In Vitro Polyploidization in Solanum lycopersicum Mill. ‘Santa Cruz Kada Gigante’

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Received January 21, 2014; accepted May 19, 2014

Summary  Solanum lycopersicum Mill. is a model plant for biological approaches and includes accessions with great agronomic relevance, such as the ‘Santa Cruz Kada Gigante’. For development of new S. lycopersicum accessions, in vitro tissue culture has proved relevant, contributing to the establishment of germplasm banks and breeding programs. In spite of the vast amount of tissue culture research already carried out in S. lycopersicum, in vitro polyploidization is still emerging. Thus, this study aimed to induce polyploid plantlets from ‘Santa Cruz Kada Gigante’ from shoot tip explants. This accession presented 2n=2x=24 chromosomes and 2C=2.00 picograms of nuclear DNA. Hence, no preexisting variation was identified, and ‘Santa Cruz Kada Gigante’ was considered appropriate for in vitro polyploidization. This procedure provided tetraploid (4x) and mixoploid (2x/4x and 2x/4x/8x) plantlets, which were identified by flow cytometry (FCM) and multiplied in vitro. Based on these results, the tissue culture procedure provided polyploid ‘Santa Cruz Kada Gigante’ plantlets, which represent a new source of variability for breeding programs of S. lycopersicum.

Key words  Tomato, Polyploidy, DNA ploidy level, Cytogenetic, Flow cytometry.

Solanum lycopersicum Mill. has been used as a model plant in phylogenetic (Filiz 2012) and morphogenetic approaches (Samach and Lotan 2007, Jian et al. 2011). This species also encompasses cultivars of great economic importance, which provide tomato fruits widely used in food and industry (Sardaro et al. 2013).

For the development of new S. lycopersicum cultivars, biotechnological in vitro techniques have proved relevant. Among these, in vitro tissue culture has provided support for breeding programs (Praça et al. 2009). From the tissue culture methods, in vitro polyploidization has been applied to distinct species aiming at the improvement of agronomic traits based on chromosome variability (Dhooghe et al. 2011). Considering that morphological and physiological changes may occur as a result of ploidy alteration (Meyer et al. 2009), relevant guidelines should be followed for success in polyploidization, such as concentration, type and time of the antimitotic agent; type of explant; genotype of the donor plant, and methods for ploidy screening (Dhooghe et al. 2011).

In vitro polyploid plantlets of S. lycopersicum were obtained from the line ‘Stupickč’ (Praça et al. 2009), which has been used as a primary standard in flow cytometry (FCM) and image cytometry measurements (Praça-Fontes et al. 2011). After polyploidization, mixoploid and tetra-
ploid plantlets were identified by FCM and successfully recovered (Praça et al. 2009).

In accordance with Dhooghe et al. (2011), the number of surviving polyploid plantlets is fundamental for choosing the most adequate polyploidization treatment. Based on this parameter, the number of polyploid plantlets obtained in distinct researches, including S. lycopersicum, has been generally considered low (Praça et al. 2009).

Since polyploidization induction for commercial accessions of S. lycopersicum is still at the initial phases, this study sought to: (a) determine the chromosome number and nuclear 2C value of S. lycopersicum ‘Santa Cruz Kada Gigante’; and (b) obtain polyploid plantlets from this access in vitro.

Materials and methods

**Plant material**

Commercial seeds of the accession S. lycopersicum ‘Santa Cruz Kada Gigante’ (Topseed Garden®) were used. This cultivar was chosen due to its great commercial interest. Seeds of S. lycopersicum ‘Stupické’ (primary standard for FCM, 2C=2.00 pg, Praça-Fontes et al. 2011) were kindly supplied by Dr. Jaroslav Doležel (Experimental Institute of Botany, Czech Republic).

**Nuclear 2C value and karyotype of ‘Santa Cruz Kada Gigante’**

The nuclear DNA content of ‘Santa Cruz Kada Gigante’ was measured according to the procedure described by Karsburg et al. (2009). The material was analyzed with a Partec PAS® cytometer (Partec GmbH, Muenster, Germany), equipped with a laser source (488 nm) and filters RG 610 nm. The generated histograms were analyzed with the FlowMax® software (Partec GmbH) to determine the nuclear genome size of the samples by comparing the G 0/G1 nuclei peak of ‘Santa Cruz Kada Gigante’ and of the standard ‘Stupické’. Five independent repetitions were performed and the average genome size of the sample was measured in picogram.

The chromosome number of ‘Santa Cruz Kada Gigante’ was determined from root tips (0.5–1.0 cm long) treated with the microtubule inhibitors oryzalin (Sigma®) or amiprophos-methyl (Sigma®) at a final concentration of 4.0 μM for 19 h at 4°C. Subsequently, the root tips were macerated in pectinase solution (Sigma®) diluted in the proportion 1:10 for 2 h at 34°C. Slides were prepared by meristematic cell dissociation, air-dried and stained with 5% Giemsa (Merck®) (Karsburg et al. 2009, Praça et al. 2009).

