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ORIGINAL ARTICLE

Gametic and somatic embryogenesis through *in vitro* anther culture of different *Citrus* genotypes

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Abstract

In vitro tissue culture represents a useful technique for advancing *Citrus* breeding and propagation. Among *in vitro* regeneration systems, anther culture is commonly used to produce haploids and doubled haploids for a fast-track producing homozygous lines, in comparison with the traditional self-pollination approach, which involves several generations of selfing. In addition, anthers culture can produce somatic embryos that can also be used for clonal propagation. In this study, two thermal shocks were applied to the anthers of six *Citrus* genotypes (two clementine and four sweet oranges), just after they were put in culture. The response obtained was different depending on the genotype: both clementines, namely Hernandina and Corsica, produced homozygous and triploid regenerants (microspore-derived embryos), whereas all of the analyzed regenerants from sweet oranges, three cultivars of Tarocco and Moro, produced heterozygous and diploid regenerants similar to the parental genotypes (somatic embryos).

Keywords: *Anther culture, cytofluorometry, homozygosity, microspore embryogenesis, ploidy level*

Introduction

At present, *Citrus* species, native to the tropical regions of southeast Asia and China, represent the largest production of fruit worldwide, with over 131 million of tons produced during 2011 (FAOSTAT 2012). 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 in *Citrus* breeding and nursery production of disease-free plants.

The production of haploids (Hs), which are plants with a gametophytic chromosome number, and of doubled haploids (DHs), which are Hs that have undergone chromosome duplication, represent a particularly attractive biotechnological method to accelerate plant breeding. Gametic embryogenesis, which allows the single-step development of complete homozygous lines from heterozygous parents, has already had a huge impact on the agricultural systems of many agronomically important crops, representing an integral part in their breeding programs (Germanà 2011a, 2011b). To induce gametic embryogenesis, it is necessary to switch the gametic cells from their normal developmental pathway toward a sporophytic pathway.

"Microspore embryogenesis" can be induced through *in vitro* anther or isolated microspore culture. Anther culture is usually the method of choice for DHs production in many crops, because of the sizable advantage of its simplicity, which allows the establishment of a large-scale anther culture and application to a wide range of genotypes (Sopory & Munshi 1996). With regard to anther culture in *Citrus* and their relatives, haploid plantlets have been

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recovered from *Poncirus trifoliata* L. Raf. (Hidaka et al. 1979) and *C. madurensis* Lour. (Chen et al. 1980); one DH plantlet has been obtained from hybrid 14 of *C. ichangensis* × *C. reticulata* (Deng et al. 1992). Moreover, homozygous plants with different ploidies and the highly homozygous embryogenic calli of *C. clementina* Hort. ex Tan. (Germanà et al. 1994, 2000a, 2005), haploid (but albino) embryos of Mapo tangelo (*C. deliciosa* × *C. paradisi*) (Germanà & Reforgiato 1997), haploid and diploid calli, embryos and leafy structures, but no green *C. limon* L. Burm. f. plants (Germanà et al. 1991), and haploid *Clausena excavata* embryos (Froelicher & Ollitrault 2000) have also been obtained via anther culture. Recently, short-lived homozygous plantlets have been recovered from the anthers of Rhode Red Valencia sweet orange (Cao et al. 2011), and Cardoso et al. (2014) obtained homozygous callus from a hybrid between *C. clementina* × *C. sinensis* "Hamlin". However, *C. sinensis* can still be considered a very recalcitrant species to gametic embryogenesis.

Numerous endogenous and exogenous factors affect the embryogenic response of anthers in culture (Atanassov et al. 1995; Smykal 2000; Datta 2005). Genotype, physiological state and growth conditions of donor plants, developmental stage of the gamete, pretreatment of flower buds, media and incubation conditions, together with the interactions between these factors, all greatly affect the anther response to *in vitro* culture (Germanà 2011a, 2011b). There is no single standard condition or protocol to obtain plant formation from anther culture, and it is possible that anthers, not only those of different species, but also from different cultivars within a species, need for very diverse requirements to undergo an embryogenic route and development.

It is necessary to develop and improve the current protocols to increase the number of genotypes that respond to *in vitro* morphogenesis. Although there are many genotypes responding very well to anther culture, many others of interest, such as *Citrus*, are still recalcitrant.

