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Analysis of the genetic stability of *Eucalyptus globulus* Labill. somatic embryos by flow cytometry

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Abstract Flow cytometry was used to measure the nuclear DNA content of *Eucalyptus globulus* Labill. somatic and zygotic embryos and leaves in order to determine if somatic embryogenesis induces DNA content and ploidy changes in this species. Mature zygotic embryos derived from open-pollination orchard families were collected from a location in the centre of Portugal. One group was kept for nuclear DNA content and ploidy analysis, and the other group was used for establishing embryogenic cultures. Mature zygotic embryos were grown on Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose and 3 mg l^{-1} α-naphthaleneacetic acid (NAA) for 3 weeks and then transferred to MS medium without growth regulators. Globular somatic embryos from approximately 8-month-old embryogenic cultures were used in the assay. DNA ploidy levels and the nuclear DNA content of mature zygotic embryos, somatic embryos and leaves from the mother field tree were determined using flow cytometry combined with propidium iodide staining. Zygotic embryos had a nuclear DNA content of 1.32 pg/2C, somatic embryos had a nuclear DNA content of 1.39 pg/2C and leaves from the field tree had a nuclear DNA content of 1.40 pg/2C. The values estimated for the somatic embryos and mother plant did not differ statistically from each other ($P \leq 0.05$), but both differed from those of the zygotic embryos ($P \leq 0.05$). These results clearly indicate that no changes were induced during the embryogenic process. However, the differences found between the field plants and zygotic embryos did suggest that some aspects must be evaluated carefully, as propidium iodide fluorescence may potentially be influenced by the presence of secondary compounds (e.g. anthocyanins, tannins) in *E. globulus* somatic embryos and mature leaves. Therefore we believe that the somatic embryogenesis methodology used did not induce major genetic changes in the somatic embryos and that our primary goal of "true-to-type" propagation was assured.

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Introduction

The genus *Eucalyptus* L'Hér. comprises approximately 700 species and varieties although only 1% of them are used for industrial purposes (Watt et al. 1999). The Eucalyptus kraft pulp industry is undoubtedly one of the most important applications and is based largely on two species, namely *E. globulus* and *E. grandis* hybrids. The natural genetic diversity within Eucalyptus species is enormous and can be further enhanced by interspecific hybridisation making it an attractive genus for breeding purposes (Eldridge et al. 1993). Due to its versatility, fast growth and fibre characteristics, *E. globulus* is grown worldwide. It was introduced into Portugal 150 years ago and at the present time represents the third most common forestry species, covering approximately 672.149 ha of forest (Direcção Geral de Florestas 2001).

Both seed and clonal propagation have intrinsic disadvantages in *E. globulus* breeding—namely the high level of heterozygosity found in seeds and the very irregular adventitious rooting behaviour (5–64%) (Marques et al. 1999) found in the vegetative propagation of some desired genotypes. Somatic embryogenesis has the capacity to provide large-scale propagation systems for many species, including forest trees, and has the advantage that both a root and a shoot meristem are present simultaneously in somatic embryos. This methodology also simplifies conservation methods as a large number of genotypes may be stored in a limited space while they are being field-tested for genotype versus environment interactions. A knowledge of these interactions, as in other

forestry species, is of particular importance in *Eucalyptus* spp. (Zobel 1993). A reproducible protocol for somatic embryogenesis in *E. globulus* from mature zygotic embryos was established by Pinto et al. (2002), thereby opening new perspectives for a large-scale application of this methodology to this economically important species. Although somatic embryogenesis in woody plants has long been regarded as a safe methodology, recent evidence has shown that this might not be the case with respect to somaclonal variation (Endemann et al. 2002). Therefore to minimize the risk of genetic variation a rapid screening protocol capable of detecting possible changes has to be applied to assure the primary goal of "true-to-type" propagation.

Numerical chromosomal changes, which are the most frequently reported changes in broadleaf and conifer species (see DeVerno 1995; Bueno et al. 1996) are not detectable by random amplified polymorphic DNA (RAPD) analysis (Fourré et al. 1997), and conventional cytogenetic techniques, such as karyotyping, are laborious and time-consuming. These drawbacks can be circumvented by using flow cytometry, provided that it is sensitive enough to detect the differences in DNA content caused by the presence or absence of one single chromosome (Pfosser et al. 1995). However, very few investigators have used this technique to assay somaclonal variation in woody plants (Awoleye et al. 1994; Bueno et al. 2000; Endemann et al. 2002) and there has been only one report of *E. globulus* Labill. plants derived from organogenesis (Azmi et al. 1997).

