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USE OF *IN VITRO* TISSUE CULTURE IN PROPAGATION AND GENETIC IMPROVEMENT OF FRUIT TREES

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ACRONYMS

2,4-D	2,4-Dichlorophenoxyacetic acid
ANOVA	Analysis of Variance
BAP	6-Benzylaminopurine
DAPI	4,6- Diamidino-2-Phenylindole
DH	Doubled Haploid
FAO	Food and Agriculture Organization
GA	Gibberellic Acid
H	Haploid
IAA	Indolo-3-acetic acid
IAM	Istituto Agronomico Mediterraneo
IBA	Indole-3-Butyric Acid
IVIA	Instituto Valenciano de Investigaciones Agrarias
MS	Murashige and Skoog
mT	meta-Topolin
NAA	1-Naphthalene Acetic Acid
PCR	Polymerase Chain Reaction
PGR	Plant Growth Regulator
RAPD	Random Amplified Polymorphic DNA
SCAR	Sequence Characterized Amplified Region
SSR	Simple Sequence Repeat
TDZ	Thidiazuron
UNIPA	University of Palermo

ABSTRACT

In vitro culture, applied to the propagation and genetic improvement of plant biodiversity, can be an effective tool to face current problems such as climate change, and new consumer needs. Moreover, it can take on a strategic role in genetic improvement and propagation of cultivars in order to obtain genotypes resistant to biotic and abiotic stresses, with fruits improved from an organoleptic point of view and plants able to adapt to climate change.

Genetic improvement through conventional methods is limited by many factors. Fruit trees are characterized by a long period of juvenitlity, high heterozygosity and, very often, by sexual incompatibility. The *in vitro* techniques of the cultivation of isolated anthers and/or microspores, to obtain haploid or double haploid plants, are able to overcome some limits of traditional genetic improvement and provide in a single step completely homozygous lines of interest for genomic studies, mutations, mapping and genetic transformation.

In addition, *in vitro* technology is a complementary method or can even support conventional methods of agamic propagation, since the plant material is protected from possible attacks by pathogens, their production is independent of seasonal conditions, this tecquique allows genetic uniformity and high multiplication rates, in a small space.

In vitro culture is made possible by the phenomenon of totipotency, that is, the ability of cells, organs or tissues to de-differentiate and acquire meristematic skills, giving rise to organs, tissues or to an entire individual, even different from the original one.

This research had as its objective the induction of gametic embryogenesis in various *citrus* genotypes through *in vitro* culture of anthers, and the development of an efficient protocol for Fig, Hop and Caper through synthetic seed technology and micropropagation, respectively.

The experiments were conducted in 2017, 2018 and 2019 at the University of Palermo (UNIPA) and the Valencian Institute of Agricultural Research (IVIA).

As far as the cultivation of citrus anthers is concerned, somatic embryogenesis was obtained in the Moro variety (2x), leading to the regeneration of embryos which after ploidy and molecular analyzes were heterozygous tetraploids; and Marisol (4x), Clemenules (4x), Moro Los Valles (4x), Sanguinelli (4x) and Sanguinelli (2) genotypes, symmetric division of the nucleus, multinucleated structures and formation of embryos that following the analysis was observed cytofluorimetric results for the most part were diploid, molecular analysis, carried out with SSR markers instead, found singular results, in fact, in the Marisol, Clemenules and Sanguinelli genotypes (2x and 4x), even if multinucleated microspores and the most regenerated were diploid, from the analysis they were all heterozygous, while in Moro Los Valles, the markers found that three regenerated diploids were homozygous, two mutated homozygotes (gametoclonal variability) and two mutated heterozygotes.

In this research, synthetic seed technology has been applied to the Houmairi variety of *Ficus carica* L., in particular, the effect of two "Plant Growth Regulators" (PGR): 6-benzylaminopurine (BAP) and meta-Topolina (mT), added to the artificial endosperm, and of the preservation at 4 ° C for 30 days of the encapsulated microcuttings. The highest percentage of viable seeds that led to a greater vegetative recovery occurred in the thesis that it did not foresee cold storage before sowing and when the artificial endosperm was added with BAP. In a different way, the highest percentage of conversion occurred, both for the stored seeds and not when the endosperm was added with mT.

In vitro propagation was applied to the Cascade, variety of *Humulus lupulus* L. with the aim of achieving an efficient micropropagation protocol.

Starting from the collection in the field of the plant material, sterilization, stabilization of the aseptic culture as well as the multiplication, rooting and acclimatization to *ex vitro* conditions were carried out. For the multiplication 3 PGR were tested: BAP, mT and Thidiazuron (TDZ) in different combinations, while for the rooting 2 PGR auxinic: indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). The TDZ / BAP interaction showed the highest germination rate even though with a greater callus development such as to consider the medium with 2 mg l^{-1} of TDZ the best despite giving a lower budding as well as for the presence of callus. As regards the greater percentage of rooting, it occurred in the medium containing 2mg l^{-1} of IBA without significant difference from the medium that contained 1 mg L of IAA.

Finally, the influence of the calcium alginate coating the cuttings along with the three different growth regulators (PGR) were evaluated: 6-benzylaminopurine (BAP), meta-topolina (mT) and zeatin (ZEA), on vitality, regrowth and the conversion of the propagules of two Sicilian genotypes of *Capparis spinosa* (L.) (Tracino and Scauri, from Pantelleria Island).

Caper microcutting have been sectioned and placed in different artificial endosperms based on Murashige and Skoog, enriched with mT or ZEA or BAP. The synthetic seeds obtained were seeded on an MS medium added with 0.4 mg / L of naphthalene acetic acid and 0.7 mg / L of gibelleric acid. After 60 days, the following parameters were detected: vitality, regrowth, number and length of shoots and roots, conversion.

The results confirm that encapsulation did not negatively affect vitality, which showed the highest percentage with BAP in Tracino and with ZEA in Scauri. Similar results were obtained in regrowth, with statistically significant differences between the three PGRs tested: Tracino showed the best regrowth in capsules enriched with BAP, Scauri with ZEA. Furthermore, synseed conversion was strongly influenced by PGR and was higher in artificial endosperm added with BAP in Tracino and ZEA in Scauri genotype.

RIASSUNTO

La coltura *in vitro*, applicata alla propagazione e miglioramento genetico della biodiversità vegetale, può rappresentare uno strumento efficace per affrontare i problemi attuali come i cambiamenti climatici, le nuove esigenze dei consumatori e indirettamente lo sviluppo delle aree rurali. Inoltre, può assumere un ruolo strategico nel miglioramento genetico e nella propagazione delle cultivar al fine di ottenere genotipi resistenti, con frutti migliori dal punto di vista organolettico e piante capaci di adattarsi ai cambiamenti climatici.

Il miglioramento genetico attraverso i metodi convenzionale è limitato da molti fattori infatti, gli alberi da frutto sono caratterizzati da un lungo periodo di giovanilità, elevata eterozigosi e molto spesso incompatibilità sessuale. La tecniche *in vitro* della coltura di antere e/o microspore isolate, per ottenere piante aploidi o doppi aploidi, riescono a superare alcuni limiti del miglioramento genetico tradizionale e fornire in un solo passaggio linee completamente omozigoti interessanti per studi di genomica, mutazioni, mappatura e trasformazione genetica.

La tecnologia *in vitro* oltre ad essere applicata al miglioramento genetico, risulta un metodo complementare ai metodi convenzionali di propagazione agamica, in quanto, il materiale vegetale risulta protetto da eventuali attacchi di patogeni, il processo è indipendente dalle condizioni stagionali, permette uniformità genetica ed elevati tassi di moltiplicazione in uno spazio ridotto.

La coltura *in vitro* è resa possibile dal fenomeno della totipotenza; cioè la capacità che hanno le cellule, organi o tessuti di de-differenziarsi e acquisire capacità meristematiche dando origine ad organi, tessuti o a un individuo intero, anche diverso da quello di partenza.

Questa ricerca ha avuto come obiettivo l'induzione dell'embriogenesi gametica in vari genotipi di agrumi, tramite coltura *in vitro* di antere. Inoltre, si è studiata la messa a punto di un efficiente protocollo di conservazione e propagazione, attraverso la tecnologia del seme sintetico, rispettivamente in Fico, Luppolo e Cappero.

Gli esperimenti sono stati condotti nel 2017, 2018 e 2019 presso l'Università degli Studi di Palermo (UNIPA) e l'Instituto Valenciano de Investigaciones Agrarias (IVIA).

Per quanto riguarda la coltura di antere di agrumi è stata ottenuta embriogenesi somatica nella varietà Moro (2x), portando alla rigenerazione di embrioni che dopo le analisi della ploidia e molecolari sono risultati tetraploidi eterozigoti; nei genotipi Marisol (4x), Clemenules (4x), Moro Los Valles (4x), Sanguinelli (4x) e Sanguinelli (2), è stata osservata divisione simmetrica del nucleo, strutture multinucleate e la formazione di embrioni che in seguito all'analisi citofluorimetrica sono risultati per la maggior parte diploidi. L'analisi molecolare, effettuata con i marcatori SSR invece, ha rilevato risultati singolari, infatti, nei genotipi Marisol, Clemenules e Sanguinelli (2x e 4x), anche se sono state osservate microspore multinucleate e la maggior parte dei rigenerati erano diploidi, dall'analisi sono risultati tutti eterozigoti, mentre in Moro Los Valles, i

marcatori hanno rilevato che dei diploidi rigenerati tre erano omozigoti, due omozigoti mutati (variabilità gametoclonale) e due eterozigoti mutati.

La tecnologia del seme sintetico è stata applicata alla varietà Houmairi di *Ficus carica* L., in particolare, è stato valutato l'effetto di due Regolatori di crescita (PGR): 6-benzilaminopurine (BAP) e meta-Topolina (mT), aggiunti all'endosperma artificiale, e della conservazione a 4° C per 30 giorni delle microtalee incapsulate. La più alta percentuale di semi vitali e che hanno portato ad una maggiore ripresa vegetativa si è avuta nella tesi che non ha previsto conservazione a freddo prima della semina e quando l'endosperma artificiale veniva addizionato con il BAP. In maniera diversa, la percentuale maggiore di conversione si è avuta, sia per i semi conservati che non quando l'endosperma veniva addizionato con mT.

La propagazione *in vitro*, è stata applicata anche, alla varietà Cascade di *Humulus lupulus* L. con l'obiettivo di realizzare un protocollo efficiente di micropropagazione.

Partendo dal prelievo in campo del materiale vegetale, si è proceduto alla sterilizzazione, stabilizzazione della coltura asettica nonché alla moltiplicazione, radicazione e acclimatazione alle condizioni *ex vitro*. Per la moltiplicazione sono stati testati 3 PGR: BAP, mT e Thidiazuron (TDZ) in diverse combinazioni, mentre per la radicazioni 2 PGR auxinici: Acido indol-3-acetico (IAA) e Acido indol-3-butirrico (IBA). L'interazione TDZ/BAP ha mostrato il più alto tasso di germogliamento anche se con un maggiore sviluppo di callo tale da considerare il mezzo con 2mg L^{-1} di TDZ il migliore nonostante dia un germogliamento inferiore così come per la presenza di callo. Per quanto riguarda la maggiore percentuale di radicazione si è avuta nel mezzo contenente 2 mg L^{-1} di IBA anche se differiva per pochi punti percentuali dal mezzo che conteneva 1 mg L^{-1} di IAA.

Infine, è stata valutata l'influenza del rivestimento di alginato di calcio e di tre diversi regolatori di crescita (PGR): 6-benzilaminopurina (BAP), meta-topolina (mT) e zeatina (ZEA), sulla vitalità, la ricrescita e la conversione dei propaguli. di due genotipi siciliani di *Capparis spinosa* (L.).

Le microtalee di cappero sono state sezionate e collocate in diversi endospermi artificiali a base di sali e vitamine Murashige e Skoog, arricchiti di mT o ZEA o BAP. I semi sintetici ottenuti sono stati seminati su un mezzo MS addizionato con 0,4 mg / L^{-1} di acido acetico naftalenico e 0,7 mg / L^{-1} di acido gibellerico. Dopo 60 giorni, sono stati rilevati i seguenti parametri: vitalità, ricrescita, numero e lunghezza dei germogli e delle radici, conversione.

I risultati confermano che l'incapsulamento non ha influito negativamente sulla vitalità, che ha mostrato la percentuale più alta con BAP in Tracino e con ZEA in Scauri. Risultati simili sono stati ottenuti nella ricrescita, con differenze statisticamente significative tra i tre PGR testati: Tracino ha mostrato la migliore ricrescita in capsule arricchite con BAP, Scauri con ZEA. Inoltre, la conversione dei semi sintetici è stata fortemente influenzata dalla PGR ed era più elevata nell'endosperma artificiale addizionato con BAP in Tracino e ZEA in Scauri.

INTRODUCTION

The genetic improvement of fruit crops conducted with traditional methods takes time and is subject to limiting factors. The cultivation of anthers is a biotechnological method that can provide in a single step, haploid plants, and in vitro micropropagation can provide, with suitable precautions, healthy plants, genetically identical to the plant mother, helping, in this way breeders and nurserymen.

The purpose of the *in vitro* culture of anthers and isolated microspores is to obtain plants with halved chromosome contents. In order to move the development of the microspore from the gametophytic to the sporophytic path it is necessary to take into account some factors such as: genotype, physiological state of the donor plants, stage of development of the microspore, composition of the culture medium, physical and chemical stresses, pre-treatments and environmental conditions used during *in vitro* culture.

In the experiments, *in vitro* culture of anthers of various citrus genotypes, , which possessed uninucleated/vacuolated microspores, a stage that responds to gametic embryogenesis, an important role has been given to thermal stress. In fact, it is known that without stress, the microspores follow the normal gametophytic pathway and form pollen (Touraev et al. 1997). Stress can be applied to donor plants or to microspores, for convenience and efficacy of the embryoggenic process, the stress in the experiments was applied as a thermal pre-treatment at -4 ° C to the flower buds, and later, the anthers already placed in culture, were subjected to -4 ° C, -20 °C and + 35 °C. The whole embryogenic process was monitored by staining with 4', 6 diamidino-2 phenylindole (DAPI) and observation under fluorescence microscopy.

The second line of research was the use of *in vitro* micropropagation as a method of multiplication, of species such as hops, and of conservation, through the technique of encapsulation, of fig.

The technique of micropropagation applied to hop, has been the multiplication starting from nodal segments equipped with axillary buds, as it is the most suitable method to guarantee the genetic stability of the plants obtained (Martins et al. 2004). The aim of this research was to evaluate the response of hops to culture medium supplemented with different concentrations of PGR and types of PGR.

Moreover, in this investigation, it was been studied if different PGRs exercise a synergistic function when placed in a culture medium.

With the encapsulation technique, the effect of two different PGRs was evaluated: BAP and mT, on the vitality, regrowth, conversion, length of the shoots and roots of encapsulated nodal segments of Houmairi, variety of *Ficus carica* L.

The tests included the sowing and the direct cultivation of the synthetic seeds in an air-conditioned cabinet and the preservation of the encapsulated nodal segments, at 4 ° C for 30 days and then the subsequent sowing and cultivation in an air-conditioned cabinet for the short-term conservation of this material.

1. Biotechnology in genetic improvement

Today, the main objectives of plant cultivation must have as their purpose, the production of foods with better organoleptic characteristics, have a low environmental impact and be able to satisfy the constant growth of the population. Alongside traditional methods of plant breeding, such as selection, hybridization, mutation, etc., biotechnological methods such as genetic engineering, early selection through molecular analysis, the production of haploid plants, etc. are increasingly affirming. The term biotechnology, was coined at the beginning of the 70s with the discovery of the restriction endonuclease, an enzyme, which allows, by cutting the DNA molecule in some very precise points, the removal of the genes of interest and the subsequent insertion into other portions of DNA (Trigiano and Gray 2003).

Since then, plant biotechnology has come a long way, to the point that, today, traditional plant improvement programs are accompanied by biotechnological methods. Therefore biotechnologies are not an alternative to traditional methods but they allow to speed up the processes to obtain and release new varieties, furthermore, they allow to preserve the genetic variability, raw material, which used and combined in the right way can produce new varieties and/or better, obtaining homozygous plants in a single generation, through androgenesis and gynogenesis, facilitating cross-breeding programs (embryo rescue, *in vitro* fertilization, etc.), makes it possible to evaluate the expression of the mostly quantitative characters in a precise manner, through molecular analysis, to keep new sprouts and rootstocks selected for resistance to biotic and abiotic stresses (Germanà 1997, 2006) and through micropropagation it is possible to obtain a high number of individuals with appropriate precautions from the phytopathological point of view and in unp small space. Therefore, both in fruit plants and in woody ones, biotechnologies provide powerful tools for plant breeding.

2. The importance of haploids (Hs) and double haploids (DHs) in plant breeding

The haploid plants (Hs) are sporophytes with the gametic number of chromosomes (n instead of 2n). When there is the spontaneous or induced duplication of the haploid chromosome, the resulting plant is called doubled haploid (DH). The interest of growers in haploids and doubled haploids, is based, on the possibility of obtaining in a single generation, homozygosity especially in woody plants that are characterized by a long reproductive cycle, a high degree of heterozygosity, large sizes and sometimes by self- incompatibility (Germanà 2006, 2011a). It has been seen that in annual plants, through six cycles of self-pollination, only a partial homozygosity is obtained, while by haploid technology only one passage is necessary (Germanà 2011b). Hs plants arouse interest not only in genetic studies but sometimes in cultivation. Haploid technology is

important for its potential use in mutation research, selection, genetic analysis, transformation and production of homozygous cultivars (Germanà 2010, 2011a). Nowadays, several genome sequencing programs are using haploid genome because of its simplified assembly, such as many fruit crops like peach, pear, apple and citrus (Dunwell 2010).

Moreover, Hs plants can be used as parental in the fusion of protoplasts with diploid plants in order to obtain triploids, particularly important in *Citrus* as they are seedless. DH play an important role in genome mapping as they can provide information on the location of economically important genes, overcome inbreeding depression problems in cross-pollinated populations, represent parents for the production of F1 hybrid seed of vegetable crops showing a heterosis and therefore a notable increase in yields (Maluszynski et al. 2001).

To exploit the advantages of Hs and DH plants, efficient and economic protocols for their production are necessary. Unfortunaletely, there are no universal protocols suitable for all species, especially for woody plants that are recalcitrant to this technology.

2.1 Status of art of Hs and Dhs in plant breeding

The renewed interest of breeders in haploid plants was registered around 2009-10, when there was a vast number of patents granted and patent applications (Dunwell 2009).

The site www.scri.sari.ac.uk/assac/COST851/default.htm provides a complete list of new varieties obtained with haploid technologies. As far as tree species are concerned, species in which the induction frequency is still low and is highly genotype-dependent, until now gametic embryogenesis has been mainly obtained starting from anthers or microspores isolated in: *Actinidia deliciosa* (Chalak and Legave 1997), *Annona squamosa* L. (Nair et al. 1983), *Cassia fistula*, *Jacaranda acutifolia* and *Poinciana regia* (Bajaj and Dhanju 1983), *Carica papaya* L. (Litz and Conover 1978; Tsay and Su 1985; Rimbeira et al. 2005), *Cocos nucifera* (Thanh-Tuyen and De Guzmán 1983), *Quercus petraea* and *Fagus silvatica* (Jörgensen 1988), *Aesculus carnea* (Radojevic et al. 1989), *Hevea brasiliensis* (Chen 1990), *Populus* spp. (Baldursson et al. 1993), *Coffea arabica* (Ascanio and Asdrúbal 1994; Carneiro 1993), *Quercus suber* (Bueno et al. 1997), *Clausena excavata* (Froelicher and Ollitrault 2000), *Phoenix dactylifera* (Chaibi et al. 2002), *Malus domestica* (Höfer 1994; 1995; 2002; 2004), *Musa balbisiana* (Dennis Thomas et al. 1999) (Assani et al. 2003; Kerbellec 1996), *Olea europaea* (Bueno et al. 2004, 2005, 2006; Salis et al. 2008; Chiancone et al. 2010), *Prunus armeniaca* (Peixe et al. 2004), *Prunus avium* L. (Seirlis et al. 1979; Höfer and Hanke 1990; Germanà et al. 2011), *Prunus persica* (L.) Batsch (Michellon et al. 1974; Seirlis et al. 1979; Hammerschlag 1983), *Pyrus collumunis* L. (Bauvier et al. 2002), *Pyrus pyrifolia* Nakai (Kadota and Niimi 2004) and *Vitis vinifera* L. (Gresshoff and Day 1974; Kim and Peak 1981; Cersosino 1986).

While haploid plants are citrus and related species, they have been obtained in:

Poncirus trifoliata L. Raf. (Hidaka et al. 1979; Deng et al. 1992), *C. madurensis* Lour. (Chen et al. 1980.), in the hybrid *C. ichangensis x C. reticulata* (Deng et al. 1992), *C. aurantium* (Hidaka et al. 1982; Germanà unpublished), *C. aurantifolia* (Chaturvedi and Sharma 1985), *C. reticulata* (Germanà et al. 1994), *C. clementina* Hort. ex Tan. (Germanà et al. 1994, 2000a, 2005. Germanà and Chiancone 2003), "Mapo tangelo" (*C. deliciosa* x *C. paradisi*) (Germanà and Reforgiato 1997); *C. limon* Burm. L. (Germanà et al. 1991), *Clausena excavata* (Froelicher and Ollitrault 2000), *C. sinensis*, (Hidaka 1984b; Cao et al. 2011; Cardoso et al. 2015).

In addition to haploids and double haploids, the culture of anthers has also succeeded in regenerating tri-haploids in clementine (Germanà et al. 2005).

2.2 Methods to obtain Hs and DHs

Since 1970, numerous studies have been carried out to obtain haploids for the genetic improvement of tree species through gametic embryogenesis (Chen 1986; Ochatt and Zhang 1996), although, as reported by Ochatt and Zhang (1996), they are not always fully satisfactory results have been obtained. Haploids can be obtained fundamentally by four methods: through spontaneous development, crossing between genetically distant species, regeneration from the female gamete and from the male gamete.

- **Spontaneous development**

Spontaneous development has been reported in more than 100 species of angiosperms. In particular, in fruit trees, spontaneous haploids have been found in apple, pear, peach, plum, apricot, citrus, etc. Since the frequency of obtaining is very low, this method cannot be used in genetic improvement (Zhang et al. 1990). The origin of spontaneous haploids may be due to parthenogenesis (the development of the embryo without the participation of the male gamete) or due to apogamy (the production of the embryo from a cell other than the egg cell).

- **Crossing between genetically distant species**

In some intersections, interspecific or intergeneric, the formation of the hybrid embryo is followed by the elimination of the paternal chromosomes. This technique is used mainly for cereals. In particular, in these hybrids, the endosperm is either not formed or is poorly developed and therefore *in vitro* culture is necessary to save the haploid embryo.

Intergeneric crosses are also applied in the potato where there is the parthenogenic induction of the egg cell.

- **Regeneration from the female gamete**

The method of gynogenesis has spread especially in those species that were recalcitrant to microspore embryogenesis. The method was developed in the late 1980s for some wheat and barley genotypes. It has also been successful in the potato, beetroot, onion and cucumber.

Gynogenesis by *in situ* pollination with irradiated pollen was also advantageous in: *Malus domestica* L. Borkh (Zhang and Lespinasse 1991; Höfer and Lespinasse 1996), *Pyrus communis* L. (Bouvier et al. 1993), *Actinidia deliciosa* (Pandey et al. 1990, Chalak and Legave 1996) and *Citrus clementina* Hort. ex Tan. cv. SRA 63 (Ollitrault et al. 1996). This method is based on pollination with pollen irradiated with gamma rays and it will be followed by *in vitro* culture of immature seeds or embryos. The selection of an effective radiation dose, the optimization of the pollination method, the time of seed collection, the development stage and the culture media are the necessary conditions for the success and the achievement of an adequate number of haploids.

In vitro culture of ovaries was also used for the formation of embryos from unfertilized ovules(Keller and Korzun 1996). This technique, to obtain haploids, has been used both in gymnosperms and in angiosperms that do not respond to the culture of anthers (Yang and Zhou 1982). A typical example was *Gerbera* spp., in fact in this species, almost 100% of the plants regenerated by the culture of unpollinated ovules turned out to be haploid (Meynet and Sibi 1984; Cappadocia et al. 1988; Tosca et al. 1990; Honkanen et al. 1991; Germanà 2006).

- **Regeneration from the male gamete**

Pollen embryogenesis, sometimes improperly called "androgenesis", is the most efficient technique for obtaining haploid or double haploid plants by exploiting the phenomenon of totipotency that also characterizes microspores.

The methods for *in vitro* regeneration of microspores are: the culture of anthers and the culture of isolated microspores. Regeneration from the male gamete has been described in more than 200 species, belonging to the family of Solanaceae, Cruciferee and Grasses ((Dunwell 1986; Hu and Yang 1986). Some leguminous plants and several tree plants, however, are still recalcitrant to this technique (Sangwan-Norrel et al. 1986; Bajaj 1990; Raghavan 1990 and Wenzel et al. 1995).

The cellular, biochemical and molecular bases that induce microspores to turn into embryos are not yet clear, but it seems that the ability to respond to this technique is inherited as well as the development stage of the microspores is of fundamental importance for induction. Usually during the first mitotic division (microspores at the uninucleated stage or at the beginning of bicellular pollen), the male gametes become competent and divide with a continuous growth differently from the gametophytic pathway. Furthermore, various stresses (physical or chemical) can be applied to the microspores in order to promote sporophytic development.

The induced microspores are characterized by an altered synthesis and an accumulation of RNA and proteins and it seems that the genes involved in this reprogramming are linked to various stresses and / or are associated with zygote embryogenesis.

Two *in vitro* phases are essentially required for this technology:

- Induction of the sporophytic phase; often preceded by pre-treatments to plants or flowers or anthers;
- Regeneration of haploid or double-haploid plants; sometimes followed by *in vitro* rooting and then by acclimatization of the seedlings to *ex-vitro* conditions.

In many species the duplication of the haploid chromosome occurs spontaneously and with a sufficiently high speed for practical purposes, in most cases, the duplication is induced through the use of chemical substances.

The development of the gametic embryo from a microspore can occur either directly or indirectly. In the first case the microspore behaves like a zygote and passes through all the typical stages of zygotic embryogenesis: globular, heart, torpedo and cotyledonary. During the globular stage, the embryos exit from the exine and continue their development up to the cotyledonary stage, and finally prepare themselves for escape from the anther. In the second case, the microspore is divided several times, generating a mass of cells that forms the callus that also emerges from the anther. Callus development appears to be due to the richness of the culture medium or the lack of polarity of the microspore. Callus can also give rise to organogenesis or embryogenesis (Reinert et al. 1977).

Often both direct and indirect embryogenesis coexist in the same crop and their proportions are due to the genotype and the cultivation conditions used.

As already mentioned, pollen embryogenesis can be performed *in vitro* or by culture of anthers or by cultivation of isolated microspores.

The cultivation of anthers has been applied to different tree species (Ochatt and Zhang 1996). The technique involves the collection of flower buds that are still closed, with anthers contain microspores at the uninucleated stage, which is the most competent for embryogenesis. Subsequently, the buds are sterilized and the anthers cultured on a medium that can be solid or liquid , under aseptic conditions. For the various species, different protocols are used with different culture media.

The culture of isolated microspores is performed by removing the somatic tissue from the anthers. It is a more tiring and laborious technique, but it turned out to be ideal for studying the mechanisms of embryogenesis because eliminating the somatic tissue of the anthers allows greater control during the process.

This technique has been applied to a few fruit species including apple trees (Oldani 1993; Hofer et al. 1999; Hofer 2004), *citrus* fruits (Germana` et al. 1996) and olives (Bueno et al. 2004). Research is underway on different genotypes, such as prickly pear, cherry, olive, medlar, etc. (Germanà et.al. 2000a). Subsequent

research, in particular on the genus *Citrus*, has shown that isolated microspores have produced multinucleated structures that have developed into small embryos that have not developed further. In the genotypes (clementines and sour orange) that had developed embryos following anther culture, the formation of white or green structures, called "pseudo bulbilli", which have already been described by Button and Kochba (1977), was also observed.

