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Variation of nuclear DNA content during somatic embryogenesis and plant regeneration of *Coffea arabica* L. using cytophotometry

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Abstract

Cytophotometric analysis of nuclear DNA was carried out in leaves of *Coffea arabica* L. plants grown in vitro. They were maintained for more than 1 year on MS media containing 0.53 μM NAA, and 2.32 μM kinetin, and embryogenic calli and somatic embryos were derived from them. Four suspension cultures of *C. arabica* differing in their embryogenic potential were also studied. In in vitro leaves used as primary explants many nuclei gave values that were hypoaneuploid, yet the somatic embryos derived from them consisted predominantly of diploid cells. As primary explants were shifted to conditioning medium (MS medium added with 0.53 μM NAA and 2.32 μM kinetin) and then to induction medium (to obtain embryogenic calli; Yasuda medium supplemented with 5 μM BAP), the frequency of hypoaneuploid values dropped. An analysis of four suspension cultures did not reveal any relationship between the cytogenetic state of cell strains and their morphogenetic potential. Of four suspensions, cultures having similar frequencies of diploid cells (60–82%), only one was capable of embryogenesis.

Keywords: *Coffea arabica*; Somatic embryogenesis; Nuclear DNA; Cytophotometry

1. Introduction

The development of methods for plant somatic embryogenesis is an important issue. Cell systems providing a mass formation of somatic embryos are useful in the study of the basic mechanisms of embryogenesis, morphogenesis and senescence. Somatic embryogenesis is effective as a method of rapid propagation and can also be applied in obtaining 'artificial' seeds.

The history of somatic embryogenesis in coffee began when Staritsky [1] induced this process by the in vitro culture of young internodes of *Coffea canefora* Pierre. Since then, great progress has been achieved in this field. Presently, somatic embryos of different species and genotypes of coffee are available that have the capacity for further development of different organs and tissues, as stems [2], leaves [3–5], hypocotyls, cotyledons [6], and integuments [7]. The methods provide a high yield of embryos [8–10] and the protoplasts isolated from their leaves has been used to produce embryogenic callus [11,12].

Not all genotypes are capable of forming somatic embryos to the same extent. An approach which allows this barrier to be overcome is the use of in vitro plant tissue as the initial explants [13], however, auxins and cytokinins present in the propagation media may effect the genetic apparatus that results in the change of the ploidy level of the plant cells cultured in vitro [14].

For these reasons it is interesting to study the interrelationship between somatic embryogenesis and the cytogenetic state of initial explants. A comparative

Abbreviations: BAP, 6-benzylaminopurine; DAPI, 4,6-diamidino-2-phenylindole; GA, gibberellic acid; NAA, α-naphthylacetic acid.

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Table 1
Composition of media and experimental conditions according to Yasuda's and Söndahl's protocols

Components and conditions	Yasuda protocol	Söndahl protocol	
		Conditioning medium	Inductioning medium
NH_4NO_3 (mg l^{-1})	412.5	1650	825
KNO_3 (mg l^{-1})	475	1900	3800
$MgSO_4 \cdot 7H_2O$ (mg l^{-1})	92.5	370	185
KH_2PO_4 (mg l^{-1})	85	170	85
$CaCl_2 \cdot 2H_2O$ (mg l^{-1})	110	440	220
Microelements	MS/2[a]	MS	MS/2
Fe–EDTA	MS	MS	MS/2
Thiamine (mg l^{-1})	10	10	10
Nicotinic acid (mg l^{-1})	1	–	–
Pyridoxine (mg l^{-1})	1	–	–
L-Cystein (mg l^{-1})	–	25.5	25.5
Myo-inositol (mg l^{-1})	100	100	100
Sucrose (g l^{-1})	30	40	20
2,4-D (μM)	–	4.52	–
NAA (μM)	–	–	0.26
BAP (μM)	4.43	–	–
Kinetin (μM)	–	1	4.64
Gelrite (g l^{-1})	2	2	2
Period of cultivation	21 days	50 days	30 days
Light	12 h photoperiod regime; 15 M m^{-2} s^{-1}	Darkness	12 h photoperiod regime; 15 M m^{-2} s^{-1}
Temperature (°C)	25	25	25

[a] According to Murashige and Skoog [15].

cytophotomeric analysis of nuclear DNA was carried out in the initial plants, callus cultures and somatic embryos derived from them. Four suspension cultures of cotyledon and leaf origin and the regenerants derived from one of them were also studied.

