiamine	5 mg	5 mg	5 mg	5 mg	5 mg	-
Pyridoxine	5 mg	5 mg	5 mg	5 mg	5 mg	-
2,4-D	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.02 mg
IAA	-	-	-	-	-	0.02 mg
Kinetin	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.5 mg	1 mg
Zeatin	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	0.5 mg
TDZ	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.1 mg
BAP	0.5 mg	-	0.5 mg	-	0.5 mg	0.5 mg
Meta-Topolin	-	0.5 mg	0.5 mg	5.3 mg	5.5 mg	-
Coconut water	100 ml	100 ml	100 ml	100 ml	100 ml	50 ml
Casein	500 mg	500 mg	500 mg	500 mg	500 mg	500 mg
Serine	100 mg	100 mg	100 mg	100 mg	100 mg	-
Glycine	2 mg	2 mg	2 mg	2 mg	2 mg	-
Glutamine	800 mg	800 mg	800 mg	800 mg	800 mg	200 mg
Malt extract	500 mg	500 mg	500 mg	500 mg	500 mg	-
pH	5.8	5.8	5.8	5.8	5.8	5.8

Table 4. Medium composition

Table 5 Influence of medium composition on *C. sinensis* L. Osbeck, cv. Moro, isolated microspore development, after seven months of culture

MEDIUM	Uninucleated (%)	Binucleated asymmetrical (%)	Binucleated symmetrical (%)	Trinucleated (%)	Multinucleated (%)	Calli (%)	Embryos/Petri dish (n°)
PC	41.2	3.0 a	14.2	13.0 a	21.9 b	6.7 ab	5.0
Pmt/BA	46.2	1.6 ab	10.0	8.2 b	28.3 a	5.7 ab	4.6
Pmt/ZEA	51.0 ns	1.5 ab	11.1 ns	6.8 b	26.6 ab	2.9 b	4.5 ns
Pmt/BA10	50.6	1.4 ab	12.1	9.9 ab	23.3 ab	2.7 b	6.8
Pmt/ZEA10	42.2	1.0 b	10.4	8.9 b	24.5 ab	12.8 a	6.3

Values within each column followed by different letters are significantly different at p≤0.01 (One way ANOVA, Tukey's test)

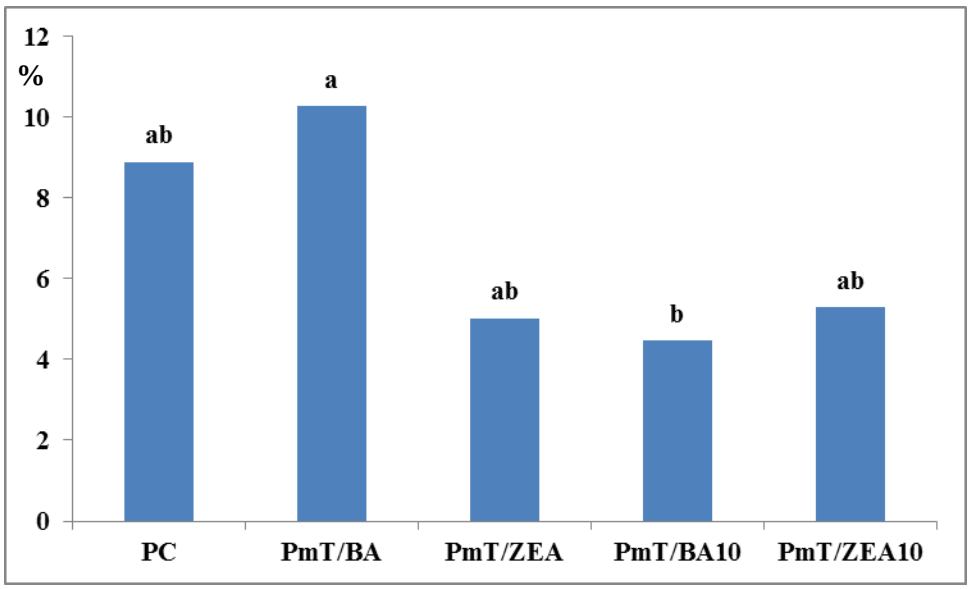
Tab. 6 Influence of cultivar and medium composition on clementine, cvs. Monreal Rosso and Nules, isolated microspore development, after seven months of culture

CV	MEDIUM	Uninucleated %	Binucleated %	Trinucleated %	Multinucleated %	Calli %	Embryo/ Petri dish (n°)
Monreal Rosso	PC	22.4	18.6	6.4	10.4	45.4	2.2
	Pmt/BA	24.2	21.7	8.9	10.1	41.0	2.6
	Pmt/ZEA	8.8	17.3	5.6	6.4	70.8	3.4
	Pmt/BA10	13.2	14.3	4.7	3.0	68.1	4.0
	Pmt/ZEA10	18.6	14.9	4.5	2.8	61.1	1.8
Nules	PC	22.6	19.8	6.4	7.4	45.6	1.4
	Pmt/BA	28.1	17.0	7.5	10.5	36.3	2.0
	Pmt/ZEA	29.2	18.4	3.2	3.7	43.0	0.6
	Pmt/BA10	31.7	20.4	7.4	6.0	33.1	2.0
	Pmt/ZEA10	33.9	24.5	5.9	7.8	30.0	2.0
CV		0.000	0.002	0.673	0.453	0.000	0.013
MEDIUM		0.002	0.231	0.063	0.018	0.001	0,328
CVXMEDIUM		0.000	0.005	0.206	0.141	0.000	0.200

Two-way ANOVA, $p<0.05$

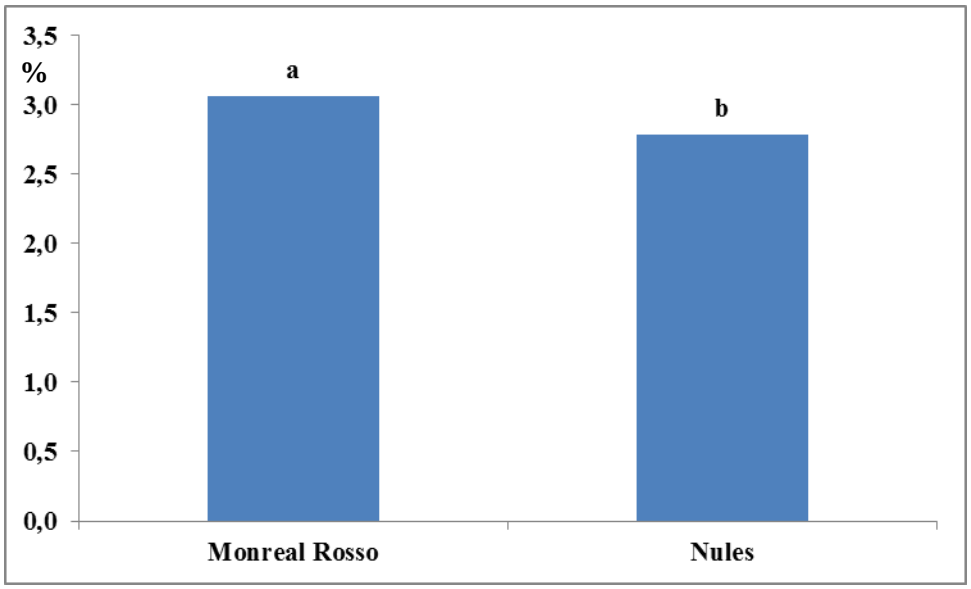
Graphics

Graph. 1 Influence of medium composition on the percentage of multinucleated microspores, after seven months of culture



One-way ANOVA, Tukey's test ($p \leq 0.05$)

Graph. 2 Influence of genotype on the number of microspore-derived embryos, after seven months of culture



One-way ANOVA, Tukey's test ($p \leq 0.05$)

Figures

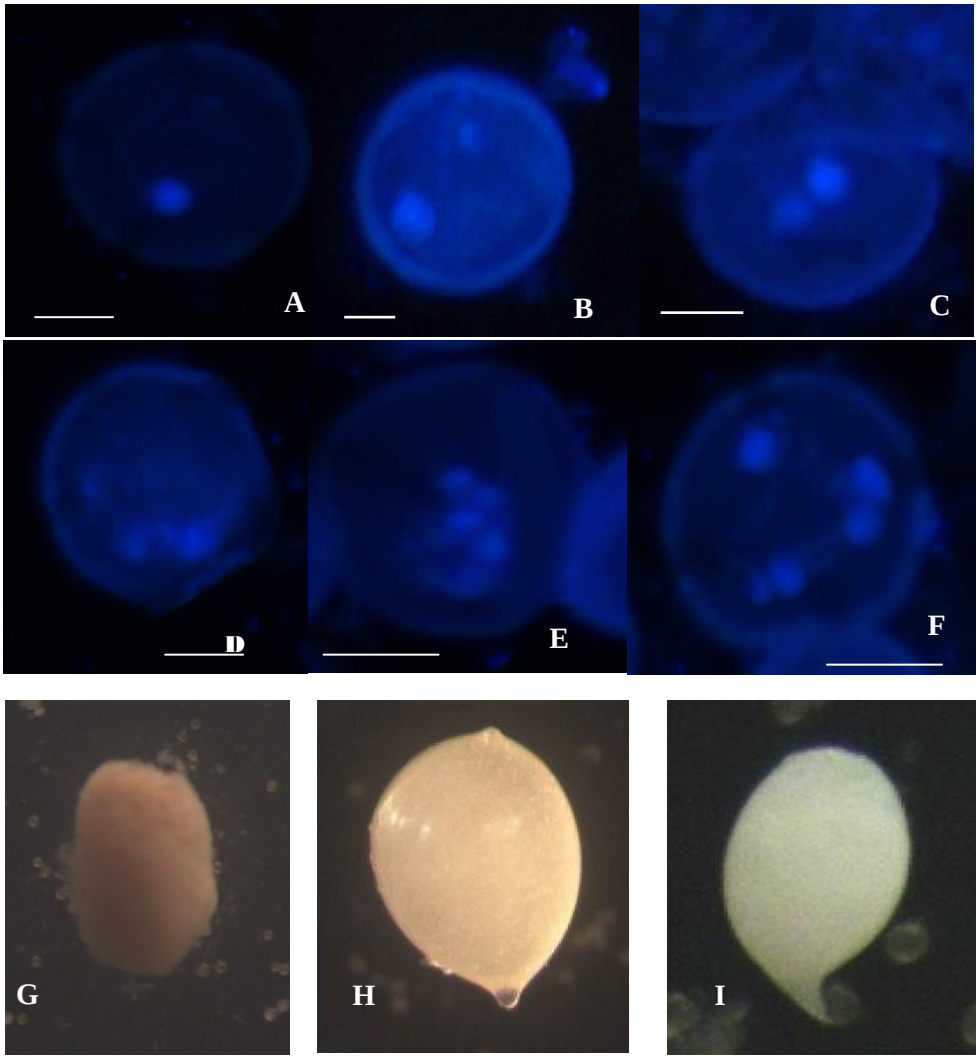


Fig. 7 Gametic embryogenesis, through isolated microspore culture of *Citrus sinensis* (L.) Osbeck, cv. Moro, and *Citrusclementina* Hort. ex Tan, cvMonreal Rosso and Nules. **A:** uninucleate microspore of Moro (DAPI staining); **B**: bicellular pollen, asymmetrical division (DAPI staining) of Moro;**C**: bicelluar microspore, symmetrical division (DAPI staining), Nules; **D**: tricellular microspore (DAPI staining) of Moro; **E-F**: multicellular microspore (DAPI staining) of Moro (**F**) and of Monreal Rosso (**E**); **G**: Microspore-derived callus of Moro; **H**:embryos of Moro (left) and Monreal Rosso(**I**) (right). (**Bars represent in A-C: 10µm**)

1.4 Conclusions

Regeneration through somatic embryogenesis is rather common in different species of *Citrus* using stem and epicotyls segments, part of flowers such as unfertilized ovule, anthers, stigma and style as explant material. This embryogenic material can be genetically manipulated by protoplast fusion and transformation (Kochba et al., 1978), but it is also valuable for clonal propagation, synthetic seed production and germplasm storage (Germanà 2003; 2005; Germanà et al, 2011).

Numerous research, carried out to obtain haploids, resulted instead in producing heterozygous somatic plantlets. Particularly, anther culture produced somatic regenerants in *C. aurantium*, (Hidaka et al., 1981; Germanà 2003; 2005), *C. sinensis* (Hidaka, 1984b), *C. aurantifolia* (Chaturvedi and Sharma, 1985), *C. madurensis* (Ling et al., 1988), *C. reticulata* (Germanà et al., 1994; Germanà, 2003; 2005), *Poncirus trifoliata*, the hybrid No. 14 of *C. ichangensis* x *C. reticulata* (Deng et al., 1992) and *C. paradisi* (unpublished). In these cases, anther culture can be regarded as a further method of obtaining somatic embryogenesis.

Despite progress has been achieved, many *Citrus* genotypes are still considered recalcitrant to anther culture. Since the first haploid embryogeniccalli production and plantlet regeneration by anther culture of *C. clementina*, cv. Nules, was reported (Germanà et al 1994), many studies have been carried out in *Citrus*, focusing on increasing the frequency of embryogenesis with responsive species and on developing new protocols for recalcitrant ones (Germanà and Reforgiato 1997; Germanà et al 2000a; 2005; Germanà and Chiancone 2003).

This study represents an advancement in the knowledge of pollen embryogenesis via isolated microspore culture technique in blood sweet orange *C. sinenesis* Osbeck cv. Moro and in clementine *Citrus clementina* Hort. ex Tan., cvs. Monreal Rosso and Nules, reporting for the first time, the regeneration of pollen-derived embryos in *Citrus*.

Thank to this study, new protocols have been set up inducing an improvement of haploid induction rate (the frequency of anthers which form embryoids) and an enlargement of responsive genotypes.

This study is important because it increased the number of genotype responding to gamete embryogenesis. In fact, for the first time, anther culture of the cultivars Hernandina and Corsica produced triploid homozygous calli and plantlets. On the

other hand, for the first time, blood oranges produced, via anther culture, somatic embryogenic callus and regeneration of plantlets were obtained, very important for the numerous applications in the fields of the clonal propagation and of the breeding.

These studiesconfirmed the strong influence of the genotype on the type of the response of anthers *in vitro* cultured. Actually, the same treatments applied to the explants in culture resulted in the production of gametic embryos in clementines and of somatic embryos in sweet oranges. Clementine confirms its tendency to regenerate homozygous and tri-haploids embryos and plantlets.

