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Artigo/Article

Gametic embryogenesis through isolated microspore culture in mandarin (*Citrus reticulata* Blanco), Mandarino Tardivo Di Ciaculli: effect of meta-Topolin and temperature treatments

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SUMMARY

Haploid technology, allowing the single-step development of complete homozygous genotypes from heterozygous plants through gametic embryogenesis, has already an enormous impact on breeding programs of many important crops. Microspore embryogenesis can be carried out through *in vitro* culture of anther or isolated microspore. Experiments have been carried out via isolated microspore culture in *Citrus reticulata* (Blanco), Mandarino Tardivo di Ciaculli, investigating the influence of two culture media, of two plant growth regulator types and of two temperature treatments applied before the culture or just after the culture. After ten months of culture, for all the media tested, different structural features have been observed and registered: uninucleated, binucleated, multinucleated microspores, and, for the first time in mandarin isolated culture, calli and microspore-derived embryos. These results represent advancement in the knowledge of microspore embryogenesis in mandarin. Actually, this is the first time that embryo regeneration from isolated microspore cultures has been reported in mandarin, a genotype very recalcitrant to microspore embryogenesis.

Index terms: breeding, haploid, homozygosity, microspore-derived embryos.

Embriogênese gamética através do cultivo de isolado microspórico em tangerina (*Citrus reticulata* Blanco), Mandarino Tardivo Di Ciaculli: efeito de meta-Topolins e tratamentos de temperatura

RESUMO

A tecnologia de haploidia, que permite o desenvolvimento em um único passo de genótipos homozigotos completos de plantas heterozigóticas por meio de embriogênese gamética, já teve um enorme impacto nos programas de melhoramento de muitas culturas importantes. A embriogênese por micrósporo pode ser realizada através da cultura *in vitro* de uma antera ou micrósporo isolado. Os experimentos foram realizados por meio da cultura de micrósporos isolados em *Citrus reticulata* (Blanco), Mandarino Tardivo di Ciaculli, investigando a influência de dois meios de cultura, dois tipos de regulador de crescimento e de dois tratamentos de temperatura aplicados antes ou logo

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após a cultura. Após dez meses, para todos os meios de cultura testados, diferentes características estruturais foram observadas e registradas: micrósporos uninucleados, binucleados e multinucleados e, pela primeira vez em culturas isoladas de tangerina, calos e embriões derivados de micrósporos. Esses resultados representam um avanço no conhecimento da embriogênese de micrósporos em tangerina. Na verdade, esta é a primeira vez que a regeneração embrionária de culturas de micrósporos isolados foi relatada em tangerina, um genótipo muito recalcitrante para embriogênese de micrósporo.

Termos de indexação: melhoramento, haploidia, homozigozidade, embriões derivados de micrósporos.

INTRODUCTION

Citrus are native to the tropical regions of southeast Asia and China and represent the largest production of fruit worldwide, with over 136 million of tons produced during 2013 (FAOSTAT, 2014). In particular, the FAO estimated the total mandarin production of 2.5 million of tons (FAOSTAT, 2014), characterized by different species and hybrids throughout the world, including Clementines in the Mediterreanean, 'Satsumas' in Japan, Ponkan in China and hybrids in California. In the Mediterranean regions, Spain is the main producing country, followed by Italy and Turkey.

The first formal *Citrus* breeding program was started in 1893 by the United States Department of Agriculture (USDA) and now, one of the largest breeding programs, is located at the Citrus Research and Education Center (CREC), Lake Alfred (Khan & Kender, 2007). Biotechnology methods can be used to enhance the efficiency of traditional breeding programs. Haploid technology allows to achieve completely homozygous lines from heterozygous parents just in one step, significantly reducing the time required to obtain the same result with the conventional method, which involves several generations of selfing. It is particularly useful for woody species characterized by long juvenility, a high degree of heterozygosity and often self-incompatibility (Germanà, 2009). Gametic embryogenesis leads to the production of Haploids and Double-Haploids, as a way for rapid production of homozygous lines. They can be used for important breeding applications such as mutation, transformation, genetic analysis and gene sequencing (Germanà et al., 2013).

