

## Full Length Research Paper

## Multiploidy occurrence in tomato calli from anther culture

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Anther culture has long been used for the production of fully homozygous lines in order to produce, mainly, doubled haploid plants, which are of great interest in plant breeding. For tomato, a recalcitrant species for androgenesis production protocols have not been standardized. It is known that the genotype, anther size, the developmental stage of the microspore, and the medium composition are some factors that can influence the calli production. The present study aimed to adapt flow cytometry methodology to verify the microsporogenesis phases of anthers in order to assess the anther responsiveness of different tomato genotypes in an androgenesis-induction culture medium and to analyze the DNA ploidy level of calli produced by flow cytometry. Anthers from flower buds of length 1.0 to 5.9 mm, corresponding to the size range as analyzed by flow cytometry and cytogenetic methods, were inoculated into Murashige and Skoog (MS) basal medium containing the growth regulators 6-(*γ*,*γ*-dimethylallylamino) purine and indole-3-acetic acid. The obtained calli were subsequently analyzed by flow cytometry to determine the DNA ploidy level. Surprisingly, despite no pretreatment with microtubule-depolymerizing agents, five classes of multiploid calli were observed, as follows: class I (2C-4C-8C-16C), class II (2C-4C-8C-16C-32C), class III (4C-8C), class IV (4C-8C-16C) and class V (8C-16C-32C). Multiploid calli were identified in short-term (two month) culturing, suggesting that the variable culture duration did not directly influence the occurrence of endoreduplication. In this work, this type of somaclonal variation has been reported for the first time in tomato anther culture, and their possible origin has been discussed.

**Key words:** Callogenesis, flow cytometry, polyploidy, *Solanum lycopersicum*, somaclonal variation.

### INTRODUCTION

*In vitro* androgenesis is a remarkable example of cell totipotency in plants (Reynolds, 1997; Góralski et al., 2005), and it can induce male gametophyte cells or their precursors to deviate their route toward a sporophytic

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**Abbreviations:** FCM, Flow cytometry; PMCs, pollen mother cells.

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program, resulting in the development of haploid embryos (Góralski et al., 1999; Bárány et al., 2005; Seguí-Simarro, 2010). Haploid plants are interesting in fundamental research owing to the development of sporophytes with the gametophytic number of chromosomes. Nevertheless, their main practical utility is because of the generation of doubled haploid (DH) plants obtained by duplication of the chromosomes of haploid lineage either spontaneously or through induction by a microtubule-depolymerizing agent (Murovec and Bohanec, 2012). The DH generation is an exciting and powerful tool of pure line production (100% homozygous) for breeding programs (Kasha and Maluszynski, 2003; Dunwell, 2010; Germanà, 2010, 2011). Despite the knowledge obtained through model systems to study androgenesis such as in rapeseed (*Brassica napus* L.), tobacco (*Nicotiana tabacum* L.), or barley (*Hordeum vulgare* L.), the production of DH plants by androgenesis remains to be effectively standardized in tomato (*Solanum lycopersicum*,  $2n = 2x = 24$  chromosomes). The main hindrance in standardization is probably the recalcitrant nature of the crop (Bal and Abak, 2007; Seguí-Simarro and Nuez, 2007; Seguí-Simarro et al., 2011). *S. lycopersicum* has great economic importance in the horticultural industry (Wang et al., 2005), and it is considered as a good model for genetic studies of the Solanaceae family members (Hay et al., 2004; Koo et al., 2008).

The genotype dependency is a critical factor affecting the response in tomato androgenesis (Asoliman et al., 2007), and it has been reported that different cultivars of the same species exhibit diverse responses to another culture. For example, Zagorska et al. (1998) tested the androgenetic ability of 85 tomato cultivars, of which 53 were responsive and produced calli and a smaller number of cultivars developed regenerants. According to Seguí-Simarro and Nuez (2007), tomato genotypes can be divided into two categories: those inducible to form callus from meiocytes and those inducible to form embryos from microspores. Another critical aspect in androgenesis induction is the stage of microspore development at the time of anther excision and plating for *in vitro* culturing (Seguí-Simarro and Nuez, 2008a). In nearly all responsive crops, including model species such as rapeseed and tobacco, the inducible stage revolves around the first pollen mitosis, that is, from vacuolate microspore to early bicellular pollen (Touraev et al., 2001). In most tomato, the meiocyte is the most suitable developmental stage for androgenesis induction (Zamir et al., 1980; Summers et al., 1992; Shtereva et al., 1998; Brasileiro et al., 1999; Seguí-Simarro and Nuez, 2005; 2007). However, according to Seguí-Simarro and Nuez (2005), the stage with highest response in terms of callus generation in tomato is metaphase I to telophase II.

Hence, it is necessary to identify the appropriate anther developmental stage. Generally, traditional cytogenetic procedures such as squashing have been used for this

purpose (Seguí-Simarro and Nuez, 2005). However, an accurate determination of microspore/pollen stage requires a complex cytogenetic analysis. Nevertheless, external morphological indicator, such as the corolla length, has been used because it is a simple procedure for measuring large-scale cases, although it is a less reliable method (Dunwell, 2010). An alternative method used to determine the microspore/pollen development stage is flow cytometry (FCM), which is a practical and efficient methodology for quantify (screening) nuclear DNA content. This technique allows analyzing the individual nuclei faster than the cytogenetic traditional methods. Moreover, this methodology can be also used for evaluating the DNA ploidy level of calli, and these results can be related to any effect on androgenetic potential of anthers. The loss of embryogenic and organogenic competence is one of the major problems encountered when developing *in vitro* regeneration systems for propagation and genetic manipulation of plants (Winkelmann et al., 1998).

