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Nuclear genome stability of *Mammillaria san-angelensis* (Cactaceae) regenerants induced by auxins in long-term in vitro culture

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Abstract

Prolonged cultivation and the presence of exogenous growth regulators are factors suspected to induce genetic instability in vitro. In our previous work, we have achieved regeneration of a severely endangered cactus *Mammillaria san-angelensis* Sánchez-Mejorada from a long-term culture in the presence of auxins. The aim of this work was to investigate the cytogenetic characteristics of in vitro derived regenerants, analyzing nuclear DNA content, ploidy level and the extent of endopolyploidy. Plantlets grown for up to 7 years in MS medium alone were used as a source of explants which were cultured on MS in the presence of NAA, IAA, IBA, 2,4-D or Picloram at 2, 4, and 6 mg l^{-1}. In vitro plantlets regenerated without auxins were used as controls. Adult plants grown in a greenhouse and in vitro young plantlets were both found to be diploids ($2n = 22$) with the same karyotype, and no differences in DNA content were found between these two groups, both having a 2C DNA content = 3.20 pg. However, differences in the frequency of endopolyploid cells were found between young and adult plants. On the other hand the extent of endopolyploidy (the frequency of cells with 2C, 4C, 8C, and 16C DNA content) in differentiated tissues was basically the same in the control as in plants regenerated in the presence of auxins, and only marginal differences were detected in five cases, without any pattern. Meiosis in adult plants was a normal behavior with eleven bivalents ($n = 11$). This study demonstrated karyological stability of tissue cultured *M. san-angelensis* despite its origin from long-term subculture and the presence of auxins.
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Keywords: *Mammillaria san-angelensis*; Cacti; DNA content; Ploidy level; Endopolyploidy; Flow cytometry

1. Introduction

Mammillaria san-angelensis Sánchez-Mejorada is a cactus near to extinction which has been successfully micro-propagated in vitro [1]. In vitro derived plants were restored to the natural site where they were able to grow to maturity, flower and produce fertile seeds [2]. The in vitro derived population was obtained from just eight seeds coming from the last five individuals left in the wild. Consequently, the genetic make-up of this severely endangered species in very narrow. Mass propagation of this valuable material was achieved in long term (up to 7 years) subcultures, and in a subsequent work, the influence of several auxins on plant regeneration was investigated (Rubluo et al., to be published elsewhere).

Abbreviations: 2,4-D,2, 4-dichlorophenoxyacetic acid; MS, Murashige-Skoog medium; NAA, 1-naphthalene acetic acid; IAA, 3-indolyl-acetic acid; IBA, 3-indolebutyric acid; Picloram, 4-amino-3,5,6-trichloropicolinic acid; BAP, 6-benzylaminopurine; DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide; MI, metaphase I; AI, anaphase I; PMC's, pollen mother cells; RI, recombination index; CV, coefficient of variation.

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In vitro tissue cultures are known to be genetically unstable and may be used as a potential source of variation [3]. Regenerated plants have been reported with altered chromosome number, polyploidy being the most frequently observed chromosomal abnormality [4]. Several factors have been correlated with the instability [3]. The length of time that a culture has been maintained in vitro is among the most important, as well as the exposure to growth regulators like auxins and particularly 2,4-D, due to their capacity to stimulate disorganized growth affecting mitosis during the callus proliferation [5].

Endopolyploidization, i.e. chromosome duplication without subsequent karyokinesis and cytokinesis, is a phenomenon frequently found in differentiated tissues of angiosperms. Its biological function is not well understood, although it has been linked to higher metabolic activity or storage function of differentiated tissues [6]. Most species belonging to Angiosperm families display endopolyploidy in nearly all their cells and values between 75–80% has been reported [6]. The pattern of endoreduplication seems to be genetically regulated and characteristic for a given tissue and growth phase [6,7]. To our knowledge, no attention has been paid so far to the effect of in vitro culture on the pattern of endopolyploidization in regenerated plants.