Anthers culture is often the method to induce gametic embryogenesis in many crops (Germanà & Chiancone, 2003; Germanà, 2006; Murovec & Bohanec, 2011; Germanà, 2011a; Cardoso et al., 2014, 2016; Chiancone & Germanà, 2016; Cimò et al., 2017). However, isolated microspore culture is a better way to investigate the processes at the cellular, physiological, biochemical, and molecular levels. Actually, microspore embryogenesis provides a unique system to understand totipotency and cellular early fate decisions (Seguì-Simarro & Nuez, 2008; Soriano et al., 2013). This technique, although more laborious, is ideal avoiding the influence of anther somatic tissues and allowing a greater control over the culture process. Moreover, other advantages are that the isolated microspore culture can be exposed directly to treatments, without interfering of the maternal tissue, and that the culture conditions can be more rigorously controlled (Germanà, 2011a, 2011b).

With regard to *Citrus* species, haploid plantlets have been recovered from *C. madurensis* Lour. (Chen et al., 1980) and *Poncirus trifoliata* L. Raf. (Hidaka et al., 1979); homozygous plants and embryogenic calli with different ploidies have also been obtained via anther culture of *C. clementina* Hort. ex Tan. (Germanà et al., 1994, 2000; Germanà, 2005), as well ws haploid embryos of Mapo tangelo (*C. deliciosa* × *C. paradisi*) (Germanà & Reforgiato, 1997) and haploid and diploid calli, embryos and leafy structures of *C. limon* L. (Germanà et al., 1991). Moreover, one DH plantlet has been obtained from *C. ichangensis* × *C. reticulata* (Deng et al., 1992). More recently, via anther culture, doubled haploid callus and homozygous lines from sweet orange cultivars (Cao et al., 2011; Wang et al., 2015), homozygous callus from (*C. clementina* × *C. sinensis* cv. Hamlin) (Cardoso et al., 2014) and homozygous and triploid regenerants of Hernandina and Corsica clementines (Cardoso et al., 2016), were also recovered. Homozygous microspore-derived embryos were produced through isolated microspore culture from cvs. Monreal Rosso and Nules (Chiancone et al., 2015). However, mandarin (*C. reticulata* Blanco) can be considered a very recalcitrant species to gametic embryogenesis (Germanà, 2006, 2009).

Microspore embryogenesis response is affected by several factors, such as the thermal shock applied and the culture medium composition, particularly, the type and the concentration of plant growth regulators. Meta-Topolin, a natural aromatic cytokinin, is considered an alternative to benzyl-adenine (BA), zeatin (ZEA), kinetin (KIN) in plant tissue culture (Aremu et al., 2012) and it has

been previously used to early embryo achievement through isolated microspore culture in *Citrus clementina* (Chiancone et al., 2015).

The aim of this study was to induce gametic embryogenesis in mandarin via isolated microspore culture, investigating the effect of mT and of temperature treatments for switch microspore development from gamethophytic to sporophytic-way.

MATERIAL AND METHODS

Plant material and microspore developmental stage

Flower buds were harvested from trees of mandarin cultivar Mandarino Tardivo di Ciaculli (MTC), grown in the collection orchard (Campo d'Orlèans, Palermo 38°N) of the Università degli Studi di Palermo, Italy. Anthers from bud flowers of different sizes were squashed in a few drops of 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) solution (1 mg mL^{-1}) and observed under a fluorescent microscope (Zeiss, Axiophot, Germany) to determine the microspore developmental stage. Only flower buds of the size, containing anthers with microspores at the uninucleated-vacuolated stage, were selected for the culture. In *Citrus,* this stage was previously identified as the most responsive for gametic embryogenesis (Cardoso et al., 2014; Chiancone et al., 2015; Chiancone & Germanà, 2016).