However, the presence of multinucleate pollen grains developed in sweet oranges by this *in vitro* system, indicated that, also in this species, the induction of microspore nucleus division, the switch of developmental programme and their commitment to the embryogenic pathway, occurred. This constitutes a crucial step to design new protocols for the regeneration of microspore-derived embryos and plants.

Further studies should be performed, also, in other *Citrus* genotypes aimed to obtain *in vitro* regeneration procedures, suitable for different applications, as new opportunities for genetic improvement and for the innovation in propagation methods.

2 Olive (*Olea europaea* L.)

2.1 Introduction

The olive tree (***Olea europaea*** L.), family *Oleaceae*, with more than 2000 cultivars identified and gathered in germplasm collections, is one of the species that most characterize the Mediterranean regions (Pintos et al., 2007).

The 99% of the world olive production (20.59 Million tons) is produced in Mediterranean Basin, performing an important role in the economy of these countries, such as Spain, Italy, Greece, Turkey, Morocco and Egypt (FAOSTAT, 2013). Olives is one of the oldest cultivated tree, it is part of the history (more than 4000 years), the gastronomy and other several aspects of life in many Mediterranean countries.

Olive is adapted to extreme arid conditions because of its special leaf structure and ramified root system. The olive tree is an evergreen, the flowers are borne in racemes which emerge from the axils of the leaves and produce large quantities of pollen, the fruit is a drupe.

Olive trees are characterized by a long reproductive cycle with several years of a juvenile phase, tendency to allogamy and a large tree size.

The genetic improvement in olive, by conventional methods is time consuming and needs lots of space for field experiments.

High heterozygozity, long juvenile phase, self incompatibility and other factors don't allow to reach homozigosity by conventional methods.

Gametic embryogenesis, through pollen embryogenesis, can help to speed up the breeding programs.Few are the studies on gametic embryogenesis in olive (Bueno et al., 2005, 2006; Solıs et al., 2008; Chiancone et al., 2013) and they report the switch of the microspore development pathway and the formation of microspore derived multicellular structures, proembryos and cytochemical analysis in the early stages of isolated microspore culture of several Spanish and Italian cultivars.

To exploit the enormous potential of gametic embryogenesis, it is important to study the factors influencing this process, particularly, the culture medium composition.

Aliphatic polyamines (PAs), such as putrescine (PUT) and spermidine (SPE) are low molecular mass polycations present in all living organisms (Martin-Tanguy, 2001; Bagni and Tassoni, 2001). These growth regulators play a very important role in flower initiation, pollen tube growth, cell division (Bagni and Tassoni, 2001).

The addition of PAs to the medium stimulated gametic embryogenesis in *Citrus clementina* Hort. ex Tan (Chiancone et al., 2006), in some wheat cultivars (Rajyalakshmi et al., 1995) and in cucumber (Ashok Kumar et al., 2004).
In this study, *in vitro* anther and isolated microspore culture of ten Sicilian cultivars of olive has been carried out, investigating the influence of two polyamines (putrescine and spermidine) and of two medium compositions on pollen embryogenesis induction.

2.2 Anther culture

2.2.1 Material and methods

Plant material

Flower buds were collected in May, from a collection field, located in Scillato (Palermo, Italy). The following ten olive cultivars were used for both anther and isolated microspore culture: Biancolilla Napolitana, Cerasuola Sciacca, Galatina, Nocellara del Belice and Verdello. Flower buds from small branches of olive were harvested and wrapped in aluminum foil and maintained in darkness at 4°C for 9 days, as a pre-treatment (Bueno et al., 2006).

Microspore stage correlation with bud size and development of microspores in the anthers during the culture

The relationship between the bud size and the microspore development stage was studied before the culture. Flower buds were collected randomly from the plant and were graded into three bud size range and then they were measured.
In order to study the effect of microspore development before the culture and the microspore development during the culture, anthers 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).

Anther culture technique

Flower buds were surface sterilized by immersion for 3 min in 70% (v/v) ethyl alcohol, followed by immersion for 20 min in 25% (v/v) commercial bleach (about 0.5% active chlorine in water). Petals were aseptically removed with small forceps and anthers were placed in 60 mm diameter Petri dishes containing 10 ml solid medium. Media called P and N6 (Germanà et al., 1996 ; Germanà and Chiancone, 2003) (Table 7) were tested. Petri dishes were placed in the dark at 26±1˚C for the first 30 days, then placed under cool white fluorescent lamp (Philips TLM 30W/84, France) with photoperiod of 16 light hours.

Experimental design

The influence of the presence in two basic media (P and N6) of two polyamines (Putrescine and Spermidine) in different combinations was studied.

In particular, media tested were the following:

1) **P control (without polyamines).**
2) **P + 2 mM putrescine (PUT).**
3) **P + 2 mM spermidine (SPE).**
4) **P + 1 mM putrescine + 1 mM spermidine (PUT + SPE).**
5) **N6 control (without polyamines).**
6) **N6+ 2 mM putrescine (PUT).**
7) **N6 + 2 mM spermidine (SPE).**
8) **N6 + 1 mM putrescine + 1 mM spermidine (PUT + SPE).**

The pH of all media was adjusted to 5.8 with 1M NaOH or 1M HCl, before adding 8.5 gl^{-1} Plant Agar (Micropoli) as a solidifying agent and then, media were autoclaved at 121 C and 0.1 MPa for 21 min.

Per each cultivar, 40 anthers were put in each Petri dish. 10 Petri dishes per each medium were disposed for a total of 400 anthers per medium and 3200 anther per cultivar. After 8 months of culture, per each Petri dish, the number of not developed anthers (Fig. 8A), swollen anthers (Fig. 8B) and anthers with callus (Fig. 8C) were recorded. The statistical analysis was conducted in order to study, firstly, the influence of the genotype by one-way ANOVA. Means were separated by the Tukey's test. After that, the influence of polyamines on the *in vitro* response of anthers was studied per each cultivar by one-way ANOVA; because data were not normally distributed.

2.2.2 Results

Correlation of floral and male gametophyte development

In all the cultivars observed, unopened floral buds measuring approximately 2 mm long, with yellow-greenish anthers, were identified as those with the higher proportion of microspores at the late uninucleate to the bicellular pollen stage, that means the optimal stages for the embryogenesis induction.

Influence of the genotype on the in vitro cultured anthers, after height months of culture

After 1 week of culture, most of the anthers were swollen and after 1 monthtwo types of calli, yellowish (Fig. 8D) and green (Fig. 8E). The green calli appeared compact, whereas the yellowish were friable. Unfortunately none of them differentiated in embryos, but the sporophytic pathway undertaken by the microspores is demonstrated by the presence of multinucleated microspores observed at the fluorescent microscope, after DAPI staining. Microspores with several nuclei in the cytoplasm, still surrounded by the exine were observed (Fig. 8F and G).

The statistical analysis showed that, as reported in other species (Germanà et al., 2011), the response of the anthers in culture is highly genotype-dependent (Table 8).

In fact, after eight months of culture, some cultivars, such as Galatina and Nocellara del Belice, showed the statistically lowest percentage of not developed anthers (respectively, 45.0% and 41.0%), but also the statistically highest percentage of swollen anthers (respectively, 37.3% and 34.1%) and of anthers with callus (respectively, 17.7% and 24.8%), testifying the microspore switching toward the sporofhytic pathway.

On the other side, cv. Cerasola Sciacca gave the statistically worst performance, with the statistically highest percentage of not developed anther (91.0%) and the lowest percentage of anthers swollen and with callus (respectively, 6.9% and 2.0%).

Because, statistical analysis demonstrated that each cultivar responded differently, the response to the medium composition was studied separately.In particular, Biancolilla napoletana (Table 9) and Nocellara del Belice (Table 12), no statistically significative differences was observed for any of the considered parameters, for the others there was a significative correspondence between the percentage of anthers not developed and the percentage with callus; and for these cultivars it was possible to individuate which medium was the best in inducing callus production incultured anthers.

For example, Cerasola Sciacca gave the best response, when anthers were cultured on the medium with Putrescine and Spermidine (P PUT+SPE) (Table 10), and similar results were reported for cv Verdello.Moreover, for this cultivar, also the medium P SPE gave good results (Table 13).

Finally, the cultivar Galatina had an intermediate behavior; in fact, no statistically significative differences were observed for the parameters anthers not developed and anthers with callus, while significative differences were recorded for the swollen anthers. Even though, it was not possible to individuate which was the best medium for this cultivar, because very slightly are the differences in the response to each medium (Table 11).

Tables

Table 7 Medium composition

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

Table 8 Influence of the genotype on the *in vitro*cultured anthers, after eight months of culture.

Cultivar	Not developed anthers (%)	Swollen anthers (%)	Anthers with callus (%)
Biancolilla napoletana	67,4 b	32.1 a	0.5 D
Cerasola Sciacca	91,1 a	6.9 b	2.0 C
Galatina	45.0 c	37.3 a	17.7 A
Nocellara del Belice	41.0 c	34.1 a	24.8 A
Verdello	61.9 b	28.7 a	9.4 B

Values followed by different letters are statistically different at $p \leq 0.05$ (one-way ANOVA, Tukey's test).

Table 9 Influence of the medium composition on the *in vitro*cultured anthers of Biancolilla napoletana, after eight months of culture.

Cultivar	Not developed anthers (%)	Swollen anthers (%)	Anthers with callus (%)
P C	64.0	34.2	1.8
P PUT	63.1	356	1.2
P SPE	63.0	37.0	1.0
P PUT+SPE	63.8	35.8	0.0
N6 C	70.8 ns	29.2 ns	0.0 Ns
N6 PUT	72.0	28.0	0.0
N6 SPE	71.9	28.1	0.0
N6 PUT+SPE	69.8	30.2	0.0

Values followed by different letters are statistically different at $p \leq 0.05$ (one-way ANOVA, Dwass-Steel-Chritchlow-Fligner's test, at $p \leq 0.05$).

Table 10 Influence of the medium composition on the *in vitro*cultured anthers of Cerasola Sciacca, after eight months of culture.

Cultivar	Not developed anthers (%)	Swollen anthers (%)	Anthers with callus (%)
P C	93.4 a	1.6 b	2.5 A
P PUT	87.7 a	9.8 a	2.5 A
P SPE	94.5 a	5.5 ab	0.0 B
P PUT+SPE	85.5 b	11.5 a	3.0 A
N6 C	94.0 a	5.0 ab	1.0 Ab
N6 PUT	93.3 a	5.0 ab	1.7 Ab
N6 SPE	98.3 a	1.7 b	0.0 B
N6 PUT+SPE	100.0 a	0.0 b	0.0 B

Values followed by different letters are statistically different at $p \leq 0.05$ (one-way ANOVA, Dwass-Steel-Chritchlow-Fligner's test).

Table 11 Influence of the medium composition on the *in vitro*cultured anthers of Galatina, after eight months of culture.

Cultivar	Not developed anthers (%)		Swollen anthers (%)		Anthers with callus (%)	
P C	58.5		22.0	b	19.5	
P PUT	45.9		44.1	a	10.0	
P SPE	53.0		25.0	b	22.0	
P PUT+SPE	37.5	ns	32.0	ab	30.5	Ns
N6 C	41.3		44.4	a	14.4	
N6 PUT	38.8		43.8	a	17.5	
N6 SPE	40.8		43.5	a	15.7	
N6 PUT+SPE	42.0		46.5	a	11.5	

Values followed by different letters are statistically different at $p \leq 0.05$ (one-way ANOVA, Dwass-Steel-Chritchlow-Fligner's test).

Table 12 Influence of the medium composition on the *in vitro*cultured anthers of Nocellara del Belice, after eight months of culture.

Cultivar	Not developed anthers (%)		Swollen anthers (%)		Anthers with callus (%)	
P C	38.3		22.5		39.2	
P PUT	39.4		32.5		28.1	
P SPE	55.8		25.0		19.2	
P PUT+SPE	38.3	ns	29.2	ns	32.5	Ns
N6 C	41.3		39.1		19.6	
N6 PUT	33.8		32.5		33.8	
N6 SPE	37.5		47.5		15.0	
N6 PUT+SPE	42.0		36.5		21.5	

Values followed by different letters are statistically different at $p \leq 0.05$ (one-way ANOVA, Dwass-Steel-Chritchlow-Fligner's test).

Table 13 Influence of the medium composition on the *in vitro*cultured anthers of Verdello, after eight months of culture.

Cultivar	Not developed anthers (%)		Swollen anthers (%)		Anthers with callus (%)	
P C	57.0	ab	33.1		7.4	B
P PUT	50.5	ab	33.0		16.5	Ab
P SPE	44.3	b	33.3		22.4	A
P PUT+SPE	46.9	b	23.9		29.3	A
N6 C	65.0	ab	35.0	ns	0.0	C
N6 PUT	72.8	a	27.2		0.0	C
N6 SPE	76.5	a	23.5		0.0	C
N6 PUT+SPE	78.5	a	21.5		0.0	C

Values followed by different letters are statistically different at $p \leq 0.05$ (one-way ANOVA, Dwass-Steel-Chritchlow-Fligner's test).