Flow cytometry was originally developed as a method for rapidly counting and analysing blood cells (Ayele et al. 1996). At the present time, it is being applied in the fields of plant science mostly focused on DNA ploidy and nuclear DNA content analysis. Flow cytometry analysis of the nuclear DNA content is based on the use of DNA-specific fluorochromes and on the analysis of the relative fluorescence of the stained nuclei (Dolezel 1991). In most plants, analysis of relative DNA content of nuclei isolated from young tissues yields a histogram showing a dominant peak corresponding to nuclei at the G_0/G_1 phase of the cell cycle and a minor peak corresponding to G_2/M nuclei. For the ploidy level of an unknown sample to be estimated, the position of the G_1 peak of that sample on a histogram is compared to that of a reference plant with known ploidy (Dolezel 1997). The flow cytometric assay has some important advantages over chromosome counting. It is convenient (sample preparation is easy), rapid (several hundreds of samples can be analysed in one working day), it does not require dividing cells, sample preparation requires only a few milligrams of tissue and it can detect mixoploidy (Dolezel 1997).

In the investigation reported here, mature zygotic embryos from several open-pollinated orchard families and a field plant were used to quantify the DNA content of *E. globulus*. Also, in order to determine if somatic embryogenesis induced DNA content and/or ploidy changes, we compared somatic embryos obtained from one of the families studied with zygotic embryos and with mature leaves from the mother plant. To complement these analyses, we estimated the nuclear DNA content of leaves of in vitro-micropropagated plantlets derived from mature zygotic embryos of the same family and compared these with the values found for this family.

Materials and methods

Induction of somatic embryogenesis

Half-sib seeds of *Eucalyptus globulus* ssp. *globulus* Labill. (StoraEnso, Celbi, Leirosa, Portugal) collected in the centre of Portugal from open-pollinated orchard families (EG01, EG02, EG04, EG06, EG09, EG10, EG11, EG12, EG13) were sterilised as described by Pinto et al. (2002). Somatic embryogenesis cultures were initiated from zygotic embryos following the protocol established by Pinto et al. (2002). Briefly, the seed coat was removed from the mature zygotic embryos and they were transferred to Murashige and Skoog (1962) medium (MS) supplemented with 3% (w/v) sucrose and with 3 mg l^{-1} NAA to induce somatic embryogenesis. After 3 weeks on the induction medium, the explants were transferred to MS medium without growth regulators (MSWH) on which they were maintained. Eight-month-old somatic embryos were used for ploidy comparison with the mature zygotic embryos.

Laser flow cytometry analysis

Nuclear suspensions from mature zygotic embryos dissected from the seeds, from somatic embryos and from in vitro and field leaves were prepared according to Galbraith et al. (1983). In brief, nuclei were released from the cells by chopping samples of this material with a razor blade together with a young leaf of the internal reference standard *Lycopersicon esculentum* cv. Stupicke (2C=1.96 pg DNA; kindly provided by J. Dolezel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic) in Marie's isolation buffer (50 m*M* glucose, 15 m*M* NaCl, 15 m*M* KCl, 5 m*M* EDTA Na_2, 50 m*M* sodium citrate, 0.5% Tween 20, 50 m*M* HEPES, pH 7.2; Marie and Brown 1993). The suspension of nuclei was first filtered through a 50-μm nylon filter to remove cell fragments and large tissue debris and then 50 μg ml^{-1} of propidium iodide (PI) (Fluka, Buchs, Switzerland) and 50 μg ml^{-1} of RNAse (Sigma, St. Louis, Mo.) were added to the samples to stain the DNA. Samples were analysed within a 15-min period in a flow cytometer.