Analysis of the genetic make-up of *M. san-angelensis* from the in vitro system is urgently needed to detect the presence of severe gross genetic changes that could impair the reintroduction of this recovered species into the wild. It is intended that weak mutagenic treatments will be applied to this genetically stable material with the aim of slightly broadening the genetic variability for this endangered species. However, the analysis of ploidy level stability is complicated by the random occurrence of aberrations and thus a necessity to analyse large samples. Flow cytometry allows rapid and precise determination of nuclear DNA content [8,9] and was shown to be suitable for analysis of karyological stability in vitro [10–13]. Furthermore, it allows rapid estimation of the extent of endopolyploidy in differentiated plant tissues [8,9].

The aim of this work was to investigate how the nuclear genome of *M. san-angelensis* was influenced by the long term subculture conditions, together with the action of various auxins. Flow cytometry was used to estimate the genome size, analyze the stability of nuclear DNA content, ploidy levels and the extent of endopolyploidy in differentiated tissues. Additionally, karyotypes and meiotic behavior on this endangered cacti were also analysed.

2. Materials and methods

2.1. Plant material

Plantlets of *M. san-angelensis* regenerated on MS [14] basal medium enriched with BAP at 0.1 mg l^{-1} were used as the original source of explants [1]. They were subcultured every 6 months for 7 years in MS basal medium with no growth regulators in 30 ml baby food jars, and maintained in a growth chamber at 27°C $\pm$ 2 constant temperature under cool-white fluorescent lamps with 14.8 μmol m^{-2} s^{-1} light and a 16 h photoperiod. Sections of shoots of this long-term subcultured material were transferred to MS alone (control) as well as supplemented with the auxins NAA, IAA, IBA, 2,4-D, or Picloram at 2, 4, and 6 mg l^{-1} and incubated as stated before. A factorial experiment with four replicates was used with baby food jars (four explants each as experimental units). Plantlets of the same size ($\approx$1.5 cm high) and regenerated from this experiment were analyzed using flow cytometry for ploidy level determination.

In vitro, derived adult plants that had grown in a greenhouse for seven years were analyzed for determination of genome size.

2.2. Flow cytometric determination of nuclear genome size

Suspensions of intact nuclei were prepared according to Otto [15] with some modifications as described by Dolezel and Göhde [16]. *Lycopersicon esculentum* cv. 'Stupické polní rané', 2C = 1.96 pg DNA, [17] was used as an internal reference standard. Briefly, 300 mg of *Mammillaria* tissue from adult plants or regenerated plantlets in vitro, and 50 mg of young leaves from *L. esculentum* were simultaneously chopped with a razor blade in a petri dish containing 1 ml of 0.1 M citric acid and 0.5% Tween 20. The chopped material was filtered through a 50 μm nylon filter and incubated

for 10 min at room temperature. Then the nuclei in the filtrate were pelleted by centrifugation (1000 rpm per 5 min), resuspended in 200 μl of the citric acid/Tween 20 solution and incubate for 10 min at room temperature. Subsequently, 1 ml of the staining solution (0.4 M sodium hydrogen phosphate, 50 μg ml^{-1} PI and 50 μg ml^{-1} RNase) was added to stain the DNA. PI was used because its binding to DNA is not influenced by the AT/GC ratio of nuclear DNA [17,18].

Fluorescence intensity of stained nuclei was analysed using a Partec CA II flow cytometer (Partec GmbH, Münster, Germany) at a rate of 20–50 nuclei s^{-1}. In each sample, 5–20 000 nuclei were analysed. Peak positions, their areas and CV were calculated using DPAC software (Partec GmbH). Nuclear genome size was then calculated according to Dolezel [19] using the formula:

$$2C \text{ DNA content of } M.s. = \frac{M.s.\ G_1 \text{ peak mean}}{L.\ esculentum\ G_1 \text{ peak mean}} \times 1.96 \text{ (pg)}$$

These measurements were repeated six times for adult plants (control) and nine times in young in vitro plantlets.

2.3. Flow cytometric analysis of DNA ploidy levels

Determination of DNA ploidy levels was done using *Glycine max* as an internal reference standard. The procedure was described above with the exception of the staining solution which contained 5 μM of DAPI instead of PI.

Ploidy of regenerated plantlets was determined based on a position of the leftmost peak of *M. san-angelensis* relative to G_1 peak of internal standard. To analyse the extent of endopolyploidy, the frequency of *M. san-angelensis* nuclei with different DNA content was calculated for each of the four replicates of each treatment combination and also nine replicates of adult plants and of young in vitro plantlets.