Microspore isolation and culture

Firstly, flower buds were surface sterilized under a laminar flow hood by immersion in 70% (v/v) ethyl alcohol for 5 min, followed by immersion in sodium hypochlorite solution (0.5% active chlorine) with few drops of Tween-20 for 20 min and finally rinsed in sterile distilled water three times. After sterilization, petals were aseptically removed using small forceps and anthers were carefully excised. Microspores were isolated employing a Waring blender (Eberbach, Ann Arbor, MI, USA), as described by Karasawa et al. (2016) and cultured at the concentration of 100,000 microspores per mL in four liquid media: P (Germanà et al., 1996), N6 (Karasawa et al., 2016) and replacing 0.5 mg L^{-1} of BA with 0.5 mg L^{-1} of meta-Topolin (mT), in both of them (PmT and N6mT). Media composition (in g L^{-1} or mg L^{-1}) is reported in Table 1. Petri dishes (3001-type, 35 mm × 10 mm, BD Biosciences) were employed. All Petri dishes were put in the dark for the first 30 days, and then placed under cool white fluorescent lamp (Philips TLM 30W/84, France), with a photosynthetic photon flux density of 35 µmol m^{-2} s^{-1} and a photoperiod of 16 h . In addition, two thermal treatments were tested: half of the Petri dishes were directly put at 26 ± 1 °C (Control, C), the other half were placed at 35 °C per 30 days (H). The experiments were repeated twice: in the first one, the flower buds were processed immediately after the collection (T0). In the second one they were subjected to a cold (4 °C) pre-treatment for a week (T7). For each test, fifteen Petri dishes were prepared.

Table 1. Media composition (in g L^{-1} or mg L^{-1})

Components	P	N6	PmT	N6mT
Chu Salts	1X	1X	1X	1X
N&N Vitamins	1X	1X	1X	1X
Galactose	18 g	9 g	18 g	9 g
Lactose	36 g	18 g	36 g	18 g
Ascorbic Acid	500 mg	500 mg	500 mg	500 mg
Myoinositol	5 g	-	5 g	-
Biotin	500 mg	500 mg	500 mg	500 mg
Thiamine	5 mg	-	5 mg	-
Pyridoxine	5 mg	-	5 mg	-
2,4-Dichlorophenoxyacetic acid	0.5 mg	0.02 mg	0.5 mg	0.02 mg
1-Naphthaleneacetic acid	-	0.02 mg	-	0.02 mg
Kinetin	0.5 mg	1 mg	0.5 mg	1 mg

P modified (Germanà et al., 1996); **N6** (Karasawa et al., 2016); **Chu salts** (Chu, 1978); **N&N vitamins** (Nitsch & Nitsch 1969).

Table 1. Continued...

Components	P	N6	PmT	N6mT
Zeatin	0.5 mg	0.5 mg	0.5 mg	0.5 mg
Gibberellic acid	0.5 mg	0.5 mg	0.5 mg	0.5 mg
Thidiazuron	0.5 mg	0.1 mg	0.5 mg	0.1 mg
Coconut water	100 mL	50 mL	100 mL	50 mL
Casein	500 mg	500 mg	500 mg	500 mg
Serine	100 mg	-	100 mg	-
Glycine	2 mg	-	2 mg	-
Glutamine	800 mg	200 mg	800 mg	200 mg
Malt extract	500 mg	800 mg	500 mg	800 mg
Benzyladenine	0.5 mg	0.5 mg	-	-
Meta-Topolin	-	-	0.5 mg	0.5 mg

P modified (Germanà et al., 1996); **N6** (Karasawa et al., 2016); **Chu salts** (Chu, 1978); **N&N vitamins** (Nitsch & Nitsch 1969).

Evaluation of the microspore response

After ten months in culture, per each treatment, 450 microspores (three replicates with around 150 microspores each) were observed under a fluorescence microscope (Zeiss, Axiophot, Germany) after DAPI staining to check their development. Moreover, the number of embryos produced per each Petri dish was registered using a binocular microscope (Leica MZ 125). These values were used to calculate means. Statistical analysis was carried out using Statgraphics software. Three factors were considered: 'Cold treatment', 'Hot treatment' and 'Culture medium'. Differences between them were tested by analysis of variance (ANOVA) at $p \leq 0.05$ level. Tukey's test was, then, used to separate means.