Figures

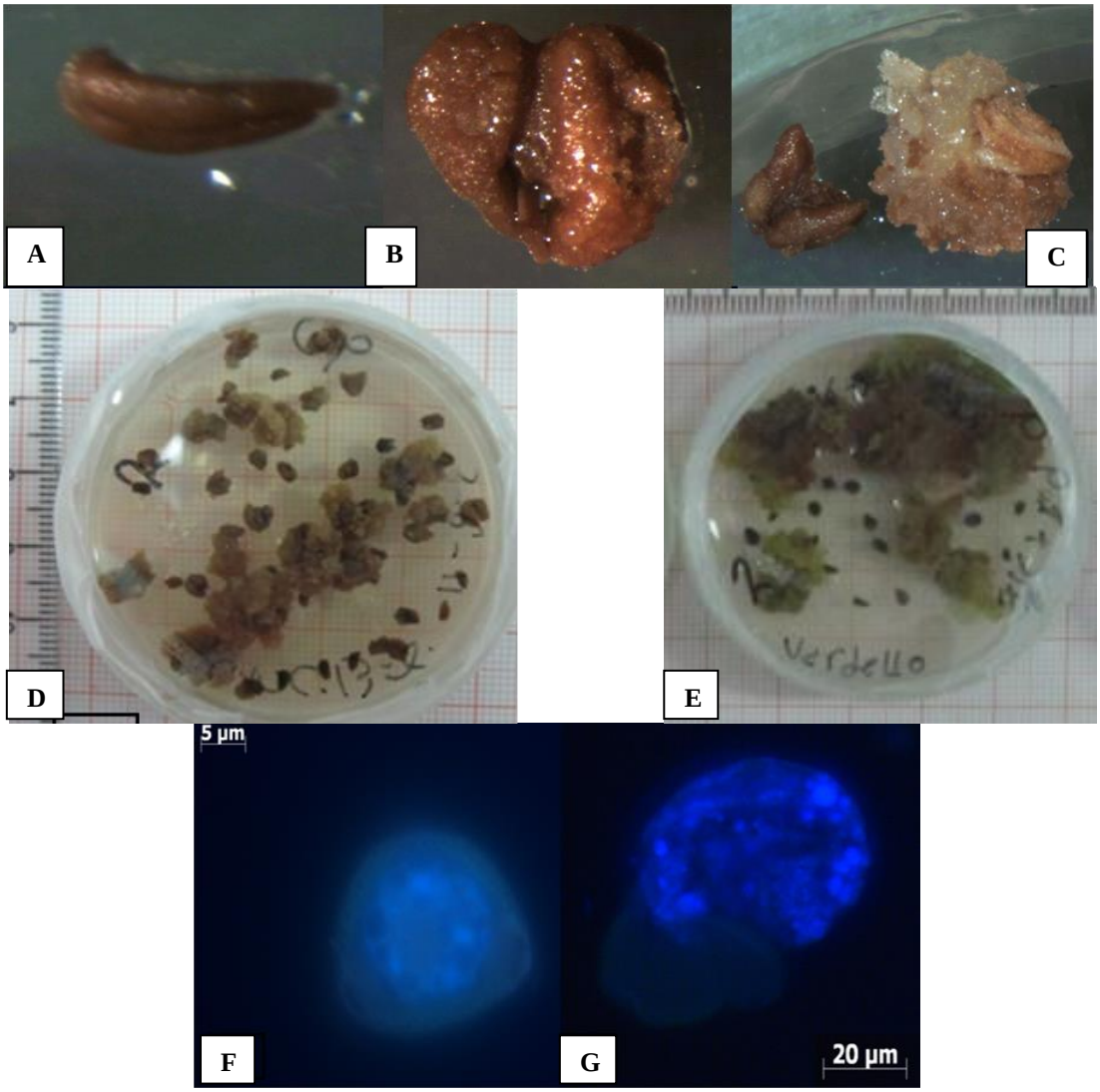


Fig. 8. Anther culture in olive: **A**: Not developed anthers after 3 months in culture; **B**: Swollen anther, after three months in culture cv. Galatina; **C**: Anther with callus after three months in culture cv. Nocellara del Belice; **D, E:** Anther producing yellowish (cv. Nocellara del Belice, **D**) or green callus (cv. Verdello, **E**);.**F, G**: multinucleated like proembryo structure, DAPI staining after three months of anther culture cv. Cerasola Sciacca (**F**) and cv. Galatina (**G**).

2.3 Isolated microspore culture

2.3.1 Material and methods

Plant material and correlation of microspore gametophyte development

Flower buds were collected in May from a collection field, located in Scillato (Palermo, Italy) (Fig 9A). Four cultivars were studied: Biancolilla napoletana, Nocellara del Belice, Tonda Iblea and Verdello. As a pre-treatment, the harvested olive branches (50 cm long) were wrapped in aluminum foil, with damp cotton wool at the base of the stem and maintained in darkness at 4 °C for one week.

Anthers from different flower bud sizes (Fig. 9A), before culture and isolated microspore at different times during the culture, were stained with 4',6-diamidino-2-phenylindole (DAPI) to determine the microspore development stage and observed under fluorescence microscope (Zeiss, Axiophot, Germany).

Flower bud sterilization and isolated microspore culture

Flower buds were surface sterilized as above reported. The microspore isolation protocol was performed following the protocol reported by Kuhmlen (2006) and set up for olive by Chiancone et al. (2013). Per each cultivar, an average of 400 flower buds were used, as starting material.

Isolated microspores were cultivated on eight media, containing the two polyamines in different combinations:

A) 2 Mm putrescine (PUT).

B) 2 mM spermidine (SPD).

C) 1 mM each of putrescine and spermidine (PUT + SPD).

D) control without polyamines (C).

The four growth regulator combinations were added to two media employed in previous experiments on gametic embryogenesis of other woody plant species: P and N6 (Germanà et al., 1997; Germanà and Chiancone, 2003). Eight replicates for each medium type were used (64 Petri dishes /cultivar).

Influence of the genotype and of the medium composition on the microspore development process

After four weeks in culture, per each cultivar and per each medium, 450 microspores were observed (three replicates with around 150 microspores each) under fluorescence microscope (Zeiss, Axiophot, Germany). The number of microspores uninucleated, with no development, binucleated, trinucleated, tetranucleated, multinucleated and forming calli was counted.

These values were used to calculate means. Differences among cultivars and media were tested by two-way analysis of variance (ANOVA). Differences among means were tested by Tukey's multiple comparison test or both analysis.

Per each cultivar, a one-way ANOVA was performed to isolate the influence of the media composition. Differences among means were tested by Tukey's multiple comparison test at $p \leq 0.05$.

Fixation and processing for microscopical analysis

Isolated microspores before the culture and every month for ten months were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight at 4°C then samples were stored in 0.1% paraformaldehyde at 4°C. After fixation, the samples were centrifuged and the pellets were embedded in 15 % gelatin (Solis et al., 2008).

After washing in PBS, the embedded pellets 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.

Toluidine blue staining and iodide–potassium-iodide (I_2KI) cytochemistry for starch were employed.

Toluidine blue staining was used to observe the cellular organization under light microscopy. Iodide-based cytochemistry was performed after ten months of culture 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.

2.3.2 Results

Influence of the genotype and the medium composition on the microspore development process

Different features of microspores have been observed: uninucleated with no development (Fig. 9B), binucleated with two asymmetrical nuclei (normal gametophytic pathway: one vegetative and one generative nucleus) (Fig. 9C), binucleated with two equal-size vegetative-type nuclei that had just started their sporophytic pathway (Fig. 9D), trinucleated (Fig. 9E), tetranucleated, multinucleated (Fig. 9F) and callus formation (Fig. 9G), indicating steps towards the gametic embryogenesis development.

Differently than in *Citrus clementina* Hort ex Tan anther culture (Chiancone et al., 2006), where the medium N6 SPE induced the highest regeneration, in this experiment conducted on olive microspores, there are no univocal results (Table 14).

A strong Genotype-Medium interaction has been observed per each parameter; in fact, each cultivar responded differently depending on the medium tested. The best performances in terms of callus production was observed, for the cultivars Biancolilla napoletana and Tonda Iblea, with the N6 PUT medium (respectively 52.5% and 41.2%), for the cultivar Verdello, with the P C medium (52.6%). Very low percentage of callus regeneration was observed in the cultivar Nocellara del Belice, for all media tested; the highest percentage was recorded with the medium N6 PUT + SPE (7.4%) (Table 14).

The statistical analysis performed per each cultivar revealed that in Biancolilla napoletana trinucleated microspores were observed in highest percentage in media P SPE and N6 C. While the lowest in the media P PUT+SPE and N6 PUT. Also multinucleated microspores were observed mainly in the medium P, in percentage similar for P C, P PUT and P SPE.

The worse result was observed in the medium N6 C. N6 media do not seem to induce the formation of multinucleated microspores. Even if the addition of PUT+SPE in both type of medium (P and N6) induced the formation of a certain percentage of multinucleated microspores (respectively 3.2 and 3.1)

In Nocellara del Belice, trinucleated microspores were observed in highest percentage in media P C and N6 PUT and N6 PUT+SPE, while the lowest in the media P SPE.

Also multinucleated microspores were observed in the media P C, P SPE and N6 C. The worse result was observed in the medium P PUT + SPE,but also very low percentages were observed in media N6 SPE and N6 PUT+SPE.

Regarding Tonda Iblea, trinucleated microspores were observed in highest percentage in medium P PUT, while the highest percentage of multinucleated microspores were observed in the medium P PUT + SPE.

The lowest percentages were observed in medium N6 for both type of microspores (trinucleated and multinucleated). In particular, N6 medium, without polyamines (C) and in combination with SPE induced the worse response. N6 PUT induced a slightly higher percentage of trinucleated and multinucleated microspores (respectively 4.1% and 1.4%). Regarging multinucleated microspores, also N6 PUT + SPE gave intermediate results (1.7%).

Finally, in Verdello, statistical differences were observed among all media tested.

Trinucleated microspores were observed in highest percentage in medium P C, while the highest percentage of multinucleated microspores was observed in the medium P PUT.

The lowest percentages were observed in medium N6 (except for N6 PUT) for both type of microspores trinucleated and multinucleated. But also the medium P SPE did not induce high percentage of both trinucleated and multinucleated microspores (respectively 8.2% and 0%).

Iodine–Potassium-Iodide (I_2KI): Cytochemistry for starch

In several woody species, the analysis of starch accumulation is considered a method to individuate microspores that switched from the gametophytic toward the sporophytic pathway (Ramirez et al., 2003).

In this study, the analysis carried out after 10 months of culture, showed that the starch accumulation varied greatly in dependence of the developmental stage of microspores. In fact, monitoring the microspores cultured in the medium that induced the highest percentage of multicellular structures, it was possible to observe that the accumulation of starch was low. On the contrary, microspores cultured on medium that induced a very low percentage of multinucleated structures showed a high starch accumulation (Fig. 9 H and I).

These results confirmed what reported in *Olea europaea* L. by Solıs et al. (2008). In fact, in their work, no starch-staining was detected in the vacuolated microspore,

whereas at later stages an increasing number of stained inclusions were found in the vegetative cytoplasm, indicating a progressive accumulation of starch deposits during pollen maturation. The presence of starch deposits constituted an early marker of the non responsive microspores which can follow, at least in part, a gametophytic-like pathway (Solıs et al., 2008).

On the other hand, in *Citrus clementina* Hort ex Tan (Ramirez et.al., 2003) and in *Eriobotrya japonica* and *Prunus armeniaca* L.. Germanà et al. (2006; 2010) observed a high starch accumulation in the bicellular pollen grain as an early microspore embryogenic pathway after about 6 months of culture. Starch accumulation and deposition has been observed and suggested to be associated with polarity establishment and cell differentiation (Testillano et al., 2000).

Tables

Table 14 Influence of genotype and several medium composition on the olive microspore development, after one month of culture.

Genotype (G)	Medium (M)	Microspore features				
		Uninucleated (%)	Binucleated (%)	Trinucleated (%)	Multinuclated (%)	Calli (%)
Biancolilla napoletana	P C	36.3	8.8	10.6	7.1	37.2
	P PUT	29.4	2.5	11.8	7.6	48.7
	P SPE	41.0	9.7	13.4	7.5	28.4
	P PUT+SPE	33.3	10.3	5.6	3.2	47.6
	N6 C	43.7	10.6	12.1	1.0	32.6
	N6 PUT	35.8	5.8	4.2	1.7	52.5
	N6 SPE	58.6	6.3	6.3	1.6	27.3
	N6PUT+SPE	46.6	6.1	9.9	3.1	34.4
Nocellara del Belice	P C	46.6	18.6	28.0	3.4	3.4
	P PUT	51.4	23.8	19.0	2.9	2.9
	P SPE	61.9	14.3	14.3	3.8	5.7
	P PUT+SPE	65.4	13.1	20.6	0.9	0.0
	N6 C	53.6	18.8	22.3	3.6	1.8
	N6 PUT	44.9	19.7	29.9	3.1	2.4
	N6 SPE	72.8	6.8	17.5	1.9	1.0
	N6PUT+SPE	35.2	24.1	31.5	1.9	7.4
Tonda Iblea	P C	50.9	15.7	8.3	3.7	21.3
	P PUT	48.7	8.7	13.0	3.5	26.1
	P SPE	45.3	6.5	5.8	2.9	39.6
	P PUT+SPE	62.3	4.4	4.4	6.1	22.8
	N6 C	65.1	4.6	2.8	0.9	26.6
	N6 PUT	47.3	6.1	4.1	1.4	41.2
	N6 SPE	64.2	3.0	2.2	3.0	27.6
	N6PUT+SPE	69.8	1.7	2.6	1.7	24.1
Verdello	P C	37.7	1.8	6.1	4.2	52.6
	P PUT	54.4	2.6	0.9	8.5	42.1
	P SPE	57.8	3.4	2.6	0.0	35.3
	P PUT+SPE	61.0	7.3	0.8	1.8	30.9
	N6 C	51.0	1.9	0.6	0.9	46.5
	N6 PUT	56.1	2.3	2.3	2.0	37.2
	N6 SPE	72.9	8.9	2.7	3.0	12.5
	N6PUT+SPE	70.3	2.7	0.0	1.0	26.0
GENOTYPE		<0.001	<0.001	<0.001	<0.001	<0.001
MEDIUM		<0.001	<0.001	<0.001	<0.001	<0.001
GENOTYPE X MEDIUM		<0.001	<0.001	<0.001	<0.001	<0.001