2.4. Statistical analysis of ploidy levels

Differences in the frequency of cells with various DNA contents (2C, 4C, 8C and 16C) in plantlets regenerated in the presence of different auxins were evaluated according to the 3×4 factorial design with four replicates (four auxins with three concentrations) and two additional treatments; Picloram at 2 mg l^{-1} (the only concentration that produced plantlets) and in vitro control plantlets (no auxins).

Data obtained were analyzed through an ANOVA for each ploidy level. Since the dependent variable is a percent, the Shapiro–Wilk test for normality was done for the standardized residuals from the ANOVA. In all cases the test was non-significant, therefore transformation of the data was not necessary. Afterwards a multivariate ANOVA considering the four ploidy levels frequencies as a vector of response for each plant was performed. A further analysis as a split-plot ANOVA was made using the ploidy levels as a nested factor within treatments, with adjusted degrees of freedom by Greenhouse–Geisser technique. When a significant effect was detected a Tukey test was done for each concentration and ploidy level. On the other hand ploidy levels of in vitro young plantlets were compared with the adult plants growing in the greenhouse with a '*t*' and Kruskal–Wallis test. All statistical computation were performed using the JMP version 3.2.1 of SAS company program, using a Pentium PC.

2.5. Meiotic chromosome analysis

Observations were made in young flower buds of ten adult plants. Fresh anthers were squashed in 1.8% acetorcein. For MI and AI analysis, 40 PMC's were analysed for ten adult plants.

2.6. Mitotic chromosome analysis

Observations were done on root tip cells of seven adults plants and ten 6 month old in vitro plantlets. Elongating secondary root tips were placed in a saturated solution of 1-Bromonaphtalene for 5 h at 18°C, fixed in fresh Farmer's solution (three parts absolute alcohol: one part acetic acid) and stained following Feulgen technique modified by Cid and Palomino [20]. For each group of plants analyzed, ten cells were selected for study. Three of the best cells were analyzed in order to make the idiogram using a Zeiss Drawing Apparatus. The chromosomes were classified according to Levan, Fredga and Sandberg [21].

3. Results

3.1. Genome size stability

For estimation of nuclear genome size, the relative fluorescence intensity of PI stained nuclei isolated from *M. san-angelensis* and *L. esculentum* was compared (Fig. 1).

No differences in nuclear genome size were found between young plantlets and in vitro derived adults. 2C DNA content was equal to 3.2 ± 0.01 pg (mean $\pm$ S.E.). Assuming 1 pg DNA = 965 Mb [22] this is equivalent to 1C genome size of 3088 Mb for *M. san-angelensis*.

3.2. Ploidy level

Fig. 2, shows a typical histogram obtained after a simultaneous analysis of relative DNA content of nuclei isolated from *M. san-angelensis* in vitro plantlets and *G. max* (internal standard). Most of the nuclei were at the G_0/G_1 phase, i.e. having 2C DNA content, with their peaks on channel 42.5 for *G. max* and channel 50 for *M. san-angelensis*. In addition, peaks correspoding to G_2 nuclei (4C DNA content) were noted (channels 85 and 100, respectively). In *M. san-angelensis*, peaks corresponding to 8C and 16C nuclei were also apparent. Our analyses showed that the position of the leftmost peak of *M. san-angelensis* relative to G_1 peak of *G. max* was constant and the same in control as well as in all regenerated plants. This results clearly indicated that all regenerated plants remained predominantly at the diploid level, irrespective of their origin (with or without auxins).

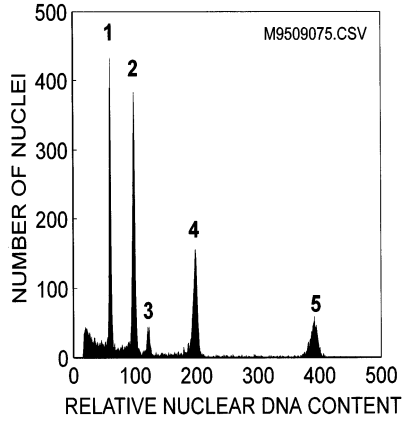

Fig. 1. Distribution of nuclear DNA content obtained after flow cytometric analysis of propidium iodide-stained nuclei isolated from whole plantlets of *M. san-angelensis*. Peaks 2, 4, and 5 represent nuclei with 2C, 4C and 8C DNA content, respectively. Peaks 1 and 3 represent G_1 (2C) and G_2 (4C) nuclei of *L. esculentum* which were analysed simultaneously and served as internal standard. Genome size was estimated based on the ratio of 2C peaks of *M. san-angelensis* and *L. esculentum*.