The relative fluorescence intensity of PI-stained nuclei was measured by a Coulter EPICS XL (Coulter Electronics, Hialeah, Fla.) flow cytometer. This instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. Integral fluorescence together with fluorescence pulse height and width emitted from nuclei were collected through a 645-dichroic long-pass filter and a 620-band-pass filter and converted on 1,024 ADC channels. Prior to analysis, the instrument was checked for linearity with fluorescent check beads (Coulter Electronics), and the amplification was adjusted so that the peak corresponding to *E. globulus* nuclei was positioned approximately at channel 200. This setting was kept constant. The results were obtained in the form of three graphics: linear-fluorescence light intensity (FL), forward angle (FS)- versus side angle (SS)-light scatter and FL pulse integral versus FL pulse height. This last cytogram was used to eliminate partial nuclei and other debris, nuclei with associated cytoplasm and doublets (these events have a higher pulse area but the same pulse height as single nuclei) (Price and Johnston 1996). In this cytogram an "interest zone" was defined such that only single intact nuclei were included in the FL histogram (Brown et al. 1991).

To estimate ploidy level, we compared the position of the G_0/G_1 peak of the sample on a histogram with that of the internal reference plant with known ploidy. For each sample at least 5,000–

10,000 nuclei were analysed. The size of the nuclear genome of *E. globulus* Labill. was calculated according to the following formula:

$$E.\ globulus\ \text{2C nuclear DNA content (pg)} = \frac{E.\ globulus\ G_0/G_1\ \text{peak mean}}{L.\ esculentum\ G_0/G_1\ \text{peak mean}} \times 1.96$$

Between 4 and 15 zygotic embryos of each family (EG01, EG02, EG04, EG06, EG09, EG10, EG11, EG12, EG13) were analysed in our assay of *E. globulus* nuclear DNA content. To detect possible changes induced by somatic embryogenesis, we analyzed the ploidy levels of 21 somatic embryos and 15 mature zygotic embryos from the same family (EG12). The nuclear DNA content of leaves from two in vitro-germinated plantlets (EG12) and nine leaves from a field tree (EG12) was also assayed, and the values obtained were then compared with the ones obtained for zygotic and somatic embryos.

Test for inhibitors

Experiments were carried out in order to determine if *E. globulus* Labill. extracts contain compounds that may affect PI fluorescence; see Price et al. (2000). Briefly, in the first experiment (A), nuclei suspensions were obtained from one-half of a tomato leaf simultaneously processed (co-chopped) with: (a) mature zygotic embryos, (b) somatic embryos or (c) field leaves. These samples were then stained with PI. The second experiment (B) consisted of nuclei suspensions that were obtained from the other half of the tomato leaf processed independently. These samples were also stained with PI. After staining for 15 min, samples from experiments A and B were analysed on the flow cytometer for mean PI fluorescence, after which they were mixed and re-analysed.

Statistical analysis

Statistical analyses were performed using a one-way ANOVA (SIGMASTAT FOR WINDOWS ver. 2.03, SPSS, USA) to analyse possible differences between mature zygotic embryos of open-pollinated trees of *E. globulus* and to analyse possible differences between zygotic embryos, somatic embryos, leaves of in vitro-germinated plantlets and field leaves. A multiple comparison Tukey-Kramer test was applied to determine exactly which groups were different.

Results and discussion

FS is proportional to cell-surface area or size, and SS is proportional to cell granularity or internal complexity. A graphic representation of these two parameters provides useful information about the nuclei, and most of the flow cytometry reports on plant material that are available do not take these factors into consideration. Figure 1a–d shows that there is a general homogeneity among nuclei derived from zygotic embryos (Fig. 1a), somatic embryos (Fig. 1b), leaves of in vitro-germinated plantlets (Fig. 1c) and field leaves (Fig. 1d) with respect to FS and SS and that eucalyptus and tomato nuclei have almost the same size as is clearly visible from the overlapping between particles of both species.