Allelic pattern detection by SSR analysis

To check the homozygosity of the embryos, DNA was extracted from leaves of the mother plant and from the embryos obtained by *in vitro* culture. The leaves were frozen in liquid nitrogen and ground with mortar and pestle; the embryo was collected from the culture medium by an insulin syringe, placed in a 1.5 mL eppendorf with a small amount of quartz powder and ground with 600 µL of extraction buffer directly in the eppendorf. DNA extraction was then performed as described in Doyle & Doyle (1987). The parent DNA was resuspended in 60 µL TE buffer (Tris-EDTA, pH 8.0) and then diluted to 10 ngμL^{-1}. Embryo DNA was resuspended in 25 µL TE.

Ten SSR (Simple Sequence Repeats) loci isolated by Froelicher et al. (2008) from *C. reticulata* and Novelli et al. (2006) from *C. sinensis* were screened on the DNA from the leaves and 3 were selected for their heterozygosity in parental genotype: Ci08C05, mCrCIR01B10 (Froelicher et al., 2008) and CCSM147 (Novelli et al., 2006). These loci were used for assessing the allelic pattern of the embryo, as described in Chiancone et al. (2015).

Polymerase chain reactions (PCRs) were performed in two steps in a total volume of 10 µL containing 3µL DNA (corresponding to 30 ng of DNA for the parent plant), 0.25 U of KAPA Taq DNA polymerase (KAPABIOSYSTEMS, Wilmington MA, USA), 1 µL of 10X PCR buffer, 200 µM nucleotide mix and 0.5 µM of each primer. PCR conditions were as follows: an initial denaturation step at 95 °C for 3 min followed by 34 cycles of denaturation (30 s at 95 °C), annealing (45 s at 50 °C), and extension (90 s at 72 °C). The final elongation step was at 72 °C for 30 min. Four µL of the product obtained from the first amplification were then used as template for a second PCR, carried out for 28 cycles with the same conditions of the first one. PCR products were then analyzed by a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA,USA). Data were processed using GeneMapper Software (ver. 4.0; Applied Biosystems) and alleles were defined by their size in base pairs, by comparison with the standard size (GeneScan-500 LIZ, Applied Biosystems).

RESULTS AND DISCUSSION

The microspore culture response can be strongly affected by the microspore development stage. The bud size and the petal length are very often used as an external morphological indicator of the microspore stage for large-scale tests. Results of preliminary investigation with DAPI staining showed a correlation between the floral bud size and the microspore developmental stages. Bud sizes of 4.0 to 4.5 mm in length (Figure 1a), corresponded to the highest percentage (>80%) of the appropriate uninucleate-vacuolated stage (Figure 1b). This stage was previously identified as the most responsive for gametic embryogenesis in *Citrus* (Cardoso et al., 2014; Chiancone et al., 2015; Chiancone & Germanà, 2016).

After ten months of culture, for all the media tested, different structural features were observed. Among them, microspore with two identical nuclei equal in size and chromatin condensation patterns, derived by a symmetrical nucleus division (SND). The SND is considered one of the first signs of the beginning of the embryogenic program (Seguì-Simarro & Nuez, 2008). Furthermore, multinucleated structures (Figure 1c), and, for the first time in mandarin isolated microspore culture, calli and microspore-derived embryos (Figure 2a, 2b) have been detected. These features indicate that microspores switched their developmental program from the normal gametophytic pathway toward the sporophytic pathway (Seguì-Simarro & Nuez, 2008). The *in vitro* isolated microspore culture response after ten months of culture is showed in Table 2.