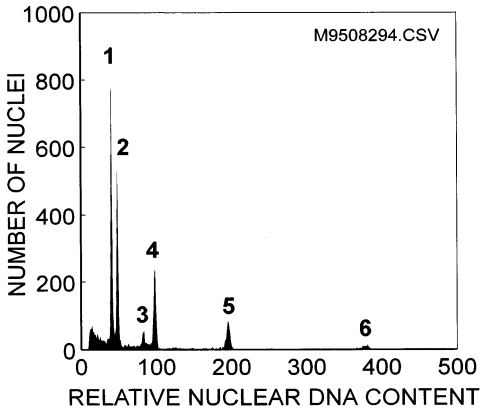

Fig. 2. Distribution of nuclear DNA content obtained after flow cytometric analysis of DAPI-stained nuclei isolated from young whole plantlets of *M. san-angelensis*. Peaks 2, 4, 5, and 6 represent nuclei with 2C, 4C, 8C and 16C DNA content, respectively. Peaks 1 and 3 represent G_1 (2C) and G_2 (4C) nuclei of *G. max* which were analysed simultaneously and served as internal standard. Ploidy of *M. san-angelensis* was estimated based on a position of the leftmost peak 2 of *Mammillaria* relative to G_1 peak 1 of the internal standard.

3.3. Endopolyploidization pattern

As can be seen on Fig. 2, a large proportion of nuclei isolated from young plants of *M. san-angelensis* nuclei had ploidy levels up to 16C. Adult plants gave similar distribution (data not shown). In order to check whether there were nuclei with a ploidy level higher than 16C, we also performed analyses with a lower instrument gain. Fig. 3 shows an example of this assay. An inconspicuous peak in channel 300 representing less than 3% of total count of nuclei was detected just in a few cases. This peak corresponds to a ploidy level of 32C. No nuclei with higher DNA content ploidy were detected. Based on these observations, we have not included cells at 32C level of ploidy in our analyses.

3.4. Statistical analysis of ploidy levels

Table 1 shows the ploidy level frequencies in young in vitro plantlets and adult plants. Some dissimilarities were observed for the two kinds of analyzed individuals. The differences in the pro-

Table 1
Means frequency of cells with various DNA of ploidy levels in young plantlets and adult plants of *M. san-angelensis*

Ploidy level	In vitro young plantlets	In vitro derived adult plants growing in greenhouse for 7 years	*t*-test		Kruskal–Wallis test	
			t	*P* values	*H*	*P* value
2C	40.4	31.8	−2.28	0.063	3.04	0.081
4C	30.0	28.3	−0.76	0.478	0.33	0.564
8C	19.7	17.2	−1.04	0.338	0.75	0.387
16C	9.9	22.7	4.38	0.005	5.33	0.021

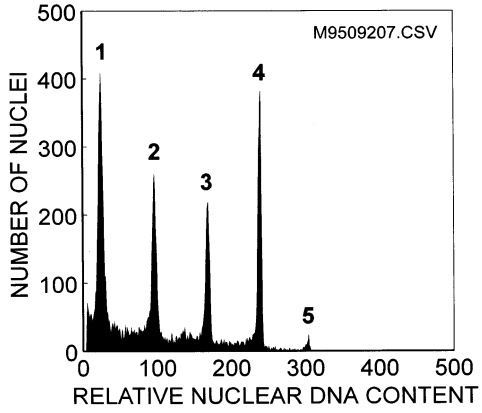

Fig. 3. Distribution of nuclear DNA content obtained after flow cytometric analysis of DAPI-stained nuclei isolated from whole plantlets of *M. san-angelensis*. Peaks 1, 2, 3, 4, and 5 represent nuclei with 2C, 4C, 8C, 16C, and 32C DNA content, respectively. To compress the *X* scale, the analysis was performed with a logarithmic amplification. The analysis clearly demonstrates the absence of nuclei with DNA content higher than 32C.

portion of 2C nuclei was slightly significant ($P = 0.06$) 40 vs. 31% in young regenerants compared with older ones. On the other hand the frequency of 16C nuclei was more than double (22% vs. 9%) in adult plants compared to young ones, a highly significant change ($P = 0.005$). Intermediate ploidy levels (4C and 8C) were very similar in both groups and no statistical significance was found (Table 1).These results were supported also by the non parametric Kruskal–Wallis test (Table 1).