Höfer et al. (1999) reported for the first time the induction of embryogenesis and the formation of plants from microspores isolated from the apple cultivar “Renè”, and in a subsequent research, the improvement of the induction phase was reported, using the in-depth pretreatment studies, carbon source concentration and microspore density (Höfer 2004).

Finally Bueno et al. (2004) obtained sporophytic division, multinucleated microspores and multicellular structures with the cultivation of isolated olive microspores in the Arbequina and Picual cultivars.

2.3 The factors influencing gametic embryogenesis

The response of the microspores to embryogenetic induction is conditioned by several factors, both endogenous and exogenous, such as: genotype, physiological state and rowth conditions of the donor plants, pollen development phase, pre-treatment, medium composition and culture conditions.

These factors, besides acting individually, also interact with each other.

- **Genotype**

Many researchers working on pollen embryogenesis attribute a very important role to the genotype. In fact, it has been reported several times that within the same species, the different cultivars have different responses to the cultivation of anthers. Genotype, developmental stage, and culture conditions are the most important factors affecting the microspore developmental fate (Seguı`-Simarro and Nuez 2008a, b).

Research conducted simultaneously on anthers of a large number of different citrus genotypes (4 cultivars of clementines, 2 of mandarins, 4 of sweet orange, 4 of bitter orange, 5 of lemon, 4 of grapefruit), using the same culture conditions, the same pre-treatment and eleven culture media led to the discovery that haploid callus developed only in a cultivar of clementine and in a lemon (Germanà 2007). It appears that the formation of the competent microspores is conditioned by the interaction between cytoplasmic and nuclear genes modified by the environment (Heberle-Bors 1995) and that the ability to undergo embryogenesis, on the part of the microspora, results in an inheritable trait controlled by recessive genes.

- **Physiological state and the growth conditions of the donor plants**

  The physiological conditions of the starting material, the endogenous levels of hormones and the phytosanitary state of the anther tissue, determine the success of gametic embryogenesis. Even if the ideal conditions, so that gametic embryos can develop, vary within the species and in the different genotypes. The intensity of the light, the duration of the photoperiod, the temperature and the humidity are all affecting factors, as well as the various stresses and the various infections to which donor plants may be affected.

  Vasil (1980) observed that anthers taken from plants grown in the field gave a better response than those taken from plants grown in greenhouses; instead Dunwell (1981) showed that both the photoperiod and the intensity of the light influence the yield in embryos.

  Even the age of the plant material is very afffecting. In fact, as has been demonstrated for barley, the younger inflorescences showed a greater embryogenic capacity than the older inflorescences (Powell 1990; Chupeau et al. 1998). Marinkovic and Radojevic (1992), studying the species *Aesculus carnea*, noted that the best embryogenic response was given by the anthers taken from trees between 60 and 100 years old compared to the anthers taken from trees with an age included between 20 and 40 years.

  From these studies, it appears obvious that the conditions of the donor plants from which to take the starting material cannot be standardized, but it is necessary that for each species the age of sampling, the cultural conditions, the phytosanitary state, etc. are determined.

- **Pollen development stage**

  This factor strongly influences the success of pollen embryogenesis. It has been observed that, in general, the period of sensitivity to inductive treatments is around the first mitotic division of the pollen, that is, between the beginning of the vacuolated microspora phase up to the bicellular pollen phase (Raghavan 1986; Pechan and Keller 1988; Smýkal 2000; Telmer et al. 1993; Touraev et al. 1996b; Gonzalez-Melendi et al. 1995 and 1996 a,b). In the phases in which pollen grains begin to accumulate reserves, they usually lose their embryogenic capacity and follow the gametophytic pathway (Heberle-Bors 1985; Raghavan 1990). However, from some experiments, it has been observed that applying various stresses (thermal or chemical) it is possible to reprogram the microspores and obtain embryogenic induction.

  The stage of pollen development, for observation by light microscope, is usually carried out with acetocarmine (1% acetocarmine and 45% acetic acid) or with the fluorescent DAPI (Germanà 2011; Hidaka et al. 1979, 1981; Chen 1985; Vasil 1967; Shull and Menzel 1977).

- **Pre-treatment**

In some plant species, the microspores possess an intrinsic embryogenic potential (Guha and Maheshwari 1964; Chuang et al. 1978; Heberle-Bors 1985; Raghavan 1986; Ziauddin et al. 1990; Zhou et al. 1991), but, without pre-treatments, the frequency of spontaneous embryogenesis is very low. Therefore, to have an efficient production of haploids, the microspores must be subjected to different pre-treatments (Touraev et al. 1996a, 2001; Reynolds 1997; Zheng et al. 2001).

These can be performed both *in vivo* and *in vitro* and applied either to flower buds, or to buds or anthers. Recently Shariatpanahi et al. (2006), classified them into three categories: widely used, little used and new. Another method, classifies pre-treatments as: thermal, chemical, physiological and physical.

- *Thermal pre-treatments:* thermal shock is considered the most effective and more simple method to induce embryogenesis in microspores (Smýkal 2000). Nitsch and Norreel (1973) were the first to suggest the efficacy of thermal shock, obtained at low temperatures, for the purpose of embryogenesis from isolated microspores of *Datura innoxia* which, under such conditions, undergo an alteration of the normal polar division in the first haploid mitosis. Similar results were also obtained from Rashid and Reinert (1983) in tobacco and from Huang and Sunderland (1982) in barley. Cold pre-treatment (4 °C for 2 - 3 weeks) is used routinely in the cultivation of anthers of many cultures, and it has been observed that its effect is genotype-dependent. In other species, high temperature thermal shock was more effective as reported by Gonzales-Melendi et al. 1995 and 1996; Sapute et al. 2005; Bueno et al. 2003 and 2006. In fact, in different species of *Brassica*, short periods at a temperature of 30-35ºC before cultivation at 25ºC, are essential for inducing embryogenesis from isolated microspores.

- *Chemical pre-treatments*: it has been seen that ethanol and 2-hydroxynicotinic acid (2-HNA) can induce embryogenesis (Zheng et al. 2003).

- *Physiological pre-treatments*: represent physiological stress, which can induce embryogenesis:ack of water, air, saturation of the environment with high humidity, but also the use of different media with different concentrations and types of sources of sugar and nitrogen (starvation treatment). In tobacco, (as well as in other species and from studies carried out by other researchers), Kyo and Harada (1985 and 1986), obtained a high embryogenesis by raising the microspores for three days in a medium poor in sucrose and subsequently transferring them into a medium rich in carbon and nitrogen.

- *Physical pre-treatments*: those used result in irradiation with γ rays, carried out in some species of brassicace by Pecham and Keller (1988), centrifugation and lowering of atmospheric pressure.

Although it is still not clear how these stresses work, they seem to alter the polarity of the mitotic division, allow a cytoskeleton reorganization, delay and modify the pollen-mitosis, blocking starch production or dissolving microtubules or maintaining the viability of the pollen grains in culture.

- **Composition of medium**

  For the purposes of embryogenetic induction, the composition of the culture medium is extremely important. The different genotypes show, in fact, different requirements in the composition, which is simpler for the cultivation of anthers than the culture of isolated microspores. The most commonly used basic medium for the cultivation of anthers are: N6 (Chu 1978), MS (modified) (Murashige and Skoog 1962), Nitsch and Nitsch (1969) and B5 (Gamborg et al. 1968), but many others are also used. The main sugars used for the cultivation of anthers or microspores isolated, *in vitro*, are: sucrose, maltose, glucose, galactose and lactose. The influence of different carbohydrates has been tested on anther culture of *C. clementina* and *C. reticulata* (Germanà et al. 1994).

  In addition to the various carbon sources present in the various media an important role is played, for embryogenetic purposes, by growth regulators (auxins, cytokinins or a combination of these), especially for those recalcitrant species, and by other substances, such as glutamine, casein, proline, biotin, myo-inositol,, coconut water, silver nitrate (ethylene antagonist) etc. Furthermore, as reported by various authors (Bruins et al. 1996; Puolimatka et al. 1996; Hu and Kasha 1997; Devreaux and Li 2001; Zheng et al. 2001, 2002; Liu et al. 2002), it is also important to add ovaries to the culture medium. It seems that these release some regular growths that accelerate and maintain the rate of microspore cell division (Puolimatka et al. 1996; Hu and Kasha 1997; Zheng et al. 2002). PH is another factor that can influence gametic embryogenesis; the range is adjusted, before autoclaving, to 5.7 - 5.8. Usually the culture medium for anthers is solidified with agar, or with other solidifying agents, while that for the culture of isolated microspores is liquid.

- **Culture conditions**

  The anthers are usually incubated at 24 - 27 ° C and exposed to an intensity of about 3000 lux for 16 hours a day (Reinert and Bajaj 1997), but other conditions have also been reported. In fact, it is appropriate to determine these parameters for each individual species. In other tests, the absence of light was also indispensable at the end of the induction as well as the alternation of light and dark, as reported in: *Hyoscyamus niger* (Courduan 1975), *Datura innoxia* (Sopory and Maheshwari 1976), *Nicotine tabacum* (Sunderland 1971) and in *Citrus clementina* Hort. ex Tan. (Germanà et al. 2005b). The composition of the atmosphere and the density both of the microspores and of the anthers inside the culture plates have also been the object of study, because at certain densities, the absorption of nutrients and oxygen is facilitated, and spaces are guaranteed adequate for important cell division for embryogenetic purposes (Ziauddin et al. 1990; Olsen 1991; Hoekstra et al. 1993; Gustafson et al. 1995).

2.4 Characterization of Hs and DHs

The first step, to characterize the regenerates obtained from the *in vitro* anthers culture and isolated microspores, is to detect their ploidy level. There are several approaches used for chromosome counting: there are methods that do not require expensive equipment such as the count of chloroplasts in the stomatal guard cells or the determination of plastid sizes (Qin and Rotino 1995) or there are methods that exploit cytological techniques such as chromosome counts (Maluszynka 2003) or through the use of flow cytometry (Bohamec 2003).

It has been seen, however, that not only haploids and double haploids are obtained with gametic embryogenesis, but also diploids, triploids, tetraploids, pentaploids and hexaploids (D'Amanto 1987; Dunwell 2010). Flow cytometry allows the analysis of a large number of target plants in a shorter period of time (Ochatt 2008), enabling detection of mixoploid regenerants (having cells with different ploidy) and the determination of their proportion, according to position of peaks representing the size of the nuclei. However, with the analysis of ploidy, it is not possible to know the origin of the regenerated in fact, the embryos besides the gametic cells can regenerate from the somatic ones. For this reason, there is a need for analyzes to ascertain 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 to determine the gametic origin of calluses and regenerated plantlets, irrespective of their ploidy (Germanà 2011a).

3. *In vitro* micropropagation

Micropropagation is an *in vitro* vegetative propagation technique which consists in the cultivation of vegetable explants (organs, tissues, cells or protoplasts) on known nutrient substrates, in sterility and in controlled environmental conditions (especially light and temperature). This form of propagation is made possible by the "totipotency" of plant cells: that is, their ability to differentiate and acquire meristematic abilities, giving rise to organs, tissues or even to an entire individual even starting from single cells or from organs and tissues of different departure from the final ones (Ammirato 1985; Donnely and Vidaver 1988; Steward 1997). It is a method of clonal propagation that allows the production and conservation *in vitro* of plants genetically identical to the mother plant. This technique, in addition to being of considerable importance for propagation purposes commercial plant,

representing a valid alternative to the traditional systems of multiplication by seed or cuttings, can also have a strategic role in conservation *ex situ* of plant biodiversity.

The starting material most used for the preparation of aseptic culture is represented mainly by apical gems and axillary buds, but it is possible also use leaves, floral organs, immature embryos and fragments of cotyledons (Paunescu 2009).

The advantages of micropropagation are numerous and include:

• High multiplication rate,

• Independence from seasonal conditions,

• Need for reduced material to start the crop,

• Genetic uniformity, except for somaclonal variability,

• Need for reduced spaces,

• Possibility of viral recovery (meristem culture),

• Conservation of germplasm.

However, this technique may also present disadvantages such as:

• Somaclonal variability,

• Risks of contamination,

• High costs for equipment,

• Need for trained personnel,

• Problems in the transition from *vitro* to *vivo*,

• Difficulty in micropropagating recalcitrant species to *in vitro* culture.

3.1 The stages of the micropropagation

In micropropagation five stages are distinguished:

Stage 0: selection and preparation of the mother plant

The quality of the explant (the type, age, when and how it is taken) and the subsequent response *in vitro* are significantly influenced also by the phytosanitary

and physiological conditions of the mother plant (Debergh and Maene 1981; Read 1988). The size of the explant taken to start the culture *in vitro* can influence the response and possible recovery from pathogenic plant pathogens.

Stage I: implantation of aseptic culture

During this stage, a culture of buds or terminal or lateral meristems is started and stabilized, possibly without pathogens. The harvested explants of mother plants must be sterilized on the external surface. The presence of microbial contamination negatively affects the survival of the shoots, their growth and subsequent proliferation.

Stage II: proliferation of axillary shoots

The stage II is characterized by repeated cycles of proliferation of axillary shoots from apical or lateral shoots, raised in a culture medium containing higher cytokinin levels to inactivate the apical dominance of the terminal bud. In stage II the crops are regularly divided into portions of smaller shoots, single apical shoots or nodal segments, which are used as propagules for further proliferation.

Stage III: pre-transplantation (rooting)

This stage consists of preparing the sprouts obtained in stage II to the soil transfer phase. The process may include: 1) lengthening of the shoots before rooting; 2) rooting of single shoots or groups of shoots; 3) satisfaction by cold treatment of the dormancy of the reserve organs; 4) "pre-hardening" of crops to reduce water shocks and increase their survival. There are many reasons for the elimination of this stage. The cost of stage III is estimated at 35 to 75% of the total production cost of the micropropagated plant. The elimination of stage III would therefore result in considerable savings. Furthermore, the *in vitro* root system is often largely non-functional and destined to die after transplantation.

Stage IV: transfer to the external environment

The success of the cultivation of sprouts or nodal segments depends on the ability to transfer the seedlings from the containers to the air-conditioning greenhouses and to re-establish their active growth. This implies acclimatizing or preparing the seedlings in conditions of significantly lower relative humidity and higher luminous intensity. However, even when the acclimatization procedure is applied carefully, it is not uncommon to find a low survival rate of the seedlings. The difficulties that micropropagated plants pose at the time of transplantation are mainly due to two causes:

1) the nutrition heterotrophic system

2) the low level of water self-regulation.

Plants grown *in vitro* in the presence of carbon sources, in conditions of low brightness and reduced gas exchange, show a limited or almost no photosynthetic capacity. During acclimatization, plants need to pass quickly from a heterotrophic

state to an autotrophic state (Preece and Sutter 1991). Unfortunately, this step is not very fast. To overcome these limitations, the plants are transplanted into a sterile and well-drained substrate and maintained in conditions of high relative humidity, reduced light intensity and a temperature between 20 and 27 °C.

The plants obtained by micropropagation must be carefully followed until they are marketed and used. This therefore entails problems linked, for example, to the transport of plants that must be protected from possible damage so as not to compromise their integrity and health. All this seems to place limits, above all economic, on the use of this type of propagating material in comparison to the use of gametes, which contain in a single product the advantage of the smallest dimensions, the best handling, the easiest transportability and of the easier preservation (Standardi and Micheli 2010a). The encapsulation technology allows to combine some advantages of *in vitro* multiplication (high production efficiency, material homogeneity, rapidity of the propagation cycle), with the ease of handling, the possibility of storage and the simplification of the operations connected to it, typical of the gamic propagation (Standardi et al. 1998).

In vitro propagation protocols have been developed for several hundred species (Geoge 1996), including numerous rare and endangered species (Fay 1992; Sarasan et al. 2006).

3.2 *In vitro* propagation via synthetic seed

The term "synthetic seed" dates back to 1977 when, during the Symposium on Tissue Culture for Horticultural Purposes, held in Ghent (Belgium), Murashige suggested the possibility of using somatic embryogenesis to obtain bipolar vegetative propagules (somatic embryos or embryoids), functionally similar to zygotic embryos. Furthermore, in 1978 he introduced the concept of "synthetic seed", calling it "a single encapsulated somatic embryo capable of maintaining the ability to evolve into a seedling".

This definition has undergone some changes over the years. For example, Bapat et al. in 1987, proposed the production of synthetic seeds also through the encapsulation of other *vitro*-derived propagules as well as somatic embryos such as microbulbs, microtubes, rhizomes, meristemoids, tissue or organ fragments. Subsequently, in 1995, Aitken-Christie and collaborators proposed to define it “a structure consisting of a somatic embryo, bud or other tissue that, covered with an artificial protective capsule, can be used for sowing *in vitro* or *ex vitro* conditions", thus extending the concept of synthetic seed to any *vitro*-derived propagule. In recent years, the possibility of using even small portions of micropropagated shoots of 2-4 mm in length, with apical or axillary (microcutting) buds, is arousing great interest (Micheli et al. 2007). Next to the synthetic seed itself, can therefore place, as a further product of encapsulation technology, the "capsule" properly so called which can be defined as "a portion of

vitro-derived plant tissue that can be used for micropropagation after storage and / o transport"(Micheli and Standardi 2009).

To make the synthetic seeds, or the capsules, the procedure is identical and uses three phases: coating, complexation and washing.

The coating phase consists of the immersion of the propagule, taken from culture *in vitro*, in a solution containing a natural polysaccharide that gives a viscous consistency able to "trap" the propagule. The substance most commonly used in this phase is sodium alginate. It has many advantages including the absence of phytotoxicity, excellent tendency to gelation, simplicity of use and low cost (Barbotin et al. 1993). Moreover, unlike other polysaccharides or other gelling substances such as polyacrylamide, it does not need heat treatment to allow a homogeneous distribution of the molecule in the solution (and therefore around the propagule), a step that, by causing the release of phytotoxic radicals, could compromise the viability of the explant itself (Sakamoto et al. 1995).

The complexation occurs with the passage of the "drop" of alginate containing the propagule in a complexing solution, where the hardening of the capsule occurs through an ion exchange reaction between the sodium present in the coating solution and a bivalent ion present in the solution of complexation. The ion most commonly used in this step is the Ca^{++} ion and therefore a CaCl solution is used (Sakamoto et al. 1995).

During the complexation, calcium replaces sodium and gives rise to ionic bonds with carboxyl groups of some polymeric chains of alginate: it is realized, so a network whose structure is called "egg-box", for the numerous cavities in which calcium ions are arranged.

The capsules are left immersed in the complexing solution for a time interval ranging from 20 to 40 minutes. The complexation times and the concentrations of sodium alginate and calcium chloride greatly affect the hardness of the capsule: a greater consistency of the capsule corresponds to a greater protection from mechanical damage (protective function), but at the same time a greater difficulty of the sprout in escaping from the capsule. The third and final step consists in a repeated washing in sterile endosperm to remove the ionic residues.

After washing, the capsules can be stored or cultured to start new micropropagation cycles. After a certain interval from the cultivation, three fundamental parameters must be observed:

• vitality (% of green-looking explants, without necrosis or yellowing);

• vegetative restart (regrowth) (% of encapsulated microtalee producing shoots longer than 4 mm);

• conversion (emergence of shoots and roots at least 4 mm long from the encapsulated microcuttigs).

To allow this development, the two solutions used for coating and complexation, in addition to rinsing water, are enriched with nutrients and growth regulators (Capuano et al. 1998). The composition of the nutrient solution is generally similar to that used for the *in vitro* proliferation phase. To resume the analogy with the naturally occurring seed, this solution is called "artificial endosperm" and provides nourishment to the propagule in the storage and recovery phases (trophic function) (Carlson and Hartle, 1995; Gardi et al. 1999).

Many research groups are working on the encapsulation of somatic embryos of different species including cereals, fruit trees, vegetable species, ornamental plants, medicinal plants, and forestry species. At present, efficient protocols are available for producing and encapsulating somatic embryos of alfalfa, cabbage, carrot, celery, tomato, lettuce, cotton, wheat, pine, rice, soy, watermelon, citrus fruits, which may be of support for the commercial application in nurseries, especially when used for the diffusion of new or valuable genotypes (Bapat and Rao 1988; Suehara et al. 1995; Onay et al. 1996; Akhtar 1997; Castillo et al. 1998; Ara et al. 1999; Germanà et al. 1999, 2007; Wu et al. 2003; Das et al. 2006; Cangahuala-Inocente et al. 2007; Singh et al. 2007; Aquea et al. 2008; Pintos et al. 2008; Rai and Jaiswal 2008; Rai et al. 2008; Sharma et al. 2013; Teixeira da Silva 2012; Utomo et al. 2008; Cartes et al. 2009).

4. The studied genotypes

4.1 *Citrus* spp.

Citrus fruits are the most cultivated fruits in the world, cultivated in more than 80 countries (Chang 1992). They cover a worldwide area of 9.275.925 ha (2017) (FAOSTAT, 2019). The center of origin is considered to be South East Asia, China, the north east of India and the limtrofe areas.

Belong to the *Rutaceae* family which includes about 160 genera. The number of chromosomes is 2n = 18. Based on morphological descriptors and molecular data, there are three basic taxa: *Citrus maxima* (pummelo), *Citrus medica* (citron) and *Citrus reticulata* (mandarin); the other species have appeared crosses and genetic recombination of these three basic taxa (Ollitrault and Navarro 2012).

In temperate climates, growth and fruiting take place at regular intervals and usually once a year while in tropical environments, these processes can occur continuously.

The root system consists of a main root and lateral secondary roots that develop in all directions. In Mediterranean climates the development of roots alternates temporally with that of the epigeal part. In commercial culture the trunk carries 3-4 main branches on which are inserted secondary and lower-order branches that go to make up the crown that tends to assume a globular shape.The leaves are simple and persistent with the exception of *Poncirus trifoliata* where they are deciduous and trifoliate. This characteristic is a heritable trait and it is also found in its hybrids. The leaves have an elliptical or lanceolate shape wrapped with wings, this last characteristic pronounced in pummelo, grapefruit and bitter orange, less in mandarin and sweet orange.

The buds are found in the axil of the leaves together with a thorn that can be more or less developed based on the vigor or the juvenility of the plant.

The hermaphrodite flowers are carried by the branches of the previous year, they can be single or grouped in corimbiform inflorescences.

The fruit is called esperidio and originates following the growth of the ovary. Many *citrus* species are characterized by the phenomenon of polyembryony (apogamy): a single embryo develops from the fecundation process while the others develop from the diploid tissue of the nucella of maternal origin. This phenomenon has considerable importance for the propagation of the species, being present in most citrus fruits with the exception of pummelo, citron , clementine which are defined as monoembryonic.

A fundamental prerequisite for fruit setting in fruit plants is fertilization with the consequent formation of the seeds, but very frequent in *citrus* fruits is the phenomenon of parthenocarpy, that is the formation of the fruit without fertilization. These parthenocarpic fruits are characterized by the absence of seeds,

requirement required by consumers and which entails a considerable merit for these fruits.

Sweet orange, *Citrus sinensis* (L.) Osbeck, is bred both for the production of fruits for fresh consumption and for processing into juices. This species is a natural hybrid of pummelo and mandarin, and, among citrus fruits worldwide, it is the most cultivated.

Within the sweet orange species, three main groups are found: common blondes (they can have seeds, high juice yield and low limonine values); navel group (no seeds, high limonin values and low juice yield); finally, there is the group of pigmented that have red flesh due to the high presence of anthocyanins and higher levels of vitamin C.

Most of the cultivated varieties of this species derive from spontaneous bud mutations that are very frequent in sweet orange, but great importance was given to the identification of cultivars from nucellar selection, a technique that exploits the phenomenon of polyembryony and which also constitutes a method for the restoration of the species. However, with this route we obtain plants with a long juvenile phase in fact, today, for the remedation the technique of micro-grafting has been applied (Navarro 2007).

From a taxonomic point of view, the mandarin group is the most complex of citrus fruits. The classification of Swingle identifies only one species, *Citrus reticulata*, but is too restrictive.Otherwise, Tanaka, identifies 36 species resulting more respectful of the botanical and morphological features, while Hodgson classifies the mandarins in *C. deliciosa* Tenore, *C. nobilis* Loureiro, *C. reticulata* Blanco, *C. unshiu* Marcowiez and mandarins with small fruit. To these are added the hybrids, both obtained in nature and made up of humans such as tangors (mandarin x orange) and tangeli (mandarin hybrid x pummelo or grapefruit).

4.2 Fig (*Ficus carica* L.)

The genus *Ficus* belongs to the Moraceae family and includes about 800 species spread from the tropics to temperate countries, of which 500 in Australia and Asia and more than 100 in Africa. The most known and cultivated species in the Mediterranean basin is *Ficus carica* L., of which about 315.530 (2017) hectares are cultivated worldwide. The major fig producers are: Turkey, Egypt, Iran, Greece and Algeria (FAOSTAT, 2019). The species is native to western Asia and Mediterranean and has been spread over time to the temperate and subtropical belt of the whole world.

The fig is a tree that can reach considerable dimensions, from 3 to 10 m, depending on the pedoclimatic conditions and the cultivated variety. It has superficial, expanded and ramified roots, capable of colonizing even soils of little thickness, and which have high penetration force, a characteristic that allows them to search for water even at considerable distances. Adventitious roots can be emitted very easily from branches and stems.

The cultivated plants, generally, have expanded crown tending to the globular form, with single stem, while spontaneous or non-cultivated plants take on the appearance of bush due to the numerous suckers that develop from the base of the trunk.

The trunk and the large branches have a thick gray ash bark that tends to green in the young branches.

The leaves are deciduous, with a long petiole and broad lamina, are thick and sclerified, of variable shape in the same plant (pleomorphic).In fact, they can be cordiform (tending to heart-shaped) or variously webbed, with 3-7 lobes and more or less expanded intralobar sinuses. The shape of the leaves and the degree of pleomorphism are varietal characters. In the drier periods, transpiration is limited by the closure of the stomata which are also protected by a thick down, even on the upper side there are short and stiff hairs that are stinging for the human skin.

Branches, leaves and fruits possess cells that secrete, when they cause wounds, abundant whitish exudate called latex.This contains proteins that would be associated with forms of resistance to pathogenic fungi in addition to being repellents and irritants for insects, herbivorous animals and for the same man.

Depending on their location, the buds of the fig tree can be: apical, axillary, or adventitious.

In addition to the buds of the year, there are numerous dormant, less visible or barely visible vegetativebuds. These are found on branches of 2 - 3 years, on large branches and on the trunk.

The flowers are contained in a pear-shaped inflorescence with an inner receptacle provided with an axial distal opening called the ostiol. The domestic fig is devoid of male flowers while the female ones, are constituted by an enlarged club-shaped pedicel in the distal part where the ovary is located from which a long stylus originates.

The fig fruit is a false fruit, called syconium, of a more or less elongated pear-shaped shape with a peduncle attached to the branch and a neck between peduncle and fruit. The syconium has a thin outer skin of various colors covering the whitish skin, under the peel is the receptacle on whose wall are attached numerous juicy vesicles deriving from the female flowers, at the apex of which the ovary is located and to its internal the achene that carries the seed in the case of fertilization. Around the ostiolo are small and narrow protective scales that allow the pollinator to pass through. Therefore, the real fruits are represented by the achenes which, based on their size, finesse and crunchiness, characterize the variety. Generally they are larger and harder if fertilization has occurred, small and fine in non-fertilized cultivars.

In the fig tree there are two types of syconium:

- Fioroni, are pantenocarpic, found on the branches of the previous year, located in the distal and lateral part of the leaf scars but also in the basal part of a fruiting branch of 2 - 3 years;

- Provided, they are brought to the armpit of the leaves of the branches of the year and mature from late July to November depending on the variety.

In addition to the type of syconium , in the fig tree we can distinguish, based on the number of fruiting, varieties:

- "Unifere types" that produce only once or usually florons or supplied;

- "Bifera types " that produce twice, the florons and the supplied

- "Trifere types" that produce three times.

However, that the frequency of fruiting depends essentially on the varietal characteristics, environmental conditions, spontaneous caprification and cultivation techniques.