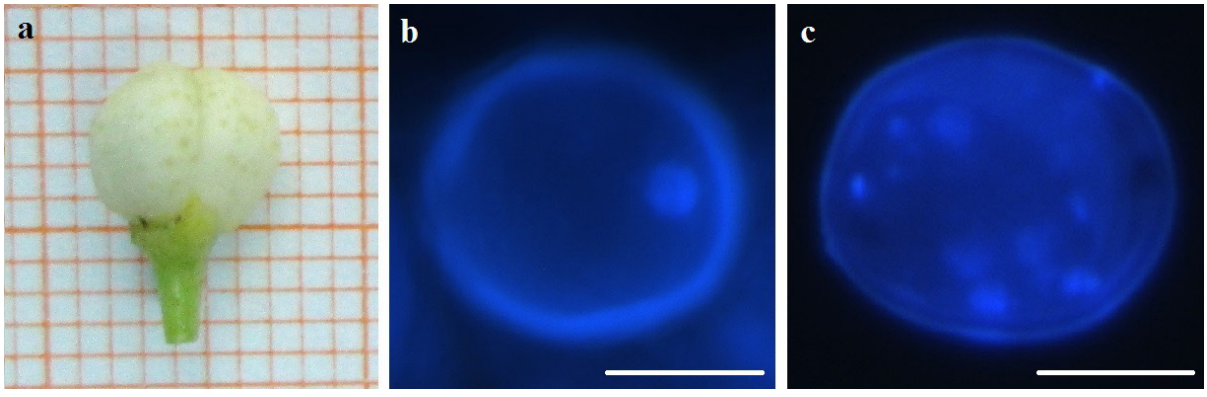

Figure 1. (a) Phenological stages of flower bud of MTC selected in relation to the microspore developmental stage (best stage = vacuolate stage); (b) Uninucleate-vacuolate microspore of MTC; (c) Multinucleated structure of MTC (*Bars* represent 10 µm).

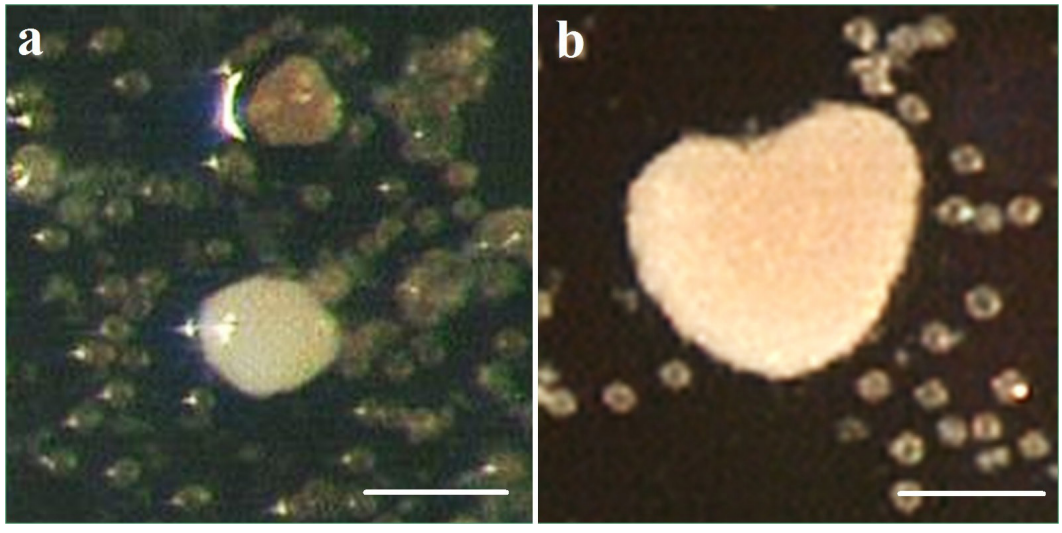

Figure 2. (a) Miscrospore-derived calli and globular embryo of MTC; (b) Heart-shaped embryo of MTC (*Bars* represent 150 µm).

Table 2. *In vitro* isolated microspore culture response after 10 months of culture

Factors		Embryo/Petri dish	
Cold treatment	T0	0.11	a
	T7	0.25	a
p value		*0.304*	
Hot treatment	C	0	a
	H	0.35	b
p value		*0.016*	
Medium	N6	0.02	a
	N6mT	0	a
	P	0.11	ab
	PmT	0.59	b
p value		*0.012*	

T0: No treatment; **T7**: 4 °C for 7 d; **C**: 26 ± 1 °C; **H**: 35 °C for 30 d.