Metaphase images were captured with a Media Cybernetics® Camera Evolution™ charge-coupled device (CCD) video camera, mounted on a Nikon 80i microscope (Nikon, Japan).

**In vitro polyploidization of ‘Santa Cruz Kada Gigante’ and FCM screening**

In a laminar flow hood, the seeds were disinfested with 70% ethanol (Merck®) for 20 min, 2.5% sodium hypochlorite (Merck®) and 0.1% Tween 20 (Merck®) solution for 20 min, and then rinsed five times with sterile distilled water. Seeds were germinated in 500-mL flasks containing MS (Murashige and Skoog 1962) salts (Sigma®) supplemented with MS vitamins (Sigma®), 3.0% sucrose (Sigma®) and 0.7% agar-type A (Sigma®). The pH of this medium was adjusted to 5.7 prior to autoclaving. The cultures were maintained at 27°C under a 16/8 h light/dark regime with 36 μmol m⁻² s⁻¹ light radiation provided by fluorescent lamps (20 W, Osram®) for 30 days (Praça et al. 2009).

After one month, shoot tip explants were excised and placed in Erlenmeyer flasks containing 10 mL of polyploidization medium (Praça et al. 2009) with different colchicine (Sigma®) concentrations (0.0–control, 0.5, 1.5, 2.5, 5.0, 6.5, 8.0 mM).

The Erlenmeyer flasks were shaken (40 rpm) with 96 or 120 h pulses in a growth room. Each flask contained three shoot tip explants, with four replicates for each treatment. Subsequently, the
Explants were washed three times with sterile distilled water and placed in semi-solid medium, as described above, but deprived of colchicine (Praça et al. 2009). Cultures were maintained at 27°C under a 16/8 h light/dark regime with 36 μmol m⁻² s⁻¹ light radiation provided by fluorescent lamps (20 W, Osram®).

After six months, leaf samples were collected for analysis by FCM, according to Praça et al. (2009) and CyStain UV Ploidy Partec® protocol. The nuclei suspensions were analyzed with a Partec PAS® flow cytometer (Partec® GmbH), equipped with a UV lamp emitting at 388 nm and a TK 420 filter. The FlowMax® software (Partec® GmbH) was used for data analyses. The Tukey test was used to compare treatments at a level of significance of 1%, using the software ASSISTAT 7.6 beta (Silva 2012).

**Results**

**Nuclear 2C value and karyotype of ‘Santa Cruz Kada Gigante’**

The fluorescence peaks of the G₀/G₁ nuclei of ‘Santa Cruz Kada Gigante’ and ‘Stupické’ showed a coefficient of variation (CV) lower than 5%. These G₀/G₁ peaks overlapped (Fig. 1a), indicating that the mean nuclear DNA content of ‘Santa Cruz Kada Gigante’ was the same in relation to the internal standard, 2C=2.00 pg.

Root tips of ‘Santa Cruz Kada Gigante’ treated with 4.0 μM oryzalin for 19 h and macerated in 1:10 pectinase solution for 2 h at 34°C provided morphologically defined metaphase chromosomes without overlaps. The chromosome complement of ‘Santa Cruz Kada Gigante’ was 2n=2x=24 (Fig. 1b).

**In vitro polyploidization of ‘Santa Cruz Kada Gigante’ and FCM screening**

Owing to the disinfestation procedure of the seeds, no contamination was detected during the in vitro process. Consequently, plantlets were successfully multiplied and propagated in vitro.

The survival rate of the explants after polyploidy induction varied among the treatments. The control explants (0% colchicine) showed 100% survival, whereas all explants died in the treatments with 0.5 mM/96 h, 0.5 mM/120 h, 2.5 mM/120 h and 8.0 mM/120 h (Table 1). Moreover, explants treated with colchicine showed a lower growth rate than the control explants. Given this fact, after

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**Fig. 1.** (a) FCM histogram obtained from the analysis of nuclear suspensions stained with propidium iodide (PI). Peak representing the G₀/G₁ nuclei of *S. lycopersicum* ‘Santa Cruz Kada Gigante’ (sample) and ‘Stupické’ (standard) positioned on channel 200. The nuclear 2C value of the sample was 2.00 pg. (b) Karyotype of *S. lycopersicum* ‘Santa Cruz Kada Gigante’ showing 2n=2x=24 chromosomes. Bar=5 μm.
six months of subculture the DNA ploidy level was verified for each plantlet using FCM (Table 1).

