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TECHNICAL ARTICLE

# Two New Nuclear Isolation Buffers for Plant DNA Flow Cytometry: A Test with 37 Species

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• *Background and Aims* After the initial boom in the application of flow cytometry in plant sciences in the late 1980s and early 1990s, which was accompanied by development of many nuclear isolation buffers, only a few efforts were made to develop new buffer formulas. In this work, recent data on the performance of nuclear isolation buffers are utilized in order to develop new buffers, general purpose buffer (GPB) and woody plant buffer (WPB), for plant DNA flow cytometry.
• *Methods* GPB and WPB were used to prepare samples for flow cytometric analysis of nuclear DNA content in a set of 37 plant species that included herbaceous and woody taxa with leaf tissues differing in structure and chemical composition. The following parameters of isolated nuclei were assessed: forward and side light scatter, propidium iodide fluorescence, coefficient of variation of DNA peaks, quantity of debris background, and the number of particles released from sample tissue. The nuclear genome size of 30 selected species was also estimated using the buffer that performed better for a given species.
• *Key Results* In unproblematic species, the use of both buffers resulted in high quality samples. The analysis of samples obtained with GPB usually resulted in histograms of DNA content with higher or similar resolution than those prepared with the WPB. In more recalcitrant tissues, such as those from woody plants, WPB performed better and GPB failed to provide acceptable results in some cases. Improved resolution of DNA content histograms in comparison with previously published buffers was achieved in most of the species analysed.
• *Conclusions* WPB is a reliable buffer which is also suitable for the analysis of problematic tissues/species. Although GPB failed with some plant species, it provided high-quality DNA histograms in species from which nuclear suspensions are easy to prepare. The results indicate that even with a broad range of species, either GPB or WPB is suitable for preparation of high-quality suspensions of intact nuclei suitable for DNA flow cytometry.

**Key words:** Cytosolic compounds, flow cytometry, general purpose buffer, genome size, lysis buffers, nuclear DNA content, nuclear DNA staining, propidium iodide, woody plant buffer.

## INTRODUCTION

Since the introduction of flow cytometry to plant sciences in the 1980s, estimation of nuclear DNA content has been the major application of flow cytometry in research, breeding and production (Doležel and Bartoš, 2005). The spread of the method was encouraged by the relative simplicity of sample preparation, which typically involves mechanical homogenization of plant tissues in a nuclear isolation buffer (Galbraith *et al.*, 1983). The buffer should facilitate isolation of intact nuclei free of adhering cytoplasmic debris, maintain nuclei stability in liquid suspension and prevent their aggregation. It ought to protect nuclear DNA from degradation and provide an appropriate environment for specific and stoichiometric staining of nuclear DNA, including the minimization of negative effects of some cytosolic compounds on DNA staining.

With the aim to fulfil these needs and to analyse nuclear DNA content with the highest resolution, many laboratories developed their own nuclear isolation buffer formulas. The current release of the FLOWER database (http://flower.web.ua.pt/) lists 27 lysis buffers with different chemical compositions (Loureiro *et al.*, 2007*a*). The usefulness of some of the buffers is difficult to judge as their performance has not been analysed thoroughly, nor have they been compared with other buffers. However, there are some exceptions and these are mainly the most popular buffers. Thus, de Laat *et al.* (1987) compared their buffer with a commercial solution, analysing the coefficient of variation (CV) of DNA peaks and the amount of debris background. Doležel *et al.* (1989) introduced the LB01 buffer by analysing the nuclear DNA content of leaves and *in vitro* cultured calli of several plant species. Arumuganathan and Earle (1991*a*) proposed a buffer containing $MgSO_4$ and used it to estimate genome size in over 100 plant species (Arumuganathan and Earle, 1991*b*). Marie and Brown (1993) tested their new buffer in approx. 70 plant species. Ulrich and Ulrich (1991) and Doležel and Göhde (1995) showed the usefulness of so-called Otto solutions (Otto, 1990) for high resolution analyses of DNA content. Finally, Pfosser *et al.* (1995) tested Tris.$MgCl_2$ buffer by evaluating the sensitivity of DNA flow cytometry to detect aneuploidy in wheat.