This study has been performed to achieve regeneration in several *Citrus* genotypes (two clementine and four sweet oranges) through anther culture, testing different treatments applied to the anthers in culture.

Materials and methods

Plant material

Flower buds of blood sweet oranges and clementines were collected in March from adult *Citrus* trees. Flower buds of *C. sinensis* cv. Moro were collected from trees cultivated in Palermo (Italy), whereas flower buds of sweet oranges Tarocco Meli, Tarocco TDV, and Tarocco S. Alfio, and of *C. clementina* cvs. Corsica and Hernandina were harvested in Lentini (Italy). All flower buds were stored at 4°C for 7 days as a cold pretreatment.

The pollen development stage was determined in one anther per bud size by 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Anthers were squashed in a few drops of DAPI solution (1 mg mL^{-1}) and observed under a fluorescent microscope Zeiss Axiophot (Zeiss, Oberrkochen, Germany) to identify the flower bud size containing the highest percentage of vacuolated microspores (5–7 mm in length for oranges, 3–6 mm for clementines: Figure 1(B),(C)). This stage (Figure 1(A)) was previously identified as the most responsive for gametic embryogenesis in *Citrus* (Germanà 2007; Cardoso et al. 2014) and were selected for culture. DAPI staining and observation by

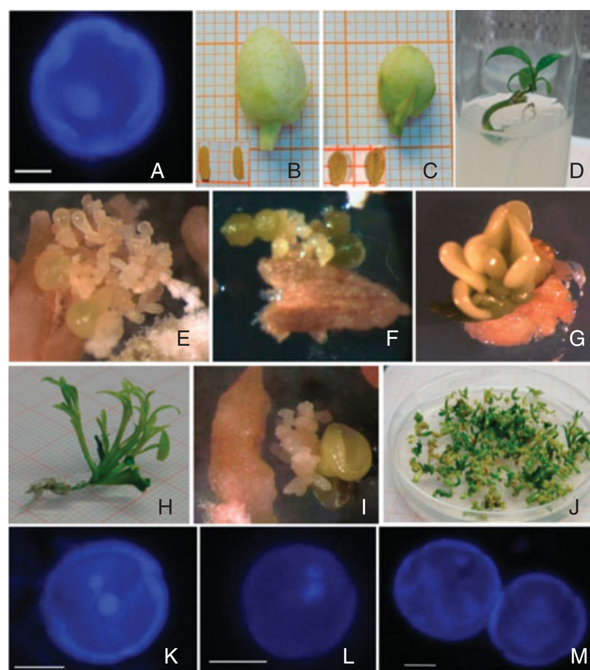

Figure 1. Gametic and somatic embryogenesis in *C. sinensis* and *C. clementina*. (A) Vacuolated microspore of cultivar Hernandina. (B) Flower bud and anthers of Moro sweet orange, with vacuolated microspores. (C) Flower bud and anthers of Corsica, with vacuolated microspores. (D) *In vitro* grafting of small shoot apexes (2–3 mm) of homozygous clementine regenerants onto etiolated 20-day-old Troyer citrange seedlings. (E) Highly embryogenic callus of Moro sweet orange. (F) Embryogenic callus and embryos emerging from an anther of Hernandina. (G) Direct embryogenesis from a Corsica. (H) Organogenesis from a somatic embryo obtained in anther culture from Tarocco S. Alfio sweet orange. (I): Secondary embryogenesis was observed at the base of an embryo in Hernandina. (J) Multiplication of highly embryogenic calli from Tarocco Meli. (K) Bicellular pollen grain, containing nuclei with different chromatin condensation patterns. (L) Pollen grain with two similar nuclei, formed by a symmetrical division. (M) Multinucleated pollen grains of Corsica. Bars in A: 5 μm, in K, L: 20 μm, in M: 10 μm.

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light and fluorescence microscopy were also performed at different times during the culture to observe changes in microspore development inside anthers.