FL represents the intensity of the PI-stained linear fluorescence (Fig. 1e–h), and a cytogram with pulse integral versus pulse height can be computed to define a gating region that excludes doublets and particles of plant debris (present in the bottom left corner of the cytogram) (Fig. 1i–l). Throughout the experiment, and as expected, the internal standard had a tight 2C distribution (mean coefficients of variation (CV)=2.5%; Fig. 1). The histograms of relative nuclear DNA content showed distinct G_0/G_1 peaks with the CV ranging from 2.2% to 4.0% for zygotic embryos (mean CV=3.03%; Fig. 1e), from 2.2% to 4.5% for somatic embryos (mean CV=3.16%; Fig. 1f), from 3.5% to 5.5% for leaves of in vitro-germinated plantlets (mean CV=4.51%; Fig. 1g) and from 4.6% to 6.8% for field leaves (mean CV=5.72 %, Fig. 1h). The importance of showing CV values in this type of study was pointed out by Marie and Brown (1993) who consider it an elementary criterion for assessing a cytological method. These authors also stated their reference guideline was a range of 1–2% for top-quality analyses in plant cells and around 3% as a routine value (Marie and Brown 1993). On the other hand, Galbraith et al. (2002) defined a CV of less than 5% as the acceptance criterion. However, for most recalcitrant species, such as woody plants, these recommended values are at the present time very difficult or in some cases impossible to obtain Grattapaglia and Bradshaw (1994) reported CV values ranging from 6.3% to 12.8% in their investigation on *Eucalyptus* spp. In oak species, Zoldos et al. (1998) reported CV values ranging from 3.3% to 6.9%. There is usually a correlation between higher CV values and higher background noise (Emshwiller 2002). High background noise is not unusual in the lower channel numbers (Kudo and Kimura 2001) and has been alternatively ascribed to broken cells damaged during the extraction procedure or to autofluorescence of the chloroplast in the cytosol (Emshwiller 2002). With respect to this latter point, Galbraith et al. (2002) reported that the contribution of chlorophyll autofluorescence is more critical in species where the nuclear DNA content is smaller, the consequence of which is that this autofluorescence can overlap PI-induced nuclear fluorescence on one-dimensional histograms, thereby affecting CV values. Unfortunately, many reports (some of them concerning *E. globulus*) do not contain any information on this point, leading to practical questions concerning the methodology used and constraining interpretation of the results.

In general, a small G_2/M peak was observed for *E. globulus*. Similar results have been obtained in woody plants, such as in *Quercus robur* embryos (Endemann et

Fig. 1 Histograms of forward angle (*FS*)- versus side angle (*SS*)-light scatter **(a–d)**, relative fluorescence intensity (*FL*) **(e–h)** and relative FL pulse integral versus relative FL pulse height **(i–l)** obtained after simultaneous analysis of nuclei isolated from *Lycopersicon esculentum* cv. Stupicke (2C=1.96 pg DNA, as an internal reference standard) and *Eucalyptus globulus*: **a**, **e**, **i** Mature zygotic embryo of the family EG12, **b**, **f**, **j** somatic embryo of the family EG12, **c**, **g**, **k** leaves of in vitro-germinated plantlets, **d**, **h**, **l** leaves of field plant of the family EG12. In all of the FL histograms **(e–h)** four peaks were observed: *1* nuclei at the G_0/G_1 phase of *E. globulus*, *2* nuclei at G_0/G_1 phase of *Lycopersicon esculentum* leaves, *3* nuclei at G_2/M phase of *E. globulus*, *4* nuclei at G_2/M phase of *L. esculentum* leaves. In FL pulse integral versus relative FL pulse height cytograms **(i–l)** a gating region was defined to exclude doublets and particles of plant debris (present in the *bottom left corner* of the cytogram)

