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Research Note

Flow cytometry - a simple method for nuclear DNA content evaluation of *Vitis vinifera* cv. Periquita somatic embryos obtained from anther cultures

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K e y w o r d s : DNA content, flow cytometry; somatic embryos, zygotic embryos, *Vitis vinifera* L.

Introduction: The process of *in vitro* somatic embryogenesis obtained from cultured anthers of the Portuguese grapevine cv. Periquita was mediated by *callus* formation (Lima *et al.* 1999). A common problem of this type of culture is the determination of ploidy level among arising embryos. It is known that anther-derived *calli* may contain haploid nuclei, but derivatives of these cells do not seem to participate in embryo formation (Rajasekaran and Mullins 1983). *In vitro* anther tissues of cv. Periquita produced callus that allowed both somatic embryo formation and plant regeneration (Lima *et al.* 1999). The ploidy level of these regenerated plants was determined using root tips and the Feulgen technique for chromosome counting on several metaphasic plates (Lima, unpubl.). Due to the relatively high diploid chromosome number of *Vitis vinifera* L. (2n=38) and to their small size, these results (data not shown) not always clearly elucidated their ploidy level. Consequently, the ploidy status was evaluated by flow cytometry and correlated with previously performed cytogenetic evaluations. Although flow cytometry has originally been developed for medical purposes (Winkelmann *et al*. 1998) it has been applied also in plant science offering a reliable method to evaluate nuclear DNA contents.

Material and Methods: P l a n t m a t e r i a l : Somatic embryos were obtained from *calli* derived from *in vitro*-cultured anthers of *Vitis vinifera* L. (cv. Periquita) (Lima *et al.* 1999) that exhibited embryos at torpedo stages (length: 1-1.5 mm). They were manually selected under a dissecting microscope, frozen into liquid nitrogen and kept in Eppendorf tubes at -80 ºC. Zygotic embryos were obtained from mature dormant seeds sterilized for 5 min in 45 % (v/v) commercial bleach (2.4 % w/v NaOCl) containing Tween 20, and washed in sterile distilled water until all visible traces of detergent were removed. Seeds were soaked in sterile distilled water for 72 h (in some cases for one week) on a rotating shaker (80 rpm) at room temperature. Excised zygotic embryos were white (length: 0.7-1.5 mm) and consisted of a root axis, hypocotyl, and two cotyledons. Leaves from *Lactuca sativa* and *Prunus persica,* used as standards, were washed in sterile distilled water, ground in liquid nitrogen and kept in Eppendorf tubes at -80 ºC.

S o l u t i o n s : Stock solutions of $MgSO_4$ buffer, PI and Triton X-100 were prepared according to Arumuganathan and Earle (1991 b). Our RNAase (DNAase free) was from Sigma-R 6513 (stock solution 1 mg·ml^{-1} TE buffer). Solutions A and B were prepared freshly: solution A (15 ml for up to 12 samples contained 14.3 ml ice-cold $MgSO_4$ buffer, 15 mg DTT, 300 µl propidium iodide (PI) stock solution and 375 µl Triton X-100 stock solution). For the modified solution B (3 ml for up to 12 samples) 3 ml of solution A were added to 7.5 µl RNAase without CRBC solution (used as an internal standard by Arumuganathan and Earle 1991 b); instead human peripheral blood (HPB) available from daily collections at the Histocentre laboratory was added. The leucocyte nuclear DNA content value of this standard is 6.54 ± 0.21 pg/2C (Arumuganathan and Earle 1991 b).

P r e p a r a t i o n o f s u s p e n s i o n s o f n u c l e i : The samples (about 50 mg each) were placed on plastic Petri dishes (35 mm x 10 mm) on ice; 1 ml of solution A was added for slicing and chopping the tissue into small pieces using a sharp scalpel; the homogenate was filtered through a 33 µm nylon mesh into a microcentrifuge tube which was centrifuged at 15,000 rpm for 15-20 s. Thereafter, the supernatant was discarded, the pellet resuspended in 200 µl of solution B and incubated for 15 min at 37 ºC. To evaluate the 2C DNA content of each plant sample by flow cytometry, 50 µl of HPB were added to the nuclei suspension.