Table 2 shows mean frequencies of nuclei with different ploidy levels obtained after analysis of *M. san-angelensis* plantlets regenerated in presence of several auxins. Values of young plantlets growing in vitro in the absence of auxins shown in Table 1 were used as the control. Explants exposed to Picloram regenerated only at 2 mg l^{-1} (Rubluo et al., to be published elsewhere), thus the extent of endopolyploidy in plants regenerated on this auxin was analysed only at this concentration. A multivariate ANOVA analysis was performed with these data (not shown) demonstrating that there were differences ($P < 0.05$) between all treatments and the control. An ANOVA was also done for each ploidy level, and because significant differences were detected a Tukey test was performed contrasting young plantlet values (control) against ploidy levels registered for every auxin tested at each concentrations. No differences were detected at 4C and 16C, however at 2C and 8C ploidies some differences were noted. Table 3 shows the results of this Tukey's multiple range test on mean of ploidy levels and auxin concentrations only for those values with significant differences. From these results it is apparent that only in five cases clear differences were noted; at 2C ploidy level higher percentage for IBA at 2 mg l^{-1}; IAA at 4 mg l^{-1} and NAA at 6 mg l^{-1}; and for the 8C level for 2,4 – D and IBA at 4 mg l^{-1} However no statistical differences with the control were detected at the 8C level (Table 3).The rest of the values did not have statistical differences.

3.5. Meiotic chromosome analysis

Meiotic chromosome behavior on in vitro derived adult plants showed eleven bivalents ($n = 11$). At MI individual plants presented ten combinations of rod and ring bivalents, the most frequent being 8II rod + 3II ring. The average

Table 2
Mean frequency of cells with various DNA ploidy levels in long – term *M. san-angelensis* regenerated in presence of auxins

Concentration mg l^{-1} of each auxin	Auxins/ploidy level	NAA	IAA	IBA	2,4-D	Picloram
2	2 C	41.0	39.7	46.6	40.7	39.5
	4 C	29.9	31.4	29.4	31.1	30.7
	8 C	16.9	18.4	16.0	20.1	19.1
	16 C	12.3	10.2	8.0	8.2	10.7
4	2 C	43.8	45.3	40.3	42.3	Not grown
	4 C	29.1	31.8	31.2	29.4	"
	8 C	16.7	15.7	20.7	20.7	"
	16 C	10.4	7.2	7.8	7.5	"
6	2 C	47.4	44.7	42.9	42.6	Not grown
	4 C	28.9	30.6	31.1	30.5	"
	8 C	15.7	17.5	19.0	18.3	"
	16 C	8.0	7.2	6.9	8.6	"

Table 3
Results of Tukey's multiple range test on mean of ploidy levels[a] (%) in *M. san-angelensis* as affected by the presence of several auxins[b,c]

Concentration mg l^{-1}	Ploidy level	Auxin	Means Tukey's grouping
2	2 C	IBA	46.6 b
	2 C	NAA	41.0 a
	2 C	2,4-D	40.7 a
	2 C	Control	40.4 a
	2 C	IAA	39.7 a
	2 C	Picloram	39.5 a
4	2 C	IAA	45.3 b
	2 C	NAA	43.8 a b
	2 C	2,4-D	42.3 a b
	2 C	Control	40.4 a
	2 C	IBA	40.3 a
6	2 C	NAA	47.4 b
	2 C	IAA	44.7 a b
	2 C	IBA	42.9 a b
	2 C	2,4-D	42.6 a b
	2 C	Control	40.4 a
4	8 C	2,4-D	20.7 b
	8 C	IBA	20.7 b
	8 C	Control	19.7 a b
	8 C	NAA	16.7 a b
	8 C	IAA	15.7 a

[a] Ploidy levels and concentrations considered only those with significative differences.
[b] Same letters indicate no statistical difference using $\alpha = 0.05$.
[c] Control was the in vitro young plantlets.

chiasmata numbers per nucleus corresponded to 16.74 ± 0.01, with a RI = 27.74. In these plants AI cells exhibited normal segregation of 11:11 chromosomes. The percentage of pollen sterility was 7.4%.