Considering that different genotypes can trigger different androgenetic responses, the difficulty of selecting responsive anthers to the culture and solving why calli have not develop in embryogenic calli, the present study focused on (i) the introduction of a procedure to select microsporogenesis stages is potentially more responsive for callus induction, associating cytogenetic, and FCM technique; (ii) the use of a culture medium previously described as effective in the literature for callus induction in different genotypes; (iii) evaluation of responsiveness of different genotypes by callus production, and (iv) screening of the DNA ploidy level profile in the calli.

## MATERIALS AND METHODS

### Plant materials

Flower buds belonging to four genotypes of *S. lycopersicum*: 'Debora', 'Santa Clara', 'Alambra', and 'Pizza Doro' were provided by Dr. Derly J. H. da Silva, Vegetable Germplasm Bank Curator [Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil]. Flower buds were collected in the morning at the experimental field of UFV; some of the collected buds were immediately fixed in a solution of methanol:acetic acid (3:1 v/v) for cytogenetic and FCM analyses, while the remaining were used for anther culturing. The fixative was changed three times, and the samples were stored at -20°C (Carvalho and Saraiva, 1997). The experiments were conducted at the Laboratório de Citogenética e Citometria, Departamento de Biologia Geral, UFV.

### Monitoring of anther maturation by FCM

A total of 15 flower buds (1.0 to 6.0 mm) of all genotypes were removed from the donor plants in the morning, and their anthers were classified according to their size as small (0.5 to 0.7 mm), medium (0.8 to 1.5 mm), or large (1.6 to 1.9 mm). Four anthers of each bud were subjected to FCM procedure (total: 60 anthers) and one anther was reserved for cytogenetic analysis (total: 15 anthers). First, the anthers were softened by enzymatic maceration

at 36°C as follows: the anthers were placed on a 70 µm mesh nylon filter submerged into a 2.0 mL microtube (Eppendorf) containing enzyme solution consisting of 2% cellulase (obtained from *Trichoderma viride*; Onozuka Yakult Honsha Co<sup>®</sup>), 1% cellulase (obtained from *Aspergillus niger*; Sigma<sup>®</sup>), 2.5% macerozyme (Yakult Honsha Co<sup>®</sup>), 2.5% pectinase powder (Sigma<sup>®</sup>), and 1% hemicellulase. The enzymatic maceration process was performed in two steps: the anthers were maintained in the enzymatic solution for 2 h and the softened anthers were macerated with a pin head so as to release the pollen mother cells (PMCs), followed by incubation of the PMCs in the same digestion solution for 30 min. Finally, the tubes were centrifuged for 5 min at 100 ×g, and the enzyme solution was discarded. Subsequently, the pellet was resuspended in 1 mL of OTTO-I lysis buffer (Otto, 1990) and the solution was homogenized with 12 pulses (approximately 1 s per pulse) by using a commercially available mini-handheld mixer to provide nuclei suspensions (Silva et al., 2010). The suspensions were filtered through 30 µm mesh nylon filter and centrifuged for 5 min at 100 ×g. The supernatant was discarded, and the pellet was resuspended in 100 µL of OTTO-I and incubated for another 10 min at the room temperature.

The nuclei suspension was stained with 1.5 mL of the OTTO-II buffer (Otto, 1990) supplemented with 1.5 mM 4'6'-diamino-2-phenylindole (DAPI; Sigma<sup>®</sup>), filtered through a 20 µm mesh nylon filter, and maintained in the dark for 30 min. The nuclei suspensions were analyzed by PAS-III flow cytometer (15-01-1000; Partec GmbH) equipped with a UV lamp (with emission at 378 nm), excitation filters (KG 1, BG 38, and UG 1), a GG 435 long-pass barrier filter for blue fluorescence, and a TK 420 nm dichroic mirror to supply epi-illumination. The equipment was carefully calibrated and aligned using microbeads and standard solutions as per the manufacturer's recommendations. The FlowMax software (Partec GmbH) was used to process the data. The instrument gain was set up in such a way that the G<sub>0</sub>/G<sub>1</sub> peak was positioned on channel 200 using nuclear suspension from leaves of the same plant.

### Cytogenetic analysis

The anther that was separated in each bud was macerated in the same enzymatic solution used in the FCM analysis. Each anther was slightly fragmented with the tip of a scalpel blade in 50 µL of 45% acetic acid solution (v/v). The excessive material (wall anther) was removed from the slide and, on a hot plate, the solution containing the anther cell suspension was spread on the slide using a hair dryer. The slides were stained with 4.0 mM DAPI (Sigma<sup>®</sup>) and observed under the Olympus TM BX-60 fluorescence microscope.