The application of different stress treatments, such as cold or high temperatures, before or during the culture, is commonly employed to improve the microspore embryogenesis response (Shariatpanahi et al., 2006; Germanà, 2011a, 2011b; Islam & Tuteja, 2012). Indeed, temperature shock is considered to be the most effective treatment to induce microspore embryogenesis development, acting as a trigger for inducing the sporophytic pathway and preventing the development of mature pollen (gametophytic pathway) (Germanà, 2011a, 2011b). Hot inductive treatments can generate different cellular responses, such as the synthesis of heat-shock proteins (HSPs). An increase in the levels of different HSPs has been observed in response to heat during microspore embryogenesis (Seguì-Simarro et al., 2003). At first it was thought that HSPs would have a prominent role in microspore induction, but it was found that microspores also exposed to heat shock but not induced do accumulate HSPs as well (Seguì-Simarro et al., 2003). Currently, HSPs seem to provide a role more directly related to stress tolerance. Indeed, the involvement of HSPs in the androgenic switch is probably indirect and related to the inhibition of apoptotic events or allowed for the proper localization of other players more directly involved (Seguì-Simarro & Nuez, 2008). Indeed, once induced, the microspore undergoes numerous changes at different levels, from morphology to gene expression. The stress acts at the first haploid mitosis, by altering the polarity of the division, involving reorganization of the cytoskeleton, blocking starch production and dissolving microtubules. These rearrangements provide, in induced microspores, a 'star-like' morphology, that was considered the early morphological marker of embryogenic commitment (Seguì-Simarro & Nuez, 2008).

In this experiment, a marked and significant ($p \leq 0.05$) 'Hot treatment' effect was observed in the embryo production. In fact, only isolated microspores cultured at 35 °C for 30 days (H) finally produced embryos (0.35 embryos/Petri dish). On the contrary, no significant statistically differences were observed among microspores immediately processed (T0) or subjected to the cold (T7) pre-treatment (0.11 and 0.25 respectively) (Table 2). Germanà (1997) already reported the influence of pretreatments to promote *in vitro* embryogenesis in *Citrus*. However, the microspore culture responses to the temperature treatments were, as previously reported in other fruit crops, highly genotype-dependent (Germanà, 2011a, 2011b; Cimò et al., 2017).

Regarding the four culture media tested, 'P' and 'PmT' induced a higher average number of regenerated embryos per Petri dish (0.11 and 0.59 respectively), while the 'N6mT' medium the lowest. In addition, Tukey's test evidenced that 'PmT' medium produced a statistically significant ($p \leq 0.05$) higher number of embryos per Petri dish when compared with 'N6' and 'N6mT' media (Table 2). Chiancone et al. (2015) previously reported the production of microspore-derived embryos obtained through isolated microspore culture in *Citrus clementina* Hort. ex Tan., cvs. Monreal Rosso and Nules. An higher (when compared to mandarin results) embryo production was previously observed for both clementine cultivars, with the Nules cultivar showing a higher average number of embryos/Petri dish regenerated than in Monreal Rosso (1.5 vs. 1.0). However, also in this case, it appears that the application of mT, replacing BA, was not statistically significant for the embryo induction. Furthermore, the response to mT in the culture media appears to be genotype-dependent. For example, Chiancone et al. (2015) reported that microspore-derived embryo production seemed to be favored by mT addition in cv. Nules but not in Monreal Rosso.

Although the microspore-derived embryos obtained were transferred to different solid media to achieve conversion and plantlet production, no germination was achieved, probably due to immaturity and lack of accumulation of the storage compounds or a deficiency in the media compositions, as already observed in somatic embryogenesis process, that prevents maturation and germination (Ochatt & Revilla, 2016). Since the embryos were of reduced dimensions, ploidy level could not be determined by flow cytometry at this stage.