<table>
<thead>
<tr>
<th>Colchicine concentration (mM)</th>
<th>Duration (h)</th>
<th>No. of individuals treated</th>
<th>Survival rate (%)</th>
<th>No. of regenerated plantlets*</th>
<th>Number of individuals (%) by ploidy level</th>
</tr>
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<tr>
<td></td>
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<td>Diploid Tetraploid Mixoploid</td>
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<tr>
<td>0.0</td>
<td>96</td>
<td>12</td>
<td>100a</td>
<td>12 (100)</td>
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<td>12</td>
<td>100a</td>
<td>12 (100)</td>
<td>0a 0b</td>
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<tr>
<td>0.5</td>
<td>96**</td>
<td>12</td>
<td>0b</td>
<td>0</td>
<td>0b</td>
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<tr>
<td></td>
<td>120**</td>
<td>12</td>
<td>0b</td>
<td>0</td>
<td>0b</td>
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<tr>
<td>1.5</td>
<td>96</td>
<td>12</td>
<td>33.33b</td>
<td>9d,e</td>
<td>5 (55.55) 0b 4b (44.44)</td>
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<td></td>
<td>120</td>
<td>12</td>
<td>41.66b</td>
<td>27e</td>
<td>13 (48.14) 0b 14b (51.85)</td>
</tr>
<tr>
<td>2.5</td>
<td>96</td>
<td>12</td>
<td>16.66b</td>
<td>9d,e</td>
<td>7 (77.77) 0b 2b (22.22)</td>
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<tr>
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<td>120**</td>
<td>12</td>
<td>0f</td>
<td>0</td>
<td>0b</td>
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<tr>
<td>5.0</td>
<td>96</td>
<td>12</td>
<td>100b</td>
<td>26 a</td>
<td>11 (42.30) 0b 15b (57.69)</td>
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<tr>
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<td>12</td>
<td>25b</td>
<td>24f</td>
<td>3 (100) 0b 0b</td>
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<td>96</td>
<td>12</td>
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<td>21d,e,f</td>
<td>3 (14.28) 14b (66.66) 4b (19.04)</td>
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<td>41.66b</td>
<td>12g,h</td>
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<td>8.0</td>
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<td>12</td>
<td>41.66b</td>
<td>15g,h</td>
<td>4 (26.66) 0b 11b (73.33)</td>
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<td></td>
<td>120**</td>
<td>12</td>
<td>0f</td>
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<td>0b</td>
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In the columns, means followed by the same letter are not significantly different at the $p \leq 0.01$ by the Tukey test.

* Plantlets regenerated after seven subcultures totaling 210 days.

** All explants treated with 0.5 (96/120 h), 2.5 (120 h) and 8.0 mM (120 h) colchicine concentration died.


discussion

Nuclear 2C value and karyotype of ‘Santa Cruz Kada Gigante’

The control plantlets (0% colchicine) and *S. lycopersicum* ‘Stupické’ grown in greenhouse (2C=2.00 pg; 2x=24 chromosomes) exhibited G0/G1 peaks in the same channel (Fig. 2a). Thus, these plantlets were considered to be diploid (2x, Fig. 2b). Tetraploid plantlets were only obtained with the treatment 6.5 mM/120 h (Table 1); these plantlets exhibited G0/G1 peaks equivalent to nuclei 2C=4x (Fig. 2c, d). FCM also identified mixoploid plantlets from the treatment combinations 1.5 mM/96 h or 1.5 mM/120 h, 2.5 mM/96 h, 5.0 mM/96 h, 6.5 mM/120 h, and 8.0 mM/96 h (Table 1). These histograms exhibited G0/G1 peaks equivalent to 2C=2x/4x, with sporadic seedlings showing 2C=2x/4x/8x (Fig. 2e, f).

Based on these results, a second *in vitro* polyploidization procedure was conducted using the most effective colchicine concentration to induce tetraploid plantlets. As a result, 41.50% of tetraploids were generated.

In this study, the accession ‘Stupické’ (2C=2.00 pg) was used as an internal standard. The use of an internal standard minimizes FCM errors, as the leaves of the sample and standard are processed simultaneously (Doležel and Bartoš 2005).

As recommended by Oliveira et al. (2013), in addition to 2C value, ‘Santa Cruz Kada Gigante’ was also characterized in relation to chromosome number. This accession presented 2n=2x=24
chromosomes, agreeing with other reports (Peterson et al. 1996, Karsburg et al. 2009, Praça et al. 2009) and corroborating with FCM results. For ‘Santa Cruz Kada Gigante’, oryzalin was the most appropriate anti-tubulin agent, yielding morphologically defined metaphase chromosomes (Fig. 1b). Differently, Karsburg et al. (2009) and Praça et al. (2009) used amiprophos-methyl to obtain metaphase chromosomes from root tips of *S. lycopersicum* ‘BGH 160’ and ‘Stupické’, respectively.