### Anther culture

Flower buds of *S. lycopersicum* genotypes 'Debora', 'Santa Clara', 'Alambra', and 'Pizza Doro' were sorted manually according to the size, from 1.0 to 6.0 mm at an interval of 1.0 mm. Next, the buds were pre-treated with 1% polyvinylpyrrolidone (PVP; Sigma<sup>®</sup>) solution in order to minimize the oxidation of the anthers and were maintained at 4°C for 48 h. After a cold shock, buds were sterilized under laminar flow hood with 70% (v/v) ethanol (Merck<sup>®</sup>) for 20 s, followed by immersion in 1.0% (v/v) solution of sodium hypochlorite (Merck<sup>®</sup>) for 5 min, and washed three times (3 min, each) with autoclaved distilled water (Zagorska et al., 1998). The anthers were aseptically removed; four anthers were inoculated into 60 × 15 mm Petri dishes (J. Prolab<sup>®</sup>) containing callus induction medium, and one anther was reserved for cytogenetic analysis to determine the stage of meiotic development. Induction medium consisted of MS salts and vitamins (Murashige and Skoog, 1962; Sigma<sup>®</sup>) supplemented with 2.8 g L<sup>-1</sup> phytigel, 20 g L<sup>-1</sup> sucrose, 0.1 g L<sup>-1</sup>

myo-inositol, 0.04 g L<sup>-1</sup> cysteine, 1 mg L<sup>-1</sup> 6-(γ,γ-dimethylallylamino) purine (2iP; Sigma<sup>®</sup>) and 2 mg L<sup>-1</sup> indole-3-acetic acid (IAA; Sigma<sup>®</sup>). The pH was adjusted to 5.7 prior to autoclaving. The growth regulators were filter-sterilized and added after autoclaving of the medium (adapted from Shtereva et al., 1998).

Petri dishes were sealed and kept in darkness for 1 month at 25 ± 2°C and subsequently transferred to a 16/8 h light/dark photoperiod regime with 36 µmol m<sup>-2</sup> s<sup>-1</sup> light radiation. Subcultures were made at 30 day intervals. The percentage of callus production was evaluated 60 days after inoculation. Anthers that did not respond to culture were discarded. The experiment followed a completely randomized design in a factorial 4 × 5, with a total of 20 treatments, corresponding to four genotypes and five bud sizes. Each treatment consisted of six Petri dishes containing four explants, and the parameter evaluated was the average of explants exhibiting calli. The data were submitted to analysis of variance (ANOVA) and Tukey's test at 5% probability level, both performed by using the statistical program Genes (Cruz, 1997).

### Calli screening by FCM

We analyzed the ploidy level of young calli (2 month old) by FCM, on six calli in each treatment, one per plate, except the plates where the anthers did not respond to induction of callus. Random pieces of each callus were removed and processed to release the nuclei, as described by Galbraith et al. (1983). Approximately 50 mg of callus was chopped in 100 µL of OTTO-I lysis buffer, followed by the addition of 400 µL of the same buffer. Then, the nuclei suspension was adjusted to 1 mL with the same buffer, filtered through 30 µm-mesh nylon filter into 2-mL microcentrifuge tubes, and centrifuged at 100 ×g for 5 min. The pellet was then incubated in 100 µL of OTTO-I lysis buffer for 10 min. The nuclei suspension was stained with 1.5 mL of the OTTO-II buffer supplemented with 1.5 mM DAPI (Sigma<sup>®</sup>), filtered through the 20 µm-mesh nylon filter, and maintained in the dark for 30 min. The suspension was analyzed by the Partec PAS<sup>®</sup> flow cytometer. For DNA ploidy level characterization of the calli, nuclear suspension from leaves of the correspondent plants (2C ploidy standard) was used to determine the G<sub>0</sub>/G<sub>1</sub> peak. The peak relative to the 2C standard nuclei was set to channel 200. The histograms were obtained from the semi-logarithmic scale (log X-axis).

### Cytological analysis

In order to confirm the nuclei isolation efficiency and to eliminate the possibility of nuclei aggregate formation, slides were prepared after the cytometric analysis. Nuclei suspensions were transferred to 2 mL of the microcentrifuge tubes and centrifuged at 100 ×g for 5 min. The supernatant was discarded, and 30 µL of the pellet were resuspended and then placed on a glass slide and covered with a coverslip. The images were captured by the DP71 digital camera coupled to the Olympus TM BX-60 fluorescence microscope using WU filter.

## RESULTS

### Monitoring of anther maturation by FCM and cytogenetic methods

For the first time, FCM approach was used to screen the meiotic progression in tomato anthers. Anthers of different sizes were evaluated by FCM in the four genotypes of tomato. The generated histograms showed

coefficient of variation (CV) of < 5%, which is considered acceptable in plant FCM studies (Doležel and Bartoš, 2005). Histograms with low CV indicate that nuclei suspensions have intact, isolated, and stoichiometrically stained nuclei. Among the 60 anthers of different sizes analyzed, two histogram patterns were identified. The small anther profile consists of two histogram peaks, corresponding to the nuclei with 2C and 4C DNA content (Figure 1a). The large anther profile showed three peaks (1C, 2C and 4C ploidy level) (Figure 1b). Differences in the relative nuclear DNA content in PMCs and microspores within anthers were clearly evident from FCM analysis; young anthers (small) did not show *n* cells. In parallel to FCM analysis, cytogenetic analysis was performed to identify the meiotic phases correlated to the evaluated anther sizes. The developmental stages of microsporogenesis demonstrated that the (a) anthers of 0.5 to 0.7 mm size contained cells at interphase and prophase I; (b) anthers of 0.8 to 1.5 mm size contained meiocytes at metaphase I, anaphase I, telophase I, and meiosis II phases; and (c) anthers of > 1.6 mm size presented cells as tetrads and microspores (data not shown).