The pollination of the fig is entomophilous, it is called caprification because the pollen is provided only by the caprificus. Pollen is transported by a specialized hymenoptera, *Blastophaga psenes*, a wasp that hibernates as an egg or larva in caprific mothers, syconia that form in autumn and remain on the plants until the following spring. In spring the larvae colonize the ovaries of the female flowers they eat. In April-May, adult females abandon their mothers and colonize the profichi (syconia that are formed in spring and mature in June) in which they lay their eggs. The larvae feed the ovaries that take on a characteristic gall form.

In June the males leave these females before the females, leaving the exit holes visible, and they go to fertilize the females that are still inside other galls. The males die inside the receptacle, while the fertilized females leave the caprifichies smeared with pollen released by the dehiscent anthers located around the ostium and go to colonize the mammons or supplied in which they will lay the eggs of the next generation.

From what has been said the caprification, in the domestic fig, is not necessary in the florons and in the cultivars that have long sterile female flowers, in fact, the fruiting are pantenocarpic, instead, it is indispensable, in some late-cultivated cultivars spread mostly in environments warm Mediterranean, which provided fertile female long styled flowers that, if not fertilized, lose their fruit.

The varieties that require pollination, to produce supplied, have been grouped into two types, based on the production of fioroni:

- Smyrna, with little or no fioroni;

- San Pietro, with fioroni as main production and supplied of inferior quality.

According to Condit (1955) about 80% of the cultivated varieties are of the pantenocarp type, 16% are of the Smyrna type, while 4% are of the San Pietro type.

4.3 Hop (*Humulus lupulus* L.)

The genus *Humulus* belongs to the Cannabaceae family, together with the genus *Cannabis*. Originally included in the order *Urticales*, they were later merged with the *Rosales* order (Bremer et al. 2003). Three species belong to the genus *Humulus: Humulus lupulus* L., *Humulus japonicus* Siebold and Zucc., known as wild hops, and *Humulus yuannensis* Hu (Small 1978; Neve 1991). It is estimated that in the world there is a surface, planted with hops for a total of 91,881 ha (2017), of which 30,215 ha only in Europe (FAOSTAT, 2019). The world's leading producers of dry hop cones are: Germany and the USA with around 2/3 of world production.

The origin of the genus *Humulus*, to which the species *Humulus lupulus* belongs, is uncertain but it is assumed that the first species of hops appeared in China and that from there they spread eastwards, towards North America and west towards Europe, thus leading to the formation of two distinct populations.

Hops is a herbaceous, perennial, and normally dioecious plant, although some monoecious individuals have been found in some wild populations in North American (Haunold et al. 1993). It prefers fresh environments, fertile, well-worked and slightly acid soils, with a pH between 6 and 6.2. Strongly clay soils cause problems of radical asphyxia and difficulty in the absorption of microelements (Neve 1991). It grows spontaneously in most of the temperate zones of the northern hemisphere, between 30 and 50 degrees of latitude. The epigeal part of the plant perishes every winter, while the hypogean part, called ceppaia, rhizomatous, is perennial and can survive for many years. This species is able to regenerate itself every year, thanks to the numerous buds present in the apical part of the rhizome that, at the beginning of each spring, sprout producing numerous annual stems. These are first herbaceous, then woody and due to their vertical development they need support supports on which to twist.

Hop is a long-day species, which means that the plant differentiates flower buds only in the presence of a photoperiod of at least 16 hours, below which it is not possible to induce differentiation of flower buds (Villacorta et al. 2008). Below a photoperiod of 8-10 h, the plant ceases vegetative growth and tends to form dormant apical buds.

From the cytological point of view, the species Humulus lupulus L., appears to be diploid (2n = 20) with heteromorphic sex chromosomes; female individuals possess XX sex chromosomes, while male XY chromosomes (Danilova et al. 2006).

Based on the morphological characteristics and geographical location, Small (1978) classified the species of Humulus lupulus in five taxonomic varieties: var. lupulus Small (European hops), var. cordifolius Small (Japanese hops), var. neomexicanus Nelson and Cockerell, var. small pubescens and var. Small lupuloides (North American hops).

The permanent root system, very developed, forms two types of roots: those with horizontal development, which propagate around the plant and those with vertical development, which have thicker bark and few branches, performing important functions of water supply and nourishment (Biancardi and Wagner 1989). Hop

plants differentiate both rhizomes and true roots. True roots become woody and do not produce buds. The rhizome, which can be defined as an enlarged stem with a reserve function, tends to grow just below the surface of the soil and differentiates both vegetative and root buds (Neve 1991).

The stem has a hexagonal section, herbaceous at birth and subsequently woody, it is hollow, without tendrils and creeper; it coils, in a left-hand direction, around each available support, using hooked trichomes placed at the corners of the stem. The stem can reach a height of 6 - 9 m. (Krištin 1987; Rybaček 1980).

At each node, opposite leaves develop, initially palmate then palmate lobate, with 3, 5 or 7 lobes on a serrated edge. Rough as covered with hair, short and thin. From the axilla of the leaves, especially in the highest part of the plant, secondary shoots develop, on which subsequently the dioecious inflorescences will be formed starting also from the axillary buds of the leaves (Biancardi and Wagner 1989). The pistillifere inflorescences constitute the part of the plant of highest commercial interest; for this reason only female plants are cultivated while the male inflorescences are used for breeding interventions to constitute new hybrid varieties (Rybaček 1980; Neve 1991). The inflorescences of the hops are typically called cones, they are ovoid in shape and are formed by bracts and bracts of herbaceous consistency, inserted on a central spine. Each bract has two bracts and a variable number of flowers located near the central axis and carrying two stigmas each. The number of bracts present in each cone is selected and is higher in the cultivated varieties, so that the inflorescence is more compact, richer in useful substances and more resistant to mechanical stress. The male flowers are gathered in pendulous panicles and each presents 5 tepals fused at the base and 5 stamens (Biancardi and Wagner 1989).

During maturation, resinous particles of a more or less intense yellow color containing lupulin form in the lower part of the bracts, secreted by appropriate glands (Rybaček 1980). This resin contains the aromatic and bittering substances used in the production of beer. In the presence of pollen the ovary develops the seed (achene). Cone fertilization greatly reduces their technological quality, especially due to the lower lupulin content (Biancardi and Wagner 1989).

Since the species is dioecious, self-pollination is impossible and hop plants are highly heterozygous. This characteristic means that the hop plants derived from seed have extremely variable offspring and therefore do not have commercial value (Neve 1991).

4.4 Caper (*Capparis spinosa* L.)

The genus *Capparis* belongs to the family of the Capparaceae and includes about 250 species (Fici 2001). The populations of the European continent can be traced back to one species, *Capparis spinosa* L. with two subspecies, subsp. *spinosa* and subsp. *rupestris* (Sibth & Sm.) Nyman (Heywood 1993).

The caper grows throughout the Mediterranean and is extremely resistant to drought. Cornelini et al. (2008) in a recent study states that typical plants of the Mediterranean scrub like those of the caper are able to reduce the erosion of the soils in rocky and steep environments and to preserve the water reserves from the soil, thanks to the extensive and deep root system.

The caper has erect branches up to 3 m long, dark green with internodes of 1.5 - 3.5 cm. The leaves are ovate or obovate herbaceous with not prominent veins, the petiole is 0.7 - 1 cm long, the flower buds are carried by a thick floral pedicel 5 - 6.5 cm long, the flowers are zygomorphic with 1.8 - 2.4 cm long sepal and 0.6 - 1.1 cm wide; the stamens are numerous, from 100 to 150, the fruit is oblong with the pulp red with numerous seeds that are brown at maturity; flowering occurs from May to October.

The floral button of the plant, commonly called "caper", following treatments with sea salt and sometimes with vinegar, is used in the preparation of Mediterranean dishes, thanks to its unique aromatic properties. The fruit, of similar taste but more delicate, it is called "cucuncio" or "capperone" and is traditionally used in the Aeolian cuisine. (Legua et al. 2013). The caper, due to its antioxidant, antifungal, anti-inflammatory and anti-diabetic properties (Tesoriere et al. 2007), is used in the food industry and for the production of traditional medicines.

The organoleptic characteristics of this shrub have made it of great economic importance in the Mediterranean basin both for the local market and for export.

OBJECTIVES

The studies were conducted at the Università degli Studi di Palermo (UNIPA) and at the Valencian Institute of Agricultural Research (IVIA). The experiments involved the use of the anther culture technique, in order to obtain regenerantes in various *citrus* genotypes, the application of synthetic seed technology and *in vitro* micropropagation, to conserve and multiply explants, respectively in fig, hop and caper.

During the first year, the research focused on the evaluation of the effect of two different growth regulators (PGR): BAP and mT, on vitality, regrowth, conversion, shoot length and root of encapsulated nodal segments of the Lebanese variety of fig Houmairi and in two Sicilian genotypes of *Capparis spinosa* (L.). Furthermore, the experiment provided for the evaluation of cold seed storage, in fact, a part of the synthetic seeds was sown directly in an air-conditioned cabinet, and a part was subjected to 4 °C for 45 days and then sent to an air-conditioned cabinet At the same time, in the same year, an *in vitro* culture protocol was developed for anthers of *Citrus sinensis* L. Osbeck, variety Moro. In this experiment, great importance was given to the pre-treatment of flower buds and thermal stresses applied directly to the anthers, since, together with other factors such as: genotype, physiological conditions of donor plants, culture media, etc., influence the embryogenic response of the anthers.

The research activity of the second year concerned the analysis of the ploidy and molecular of the embryos that regenerated from the anthers culture of the Moro variety. Later, the same protocol was applied to various genotypes, tetraploids and diploids, of citrus fruits, since the embryogenesis technology is a good method to obtain homozygous regenerates and with halved chromosomal contents, useful for genetic improvement.

Cross-over between the second and third years, I worked on the development of an efficient sterilization protocol and then *in vitro* multiplication and rooting of the Cascade variety of *Homulus lupulus* L. For the multiplication phase, three PGRs were tested: TDZ, BAP and mT in different concentration and combination; while for the rooting phase two auxinic PGRs: IAA and IBA. Subsequently, the ploidy analysis of the regenerates obtained from the *in vitro* anthers culture of the various *citrus* genotypes were carried out and then the molecular analyzes, subjecting the DNA extracted from the embryos to six different molecular markers.

EXPERIMENTS

Chapter 1. Somatic embryogenesis trough *in vitro* anther culture of *Citrus sinensis* L. Osbeck, cultivar Moro.

In many crops, anther culture is the most used method to induce gametic embryogenesis, aimed to regenerate homozygous plants. However, also somatic embryogenesis can be obtained by this method, when somatic tissue is involved in the regeneration process. Many factors can affect this procedure, such as genotype, temperature pre-treatment applied to floral buds, pollen developmental stage, donor plant state, culture media composition and culture conditions. Anthers of *Citrus sinensis* L. Osbeck 'Moro', were collected at the vacuolate stage, and after a chilling (4°C) pre-treatment of 7 days, were placed on the same medium, evaluating different temperature stresses applied after placing them in culture. In this study, the effect of three thermal treatments, compared with direct in vitro culture of the anthers (after the pre-treatment of the floral buds at 4°C for 7 days), was observed in cultivar 'Moro'. Embryo regeneration has been obtained and their characterization, through ploidy and molecular analyses, showed that they were heterozygous tetraploids.

1.1 Introduction

Citrus species represent the largest production of fruit worldwide, with over 140 million tons produced during 2016 (FAOSTAT Database 2018). Important advancements have been made in the genetic improvement and vegetative propagation of *Citrus* spp. through the application of biotechnology and, in particular, through tissue culture. Embryo rescue and culture, somatic hybridization, genetic transformation, haploid production, and *in vitro* shoot-tip grafting are all biotechnological tools that can greatly help *Citrus* breeding and nursery production of disease-free plants.

The production of haploid and of doubled haploid plants, represents a particularly attractive biotechnological method to accelerate plant breeding. Anther culture is the most common method for haploid production, but it is also suitable for somatic embryogenesis in a number of fruit trees, including *Citrus* species (Germanà et al. 1994; Germanà 2003a). Numerous endogenous and exogenous factors affect the embryogenic and somatic response of anthers in culture (Atanassov et al. 1995; Smýkal 2000; Datta 2005). Genotype, physiological status and growth conditions of donor plants, the stage of gamete development and pre-treatment of the flower buds, culture media and conditions of incubation, and their interactions, are all factors that greatly affect the cell response to the *in vitro* culture (Germanà 2011a, b). There is no single standard condition, or protocol for obtaining plant formation by anther culture. Anthers of different species and cultivars within a species can present very different requirements for embryogenic and somatic development. For these reasons, studies of increasing embryogenesis efficiency, were focused on detecting the influence of different factors, particularly growth regulators, on anther culture and isolated microspore culture in *Citrus* spp. and other fruit crops (Germanà et al. 1996, 2006, 2011a; Höfer et al. 2002; Germanà and Chiancone 2003; Bueno et al. 2005, 2006; Chiancone et al. 2006; Padoan et al. 2011). Although in the genus *Citrus* somatic embryogenesis

was reported a long time ago (Maheshwari and Ranga Swamy 1958), a number of difficulties have been encountered in establishing reliable protocols.
This study examined the effects of three different thermal treatments on the anther culture of the cultivar 'Moro' of *Citrus sinensis* L. Osbeck. 'Moro' was originated as a spontaneous mutation from *C. sinesis* L. Osbeck 'Sanguinello Moscato' (Hodgson 1967).

1.2 Material and Methods

Plant material and pollen developmental stage

Flower buds were harvested in April 2017 from a tree of 'Moro' sweet orange grown in a collection orchard (Campo d'Orlèans, Palermo, 38°N) of the Università degli Studi di Palermo, Italy. Microspore developmental stage was determined in one anther per flower bud by 4',6-diamidino-2-phenylindole (DAPI) staining. Anthers from buds of different sizes were squashed in a few drops of DAPI solution (1 mg l^{-1}) and observed under a fluorescent microscope (Zeiss, Axiophot, Germany). For further experiments, only flower buds between 3.5-4.0 mm in length, containing most anthers with microspores at the uninucleated/vacuolated stage, were selected for *in vitro* culture.

Flower bud sterilization and anther culture

As pre-treatment, flower buds were placed at 4°C for 1 week. 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 (0.5% of active chlorine), containing few drops of Tween 20, and then rinsed three times with sterile distilled water. The petals were removed aseptically and 60 anthers from 3-4 flower buds, were placed in 6-cm Petri dishes containing 10 mL of solid culture media that were sealed with parafilm. Anther cultures were maintained at 26±1°C in the dark for the first 30 days, and then were placed under cool white fluorescent lamp (Philips TLM30W/84, France), with a photosynthetic photon flux density of 35 μmol m-1 s-1 and a photoperiod of 16 light hours.

Media composition

Anthers were cultured in vitro in a culture medium previously used in *Citrus* by Germanà et al. (1996) and described in Table 1.1 (hereafter referred to as culture media P). In order to study the influence of the thermal stress, anthers placed in medium P, were subjected to the following treatments: 1) Control, 2) -20°C for 60 min, 3) +4°C for 7 days and 4) +35°C for 7 days. Twenty-two Petri dishes were prepared containing 60 anthers for each treatment. The obtained embryos were transferred to the germination medium reported in (Table 1.2).

In vitro evaluation of the anther culture response, data processing and statistical analysis

Petri dishes containing anther in cultures were observed weekly by binocular microscope (Leica). Samples of anther were stained with DAPI and observed by a fluorescence microscope (Zeiss, Axiophot, Germany), to monitor their in vitro development, once a month, every month, during the culture. Different structural features have been observed and registered: microspores uninucleated, binucleated, trinucleated, tetranucleated, and multinucleated. Moreover, after 7 months of culture in the same Petri dishes, the number of embryos produced per each anther in every vessel was registered.

These values were used to calculate means. Statistical analysis was carried out using SYSTAT 13 software. One factor was considered: 'temperature treatment' and differences between them were tested by two-way analysis of variance (ANOVA), at $p \leq 0.05$ level. Tukey's test was, then, used to separate means.

Table 1.1. Composition of the culture medium P used for anther culture of 'Moro' sweet orange (Germanà *et al*., 1996)

Components	**Quantity**	**Components**	**Quantity**
N6 Chu salts[1]	1x	Zeatin	0,5 mg L^{-1}
N&N vitamins[2]	1x	GA_3	0,5 mg L^{-1}
Galactose	18 g L^{-1}	TDZ	0,5 mg L^{-1}
Lactose	36 g L^{-1}	BAP	0,5 mg L^{-1}
Ascorbic acid	500 mg L^{-1}	Casein	500 mg L^{-1}
Myoinositol	5 g L^{-1}	Serine	100 mg L^{-1}
Biotin	500 mg L^{-1}	Glycine	2 mg L^{-1}
Thiamine	5 mg L^{-1}	Glutamine	800 mg L^{-1}
Pyridoxine	5 mg L^{-1}	Malt extract	500 mg L^{-1}
2,4-D	0,5 mg L^{-1}	Agar	8,5 g L^{-1}
Kinetin	0,5 mg L^{-1}	pH	5,8

[1]Chu (1978); [2]Nitsch and Nitsch (1969).

Table 1.2. . Composition of the culture medium used for embryo germination.

Components	**Quantity**	**Components**	**Quantity (**mg L^{-1}**)**
MS salts[1]	1x[4]	GA[2]	1 mg L^{-1}
MS vitamins	1x	NAA[3]	0,02 mg L^{-1}
Sucrose	30 g L^{-1}	Agar	8,5 g L^{-1}
Ascorbic acid	500 mg L^{-1}	pH	5,8
Malt extract	500 mg L^{-1}		

[1]Murashige and Skoog (1962); [2]GA3: gibberellic acid; [3]NAA: α-naphthaleneacetic acid; x: Full strength

Ploidy evaluation

Ploidy level was determined on seven regenerated embryos, by flow cytometry according to the methodology described by Aleza et al. (2009). Flow cytometer is useful to detect and compare the ploidy of *in vitro* regenerated plantlets (Naing et al., 2014). Each sample consisted of a small piece of the germinated embryo, with a leaf piece (~0.5 mm2) collected from a diploid control plant. Samples were chopped together using a razor blade in the presence of a nuclei isolation solution (High Resolution DNA Kit Type P, solution A; Partec®, Münster, Germany).

Nuclei were filtered through a 30-μm nylon filter and stained with a DAPI (4,6-diamine-2-phenylindol) (High Resolution DNA Kit Type P, solution B; Partec®) solution. Following a 5-min incubation period, stained samples were run in a flow cytometer CyFlow® Ploidy Analyzer (Partec®), equipped with optical parameters for the detection of DAPI fluorescence. The DNA fluorochrome DAPI is excited by the UV-LED at 365 nm. Histograms were analysed using the CyView software (Partec®), which determines peak position, coefficient of variation (CV), arithmetic mean and median of the samples.

Molecular analysis

Genomic DNA was isolated from each embryo using a Plant DNeasy Kit from Qiagen Inc. (Madrid, Spain), according to the manufacturer's instructions. Nine heterozygous Simple Sequence Repeat (SSR) markers in 'Moro' sweet orange; TAA1 (Kijas et al., 1997), mCrCIR01F08a, mCrCIR06A12, mCrCIR01E02 (Froelicher et al. 2008), MEST192 (Aleza et al. 2011), mCrCIR07F11 (Kamiri et al. 2011), MEST1 and MEST104 (García-Lor et al. 2012), were used in this study. PCR amplifications were performed using a Thermocycler rep gradient S (Eppendorf®) in a 10-μL final volume containing 0.8 U of Taq DNA polymerase (Fermentas®), 2 ng μL-1 citrus DNA, 0.2 mM welled (Sigma®) dye-labeled forward primer, 0.2 mM non-dye-labeled reverse primer, 0.2 mM of each dNTP, 10×PCR buffer, and 1.5 mM MgCl2. The PCR protocol was as follows: denaturation at 94°C for 5 min followed by 40 cycles of 30 s at 94°C, 1 min at 50 or 55°C, and 45 s at 72°C; and a final elongation step of 4 min at 72°C. Capillary electrophoresis was carried out using a CEQTM 800 Genetic Analysis System (Beckman Coulter Inc.). The PCR products were initially denatured at 90°C for 2 min, injected at 2 kV for 30 s, and separated at 6 kV for 35 min. Alleles were sized based on a DNA size standard (400 bp). Genome LabTM Gap v.10.0 genetic analysis software was used for data collection.

1.3 Results and Discussion

Anther culture response and ploidy evaluation

Using the staining method facilitated the observations of whole process of embryogenesis in anther culture. Monitoring of the culture by samples stained with DAPI (to show the nuclei) revealed that initially microspores of the genotype were uninucleated (Figure 1.1a).

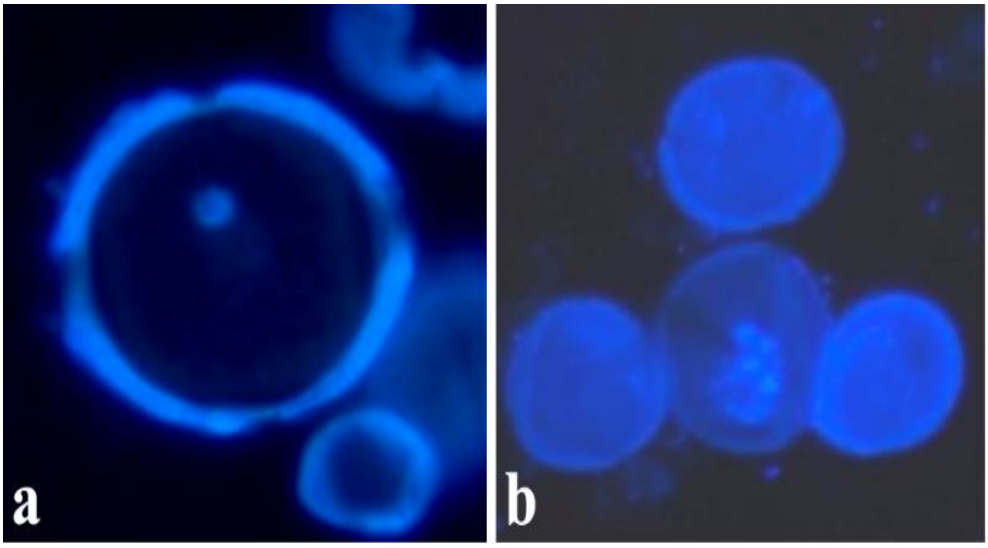


Figure 1.1. Uninucleated (a) and multinucleated (b) microspores of 'Moro'.

This is the developmental stage reported as being the most responsive for embryogenesis induction in citrus and many other woody and herbaceous species (Germanà and Chiancone 2003; Germanà et al. 2011a; Gaitán-Solís et al. 2008; Prem et al. 2008). It was possible to observe that some microspores did not show any change in the nuclei number or shape. In other microspores, the nucleus started to symmetrical division (Figure 1.1b). This type of division was considered the first step of the sporophytic pathway followed by the gametic embryogenesis (Germanà 2011a, b).

After a week in culture, many anthers were enlarged and after about a month, they began to produce callus. Most of the calluses were non-embryogenic. The morphogenic callus at first appeared crumbly and white, later embryos were differentiated. Results recorded after 7 months of anther culture, and their statistical analysis are reported in Table 1.3.

Direct embryo formation was observed (Figure 1.2a); in particular seven plants were obtained, both with the same level of ploidy. The embryos obtained were moved to the germination medium, continued to develop, germinate and to form plantlets that were micrografted *in vitro,* following the method described by Navarro and Juàrez (2007) (Figure 1.2b).

The statistical analysis carried out on the data registered after 7 months of culture and regarding: undeveloped anthers, swollen anthers, anthers with a callus and with an embryogenic callus or with embryos, pointed out that the treatment factor had a greater effect than the genotype factor and medium of culture. Already since the first month of cultivation, the first anatomical changes of the anthers could be observed, the enlargement of these is the first, while many anthers, especially those subjected to the treatment "+35°C for 7 days" (65.8%), maintained their initial dimensions since the beginning of the crop. Thus, anatomical changes in anthers consist of: increased turgidity, development of calluses and development of embryos. As opposed to the other treatments, it is possible to notice how the treatment "+35°C for 7 days" has recorded the highest percentage of anthers that have produced callus compared to the "control" "+4°C for 7 days" and "-20°C for

60 min" treatments (30 *vs*. 11.93%; 11.35%; 21.9%), even if the highest percentage of anthers not developed has been observed. From this, it is possible to deduce that the heat treatment "+35°C for 7 days" in part, supplies a better input than the other treatments to the anatomical change of the anthers, even if it did not lead to the development of embryos.

Regarding the anthers that developed embryos, the best treatment was the "control" followed by treatment "-20°C for 60 min" (0.37 *vs*. 0.15%).

Table 1.3. Influence of three thermal treatments (in darkness) on the anther culture of *C. sinensis* L. Osbeck 'Moro', registered after 7 months of culture. Each value is the average of 1320 anthers per treatment (22 Petri dishes with 60 explants).

Medium	Treatments	Percentage of anthers without development (%)	Percentage of swollen anthers (%)	Percentage of anthers produced callus (%)	Percentage of anthers produced embryoids and/or embryogenic callus (%)
P	**Control**	21.4	66.3	11.9	0,37
	-20°C for 60 min	31.3	57.2	11.3	0.25
	+4°C for 7 days	29.6	48.5	21.9	0
	_35°C for 7 days	65.8	4.2	30.0	0
		p= 0.000	p= 0.000	p= 0.000	p= 0.0224

Values within each column are significantly different at p≤0.05. Two-way ANOVA, Tukey's test, p≤0.05.

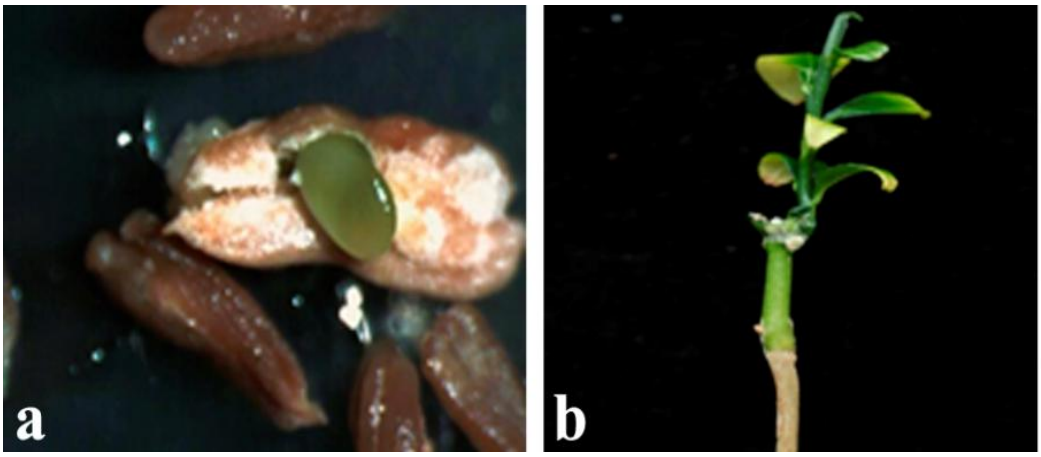


Figure 1.2. Embryos emerging from an anther (a) and in vitro micrografting of tetraploid plant (b) of 'Moro' regenerated from anther culture.

Flow cytometry analysis has been employed to detect the ploidy of regenerants. For all regenerants, the histogram of the flow, shows two peaks, one that refers to the mother plant, and the other showing a DNA content two times higher than that of the diploid control plant (Figure 1.3). The obtained tetraploids are not an exceptional fact for citrus fruits. In fact, there are many regenerants with different levels of ploidy found with the anther culture in the *citrus* species (Germanà et al. 2005; Germanà 2007). Non-haploid (diploid, triploid, tetraploid, pentaploid, or hexaploid) embryos and plantlets have been obtained also from anther culture of other genotypes (D'Amato 1977).

Figure 1.3. Flow citometry analysis that demonstrates tetraploid state of the regenerated embryo. 1) diploid peak of the mother plant (2*n*); 2) tetraploid peak (4*n*) of the regenerated embryo from anther of 'Moro'.