Two-way Anova, p≤0.01

Figures

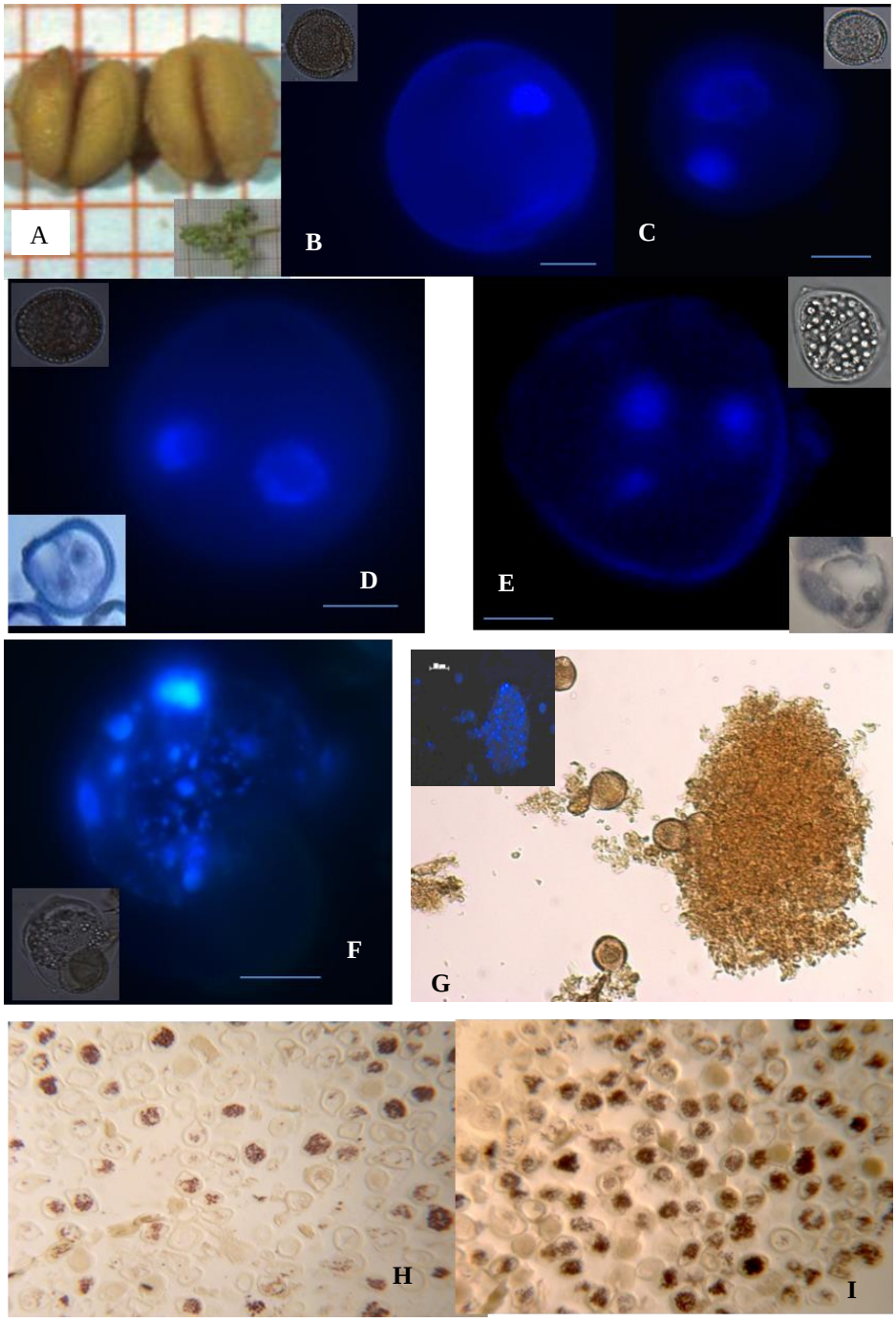


Fig. 9 Gametic embryogenesis in olive, cv Biancolilla napoletana.:**A**: flower bud and anthers at the beginning of culture; **B**: uninucleate microspore (DAPI staining); **C**: bicellular pollen, asymmetrical division (DAPI staining). (**D-G**) Gametophytic pathway: **D**: bicelluar microspore, symmetrical division (DAPI and toluidine blue staining); **E**: tricellular microspore (DAPI and toluidin blue staining); **F**: multicellular microspore (DAPI staining); **G**) callus regenerated from isolated microspore in culture. (**H-I**) I2KI cytochemistry: **H**: low starch accumulation (Verdello on medium P PUT); **I**: high starch accumulation (Verdello on medium P SPE). (Bars represent in B-F: 10µm)

2.4 Conclusions

Pollen biotechnology represents a powerful tool not only in olive breeding, but also in genome sequencing and gene mapping studies. The exploitation of haploid and doubled haploids, as a powerful breeding tool, requires the availability of reliable and cost-effective tissue culture experimental procedures.

There is not yet a universal protocol, suitable for all crops and the development of new techniques for doubled haploid induction is still required for many recalcitrant genotypes.

To investigate the influence of the factors affecting the microspore response to the culture. In these researches, the influence of the medium composition in particular, of two polyamines added alone or in combination were studied. Although many researches have been conducted on the effect of polyamines in plant tissueculture, only a few have studied their influence on gametic embryogenesis (Chiancone et al., 2006).

In olive, the positive influence of polyamines is dependent on which cultivar is considered. For this reason, it could be interesting to devlop the investigations in this subject, testing new polyamines, such as the spermine, and adding them at different concentrations and combinations.

In any case, this research representsan advancement in the knowledge of gametic embryogenesis in olive. In fact, by both techniques it was possible to produce multicellular structures and calli, and that represents the first step towards haploid olive embryos production.

3 Hazelnut (*Corylus avellana* L.)

3.1 Introduction

Hazelnut (*Corylus avellana* L.), native of the European regions, is the fourth tree nut worldwide, with total production more than 742,000 ton/year (FAOSTAT 2013), behind cashew, *Anacardium occidentale* L., almond, *Prunus dulcis* and Persian walnut, *Juglans regia* L.

All cultivated forms of hazelnut are diploid with a monoploid number of chromosomes (n=x=11). Hazelnut is a self-incompatible and wind pollinated plant (Rovira et al. 1993).

Self-incompatibility is a mechanism that prevents self-fertilization and encourages fertilization by genetically unrelated individuals. The control of self-incompatibility is generally attributed to a single S-locus, expressing multiple alleles. In hazelnut, incompatibility is determined sporophytically and it is controlled by a single locus with multiple alleles (Martins 2010). These factors donot allow to reach easily homozygozity and they limit the genetic improvement in hazelnut by conventional methods.

Among all the biotechnological *in vitro* regeneration systems, anther and isolated microspore culture are the most commonly used method to produce haploids (Hs) and doubled-haploids (DHs), useful for fruit breeding programs to shortthe time required to produce homozygous lines comparing with the conventional breeding methods.

In our knowledge, This is the first investigation carried out to obtain haploids through anther culture and isolated microspore culture in hazelnut

This study has been carried out to obtain regeneration through anther and isolated microspore culture of several hazelnut cultivars, testing different temperature stresses applied to the explants in culture.

3.2 Anther culture

3.2.1 Materials and Methods

After a morphological characterization of flower buds (catkins) of six hazelnut cultivars (Carrello, Gentile romana, Imperatrice Eugenia, Meraviglia De Bollwiller, Nostrana and Tonda romana) (Fig. 10A) and after a pre-treatment, flowers catkins

were surface sterilized by immersion for 3 min in 70% (v/v) ethyl alcohol, followed by immersion in sodium hypochlorite solution (about 1.5% active chlorine in water) containing a few drops of Tween 20 for 20 min, then rinsed three times for five minutes with sterile distilled water. Finally, they were maintained in gentamicin antibiotic solution (0.2%) for 30 min. After the sterilization, anthers (Fig. 10B) were carefully dissected and placed in 60 mm diameter Petri dishes in the culture medium P (Germanà et al., 1996) and, just after, they were subjected to two different thermal shocks: 60 min at 35°C (PH) and 30 min at -20°C (PF). Per each treatment, 12 Petri dishes were prepared (720 anthers/treatment). Moreover, as control, 12 Petri dishes were incubated directly in the growth chamber (PC). Cultures were incubated at 27±1°C, for thirty days in the dark, and then placed under cool white fluorescent lamps (Philips TLM 30W/84) with a Photosynthetic Photon flux Density of 35 μmol m^{-2} s^{-1} and a photoperiod of 16 hrs.

Observations under light and fluorescence microscopy were performed at different times, before and during the culture, to observe the changes of microspore development inside the anthers by 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) staining.

After 7 months, for all the genotype and treatment tested, the number of anthers that did not develop, swollen, or producing calli were observed and registered per each Petri dish.

These values were used to calculate means and two-way ANOVA, performed to individuate the influence of the two factors, genotype and thermal shocks, and their interaction on the response of anthers.

3.2.2Results

Observations carried out after 7 months of culture showed that during the culture some anthers did not change their feature (not developed), but most of them increased their volume and some started to produce white/yellowish callus.

Studies carried out at microscopical level showed that, at the beginning of the culture, anthers contained mostly vacuolated microspores (Fig. 10C), the developmental stage considered the most responsive for gametic embryogenesis. During the culture, it was possible to observe that microspores undertook the sporophytic pathway with the

presence of binucleated (with symmetrical nucleus division) (Fig. 10D), trinucleated (Fig. 10E) and multicellular structures (Fig. 10F).

Results of statistical analysis are reported in Table 15.

The statistical analysis of recorded data demonstrated that, for the not developed anthers (Fig. 10G) there was a strong interaction between the genotype and the treatment. In fact, each treatment had a significant influence on the anther response, in dependence of the cultivar considered. For example, the cold treatment (PF) did not stimulate the development of anther for the cultivars Nostrana (24.2%) and Tonda romana (7.3%), while in the cv. Carrello gave the lowest results (3%). The PH treatment determined the highest percentage of not developed anthers in the cvs Carrello (6.1%) and Gentile romana (5.3%). Regarding the swollen anthers (Fig. 10H arrow), the only factor that significatively influenced the anthers response was the genotype: for three cultivars was observed the highest percentage of swollen anthers, Carrello, Gentile romana and Tonda romana, (respectively, 89.4%, 90.3 and 89.8%) (Graph. 3). These cultivars are not the same that produced the statistically highest percentage of anthers with callus, and this could be explained by the different requirements of stress that each cultivar presents. In fact, a strong correlation between the two factors was observed:some cultivars such as Gentile romana, Imperatrice Eugenia and Tonda romana, gave the best results if anthers undergone the cold treatment (respectively, 5.7%, 14.2% and 6.6%), others produced more callus in the control (Carrello, 8.1% and Nostrana, 6.5%), only in Meraviglia de Bollwiller, the highest percentage of anthers with callus (Fig. 10H) was observed in the hot treatment (11.7%) (Table 15).

Tables

Table 15 Effect of the temperature treatments on the response of hazelnut (*Corylus avellana* L.), after seven months in culture.

CULTIVAR	TREATMENT	Not developed anthers %	Swollen anthers %	Anthers with callus %
Carrello	**C**	5.4	86.5	8.1
	PF	3.0	90.5	6.5
	PH	6.1	91.2	2.7
Gentile romana	**C**	4.5	90.7	4.9
	PF	4.8	89.5	5.7
	PH	5.3	90.7	3.9
Imperatrice Eugenia	**C**	13.9	73.7	12.4
	PF	11.8	74.0	14.2
	PH	12.6	77.2	10.3
Meraviglia de Bollwiller	**C**	16.5	74.0	9.5
	PF	16.0	76.9	7.1
	PH	12.8	75.5	11.7
Nostrana	**C**	22.4	71.1	6.5
	PF	24.2	69.6	6.2
	PH	19.7	76.1	4.3
Tonda romana	**C**	6.5	90.0	3.5
	PF	7.3	86.2	6.6
	PH	3.1	93.1	3.8
CULTIVAR		0.000	0.000	0.000
TREATMENT		0.031	0.628	0.126
CULTIVARX TREATMENT		0.035	0.077	0.000

Two-way Anova, $p \leq 0.01$ (Tukey's test)

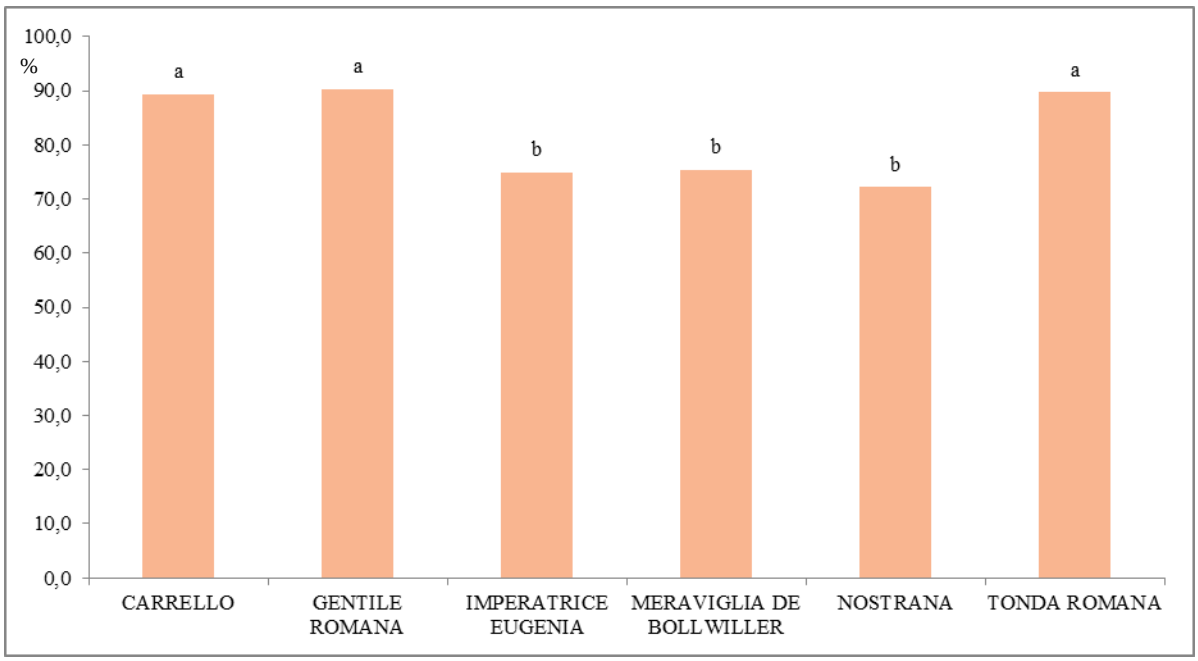
C: Control

PF: 30 min at -20°C;

PH:60 min at 35°C.