### Anther culture

The four genotypes used in this study were tested for their androgenetic response by anther culture under the same experimental conditions. The FCM results in anther contributed to the choice of flower buds of the optimal size for inoculation of anthers, as suggested in the literature. Thus, anthers containing meiocytes (1.0 to 6.0 mm sized flower bud) were inoculated into the induction medium. Calli formation occurred 30 days after the beginning of each culturing. During the initial culturing, in the dark, the calli presented whitish color, which turned to green under 16/8 h photoperiod. Some shoots were formed on the generally compact calli (Figure 2). The evaluation of genotype  $\times$  anther size interaction showed significant differences in some treatments for callus production, as can be visualized in Table 1. The callus production for 'Pizza Doro' genotype was influenced by the anther size (2 to 3 mm), and the genotype 'Alambra' influenced both 4 to 5 and > 5 mm anther lengths.

### Calli screening by FCM

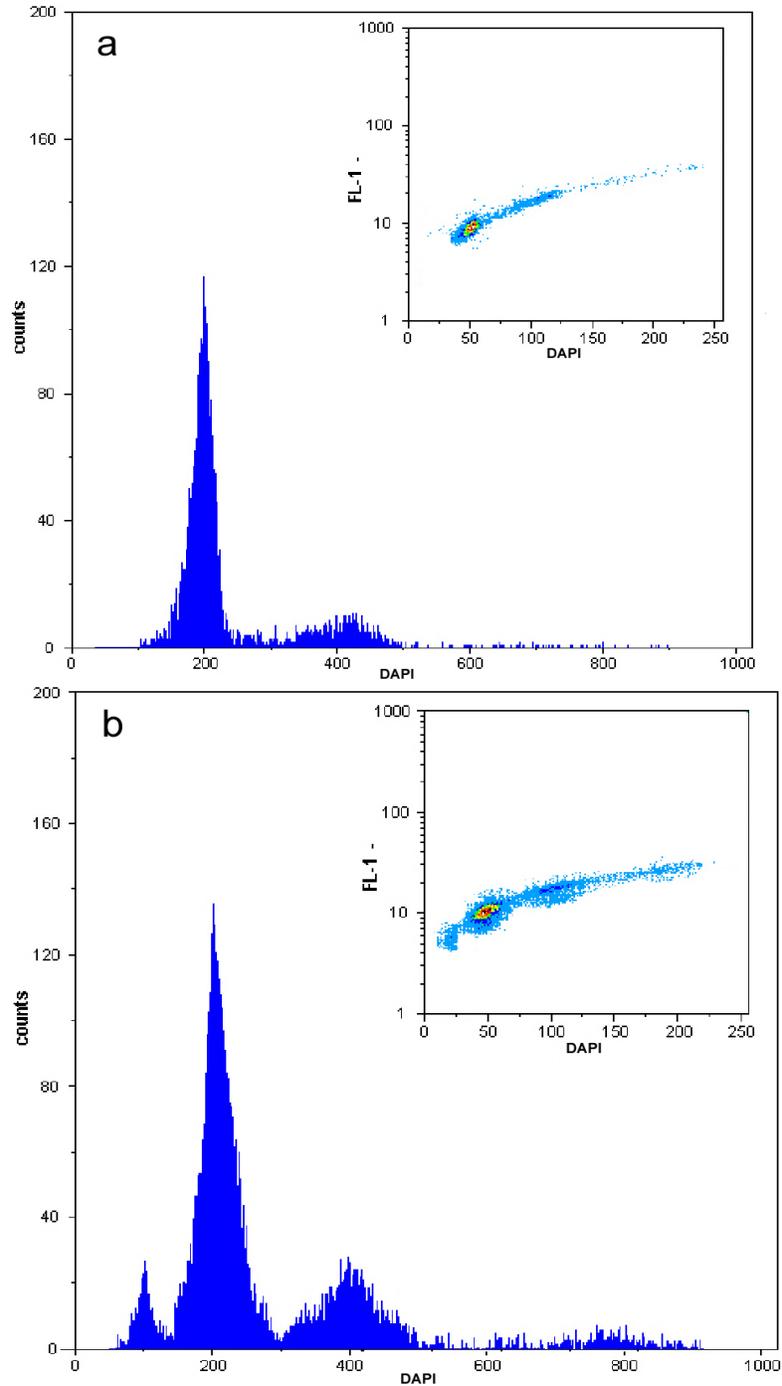
The procedure adopted here for nuclei extraction from calli was effective. The microscopic examination of nuclei suspensions revealed isolated nuclei, with only few nuclear aggregates. Moreover, the FCM analyses results were consistent in showing low CVs. Surprisingly, these FCM analyses showed that all calli were multiploid, a term suggested here to denote calli with a complex type of mixoploidy, presenting multiple DNA ploidy levels in

the same calli and different ploidy levels in different calli. These multiploid calli were divided in five classes of DNA ploidy levels: class I (2C-4C-8C-16C), class II (2C-4C-8C-16C-32C), class III (4C-8C), class IV (4C-8C-16C-32C), and class V (8C-16C-32C) (Figure 3). We have presented 'Débora' FCM data to illustrate the multiploid event; however, the same classes were observed for the other genotypes as well. Each analyzed callus presented one of these classes. The calli showed the following distribution: 44.4% of the total belonging to class II, 27.78% to class I, 16.67% to class IV, and 5.55% to classes III and V. Class II was also analyzed cytologically to examine the nuclear size profile. Figure 4 illustrates five nuclear types in the ascending order of size that was compatible with five types of DNA ploidy levels as detected by FCM.