Anther culture technique

After pretreatment, flower buds were surface-sterilized by immersion in 70% (v/v) ethanol for 3 min, followed by immersion in sodium hypochlorite solution (~1.5% active chlorine) with few drops of Tween 20 for 20 min, and finally rinsed three times, in sterile distilled water. Petals were aseptically removed using a small forceps. Anthers were carefully dissected and placed in Petri dishes (6 cm in diameter) containing 10 mL of solid medium. Sixty anthers were placed in each dish. Petri dishes were sealed and incubated at 27 ± 1°C in the dark for 30 days and, then, transferred under cool white fluorescent lamps Philips TLM 30W/84 (Philips, Chalon-sur-Saône, France) conditions with a photosynthetic photon flux density of 35 μmol m^{-2} s^{-1} and a photoperiod of 16 h.

Culture media

The induction medium composition was the following: N6 mineral salts (Chu 1978) supplemented with Nitsch and Nitsch vitamins (1969), 36 g L^{-1} lactose, 18 g L^{-1} galactose, 10% coconut water (Sigma, Saint Louis, MO, USA), 500 mg L^{-1} casein, 0.5 mg L^{-1} biotin, 500 mg L^{-1} ascorbic acid, 5 g L^{-1} myo-inositol, 800 mg L^{-1} glutamine, 0.1 mg L^{-1} serine, 5 mg L^{-1} thiamine, 5 mg L^{-1} pyridoxine, and 2 mg L^{-1} glycine. Growth regulators were added to the culture medium before autoclaving (in mg L^{-1}): 0.5 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 kinetin, 0.5 6-benzylaminopurine, 0.5 zeatin, 0.1 thidiazuron, 0.5 gibberellic acid (GA_3), and 0.8% agar (Germanà et al. 1996). The pH was adjusted to 5.8 before autoclaving (20 min, 120°C).

Embryogenic calli were multiplied on Murashige and Skoog medium (MS) (1962) supplemented with 5% sucrose, 0.02 mg L^{-1} α-naphthaleneacetic acid (NAA), 1 mg L^{-1} GA_3 and 0.8% agar.

Temperature treatments: experimental design and data collection

The thermal shock response was studied applying the following treatments: −20°C for 30 min (F) and 37°C for 60 min (HT) to *in vitro* cultured anthers of the six cultivars (just after inoculating the medium). Ten Petri dishes were prepared for each treatment (600 anthers/treatment). Moreover, 10 Petri dishes were incubated directly in the growth chamber under the same conditions as those mentioned above as controls.

Anthers in culture were observed every month to follow their developmental process. Ten months after the isolation, the number of undeveloped anthers, anthers that swelled, or that produced embryoids and calli were recorded for each Petri dish.

These data were used to calculate means. The genotype effects on the recorded data were tested by one-way analysis of variance. Because the values for number of undeveloped anthers, anthers that swelled, or that produced embryos and calli were not normally distributed, these data were transformed by arcsin square root. Games-Howell's ($p \leq 0.05$) test was used to compare the means for all parameters, with the exception of anthers with calli, for which Tukey's ($p \leq 0.05$) test was used. Moreover, for the parameter anthers with embryos or embryogenic callus, the means ± standard errors (SE) were presented.

Germination medium

Regenerated embryos were transferred to test tubes with MS medium containing 3% sucrose, 1 mg L^{-1} GA_3, 0.01 mg L^{-1} NAA, and 0.75% agar as soon as they appeared, in order to induce germination and to convert them into plantlets.

Plant recovery

Small shoot apexes (2–3 mm) of clementines were *in vitro* micrografted onto etiolated 20-day-old Carrizo citrange seedlings to obtain more vigorous plantlets (Figure 1(D)) because it was previously observed that the homozygous plantlets grew slowly in soil.

After 3–4 months, grafted plantlets with ~4–5 cm high were acclimatized to greenhouse conditions in plastic pots containing sterilized peat moss, sand, and soil, in a 1:1:1 ratio, for the hardening phase.

Characterization of regenerants: ploidy analysis and analysis of heterozygosity

The ploidy levels of the regenerants were evaluated by flow cytometry analysis. Tissues from calli, embryoids or plantlets were chopped with a razor blade in 1 mL of nuclear extraction buffer (Partec GmbH, Münster, Germany), and with a diploid sample being used as the control. Each nuclear suspension was filtered through a 30-μm nylon filter and mixed with 4 mL of DAPI staining solution. The relative DNA content of each sample was determined by using a Partec Cell analyzer PA II (Partec GmbH) and a diploid control sample as a reference.