2. Materials and methods

2.1. Plant materials

An in vitro plant of *Coffea arabica* cv. Caturra, derived from the single somatic embryo, was maintained for 1 year by micropropagation on the medium G consisting of MS macro- and microelements [15], 10 mg l^{-1} thiamine, 0.01 mg l^{-1} biotin, 25 mg l^{-1} cysteine, 0.53 μM NAA, and 2.32 μM kinetin. The sub-culturing period was 1 month. The plant was then cultured on the same medium but without phytohormones for two more periods and after this it was placed on medium with 62 μM BAP for the induction of the mass formation of shoots. After cutting, shoots were again planted on medium with 0.53 μM NAA and 2.32 μM kinetin for the induction of in vitro plants. After a month of cultivation, mature leaves from these plants were used for direct Yasuda's protocol [16] and indirect embryogenesis protocol ([4,17], Table 1).

Three in vitro plants of *C. arabica* cv. Catuai developed from isolated zygotic embryos were maintained on medium G which contained in addition 2.89 μM GA. They were then cultured on phytohormone-free medium for two periods. Mature leaves from these plants were used for obtaining the somatic embryos according to Yasuda's protocol [16] and embryogenic calli according to Söndahl's protocol ([4,17], Table 1). Somatic embryos at the globular stage were obtained according to Yasuda's protocol.

The embryogenic calli induced on the Söndahl medium (conditioning medium) were maintained under these conditions for 6 weeks and then transferred to the induction medium for 3 weeks (Table 1) after which the cytophotometric analysis was performed.

The cell strains IV-HC2s (low embryogenic potential suspension culture) and IV-HC5s (non-morphogenic suspension culture) were of cotyledonary origin while IV-HV7s and IV-HV9s (non-morphogenic suspension cultures) were obtained from calli derived from mature leaves of *C. arabica* cv. Catuai. All cell strains were maintained on medium G without biotin but containing 30 g l^{-1} sucrose, 13.56 μM 2,4-D and 4.43 μM BAP. Sub-culturing was carried out after 2 weeks on a shaker (100 rpm) and in darkness at 25 °C. The inoculum size was 1 g of fresh weight per 100 ml of medium. For embryogenesis induction, cells of the strain IV-HC2s were transferred onto medium G with 4 mg l^{-1} thiamine and 20 g l^{-1} sucrose but the inoculum was halved.

We also tested the regenerants that were derived from the cell suspension IV-HC2s.

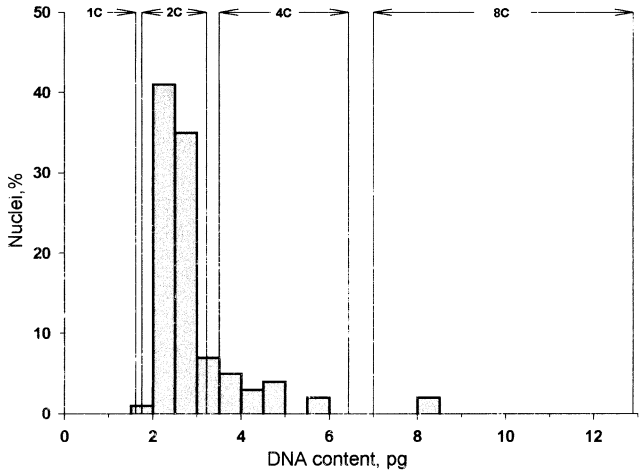

Fig. 1. The distribution of nuclear DNA values in IV-HC5s suspension cells. Vertical lines show the boundaries for the ploidy classes discriminated at the 95% confidence level.