3.6. Karyological analysis

All analysed adult plants and in vitro regenerated plantlets of *M. san-angelensis* were found to be diploids with $2n = 22$. Karyotypes of both, adult plants and young plantlets in vitro were similar, consisting of eleven pairs of metacentric chromosomes, two of them carrying a satellite

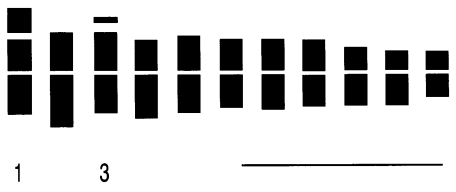

Fig. 4. Idiogram of *M. san-angelensis* adult plants, showing 11 pairs of metacentric chromosomes. Numbers 1 and 3 indicate chromosomes with satellites. Scale equals 10 μm.

(Fig. 4). This finding confirmed that all plants classified as diploids using flow cytometry were indeed diploids thus confirming stability at chromosomal level in both lots.

4. Discussion

Chromosomal instability of plant tissue cultures is a frequent phenomenon [23–25], particularly if regeneration occurs from long- term callus cultures [3]. Genetic instability may be pronounced especially if in vitro culture involves a callus phase [26].

The usefulness of flow cytometry for ploidy screening in regenerated plants is well documented [27–29]. Our results confirmed the usefulness of the technique for the analysis of ploidy levels in *M. san-angelensis* regenerants.

Using flow cytometry, we have also determined nuclear genome size of *M. san-angelensis* for the first time (Fig. 1), and analysed the stability for this parameter in regenerated plants, no variation in genome size being detected. Recent reviews

[22,30] stated that the genome size has been estimated only in about 2500 angiosperms (1% of the reported world's total angiosperm flora). Among them, nine cacti have been analysed by Barlow (cited by [31]). The authors reported 2C DNA values ranging between 3.1 and 4.1 pg for eight of them, all with $2n = 22$. Similarly [11] estimated the 2C DNA content of 2.05 pg for *Pereskia grandifolia* a primitive member of the Cactaceae family. Our estimation of 2C DNA content of *M. san-angelensis* (3.2 ± 0.01 pg) thus agrees well with the reported range for Cactaceae. Interestingly, 2C DNA content of 14.2 pg estimated for *Weberbauerocereus winterianus* (Barlow, cited by [31]) falls out of the range known for Cactaceae so far. This discrepancy can be explained by its polyploid nature ($2n = 88$). However, recently Mohanty, Das and Das [32] reported 2C DNA content of eight diploid ($n = 11$; $2n = 22$) species of *Mammillaria* ranging between 9.1 pg in *M. klissingiana* to 12.1 pg in *M. brevispina*. These authors used Feulgen cytometric estimation and their values are in disagreement with ours as well as for those others previously mentioned (Barlow cited by [31,11]) from Cactaceae species, including *M. san-angelensis*.

Considering the known range of genome size in angiosperms (1C ranging from 0.2 to 127.4 pg DNA [30] the results from most of the above mentioned authors as well as our own results indicate that members of the Cactaceae family belong to species with a small genome. Thus, their nuclear genomes presumably contain relatively small amounts of non-coding repetitive sequences and should be amenable for analysis at molecular level. Exact knowledge of genome size is important in many areas of research including genome organization, plant evolution and ecological adaptation of germplasm [33,34] and thus of prime interest for endangered species such as *M. san-angelensis*. A correlation between DNA-C value and the effect of ionizing radiation has also been observed [35,36]. In the light of an ongoing project on broadening the narrow genetic variability using mutation induction, the knowledge of genome size of *M. san-angelensis* will be important to facilitate planning of irradiation experiments.