A total of 96 regenerants were analyzed; 30 of these came from blood sweet orange regenerants (9

from Moro, 12 from Tarocco S. Alfio, 3 from Tarocco TDV, and 6 from Tarocco Meli) and other 66, from clementine regenerants (51 from Hernandina and 15 from Corsica).

Simple Sequence Repeat (SSR) markers were adopted to assess the heterozygosity and to determine the origin (gametic or somatic) of the calli and the regenerated plantlets.

Total DNA was extracted from 0.2 g of plant material (young leaves or calli) from randomly selected regenerant samples as follows: 3 regenerants from Moro, 3 from Tarocco Meli, 3 from Tarocco S. Alfio, 1 from Tarocco TDV, 1 from Corsica, and 5 from Hernandina, using a Qiagen Plant DNA Mini Kit according to the manufacturer's protocol. DNA concentration of the samples was determined by fluorometer (Hoefer DyNA Quant 200, Hoefer Inc., Holliston, MA, USA) by using Hoechst H 33258 fluorescent dye, with human DNA (50–250 ng μL^{-1}) as a standard.

Fourteen trinucleotide microsatellites developed by Kijas et al. (1997) from *C. limonia* (Rangpur lime) × *P. trifoliata* hybrid were screened in a preliminary test and four of them (TAA1, TAA15, TAA41, and CAC15) were selected for analysis because of their polymorphism and easily scorable patterns, and little stuttering.

PCRs were carried out in an 8 μL volume containing 10 mM Tris–HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.3 μM of each primer (the forward primer was labeled with either FAM or HEX fluorescent dye), 20 ng of genomic DNA, and 0.3 U of Taq polymerase (Amersham Biosciences, Zurich, Switzerland) in a PT 100 thermal cycler (MJ Research, Waltham, MA, USA). PCR thermal profile was as follows: 1 cycle at 94°C for 5′; 32 cycles at 94°C for 60″, 55°C for 30″, 72°C for 60″, and 72°C for 5′.

One microliter of desalted PCR product was mixed with 2.75 μL of loading solution (70% formamide and 1 mM EDTA), 0.25 μL of ET-ROX dye (Et400-R size standard, Amersham Biosciences), and 1.0 μL of deionized H_2O, centrifuged at 900 rpm for 2′, denatured at 95°C for 4′, and cooled on ice. Electrophoresis was performed on a MegaBACE 500 capillary sequencer (Amersham Biosciences). The fragment profile was analyzed by Genetic Profiler v2.0 (Amersham Biosciences). Each PCR/EF run was repeated 4– 6 times.

Results and discussion

Anther culture, embryo, and plantlet regeneration

After 1 week of culture, many anthers had already started to swell and to produce calli. There were two types of anther-derived calli: a hard white-greenish nonmorphogenic callus, similar to ones obtained by Cardoso et al. (2014), or highly embryogenic type (Figure 1(E),(F)) with a soft and friable aspect, and with small immature embryos. Rarely, the route of regeneration took place through direct embryogenesis (Figure 1(G)), organogenesis (Figure 1(H)), or through secondary embryogenesis starting at the base of an embryo (Figure 1(I)). Embryogenic calli usually differentiate into a clump of embryos (Figure 1(E)).

The embryogenic calli were multiplied (Figure 1 (J)), and most of the resulting embryos were well-structured. They developed as zygotic embryos through the globular, heart, torpedo, and cotyledonary stages.

Anthers collected at different times during the culture exhibited differences in microspore developmental responses after DAPI staining as observed by fluorescence microscopy. In fact, bicellular pollen grains containing nuclei with different chromatin condensation patterns, such as the larger vegetative nucleus and the smaller generative one (Figure 1(K)) were observed, denoting an asymmetrical division, which is typical of pollen maturation (Pacini 2012). In addition, pollen grains with two similar nuclei (Figure 1(L)) were noted after a symmetrical division, as well as multinucleate pollen grains in all genotypes (Figure 1(M)). This feature indicates that these microspores switched their developmental program toward gametic embryogenesis. In some anthers, large multicellular structures or proembryos were observed after 6 months in culture. In fact, an association between the polarity of the first pollen division and a capacity for embryogenesis induction from the microspore was observed (Twell & Howden 1998), and the symmetric division of the pollen nucleus is considered the first indication of the onset of the embryogenic program (Bárány et al. 2005; Seguí-Simarro & Nuez 2008).