### DISCUSSION

In this study, we show that flow cytometry can be a useful tool to screen the developmental stage of anthers. Brasileiro et al. (1999) also analyzed the correlation between tomato anther length and meiotic phase. These authors reported that flower buds of size 5.0 to 6.0 mm and anthers of size 2.0 to 2.3 mm showed cells in meiosis. Furthermore, Seguí-Simarro and Nuez (2005) observed that meiocytes were concentrated in the buds of length 4.0 to 5.9 mm. However, according to Zamir et al. (1980), anther and flower bud sizes are not directly correlated to the microsporogenesis stage. In the present study, different developmental stages were identified for similar bud sizes in the same plant, anthers in the same flower, and even within a single anther. Furthermore, the difference in the results of the present study and other literature reports can be attributed to the different genotypes analyzed in each experiment as well as the environmental and physiological conditions in which the plants were grown. The cytogenetic and FCM techniques allowed differentiation of the anthers at the interphase/prophase and the tetrad/microspore stages. Small anthers at the interphase/prophase I displayed a histogram profile similar to that of the standard tomato leaf, whereas large anthers in the tetrad stage and microspore stages showed a peak corresponding to the haploid DNA content of microspores. Based on these results, flower buds of size 1.0 to 6.0 mm and anther size similar to that analyzed by FCM and cytogenetic procedures were inoculated into the induction medium.

The FCM analysis presented here represents a relatively simple, rapid, and reliable method to analyze and discriminate the development stage of anthers. The sample preparation in this study requires a small amount of anthers and the analysis is rapid. Moreover, a significant number of nuclei can be measured in a few minutes, which makes the results statistically robust. This procedure is interesting to accelerate the meiotic



**Figure 1.** Representative FCM histograms of nuclei isolated from DAPI-stained tomato 'Deborá' anthers. a) Small anthers (0.5 to 0.7 mm) with  $G_0/G_1$  peak of anther wall cells and microspore mother cells set to channel 200. The  $G_2/M$  peak of tapetal cells,  $G_2$  microspore mother cells and meiocytes at prophase I is represented in channel 400. b) Large anthers (1.6 to 1.9 mm) with  $G_0/G_1$  peak of anther wall cells and meiocytes II set to channel 200. The  $G_2/M$  peak of anther wall cells and meiocytes I is represented in channel 400. The peak in channel 100 corresponds to haploid nuclei released from tetrads. The  $G_2/M$  peak in channel 800 corresponds to endoreduplicated tapetal cells. In the upper right panel of both histograms, density dot plots (cytograms) show the distribution frequencies of nuclei corresponding to the peaks based on two fluorescence parameters.



**Figure 2.** Calli originated from anther culture of tomato ‘Debora’. Left: initial callus maintained in the dark. Center: callus transferred to light, showing greenish color and early shoot. Right: shoot at a more advanced developmental stage. The brownish calli pieces correspond to vestiges of anthers in oxidation process. Scale bar = 5 mm.

**Table 1.** Average number of anthers per Petri dish showing calli in accordance with the flower bud size at 60 days after inoculation.