Regeneration through somatic embryogenesis is also rather common in different citrus species, and it is valuable method for clonal propagation, synthetic seed production, and germplasm storage (Germanà 2005; Germanà et al. 2011a). Numerous investigations have been performed to obtain homozygosity, but they resulted instead in heterozygous somatic plantlets. In particular, anther culture has produced somatic regenerants in *C. aurantium* (Hidaka et al. 1981; Germanà 2005); *C. sinensis* (Hidaka 1984; Cardoso et al. 2016); *C. aurantifolia* (Chaturvedi and Sharma 1985); *C. madurensis* (Ling et al. 1988); *C. reticulata* Blanco (Germanà et al. 1994; Germanà 2005); *Poncirus trifoliata*, and the 14 hybrids of *C. ichangensis* × *C. reticulata* (Deng et al. 1992). In these cases, anther culture can be regarded as an additional method for achieving somatic embryogenesis.

The microsatellite analysis was performed because the ploidy level analysis cannot discriminate between diploids with gametic origin (DHs) and diploids of somatic origin.

The genetic, biochemical and molecular markers provide a very useful tool to demonstrate the androgenetic nature of the regeneration from the cultivation of anthers of *Citrus* (Deng et al. 1992; Germanà et al. 1992, 1994, 2000a; Germanà and Reforgiato 1997).

In previous research, isoenzymes were used to distinguish between tissues of gametic and somatic origin. In fact, due to spontaneous doubling or spontaneous polyploidization of the haploid calluses, cytological analysis becomes unusable for the identification of homozygous regenerants (Germanà et al. 1992, 1994, 2000a; Germanà and Reforgiato 1997).

Genetic origin of regenerated plants

Two tetraploid recovered plants and 'Moro' sweet orange were analysed with nine heterozygous SSR markers. The tetraploid plants displayed the same heterozygous profiles as 'Moro' sweet orange for all molecular markers (Figure 1.4). These results suggest that tetraploid plants originated as a consequence of chromosome duplication from somatic cells during the *in vitro* culture process. In order to discriminate between these two types of regenerants, SSR markers being heterozygous for the parental genotypes, were selected from a preliminary screening. This type of regeneration through somatic embryogenesis from anther culture has been observed previously in *Citrus*, being a valuable issue for clonal propagation, synthetic seed production, elimination of pathogens and *in vitro* and germplasm storage (Germanà 2005; Germanà et al. 2011a).

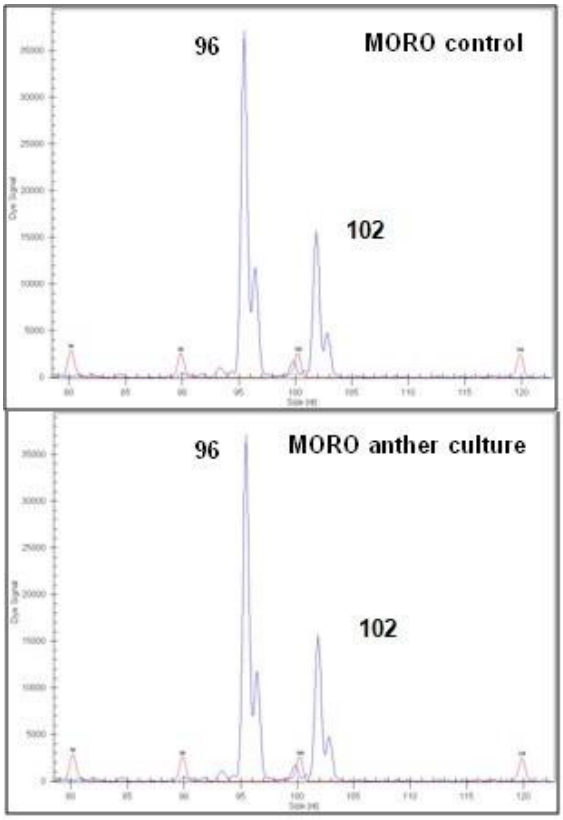


Figure 1.4. Amplicons of the SSR mCrCIR06A12 locus of the parental genotype (top) and the embryo (bottom) 'Moro'. Values in the box next to each peak represents the size of the observed alleles (bp).

1.4 Conclusion

Obtaining tetraploid genotypes from a blood sweet orange cultivar represents an important result for numerous applications in breeding. The results reported in this study confirm that the response depends, not only on the genotype, but also on the identification of the best treatment for the anthers.

The presence of multinucleate pollen grains, also developed in *C. sinensis* 'Moro' from this *in vitro* system, indicates the induction of microspore nucleus division and the switch of the gametophytic developmental program to the embryogenic pathway, even the regeneration does not regard the gametic embryos, but only the somatic ones.

For this reason, in conclusion, it can be stressed that the study of the factors influencing the phenomenon of gametic embryogenesis is extremely important, in order to clarify aspects that can lead to an increase in the frequency of microspores forming embryos and the number of plantlets anther obtained above all in those genotypes such as *Citrus* and all the woody plants which are recalcitrant to gametic embryogenesis.

Chapter 2. Anther culture of tetraploids *Citrus* genotypes and dihaploids regeneration.

Microspore embryogenesis through *in vitro* culture is a widely used method to obtain haploid plants (sporophytes with gametic chromosome number, n instead of 2n) and doubled haploids (DH, haploid subjected to chromosomal duplication, spontaneous or induced). Anther culture can also be performed in tetraploid genotypes to halve chromosomes, as previously made in tetrasomic somatic hybrids in *Solanum.*

This work reports the formation of multicellular pollen and embryos in four tetraploid *Citru*s cultivars (Marisol, Clemenules, Moro Los Valles and Sanguinelli), obtained in Spain at IVIA from colchicine or from spontaneous duplication. Also anthers of diploid Sanguinelli were cultured.

Anthers were *in vitro* cultivated at the uninucleated/vacuolated stage. Five genotypes, one culture medium, three (two temperature treatments and a control treatment) treatments were evaluated. During the investigation, the symmetrical division of the nucleus was observed and multinucleated structures were observed in all the genotypes and in all the temperature treatments tested, as well as the regeneration of embryos were observed. The embryos characterization involved flow cytometric analysis and showed that most regenerants were diploids. The molecular marker analysis, carried out through SSR markers, revealed singular results. Particularly, in the cultivar Marisol, Clemenules and Snguinelli (2x and 4x), even if multinucleated microspores were observed and mostly diploids were regenerated, the molecular analysis showed all heterozygous regenerants, while in Moro Los Valles, the analysis revealed three regenerating homozygotes, two mutants homozygotes (gametoclonal variabitity) and two heterozygous mutants were obtained.

This study reports genome halving by microspore embryogenesis, in tetraploid Citrus genotypes, even if further investigations have to be carried out to easier select genes of interest.

2.1 Introduction

Most species of the genus *Citrus* are diploid (2n = 18) with a basic number of chromosome x = 9. However, plants with a different number of chromosomes, such as triploids (2n = 3x = 27) and tetraploids (2n = 4x = 36) (Lee 1988), may originate, albeit rarely, naturally. Most of the commercially important cultivars of sweet orange (*C. sinensis*), grapefruit (*C.* × *paradisi*), mandarin (*C. reticulata*), and lemon (*C. limon* [L.] Burr.) are seedless as main breeding objective for Citrus scion cultivar development programs (Soost and Cameron, 1975), because seedlessness is requred for fresh consumption. Citrus triploids have been produced most commonly by interploid hybridization, mostly using tetraploids as seed parents (Lee, 1988). 'Oroblanco' and 'Melogold' hybrids (diploid *C. grandis* [L.] Osb. × tetraploid *C.* × *paradisi*) are seedless obtained by this method (Krug and Bacchi 1943; Soost and Cameron 1969, 1980, 1985). They are also of potential interest for direct use as dwarfing rootstocks (Cameron and Frost 1968;

Lee 1988; Ollitrault et al. 2008), thanks to their strength and robustness. There are too few available tetraploid Citrus selections representing too small a fraction of the gene pool to exploit fully the potential to produce seedless cultivars by interploid hybridization (Barrett 1974). As reported by Gmitter, Jr. and Ling, (1991), tetraploid Citrus plants can originate:
1) as spontaneous nucellar seedlings (Barrett and Hutchison 1978),
2) by colchicine treatment of axillary meristems (Barrett 1974),
3) following 2x × 4x hybridization (Cameron and Soost, 1969),
4) as spontaneous tissue culture-derived variants (Vardi 1981 Zhang, 1985), and
5) by somatic hybridization (Grosser and Gmitter 1990; Kobayashi and Ohgawara 1988).
The altenative method used to obtain polyploid plants is the treatment with colchicine in undeveloped ovule or in micro-grafting (Gmitter and Ling 1991; Zhang et al. 2007; Aleza et al. 2009; Juárez et al. 2015; Rousis et al. 2017), and embryo rescue was performed successfully in citrus seeds, collected from unripe fruits. In Citrus the frequency of spontaneous polyploid plants is variable and it appears to be under genetic and environmental control (Barrett and Hutchison 1978; Hussain et al. 2012). The polyploidy can be obtained through the fusion of a non-reduced gamete (2n) with a normal haploid or two non-reduced gametes. Polyploid plants of somatic origin, or "spontaneous polyploids", can appear through natural processes of mutation, via the duplication of chromosomes in the nucella and the lack of division during mitosis, originating tetraploid plants (Cameron and Frost 1968; Lee 1988; Omar et al. 2016; Zeng et al. 2006). Given the low frequency of spontaneous tetraploids, to find new techniques for producing stable tetraploids has become necessary.
The enormous potential of homozygotes is now universally known in the breeding of fruit crops. Unfortunately, the possibility of exploiting this potential for practical purposes is considerably hampered by the absence of efficient and universal protocols for the production of haploids and doubled haploids through gametic embryogenesis. In fact, the embryogenic response is highly genotype-dependent (Germanà 2007; Heberle-Bors 1985) and it is therefore necessary, for each species, and within each species, for each variety to adapt protocols already set up. In order to exploit homozygotes for application purposes, it is necessary not only to improve the efficiency of regeneration from gametes, but also to increase the genotypes responding to gametic embryogenesis.
This study used gametic embryogenesis through anther culture to induce genome halving in *Citrus* tetraploid cutivars, to isolate genes of interest, such as phytopathic resistance genes (ex. resistance to the *Alternaria* fungus), or genes that improve the organoleptic characteristics of the fruits (ex. gene of the red orange), transferring genetic advantages in a single generation of crosses.

2.2 Material and Methods

Plant Material

Flower buds were collected in April 2018 from five different genotypes of Citrus, four tetraploids and one diploid: Moro Los Valles 4x, Sanguinelli 2x, Sanguinelli

4x (sweet orange: *Citrus sinensis* L. Osbeck), Marisol 4x and Clemenules 4x (clementine: *Citrus clementina* Hort. ex Tan.), cultivated in an collection orchard of the Instituto Valenciano de Investigaciones Agraria (IVIA), Moncada, Valencia, Spain. Marisol 4x and Clemenules 4x genotypes were obtained through colchicine treatment (Aleza et al. 2009) while Moro Los Valles 4x and Sanguinelli 4x through spontaneous double (Aleza et al. 2011).

Bud size

Stage of development of microspores was determined in an anther for flower bud with 4 ', 6-Diamidino-2-phenylindole (DAPI) staining. Anthers from different sized buds have been separately crushed in a few drops of DAPI solution (1 mg/mL) and they were observed under a fluorescent microscope (Zeiss, Axiophot, Germany). Only flower buds between 3.5 (clementine) and 4.0 mm (orange) were selected for *in vitro* culture, because most anthers contained microspores in the uninucleated/vacuolate phase (Fig. 2.1).

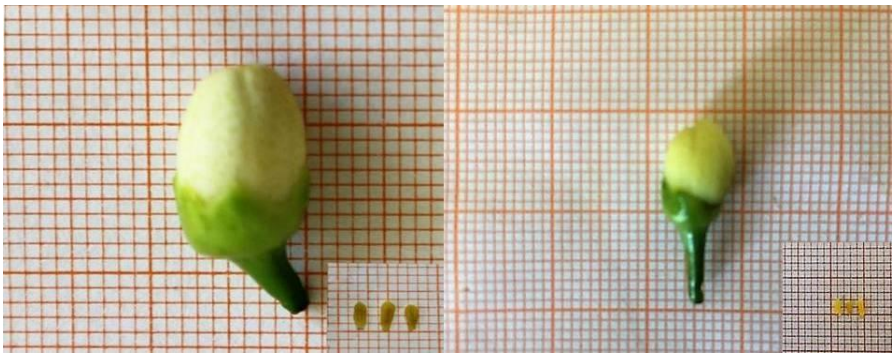


Figure 2.1. Flower bud and anthers of Sanguinelli 4x sweet orange (left) and Clemenules 4x (right) with vacuolated microspores at the development stage of culture.

Flower Bud Sterilization and Anther Culture

As pre-treatment, flower buds were placed at 4°C for 1 week. After that, 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 (0.5% of active chlorine), containing few drops of Tween 20, and then, they were rinsed three times with sterile distilled water. The petals were removed aseptically and 60 anthers from 3-4 flower buds, were placed in 6-cm Petri dishes containing 10 ml of solid culture media and they were sealed with parafilm. Anther cultures were maintained at 26 ± 1 °C in the dark for the first 30 days, and then were placed under cool white fluorescent lamp (Philips TLM30W/84, France), with a photosynthetic photon flux density of 35 μmolm−1 s−1 and a photoperiod of 16 light hours.

Culture media and temperature treatments

Culture media

Anthers were cultured *in vitro* in a culture medium previously used in *Citrus* by Germanà et al. (1996) and described in Table 2.1 (hereafter referred as culture medium P).

Temperature treatments

In order to study the influence of the thermal stress, anthers placed in medium P, were subjected to the following treatments:

- ✓ Control: directly at 26 ± 1 °C in the dark,
- ✓ -20 °C for 120 minutes in the dark,
- ✓ +35 °C for 5 days in the dark. The treatment at +35 °C required the passage of the anthers in a new culture medium to avoid degradation of components P.

Table 2.1. Culture medium P used for anther culture of citrus trees (Germanà et al. 1996).

Components	**Quantity**	**Components**	**Quantity** mg L^{-1}
N6 Chu salts[1]	1x	Zeatin	0,5 mg L^{-1}
N&N vitamins[2]	1x	GA_3	0,5 mg L^{-1}
Galactose	18 g L^{-1}	TDZ	0,5 mg L^{-1}
Lactose	36 g L^{-1}	BAP	0,5 mg L^{-1}
Ascorbic acid	500 mg L^{-1}	Casein	500 mg L^{-1}
Myoinositol	5 g L^{-1}	Serine	100 mg L^{-1}
Biotin	500 mg L^{-1}	Glycine	2 mg L^{-1}
Thiamine	5 mg L^{-1}	Glutamine	800 mg L^{-1}
Pyridoxine	5 mg L^{-1}	Malt extract	500 mg L^{-1}
2,4-D	0,5 mg L^{-1}	Agar	8500 mg L^{-1}
Kinetin	0,5 mg L^{-1}	pH	5,8

[1]N6 Chu salts: Chu (1978); [2]N&N vitamins: Nitsch and Nitsch (1969).

Experimental design

For each genotype, 39 Petri dishes were prepared, divided by the two heat treatments and the control, each Petri dish contained 60 anthers (total per genotype 2340, 780 for treatment).

Germination medium

The embryos obtained were transferred to the germination medium reported in (Table 2.2). To obtain plants more quickly, embryos were subjected to micro-grafting, according to the protocol of Navarro and Juàrez (2007).

Evaluation of anthers response, data processing and statistical analysis

Petri dishes containing anther in cultures were weekly observed by binocular microscope (Leica). Samples of anthers were stained with DAPI and observed by a fluorescence microscope (Zeiss, Axiophot, Germany), to monitor their *in vitro*

development, every month during the culture. Different structural features have been observed and registered: uninucleated, binucleated, trinucleated, tetranucleated, and multinucleated microspores. Moreover, after 7 months of culture in the same Petri dishes, the number of embryos produced per each anther in every vessel was registered. These values were used to calculate means. Statistical analysis was carried out using SYSTAT 13 software. Two factors have been considered: "Temperature treatment ", "genotypes" and in the case of the Sanguinelli genotype, "ploidy" has also been taken into account. The differences between them were tested by bidirectional variance (ANOVA) analysis, at $p \leq 0.05$ level. The Tukey test was, therefore, used to separate the means.

Table. 2.2. Culture medium used for embryo germination of *Citrus* spp.

Components	Quantity	Components	Quantity
MS salts[1]	1x	GA[2]	1 mg L^{-1}
MS vitamins	1x	NAA[3]	0,02 mg L^{-1}
Sucrose	30 g L^{-1}	Agar	8500 mg L^{-1}
Ascorbic acid	500 mg L^{-1}	pH	5,8
Malt extract	500 mg L^{-1}		

[1]Murashige and Skoog (1962); [2]GA_3: gibberellic acid; [3]NAA: α-naphthaleneacetic acid

Characterization of regenerants

Ploidy analysis

The ploidy was determined on all the regenerated embryos, by flow cytometry, according to Aleza et al. (2009). The flow cytometer is useful for detecting and comparing the ploidy of *in vitro* regenerated plantlets (Naing et al. 2014). Each consisted of a small piece of the germinated embryo, with a piece of leaf (~ 0.5 mm^2) harvested from a tetraploid or diploid control plant. The samples were chopped together using a razor blade in the presence of a core isolation solution (high resolution DNA Kit type P, Solution A; Ment®, Münster, Germany). The nuclei were filtered through a 30 μm nylon filter and stained with a drop of DAPI (4′,6-diamidino-2-phenylindole) (High-resolution DNA kit type P, Solution B; ®). After a 5-minute incubation period, the coloured were performed in a CyFlow® Ploidy Analyzer (ment®) flow cytometer with optical parameters for DAPI fluorescence detection. The DNA fluorochrome DAPI is excited by the UV LED at 365 nm. The histograms were analyzed using the CyView (ment®) software, which determines the peak position, the coefficient of variation (CV), the arithmetic mean and the median of the samples.

DNA extraction

Genomic DNA was isolated from each embryo using a Qiagen DNeasy settlement kit (Madrid, Spain), according to the manufacturer's instructions.

Molecular analysis

Six Simple Sequential repeat (SSR), heterozygotes in mother plants of sweet orange and *citrus clementine* from which anthers were collected, were chosen as markers. The following SSR, named MEST470 (Ollitrault et al. 2012), ACA01 (Ollitrault et al. 2012), MEST308 (Ollitrault et al. 2012), mCrCIRO3B07 (Cuenca et al. 2011), MEST15 (Garcia-Lor et al. 2012) and MEST88 (Garcia-Lor et al. 2012), were used in this study. The PCR amplifications were performed with a Rep gradient S (Eppendorf®) in a final volume of 10 µL containing 0.8 U of Taq DNA polymerase (Fermentas®), 2 ng µL of Citrus DNA, 0.2 mM background (SIGMA®), primer forward with dye label, 0.2 Reverse Primer not dyed in mM 0.2 mm of each dNTP, 10 × PCR buffer and 1.5 mM MgCl2. The PCR protocol was as follows: Denaturation at 94 °C for 5 min followed by 40 Cycles of 30s at 94 ° C, 1 min at 50 or 55 °C and 45s at 72 °C, and a final step of stretching 4 Min at 72 °C. Capillary electrophoresis was performed using a CEQTM 800 genetic analysis System (Beckman Coulter Inc.). The PCR products were initially denatured at 90 °C for 2 min, injected at 2 kV for 30 s, and separated at 6 kV for 35 min. Alleles were dimensioned according to the DNA Standard Dimensions (400 BP). The LabTM Gap v. 10.0 Genome Genetic analysis software was used.

2.3 Results and Discussion

Evaluation of anthers response, data processing and statistical analysis.

The use of the staining method facilitated the observations of the whole process of embryogenesis in anthers culture. Monitoring of culture from coloured samples with DAPI (to observe or not the symmetrical division of nucleus) revealed that initially most microspores of all the genotypes were uninucleated (Fig.2.2).

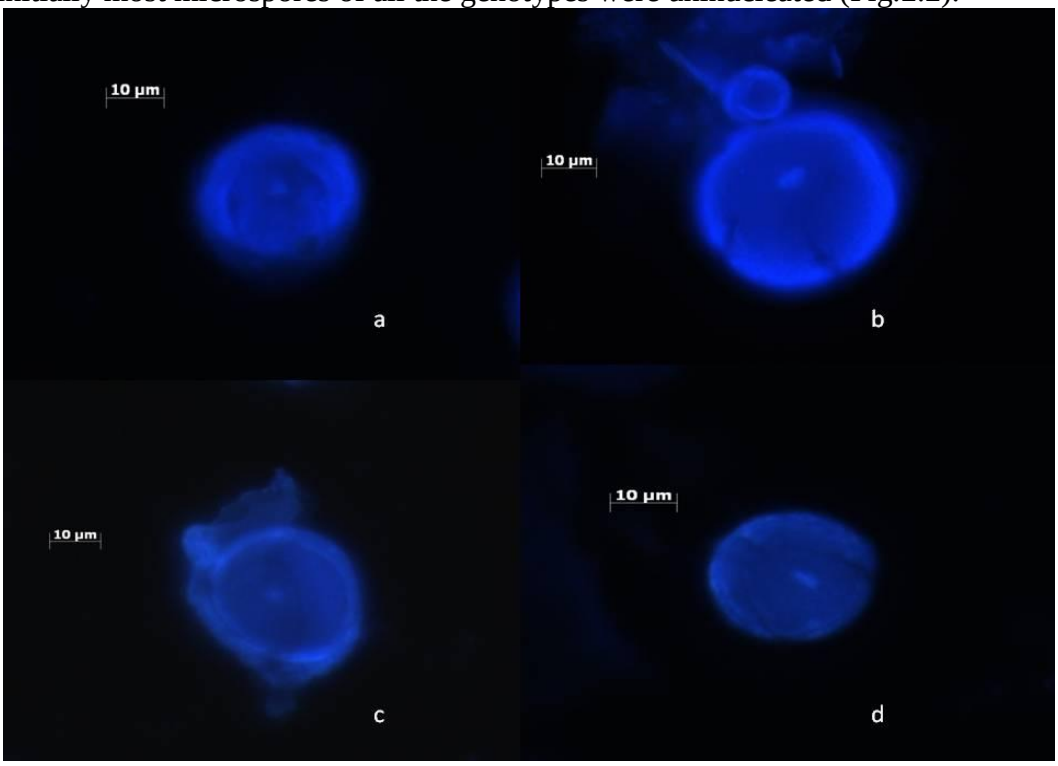


Figure 2.2. Uninucleated microspores of: (a) Clemenules 4x, (b) Moro Los Valles 4x, (c) Sanguinelli 4x and (d) Sanguinelli 2x.

This is the developmental stage reported as being the most responsive for embryogenesis induction in citrus and many other woody and herbaceous species (Germanà and Chiancone 2003; Germanà et al. 2011a; Gaitán-Solís et al. 2008; Prem et al. 2008). It was possible to observe that some microspores did not show any change in the nuclei number or shap, while other microspores, showed the nucleus starting the symmetrical division (Figure 2.3). This type of division was considered the first step of the sporophytic pathway, followed by the gametic embryogenesis after induction (Germanà 2011a, b). After a week in culture, many anthers were enlarged and after about a month, they began to produce callus. Most of the calluses were non-embryogenic. The morphogenic callus at first appeared crumbly and white, later embryos were differentiated. Results recorded after 7 months of culture, and their statistical analysis are reported in Table 3, 4, 5, 6.

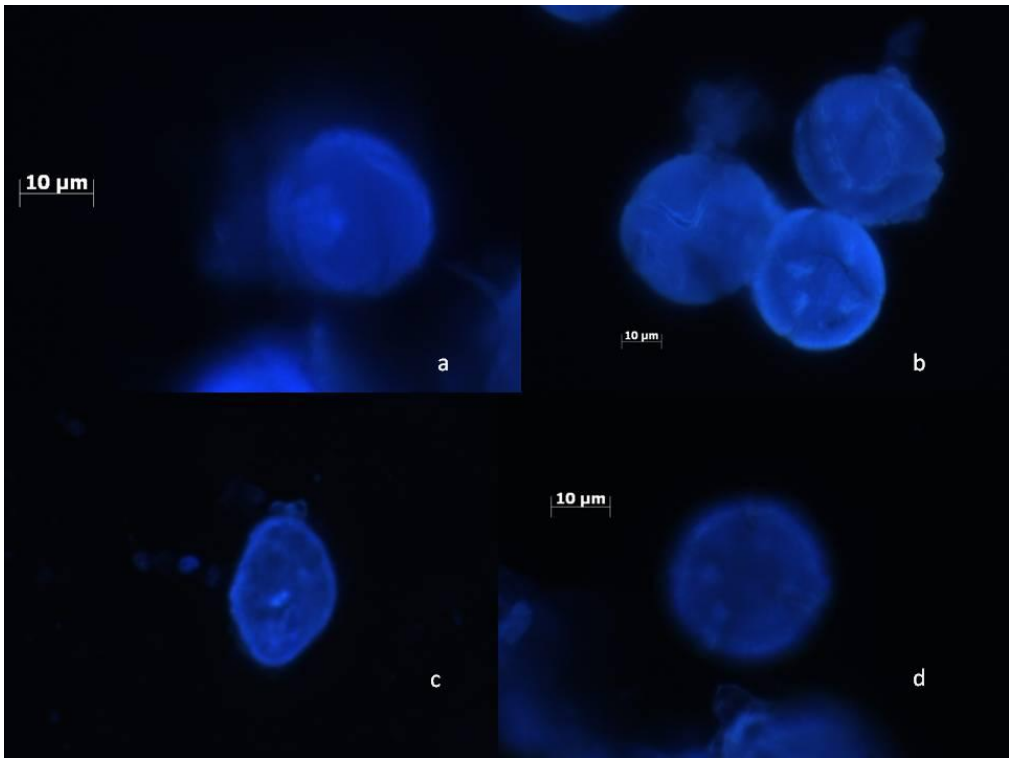


Figure 2.3. Change in microspore development towards gametic embryogenesis: (a) multinucleated miscrospore of Clemenules 4x, (b) multinucleated miscrospore of Moro Los Valles 4x, (c) multinucleated miscrospore of Sanguinelli 4x and (d) multinucleated miscrospore of Sanguinelli 2x.

Mainly, direct formation of the embryo has been observed, but also regeneration through the callus phase has been noticed. Specifically ninety-four embryos were produced in Marisol genotype 4x, twenty-two in Clemenules 4x, forty-four in Moro Los Valles 4x, forty-two in Sanguinelli 4x and nine in Sanguinelli 2x (Fig.2.4).

Figure 2.4. Emerging embryos from an anther of: (a) Marisol 4x, (b) Clemenules 4x, (c) Sanguinelli 4x and (d) Sanguinelli 2x.

All the cultivars gave origin to the morphogenic process *in vitro*, despite differences linked to the genotype were observed. In fact, a strong genotypic effect was observed on all the recorded parameters (anthers without development, swollen anthers, anthers with callus and with embryos). Significant differences have been recorded. Zero values of undeveloped anthers were recorded in the Clemenules 4x and Sanguinelli 4x cultivars. Regarding the number of undeveloped anthers, the highest value was observed in Marisol 4x, although it was observed in this genotype, the highest percentages of anthers producing embryos. For all the genotypes, the anthers that produced non-embryogenic callus there was a percentage ranging between 81.4 in Marisol 4x and 93.9 in Clemenules 4x. Considering the most important data, the percentage of anthers that produced embryos, the highest values were recorded in clementine Marisol 4x (3.6 %), followed by blood oranges, Sanguinelli 4x and Moro Los Valles 4x (1.7-1, 6%), while the lowest one was noticed in Clemenules 4x (0.97%) and Sanguinelli 2x (0.10%), which unlike the other cultivars, has a diploid chromosomal number (Tab. 2.3).
Regarding the temperature treatment, pretreatment at 4°C is the most commonly used stress employed in citrus anther culture (Germana` 2006). In response to the temperature treatment, for anthers without development, swollen and embryos, there was no difference in behaviour between the sweet orange and the clementine, unlike Cardoso et. al. (2016). Only differences were observed in the anthers that have produced non-embryogenic callus. Except for Clemenules 4x, heat treatment, both cold than hot, differs from control. Marisol 4x was the only

genotype in which there have been statistically significant differences for the anther without development, swollen and with non-embryogenic callus (Tab. 2.4). Taking into consideration the thermal treatments, independently of the genotype, significant statistical differences, were found, only in the percentage of anther without development, where, the treatment -20° for 120 minutes differed with the control. In percentage of anther producing non-embryogenic callus, where, the treatment + 35 °C for 5 days, also differed with control (Tab. 2.5).
For the genotype Sanguinelli, the ploidy factor was also considered, considering anthers of diploid and tetraploid plants. Statistically significant differences are detected only in anthers that have produced embryogenic callus and or embryos, raising higher percentages in tetraploid genotypes (Tab. 2.6).