Anther culture responses after 10 months of culture in the two *C. clementina* cultivars, namely Hernandina and Corsica, and the four cultivars of *C. sinensis*, namely Moro, Tarocco S Alfio, Tarocco TDV, and Tarocco Meli, are reported in Table I. All gave rise to a morphogenetic process from *in vitro*-cultured anthers, in spite of strong genotypic differences.

In fact, a strong genotype effect was observed for all registered parameters (undeveloped anthers, swollen anthers, anthers with a callus and with an embryogenic callus or with embryos). Significant differences were observed in the percentages of undeveloped anthers or those that were swollen and had calli; Tarocco S. Alfio and Meli showed the lowest amounts of the first type and the highest amounts of the second one. Regarding the anthers with calli, the highest percentages were observed in Corsica clementine and in Tarocco S. Alfio and Meli

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Table I. The developmental response after 10 months of anther culture in two cultivars of *C. clementina*, cvs. Hernandina and Corsica, and from four cultivars of *C. sinensis*, cvs. Moro, Tarocco S Alfio, Tarocco TDV, and Tarocco Meli.

Cultivar	Undeveloped anthers (%)	Swollen anthers (%)	Anthers with calli (%)	Anthers with embryos or embryogenic callus (%)
Hernandina	58.5^{a}	27.5^{b}	10.6^{b}	3.3^{a}
Corsica	49.9^{a}	31.4^{b}	16.2^{a}	2.4^{a}
Moro	60.4^{a}	29.3^{b}	10.0^{b}	0.3^{b}
Tarocco S. Alfio	12.8^{b}	65.0^{a}	21.4^{a}	0.8^{b}
Tarocco TDV	47.9^{a}	42.8^{b}	9.1^{b}	0.2^{b}
TaroccoMeli	19.1^{b}	63.9^{a}	16.8^{a}	0.3^{b}

Note: The values within each column followed by different letters are significantly different at $p \leq 0.05$ (Games-Howell's test was used for undeveloped, swollen anthers and anthers with embryoids or embryogenic calli; Tukey's test was used for anthers with calli).

sweet oranges, although calli were not always embryogenic. The percentage of anthers producing embryogenic calli, which was the most interesting data, was higher in clementines than in blood oranges (Table I).

There was a difference in behavior between sweet oranges and clementines in response to temperature treatments. Pretreatment at 4°C is the most commonly used stress employed in anther culture (Germanà 2006). In our knowledge, this is the first time that a so low temperature has been applied to anthers in culture. Cold treatment (−20°C) induced a noticeably higher production of embryogenic calli in Hernandina (5.6%) and Corsica (3.7%) anthers, but it had no clear effect on the development of sweet orange explants. Among blood oranges, the Tarocco S. Alfio cultivar exhibited the highest response for anthers with calli and with embryogenic calli, regardless of the temperature treatment, followed by the Tarocco Meli cultivar (Figure 2). Higher values of SE were due to the nature of data, caused by high number of Petri dishes with anthers that not produced embryos (zero values).

In recalcitrant genotypes, application of stress may be required to switch the gamete development toward the sporophytic pathway. In fact, when the treatments (both physical or chemical) of many genotypes were applied to excised flower buds, whole inflorescences, or isolated anthers before the start of a culture, they acted as a trigger to induce the sporophytic pathway, preventing pollen maturation (gametophytic pathway) (Germanà 2011a, 2011b). Among these treatments, chilling, high temperatures, high humidity, water stress, anaerobic treatment, centrifugation, sucrose and nitrogen starvation, addition of ethanol or a microtubule-disrupting agent, γ-irradiation treatment, electro-stimulation, culturing at a high pH, heavy metal treatment, etc., have been used in anther and microspore cultures. Shariatpanahi et al. (2006) classified these treatments into the following three categories: widely used, neglected, and novel methods. Stress seems to act in different ways, by altering the polarity of the division at the first haploid mitosis, being involved in the reorganization of the cytoskeleton (Nitsch & Norreel 1973; Reynolds 1997), delaying and modifying pollen mitosis (two equal-sized vegetative-type nuclei instead of one