A systematic comparison of nuclear isolation buffers was done only recently by Loureiro *et al.* (2006*a*) who

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compared four of the most common buffers differing in chemical composition: Galbraith (Galbraith *et al.*, 1983), LB01 (Doležel *et al.*, 1989), Otto (Ulrich and Ulrich, 1991; Doležel and Göhde, 1995) and Tris.$MgCl_2$ (Pfosser *et al.*, 1995) buffers. Among others, the results confirmed the until then empirically known fact that due to the diversity of plant tissues in structure and chemical composition, no single buffer works well with every species (Doležel and Bartoš, 2005). Nonetheless, Loureiro *et al.* (2006*a*) showed that some lysis buffers consistently yielded better results than others, at least in unproblematic species in which high quality suspensions of isolated nuclei suitable for DNA flow cytometry could be prepared. The same set of buffers was evaluated while studying the effect of tannic acid, a common phenolic compound, on isolated plant nuclei and estimation of DNA content (Loureiro *et al.*, 2006*b*). The study revealed that tannic acid affected fluorescence and light scatter properties of nuclei in suspension regardless of the isolation buffer. However, the extent of the negative effect of tannic acid was different for each buffer.

Stimulated by the results of Loureiro *et al.* (2006*a*, *b*), we decided to develop nuclear isolation buffers suitable for a broader range of plants. This paper reports on two new nuclear isolation buffers: general purpose buffer (GPB) and woody plant buffer (WPB). The performance of these buffers was evaluated by analysing a wide set of plant species representing 37 taxa belonging to 24 different families, including herbaceous and woody plant species, with tissues differing in structure and chemical composition. Also the genome size of 30 out of the 37 taxa was estimated using the buffer that performed better in a given species of which ten are new estimations.

## MATERIALS AND METHODS

### *Plant material*

Plants of *Coriandrum sativum* (commercial lot), *Solanum lycopersicum* 'Stupické', *Pisum sativum* 'Ctirad' and *Vicia faba* 'Inovec' were grown from seeds (seeds from the latter three taxa were provided by the Institute of Experimental Botany, Olomouc, Czech Republic). Plants of *Festuca rothmaleri*, *Oxalis pes-caprae* and *Pterospartum tridentatum* were kindly provided by Prof. Paulo Silveira, Dr Sílvia Castro and Eng. Armando Costa (Department of Biology, University of Aveiro, Portugal), respectively. Plants of *Olea europaea*, *Quercus robur*, *Saintpaulia ionantha* and *Vitis vinifera* were available from previous studies in the Laboratory of Biotechnology and Cytomics at University of Aveiro. Plants of *Sedum burrito* were obtained from Flôr do Centro Horticultural Centre (Mira, Portugal). All plants were maintained in a greenhouse at $22 \pm 2$ ºC, with a photoperiod of 16 h and a light intensity of $530 \pm 2$ μmol $m^{-2}$ $s^{-1}$. Leaves from the remaining taxa were collected directly from field-grown individuals in Aveiro and Oporto districts, Portugal, and either analysed immediately or maintained in a refrigerator on moistened paper for a maximum of 2 d until use.

### *Sample preparation*

In each species, 40–50 mg of young leaf tissue was used for sample preparation. However, in *Sedum burrito* the quantity of leaf material required to release a sufficient number of nuclei had to be increased to approx. 500 mg (Loureiro *et al.*, 2006*a*). Nuclear suspensions were prepared according to Galbraith *et al.* (1983) using our isolation buffers, GPB and WPB (Table 1). In each case, 1 mL of buffer solution was added to a Petri dish containing the plant tissue, which was chopped using a sharp razor blade for approx. 60 s. For genome size estimations, the buffer that performed better in a particular species was chosen and leaf tissue from both the sample and DNA reference standard (Table 2) were chopped simultaneously. The resulting homogenate was filtered through an 80-μm nylon filter to remove large debris. Nuclei were stained with 50 μg $mL^{-1}$ propidium iodide (PI; Fluka, Buchs, Switzerland), and 50 μg $mL^{-1}$ RNase (Sigma, St Louis, MO, USA) was added to nuclear suspension to prevent staining of double-stranded RNA. Samples were incubated on ice and analysed within 10 min.