Table 2.3. Influence of three temperature treatments (in the dark) on the anther culture of the various genotypes tested, recorded after 7 months of culture. Each value is the Average of 780 anthers for treatment (13 Petri dishes with 60 explants).

Genotypes	Percentage of anthers without development (%)	Percentage of swollen anthers (%)	Percentage of anthers producing callus (%)	Percentage of anthers producing embryoids (%)
Clemenules 4x	0 b	2.4 ab	93.9 a	0.9 cb
Marisol 4x	1.5 a	13.2 a	81.3 b	3.6 a
Moro Los Valles 4x	0.3 b	10.6 ab	85.1 b	1.5 b
Sanguinelli 4x	0 b	3.9 b	94.3 a	1.6 b
Sanguinelli 2x	0.1 a	6.5 cb	93.1 a	0.1 c
p	0.0002	0.0079	0.0009	0.000

Values within each column are significantly different a $p \leq 0.05$. Two-way ANOVA, Tukey's test, $p \leq 0.05$

Table 2.4. Influence of three heat treatments (in the dark) on the anther culture of the various genotypes tested, recorded after 7 months of culture. Each value is the Average of 780 anthers for treatment (13 Petri dishes with 60 explants).

Genotypes	Treatments	Ploidy	Percentage of anthers without development (%)	Percentage of swollen anthers (%)	Percentage of anthers producing callus (%)	Percentage of anthers producing embryoids (%)
CLEMEULES 4X	control	4x	0 a	1.3 a	86.9 a	0.5 a
	-20°C for 120 min.	4x	0 a	0.7 a	98.0 a	1.1 a
	+35°C for 5 days	4x	0 a	5.0a	94.0 a	0.8 a
p			0	0.1489	0.3144	0.5399
MARISOL 4X	control	4x	0.8 b	8.2 b	86.6 a	4.2 a
	-20°C for 120 min.	4x	3.6 a	28.3 a	64.7 b	2.6 a
	+35°C for 5 days	4x	0.1 b	3.0 b	92.7 a	4.0 a
p			0.0167	0.0001	0.0000	0.4036
MORO LOS VALLES 4X	control	4x	1.0 a	19.8 a	70.4 a	1.5 a
	-20°C for 120 min.	4x	0 a	0.5 a	98.3 ab	1.2 a
	+35°C for 5 days	4x	0 a	8.1 a	90.0 b	1.8 a
p			0.1675	0.1892	0.0562	0.7752
SANGUINELLI 4X	control	4x	0 a	0 b	99.3 a	0.6 a
	-20°C for 120 min.	4x	0 a	7.6 a	91.3 b	0.9 a
	+35°C for 5 days	4x	0 a	2.4 ab	94.3 ab	3.2 a
p			0	0.0250	0.0403	0.0924
SANGUINELLI 2X	control	2x	0.5 a	14.1 a	85.1 b	0 a
	-20°C for 120 min.	2x	0 a	2.3 b	97.3 a	0.1 a
	+35°C for 5 days	2x	0 a	5.8 b	94.0 a	0 a
p			0.2538	0.0101	0.0063	0.7608

Values within each column are significantly different at $p \leq 0.05$. Two-way ANOVA, Tukey's test, $p \leq 0.05$

Table 2.5. Influence of only heat treatments on the anther culture, recorded after 7 months of culture. Each value is the average of 780 anthers for treatment (13 Petri dishes with 60 explants).

Treatments	Percentage of anthers without development (%)	Percentage of swollen anthers (%)	Percentage of anthers producing callus (%)	Percentage of anthers producing embryoids (%)
control	0.5 ab	9.7 a	85.1 a	1.4 a
-20°C for 120 min.	0.6 a	7.4 a	90.7 ab	1 a
+35°C for 5 days	0 b	4.9 a	93.1 a	1.8 a
p	0.0714	0.1861	0.0296	0.2072

Values within each column are significantly different a p≤0.05. Two-way ANOVA, Tukey's test, p≤0.05

Table 2.6. Influence of ploidy on the anther culture of *C. sinensis* L. Osbeck 'Sanguinelli', recorded after 7 months of culture. Each value is the average of 780 anthers for treatment (13 Petri dishes with 60 explants).

Ploidy	Percentage of anthers without development (%)	Percentage of swollen anthers (%)	Percentage of anthers producing callus (%)	Percentage of anthers producing embryoids (%)
Sanguinelli 2x	0.1 a	6.5 a	93.1 a	0.1 b
Sanguinelli 4x	0 a	3.9 a	94.3 a	1.6 a
p	0.3220	0.2093	0.5620	0.0010

Values within each column are significantly different a p≤0.05. Two-way ANOVA, Tukey's test, p≤0.05

In vitro micrografting

The obtained embryos were transferred to the germination medium, but not all continued to develop and most were used for ploidy and molecular analysis. In particular, all the embryos obtained were used for the analysis of ploidy and molecular analysis in Marisol 4x and Sanguinelli 4x, while fifteen in Clemenules 4x, forty-one in Moro Los Valles 4x and eight in Sanguinelli 2x . Twelve embryos were in vitro micro-grafted to facilitate the plant obtainment, following the method described by Navarro and Juàrez (2007) (Fig. 2.5). 7 plants (2 clemenules, 4 Moro Los Valles and 1 Sanguinelli) were obtained.

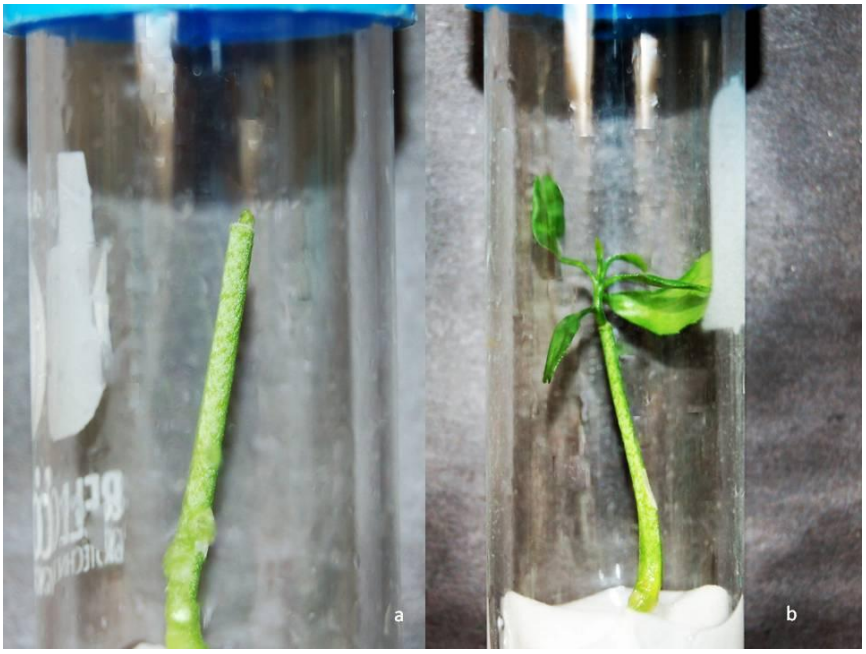


Figure 2.5. *In vitro* micrografting of regenerants from anther culture: (a) Sanguinelli 2x, (b) Clemenules 4x.

Characterization of regenerants

Ploidy analysis

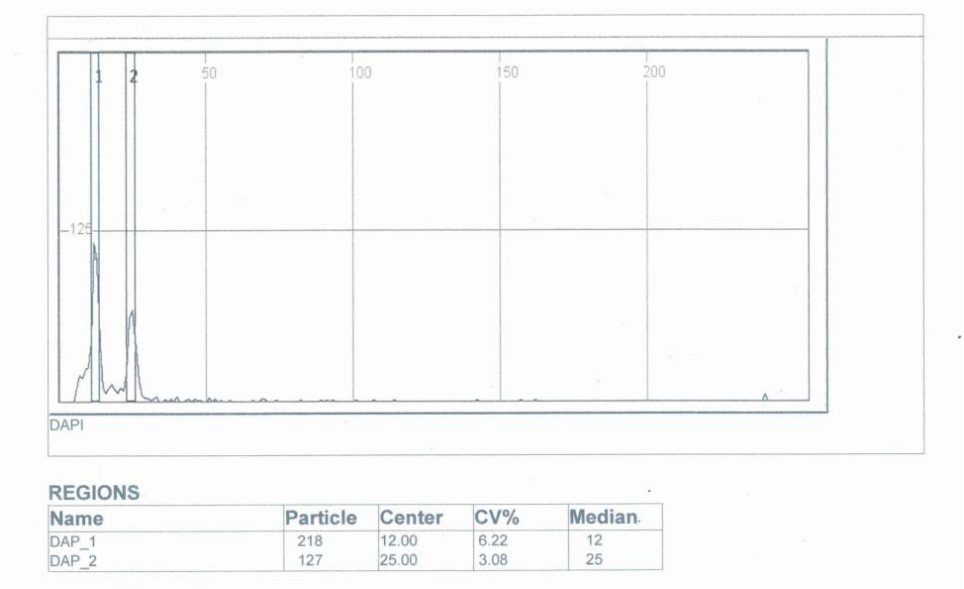
The analysis of ploidy, through flow cytometry, realized that all 109 regenerated (embryos and/or plants), of the species C. clementina, were diploid, obtained through anthers culture of the Marisol 4x and Clemenules 4x genotype, independently of the genotype and heat treatment (Tab. 7) (Fig. 2.6).

In the case of the sweet oranges Moro Los Valles 4x and Sanguinelli 4x, by anther culture, respectively 41 and 39 embryos were produced. 34 dihaploids and 7 tetraploids embryos were obtained for the Moro Los Valles 4x and 29 dihaploids and 6 tetraploids for the Sanguinelli 4x. In the analysis of regenerated tetraploids (only in 13 cases of sweet orange), the histogram of the fluorescence of the nuclei, showed that the peak of the tissue of the mother plant leaf corresponds with that of the tetraploid regenerants (Fig. 2.7).

A comparison of ploidy was made for embryos regenerated from Sanguinelli variety (4x, number of embryos 35), and the anthers collected from diploid mother plant (2x, number of embryos 8), that both were *in vitro* cultivated into the same conditions. The analysis showed that the number of embryos that developed fom Sanguinelli 2x was significantly lower and embryos were not regenerated in the +35 °C for 5 days treatment (Tab. 7). By the flow cytometry analysis, it has emerged that the 8 embryos regenerated from anthers collected from 2x plants analysed had a level of ploidy equal to that of the diploid mother plant (Fig. 2.8).

Table 2.7. Ploidy level of regenerated obtained through the culture of anthers

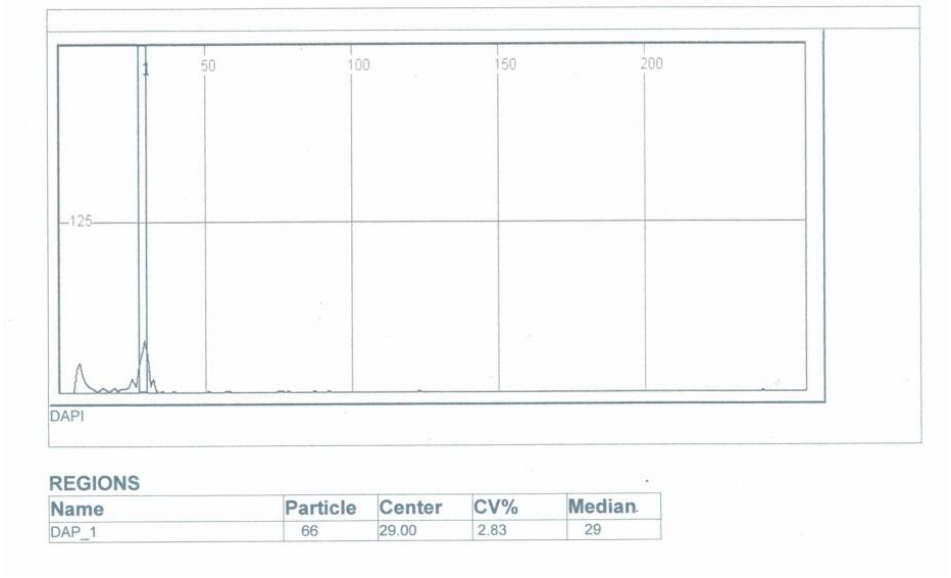
Genotype	Treatments	Number of diploid regenerates	Number of tetraploid regenerates
Marisol 4x	control	33	0
Marisol 4x	-20°C for 120 min.	26	0
Marisol 4x	+35°C for 5 days	35	0
Embryos from Marisol 4x		94	0
Clemenues 4x	control	3	0
Clemenues 4x	-20°C for 120 min.	11	0
Clemenues 4x	+35°C for 5 days	8	0
Embryos from Clemenules 4x		22	0
Moro Los Valles 4x	control	15	2
Moro Los Valles 4x	-20°C for 120 min.	3	1
Moro Los Valles 4x	+35°C for 5 days	16	4
Embyos from Moro Los Valles 4x		34	7
Sanguinelli 4x	control	4	1
Sanguinelli 4x	-20°C for 120 min.	11	2
Sanguinelli 4x	+35°C for 5 days	14	3
Embryos from Sanguinelli 4x		29	6
Sanguinelli 2x	control	4	0
Sanguinelli 2x	-20°C for 120 min.	4	0
Sanguinelli 2x	+35°C for 5 days	0	0
Embryos from Sanguinelli 2x		8	0



REGIONS

Name	Particle	Center	CV%	Median
DAP_1	218	12.00	6.22	12
DAP_2	127	25.00	3.08	25

Figure 2.6. Characterization of regenerants from anther cultures. Cytofluorometric analysis of ploidy: histograms of the intensity of the fluorescence in the nuclei of the diploid embryonic tissue of C. clementina cv. Marisol 4x of anther culture (peak 1) and from tetraploid leaf tissue of C. clementina mother plant (peak 2).



REGIONS

Name	Particle	Center	CV%	Median
DAP_1	66	29.00	2.83	29

Figure 2.7. Characterization of regenerants of anther culture. Cytofluorometric analysis of ploidy: histograms of the intensity of fluorescence in the nuclei of the tetraploid embryonic tissue, regenerated by anthers culture of the Sanguinelli 4x genotype, corresponds with that of the tissue of the mother plant leaf.

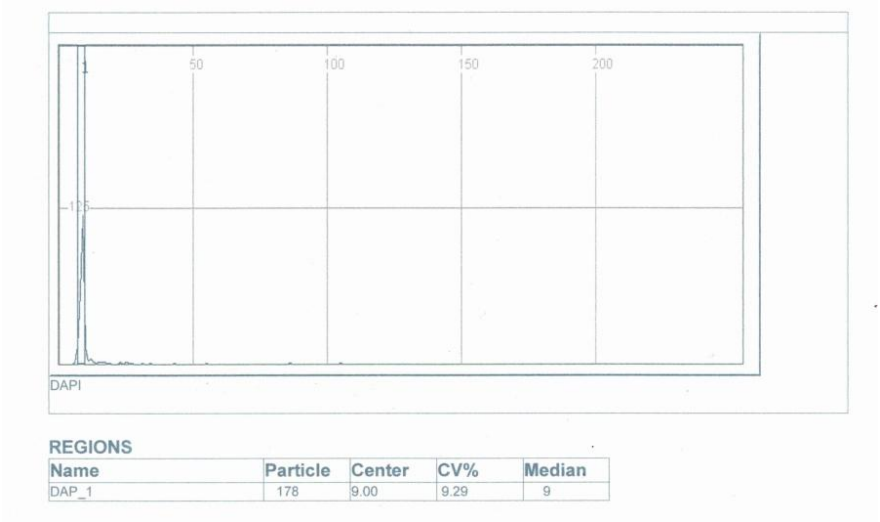


Figure 2.8. Characterization of regenerants of anther culture. Cytofluorometric analysis of ploidy: histograms of the intensity of fluorescence in the nuclei of the diploid embryonic tissue, regenerated by anthers culture of the Sanguinelli 2x genotype, corresponds with that of the tissue of the mother plant leaf.

Molecular analysis of regenerated embryos

The tetraploid *citrus* varieties are of little commercial interest but they are important for use as rootstocks or in breeding programs, to obtain, trough crossing with diploid genotypes, triploid varieties, producing seedless fruits, accepted by consumers. Anthers culture can also be used to in tetraploid genotypes to halve the genome, as previously made in tetrasomic somatic hybrids in Solanum (Rizza et al. 2002; Rotino 2005; Barchi et al. 2010). To shorter DNA sequences allow, in fact, more easily transferring genes of interest into progenies subject to genetic improvement programs. The microsatellite analysis was performed to discriminate between dihaploids that have gametic origin (DHs) and diploids of somatic origin. To discriminate between these two types of regenerants, single sequence repeat (SSR) markers, heterozygous for parental genotypes, were used in screening. Particularly, six SSRs were selected, namely MEST15, MEST470, MEST88, MEST308, ACA01, and mCrCIRO3B07, and they were heterozygous in both sweet orange and clementine mother plants.

All regenerated Marisol 4x and Clemenules 4x cultivars, even if the level of ploidy was diploid, showed allelic models identical to those of the original mother plants from which the regenerates were obtained, so that from a genetic point of view they were heterozygous (Fig. 2.9).

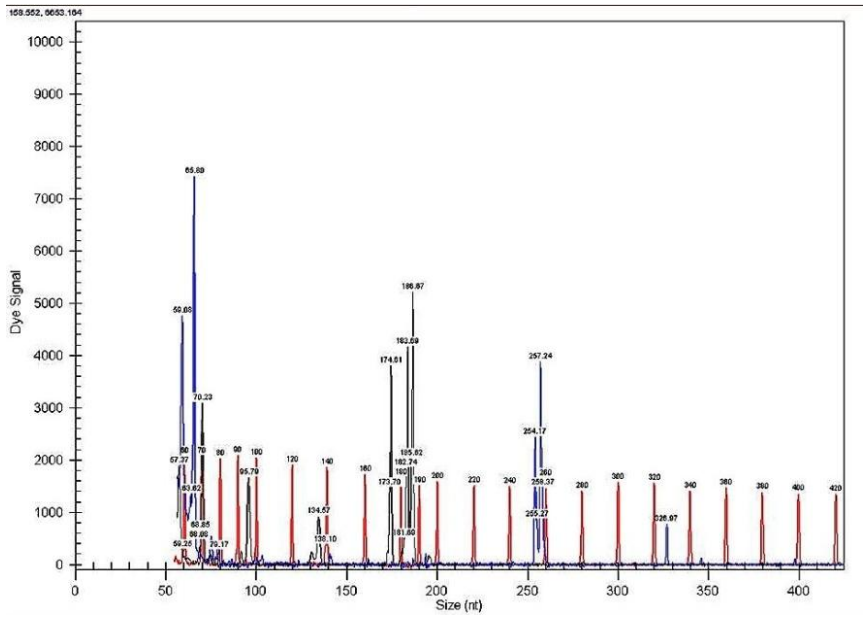


Figure 2.9. Amplification of the SSRs MEST15 and MEST470 of the embryo "Marisol"; For both markers, the peaks correspond to those of the heterozygous mother plant.

In the Moro Los Valles 4x variety, of the 41 regenerated obtained from the anthers culture, 7 tetraploids had an allele profile identical to that of the heterozygous mother plant, suggesting the somatic origin of these regenerated. This last type of regeneration through somatic embryogenesis from anther culture has been observed previously in *citrus*, being a valuable issue for clonal propagation, synthetic seed production, *in vitro* elimination of pathogens and germplasm storage (Germanà 2005; Germanà et al. 2011a). The remaining embryos analysed (34) resulted diploid, of which, for the allelic profile investigated: 5 homozygotes dihaploids, of which 3 with only one mutant allele (Fig. 2.10) and 2 heterozygotes mutated in one allele (Fig. 2.11), 27 heterozygotes with the same allelic profile of the mother plant.

The regenerated from Sanguinelli 4x cultivar, that were: 6 tetraploids and 33 diploids, in both cases the allelic profile was identical to that of the heterozygous mother plant. In the comparison with the Sanguinelli 2x cultivar, all the regenerated were diploid heterozygous to the allelic profile and therefore of somatic origin (Tab. 2.7; Fig. 2.12).

Regeneration through somatic embryogenesis is rather common in different *Citrus* species, and it is also valuable for clonal propagation, synthetic seed production, and germplasm storage (Germanà 2005; Germanà et al. 2011).

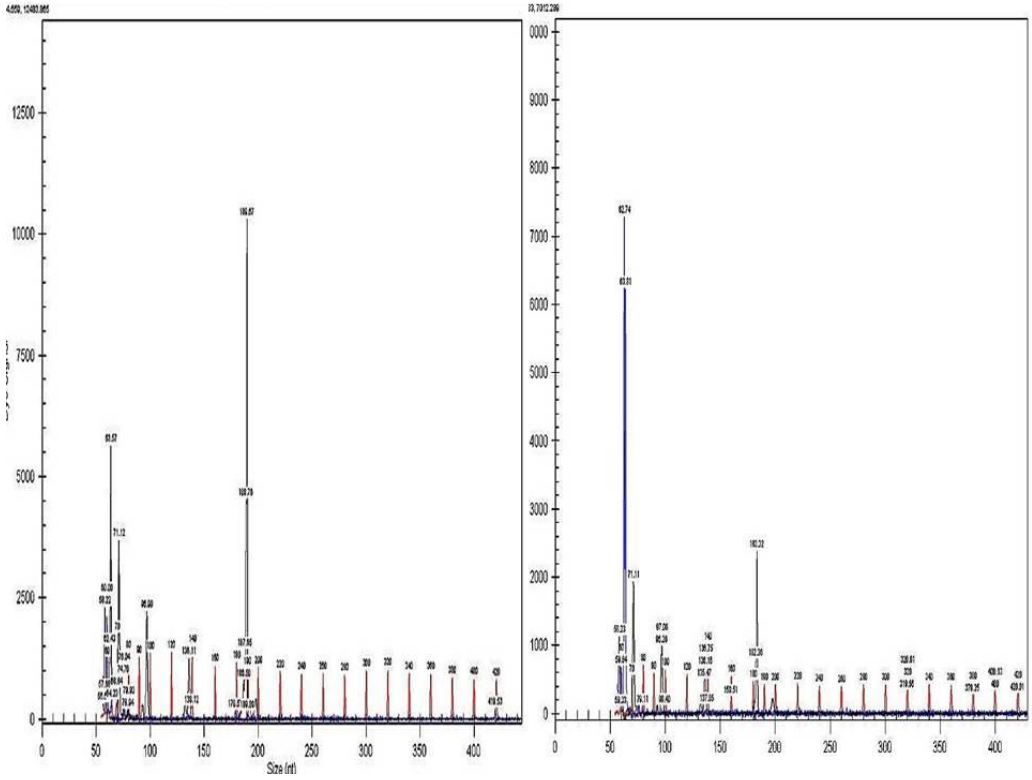


Figure 2.10. Microsatellite analysis: Pherograms of the microsatellite marker MEST15, for two different embryos, obtained from the anthers culture of the Moro Los Valles 4x variety. The respective phenograms indicate a mutated homozygous allele, "189" in the left phenogram and a double haploid in the right right one.

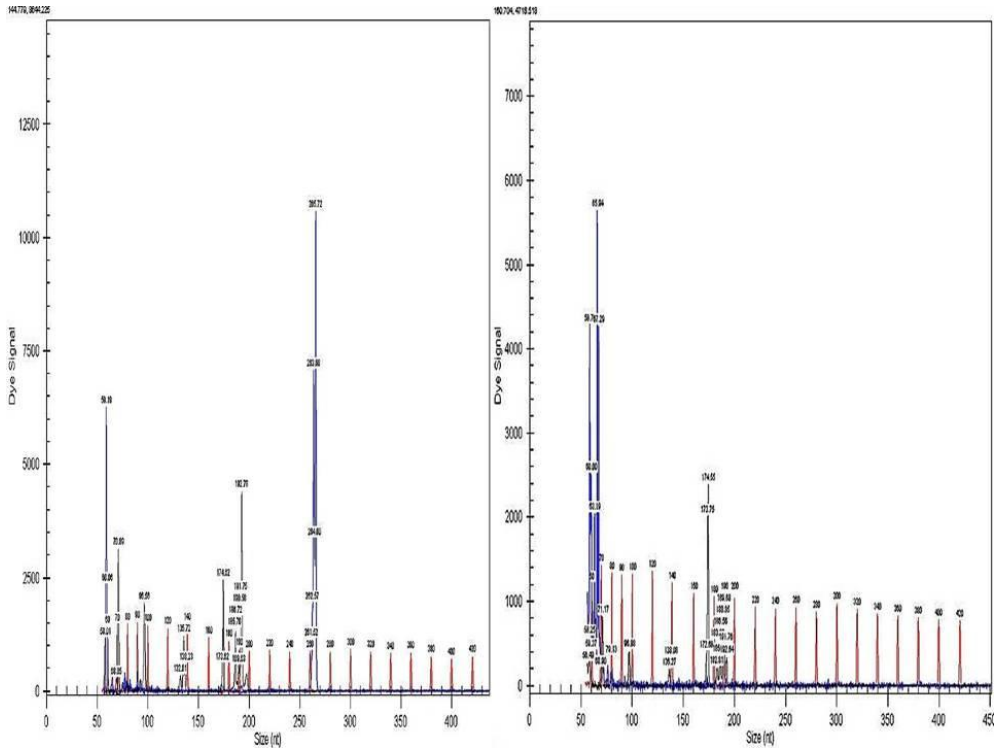


Figure 2.11. Microsatellite analysis: pherograms of the microsatellite marker, for two different embryos, obtained from the anthers culture of the Moro Los Valles 4x cultivar. The left phenogram indicates the analysis of an embryo analyzed with two respective markers; for MEST15, the regenerated is heterozygous with a mutated allele, while for mCrCIRO3B07, the allelic profile is heterozygous as in the mother plant. In the right pherograms, the embryo was analyzed with only the MEST15 marker, from the analysis it emerged that the regenerated is heterozygous with a mutated allele. The presence of alternative alleles from the mother plant has been considered as support for the gametic origin of regenerants.

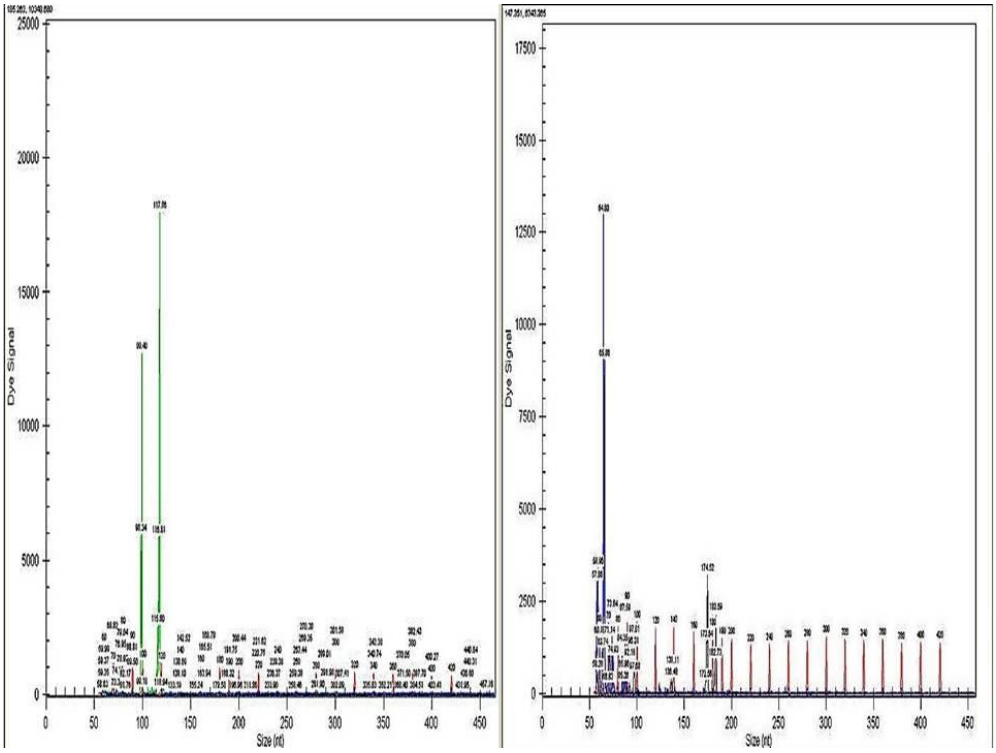


Figure 2.12. Microsatellite analysis: pherograms of the microsatellite marker, for two different embryos, obtained from the anthers culture of the Sanguinelli 4x (left) and Sanguinelli 2x (right). Phenogram of microsatellite markers: MEST88 (left) and MEST15 (right) of the profiles of two respective embryos generated by Sanguinelli 4x and Sanguinelli 2x cultivars Although the mother plant is heterozygous and carries two alleles, the regenerants will display either allele of their mother plant.