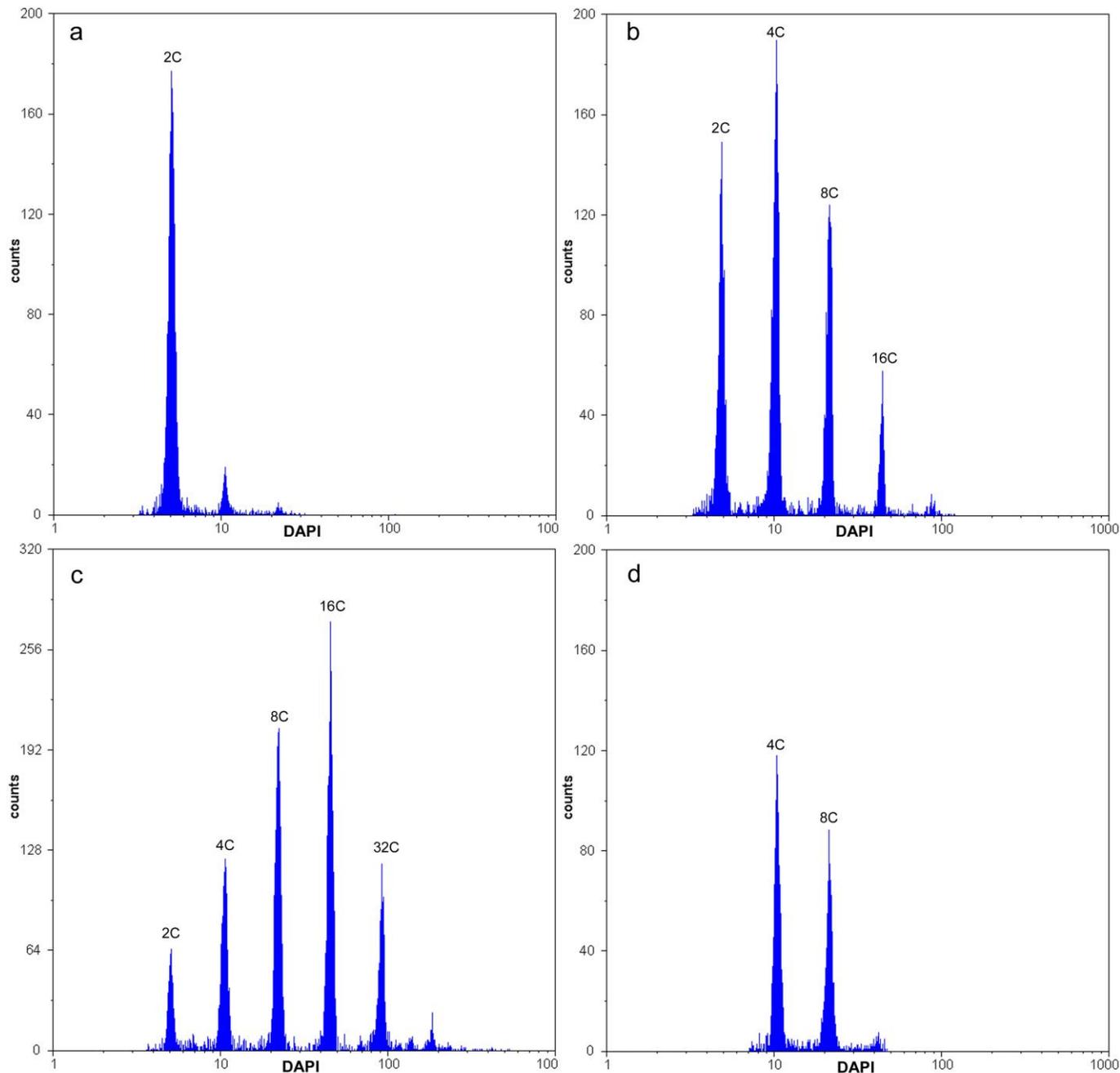
Budsize (mm)	Genotype			
	Débora	Santa Clara	Alambra	Pizza Doro
1-2	0.667 <sup>Aa</sup>	1.667 <sup>Aa</sup>	0.00 <sup>Ab</sup>	0.00 <sup>Ab</sup>
2-3	1.667 <sup>Aa</sup>	2.50 <sup>Aa</sup>	2.667 <sup>Aa</sup>	3.00 <sup>Aa</sup>
3-4	1.33 <sup>Aa</sup>	1.00 <sup>Aa</sup>	2.33 <sup>Aa</sup>	1.33 <sup>Ab</sup>
4-5	0.667 <sup>Ba</sup>	1.00 <sup>Ba</sup>	3.33 <sup>Aa</sup>	1.33 <sup>Bb</sup>
≥ 5	0.00 <sup>Ba</sup>	1.50 <sup>Aa</sup>	2.33 <sup>Aa</sup>	1.00 <sup>Bb</sup>

\*Means followed by the same capital letters at the horizontal group are statistically homogeneous as determined by Tukey's test at 0.05% probability level.

analysis, especially in plants in which no correlation exists between bud size or anthers with the meiotic stage. We have identified the response of four different tomato genotypes with different anther sizes in relation to calli production that were induced in an induction medium previously tested. Most studies on tomato anther culture have reported the occurrence of callogenesis ((Zamir et al., 1980; Shtereva et al., 1998; Brasileiro et al., 1999; Seguí-Simarro and Nuez, 2007; Corral-Martínez et al., 2005; 2011). However, considering the difficulty in achieving plant regeneration from calli in the test species besides the high recalcitrance of tomato to another culture (Seguí-Simarro and Nuez, 2005; Corral-Martínez et al., 2011), very little progress has been made in this context, with no standardized method being available for the generation of haploid and DH tomato plants (Seguí-Simarro and Nuez, 2007).

Various culture media have been tested for the induc-

tion of callus in tomato anthers. In this study, the induction medium suggested by Shtereva et al. (1998) was efficient for calli production in four genotypes of tomato anthers tested. Using the same medium, Asoliman et al. (2007) obtained a high frequency of somatic embryos from tomato anther culture, whereas Seguí-Simarro and Nuez (2005) obtained a high percentage of calli. Identification of the microsporogenic phase is considered as an important step for the successful anther culture. In this study, anthers were selected according to the size of flower buds and analyzed by FCM and cytogenetic procedures. In order to select the desired anther phase, Seguí-Simarro and Nuez (2005) and Góralski et al. (2005) identified the stage most responsive to androgenesis in tomato and maize plants, respectively, based on the relationship between *in vitro* anther development and cell characterization of the microspore/pollen.



**Figure 3.** Representative histograms, in semi-logarithmic scale (log X-axis), of tomato 'Deborá' nuclei stained with DAPI. The X-axis represents the relative DNA content; the Y-axis indicates the number of examined particles. The channel numbers are proportional to the log of fluorescence intensities. a) Donor plant leaf used as diploid DNA content standard. (b-f) Multiploid calli originated from anthers. The last peak with unidentified ploidy corresponds to the  $G_2/M$  phase. The various peaks corresponding to multiploid calli were identified by comparison of their nuclear DNA content with that of the diploid leaf.