About 90% of angiosperms are though to be polysomatic, i.e. contain endopolyploid cells in their differentiated tissues [37]. While meristematic tissues remain at the diploid level, cells in differentiated tissues may undergo chromosome endoreduplication giving rise to subpopulations with higher DNA content levels (8C, 16C, etc). As a consequence, so called polysomatic tissues are mosaics containing cells with different ploidy levels [6]. Our results showed that the differentiated leaf tissues of *M. san-angelensis* contain endopolyploid cells and that their frequency is precisely regulated, as all regenerated plantlets had similar patterns of endopolyploidy (Figs. 1 and 2). It has been proposed that nuclear DNA content of $G_{0/1}$ cells reflects the ploidy status of a plant [38,39]; in our results we found that *M. san-angelensis* presents the highest values of ploidy level in 2C, so we conclude that this species is a diploid (Figs. 1 and 2).

We have observed differences in the frequency of endopolyploid cells between young and adult plants, respectively (Table 1). The association of polyploidy with developmental stages was previously observed by De Rocher et al., [11] who described a developmental gradient in ploidy levels correlated with the age of leaves in *Mesembryantenemum erystillinum*. These plants showed eight multiple ploidy levels with the 2C nuclei making up only a minor part of the total nuclei population. Multiploidy has been observed also in other succulent species [11]. *M. san-angelensis* is a diploid species with an endopolyploidy pattern including 4C, 8C and 16C nuclei, with 2C the most abundant. It is interesting to note that the maximum level of endopolyploidy in *M. san-angelensis* (16C) was the same for young and adult plants and that with the exception of few 32C nuclei, higher levels of endopolyploidy were not found (Fig. 3).

From all the ploidy level values and treatments analysed (Table 2), only five showed statistical differences (Table 3), and compared with the control, only those at the 2C level; moreover, no pattern was detected as influenced by the different growth regulators at the concentrations tested and the changes detected were induced in an apparently random way. This result indicates that basically the process of endopolyploidization during cell and tissue differentiation was not disturbed by long term in vitro subculture and by the presence of auxins. Auxins were present only at the final stage during which regeneration was induced and this could count the marginal effect they exercised on the level of endopolyploidy in some of the

regenerants (Table 3). It is known that a positive correlation exists between chromosomal stability and regenerative capacity of the cultured cells [24] and this seems to be the case also for our in vitro system of *M. san-angelensis*, because no impairment into the regeneration capacity was detected in any case, except for high doses of Picloram (Rubluo et al., to be published elsewhere).

The chromosomal constitution of original explants is known to influence the karyological status of in vitro culture and ploidy level of regenerated plants [40]. Endopolyploid cells may be induced to divide and give rise to subpopulations of polyploid cells [41]. It is interesting that of *M. san-angelensis* regenerants analyzed in this study were diploid ($n = 11$, $2n = 22$; Figs. 2 and 4,) even if the original explants contained a large proportion of endopolyploid cells (Figs. 1–3). This can be explained either by an irreversible loss of totipotency in endopolyploid cells or by a selective advantage of diploid cells during culture and regeneration.

Because flow cytometry analyses DNA content and not chromosome number, we have confirmed the results obtained by flow cytometry by chromosome number counting in randomly selected regenerants.

Most of the reported chromosome counts of the *Mammillaria* species [42] are diploids with $n = 11$ and $2n = 22$ and a basic number of $x = 11$ chromosomes [42,43].

Meiotic behavior in the diploid taxa of *Mammillaria* is usually regular with eleven bivalents and somatic chromosomes in diploid and polyploid species are mostly metacentrics with one pair of chromosomes presenting satellites in diploid plants, and at least two of them in polyploids [42,44]. Our results fit well with those mentioned, and show that *M. san-angelensis* is the same as that reported, in despite of its in vitro origin; however *M. san-angelensis* presented two pairs of chromosomes with satellites (Fig. 4). Other authors have also reported the presence of more than one pair of chromosomes with a satellite in other species of diploid Cactaceae [20,45,46].

To conclude, our results clearly showed that despite a long-term culture and the presence of auxins in nutrient media, mass propagated individuals of severely endangered cacti *M. san-angelensis* did not demonstrate any gross karyological changes as they present a stable genome size, ploidy and endopolyploidization pattern. These findings are important as in vitro propagated plants are being reintroduced to the nature. Furthermore, these micropropagated plants can be used for mutagenic induction experiments with the aim to slightly broaden, the extremely narrow genetic make-up of the rescued population. Altogether our work expands survival chance of *M. san-angelenis* as a species.

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