Numerous investigations have been performed to obtain DHs, but they resulted instead in heterozygous somatic plantlets. In particular, anther culture has produced somatic regenerants in *C. aurantium* (Hidaka et al. 1981; Germanà 2005); *C. sinensis* (Hidaka 1984); *C. aurantifolia* (Chaturvedi & Sharma 1985); *C. madurensis* (Ling et al. 1988); *C. reticulata* (Germanà et al. 1994; Germanà 2005); *P. trifoliata*, and the hybrid 14 of *C. ichangensis* x *C. reticulata* (Deng et al. 1992).

2.4 Conclusion

Anther culture showed to be a suitable method to whole genome halving in *citru*s tetraploids, because most of obtained regenerants were diploids. Dihaploids embryos were produced in fact through anther culture and analysed by flow cytometer and single sequence repeat (SSR) markers. Unfortunately, the dihaploids, deriving from microspores (gametes generated after meiosis), exhibited the occurrence of recombination between chromosome.

This, as previously achieved in other genotypes, could be interesting for introgression of useful traits.

Chapter 3. Application of the synthetic seed technique for *in vitro* conservation of the Lebanese fig (*Ficus carica* L.), variety Houmairi.

In this study, the effect of two plant growth regulators: 6-benzilaminopurine (BAP) and meta-Topoline (MT), was evaluated on viability, regrowth, conversion, shoot and root length, of encapsulated uninodal segments of the Lebanon variety Houmairi of *Ficus carica* L.. The results confirmed the practical applicability of encapsulation technology for propagation of this species. Although the conversion rates were not high, plantlet production for treatment with cold storage (4 °C for 45 days) and the addiction of meta-topoline to endosperm, provided a satisfactory conversion rate (23%). To our knowledge, this is the first report of rooting achievement in a single step of medium-term storage of encapsulated *Ficus carica,* cv. Houmairi nodal segments.

3.1 Introduction

The genus *Ficus* belongs to the family of the *Moraceae* and includes about 800 species spread by the Tropics to temperate countries. The most known species is cultivated in the Mediterranean basin is *Ficus carica* L., of which about 364,108 hectares are cultivated in the whole world (FAOSTAT 2014). The species is native of western Asia and the Mediterranean and has spread over time in the temperate and subtropical zone of Worldwide. Syconium can be eaten fresh or dried. In the Italian fruit-growing panorama, fig is today a minor species. Moreover, the fig tree suffers of poor innovation in propagation methods and of a growing spread of viral and non-viral diseases that affect production. Among the propagation methods undoubtedly those employed for the fig tree vegetative, they are more important. These methods, however, contribute significantly to spread of pathogens, which break down the potential productive of the varieties. Among the innovative methods of propagation, seeds synthetic or semi artificial, are able to combine the advantages of micropropagation with the ease of handling, storage possibilities and ease of transport of gamic seeds (Redenbaugh 1993). From several decades, several studies have allowed the identification of appropriate encapsulation protocols, either with unipolar propagules (microcutting), that with embryos somatics (Germanà et al. 2010). Regarding *Ficus carica* L., a study was performed by Sharma et al. 2015, in which a efficient regeneration and conservation system a short term of encapsulated nodal segments.

This protocol has been improved by Yahyaoui et al. (2016), who managed to get rooted in a single phase, instead of changing the medium of culture.

The objective of the present work is the verification of the validity of calcium alginate encapsulation for medium-term conservation of the Lebanese variety of fig Houmairi. In particular, they have been encapsulated uninodal cuttings deriving from proliferation *in vitro* buds, evaluating the influence of two regulators of growth (PGR): 6-Benzylaminopurine (BAP) e meta-Topolina (mT), added to the artificial endosperm, and recording the parameters of vitality, recovery vegetative and conversion of synthetic seeds directly or after 45 days of storage at 4 ° C.

3.2 Material and Methods

Plant material and sterilization

The plant material used is constituted by the Houmairi cultivar of *Ficus carica* L. coming from collection of the Mediterranean Agronomic Institute, (IAM-Bari), Puglia. The propagules used for the realization of the synthetic seed were taken from sprouts multiplied *in vitro* and obtained by proliferation of apical and lateral dormant buds. The explants, taken from the mother plant in December, they were subjected to sterilization before being cultured, following the Yahyaoui et al. (2016) protocol.

Multiplication

The sterilized buds of Ficus carica L. were placed on a stabilization and multiplication medium agarized composed of salts and MS vitamins (Murashige and Skoog 1962), added with 30 g / L of sucrose, 4 mg / L of BAP and 0.4 mg / L of NAA (kumar et al., 1998) and solidified with 6.5 g / L of agar.

Encapsulation

Microtalee (3-4 mm long) without leaves and with an axillary bud, they were taken from buds in proliferation and subjected to encapsulation technology, as reported by Yahyaoui et al. (2016). They have been used as artificial endosperm, two different compound means from salts and vitamins MS (Murashige and Skoog 1962), added with 30 g / L of sucrose, 0.4 mg / L of NAA, 0.7 mg / L of GA_3 and solidified with 6.5 g / L of agar, and each contained 0.5 mg / L of a regulator of different growth: BAP or MT (Yahyaoui et al., 2016). In each Petri dish, 5 capsules were placed and per each treatment was used 30 (fig. 3.1). The present study also included two treatments (T):

• direct cultivation of encapsulated cuttings in cabinets of growth at a temperature of 25 ± 1 ° C, with light / dark photoperiod of 16/8 hours and white light supplied by fluorescent lamps (TMN 30W / 84; Philips, Surenses, France), at flux density photosynthetic photon (PPFD) of 35 μmol m-2 s-1.

• the conservation of synthetic seeds before at 4 ° C of temperature for forty-five days and then the crop in growth cabinets with the same conditions of temperature and brightness of the previous thesis.

Recorded data and statistical analysis

After 45 days the following parameters were recorded: vitality (% of green-looking explants, without necrosis or yellowing), vegetative growth (% of encapsulated micro cutting that produces shoots or roots longer than 4 mm) and conversion (% of emergence of shoots and roots at least 4 mm long of the

encapsulated microtalee). The data was analyzed using the two-way ANOVA test, using the version 17 SPSS (SPSS Inc, Chicago, IL, USA), followed from the Tukey test, for P <5%. The factors considered are states: (T), type of test (seeds stored at 4 ° C for 45 days or directly seeded) and (P), different regulators of growth: BAP or mT.

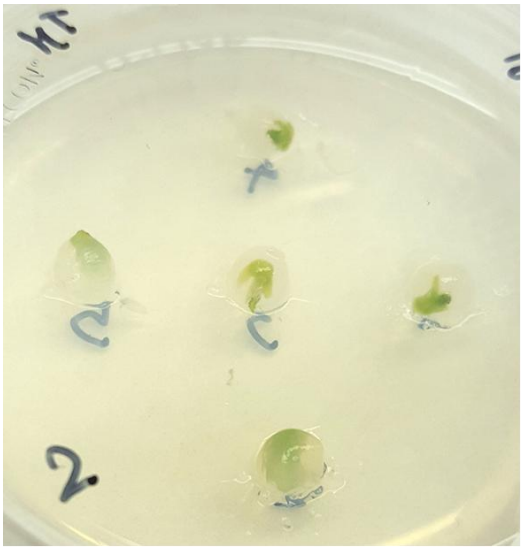


Figure 3.1. Encapsulated microcuttings of the Lebanon fig genotype Houmairi

3.3 Results and Discussion

Vitality

Synthetic seeds showed a average vitality 85.5%. Regarding the hormone content, the BAP showed a higher average vitality (83%), compared to MT (63%) with regard to conservation cold (C-S), while, seeds not conserved (N-C-S) showed, 45 days after sowing, 100% vitality when BAP was added, compared to 97% of vitality when mT was added to artificial endosperm. However, these differences do not were statistically significant (P = 0.068) (tab. 3.1).

Regrowth

The average vegetative restart of the encapsulated microtalee was 60%. In particular, the cuttings encapsulated with the BAP showed a recovery vegetative of 65% (fig. 3.2) and those encapsulated with mT of 55%. This difference did not result statistically significant (P = 0.245). For what concern comparison of seeds not stored and stored at 4 ° C for 45 days before sowing, vegetative growth of the former it was clearly higher (73% *vs* 47%). In detail, the non-preserved seeds presented a greater vegetative restart when the BAP was added to the artificial endosperm: 87% *vs* 60%. This difference was statistically significantsignificant (P = 0.019).

For preserved seeds, on the other hand, the behavior is upper state for encapsulated microtalee with endosperm artificial containing mT (50% *vs* 43%). There average length of the shoot produced by synthetic seeds unpreserved, it was 0.88 cm, statistically higher (P = 0.009) in seeds containing BAP (1.09 cm) compared to those containing mT (0.67 cm) (data not shown). The length of the shoots registered after 45 days from sowing in the stored seeds is was 0.55 cm, and statistically not different (P = 0.743) was the behavior of containing seeds mT and BAP (0.58 cm *vs* 0.53 cm) (no data show yourself).

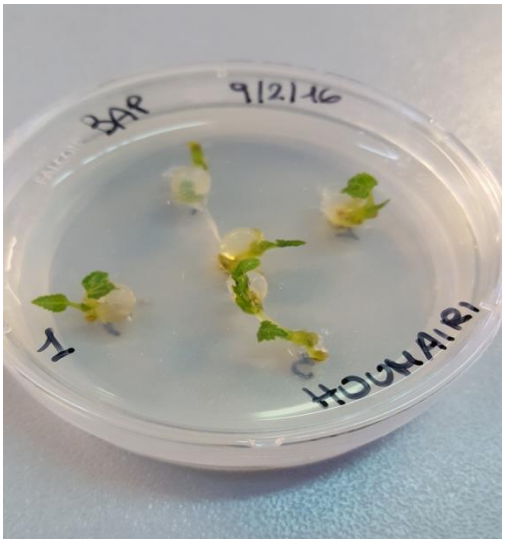


Figure 3.2. Regrowth of encapsulated nodal segment, stored at 4 °C, after 2 weeks from sowing using BAP.

Conversion

Conversion is the most interesting datum to detect as it indicates the microtalee that have sprouted and produced the root. A value has been registered statistically different conversion rate (P = 0.015) between seeds containing mT (17%) and those containing BAP (3%) (fig. 3). Relative to the comparison among the treatments among the non-preserved and preserved seeds at 4 ° C for 45 days before sowing, the first recorded a conversion of 7%, significantly lower than that found in preserved seeds (13%).

The no-preserved seeds showed, a superior conversion when the endosperm was added with mT (10%) compared to when it was added with BAP (3%). Similarly, in stored seeds, for 45 days at 4 ° C, mT induced one much higher conversion (23%) compared to 3% of the BAP and the difference was statistically significant (P = 0.023) (tab. 3.1). As for the length of the roots produced after 45 days from sowing from the encapsulated microtalee, it was recorded an average equivalent value of the seeds not stored and stored (0.61 cm *vs* 0.61 cm). In particular, both in the thesis of the no-preserved seeds that preserved, the MT has recorded higher values than the BAP: 0.83 cm *vs* 0.40 cm in the non-preserved seeds e 0.73 cm *vs* 0.50 cm in preserved seeds.

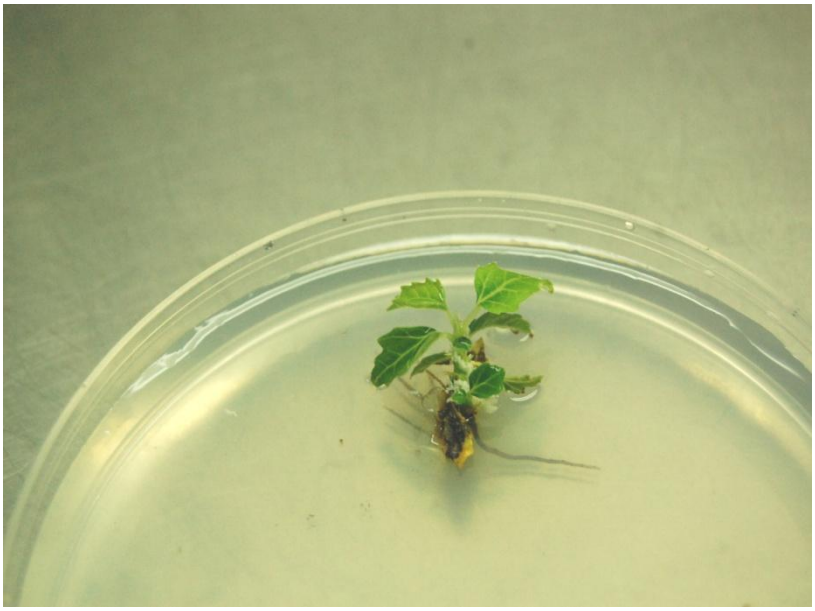


Figure 3.3. Conversion of encapsulated nodal segment, stored at 4 °C, after 6 weeks from sowing, using BAP as PGR.

Table 3.1. Viability, regrowth and conversion rates, according to the type of test (conserved or not) and PGRs used.

	Vitality (%)			**Regrowth (%)**			**Conversion (%)**		
PGR (P)	**BAP**	**MT**	**Total**	**BAP**	**MT**	**Total**	**BAP**	**MT**	**Total**
Treatment (T)									
Conserved C-S	83	63	73	43	50	47	3	23	13
No-conserved N-C-S	100	97	98	87	60	73	3	10	7
P	0.068			0.245			0.014		
T	<0.001			0.002			0.216		
P x T	0.16			0.054			0.216		

3.4 Conclusion

The results of the present encapsulation study of *in vitro*-derived microcutting of the Lebanese cultivar Houmairi of *Ficus carica* L., confirmed the applicability of this propagation method to this species. Although the conversions have not been long high, the thesis that provided for the conservation treatment cold (4 °C for 45 days) and use of the growth regulator mT, reached a suitable percentage of conversion rate (23%). For the first time, this technology has been applied to the genotype Lebanese fig tree Houmairi and the conversion of synthetic seed figs was obtained directly after the sowing them, confirming what was obtained from Yahyaoui et al. (2016), without change of means, like instead it was necessary in previous studies (Sharma et al. 2015). Encapsulation occurs, therefore, as a valid means of propagation of *Ficus carica* L., alongside the traditional protocols of

micropropagation and may represent if supported from further research aimed at obtaining percentages superior conversion, even a valid one support for commercial nursery.

Chapter 4. Influence of the compositin of medium on the micropropagation of Hop (*Humulus lupulus* L.).

In this study the technique of *in vitro* propagation was applied to the Cascade variety of *Humulus lupulus* L. starting from the collection of the plant material. The plant material was collected from a private field in Aragona (Agrigento, Sicily, Italy), it was proceeded to the sterilization of the same, in order to realize and stabilize the crop of the explants from which uninodal microtales have been taken to be cultured in nine different cultivation medium. The medium are composed of the same mineral elements and vitamins (Murashige and Skoog, MS) and the same carbon sources (sucrose and maltose), but differ in type, concentration and combination of growth regulators (PGR), in order to determine the best combination of hormones for the multiplication phase. In this study, Thidiazuron (TDZ), Benzyladenine (BAP) and meta-Topolina (mT) were tested, in different combinations in the nine medium. In all the multiplication medium there is 1 mg / l of auxin, indole-3-acetic acid (IAA). For the rooting phase, 5 types of different culture medium have been created in which binodal cuttings have been placed, coming from the previous multiplication test. The rooting medium differs from each other in the concentration and ratio of two auxin hormones: IAA and Indole-3-butyric acid (IBA). The solidifying agent used was the agar for all medium at all stages. Once rooted seedlings were obtained and determined which is the best culture medium also for the rooting phase, we proceeded with the transplantation of the seedlings, *vitro* derivatives, in transparent Microbox Round jars with agriperlite substrate, added with a solution containing MS at half concentration. As for the multiplication, better results were obtained with the H8 medium (TDZ 0.5 mg / l, BAP 0.5 mg / l) which showed 89% budding but with an excessive callus development, such to consider the best H6 medium (TDZ 2 mg / l) better since, despite giving a lower germination result (88%), it induces a lower callus development. Regarding rooting, the best rooting percentage was observed in the HR4 medium (67%) containing 2 mg / l of IBA but, with a slightly lower rooting percentage (64%), the HR1 medium, with 1 mg / l of IAA, induces the lower development of callus among the tested means.

4.1 Introduction

Hops, *Humulus lupulus* L., belonging to the Cannabaceae family, is a herbaceous, perennial, and normally dioecious plant, although some monoecious individuals have been found in some wild populations in North American (Haunold et al. 1993). It is estimated that in the world there is an area, planted with hops, of 90,653 ha (2016), of which 30,215 ha only in Europe (FAOSTAT 2018). In cultivation only female plants are used, as they produce inflorescences, called cones, within which substances that represent a precious material for the beer industry are accumulated. *H. lupulus* is propagated mainly by rhizome segments or by herbaceous cuttings. However, this species also responds well to agamic propagation through *in vitro* culture of tissues. Micropropagation, except for somaclonal variability, not only allows the production of clonal plants, but is also used as a main phase for other processes, in tissue culture, in different plant species. In hops, in fact, it is also used as a phase of recovery from viruses

(Postman et. Al. 2005), in cryopreservation (Martinez et. al. 1999) and for transformation (Harlemann et. Al. 2003). Given the importance of hops in the world, over the years, some protocols have been proposed in carrying out their propagation *in vitro*, proving to be valid and confirming the success in the micropropagation of *H. lupulus* (Batista et al. 1996, 2000, 2008; Machado et al. 2018; Roy et al. 2001; Peredo et al. 2009).
Unfortunately it is not possible to set up a universal protocol that can be used for all species. Each species has a different protocol and even within the same species different protocols may be required for each cultivar considered. It seems commonly ascertained that the protocol to be performed must limit the formation of callus in the multiplication phase, since it regenerates from the callus, can go against somaclonal variability and therefore be different from the starting genotype (Peredo et al. 2006, 2009). Different culture medium have been proposed by different authors for the *in vitro* organogenesis of *H. lupulus* (Roy et al. 2001; Skof et al. 2007; Peredo et al. 2009; Gatica-Arias and Weber 2013; Machado et al. 2018).
The micropropagation technique used in this study was the multiplication starting from nodal segments equipped with axillary gems, as it is the most suitable method to guarantee the genetic stability of the plants obtained (Martins et. al. 2004).
In the present research the response that hops shows when placed, *in vitro* culture, on culture medium, in which different plant growth regulators were used, was analyzed. In particular, the response to different concentrations of PGR and to different PGR was evaluated. Finally, an attempt was made to ascertain whether different PGRs exercise a synergistic function when placed in a culture medium.

4.2 Material and Methods

Plant material and sterilization

The plant material used during the experiment was taken from a culture of *Humulus lupulus* L. Cascade variety, coming from a collection field present in Aragona (AG), Sicily, Italy. The Cascade variety is considered an aroma hop. The microcuttings were taken, in May 2018, from herbaceous shoots of plants grown in the open field. Sterilization, before *in vitro* culture, has included: 45 minutes under running water, in order to eliminate dust and phenolic substances, immersion in a solution of water and 10% detergent and stirring for 10 minutes, rinsing under running water to 5 minutes and then immersion in a 2% fungicide solution for 10 minutes while stirring; from this moment, the sterilization procedure was carried out under a laminar flow hood. The cuttings were immersed in 70% alcohol for 5 minutes. Subsequently, they were immersed in a 30% sodium hypochlorite solution, with the addition of a few drops of Tween 20, for 20 minutes. After removing the sodium hypochlorite solution, the explants were rinsed for 3 minutes each with sterile distilled water.

Multiplication

In order to identify the best PGR for multiplication, uninodals microcuttings were cultivated *in vitro* on nine different culture media (Table 4.1). The nine culture

media are composed of the micro-, macro-elements and vitamins of Murashige and Skoog (1962) (MS), to which were added, 10 g / l^{-1} of sucrose and 10 g / l^{-1} of maltose, as a source of carbon and 8 g / l^{-1} of agar as a solidifying agent. In all media 1 mg / l^{-1} of IAA was added as auxin. The media differ only in the type, concentration and ratio of cytokinins used. The cytokinins tested in this study are: Benzylaminopurine (BAP), meta-Topolina (mT) and Thidiazuron (TDZ). For each thesis 15 Petri dishes were made with a number of uninodal cuttings equal to 150 per thesis (10 explants for Petri box).

Table 4.1. Composition of the culture medium used during the multiplication phase.

Components	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**
MS salts and vitamins[1]	x	x	x	x	x	x	x	x	x
Sucrose gr/l^{-1}	10	10	10	10	10	10	10	10	10
Maltose gr/l^{-1}	10	10	10	10	10	10	10	10	10
IAA[2] mg/l^{-1}	1	1	1	1	1	1	1	1	1
BAP[3] mg/l^{-1}	1	-	-	2	-	-	0,5	0,5	-
mT[4] mg/l^{-1}	-	1	-	-	2	-	0,5	-	0,5
TDZ[5] mg/l^{-1}	-	-	1	-	-	2	-	0,5	0,5
Agar gr/l^{-1}	8	8	8	8	8	8	8	8	8
pH	5,8								

[1]Murashige and Skoog (1962); [2]IAA: Indolo-3-acetic acid; [3]BAP: 6-benzylaminopurine; [4]mT: meta-Topolin; [5]TDZ: Thidiazuron.

Rooting

Once the best hormone was established for multiplicative purposes, and a sufficient number of sprouts were obtained, lateral bimodal microcuttings were isolated for the rooting test; in particular, five different culture media were tested, taking cuttings from all the multiplication medium (Table 4.2). The rooting medium have the same composition as the multiplication culture medium with regard to micro and macronutrients, vitamins and carbon source, while they differ for the PGR. Each medium differs from the other in the type, concentration and ratio of auxins. The auxins selected in the test are: indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). For each thesis, 10 Microbox jars were made, for a total of 80 explants for thesis.

Table 4.2. Composition of the culture medium used during the rooting phase of Cascade variety.

Components	HR1	HR2	HR3	HR4	HR5
MS salts and vitamins[1]	x	x	x	x	x
Sucrose gr/l^{-1}	10	10	10	10	10
Maltose gr/l^{-1}	10	10	10	10	10
IAA[2] mg/l^{-1}	1	2	-	-	0,5
IBA[3] mg/l^{-1}	-	-	1	2	0,5
Agar gr/l^{-1}	8	8	8	8	8
pH	5,8				

[1]. Murashige and Skoog (1962); [2]. IAA: Indolo-3-acetic acid; [3]. IBA: Indole-3-butyric acid.

Culture conditions

For both tests, multiplication and rooting, the Petri dishes and the microbox vessels were incubated at 27 ± 1 ° C and placed under fluorescent lamps (Philips TLM 30w / 84) with photosynthetic photonic flux intensity of 35 μmol m − 2 s − 1 and a 16-hour photoperiod.

Data collection

Starting from one week, from the cultivation and for the whole period of the culture, for both phases, bacterial or fungal contaminations were monitored.

As for the budding, the data found after 45 days were:

• Germination (percentage of sprouted explants);

• Number of shoots per explant;

• Length of buds issued (cm).

Regarding radicazine, the surveys involved:

• Rooting (percentage of rooted sprouts);

• Number of roots issued;

• Length of the roots;

• Possible sprouting (percentage of cuttings that have sprouted);

• Presence of callus (percentage of cuttings that have generated callus).

Statistic analysis

The data were analyzed with the ANOVA one-way test, the Tukey's test was adopted with $p \leq 0.05$. The SYSTAT 13 software was used to perform the test. Only one factor was considered: "culture medium"; the differences between the media were tested by the analysis of two-way variance (ANOVA) with $p \leq 0.05$.

4.3 Results and Discussion

The results concerning budding and callus presence, as far as the multiplication test is concerned, are shown in table 4.3, figure 4.1; while the data on the budding percentage, number of shoots, rooting percentage, number of roots, length of shoots and roots, of the Cascade variety of *H. lupulus* L., are shown in table 4.3, figure 4.1.

Multiplication

The culture media, containing different concentrations of PGRs exerted a variable significant influence on the budding percentage. The culture media H9, H6 and H8 promoted the highest germination rate (86% *vs* 88% *vs* 89%) while the culture medium H3 was the lowest (0.53) (Tab 4.3.). In particular, as reported by Roy et. al. (2001b) and Machado et. al. (2018), the media added with the cytokine TDZ, have been reported as the best to promote an efficient multiplication system for hops. In the study by Roy et al. (2001b), a quantity of 0.5 mg / l of TDZ was used which provided a good response, from the explants, to multiplication. In the present experiment, the concentration of 0.5 mg $l^{-1}$of TDZ was presented again in H8 and H9 media (Table 4.1) to which unlikely the study by Roy et al. mentioned added, respectively, 0.5 mg l^{-1} of BAP and of mT, finding an increase in the budding percentage (Tabble4.3) compared to the previous study (65% *vs* 89%). It should be noted that the H3 medium, although promoting the lowest percentage of budding, contained the same TDZ, although, at a concentration of 1 mg l^{-1}.

It should be emphasized that the H6 medium is distinguished by a statistically lower production of callus compared to H8 and H9 (70% *vs* 90% and 91%), therefore its use is to be preferred in order to avoid the probability of somaclonal variability.

Table 3 shows the result of the ANOVA analysis. The P-value of the test is less than 0.05 (in our case 0.00), so there is a statistically significant difference between the averages of the sprouted explants between one culture medium and another, with a reliability level of 95 % (Table 4.3).

Table 4.3. Germination rates and callus formation related to the different medium of multiplication.

Culture medium	budding percentage	callus development percentage
H1	81% de	93% e
H2	69% b	79% d
H3	53% a	45% a
H4	71% bc	59% b
H5	79% cd	65% bc
H6	88% e	70% cd
H7	83% de	90% e

H8	89% e	90% e
H9	86% de	91% e

The different letters within each column indicate significant differences to p≤ 0.05 for the variable considered.

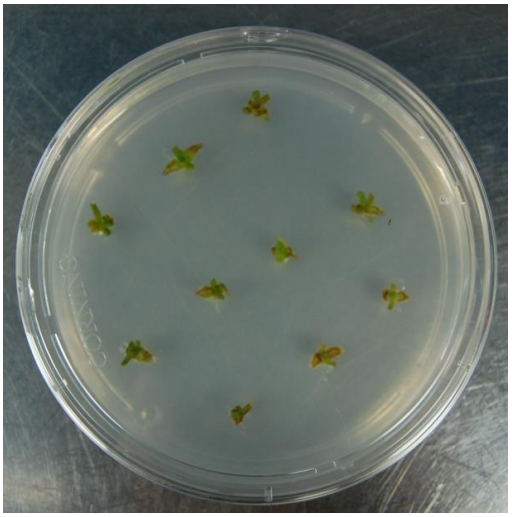


Figure 4.1. Microcuttings of *H. Lupulus* L. variety Cascade during the multiplication phase

As reported by other authors (Fortes and Pais, 2000), the presence of regulators of growth in the culture medium and, above all, the interaction between cytokinins and auxins in the *in vitro* culture of hops, it causes the callus to form at the base of the explant, with a variation between 45% (H3 medium) and 93% (H1 medium). Also for the callus formation, the statistical analysis, reports a difference statistically significant, among the average of the explants that present callus, between a medium of culture and another (tab 4.3).