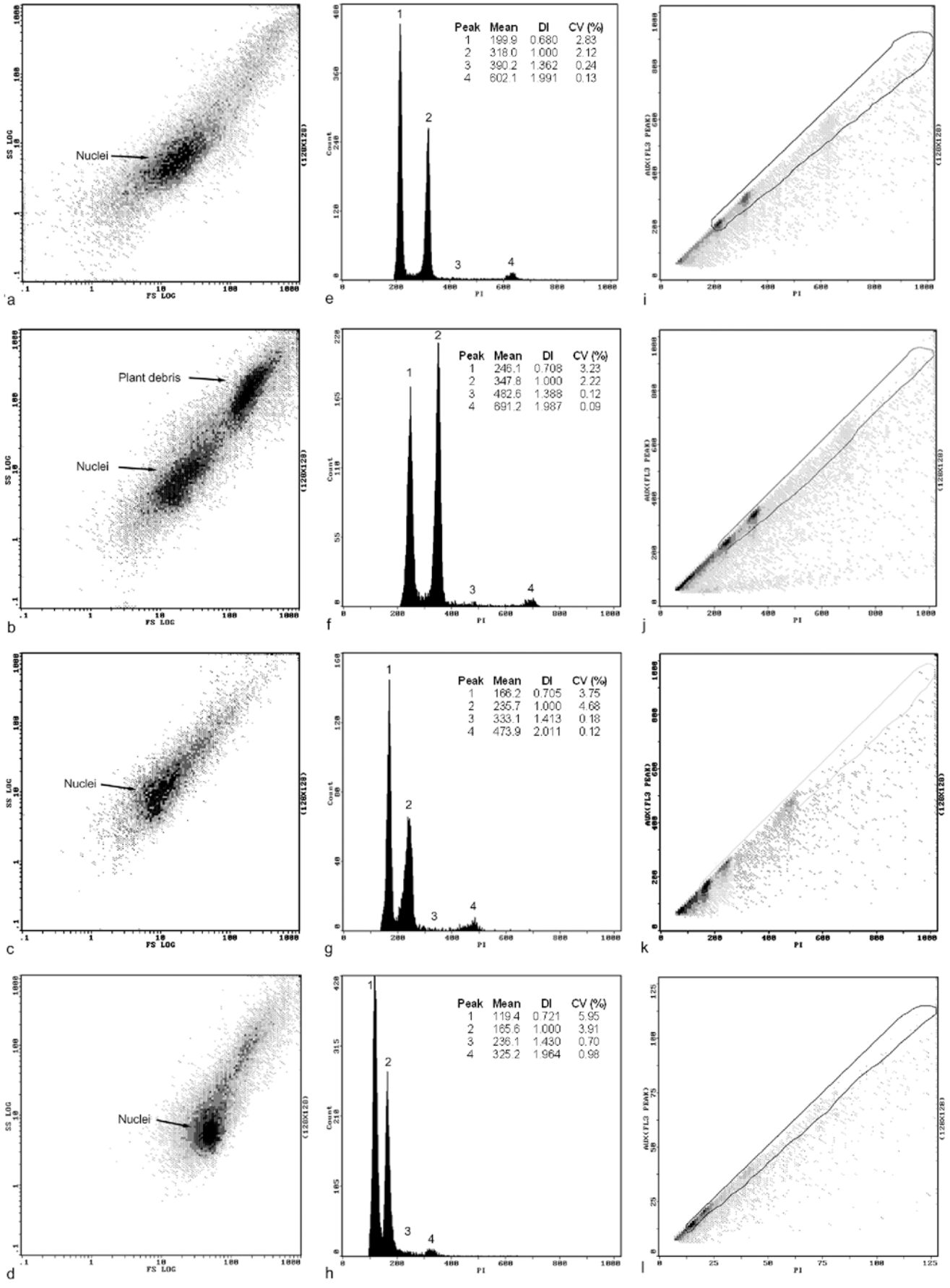
Nuclei
SS LOG
FS LOG
a
Peak Mean DI CV (%)
1 199.9 0.680 2.83
2 318.0 1.000 2.12
3 390.2 1.362 0.24
4 602.1 1.991 0.13
Count
PI
e
AUX(FL3 PEAK)
i
Plant debris
Nuclei
b
Peak Mean DI CV (%)
1 246.1 0.708 3.23
2 347.8 1.000 2.22
3 482.6 1.388 0.12
4 691.2 1.987 0.09
f
j
Nuclei
c
Peak Mean DI CV (%)
1 166.2 0.705 3.75
2 235.7 1.000 4.68
3 333.1 1.413 0.18
4 473.9 2.011 0.12
g
k
Nuclei
d
Peak Mean DI CV (%)
1 119.4 0.721 5.95
2 165.6 1.000 3.91
3 236.1 1.430 0.70
4 325.2 1.964 0.98
h
l

Table 1 Nuclear DNA content[a] of *Eucalyptus globulus* Labill. mature zygotic embryos, somatic embryos, leaves of in vitro-germinated plantlets and leaves of field plants