Graphics

Graph. 3 Influence of the genotype on % of swollen anthers, after seven months of culture



One-way ANOVA, Tukey's test ($p \leq 0.05$)

Figures

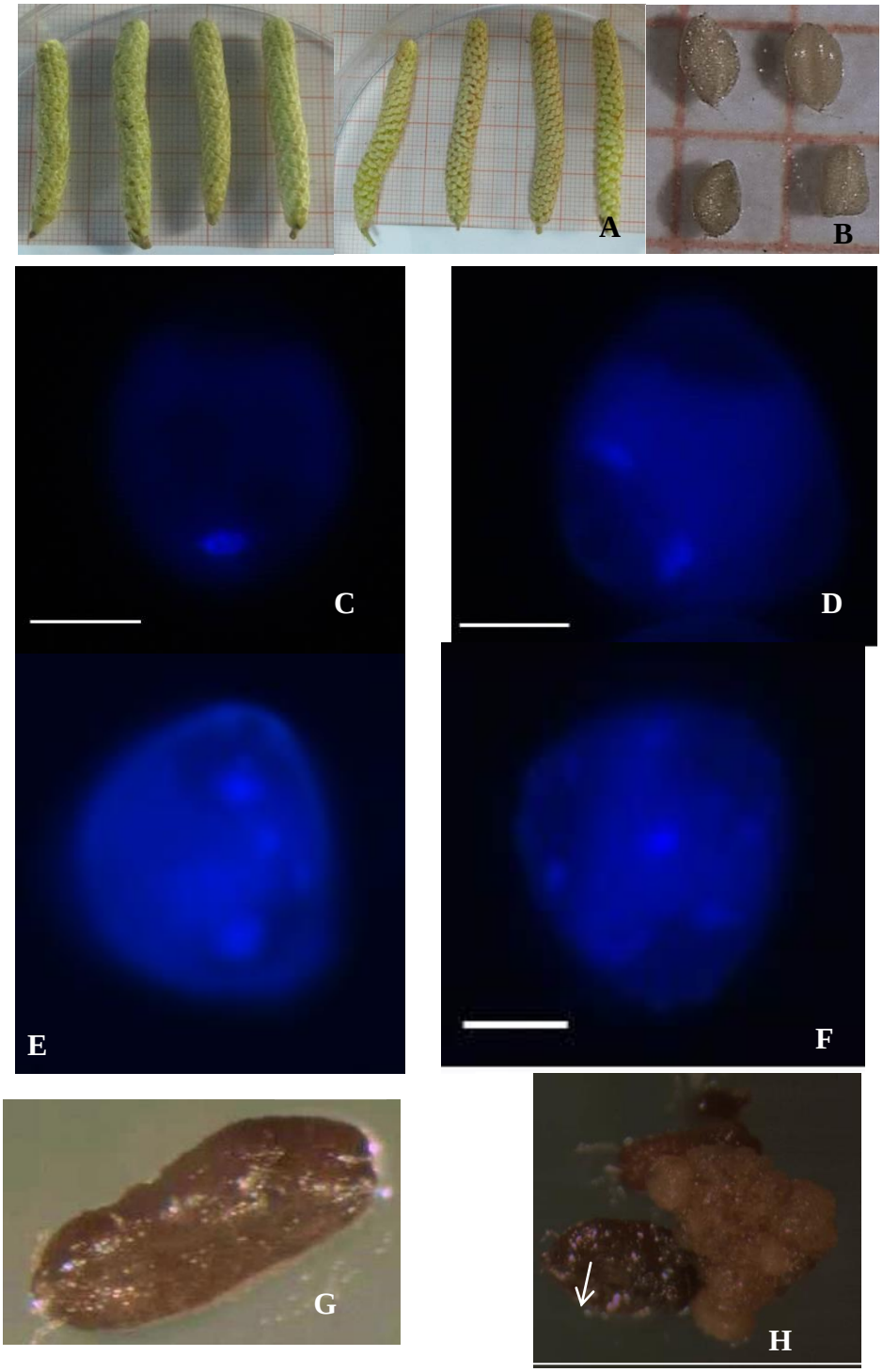


Fig. 10 Anther culture in hazelnut: **A:** Catkins of Imperatrice Eugenia (left) and Tonda romana (right); **B:** anthers of Nostrana, before the culture; **C:** Uninucleated microspore of Galatina; **D**: Binucleated, with two symmetrical nuclei, microspore of Gentile romana; **E:** trinucleated microspore of Meraviglia de Bollwiller; **F:** multinucleated microspore of Carrello; **G:** Not developed anther of Gentile romana; **H:** Anther swollen (arrow) and with callus of Tonda romana. **(Bars represent in C-F: 10µm)**

3.3 Isolated microspore culture

3.3.1 Material and methods

Plant material and sterilization protocol

Hazelnut branches of cv. Imperatrice Eugenia were collected and subjected to the same pretreatment and sterilization procedure of the ones used for anther culture. The protocol for the microspore isolation is the same used as previously described for *Citrus* spp. and Olive chapter.

Experimental design and data collection

Microspores response to two culture medium (P and N6, Table 7) and four thermal shocks were applied to the *in vitro* isolated microspore culture.

The theses studied were the followings:

1) **P C (incubated directly in the growth chamber as a control).**
2) **P H: Treated at 35°C for 30 minutes.**
3) **P H+: 40°C for 60 minutes.**
4) **P F: -20°C for 30 minutes.**
5) **P F+: -20°C for 60 minutes.**
6) **N6 C (incubated directly in the growth chamber as a control).**
7) **N6 H: 35°C for 30 minutes.**
8) **N6 H+: 40°C for 60 minutes.**
9) **N6 F: -20°C for 30 minutes.**
10) **N6 F+: -20°C for 60 minutes.**

For the ten different treatments, eight replicates for each media type were prepared (forty Petri dishes/cultivar). Microspores developmental stages were determined by placing microspores in a few drops of DAPI solution (1 mg ml^{-1}) and observed using a fluorescent microscope. Catkins with anthers with microspores at uninucleated stage were selected, assuming that, also for hazelnut, this was the most responsive developmental stage to gametic embryogenesis (Fig. 11A).

Finally, after three months of culture, 400 microspores per treatment (4000 microspores/cultivar) were observed (four replicates with around 100 microspores each under fluorescence microscope (Zeiss, Axiophot, Germany).

The number of microspores uninucleated with no development, binucleated (with symmetrical nucleus division), trinucleated, multinucleated and forming calli was counted.

These values were used to calculate means. Different treatments were tested by one-way ANOVA. Differences among means, were tested by Tukey's multiple comparison tests at ($p \leq 0.05$).

3.3.2 Results

The observations carried during the first three months of culture showed an evolution in the development of microspore; in fact, *in vitro* conditions induced the division of the nucleus, with the formation of microspores with two similar nuclei (Fig. 11B).

Trinucleated (Fig. 11C), tetranucleated (Fig. 11D) and multinucleated structures have been also observed (Fig. 11E). Moreover, it was possible to regenerate microspore-derived calli (Fig. 11F).

Statistical analysis conducted to study the influence of two factors, medium composition and thermal shock after three months of culture, is showed on table 16. It demonstrated that for the percentage of uninucleated microspores, there was a statistically significative interaction between the two factors (medium and thermal shock); in fact, the highest percentage of uninucleated microspores was recorded in medium P, after the treatment H+ (76.0%), while for N6, was obtained after the treatment F (77.7%). ANOVA results showed that the main factor influencing the percentage of uninucleated microspores was the thermal shock.

Regarding binucleated microspores, no statistically significative differences were observed. While, for the trinucleated microposres, the two factors influenced separately. For this reason, it was possible to conclude that P is the medium that better influenced the nucleus divisions (6% vs 4,2%), moreover, regarding the thermal shock, the best results were obtained in the control (8%) and secondarily, in the H+ treatment (Graph. 4).

The formation of multinucleated structures is influenced by both factors; in fact, microspores cultivated in the two media showed very different behaviors: the thermal shock H+ induced the worst result combination of P medium (2.8%) and the

best in combination with N6 (7.1%) (Graph. 5). Moreover, it was possible to observe a correspondence with the percentages of uninucleated: in fact, the combination that gave the highest percentage of multinucleated microspores was the same in which the lowest percentages of uninucleated were observed.

No statistically significative differences were observed in the percentages of microspore-derived calli: the similar tendency is showed, with the highest result for both media in the F+ treatment and the lowest in the F treatment.

3. 4 Conclusion.

Although the anther response appear to be determined independently and limited by the genotype factor, high significant interaction were recorded between the cultivar and the treatment after seven months of culture

For the first time, an experiment has been carried out to obtain regeneration through anther and isolated microspore culture of several hazelnut cultivar, testing different temperature stresses applied to the explants in culture to studying the influence of different factors affecting the gametic embryogenesis in hazelnut.

Table

Table 16. Effect of the medium composition and temperature treatments on the response of hazelnut, cv. Imperatrice Eugenia, isolated microspores, after three months in culture.

MEDIUM	THERMAL SHOCKS	Uninucleated (%)	Binucleated (%)	Trinucleated (%)	Multinuclated (%)	Calli (%)
P	C	56,8	22,9	9,6	8,7	2,0
	H	71,5	16,1	5,6	4,5	2,3
	H+	76,0	14,6	5,2	2,8	1,4
	F	73,1	16,6	5,1	4,8	0,4
	F+	69,4	18,1	4,2	4,9	3,4
N6	C	67,5	18,9	6,3	4,6	2,6
	H	75,8	18,4	1,9	2,8	1,2
	H+	65,7	17,8	7,6	7,1	1,8
	F	77,7	16,0	2,4	3,4	0,6
	F+	70,8	18,4	2,9	4,6	3,3
MEDIUM		0,285	0,865	0,039	0,410	0,988
THERMAL SHOCKS		0,002	0,180	0,004	0,120	0,988
MEDIUM x THERMAL SHOCKS		0,029	0,490	0,141	0,019	0,528

Two-way ANOVA, $p \leq 0.05$

C: Control

H: Treated at 35°C for 30 minutes.

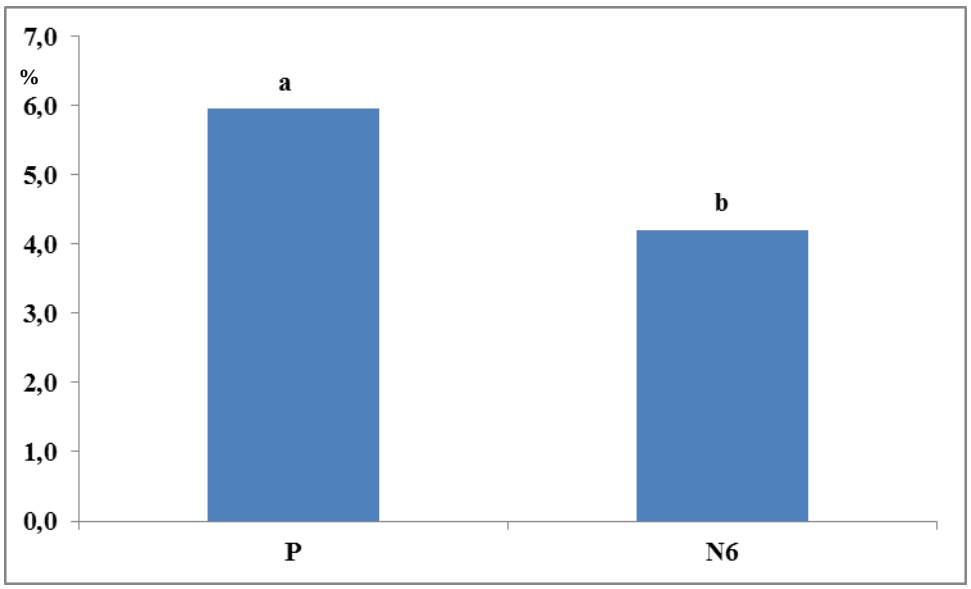
H+: 40°C for 60 minutes.

F: -20°C for 30 minutes.

F+: -20°C for 60 minutes.

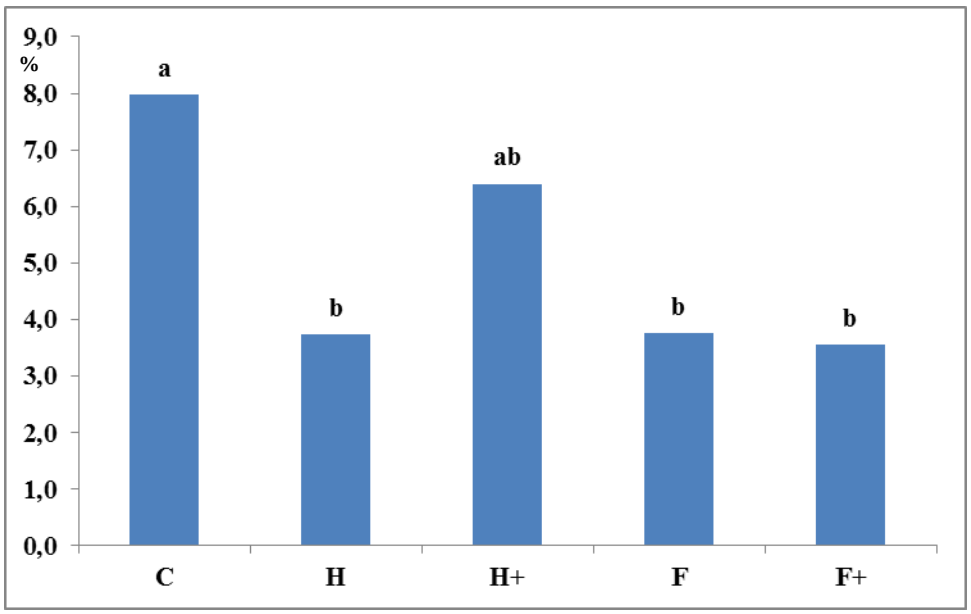
Graphics

Graph. 4 Influence of the medium composition on % of trinucleated microspore, after three months of culture.



One-way ANOVA, Tukey's test, p≤0.05

Graph. 5 Influence of the thermal shock on % of trinucleated microspore, after three months of culture.