From the data it can be deduced as to the answer of the explants, to the various cytokinin PGRs studied in the various media, the formation of callus cannot be related, in fact, the higher percentage of budding does not correspond to a higher percentage of samples that have formed callus. The answer seems to be related to the interaction "type / cytokinin concentration".

Rooting

During the experiment, the material from the multiplication test, taken from all crop media, was divided into nodal segments and cultured in rooting substrates (Tab. 4.2), in order to identify the best auxinic PGR, at order to develop an efficient *in vitro* rooting protocol.

The test was performed in microbox vessels, in which a further budding also occurred. It was therefore possible to evaluate the budding percentage, the number of shoots and their length. Table 4 indicates the average data of the parameters mentioned above. The percentage of microtalee that emitted roots was greater in

the middle HR4 (67%) in which, the length of the same, turned out to be superior to that observed in the other media. Also the HR1 medium proved to be valid, having given a rooting of 64% of the micro cutting in culture. The HR1 medium also gave a smaller percentage of cuttings to have developed callus compared to the HR4 medium (61% *vs* 65%). As regards the budding percentage, the highest value was found in the HR2 culture medium (94%) while the values observed in the other medium did not differ much from each other (Tab. 4.4).

Table 4.4. Rooting percentages, budding, and callus formation; averages obtained by number and length of roots and shoots.

Medium	HRI	HR2	HR3	HR4	HR5
Rooting percentage	64%a	59%a	54%a	67%a	53%a
n° rooting	3.6b	3.3ab	2.7ab	3.4ab	2.3a
Roots length (cm)	0.3b	0.2a	0.3a	0.4a	0.2a
Budding percentage	83%a	94%a	82%a	88%a	86%a
n° shoots	1.7a	2a	1.7a	1.6a	1.8a
Buds length (cm)	1,90b	1,28a	1,67ab	2,00b	1,95b
Callus percentage	61%a	82%b	65%a	65%a	77%ab

The different letters within each line indicate significant differences at $p \leq 0.05$ for the variable considered

Table 4 also indicates the average of the number of shoots that have developed for each half tested, showing that, as for the budding percentage, also in this case the largest number occurred in the middle HR2 (2.04). The statistical analysis, for this variable, indicates that there is no statistical difference neither within each medium nor in the comparison between them, since, the P-value is greater than 0.05 ($p = 0.45$). The statistically longer average length of the shoots, measured 45 days after being placed in culture, occurred in the middle of HR4 (2.00 cm) while the lowest in HR2 (1.28 cm) (Table 4.4). The percentage of cuttings that have emitted roots (Fig. 4.6), according to the auxinic PGR used, is shown in Table 4. The highest figure is found in the HR4 medium (67%) which has a concentration of 2 mg / l of IBA. The lowest average value (53%), instead, occurred when both IAA and IBA with the same concentration (HR5) were used together in the middle Despite this, the statistical analysis showed no statistically significant differences, which instead were found for the number of roots (Tabble 4.4).

The maximum number of roots was obtained in the medium supplemented with 1 mg / l of IAA while the lowest, as for the rooting percentage, in the HR5 medium (Table 4.4). The maximum average length of the roots was the same for the media added with IBA regardless of their concentration. As with the other variables evaluated, the HR5 half factor was the worst. Data analysis in no case showed statistically significant differences (Table 4.4).

It was noted that at the base of the explants, there was callus formation, with statistically significant values that even reached 82% levels (Table 4.4).

Figure 4.2. Microbox potted plants during rooting stage of ??.

Acclimatization

The success of the micropropagation is not only to produce a large number of healthy and genetically identical plants to the mother plant, but also, in the attention at the time of their transfer to the field. In order to minimize the cost of this stage, the derived *vitro* seedlings, having, after the rooting phase, a well-formed root system (Figure 4.3), were transferred to sterile microbox rounds, containing sterilized agriperlite and a solution liquid at half concentration, of mineral, salts and vitamins MS. After an accurate washing with sterile distilled water, in order to remove all the residues of the solid culture medium that can cause the development of fungi, these *vitro* derived seedlings were incubated under the same conditions of brightness, temperature and photoperiod of the micro propagated seedlings *in vitro*. After thirty days of culture under the same conditions previously reported, the survival rate was 97.14% (Figure 4.4).

Figure 4.3. Plant derived from *vitro* before transfer to agriperlite..

Figure 4.4. Plant transferred to microbox round with agriperlite

4.4 Conclusion

The study conducted on the Cascade variety of *Humulus lupulus L*., highlighted the great regenerative potential of this species. In testing the different means for multiplication and, subsequently, for rooting, sprouting results were detected even though variable, in any case positive (H3: 53% min. - H8: 89% max.). The species seems to show an excellent propensity to micropropagation, regardless of the hormones used. However it was possible to highlight which medium has given more satisfactory results. TDZ has been shown to provide the best results thanks to the synergistic effect obtained with the BAP. The TDZ used as the sole PGR, at a concentration of 1 mg / l, showed the worst results, while, in a concentration of 2 mg / l, it provided an excellent percentage of budding of the microtalee, with a smaller fraction of them having developed callus with respect to vehicles with both PGR. The rooting test provided, by any means, good results (HR5: 53% min. - HR4: 67% max.), Finding that the best percentage of rooted microtalee is obtained using only IBA, in a concentration of 2 mg / the. The derived vitro plants showed an excellent survival rate, with values of about 97%.

The good results obtained show that the micropropagation of hops can be a good alternative in nursery production. In a country like Italy, where hops are gaining more and more popularity, micropropagation can provide a large number of plants for the establishment of new plants in the territory, in a more efficient way than the traditional propagation methods used for the species *Humulus lupulus*.

Chapter 5. Encapsulation of *in vitro*-derived propagules of two genotypes of *Capparis spinosa* (L.) from Pantelleria Island.

Encapsulation technology, combining the advantages of zygotic or gamic seed with those of micropropagation, has recently attracted the interest of researchers as a new propagation approach, mainly due to the unsatisfying results of the traditional propagation strategies. The encapsulation of uninodal microcuttings (3-4 mm long) from two Sicilian *Capparis spinosa* (L.) genotypes (from Pantelleria Island: Tracino and Scauri) was evaluated, observing the influence of the calcium alginate coating and of three different growth regulators (PGRs): 6-Benzylaminopurine (BAP), meta-Topolin (mT) and Zeatin (ZEA), on viability, regrowth and conversion of the propagules.

Caper microcuttings were dissected and placed in different Murashige and Skoog based artificial endosperms, enriched of mT or ZEA or BAP. The synthetic seeds obtained were sown on a medium with full strength salts concentration, enriched with 0,4 mg/L of naphtalene acetic acid and 0,7 mg/L of gibelleric acid. After 60 days, the following parameters were detected: viability, regrowth, number and length of the shoots and roots, conversion.

The results confirm that encapsulation did not negatively affect the viability, which showed the highest percentage with BAP (100%) in Tracino and with ZEA (100%) in Scauri. Similar results were obtained in regrowth, with statistically significant differences among the three PGRs tested: Tracino showed the best regrowth on capsuled enriched with BAP (100%), Scauri with ZEA (100%). In addition, the synseed conversion was greatly affected by the PGR, and it was higher in artificial endosperm added with BAP in Tracino (56.6%) and ZEA in Scauri (23.3%) genotype.

5.1 Introduction

The genus *Capparis* belongs to the family *Capparaceae* and includes about 250 species (Fici, 2001). Higton and Akeroyd (1991) and Heywood (1993) included all the European populations of caper within *Capparis spinosa* L., with two subspecies, subsp. *spinosa* and subsp. *rupestris* (Sibth & Sm.). The subspecies *rupestris* grows in the Mediterranean Basin and is extremely drought-resistant. Cornelini *et al.* (2008) in a recent study states that caper, as other plants typical of the Mediterranean area, is able to reduce the soil erosion in rocky and steep environments and to preserve the water supplies from the ground, thanks to the large and far-reaching radical system. The flower bud of the plant, called "caper", is largely used in the Mediterranean food industry after a treatment with sea salt and sometimes with vinegar. The fruit of the plant, called "caper berry", is also used in the Aeolian cuisine (Legua *et al.*, 2013). The caper, for its antioxidant, anti-fungal, anti-inflammatory, anti-diabetic and anti-hyperlipidemic properties (Tesoriere *et al.*, 2007; Lo Bosco *et al.*, 2019), is used in the food industry and for the production of different traditional phytomedicines.

Its properties made the caper a crop of great economic significance in the Mediterranean Basin both for the local market and for export. In Italy, during the last three decades, the caper has been cultivated mainly in the Salina and Pantelleria islands, where it has become an important "economic culture" (Barbera

and Di Lorenzo, 1982; Giuffrida et at. 2002). The caper from Pantelleria Island received in 1996 the European designation of "protected geographical indication" (PGI), thanks to the high quality of the product.

Although there is an increasing interest in growing caper as a commercial crop, it is still difficult to propagate it by seedlings because of the poor seed germination. Sozzi and Chiesa (1995) attributed the poor germination of caper seed to dormancy, imposed probably by the seed coat. The application of cold stratification, soaking in H_2SO_4, GA_3, and KNO_3 are *in vivo* methods to increase germination percentage (Tansi 1999; Ölmez et al. 2004). Scarification through seed coat rupture has been determinant to improve germination percentage (Germanà and Chiancone 2009). Encapsulation technology, combining the advantages of zygotic seeds with those of micropropagation (Rodriguez et al. 1990), has recently attracted the interest of researchers as a new propagation approach, mainly due to the unsatisfying results of the traditional caper propagation strategies. Airò et al. (2011) tested successfully the possibility of encapsulating microcuttings of *Capparis spinosa* L., observing a different response of the propagules to different Murashige & Skoog (1962) based germination media enriched or not with IAA.

In this research, the influence of three growth regulators on caper synseeds conversion of two local genotypes from Pantelleria Island was observed.

5.2 Material and Method

Plant Material and Sterilization Protocol

For the experiment two different local genotypes of *Capparis spinosa* L. from different areas of Pantelleria Island (Trapani, Sicily): Tracino and Scauri, were used.
The propagules for the synthetic seeds were cut from *in vitro*-derived plants obtained after *in vitro* seed germination. The seeds were collected from fruits of adult *Capparis spinosa* L. plants, grown in open field and subjected to sterilization before the *in vitro* sowing.
The sterilization protocol consisted in the immersion of seeds in a solution of water and 20% commercial bleach for 10 minutes on a shaker. The plant material was subsequently rinsed under running water for 5 minutes and dried overnight on a layer of absorbent paper. The sterilization protocol was completed in aseptic conditions, soaking the seeds in 70% alcohol for 10 minutes. The seeds were then washed for 3 minutes with sterile distilled water and immersed for 20 minutes in a 20% commercial bleach solution with the addition of few drops of Tween 20. At the end, the seeds were rinsed 3 times of 3 minutes each with sterile distilled water. After the sterilization, the seeds were germinated *in vitro*.

Multiplication

From the seedlings *in vitro*-derived and for each of the two genotypes of *Capparis spinosa* L. tested, epicotyl and hypocotyl segments, 1 cm in length, were taken and placed horizontally on a MS-based multiplication medium

supplemented with naphtalene acetic acid (NAA, 0.4 mg/L^{-1}), gibelleric acid (GA_3, 0.5 mg/L^{-1}), meta-topolin (mT, 1 mg/L^{-1}) and solidified with agar (8 g/L^{-1}). The multiplication was carried out for three months, sub-culturing the shoots every three weeks, in order to have a sufficient number of contamination-free propagules for the encapsulation.

Encapsulation

Uninodal microcuttings (3-4 mm) were collected from the *in vitro* derived shoots and used for the encapsulation. To evaluate the effects of a concentration of 0.5 mg/L of BAP (6-Benzylaminopurine) or mT or ZEA (Zeatin) on viability, regrowth and conversion, three encapsulation kits, differing for their PGRs composition, were used. Each encapsulation kit consisted of a coating, a complexing and a rinsing solution with the composition shown in Tab. 5.1.

Table 5.2. Encapsulation kit composition of *Capparis spinosa* L. enriched with BAP, mT, or ZEA.

Components	**Coating solution**	**Complexing solution**	**Rinsing solution**
Artificial endosperm	1X MS[1] + vitamins, 54.0 g/L sucrose, 0.4 mg/L NAA[2], 0.7 mg/L GA_3[3]		
Sodium alginate	25.0 g/L	-	-
$CaCl_2$	-	11.0 g/L	-
BAP[4]/mT[5]/ZEA[6]	0.5 mg/L	0.5 mg/L	00.5 mg/L
pH	5,8		

[1]MS, Murashige and Skoog 1962; [2]NAA, 1-Naphthaleneacetic acid; [3]GA_3, Gibberellic acid [4]BAP, 6-Benzylaminopurine; [5]mT, Meta-topolin; [6]ZEA, Zeatin.

The kits were autoclaved at 120° for 20 minutes before being used for encapsulation. The microcuttings were first deeped in the coating solution containing sodium alginate (2.5 % w/v); then, the alginate-coated propagules were dropped into the calcium chloride ($CaCl_2$)-complexing solution (1.1% w/v) to obtain the gelling of the coating matrix; finally, to remove the ionic residues, capsules were washed for 15 minutes in the rinsing solution.

Synthetic seeds were sown on a germination medium with the composition shown in Tab. 5.2 and incubated at a temperature of 25 ± 1 °C with a photoperiod of 16/8 (light/dark).

Six Petri dishes for each treatment, with 5 synseeds/dish, were prepared.

Table 5.2. Culture medium used for synthetic seeds germination.

Components	Concentration
MS[1] Salts e vitamins mix.	1X
Sucrose	54.0 g/L^{-1}
NAA[2]	0.4 mg/L^{-1}
GA_3[3]	0.7 mg/L^{-1}
Agar	8.0 g/L^{-1}
pH	5.8

[1]MS, Murashige and Skoog 1962; [2]NAA, 1-Naphthaleneacetic acid; [3]GA_3, Gibberellic acid

Recorded data and statistical analysis

After 60 days, the following parameters were recorded: viability (n° of green encapsulated explants, without necrosis or yellowing /n° of total encapsulated explants), vegetative regrowth (n° of germinated synseeds with protruding shoots longer than 4 mm/total n° of synseeds), and conversion (n° of germinated synseeds with protruding shoots and roots longer than 4 mm/total n° of synseeds) of the synthetic seeds. In addition, the number of shoots/explant, the number of roots/explant, the length of the shoots and the length of the roots were recorded. Statistical analysis was carried out using SYSTAT 13 software. The factors considered were (G), genotypes and (P), plant growth regulation. The differences between them were tested by bidirectional variance (ANOVA) analysis, at $p \leq 0.05$ level. Tukey test was used to separate the means.

5.3 Results and discussion

In the present study, the encapsulated uninodal microcuttings of *Capparis spinosa* L., sown on the culture medium and at the conditions reported on 'Material and Methods' showed the results shown in Tab. 5.3.

Table 5.3. Effects of the three PGRs on synthetic seeds viability, regrowth and conversion of two local genotypes, after 60 days from their sowing.

Genotype	PGR	Viability (%)	Regrowth (%)	Conversion (%)
Tracino	**mT**	93.3 a	93.3 a	6.6 a
	BAP	100 a	100 a	56.6 b
	ZEA	96.6 a	96.6 a	0 a
Scauri	**mT**	76.6 a	70 a	3.3 a
	BAP	96.6 b	96.6 b	13.3 ab
	ZEA	100 b	100 b	23.3 b

Within each line and each genotype, different values followed by different letters, indicate significant differences at $p \leq 0.05$. One-way ANOVA, followed by Tukey's test, $p \leq 0.05$.

The data proved a non-toxic effect of the calcium alginate matrix on the viability (%) of the synseeds and that a sodium alginate concentration of 2.5% w/v instead of 4% w/v, reported as optimal by Airò, (2011), can be used. The highest viability (%) value was obtained by synthetic seeds enriched with BAP (100%, Tracino) and ZEA (100%, Scauri). Similar results were observed in the regrowth (%): Tracino obtained the best regrowth on capsules enriched with BAP (100%), Scauri with ZEA (100%). A strong influence of the PGRs was also found for the conversion value of the synseeds, which was higher in BAP for Tracino (56.6%) and ZEA for Scauri (23.3%) (Tab. 5.3).

Table 5.4. Effects of local genotype and PGR on synthetic seeds n° of shoots and roots per explant (average), and on shoot and root length (average in cm), after 60 days from their sowing.

		n° Shoots/explant (average)	Shoot length (average in cm)	n° Roots/explant (average)	Root length (average in cm)
Genotype	**Tracino**	0.96 a	0.21 a	0.85 a	1.22b
	Scauri	0.92 a	0.14 a	1.15 a	1.04 a
PGR	**mT**	0.85 a	0.05 a	1.00 a	1.05 a
	BAP	0.98 a	0.37 b	0.92 b	1.33 b
	ZEA	0.98 a	0.12 a	1.07 a	1.02 a

Within each line and each parameter (G and PGR), values followed by different letters are statistically different at $p \leq 0.05$. Two-way ANOVA, followed by Tukey's test at $p \leq 0.05$.

Tracino genotype showed to be the best in term of root length, obtaining an average value of 1.22 cm, unlike the other genotype Scauri tested that obtained an average value of 1.04 cm. The two genotypes did not show big differences in determining the n° of shoots per explant, obtaining approximately the same average value (Tab. 5.4). About the PGRs tested, the capsules containing BAP had an average root length of 1.33 cm, followed by those with mT (1.05 cm) and ZEA (1.02 cm). Shoot formation was observed from synthetic seeds of both genotypes as reported in Table 4. BAP seemed to be the best growth regulator for the shoot elongation with an average value of 0.37 cm, followed by ZEA (1.12 cm) and mT (0.05 cm).

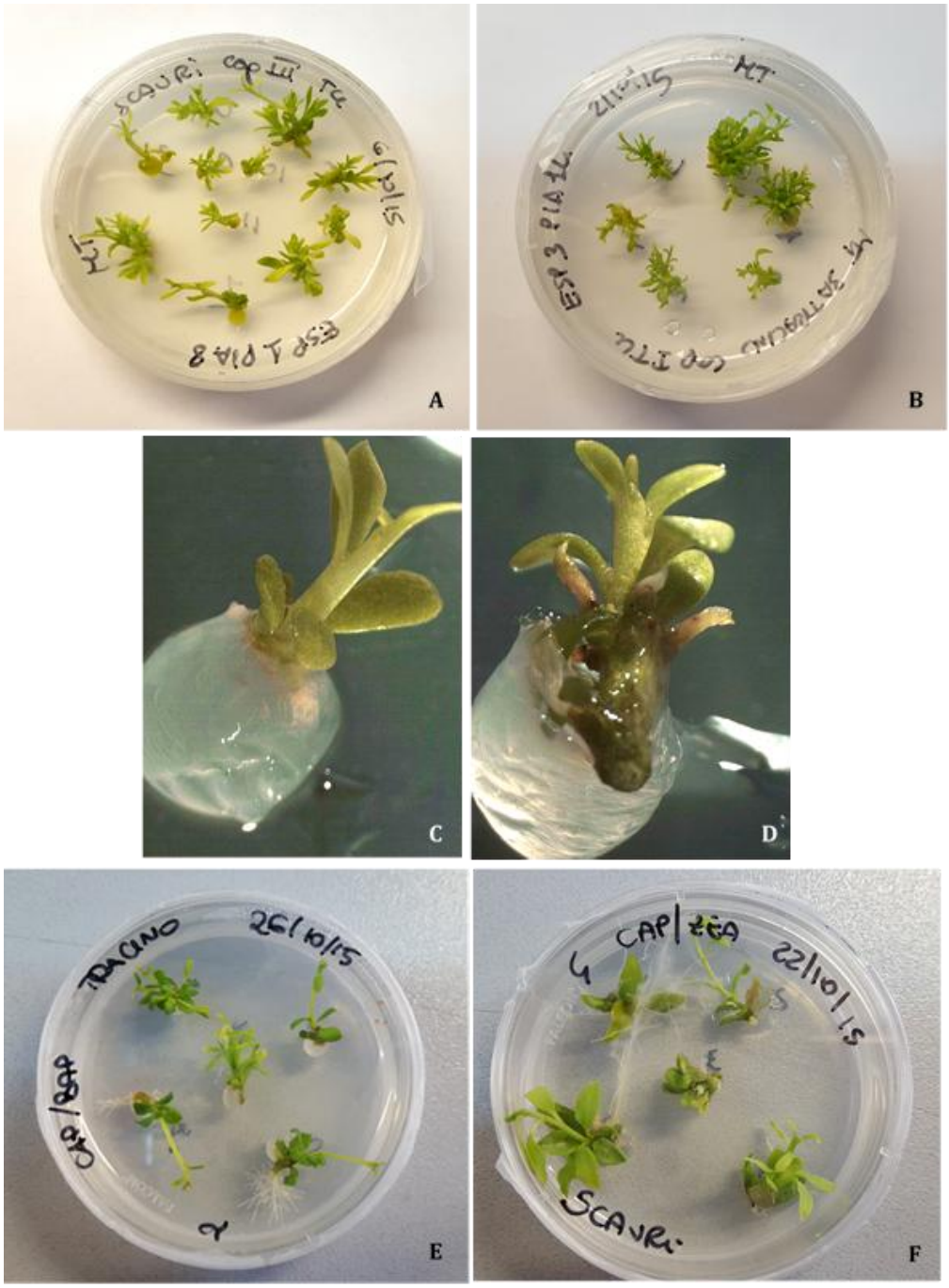


Figure 5.2. *In vitro* multiplication of Scauri (**A**) and Tracino (**B**); Germinated synthetic seed of Scauri (**C**) and Tracino (**D)** after 18 days from the sowing. Conversion and Regrowth of Tracino (**E**) and Scauri (**F**) after 60 days from the sowing, in presence of BAP and ZEA, respectively.

5.4 Conclusion

These results represent an advancement in the information of encapsulation in *Capparis spinosa* L.. In our knowledge, this is the first study in which this propagation strategy has been applied successfully in two genotypes of capers from Pantelleria Island: Tracino and Scauri. A BAP concentration of 0.5 mg/L^{-1} in the artificial endosperm of the synseed proved to be the best in terms of vitality, regrowth and conversion for the Tracino genotype. Differently, for the genotype Scauri, ZEA determined the highest values of the three main parameters observed. The encapsulation technology could be considered as a valuable micropropagation strategy of *Capparis spinosa* L. and, with further research, will be able to represent an important and cost effective support to the commercial nursery.

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Applicazione della tecnica del seme sintetico per la conservazione in vitro della varietà libanese di fico Houmairi

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Synthetic seed technology applied to in vitro conservation of Lebanon fig variety Houmairi

Abstract. In this study, the effect of two plant growth regulators: 6-benzilaminopurine (BAP) and meta-topoline (MT), was evaluated on viability, regrowth, conversion, shoot and root length, of encapsulated uninodal segments of the Lebanon variety Houmairi of *Ficus carica* L.. The results confirmed the practical applicability of encapsulation technology for propagation of this species. Although the conversion rates were not high, plantlet production for treatment with cold storage (4 °C for 45 days) and the addiction of meta-topoline to endosperm, provided a satisfactory conversion rate (23%). To our knowledge, this is the first report of rooting achievement in a single step of medium-term storage of encapsulated *Ficus carica*, cv. Houmairi nodal segments.

Key words: cytokinins, *Ficus carica*, encapsulation, micropropagation.

Introduzione

Il genere *Ficus* appartiene alla famiglia delle *Moraceae* e comprende circa 800 specie diffuse dai Tropici ai Paesi temperati. La specie più conosciuta e coltivata nel bacino del Mediterraneo è *Ficus carica* L., di cui si coltivano circa 364.108 ettari in tutto il mondo (FAOSTAT, 2014). La specie è originaria dell'Asia occidentale e del Mediterraneo ed si è diffusa nel tempo nella fascia temperata e subtropicale di tutto il mondo. Il siconio può essere consumato fresco od essiccato. Nel panorama della frutticoltura italiana il fico è oggi una specie minore. Inoltre, il fico soffre di una scarsa innovazione nei metodi di propagazione e di una crescente diffusione di patologie, virali e non, che condizionano la produzione. Tra i metodi di propagazione impiegati per il fico, senza dubbio, quelli vegetativi, rivestono maggiore importanza. Questi metodi, però, contribuiscono significativamente alla diffusione di patogeni, che abbattono il potenziale produttivo delle varietà.

Tra i metodi innovativi di propagazione, i semi sintetici o semi artificiali, sono in grado di coniugare i vantaggi della micropropagazione con la facilità di manipolazione, la possibilità di stoccaggio e la facilità di trasporto dei semi gamici (Redenbaugh, 1993). Da alcuni decenni, diversi studi hanno consentito l'individuazione di idonei protocolli di incapsulamento, sia con propaguli unipolari (microtalee), che con embrioni somatici (Germanà *et al.*, 2010). Per quanto riguarda *Ficus carica* L., è stato eseguito uno studio da Sharma *et al.* nel 2015, nel quale viene descritto un efficiente sistema di rigenerazione e conservazione a breve termine di segmenti nodali incapsulati. Tale protocollo é stato migliorato da Yahyaoui *et al.* (2016), i quali sono riusciti ad ottenere la radicazione in una sola fase, invece che cambiando il mezzo di coltura.

Obiettivo del presente lavoro è la verifica della validità dell'incapsulamento in alginato di calcio per la conservazione a medio termine della varietà libanese di fico Houmairi. In particolare, sono state incapsulate talee uninodali derivanti dalla proliferazione *in vitro* di germogli, valutando l'influenza di due regolatori di crescita (PGR): 6-Benzylaminopurina (BAP) e Meta-Topolina (MT), aggiunti all'endosperma artificiale, e registrando i parametri di vitalità, ripresa vegetativa e conversione dei semi sintetici direttamente o dopo 45 giorni di conservazione a 4°C.

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Materiali e metodi

Materiale vegetale e Sterilizzazione

Il materiale vegetale utilizzato è costituito dalla cultivar Houmairi di *Ficus carica* L. proveniente dalla collezione dell'Istituto Agronomico Mediterraneo, (IAM-Bari), Puglia. I propaguli utilizzati per la realizzazione del seme sintetico sono stati prelevati da germogli moltiplicati *in vitro* ed ottenuti per proliferazione di gemme dormienti apicali e laterali. Gli espianti, prelevati dalla pianta madre nel mese di Dicembre, sono stati sottoposti a sterilizzazione prima di essere messi in coltura, seguendo il protocollo di Yahyaoui *et al.* (2016).

Moltiplicazione

Le gemme sterilizzate di *Ficus carica* L. sono state poste su un mezzo di stabilizzazione e moltiplicazione agarizzato composto da sali e vitamine MS (Murashige and Skoog 1962), addizionato con 30 g/L di saccarosio, 4 mg/L di BAP e 0,4 mg/L di NAA (Kumar *et al.*, 1998) e solidificato con 6,5 g/L di agar.

Incapsulamento

Microtalee (3-4 mm di lunghezza) senza foglie e con una gemma ascellare, sono state prelevate da germogli in proliferazione e sottoposti alla tecnologia dell'incapsulamento, secondo quanto riportato da Yahyaoui *et al.*, (2016). Sono stati utilizzati come endosperma artificiale, due differenti mezzi composti da sali e vitamine MS (Murashige e Skoog 1962), addizionati con 30 g/L di saccarosio, 0,4 mg/L di NAA, 0,7 mg/L di GA3 e solidificati con 6,5 gr/L di agar, e ciascuno contenete 0,5 mg/L di un regolatore di crescita diverso: BAP o MT (Yahyaoui *et al.*, 2016). In ogni scatola Petri, sono state poste 5 capsule e per ogni trattamento ne sono state utilizzate 30 (fig. 1).

Il presente studio ha previsto, inoltre, due trattamenti (T):

- la coltura diretta delle talee incapsulate in armadi di crescita ad una temperatura di 25 ±1 °C, con fotoperiodo luce/buio di 16/8 ore e luce bianca fornita da lampade a fluorescenza (TMN 30W/84; Philips, Surenses, Francia), alla densità di flusso fotonico fotosintetico (PPFD) di 35 µmol $m^{-2}s^{-1}$.
- la conservazione dei semi sintetici prima a 4 °C di temperatura per quarantacinque giorni e poi la coltura in armadi di crescita con le stesse condizioni di temperatura e luminosità della tesi precedente.