D N A a n a l y s i s : Estimation of the nuclear DNA content was conducted in an Coulter -Epics XL flow cytometer (Hialeah, Florida, USA). Alignment and calibration of the instrument were performed according to instructions of the manufacturer prior to the measurements. Fluorescence was measured with a signal resolution of 1024 channels and evaluated on a linear scale. To obtain correct values of the relative and absolute DNA content, both, leucocytes from HPB and nuclei isolated from leaves of other plant species with a known DNA content were used as standards (Table). The stained nuclear suspension was aspirated into the flow cytometer; the high voltage of the photomultiplier tube was adjusted so that the signals corresponding to

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Nuclear DNA content of some plant species as determined by flow cytometry (from Arumuganathan and Earle 1991 a)

	Family	Nuclear DNA content pg/2C[a](N)
Lactuca sativa	Compositae	5.47
Prunus persica	Rosaceae	0.54, 0.55(2)
Vitis vinifera	Vitaceae	1.00

[a] Values for each cultivar were determined by two or more measurements of at least 2000 nuclei. N - number of cvs assayed.

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the populations of intact nuclei were within the scale of the log fluorescence intensity. Each measurement of at least 20,000 nuclei was repeated twice.

Results and Discussion: Assessment of DNA content by flow cytometry: It is well known that nuclei from plant tissue normally produce two distinct peaks of fluorescence intensity, one corresponding to G_0+G_1 phase cells, the so-called 2C complement of DNA and the other corresponding to G_2+M phase cells, the so-called 4C complement of DNA. The latter have twice the amount of fluorescence intensity of the G_0+G_1 nuclei because they contain twice as much DNA (Arumuganathan and Earle, 1991 b) However, the nuclei of some tissues show only the 2C component because they contain few or no dividing cells.

In our experiments all samples showed a single population of nuclei at the 2C DNA level (Figure) and no subpopulations occurred within the 2C-4C or 4C levels. The nuclear DNA content per 2C nucleus was compared on entire nuclei suspensions from *Vitis* somatic embryos, *Lactuca* leaf and HPB (Figure, A), and from somatic and zygotic embryos (data not shown). From a list of DNA contents of unreplicated haploid genomes of various plants arranged according to genome size, a value of 483 Mpb/1C is reported for *Vitis vinifera* L., and its nuclear DNA content determined by flow cytometry has a value of 1.00 pg/2C; *i.e.* 1 pg equals 965 million base pairs (Arumuganathan and Earle 1991a). However, the nuclear DNA content values for *Vitis vinifera* L. estimated by Lodhi and Reisch (1995) ranged between 0.86 and 1.00 pg/2C. According to Arumuganathan and Earle (1991 a) the DNA content of *Prunus persica* (0.54 pg) is about half that of *Vitis vinifera* (1.00 pg) and for that reason E. Earle suggested *P. persica* samples to be useful as an haploid standard of *Vitis vinifera* L., to detect haploid nuclei, if any (Figure, B). On the basis of these preliminary assays, we found that the flow cytometric analysis gives, for the somatic embryo nuclei suspension samples, a DNA content similar to the one of their zygotic embryo counterparts (data not shown), suggesting that our *in vitro* indirect somatic embryogenesis protocol in most cases may have favoured a diploid embryo formation, as no haploid or polyploid patterns were detected among the samples. However, the occurrence of minor DNA aneuploidy events (e.g., hypoploidy due to the loss of one or a few chromosomes, for a review see D'Amato 1991) leading to somatic embryo formation with variable nuclear DNA levels cannot be totally excluded. But in those cases their development may eventually be arrested at the globular stage, as was reported for another species by Cavallini and Natali (1989, cit. Faure and Nougarède 1993). According to Orfao *et al*. (1993) the term "aneuploidy" should be used for karyotypic abnormalities detected by conventional cytogenetics techniques, while terms such as "clone with an abnormal amount of DNA" or "DNA aneuploidy" should be reserved for DNA measurements made by cytometric analyses. The use of flow cytometry enables large numbers of nuclei to be analysed and so increases the chance to detect DNA aneuploidy; it facilitates a rapid and efficient distinction between different ploidy levels.

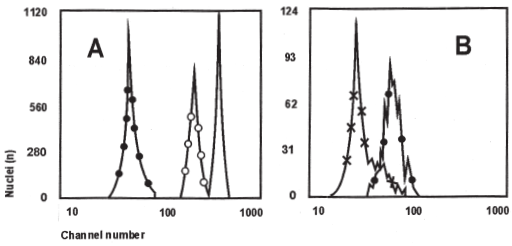

Figure: Histograms of number of nuclei per channel as a function of relative fluorescence intensity (peak position on horizontal axis). Histograms were acquired on a EPICS XL (Coulter) flow cytometer. Signals from nuclei were gated to eliminate much of debris from analysis. The channel number is proportional to the log of fluorescence intensity. **A:** (•)- *Vitis* somatic embryo; (◦)- *Lactuca sativa* leaf; (-)- human peripheral blood. **B:** (x)- *Prunus persica* leaf; (•)- *Vitis* somatic embryo.

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