Families	Index	Standard deviation	Nuclear DNA content (pg/2C)	Standard deviation	1C Genome size (Mbp)[b]	*n*	Tukey–Kramer test grouping[c]
Zygotic embryos							
EG01	0.676	0.0189	1.33	0.038	650	4	A
EG02	0.682	0.0251	1.34	0.049	655	5	A
EG04	0.668	0.0162	1.31	0.032	641	6	A
EG06	0.667	0.0044	1.31	0.008	641	4	A
EG09	0.669	0.0083	1.31	0.016	641	6	A
EG10	0.654	0.0126	1.28	0.025	626	8	A
EG11	0.682	0.0296	1.34	0.058	655	8	A
EG12	0.673	0.0225	1.32	0.044	645	15	A
EG13	0.675	0.0299	1.31	0.047	641	8	A
	0.672	0.0186	1.32	0.035	644	64	A
Somatic embryos							
EG12	0.708	0.0083	1.39	0.016	680	21	B
Leaves of in vitro-germinated plantlets							
EG12	0.710	0.0007	1.39	0.001	680	2	B
Leaves of field plants							
EG12	0.715	0.0146	1.40	0.029	689	9	B

[a] The values are given as a mean and standard deviation of the mean of the DNA index relative to the internal standard *Lycopersicon esculentum* cv. Stupicke, as a mean and standard deviation of the mean of the nuclear DNA content (pg/2C) and as 1C genome size of *E. globulus* Labill

[b] 1 pg DNA=978 Mbp (Dolezel et al. 2003)

[c] Groups followed by the same letters (A or B) are not significantly different according to the multiple comparison Tukey-Kramer test at $P \leq 0.05$

al. 2002), but these authors did not explain their finding. In other species, such as rice and wheat, no G_2/M peak was found at all (Arumuganathan and Earle 1991), a result which was attributed to the developmental stage of the leaves. No G_2/M peak was observed in *Vitis* spp. (Lodhi and Reisch 1995), and these authors attributed the obscurity of the 4C peaks to the high fluorescent background of the plant debris.

With respect to *E. globulus*, the small or even absent G_2/M peak in the zygotic embryos and field leaves can be explained in the former by seed dormancy, as low background noise was detected (Fig. 1e), and in the latter, by the mature stage of the leaves and the presence of some background noise (Fig. 1h). In somatic embryos and in in vitro leaves, although the background was not high (Fig. 1f, g) when compared that observed with other woody plant species (Lodhi and Reisch 1995; Schwencke et al. 1998; Zoldos et al. 1998), it may have been sufficient to underestimate the quantity of nuclei present in this peak.

The mean nuclear DNA fluorescence index (DI= $2C_{Eucalyptus}/2C_{Lycopersicon}$) for *E. globulus* ranged from 0.672 (mature zygotic embryos) to 0.715 (field leaves) (Table 1). Despite this range of variation the assignment of peaks to 2C and 4C level values was not affected. If tetraploid levels were to be observed, a DI of 1.344 was expected. The results obtained strongly indicated that all of the samples analysed had the same ploidy level—i.e. diploid—and that no polyploidy was present in the somatic embryos and in leaves of in vitro-germinated plantlets. Therefore we believe that there is a relative genetic stability at this level during the embryogenic process used for *E. globulus*. This result differs from those reported for other species where polyploidisation was found during somatic embryogenesis (Kubaláková et al. 1996; Kudo and Kimura 2001; Endemann et al. 2002). However, Endemann et al. (2002) found that tetraploidy occurred only in 8% of the clones tested over a culture period of 7 years. Despite the fact that no polyploidisation was found in Eucalyptus in vitro cultures, the CV values obtained and the range of variation observed could mask the possible occurrence of small differences in nuclear DNA content; consequently, the presence of a low level of aneuploidy should not be excluded. Complementary studies, such as chromosome counting analysis, to evaluate this possibility are presently being conducted in our laboratory (data not shown). Roux et al. (2003) recently combined flow cytometry and chromosome counting and showed that flow cytometry could rapidly detect aneuploidy situations in *Musa* sp.