Our results show that anthers in meiosis, up to anaphase II, were equally responsive for callus induction. Anthers containing microspores, obtained from buds with length ranging from 5.0 to 5.9 mm, did not respond to *in vitro* induction. Other studies corroborated that meiocytes is the most responsive stage for tomato anther culture

(Zamir et al., 1980; Summers et al., 1992; Brasileiro et al., 1999; Seguí-Simarro and Nuez, 2005). Gresshoff and Doy (1972), Summers et al. (1992) and Brasileiro et al. (1999) reported that anthers showed higher frequencies of callus production during prophase I. Nevertheless, Seguí-Simarro and Nuez et al. (2005) identified

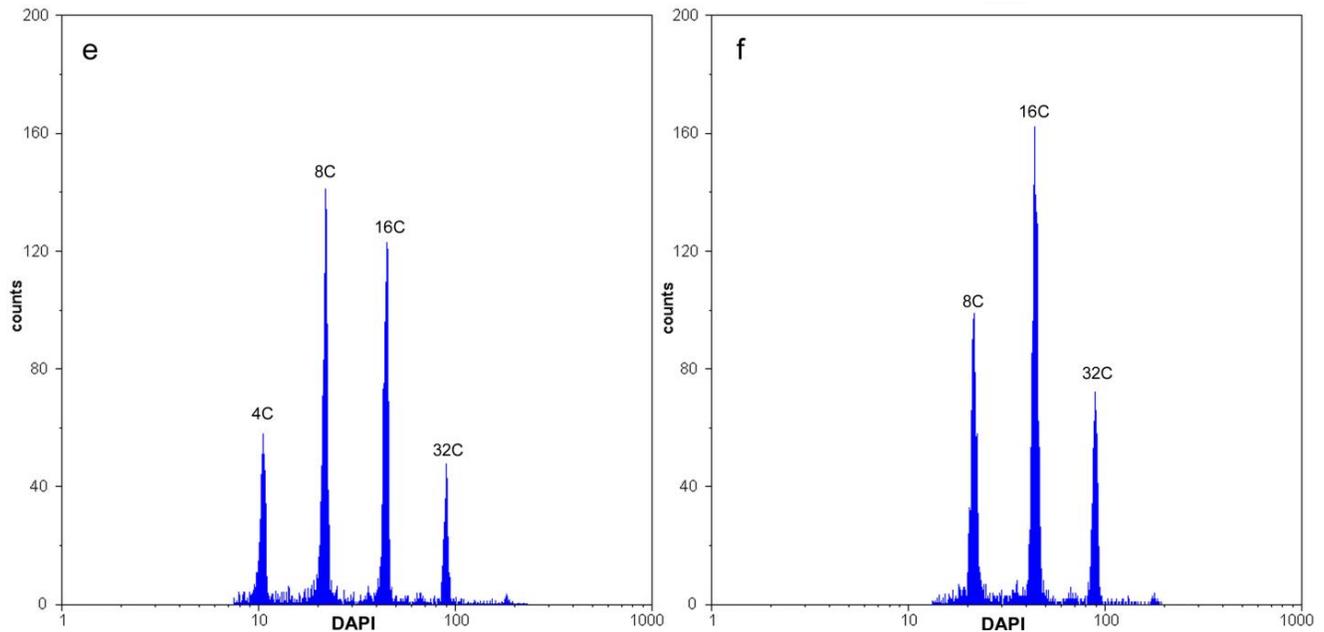


Figure 3. Contd.

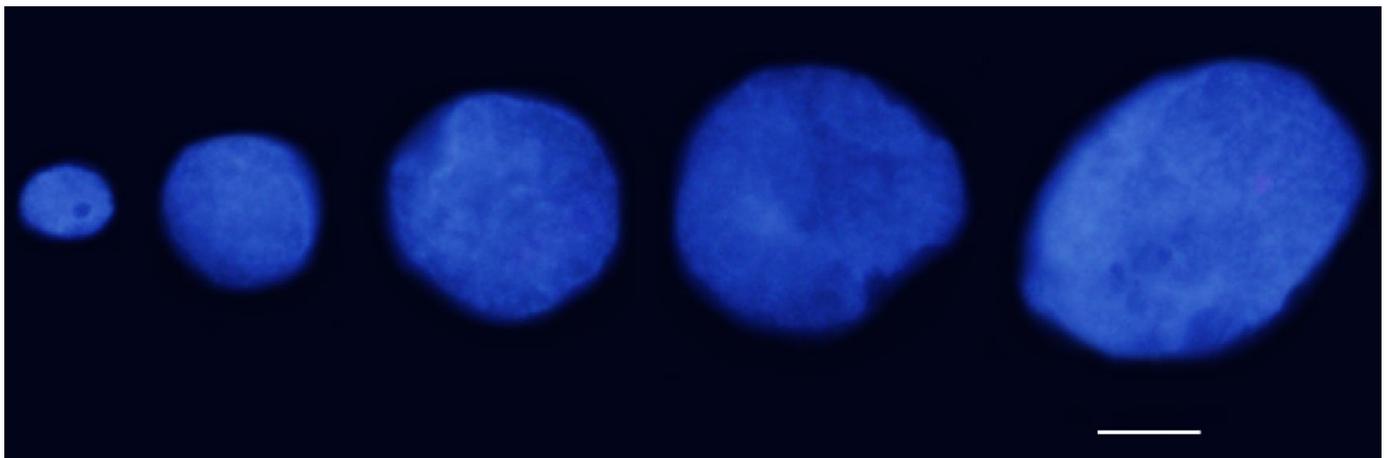


Figure 4. Cytological analysis of class II (2C-4C-8C-16-32C) nuclear suspension showing well-preserved, isolated, DAPI-stained nuclei. Scale bar = 10 µm.