2.2. Cytophotometric analysis

The following plant material was used for the cytophotometric analysis: pieces from mature leaves of in vitro cv. Caturra plants grown on medium in the presence of 62 μM BAP and on medium G containing kinetin and NAA; those for cv. Catuai grown on medium without phytohormones; pieces of the first leaf pair of the regenerants from the suspension culture IV-HC2s, embryogenic calli grown on conditioning and induction media for 6 and 3 weeks, respectively; cells of suspension cultures at the exponential stage of growth; and globular embryoids induced on Yasuda's medium. The root apical meristem cells of *Vigna radiata* cv. Berken seedlings in prophase, metaphase, anaphase, and telophase ($4C = 2.1$ pg; www: http://www.rbgkew.org.uk) were used as a standard for DNA content.

The plant materials were fixed in the mixture of 96% ethanol and glacial acetic acid (3:1, v/v) for 24 h and stored in 70% ethanol at 4 °C. Cytophotometric analysis was carried out on squashed preparations after staining according to the modified Feulgen protocol [18]. The ethanol was washed from the samples with distilled water and hydrolyzed in 5 M HCl for 35 min at 22 °C. They were then stained with Schiff's reagent for 1 h in darkness at the same temperature, followed by three washes for 5 min each in a sulphurous bath. This was followed by a further three washes in distilled water and microscopic slides were dehydrated in the series of ascending concentrations of ethanol, the mixture of ethanol and xylene, and finally in xylene. The preparations were then mounted from xylene into DePeX (Serva, Germany). The DNA content was measured on a Univar microscope (Reichert-Jung, Austria) by the two-wave length method [19] at $\lambda_1 = 500$ nm (half-maximum density) and $\lambda_2 = 550$ nm (maximum density). The ratio $D_{\lambda_1}/D_{\lambda_2}$ was calculated for each nucleus and the only cases accepted were those where the ratio was 0.5 and the deviation did not exceed 5%.

For each variant of *C. arabica*, 50–150 randomly selected nuclei from two to three slides were analyzed. For *V. radiata*, 10–15 cells of root apical meristems with mitotic figures were measured as a standard. The mathematical analysis of the results obtained was carried out using the algorithm proposed by Nosov et al. [20]. This method determines the boundaries of each ploidy class (Fig. 1). The amount of DNA corresponding to the level of $2C = 2.5$ pg was used as a criterion of cell ploidy that has been cytophotometrically demonstrated in *C. arabica* cv. Caturra stained with Shiff's reagent [21].

2.3. Statistical analyses

In order to compare between treatments a χ^2-statistical test was made. Also a paired T-test between treatments was made for all the data presented in the different tables.

3. Results

Cytophotometric analysis of the nuclear DNA content in plant leaves grown on medium with BAP (62 μM) showed a predominance of cells with the amount of DNA corresponding to $2C$. The remainder of the population (24%) consisted of cells with a decreased amount of DNA in comparison with the diploid level (Table 2).

The transition of plants from the BAP-containing medium to medium G resulted in an increase (up to

Table 2
Cytophotometric analysis of leaf material

Material	Less than $2C$ (%)	$2C$ (%)	Between $2C$ and $4C$ (%)	$4C$ (%)
Plant leaves of cv. Caturra on medium with 62 μM BAP	24	76	0	0
Plant leaves of cv. Caturra on medium with 0.53 μM NAA and 2.32 μM kinetin	56	36	0	8
Plant leaves of cv. Catuai on medium without hormones	26	70	0	4

The proportions of observations in different columns of the contingency table vary from row to row. The two characteristics that define the contingency table are significantly related ($P \leq 0.0001$).