The nuclear DNA content of *E. globulus* in absolute units ranged from 1.28 pg/2C to 1.40 pg/2C, and these estimates were reproducible for a given sample source, with low standard deviations (Table 1). The mean nuclear DNA content of *E. globulus* zygotic embryos was 1.32±0.035 pg/2C (Family EG12: 1.32±0.044 pg/2C). This value increased to 1.39±0.016 pg/2C in *E. globulus* somatic embryos and to 1.39±0.001 pg/2C in leaves of in vitro-germinated plantlets. The highest value was obtained in leaves of the field plant—1.40±0.029 pg/2C—even though fluorescence in absolute units was reduced. Statistical analysis (one-way ANOVA) showed significant differences for $P \leq 0.05$ among different groups. A multiple comparison Tukey-Kramer test showed that there were significant differences ($P \leq 0.05$) both between the values found for zygotic embryos (n=13) and those

found for somatic embryos (n=21) and between the former and the values obtained for leaves of both in vitro (n=2) and field plants (n=9) (Table 1). Despite these results a low variation of approximately 6% was found between zygotic embryos and somatic embryos and between zygotic embryos and leaves of both the in vitro and field plants.

Although our results are significant, they must be interpreted with caution, as these differences in samples do not necessarily reflect real differences in nuclear DNA content but may be due to the presence of compounds that affect PI fluorescence or to chromatin structural differences between tissues. The presence of extra-nuclear compounds that reduce nuclear PI fluorescence has been detected in some plant species, namely *Helianthus annuus* L. (Price et al. 2000) and *Coffea liberica* var. *dewevrei* (Noirot et al. 2000, 2002). Price et al. (2000) found it likely that inhibitors that decrease the fluorochrome fluorescence of plant nuclei are common in plants. Therefore, a test for naturally occurring inhibitors should be used in all flow cytometric studies. Noirot et al. (2000), working with coffee, highlighted cytosolic effects on dye accessibility to DNA and that cytosolic compounds can bias nuclear DNA content estimates by up to 20%. More recently, Noirot et al. (2003) identified two of these cytosolic compounds that modify accessibility of PI to *Petunia* spp. DNA (species used as internal standard for genome size evaluation): caffeine and chlorogenic acid (a precursor of polyphenols). These authors also draw attention to the fact that the presence of inhibitors compromises the reliability of estimations of nuclear DNA content, particularly if the detection of small differences is desired.

In general, the amount and complexity of the secondary products change with the age of the plant material and among organs, thereby interfering with the flow cytometric analyses. For this reason, it is normal procedure that experimental plant material for use in flow cytometry studies consists of young and growing tissues (Galbraith et al. 2002). This may explain the higher CV values observed in leaves with respective to embryos and also the decrease in peak resolution in the histograms of leaves from the field plants. On the other hand, Eucalyptus somatic embryos, contrary to the zygotic ones, had a yellow-brownish colour and histograms with a higher background noise were obtained, suggesting a higher content of secondary compounds (e.g. tannins, anthocyanins). The data presented here show a notorious decrease in PI fluorescence from both Eucalyptus and tomato nuclei in samples obtained from leaves of the field plants (Fig. 1h). This decrease in PI fluorescence was not observed in zygotic embryos, somatic embryos or in leaves of in vitro-germinated plantlets. Also associated with this occurrence may be the fact that PI is a DNA intercalator sensitive to chromatin structure and that, as pointed out by Biradar and Rayburn (1994), chromatin structure may vary between organs/tissues.

In the inhibitors test a decrease in PI fluorescence was observed in tomato leaves simultaneously processed with