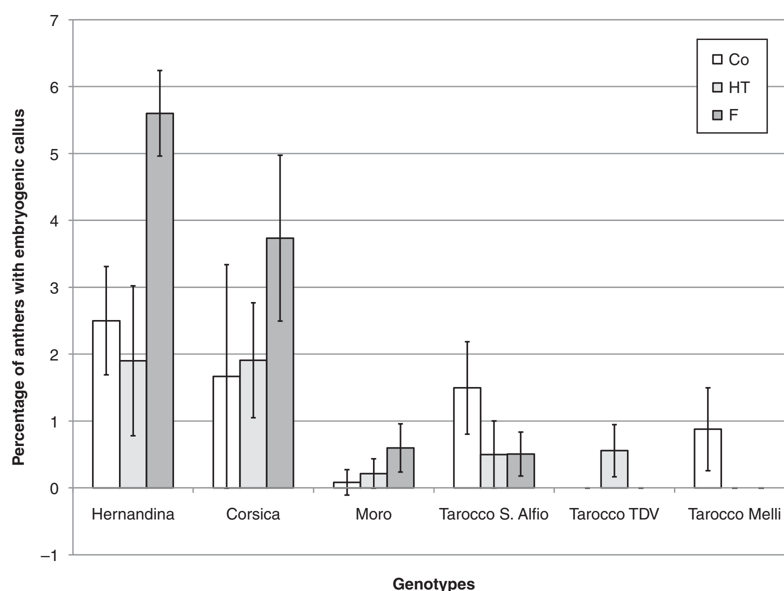

Figure 2. Effect of temperature treatments on the response of *C. sinensis* and *C. clementina* anthers with embryos or embryogenic callus. The data are the means ± SE. F, −20°C for 30 min; HT, 37°C for 60 min; Co, 27 ± 1°C constant.

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vegetative and one generative), and blocking starch production or dissolving microtubules (Nitsch 1977). Other profound cytoplasmic, nuclear rearrangements, or changes in gene expression occur before and during the induction of microspore embryogenesis (Bárány et al. 2005; Testillano et al. 2005; Seguí-Simarro & Nuez 2008).

Even if it is commonly known that thermal treatments are the most effective and easiest methods to induce pollen embryogenesis, the optimum level and duration of the shock depends on the genotype (Dunwell et al. 1983). In this study, different results were produced by the same thermal shock. Similar to other stress treatments, thermal shock is frequently associated with the biosynthesis of heat-shock proteins (HSPs), but Seguí-Simarro et al. (2003) showed that HSPs have an indirect effect on the role of microspore embryogenesis that is directly related to stress tolerance (Seguí-Simarro & Nuez 2008) and consisting in the inhibition of apoptotic-like or programmed cell death in microspore cells (Zoriniants et al. 2005). Moreover, cell death and caspase 3-like activity increased after a cold stress treatment (considered inductive to microspore embryogenesis in barley), as well as, increase in endogenous reactive oxygen species and nitric oxide were observed (Rodríguez-Serrano et al. 2012).

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Characterization of regenerants: ploidy analysis and analysis of heterozygosity

Ploidy analysis by flow cytometry revealed that all the 30 regenerants (embryos and plantlets) obtained through sweet orange anther culture were diploid (2*n*), regardless of the cultivar or temperature treatment (Figure 3(A)). Conversely, all the 66 analyzed regenerants from the clementine anther culture were triploids (Figure 3(B)). Flow cytometer is useful to detect and compare the ploidy of *in vitro* regenerated plants (Naing et al. 2014).