Table 3
Cytophotometric analysis of somatic embryos on Yasuda medium

Material	Less than 2*C* (%)	2*C* (%)	Between 2*C* and 4*C* (%)	4*C* (%)
cv. Caturra, embryo 1	0	90	6	4
cv. Caturra, embryo 2	4	68	4	24
cv. Caturra, embryo 3	5	84	0	11
cv. Caturra, embryo 4	0	95	4	1
cv. Catuai, embryo 1	2	92	4	2
cv. Catuai, embryo 2	6	91	3	0
cv. Catuai, embryo 3	8	90	0	2
cv. Catuai, embryo 4	6	90	2	2

The proportions of observations in different columns of the contingency table vary from row to row. The two characteristics that define the contingency table are significantly related ($P \leq 0.0001$).

Table 4
Cytophotometric analysis of embryogenic calli on Söndahl's media

Material	Less than 2*C* (%)	2*C* (%)	Between 2*C* and 4*C* (%)	4*C* (%)
Embryogenic callus on conditioning medium (cv. Caturra)	35	64	0	1
Embryogenic callus on conditioning medium (cv. Catuai)	9	90	0	1
Embryogenic callus on induction medium (cv. Caturra)	16	80	2	2
Embryogenic callus on enductioning medium (cv. Catuai)	4	96	0	0

The proportions of observations in different columns of the contingency table vary from row to row. The two characteristics that define the contingency table are significantly related ($P \leq 0.0001$).

Table 5
Cytophotometric analysis of suspension cultures

Material	Less than 2*C* (%)	2*C* (%)	Between 2*C* and 4*C* (%)	4*C* (%)	8*C* (%)
IV-HC2s	6	82	2	10	0
IV-HC5s	0	81	4	13	2
IV-HV7s	6	60	2	32	0
IV-HV9s	2	76	2	18	2

The proportions of observations in different columns of the contingency table vary from row to row. The two characteristics that define the contingency table are significantly related ($P \leq 0.0001$).

56%) of a proportion of cells with a reduced amount of nuclear DNA (Table 2). In addition, 8% of cells were tetraploid. Apparently they differentiated during the G_2 period. Cells with an amount of nuclear DNA between 2*C* and 4*C* that should correspond to cells in the S period were not observed, but we did not expect to see any as we used fully-grown mature leaves where there is no cell division.

Plant leaves from in vitro *C. arabica* cv. Catuai on medium without phytohormones were used as explants for obtaining somatic embryos according to Yasuda's and Söndahl's protocols. The cytophotometry of nuclear DNA revealed that 70% of the cell population were characterized with diploid DNA content, 26% had a decreased amount compared with 2*C*, and 4% of analyzed cells possessed 4*C* (Table 2). Cells having a DNA amount between 2*C* and 4*C* were not observed and therefore there were no cells in the S phase at the moment of fixation.

Cytophotometry of nuclear DNA in somatic embryos produced by direct embryogenesis showed a predominance of cells with diploid DNA content in all embryos (Table 3). The portion of cells with a DNA amount of 2*C* ranged between 68 and 95%, and cells with a DNA content of 4*C* were present with a frequency range between 1 and 24% for cv. Caturra. In contrast to mature leaves, there were cells in the tissues of somatic embryos with DNA amounts that ranged between 2*C* and 4*C*, i.e. they were in the DNA synthesis phase.

Culturing of primary explants on conditioning medium (Söndahl's protocol) for the induction of embryogenic calli resulted in an increase in the amount of diploid cells (compare 64% in callus and 36% in initial leaf explants in cv. Caturra, and 90 and 70% in cv. Catuai, respectively, Tables 2 and 4). Calli transferred to an induction medium led to a further increase in the population of diploid cells for both cv. Caturra and cv. Catuai (up to 80 and 96%, respectively).

Table 6
Cytophotometric analysis of regenerants (line IV-HC2s)

Material	Less than 2*C*	2*C*	Between 2*C* and 4*C*	4*C*
Plantlet 1	12	86	0	2
Plantlet 2	28	70	0	2
Plantlet 3	6	80	6	8

The proportions of observations in different columns of the contingency table vary from row-to-row. The two characteristics that define the contingency table are significantly related ($P \leq 0.0001$).