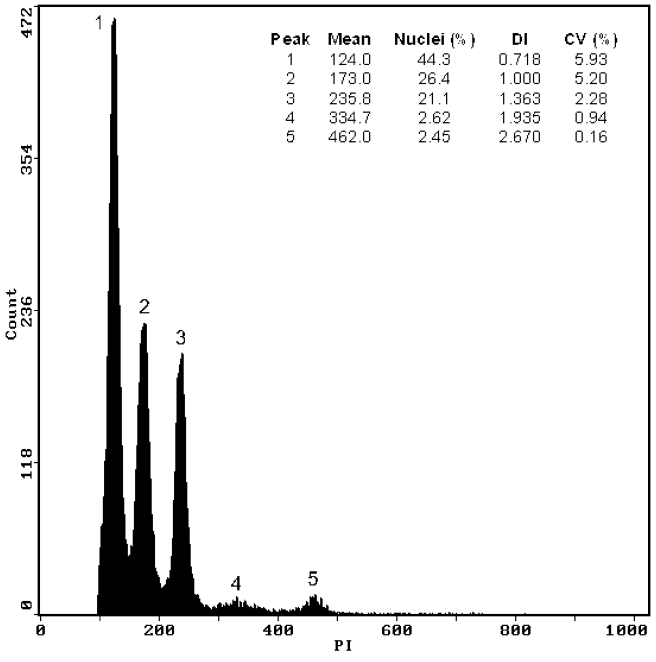

Fig. 2 Flow cytometry histogram of PI-stained nuclei from simultaneously processed *E. globulus* field leaves (*1*) and *L. esculentum* leaves (*2*) to which PI-stained nuclei from independently processed *L. esculentum* leaves (*3*) were added. Peaks *4* and *5* refer, respectively, to nuclei at the G_2/M phase of simultaneously processed *L. esculentum* leaves and independently processed *L. esculentum* leaves. Peak *3* overlaps nuclei at the G_2/M phase of *E. globulus* field leaves

Eucalyptus field leaves (experiment A) in comparison with the PI fluorescence of nuclei from independently processed tomato leaves. This result is even clearer when experiments A and B were mixed and immediately reanalysed (Fig. 2): in addition to peaks 1 and 2 that represent, respectively, nuclei at the G_0/G_1 phase of Eucalyptus and tomato leaves that were simultaneously processed, a third peak (3) (that probably had already lost some fluorescence) was observed corresponding to nuclei at the G_0/G_1 phase of the independently processed tomato leaves. In zygotic and somatic embryos this third peak was not observed. Instead, when experiments A and B were mixed an increase in the number of nuclei present in the tomato leaves peak was observed. This test reflects the presence of inhibitors in Eucalyptus field leaves that reduce PI fluorescence and that may be present in lower concentrations in other organs.

The mean values of DNA content obtained in our experiments were higher than previously reported values for this species (Table 2), although some of the latter may be less reliable because the data did not include CV values and/or statistical analysis. On the other hand, these differences may be normal among laboratories due to the use of different methodologies such as sample processing—for example, Grattapaglia and Bradshaw (1994) used lyophilised or nitrogen frozen leaves— and buffer composition and the use of different standards—for example, all investigations carried out to date on *E. globulus* have

Table 2 Estimations of nuclear DNA content for *E. globulus* Labill. using flow cytometry

Plant source	Nuclear DNA content (pg/2C)	References	Internal reference standard used
Lyophilized or frozen leaves	1.09	Grattapaglia and Bradshaw (1994)	Chicken erythrocytes (2C=2.34 pg)
Leaves	1.13	Marie and Brown (1993)	Female chicken erythrocytes (2C=2.33 pg)
Various tissues	1.13	Azmi et al. (1997)	Chicken erythrocytes (2C=2.33 pg)
Zygotic embryos	1.32	This report	*L. esculentum* nuclei (2C=1.96 pg)
Somatic embryos	1.39		
In vitro leaves	1.39		
Field leaves	1.49		

used chicken erythrocytes (Marie and Brown 1993; Grattapaglia and Bradshaw 1994; Azmi et al. 1997). Inter-laboratory differences depend on the target/standard combination and range from 4.2% for the *Arabidopsis thaliana/Rapanhus sativus* combination to 15.6% for the *Glycine max/Zea mays* combination (Dolezel et al. 1998).

Conclusion

The main objective of the investigation reported here was accomplished—we succeeded in verifying by means of flow cytometry that no major genetic instability occurred in the somatic embryos of *E. globulus* Labill. In conclusion: (1) the DNA content of *E. globulus* is higher than values previously reported for this species although this estimation (1.32–1.40 pg/2C) is the first based on the use of a plant DNA standard and the authors are aware of the constraints that methodologies involving PI staining may pose in this species; (2) homogeneity is present within the same sample source but that some heterogeneity exists between zygotic embryos and the other sample sources; (3) this heterogeneity and the decrease in PI fluorescence in leaves suggest that Eucalyptus contains extranuclear components that inhibit PI accessibility to DNA.

For all of these reasons we believe that this species, as many woody plants, has several compounds that may interfere with PI fluorescence and that the observed differences are most probably artefactual. We therefore conclude that the somatic embryogenesis methodology used did not induce major genetic changes in the somatic embryos and that our primary goal of "true-to-type" propagation was attained.

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