Dati registrati e Analisi statistica

Dopo 45 giorni sono stati registrati i seguenti parametri: vitalità (% di espianti di aspetto verde, senza necrosi o ingiallimenti), ripresa vegetativa (% di microtalee incapsulate che producono germogli di lunghezza superiore a 4 mm) e conversione (% di emergenza di germogli e radici lunghi almeno 4 mm delle microtalee incapsulate). I dati sono stati analizzati mediante il test ANOVA a due vie, usando la versione 17 SPSS (SPSS Inc, Chicago, IL, USA), seguito dal test di Tukey, per P≤5%. I fattori considerati sono stati: (T), tipo di test (semi conservati a 4 °C per 45 giorni o seminati direttamente) e (P), differenti regolatori di crescita: BAP o MT.

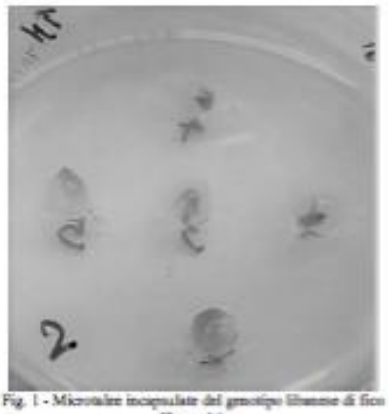

Fig. 1 - Microtalee incapsulate del genotipo libanese di fico Houmairi.
Fig. 1 - *Encapsulated microcuttings of the Lebanon fig genotype* Houmairi.

Risultati

Vitalità

I semi sintetici hanno presentato una vitalità media dell'85,5%. Relativamente al contenuto di ormoni, il BAP ha mostrato una vitalità media maggiore (83%), rispetto alla MT (63%) per quanto riguarda la conservazione a freddo (C-S), mentre, i semi non conservati (N-C-S) hanno mostrato, dopo 45 giorni dalla semina, una vitalità del 100% quando veniva aggiunto BAP, rispetto al 97% di vitalità quando veniva aggiunto MT all'endosperma artificiale. Tali differenze però, non sono risultate statisticamente significative (P=0,068) (tab. 1).

Ripresa vegetativa

La ripresa vegetativa media delle microtalee incapsulate è risultata del 60%. In particolare, le talee incapsulate con il BAP hanno presentato una ripresa vegetativa del 65% (fig. 2) e quelle incapsulate con la

Tab. 1 - Percentuali di vitalità, ricrescita e conversione in base al trattamento (conservati e no) e ai PGR utilizzati.
Tab. 1 - Viability, regrowth and conversion rates, according to the type of test (conserved or not) and PGRs used

	Vitalità (%)			Ripresa vegetativa (%)			Conversione (%)		
PGR (P)	BAP	MT	TOT	BAP	MT	TOT	BAP	MT	TOT
Trattamento (T)									
Conservati C-S	83	63	73	43	50	47	3	23	13
Non conservati N-C-S	100	97	98	87	60	73	3	10	7
Significatività									
P	0.068			0.245			0.014		
T	<0,001			0.002			0.216		
P x T	0.16			0.054			0.216		

Fig. 2 - Ripresa vegetativa della capsula, conservata a 4 °C, dopo 2 settimane dalla semina, BAP come regolatore di crescita.
Fig. 2 - Regrowth of encapsulated nodal segment, stored at 4 °C, after 2 weeks from sowing, using BAP as PGR.

MT del 55%. Tale differenza non è risultata statisticamente significativa (P=0,245). Per quanto riguarda il confronto dei semi non conservati e conservati a 4 °C per 45 giorni prima della semina, la ripresa vegetativa dei primi è risultata nettamente superiore (73% *vs* 47%). Nel dettaglio, i semi non conservati hanno presentato una ripresa vegetativa maggiore quando all'endosperma artificiale veniva addizionato il BAP: 87% *vs* 60%. Tale differenza è risultata statisticamente significativa (P=0,019).

Per i semi conservati, invece, il comportamento è stato superiore per le microtalee incapsulate con endosperma artificiale contenente MT (50 % *vs* 43%). La lunghezza media del germoglio prodotto dai semi sintetici non conservati, è stata di 0,88 cm, statisticamente superiore (P=0,009) nei semi contenenti BAP (1,09 cm) rispetto a quelli contenenti MT (0,67 cm) (dati non mostrati). La lunghezza dei germogli registrata dopo 45 giorni dalla semina nei semi conservati è risultata di 0,55 cm, e statisticamente non differente (P=0,743) è stato il comportamento dei semi contenente MT e BAP (0,58 cm *vs* 0,53 cm) (dati non mostrati).

Conversione

La conversione, è il dato più interessante da rilevare in quanto indica le microtalee che hanno germogliato ed emesso la radice. È stato registrato un valore percentuale di conversione statisticamente differente (P=0,015) tra i semi contenenti MT (17%) e quelli contenenti BAP (3%) (fig. 3). Relativamente al paragone tra i trattamenti tra i semi non conservati e conservati a 4 °C per 45 giorni prima della semina, i primi hanno fatto registrare una conversione del 7%, nettamente inferiore a quella rilevata nei semi conservati (13%). I semi non conservati, hanno mostrato, una conversione superiore quando l'endosperma era addizionato con MT (10%) rispetto a quando era addizionato con BAP (3%). Analogamente, nei semi conservati, per 45 giorni a 4 °C, la MT ha indotto una conversione molto superiore (23%) rispetto al 3% del BAP e la differenza è risultata statisticamente significativa (P=0,023) (tab. 1). Per quanto riguarda la lunghezza delle radici prodotte dopo 45 giorni dalla semina dalle microtalee incapsulate, è stato registrato un valore medio equivalente dei semi non conservati e

Fig. 3 - Conversione della capsula conservata a 4 °C, dopo 6 settimane dalla semina, BAP come regolatore di crescita.
Fig. 3 - Conversion of encapsulated nodal segment, stored at 4 °C, after 6 weeks from sowing, using BAP as PGR.

conservati (0,61 cm *vs* 0,61 cm). In particolare, sia nella tesi dei semi non conservati che conservati, la MT ha fatto registrare valori più elevati rispetto al BAP: 0,83 cm *vs* 0,40 cm nei semi non conservati e 0,73 cm *vs* 0,50 cm nei semi conservati.

Discussioni e conclusioni

I risultati del presente studio sull'incapsulamento di microtalee *vitro*-derivate della cultivar libanese Houmairi di *Ficus carica* L., ha confermato l'applicabilità di questa tecnologia di propagazione a questa specie. Sebbene le conversioni non siano state molto elevate, la tesi che ha previsto il trattamento di conservazione a freddo (4 °C per 45 giorni) e l'utilizzo del regolatore di crescita MT, ha raggiunto una percentuale di conversione adeguata (23%). Per la prima volta, questa tecnologia è stata applicata al genotipo libanese Houmairi di fico e la conversione dei semi sintetici di fico è stata ottenuta direttamente dopo la loro semina, confermando quanto ottenuto da Yahyaoui *et al.* (2016), senza cambio di mezzo, come invece era risultato necessario in precedenti studi (Sharma *et al.*, 2015). L'incapsulamento si presenta, pertanto, come un valido mezzo di moltiplicazione di *Ficus carica* L., affiancabile ai protocolli tradizionali di micropropagazione e potrà rappresentare se supportato da ulteriori ricerche finalizzate ad ottenere percentuali di conversione superiori, anche un valido supporto al vivaismo commerciale.

Riassunto

Scopo di questo studio è stato quello di valutare l'effetto di due differenti regolatori di crescita: 6-Benzilaminopurina e Meta-topolina, sulla vitalità, ripresa, conversione, lunghezza dei germogli e delle radici, di segmenti nodali incapsulati della varietà libanese Houmairi di *Ficus carica* L.. I risultati hanno confermato l'applicabilità della tecnologia a questa specie, che è risultata idonea per l'incapsulamento. Sebbene le percentuali di conversione non siano state elevate, l'ottenimento di plantule per la tesi che ha previsto il trattamento di conservazione a freddo (4 °C per 45 giorni) e l'utilizzo del regolatore di crescita Meta-topolina, ha raggiunto un soddisfacente livello di conversione (23%).

Parole chiave: citochinine, *Ficus carica*, incapsulamento, micropropagazione.

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Somatic embryogenesis through in vitro anther culture of *Citrus sinensis* L. Osbeck 'Moro'

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Abstract

In many crops, anther culture is the most used method to induce gametic embryogenesis, aimed to regenerate homozygous plants. However, also somatic embryogenesis can be obtained by this method, when somatic tissue is involved in the regeneration process. Many factors can affect this procedure, such as genotype, temperature pre-treatment applied to floral buds, pollen developmental stage, donor plant state, culture media composition and culture conditions. Anthers of *Citrus sinensis* L. Osbeck 'Moro', were collected at the vacuolate stage, and after a chilling (4°C) pre-treatment of 7 days, were placed on the same medium, evaluating different temperature stresses applied after placing them in culture. In this study, the effect of three thermal treatments, compared with direct in vitro culture of the anthers (after the pre-treatment of the floral buds at 4°C for 7 days), was observed in cultivar 'Moro'. Embryo regeneration has been obtained and their characterization, through ploidy and molecular analyses, showed that they were heterozygous tetraploids.

Keywords: molecular markers, ploidy analysis, orange

INTRODUCTION

Citrus species represent the largest production of fruit worldwide, with over 140 million tons produced during 2016 (FAOSTAT Database, 2018). Important advancements have been made in the genetic improvement and vegetative propagation of *Citrus* spp. through the application of biotechnology and, in particular, through tissue culture. Embryo rescue and culture, somatic hybridization, genetic transformation, haploid production, and in vitro shoot-tip grafting are all biotechnological tools that can greatly help *Citrus* breeding and nursery production of disease-free plants.

The production of haploid and of doubled haploid plants, represents a particularly attractive biotechnological method to accelerate plant breeding. Anther culture is the most common method for haploid production, but it is also suitable for somatic embryogenesis in a number of fruit trees, including *Citrus* species (Germanà et al., 1994; Germanà, 2003a).

Numerous endogenous and exogenous factors affect the embryogenic and somatic response of anthers in culture (Atanassov et al., 1995; Smýkal, 2000; Datta, 2005). Genotype, physiological status and growth conditions of donor plants, the stage of gamete development and pre-treatment of the flower buds, culture media and conditions of incubation, and their interactions, are all factors that greatly affect the cell response to the in vitro culture (Germanà, 2011a, b). There is no single standard condition, or protocol for obtaining plant formation by anther culture. Anthers of different species and cultivars within a species can present very different requirements for embryogenic and somatic development. For these reasons, studies of increasing embryogenesis efficiency, were focused on detecting the influence of different factors, particularly growth regulators, on anther culture and isolated microspore culture in *Citrus* spp. and other fruit crops (Germanà et al., 1996, 2006, 2011a; Höfer et al., 2002; Germanà and Chiancone, 2003; Bueno et al., 2005, 2006; Chiancone et al.,

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2006; Padoan et al., 2011). Although in the genus *Citrus* somatic embryogenesis was reported a long time ago (Maheshwari and Ranga Swamy, 1958), a number of difficulties have been encountered in establishing reliable protocols.

This study examined the effects of three different thermal treatments on the anther culture of the cultivar 'Moro' of *Citrus sinensis* L. Osbeck. 'Moro' was originated as a spontaneous mutation from *C. sinesis* L. Osbeck 'Sanguinello Moscato' (Hodgson, 1967).

MATERIAL AND METHODS

Plant material and pollen developmental stage

Flower buds were harvested in April 2017 from a tree of 'Moro' sweet orange grown in a collection orchard (Campo d'Orléans, Palermo, 38°N) of the Università degli Studi di Palermo, Italy. Microspore developmental stage was determined in one anther per flower bud by 4',6-diamidino-2-phenylindole (DAPI) staining. Anthers from buds of different sizes were squashed in a few drops of DAPI solution (1 mg mL^{-1}) and observed under a fluorescent microscope (Zeiss, Axiophot, Germany). For further experiments, only flower buds between 3.5-4.0 mm in length, containing most anthers with microspores at the uninucleated/vacuolated stage, were selected for in vitro culture.

Flower bud sterilization and anther culture

As pre-treatment, flower buds were placed at 4°C for 1 week. 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 (0.5% of active chlorine), containing few drops of Tween 20, and then rinsed three times with sterile distilled water. The petals were removed aseptically and 60 anthers from 3-4 flower buds, were placed in 6-cm Petri dishes containing 10 mL of solid culture media that were sealed with parafilm. Anther cultures were maintained at 26±1°C in the dark for the first 30 days, and then were placed under cool white fluorescent lamp (Philips TLM30W/84, France), with a photosynthetic photon flux density of 35 µmol m^{-1} s^{-1} and a photoperiod of 16 light hours.

Media composition

Anthers were cultured in vitro in a culture medium previously used in *Citrus* by Germanà et al. (1996) and described in Table 1 (hereafter referred to as culture media P). In order to study the influence of the thermal stress, anthers placed in medium P, were subjected to the following treatments: 1) Control, 2) -20°C for 60 min, 3) +4°C for 7 days and 4) +35°C for 7 days. Twenty-two Petri dishes were prepared containing 60 anthers for each treatment. The obtained embryos were transferred to the germination medium reported in (Table 2).

In vitro evaluation of the anther culture response, data processing and statistical analysis

Petri dishes containing anther in cultures were observed weekly by binocular microscope (Leica). Samples of anther were stained with DAPI and observed by a fluorescence microscope (Zeiss, Axiophot, Germany), to monitor their in vitro development, once a month, every month, during the culture. Different structural features have been observed and registered: microspores uninucleated, binucleated, trinucleated, tetranucleated, and multinucleated. Moreover, after 7 months of culture in the same Petri dishes, the number of embryos produced per each anther in every vessel was registered. These values were used to calculate means. Statistical analysis was carried out using SYSTAT 13 software. One factor was considered: 'temperature treatment' and differences between them were tested by two-way analysis of variance (ANOVA), at $p \leq 0.05$ level. Tukey's test was, then, used to separate means.

Table 1. Culture medium P used for anther culture of 'Moro' sweet orange (Germanà et al., 1996).

Components	Quantity	Components	Quantity
N6 Chu salts[1]	1×	Zeatin	0.5 mg L^{-1}
N&N vitamins[2]	1×	GA_3	0.5 mg L^{-1}
Galactose	18 g L^{-1}	TDZ	0.5 mg L^{-1}
Lactose	36 g L^{-1}	BAP	0.5 mg L^{-1}
Ascorbic acid	500 mg L^{-1}	Casein	500 mg L^{-1}
Myoinositol	5 g L^{-1}	Serine	100 mg L^{-1}
Biotin	500 mg L^{-1}	Glycine	2 mg L^{-1}
Thiamine	5 mg L^{-1}	Glutamine	800 mg L^{-1}
Pyridoxine	5 mg L^{-1}	Malt extract	500 mg L^{-1}
2,4-D	0.5 mg L^{-1}	Agar	8.5 g L^{-1}
Kinetin	0.5 mg L^{-1}	pH	5.8

[1]Chu (1978); [2]Nitsch and Nitsch (1969).

Table 2. Culture medium used for embryo germination.

Components	Quantity	Components	Quantity
MS salts[1]	1×	GA_3[2]	1 mg L^{-1}
MS vitamins	1×	NAA[3]	0.02 mg L^{-1}
Sucrose	30 g L^{-1}	Agar	8.5 g L^{-1}
Ascorbic acid	500 mg L^{-1}	pH	5.8
Malt extract	500 mg L^{-1}		

[1]Murashige and Skoog (1962); [2]GA_3: gibberellic acid; [3]NAA: α-naphthaleneacetic acid.

Ploidy evaluation

Ploidy level was determined on seven regenerated embryos, by flow cytometry according to the methodology described by Aleza et al. (2009). Flow cytometer is useful to detect and compare the ploidy of in vitro regenerated plantlets (Naing et al., 2014). Each sample consisted of a small piece of the germinated embryo, with a leaf piece (~0.5 mm^2) collected from a diploid control plant. Samples were chopped together using a razor blade in the presence of a nuclei isolation solution (High Resolution DNA Kit Type P, solution A; Partec®, Münster, Germany). Nuclei were filtered through a 30-µm nylon filter and stained with a DAPI (4,6-diamine-2-phenylindol) (High Resolution DNA Kit Type P, solution B; Partec®) solution. Following a 5-min incubation period, stained samples were run in a flow cytometer CyFlow® Ploidy Analyzer (Partec®), equipped with optical parameters for the detection of DAPI fluorescence. The DNA fluorochrome DAPI is excited by the UV-LED at 365 nm. Histograms were analysed using the CyView software (Partec®), which determines peak position, coefficient of variation (CV), arithmetic mean and median of the samples.

Molecular analysis

Genomic DNA was isolated from each embryo using a Plant DNeasy Kit from Qiagen Inc. (Madrid, Spain), according to the manufacturer's instructions. Nine heterozygous Simple Sequence Repeat (SSR) markers in 'Moro' sweet orange; TAA1 (Kijas et al., 1997), mCrCIR01F08a, mCrCIR06A12, mCrCIR01E02 (Froelicher et al., 2008), MEST192 (Aleza et al., 2011), mCrCIR07F11 (Kamiri et al., 2011), MEST1 and MEST104 (García-Lor et al., 2012), were used in this study. PCR amplifications were performed using a Thermocycler rep gradient S (Eppendorf®) in a 10-µL final volume containing 0.8 U of Taq DNA polymerase (Fermentas®), 2 ng $µL^{-1}$ citrus DNA, 0.2 mM welled (Sigma®) dye-labeled forward primer, 0.2 mM non-dye-labeled reverse primer, 0.2 mM of each dNTP, 10×PCR buffer, and 1.5 mM $MgCl_2$. The PCR protocol was as follows: denaturation at 94°C for 5 min followed by 40 cycles of 30 s at 94°C, 1 min at 50 or 55°C, and 45 s at 72°C; and a final elongation step of 4

min at 72°C. Capillary electrophoresis was carried out using a CEQ™ 800 Genetic Analysis System (Beckman Coulter Inc.). The PCR products were initially denatured at 90°C for 2 min, injected at 2 kV for 30 s, and separated at 6 kV for 35 min. Alleles were sized based on a DNA size standard (400 bp). Genome Lab™ Gap v.10.0 genetic analysis software was used for data collection.

RESULTS AND DISCUSSION

Anther culture response and ploidy evaluation

Using the staining method facilitated the observations of whole process of embryogenesis in anther culture. Monitoring of the culture by samples stained with DAPI (to show the nuclei) revealed that initially microspores of the genotype were uninucleated (Figure 1a).

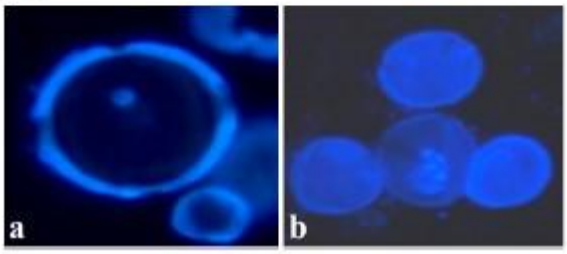

Figure 1. Uninucleated (a) and multinucleated (b) microspores of 'Moro'.

This is the developmental stage reported as being the most responsive for embryogenesis induction in citrus and many other woody and herbaceous species (Germanà and Chiancone, 2003; Germanà et al., 2011a; Gaitán-Solís et al., 2008; Prem et al., 2008). It was possible to observe that some microspores did not show any change in the nuclei number or shape. In other microspores, the nucleus started to symmetrical division (Figure 1b). This type of division was considered the first step of the sporophytic pathway followed by the gametic embryogenesis (Germanà, 2011a, b).

After a week in culture, many anthers were enlarged and after about a month, they began to produce callus. Most of the calluses were non-embryogenic. The morphogenic callus at first appeared crumbly and white, later embryos were differentiated. Results recorded after 7 months of anther culture, and their statistical analysis are reported in Table 3.

Direct embryo formation was observed (Figure 2a); in particular seven plants were obtained, both with the same level of ploidy. The embryos obtained were moved to the germination medium, continued to develop, germinate and to form plantlets that were micrografted in vitro, following the method described by Navarro and Juárez (2007) (Figure 2b).

The statistical analysis carried out on the data registered after 7 months of culture and regarding: undeveloped anthers, swollen anthers, anthers with a callus and with an embryogenic callus or with embryos, pointed out that the treatment factor had a greater effect than the genotype factor and medium of culture. Already since the first month of cultivation, the first anatomical changes of the anthers could be observed, the enlargement of these is the first, while many anthers, especially those subjected to the treatment "+35°C for 7 days" (65.8%), maintained their initial dimensions since the beginning of the crop.

Thus, anatomical changes in anthers consist of: increased turgidity, development of calluses and development of embryos. As opposed to the other treatments, it is possible to notice how the treatment "+35°C for 7 days" has recorded the highest percentage of anthers that have produced callus compared to the "control" "+4°C for 7 days" and "-20°C for 60 min" treatments (30 vs. 11.93%; 11.35%; 21.9%), even if the highest percentage of anthers not developed has been observed. From this, it is possible to deduce that the heat treatment "+35°C for 7 days" in part, supplies a better input than the other treatments to the anatomical change of the anthers, even if it did not lead to the development of embryos. Regarding the anthers that developed embryos, the best treatment was the "control" followed by treatment "-20°C for 60 min" (0.37 vs. 0.15%).

Table 3. Influence of three thermal treatments (in darkness) on the anther culture of *C. sinensis* L. Osbeck 'Moro', registered after 7 months of culture. Each value is the average of 1320 anthers per treatment (22 Petri dishes with 60 explants).

Medium	Treatments	Percentage of anthers without development (%)	Percentage of swollen anthers (%)	Percentage of anthers producing callus (%)	Percentage of anthers producing embryoids and/or embryogenic callus (%)
P	Control	21.4	66.3	11.93	0.37
	-20°C for 60 min	31.3	57.2	11.35	0.15
	+4°C for 7 days	29.6	48.5	21.9	0
	+35°C for 7 days	65.8	4.2	30.0	0
		p=0.000	p=0.000	p=0.000	p=0.0224

Values within each column are significantly different at p≤0.05. Two-way ANOVA, Tukey's test, p≤0.05.

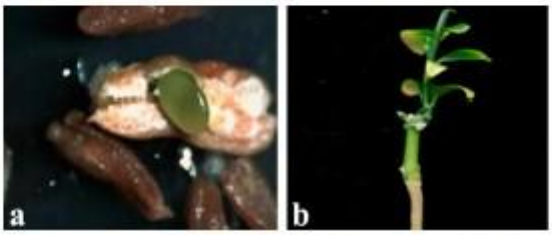

Figure 2. Embryos emerging from an anther (a) and in vitro micrografting of tetraploid plant (b) of 'Moro' regenerated from anther culture.

Flow cytometry analysis has been employed to detect the ploidy of regenerants. For all regenerants, the histogram of the flow, shows two peaks, one that refers to the mother plant, and the other showing a DNA content two times higher than that of the diploid control plant (Figure 3). The obtained tetraploids are not an exceptional fact for citrus fruits. In fact, there are many regenerants with different levels of ploidy found with the anther culture in the citrus species (Germanà et al., 2005; Germanà, 2007). Non-haploid (diploid, triploid, tetraploid, pentaploid, or hexaploid) embryos and plantlets have been obtained also from

anther culture of other genotypes (D'Amato, 1977).

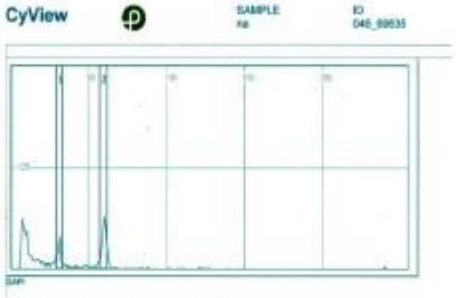

Figure 3. Flow citometry analysis that demonstrates tetraploid state of the regenerated embryo. 1) diploid peak of the mother plant (2n); 2) tetraploid peak (4n) of the regenerated embryo from anther of 'Moro'.

Regeneration through somatic embryogenesis is also rather common in different citrus species, and it is valuable method for clonal propagation, synthetic seed production, and germplasm storage (Germanà, 2005; Germanà et al., 2011a). Numerous investigations have been performed to obtain homozygosity, but they resulted instead in heterozygous somatic plantlets. In particular, anther culture has produced somatic regenerants in *C. aurantium* (Hidaka et al., 1981; Germanà, 2005); *C. sinensis* (Hidaka, 1984; Cardoso et al., 2016); *C. aurantifolia* (Chaturvedi and Sharma, 1985); *C. madurensis* (Ling et al., 1988); *C. reticulata* Blanco (Germanà et al., 1994; Germanà, 2005); *Poncirus trifoliata*, and the 14 hybrids of *C. ichangensis* × *C. reticulata* (Deng et al., 1992). In these cases, anther culture can be regarded as an additional method for achieving somatic embryogenesis.

The microsatellite analysis was performed because the ploidy level analysis cannot discriminate between diploids with gametic origin (DHs) and diploids of somatic origin.

The genetic, biochemical and molecular markers provide a very useful tool to demonstrate the androgenetic nature of the regeneration from the cultivation of anthers of Citrus (Deng et al., 1992; Germanà et al., 1992, 1994, 2000a; Germanà and Reforgiato, 1997). In previous research, isoenzymes were used to distinguish between tissues of gametic and somatic origin. In fact, due to spontaneous doubling or spontaneous polyploidization of the haploid calluses, cytological analysis becomes unusable for the identification of homozygous regenerants (Germanà et al., 1992, 1994, 2000a; Germanà and Reforgiato, 1997).

Genetic origin of regenerated plants

Two tetraploid recovered plants and 'Moro' sweet orange were analysed with nine heterozygous SSR markers. The tetraploid plants displayed the same heterozygous profiles as 'Moro' sweet orange for all molecular markers (Figure 4). These results suggest that tetraploid plants originated as a consequence of chromosome duplication from somatic cells during the in vitro culture process. In order to discriminate between these two types of regenerants, SSR markers being heterozygous for the parental genotypes, were selected from a preliminary screening. This type of regeneration through somatic embryogenesis from anther culture has been observed previously in *Citrus*, being a valuable issue for clonal propagation, synthetic seed production, elimination of pathogens and in vitro and germplasm storage (Germanà, 2005; Germanà et al., 2011a).

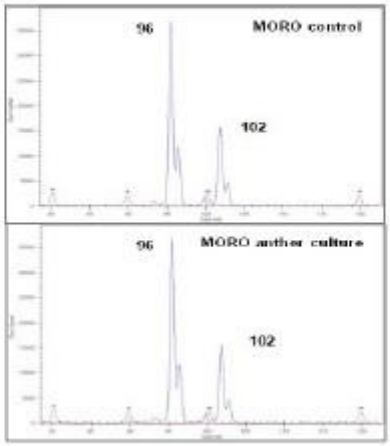

Figure 4. Amplicons of the SSR mCrCIR06A12 locus of the parental genotype (top) and the embryo (bottom) 'Moro'. Values in the box next to each peak represents the size of the observed alleles (bp).

CONCLUSIONS

Obtaining tetraploid genotypes from a blood sweet orange cultivar represents an important result for numerous applications in breeding. The results reported in this study confirm that the response depends, not only on the genotype, but also on the identification of the best treatment for the anthers.

The presence of multinucleate pollen grains, also developed in *C. sinensis* 'Moro' from this in vitro system, indicates the induction of microspore nucleus division and the switch of the gametophytic developmental program to the embryogenic pathway, even the regeneration does not regard the gametic embryos, but only the somatic ones.

For this reason, in conclusion, it can be stressed that the study of the factors influencing the phenomenon of gametic embryogenesis is extremely important, in order to clarify aspects that can lead to an increase in the frequency of microspores forming embryos and the number of plantlets anther obtained above all in those genotypes such as *Citrus* and all the woody plants which are recalcitrant to gametic embryogenesis.

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