Only one suspension culture (IV-HC2s) of four strains analyzed in this work produced embryos from which the regenerants were obtained. Analysis of the embryogenic culture IV-HC2s and non-embryogenic strains IV-HC5s, IV-HV7s, and IV-HV9s showed the predominance of diploid cells in all four strains; however, their proportions differed. In embryogenic IV-HC2s and non-embryogenic IV-HC5s strains of cotyledonary origin, the number of diploid cells was very similar, for both IV-HV7s and IV-HV9s being 60 and 76% respectively, (Table 5). In addition to the diploid cells, there were other cells with DNA content lower than 2*C*, but their proportions were not significant and did not exceed 6%. The percentage of cells of 4*C* ploidy ranged from 10% for IV-HC2s strain to 32% IV-HV7s strain. The contribution to this class was made by both tetraploid and diploid cells being at the end of S or in G_2 phase. In IV-HC5s and IV-HV9s strains, we observed small sub-populations of cells with DNA content higher than 4*C*.

The analysis of DNA content in primary leaves from regenerants from the IV-HC2s strain revealed the predominance of cells with a diploid amount of DNA (Table 6). The dosage of such cells ranged from 70 to 86%. Since the growth of primary leaves from regenerants had not yet finished, there were cells with DNA content between 2*C* and 4*C*, which corresponded to the S phase state. The percentage of cells with less than 2*C* level of DNA ranged from 6 to 28%, which is close to the values for mature leaves of in vitro plants cv. Catuai (Table 2).

4. Discussion

In this study, we used the two-wave length method due to its high accuracy, which is comparable with scanning techniques. Apical root meristem cells of *V. radiata* cv. Berken at different stages of mitosis were used as a standard for DNA content as they correspond to 4*C* and close to 2*C* in *C. arabica.* This correspondence in DNA content increases the accuracy of measurements [22]. The value of $2C = 2.5$ pg DNA per nucleus for *C. arabica* cv. Caturra as a criterion for the attribution of cells to a definite level of ploidy was chosen because different cultivars of *C. arabica* have little variation in nuclear DNA amount. Thus, when Feulgen staining was used, it ranged between 2.4 and 2.6 pg [21], in flow cytometry of nuclei stained with propidium iodide it was 2.6 pg [23], the application of DAPI gave higher values in general but for cvs. Caturra and Catuai they were very similar (2.92 and 2.91 pg, respectively) [24].

The fate of in vitro plant leaves was probably affected by exogenous growth regulators, which were introduced into the culture media. We showed that leaves of the same coffee variety cultured on the medium with a high BAP concentration contained a higher portion of diploid cells than those cultured in the presence of kinetin and NAA. We cannot rule out that the cells with lower DNA estimation could be the result of true aneuploids nuclei (improbable), or just under-assessment of DNA (probably). Since poor fixation is a drawback in Feulgen methodology [25].

In spite of the presence of cells with a lower DNA amount estimation in the initial explants used in our work, the embryoids derived by direct embryogenesis contained a high percentage of diploid cells. Calli obtained according to Söndahl's protocol had a lower portion of diploid cells at the proliferation stage (conditioning medium) than at the transition stage to embryogenesis itself (induction medium). The cells with an unchanged genome were probably necessary for the formation of embryoids.

An analysis of four suspension cultures did not reveal any relationship between the cytogenetic state of cell strains and their morphogenetic potential. In other words, diploidy cannot guarantee high morphogenic potential. Two strains of cotyledonary origin with very similar portions of diploid cells (ca. 80%) differed in their embryogenic capacity. One (IV-HC2s) was capable of regeneration while the other (IV-HC5s) was non-morphogenic. Similar results have been found in *Daucus carota* suspension cultures [26]. A number of diploid and tetraploid cell strains of *D. carota* were obtained as a result of cloning the suspension culture with low embryogenic potential. All tetraploid cultures were non-morphogenic whilst among the diploid cultures there were both highly embryogenic and fully deprived embryogenic potentials. The development of molecular techniques to study genome rearrangements in vitro is, therefore, an essential problem and application of these approaches may help to clarify the mechanisms of somatic embryogenesis and morphogenetic potential in general.

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