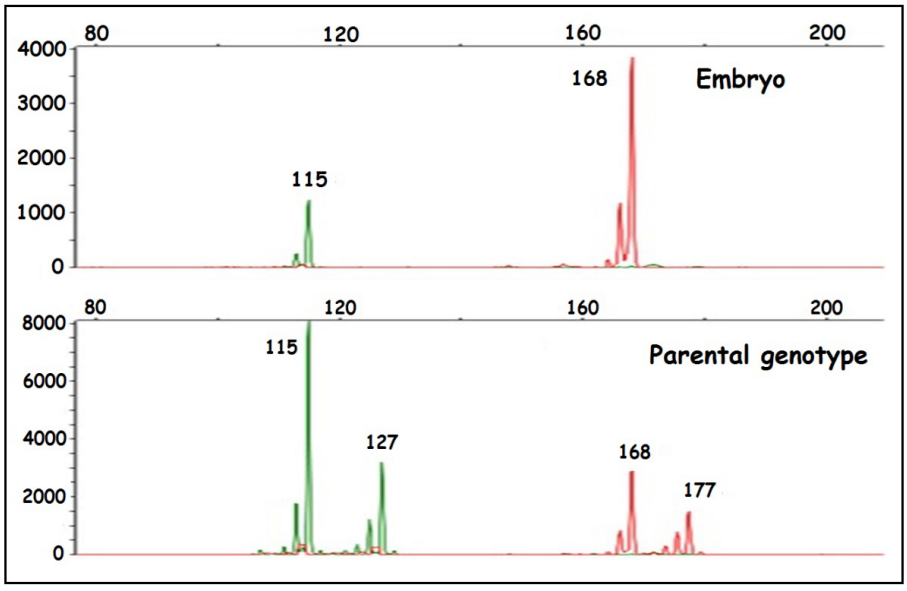

Figure 3. Amplicons of the SSR loci CCSM147 (at left) and Ci08C05 (at right) in embryo (top) and in the parental genotype (bottom) of MTC. Values above each peak represent the allele size (bp). The allelic pattern of the embryo shows a single allele, shared with the parental genotype.

To determine regenerant origins, assessing homozygosity and distinguish between spontaneously DHs and heterozygous diploids, isozyme analyses, random amplified polymorphic DNA markers and microsatellites can be used.

Isozyme analyses were used in *Citrus* to confirm the gametic origin of calluses, embryos and plantlets (Germanà & Chiancone, 2001). These 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 shows lack of an allele (Germanà, 2007). Furthermore, several DNA molecular markers are commonly adopted, such as AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), or SSR (Simple Sequence Repeat), depending on the scope of the study. Co-dominant molecular markers, such as SSRs, have the advantage that a single locus, when heterozygous in donor plants, might be used for homozygosity determination (De Vienne, 2003).

For this reasons, three SSR loci were chosen for being heterozygous in the parental genotype and were analysed to detect the allelic profile of regenerants. The allelic pattern of the embryos showed a single allele, shared with the parental genotype (Table 3; Figure 3), thus confirming the origin of the embryo from the MTC microspore. In Figure 3 the amplicons of the SSR loci CCSM147 and Ci08C05 in the embryo and in the parental genotype are showed.

Table 3. Allelic pattern in parental genotype and embryo. Allele size in bp

Parental genotype	SSR locus	Allelic pattern
MTC	Ci08C05	168-177
	mCrCIR01B10	156-160
	CCSM147	115-127
embryo	Ci08C05	168
	mCrCIR01B10	156
	CCSM147	115

MTC: Mandarino Tardivo di Ciaculli.

CONCLUSIONS

Microspore embryogenesis is a promising tool for plant breeding as fully homozygous plants can be generated with a single step starting from heterozygous parents (Germanà, 2011a, 2011b; Chiancone & Germanà, 2016).

The presence of multinucleated structures observed in this experiment, indicates that the induction of gametic embryogenesis, involving symmetrical microspore nucleus division and the switch from the gametophytic developmental program to the sporophytic embryogenic pathway, occurred. In addition, homozygous embryo regeneration was obtained, for the first time, through isolated microspore culture technique in mandarin (*C. reticulata*

Blanco), Mandarino Tardivo di Ciaculli, a genotype extremely recalcitrant to microspore embryogenesis.

In this experiment, while the application of mT (replacing BA) in the culture media was not significant, the thermal shock treatment at 35°C for 30 days (H) had a critical role in the induction of microspore embryogenesis and for triggering pollen totipotency, resulting essential for the embryo production.

These results constitutes a crucial step in designing new protocols for regenerating microspore-derived embryos and plants in mandarin, providing new opportunities for genetic improvement and for innovation in breeding methods in this species. However, further studies are necessary to better understand the gametic embryogenesis process, to optimize the rate of microspore-derived embryos and to achieve their germination into plantlets.

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