Based on FCM and cytogenetic data, no preexisting variation was identified in ‘Santa Cruz Kada Gigante’. Therefore, this material was considered appropriate for *in vitro* polyploidization.
In vitro polyploidization of ‘Santa Cruz Kada Gigante’ and FCM screening

According to Barrueto and Zimmermann (2006), the standardization of an appropriate method of disinfection has been essential to develop and conduct in vitro tissue culture. In this sense, the disinfection procedure was fundamental to establish and maintain aseptic in vitro culture of ‘Santa Cruz Kada Gigante’ plantlets, with no account of contamination in this work.

Corroborating data reported by Park et al. (2001) and Praça et al. (2009), the MS medium (Murashige and Skoog 1962) was adequate to recover S. lycopersicum plantlets in vitro. Consequently, plantlets of S. lycopersicum were propagated, proliferated and rooted, generating a satisfactory amount of explants for polyploidization.

In this research, FCM was chosen for screening of polyploid plantlets. For the same purpose, different approaches have demonstrated that FCM is practical, quick and reliable (Roy et al. 2001, Clarindo et al. 2012). Further, several samples can be analyzed on a single day (Doležel et al. 2007). Regarding these advantages, Doležel et al. (2007) and Clarindo et al. (2008) recommended FCM to determine DNA ploidy level, particularly in analyses involving many samples.

Considering the explants maintained in liquid medium supplemented with colchicine, the survival rate varied between 0 and 41.66% (Table 1). No explants survived after polyploidization treatment with 0.5 mM/96 h, 0.5 mM/120 h, 2.5 mM/120 h and 8.0 mM/120 h (Table 1). According to Allum et al. (2007), the death of explants, and consequently the success in chromosome duplication, can vary among different explant types. These authors related that tissue permeability and transport capacity of anti-mitotic agents to the explant meristem may lead to a toxic effect. In accordance with Planchais et al. (2000), when the agent enters the cell metabolism, cell cycle synchrony gradually decreases and the cell crosses numerous mitotic cycles. As a consequence, the same set of chromosomes is doubled several times to the point that the cell cannot bear all content and dies. Also, excessively high doses of colchicine are lethal to explants, often leading to death of plantlets (Allum et al. 2007, Dhooghe et al. 2011).

Praça et al. (2009) also employed the shoot tip as a source of explants for in vitro polyploidization of ‘Stupické’ with no death records for the distinct treatments. Khosravi et al. (2008) reported that the efficiency of polyploidization depends on the type of explant and its interaction with the anti-mitotic agent. According to Roy et al. (2001) and Praça et al. (2009), colchicine is adequate for in vitro induction of polyploids. This anti-mitotic agent, which is generally dissolved in ethanol, presents a smaller toxicity for the explant compared with other chromosome doubling agents and respective solvents (Dhooghe et al. 2011). However, our results support the principle that the concentration of colchicine is crucial for the success of in vitro polyploidy.

The treatment with 6.5 mM/120 h was the only one to yield tetraploid plantlets of ‘Santa Cruz Kada Gigante’ (Table 1, Fig. 2c, d). This result was confirmed by the second polyploidization approach. Praça et al. (2009) also considered the concentration of 6.5 mM/120 h to be suitable (5.55%) for tetraploid induction from the ‘Stupické’ line. However, these authors found the treatment with 8 mM/96 h to be the most efficient (11.11%). In the present work, this same treatment induced a high percentage (73.33%) of mixoploid plantlets (Table 1). Regarding these data, in vitro polyploidization success in S. lycopersicum is genotype-specific, reinforcing the hypothesis of Khosravi et al. (2008).

Mixoploid plantlets were identified in different treatments (Table 1). According to Allum et al. (2007), the occurrence of mixoploidy takes place during the polyploidization (endomitosis) process, inasmuch as the anti-mitotic agent may not act on all meristematic cells. Due to ploidy instability, many studies disregard mixoploid plantlets (Praça et al. 2009). However, for Roy et al. (2001), mixoploids can be used as explant source for callogenesis, aiming to regenerate plantlets showing a single ploidy level.

The data showed that ‘Santa Cruz Kada Gigante’ has the same chromosome number and
nuclear DNA content in relation to other S. lycopersicum accessions. From this line, tetraploid and mixoploid plantlets were obtained, increasing the genomic variability of the tomato. Therefore, the data reported here may contribute to tissue culture research concerning in vitro polyploidization in S. lycopersicum.

Acknowledgements

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil), Fundação de Amparo à Pesquisa do Espírito Santo (FAPES, Vitória, ES, Brazil), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília, DF, Brazil) for financial support.

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