callusogenic response in meiocytes from metaphase I until telophase II, whereas Shtereva et al. (1998) identified the response from prophase to telophase II as being optimal for tomato anther implantation. In most species, including model systems such as rapeseed and tobacco, the most sensitive period for inductive treatment is that near the first haploid mitosis, that is, from the vacuolated microspore to bicellular pollen stage (Touraev et al., 2001). Bal and Abak (2005) reported that the uninucleate stage was found to be suitable for the production of multicellular structure (proembryo) from

tomato-isolated microspores. Occasional shoots were observed in this study, but these shoots did not regenerate into plants, probably owing to the recurrent polyploidization noted in calli. The ploidy was examined in an attempt to find out the reason of compact callus without embryogenic competence.

According to Ochatt et al. (2000), the increase in the DNA ploidy levels in calli can significantly complicate the regeneration of plants. The low efficiency of polyploidy calli regeneration is related to the loss of totipotency due to polyploidization (Colijn-Hooymans et al., 1994).

According to Phillips et al. (1994), changes in DNA ploidy levels are the most common somaclonal variations in tissue cultures. In fact, cell polyploidization in plant tissue culture has been described in numerous experimental systems. Park et al. (2010) cultivated *Doritaenopsis* leaf, root tip and somatic embryo *in vitro* in order to investigate their degrees of endoreduplication and found that somatic embryos showed the highest level (2C-64C). Several possible origins for somaclonal variations have been suggested, among which culture time is notable. Here, it was observed that this variable did not affect polyploidization in the calli, which presented populations of polyploid cells after two months of inoculation. In contrast, Salas et al. (2011) observed that culture time was the main factor influencing chromosome doubling in embryos derived from eggplant anther culture. Moreover, Song et al. (2000), Hao and Deng (2002) and Zhang et al. (2006) reported an increase in chromosome variations throughout subculturing.

The *in vitro* environment itself is an important source of stress. Though initial culture conditions promote cell proliferation, they may also cause defects in normal cytokinesis (Seguí-Simarro and Nuez, 2008b). In the present study, we used the culture medium suggested by Shtereva et al., (1998) assuming that the medium used successfully by the authors would be adequate for the genotypes used in this study. Thus, an interaction among growth regulators added to the culture medium and the genotypes of the donor plants may have induced endoreduplication cycles, resulting in high DNA ploidy levels in calli arising from tomato anther culture. It has been reported that the influence of growth regulators on calli induction depends on the donor plant genotype (Shtereva et al., 1998), representing one of the main factors that determine the type and frequency of genetic alterations (Jain, 2001). Seguí-Simarro and Nuez (2008a) also revealed that growth regulators induce increased DNA ploidy levels in microspores during anther culturing.

In our study, no population of haploid nuclei was observed in calli analysis by FCM, but multiploid nuclei were observed. According to Salas et al. (2011), this multiploidy could have somatic origin, as reported for anther culture of eggplant and the related species. In addition, Levenko et al. (1977) mentioned that cells of the anther wall contribute to the formation of mixoploid calli in tomato plants. Corral-Martínez et al. (2011) further showed that up to 83% of the calli produced from tomato anthers were derived from anther wall tissue. Nevertheless, Seguí-Simarro and Nuez (2007) and Corral-Martínez et al. (2011) also reported calli with haploid DNA content. Moreover, we suggested the possibility of the multiploidy to be meiotic cell lineage origin, whereas only anthers with meiocytes II were responsive. However, anthers with meiocytes after phase II possess somatic tissues capable of induction and which have not responded. This study allowed us to conclude that: (i) the association of cytogenetic and FCM

techniques were effective in correlating the anther size with the meiotic developmental stage. Therefore, this method was considered to be adequate for the identification of the developmental stages of microsporogenesis that are most responsive to callus induction on a large scale; (ii) Anthers containing meiocytes in the stages of prophase I up to anaphase II (2.0 to 3.9 mm) showed a higher percentage of callus formation. However, (iii) no haploid material was obtained in all genotypes tested; (iv) multiploid calli were identified in the short-term culture (two months), suggesting that this variable (time of culturing) was not directly responsible for recurrence of endoreduplication. The occurrence of these endoreduplication cycles in tomato anther culture has been reported for the first time in the present paper. Most reports of anther culture reported that the DNA ploidy levels were not exceptionally high. Since we used different genotypes in a culture medium considered suitable for haploid production, our results indicate a greater importance of the interaction between genotype and growth regulators in the process of obtaining haploids that promoted calli with different and high DNA ploidy levels.

### Conflict of interests

The authors did not declare any conflict of interest.

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