Previous flow cytometry investigations on the ploidy of 94 regenerants, obtained through clementine anther culture, already showed that ~82% of them were triHs, rather than Hs or DHs, as expected (Germanà et al. 2005; Germanà 2007). Non-haploid (diploid, triploid, tetraploid, pentaploid, or hexaploid) embryos and plantlets have been obtained from anther culture of other genotypes (D'Amato 1977). In fruit crops, triploids have often been regenerated through anther culture in *Malus* × *domestica* (Brokh.) (Hofer et al. 2002), *Pyrus pyrifolia* Nakai (Kadota & Niimi 2004), and *Carica papaya* L. (Rimberia et al. 2006).

Triploids are important for breeding because of the seedlessness of their fruits and, for this reason, anther culture can be used to produce triploid plants that may be of great commercial importance when seedlessness is required by consumers, as in *Citrus* or table grapes.

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

To discriminate between these two types of regenerants, microsatellite DNA loci that were heterozygous for the parental genotypes in a preliminary screening were used. Three of four SSRs were selected, namely TAA1, TAA15, and TAA41, and they were heterozygous in the orange clones of both Tarocco and Moro cultivars. CAC15 was apparently homozygous with a single allele, which has been considered a homozygous state. The selected SSRs were all heterozygous for clementine cultivars Hernandina and Corsica.

The allele size ranges were compatible with the data reported in the literature (Kijas et al. 1995), with small differences in the absolute size of the base pairs depending on the size of the standard, polymer used in the capillary and/or machine used as shown in the literature (Testolin & Cipriani 2010).

All regenerants obtained from Tarocco and Moro cultivars showed allelic patterns identical to those of the original mother plants from which the regenerants were obtained. Therefore, from a genetic point of view, they were heterozygous with somatic origin.

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).

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* × *C. reticulata* (Deng et al. 1992). In these cases, anther culture can be regarded as an additional method for achieving somatic embryogenesis.

All analyzed regenerants of Hernandina and Corsica clementines showed a homozygous allelic pattern, displaying only a copy of the alleles of the mother plants. These results were absolutely consistent for all analyzed SSRs and should indicate a gametic origin for the regenerants (Figure 3(C)).

Interestingly, all Tarocco and Moro sweet orange clones showed the same allelic profiles in all analyzed SSRs. In spite of the small number of SSRs analyzed, which does not allow for definitive conclusions, the