One-way ANOVA, Tukey's test, p≤0.05

Figures

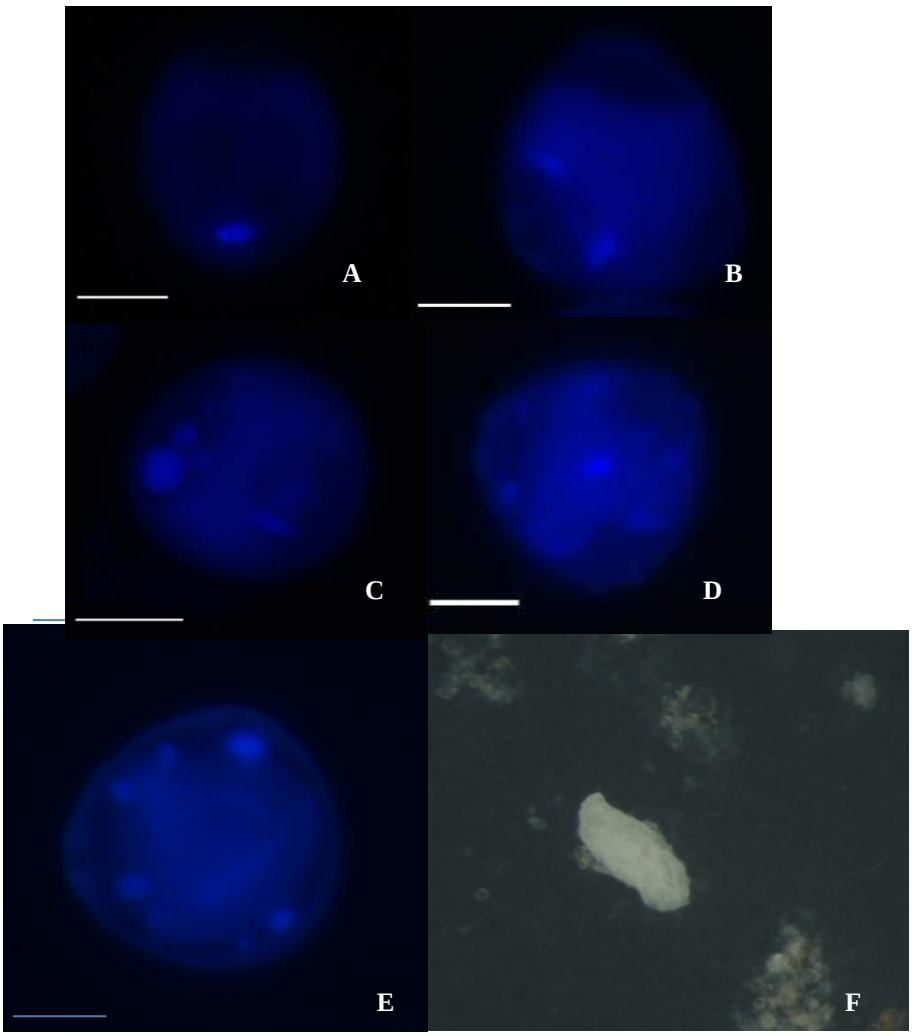


Fig. 11 Isolated microspore culture in hazelnut, cv Imperatrice Eugenia.**A:** Uninucleated microspore;**B:** Binucleated, with two symmetrical nuclei; **C:** Trinucleated microspore; **D**, **E:** Multinucleated microspore; **F:** Microspore-derived calli. **(Bars represent in A-E: 10µm)**

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-(γ,γ-Dimethylallylamino)purine
ANOVA	Analysis of variance
BA	6-Benzylaminopurine
DAPI	4,6- Diamidino-2-phenylindole
GA_3	Gibberellic acid
IAA	Indole acetic acid
IBA	Indole butyric acid
KIN	Kinetin
MS	Murashige & Skoog's medium (1962)
MT	Meta- Topolin
N6	N6 Chu (1978) induction medium
NAA	α-naphthaleneacetic acid
PAs	Polyamines
PBS	Phosphate-buffered saline
PUT	Putrescine
rpm	Rotations per minute
SPE	Spermidine
TDZ	Thidiazuron (N-phenyl-1,2,3,-thi-diazol-5-ylurea)
ZEA	Zeatin

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Coltura *in vitro* di microspore isolate per il miglioramento genetico di *Olea europaea* L.

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In vitro culture of isolated microspores for *Olea europaea* L. breeding

Abstract. Haploids (plants with the gametophytic chromosome number in the sporophyte: n instead of 2n) and doubled-haploids (haploids that doubled, spontaneously or induced, their chromosome number) have several applications in plant breeding, and, in particular, in mutagenesis, in *in vitro* selection, in trasformation, in gametoclonal variation, in genome sequencing and in functional genomic. For those reasons, they are an interesting biotechnological tool for breeding. In olive, as well as in tree crops, generally characterized by self-incompatibility, long reproductive cycle, long juvenile phase, tendency to allogamy and by high levels of heterozygosity, there isn't any possibility to achieve homozygosis by traditional breeding methods, such as several generations of self-pollinations. Gametic embryogenesis, allowing the single step production of homozygosis from heterozygous parents, is more and more a goal of breeders. In fact, it consists in gamete capability of modifying their development from gametophytic to sporophytic and it can be obtained through two strategies: "gynogenesis", when regeneration is from the female gamete and "pollen embryogenesis", when it is from male gamete (by anther culture or isolated microspore culture). In the present study, preliminary results obtained by *in vitro* isolated microspore culture of several Italian olive cultivars, are reported.

Key words: gametic embryogenesis, isolated microspore culture, olive, pollen.

Introduzione

L'olivo (*Olea europaea* L.) è una pianta arborea mediterranea tipica dei climi temperati, caratterizzati da estati calde e asciutte (Lavee, 1992). Il miglioramento genetico dell'olivo condotto con metodi tradizionali ha dovuto, da sempre, affrontare diversi problemi, come l'auto-incompatibilità, il lungo ciclo riproduttivo, la fase giovanile prolungata, una marcata tendenza all'allogamia ed un alto livello di eterosi. Come già verificato in molte specie, i metodi biotecnologici possono migliorare l'efficienza e la velocità dei programmi di miglioramento genetico.

L'embriogenesi gametica, permettendo l'ottenimento in una sola fase di linee omozigoti da parentali eterozigoti, è un metodo ampiamente usato per creare variabilità, mediante l'ottenimento di embrioni derivati dai gameti e di piante omozigoti che hanno diverse applicazioni nel miglioramento genetico. In particolare, l'embriogenesi da polline implica la riprogrammazione del polline immaturo (la microspora) verso lo sviluppo sporofitico, con la formazione di individui omozigoti (Germanà, 2006; 2011a,b). Per quanto riguarda l'embriogenesi gametica in olivo, sono stati condotti esperimenti sulla coltura *in vitro* di microspore isolate di cultivar spagnole (Arbequina e Picual), che hanno prodotto microspore multinucleate, strutture multicellulari e proembrioni, studiando, mediante analisi citochimiche e immunocitochimiche, il loro sviluppo *in vitro* (Bueno et al. 2004; 2005; 2006; Solis et al., 2008). Questo studio, che fa parte di una più ampia ricerca sull'embriogenesi gametica dell'olivo e di altre piante arboree, è stato condotto al fine di valutare l'effetto indotto da due trattamenti termici sulla coltura *in vitro* di microspore isolate di diversi genotipi italiani di olivo.

Materiali e metodi

Nel mese di maggio, sono state raccolte mignole delle varietà di olivo: Cerasuola Sciacca, Galatina, Nocellara del Belice e Verdello (Scillato, PA) e di Moraiolo (Perugia) e sono stati conservati, per due settimane, a 4°C e al buio. Prima della coltura *in vitro*,

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per studiare la correlazione tra la dimensione del bocciolo e lo stadio di sviluppo delle microspore, i boccioli sono stati divisi in tre categorie dimensionali. In seguito, le antere sono state colorate con una soluzione di 4,6- diamidino-2-phenylindole (DAPI) ed osservate al microscopio a fluorescenza (Zeiss, Axiophot, Germania), scegliendo la dimensione corrispondente allo stadio vacuolato.

Per ciascuna varietà, sono stati sterilizzati sotto cappa a flusso laminare 100 boccioli, mediante immersione per 5 minuti in una soluzione al 70% (v/v) di alcol etilico, seguita da un'immersione di 20 minuti in una soluzione di ipoclorito di sodio allo 0,5%, ed infine da tre risciacqui con acqua distillata sterile. L'isolamento delle microspore è stato svolto seguendo il protocollo di Kumlehn *et al.* (2006). Dopo l'ultimo lavaggio, le microspore sono state contate e sono state risospese in una quantità di mezzo di coltura liquido, tale da ottenere una densità di 200.000 microspore per ml di mezzo ($2x10^5$ microspore/ml).

Il mezzo di coltura usato per gli esperimenti è stato quello riportato da Germanà e Chiancone (2003), senza l'aggiunta dell'agar. Lo stesso mezzo di coltura, ma con l'aggiunta di 8,5 g/l di agar, è stato usato per la coltura del callo ottenuto dalle microspore isolate.

Le microspore isolate e poste in coltura sono state sottoposte a due trattamenti termici: 34 °C per 60 minuti (HT) e -20 °C per 30 minuti (F). Inoltre, è stato predisposto un controllo (C), in cui le microspore non hanno subito alcun trattamento termico. Le scatole Petri (35x10) con 1 ml di sospensione sono state poste al buio per i primi 30 giorni e poi poste sotto lampade bianche fluorescenti (Philips TLM 30W/84) con una densità di flusso fotosintetico di 35 µmol m^{-2} s^{-1} e con un fotoperiodo di 16 ore di luce al giorno.

Al fine di valutare l'andamento dello sviluppo delle microspore, all'inizio della coltura e per i primi tre mesi sono state effettuate delle osservazioni al microscopio a fluorescenza, ricorrendo alla stessa colorazione con DAPI riportata in precedenza. Inoltre, per ciascun trattamento e per ogni cultivar, è stato misurato il diametro di 100 microspore, calcolandone le medie. L'influenza sul diametro delle microspore del trattamento termico e del genotipo è stata valutata ricorrendo all'ANOVA a due vie. La separazione delle medie è stata effettuata mediante test Student-Neuman-Keuls (SNK) ($p \leq 0,05$).

Risultati e discussione

La scelta dell'appropriato stadio di sviluppo delle microspore è cruciale per l'embriogenesi da polline. In questo studio, è stata determinata la corrispondenza tra lo stadio di sviluppo delle microspore, da tetrade a polline maturo, con la corrispondente dimensione delle antere e del bocciolo, identificando i boccioli non ancora schiusi, di circa 2-3 mm, con antere giallo-verde, come quelli con il più alto numero di microspore allo stadio vacuolato, stadio considerato il più rispondente per l'induzione embriogenica (figg. 1 e 2) (Germanà, 2009).

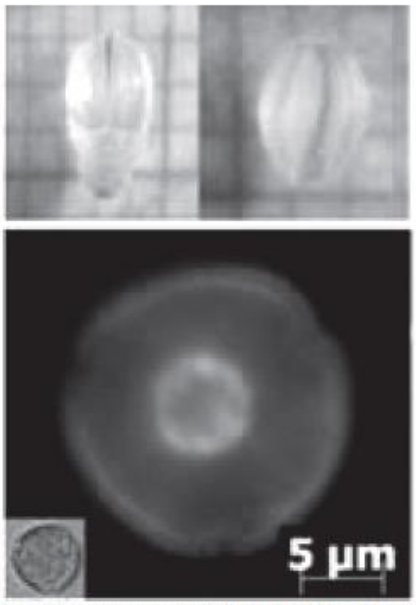

Fig. 1 - Correlazione fra lo stadio fenologico del bocciolo, dell'antera e della microspora (DAPI).
Fig. 1 - Correlation between phenological stage of flower bud, of anther and of microspore development (cv Nocellara del Belice) (DAPI).

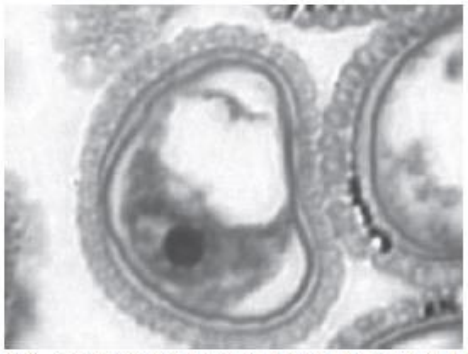

Fig. 2 - Microspora vacuolata di Nocellara del Belice (Toluidine blue).
Fig. 2 - Nocellara del Belice vacuolated microspore (Toluidine blue).

Le osservazioni effettuate al microscopio a fluorescenza durante la coltura hanno mostrato l'evoluzione delle microspore in coltura, riscontrando sia lo sviluppo gametofitico che sporofitico. Sono state rilevate, infatti, microspore che presentavano le tipiche caratteristiche del polline maturo, con due nuclei che mostravano intensità luminosa differente (nucleo generativo e vegetativo) (fig. 3) e microspore che avevano deviato dal normale sviluppo gametofitico per seguire la via sporofitica, in cui erano presenti due nuclei uguali, frutto di una divisione simmetrica (fig. 4). La divisione simmetrica del nucleo è considerata, insieme all'accumulo di amido, alla citochinesi con formazione di nuove pareti cellulari, alla presenza abbondante di ribosomi e ai cambiamenti nell'esterificazione delle pectine e nei componenti cellulosici delle pareti cellulari, una delle caratteristiche tipiche della proliferazione e della differenziazione delle microspore (Bueno *et al.*, 2005; Solìs *et al.*, 2008). Inoltre, dopo circa tre mesi di coltura, è stato possibile osservare polline maturo e germinato (fig. 5), ma anche microspore multinucleate che rappresentano un ulteriore progresso nello sviluppo embriogenico (fig. 6).

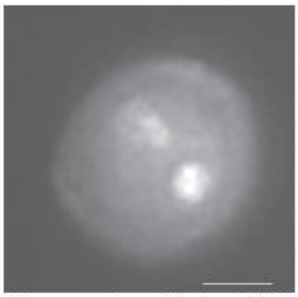

Fig. 3 - Microspora con due nuclei asimmetrici di Moraiolo (DAPI) (barra 10 µm).
Fig. 3 - Moraiolo microspore with two asymmetrical nuclei (DAPI) (scale bar 10 µm).

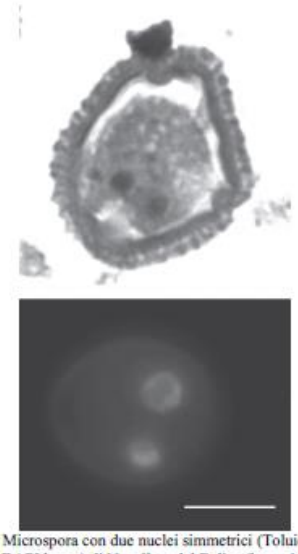

Fig. 4 - Microspora con due nuclei simmetrici (Toluidine blue alto, DAPI basso) di Nocellara del Belice (barra 10 µm).
Fig. 4 - Nocellara del Belice microspore with two symmetrical nuclei (Toluidine blue up, DAPI bottom) (scale bar 10 µm).

Le microspore misurate al momento della messa in coltura, cioè subito dopo l'isolamento, mostravano un diametro medio di 10 µm, ma le misurazioni effettuate dopo 3 mesi di coltura hanno evidenziato un aumento significativo del diametro fino ad un massimo di 23,8 µm (fig. 7). L'analisi statistica effettuata sui dati rilevati dopo tre mesi in coltura, ha evidenziato un'influenza significativa del fattore trattamento termico, ma non del fattore cultivar. Il trattamento "a freddo" (F) ed il trattamento "a caldo" hanno fornito risultati statisticamente uguali; infatti, il primo ha determinato un aumento del diametro delle microspore da 10 a 23,8 µm ed il secondo da a 10 a 20,3 µm. Differenze statisticamente significative sono state individuate fra i due trattamenti termici ed il controllo (C), in cui è stato osservato l'incremento di dimensio-

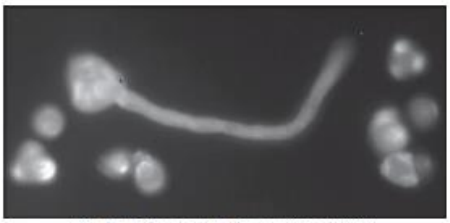

Fig. 5 - Polline di Verdello germinato (DAPI).
Fig. 5 - Verdello germinated pollen grain (DAPI).

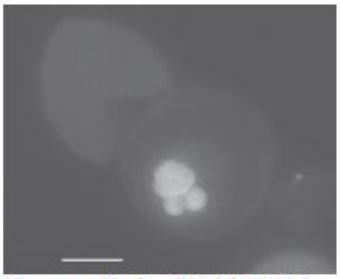

Fig. 6 - Microspora multinucleata di Verdello (DAPI) (barra 10 µm).
Fig. 6 - Verdello multinucleated microspore (DAPI) (scale bar 10 µm).

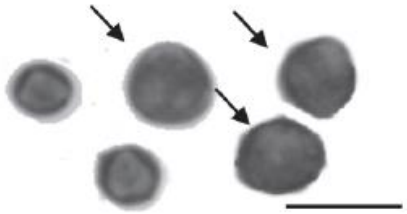

Fig. 7 - Microspore non sviluppate ed ingrossate (frecce) di Cerasuola Sciacca (barra 20µm).
Fig. 7 - Cerasuola Sciacca not developed and enlarged (arrows) microspores (scale bar 20µm).

ni minore (da 10 a 15 µm). Infine, l'analisi statistica non ha mostrato alcuna interazione significativa tra il fattore Cultivar e il fattore Trattamento. Infatti, per tutte le cultivar, eccetto che per la Cerasuola Sciacca, i valori più elevati sono stati ottenuti per le microspore sottoposte al trattamento "a freddo", seguite da quelle trattate "a caldo" ed infine da quelle non trattate (fig. 8).

Dopo 2-3 mesi di coltura, le microspore hanno cominciato a produrre un callo biancastro (fig. 9) che è stato poi trasferito sullo stesso mezzo usato per la coltura delle microspore, reso solido dall'aggiunta di agar, dove il callo si è sviluppato formando strutture proembrionali (fig. 10).

I risultati riportati in questo studio confermano quanto riferito da Zheng (2003) sull'importanza dello stress (termico o *starvation*), nell'induzione embriogenica delle microspore.

Nonostante non sia stato ancora possibile rigenerare plantule dalle strutture proembrionali e dal callo ottenuto dalle microspore, i risultati ottenuti in questo

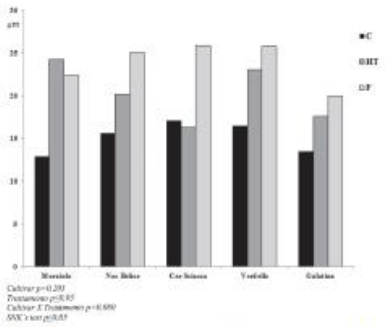

Fig. 8 - Effetto del trattamento «a caldo» e «a freddo» sul diametro delle microspore dopo 3 mesi di coltura *in vitro*.
Fig. 8 - Effect of cold and heat-treatments on the diameter of microspores after 3 months of in vitro *culture.*

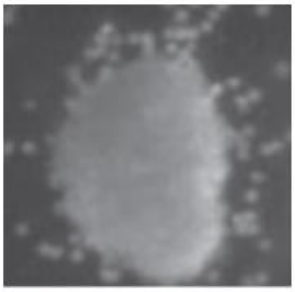

Fig. 9 - Callo ottenuto dopo due mesi di coltura *in vitro* di microspore isolate di Galatina
Fig. 9 - Callus obtained after two months of in vitro *culture of Galatina microspore culture.*

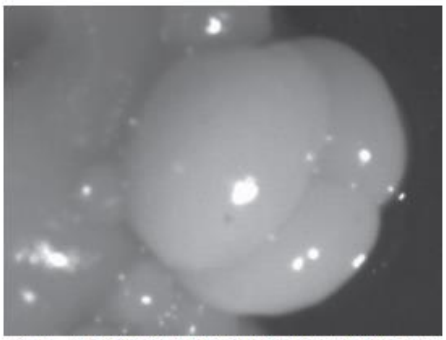

Fig. 10 - Embrioide ottenuto dopo 10 mesi di coltura di microspore isolate di Cerasuola Sciacca.
Fig. 10 - Cerasuola Sciacca embryoid developed after 10 months of isolated microspore of culture.

studio sono molto incoraggianti, poiché dimostrano che, sebbene l'olivo, come molte altre specie arboree, sia una specie recalcitrante all'embriogenesi gametica, shock termici sono in grado di indurre lo sviluppo sporofitico delle microspore.

Conclusioni

Questo studio riporta i risultati preliminari della coltura di microspore isolate di alcune cultivar italiane di olivo. Le microspore sottoposte a trattamento termico (sia "a caldo" che "a freddo") hanno presentato un maggiore incremento di dimensioni rispetto al controllo. Grazie alle osservazioni al microscopio a fluorescenza è stato possibile monitorare sia lo sviluppo gametofitico che quello sporofitico delle microspore, con la formazione di strutture multinucleate. Inoltre, ad ulteriore conferma dell'efficacia del trattamento termico, dopo tre mesi di coltura, le microspore hanno

cominciato a produrre callo che trasferito su mezzo solido ha prodotto strutture proembrionali.
Al fine di aumentare l'efficienza induttiva del processo di embriogenesi gametica e di ottenere la rigenerazione di plantule derivate da polline, è necessario continuare ad approfondire gli studi sull'embriogenesi gametica da polline in olivo.

Riassunto

Gli aploidi (piante con un numero gametofitico di cromosomi nello sporofito: n invece di 2n) ed i doppio-aploidi (aploidi che hanno raddoppiato il numero di cromosomi spontaneamente o in maniera indotta) trovano numerose applicazioni nel miglioramento genetico vegetale, ed in particolare, nella mutagenesi, nella selezione *in vitro*, nella trasformazione, nell'utilizzo della variabilità gametoclonale ed anche nel sequenziamento del genoma e negli studi di genomica funzionale. Pertanto, essi costituiscono uno strumento biotecnologico particolarmente interessante nel miglioramento genetico delle piante. Ai fini dell'ottenimento dell'omozigosi nell'olivo, come in tutte le piante arboree, caratterizzate da elevata eterozigosi, da un lungo periodo giovanile, da grandi dimensioni e spesso da autoincompatibilità, non vi è la possibilità di ricorrere a metodi tradizionali di miglioramento genetico, quali diverse generazioni di autoimpollinazione. L'embriogenesi gametica, rendendo possibile, in una sola fase, l'ottenimento dell'omozigosi da un genitore eterozigote, è sempre più oggetto di ricerca da parte dei *breeder*. Essa, infatti, consiste nella capacità dei gameti di modificare il loro sviluppo da gametofitico a sporofitico e può essere ottenuta tramite due strategie: la "ginogenesi", cioè tramite rigenerazione dal gamete femminile, e l'"embriogenesi da polline" cioè dal gamete maschile (tramite coltura *in vitro* di antere e di microspore isolate).

Nel presente studio sono riportati i risultati preliminari ottenuti dalla coltura *in vitro* di microspore isolate di diverse cultivar italiane di olivo.

Parole chiave: Coltura di microspore isolate, embriogenesi gametica, olivo, polline.

Lavoro frutto di una collaborazione tra il CSIC (Spagna) e l'Università degli Studi di Palermo (Italia) nell'ambito del progetto italiano CORI 2006 e della collaborazione tra il CNR (Italia) e il CSIC (Spagna) nell'ambito di Joint Project Spagnolo-Italiano CSIC-CNR 2008IT0046 "Embriogenesi gametica e somatica per il miglioramento genetico e la propagazione dell'olivo e di altre piante arboree mediterranee".

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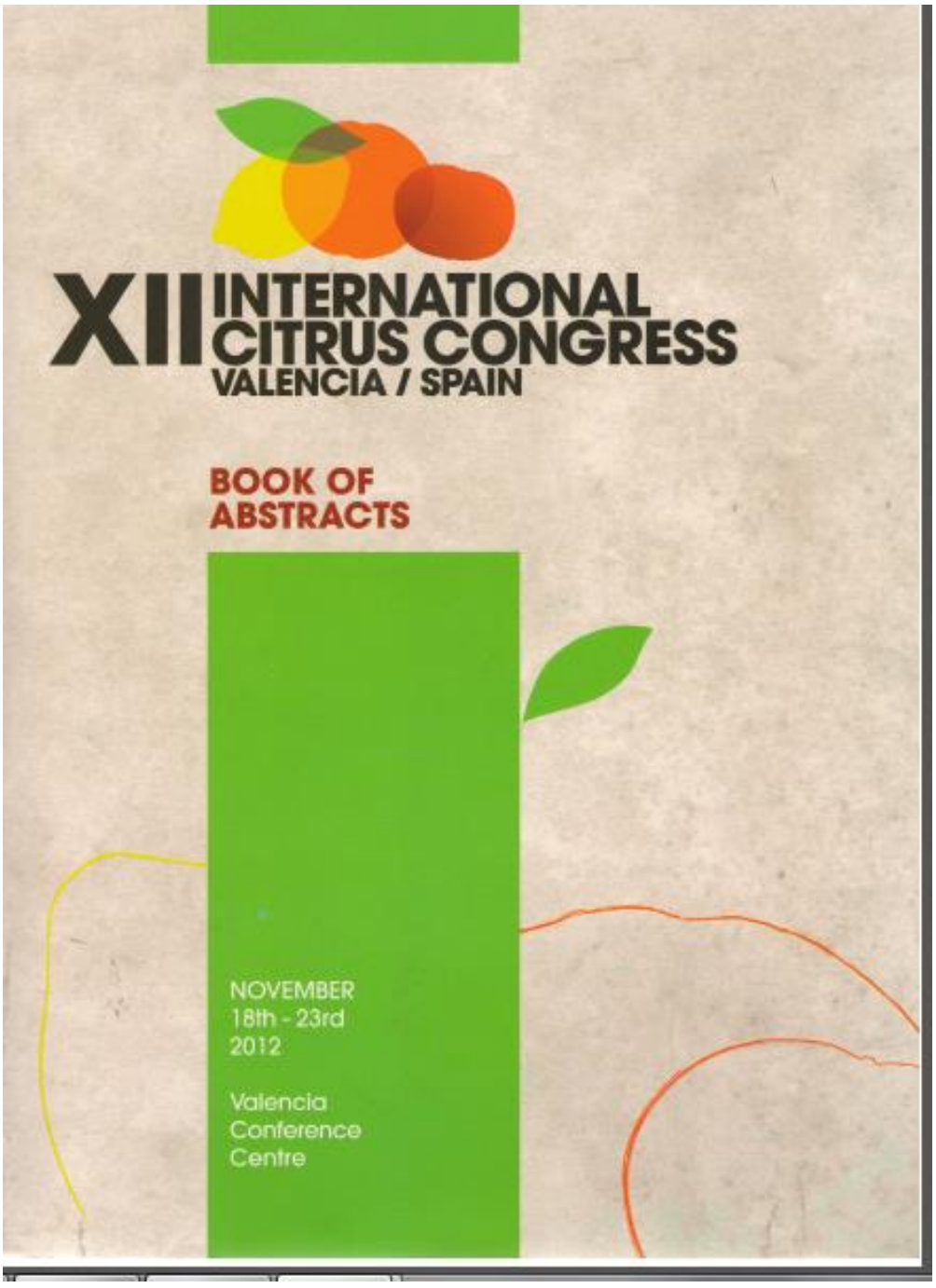
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S05O09

Targeted cybridization in citrus and transcript profiling of a male sterile cybrid pummelo containing cytoplasm from satsuma mandarin

Guo W.W.[1], Zheng B.B.[1], Xiao S.X.[1], Grosser J.W.[2], and Deng X.X.[1]

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seedlessness is an elite fruit quality trait. By symmetric fusion of embryogenic callus protoplasts of sats mandarin and mesophyll protoplasts of elite seedy cultivars, diploid cybrid plants containing sterile cytopl from satsuma were regenerated and characterized from several fusions, and the cybrid pummelo betw satsuma 'Guoqing No. 1' and 'Hirado Buntan' pummelo showed male sterile trait. To gain new insight the underlying mechanism, the nuclear gene expression profiles of floral buds of the cybrid with the fertile pummelo were compared by RNA-Seq. Gene expression profiles which identified a large numbe differentially expressed genes were captured at both petal and stamen primordia distinguishable stage addition, agreeable with flower morphology of the cybrid, expression of PISTILLATA (PI) was reduced in stam like structures, even though it was restricted to correct floral whorls. Down-regulated expression of APETA (AP3) coincided with that of PI. These finding indicated that, due to their whorl specific effects in flo development, citrus class-B MADS-box genes likely constituted 'perfect targets' for CMS retrograde signa and that dysfunctional mitochondria seemed to cause male sterile phenotype in the cybrid pummelo.