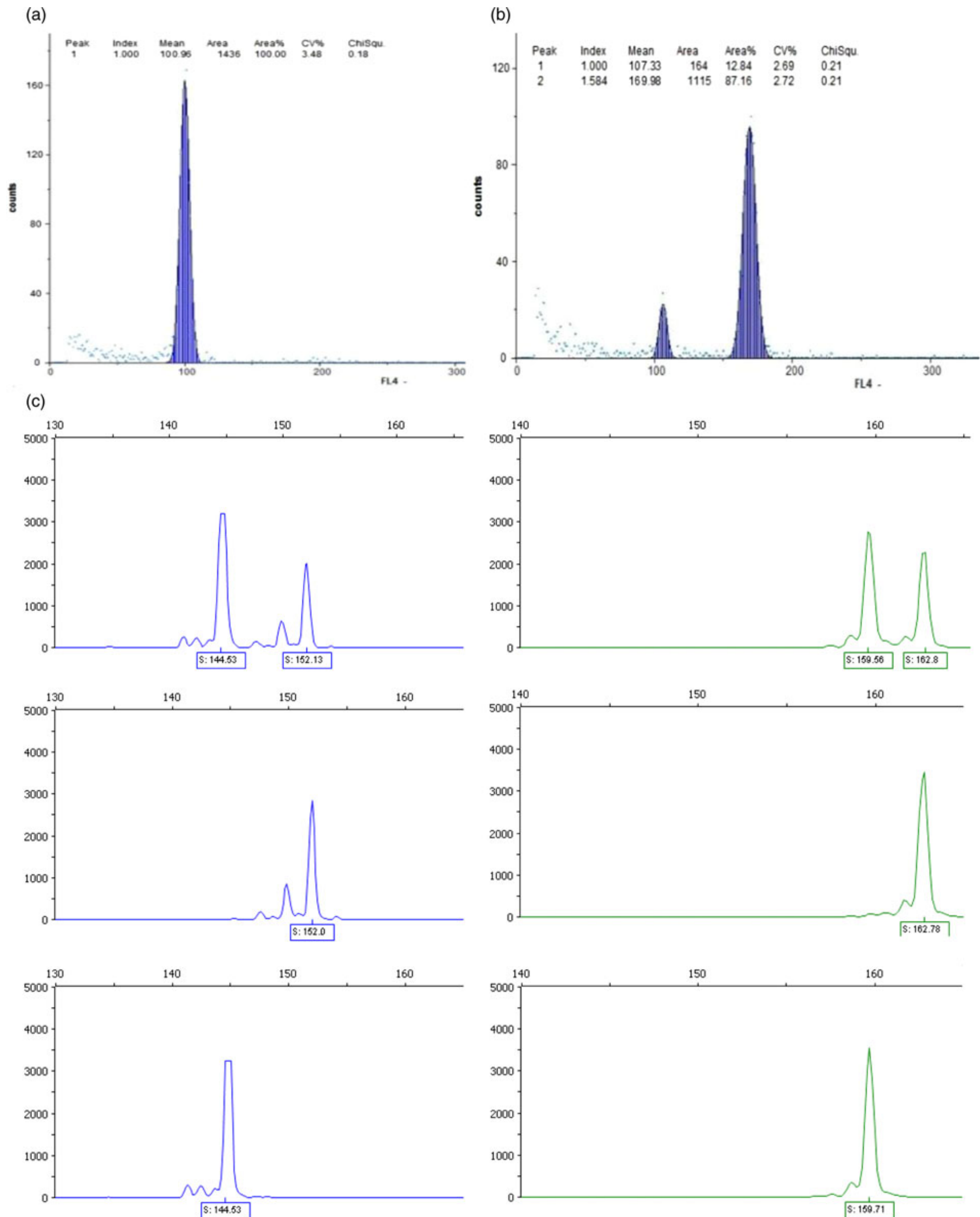

Figure 3. Characterization of anther culture regenerants. (A) Cytofluorometric analysis: histograms of fluorescence intensity in nuclei from diploid leaf tissue of *C. sinensis* and from a diploid regenerant. (B) Cytofluorometric analysis: histograms of fluorescence intensity in nuclei from diploid leaf tissue of *C. clementina* mother plant (peak 1) and from triploid embryo tissue of *C. clementina* cv. Corsica regenerated by anther culture (peak 2). (C) Microsatellite analysis: Pherograms of the microsatellite markers TAA41 (left) and TAA1 (right) profiles of the mother plant (top) and two Hernandina clementine regenerants. Although the mother plant is heterozygous and carries two alleles, the regenerants will display either allele of their mother plant. The presence of alternative alleles from the mother plant has been considered as support for the gametic origin of regenerants.

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identical profile at four loci could indicate a common somatic origin for these cultivars and clones, a common occurrence in many *Citrus* species as observed by several authors (Hodgson 1967; Cardoso et al. 2014). Microsatellites have been previously used to characterize the regenerants obtained from *Citrus* anther culture (Germanà et al. 2005, 2013).

Conclusions

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

This study is significant because it increased the number of genotypes that respond to microspore embryogenesis as well as the haploid induction rate (the frequency of anthers that recovery embryos). For the first time, anther culture of Hernandina and Corsica tangerines produced triploid homozygous calli and plantlets. Conversely, and again for the first time, blood oranges produced somatic embryogenic callus and plantlets via anther culture, which is very important for numerous applications in clonal propagation and breeding.

This paper confirms the strong influence of genotype on the type of response that can be obtained from *in vitro*-cultured anthers. Applying the same treatments to explants in culture resulted in the production of gametic embryos in clementines and somatic embryos in sweet oranges. Moreover, the clementine data confirm this plant's tendency to regenerate homozygous trihaploid embryos and plantlets.

However, the presence of multinucleate pollen grains, also developed in sweet oranges from this *in vitro* system, indicates that the induction of microspore nucleus division and the switch of the gametophytic developmental program to the embryogenic pathway. This finding constitutes a crucial step in designing new protocols for regenerating microspore-derived embryos and plants in sweet orange.

Further studies should be performed in other *Citrus* genotypes to achieve *in vitro* regeneration from anther culture, which is suitable for different applications, yielding new opportunities for genetic improvement and for innovation in propagative methods.

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Notes

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