S05O10

In vitro anther culture of several cultivars of *Citrus sinensis* and *Citrus clementina*

Cardoso J.C.[1], Abdelgallel A.[2], Chiancone B.[2], Rocha Latado R.[3], Lain O.[4], Testolin R.[4], and Germanà M.A.[2]

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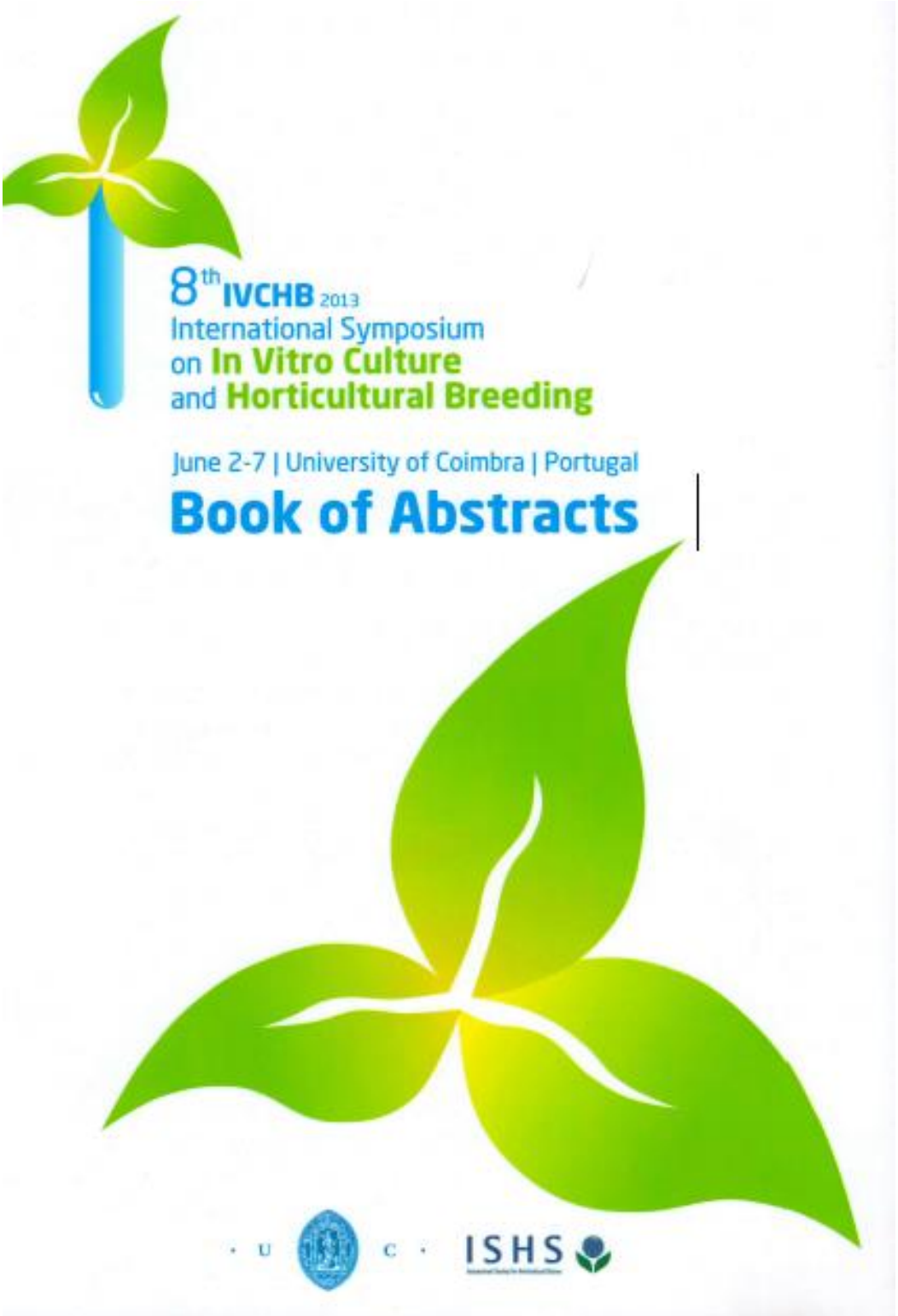
Citrus breeding goals can be achieved by traditional (hybridization, selection, mutation) or biotechnolo methods. Efficient procedures of in vitro regeneration are required, in conjunction with the recent techni of cellular and molecular biology, to achieve significant improvement in a brief time. Moreover, particular

citrus, somatic embryogenesis is an efficient method of plant regeneration and embryogenic callus is valuable for propagation as well as for genetic improvement. Anther culture, among the in vitro regeneration systems, is a widespread method to produce haploids (Hs) and doubled-haploids (DHs), drastically reducing the time needed to produce homozygous lines compared to conventional breeding methods, that include several generations of self-pollination. In addition, in vitro anther culture is also utilized to obtain somatic embryos and clonal plant propagation in many woody plants. Actually, in vitro anther culture is, in citrus, an efficient approach to obtain both gametic and somatic embryogenesis. In this study, anther culture has been applied to several citrus genotypes: four cultivars of *C. sinensis* and two of *C. clementina*, testing two temperature shocks. The strong influence of the genotype on the response of anthers in vitro cultured has been confirmed. Actually, the same treatments applied to the explants in culture resulted in the production of gametic embryos in clementines and of somatic embryos in sweet oranges. Clementine confirmed its tendency to regenerate homozygous and tri-haploids embryos and plantlets. Further studies should be performed, in other citrus genotypes aimed to obtain in vitro regeneration procedures, suitable for different applications, as new opportunities for genetic improvement and for the innovation in propagation methods.

S05O11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O28

Early in vitro development of isolated microspores of four Sicilian olive cultivars

Chiancone, B.[1]; Abdelgalel, A.M. [1]; Bárany, I.[1]; Risueño, M.C.[2]; Testillano, P.S.[2] and Germanà M.A.[1]

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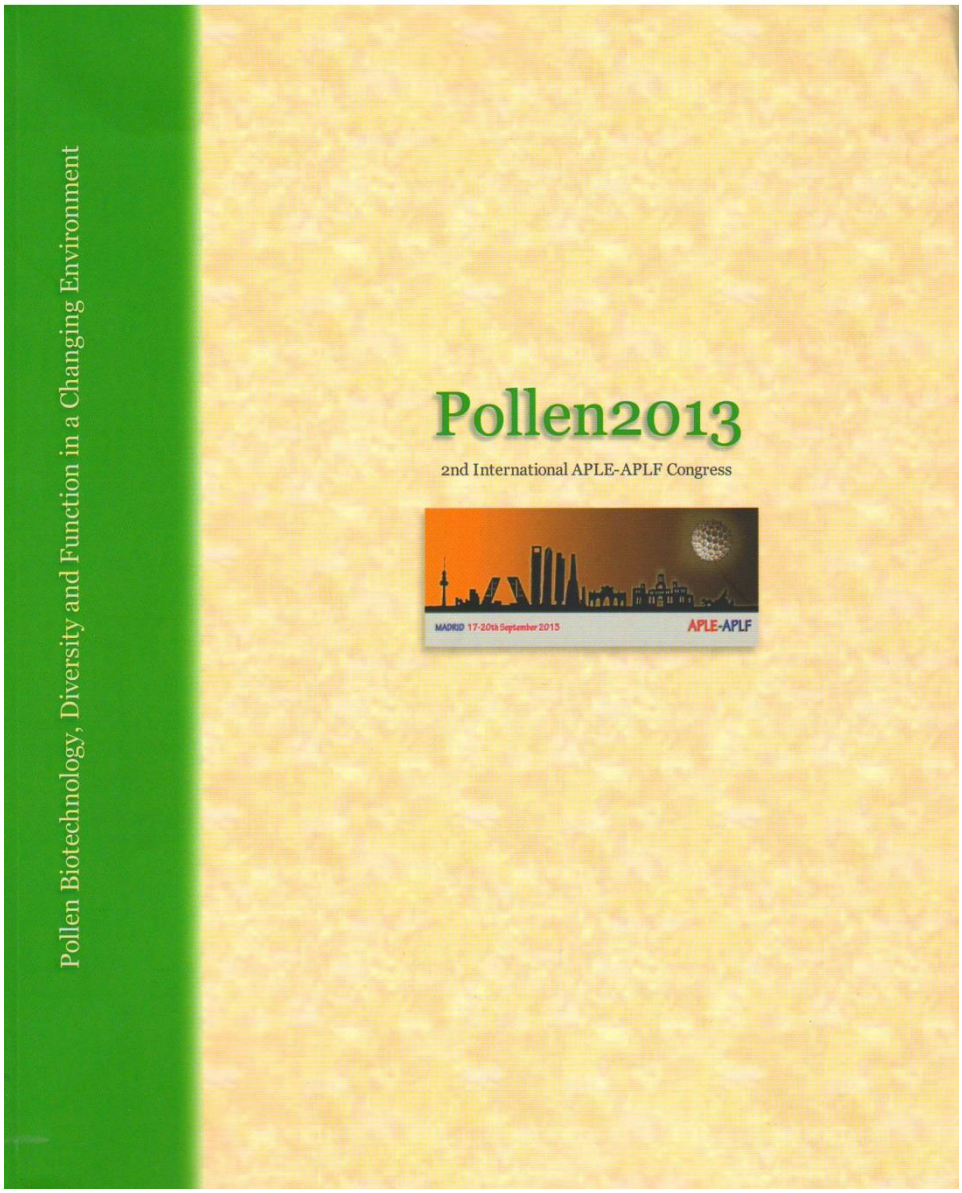
Nowadays there is a great interest in the advancement of the studies on gametic embryogenesis in olive, due to the enormous effort and relevance of its genome sequencing and gene mapping. Olive, like most of woody plants, is characterized by a long reproductive cycle, high heterozygosity, large size, and, often, by self-incompatibility; for this reason it is not possible to obtain haploidization through conventional methods, such as, several generations of selfing. In vitro anther or isolated microspore culture are the most effective and widely used methods to obtain haploids.

In this study, in vitro isolated microspore culture of four Sicilian cultivars of olive has been carried out, investigating the influence of two polyamines (putrescine and spermidine) and of two medium compositions on pollen embryogenesis induction. Particularly, microspores, isolated following the protocol set up by Kuhmlen (2006), have been cultivated on eight media, containing the two polyamines in different combinations: A) 2 mM putrescine (PUT); B) 2 mM spermidine (SPD); C) 1 mM each of putrescine and spermidine (PUT + SPD); D) control without polyamines (C). The four growth regulator combinations were added to two media employed in previous experiments on gametic embryogenesis of other woody plant species (P and N6) (Germanà et al., 1997; Germanà and Chiancone, 2003). After one month in culture, microspores have been observed by a fluorescent microscope after DAPI staining (100 microspores per each thesis). Structural analyses have been performed at different times of the culture to monitor the main cellular changes in the olive microspores.

Different features of microspores have been observed: uninucleated with no development, binucleated with two asymmetrical nuclei (normal gametophytic pathway: one vegetative and one generative nucleus), binucleated with two equal-size vegetative-type nuclei that had just started their sporophytic pathway, trinucleated, tetranucleated and multinucleated, indicating steps towards the gametic embryogenesis development. Moreover, the presence of several calli has been detected. A strong genotype-medium interaction has been observed; in fact, each cultivar responded differently depending on the medium tested.

This research represent an advancement in the knowledge of gametic embryogenesis in olive, towards the regeneration of homozygous olive plants.

Key words: gametic embryogenesis, isolated microspores, olive, polyamines, putrescine, spermidine.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LATE ABSTRACT

S5-O-01

Session 5: Pollen Biotechnology and Genetics
ORAL COMMUNICATION

Embryo production through isolated microspore culture in *Citrus clementina* Hort. ex Tan., cv. Monreal Rosso

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Key words: isolated microspore culture; homozygosity; pollen embryogenesis

Haploidy technology, that is the single-step development of complete homozygous genotypes from heterozygous parents through gametic embryogenesis, has already a huge impact on many relevant crops, representing an integral part in their breeding programmes (Germanà 2011a; 2011b). *In vitro* anther or isolated microspore culture are the most effective and widely used methods available to obtain haploids (Hs) and douled-haploids (DHs). In many crops anther culture is often the preferred method for doubled haploid production because of the big advantage of its simplicity. However, the technique of the isolated microspore culture, performed by removing somatic anther tissue, although requiring better equipment and more skills compared to anther culture, is more and more carried out, since it provides a better way of investigating the cellular, physiological, biochemical, and molecular processes involved in pollen embryogenesis.
In this study, in vitro isolated microspore culture of the clementine cultivar Monreal Rosso has been carried out, investigating the influence of five different media on pollen embryogenesis induction. During in vitro development, different structural features have been observed: uninucleated microspores, with no development, binucleated with two asymmetrical nuclei (normal gametophytic pathway: one vegetative and one generative nucleus), binucleated with two equal-size vegetative-type nuclei that had just started their sporophytic pathway, trinucleated, tetranucleated and multinucleated, indicating steps towards the sporophytic development. Moreover, the presence of calli has been detected and, for the first time in *Citrus* genus, embryos have been obtained through isolated microspore culture.

ACKNOWLEDGEMENTS
This study has been partially supported by the projects: PON01_01623 IT – CitrusGenomics and thanks are due to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarship to Marines Marli Gniech Karasawa.

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Currently, we are analysing a set of promising and preferentially female gamete-specific expressed candidate genes regarding their expression, subcellular localization, and impact on gamete fusion. These include receptor(-like) kinases, glucosidases, and proteins of unknown function.

Abstract P29

Advancement on isolated microspore culture in Citrus clementina Hort. ex Tan., cvs. Monreal Rosso and Nules

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Haploid (Hs) and douled-haploid (DHs) technology represents a valuable and powerful tool for breeding programs; in fact, it allows, in one step, the obtaining of complete homozygous lines from heterozygous parents, through gametic embryogenesis (Germanà 2011b). This technology is particularly useful for woody species, like *Citrus*, characterized by a long juvenile stage, high degree of heterozygosity and often by self-incompatibility (Germanà 2006, 2009, 2011).

In vitro anther or isolated microspore culture are the most effective and widely used methods to induce gametic embryogenesis. Even if isolated microspore culture is more time consuming and requires better equipment than anther culture, it permits a better understanding of the pollen embryogenesis process from cellular, physiological, biochemical, and molecular point of view, without the influence of the anther somatic tissue.

The efficiency of gametic embryogenesis is influenced by several factors, among others, by the culture medium composition and in particular, by the type and the concentration of plant growth regulators. Meta-topolin, a naturally occurring aromatic cytokinins, is considered an alternative to benzyladenine (BA), zeatin (ZEA) and kinetin (KIN) in plant tissue culture (Aremu et al., 2012). It has been used, mainly, to increase the efficiency in *in vitro* plant propagation of several species, among them also *Citrus (*Niedz and Evens, 2011), but never, in our knowledge, to induce gametic embryogenesis from isolated microspores.

In this study, carried out on *Citrus clementina* Hort. ex Tan., cultivars Monreal Rosso and Nules, the effect of the presence in the culture media of meta-topolin in substitution of BA or ZEA, on pollen embryogenesis induction, through isolated microspore culture, was investigated.
After five months of culture, for both genotypes and for all the media tested, different structural features have been observed and registered: microspores uninucleated, with no development, binucleated with two asymmetrical nuclei (normal gametophytic pathway: one vegetative and one generative nucleus), binucleated with two equal-size vegetative-type nuclei that had just started their sporophytic pathway, trinucleated, tetranucleated and multinucleated. The sporophytic development of isolated microspores in culture has been confirmed by the presence, in the culture, of calli and embryos at different stage.

The results presented represent an advancement in the knowledge of pollen embryogenesis in *Citrus clementina* Hort. ex Tan., in fact, this is the first time that the regeneration of embryos from Monreal Rosso and Nules isolated microspores is reported.

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