### *Flow cytometric analyses*

Samples were analysed with a Coulter EPICS XL (Beckman Coulter®, Hialeah, FL, USA) flow cytometer equipped with an air-cooled argon-ion laser tuned to 15 mW and operating at 488 nm. Fluorescence was collected through a 645-nm dichroic long-pass filter in reflecting mode and a 620-nm band-pass filter. The results were acquired using the SYSTEM II software (version 3·0, Beckman Coulter®). The instrument settings (amplification and sample rate) were kept constant throughout the experiment and, for the species which had been analysed in Loureiro *et al.* (2006*a*), they were the same as those used in that report.

The following parameters were analysed in each sample: forward scatter (FS) as a rough measure of particle's size, side scatter (SS) as a measure of particle's optical complexity, fluorescence intensity of PI-stained nuclei (FL), CV of $G_0/G_1$ peaks as a measure of nuclear integrity and variation in DNA staining, a debris background factor (DF) as a measure of sample quality, and a nuclear yield factor (YF) in order to compare the quantity of nuclei in suspension

TABLE 1. *Chemical composition of our nuclei isolation buffers, GPB and WPB*

| Buffer | Composition* |
|---|---|
| GPB | 0·5 mM spermine.4HCl, 30 mM sodium citrate, 20 mM MOPS, 80 mM KCl, 20 mM NaCl, 0·5 % (v/v) Triton X-100, pH 7·0 |
| WPB | 0·2 M Tris.HCl, 4 mM $MgCl_2.6H_2O$, 2 mM EDTA $Na_2{\cdot}2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % PVP-10, 1 % (v/v) Triton X-100, pH 7·5 |

* Final concentrations are given. Both buffers should be stored in aliquots at 4 °C and remain stable for up to 3 months.

MOPS, 4-Morpholinepropane sulfonate; Tris, tris-(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

TABLE 2. *Estimation of genome size in selected plant species*

| | | Genome size | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | This work | | | | Previous reports | | |
| Species | Family | 2C (pg) | 1C (Mbp)[1] | Peak CV (%) | Stand. | 2C (pg) | Method | Reference |
| *Acer negundo* | Aceraceae | 1·07 ± 0·03 | 525 | 3·14 | *S.l.* | N.D. | | |
| *Actinidia deliciosa* | Actinidaceae | 4·80 ± 0·06 | 2349 | 2·77 | *P.s.* | 4·45 | FCM:PI | Hopping, 1994 |
| | | | | | | 3·97 | FCM:PI | Ollitrault *et al.*, 1994*b* |
| *Allium triquetrum* | Alliaceae | 38·15 ± 0·38 | 18655 | 2·02 | *V.f.* | 36·30 | Fe | Jones and Rees, 1968 |
| | | | | | | 39·30 | Fe | Labani and Elkington, 1987 |
| *Aloysia triphylla* | Verbenaceae | 1·47 ± 0·01 | 720 | 2·79 | *S.l.* | N.D. | | |
| *Chamaecyparis lawsoniana* | Cupressaceae | 21·01 ± 0·15 | 10274 | 2·95 | *V.f.* | 23·05 | FCM:PI | Hizume *et al.*, 2001 |
| | | | | | | 30·10 | Fe | Ohri and Khoshoo, 1986 |
| *Citrus limon* | Rutaceae | 0·84 ± 0·005 | 409 | 3·74 | *S.l.* | 0·80 | FCM:PI | Ollitrault *et al.*, 1994*a* |
| | | | | | | 0·77, 0·80 | FCM:PI | Kayim *et al.*, 1998 |
| | | | | | | 0·77–1·15 | FCM:PI | Iannelli *et al.*, 1998 |
| | | | | | | 1·24, 1·30 | FCM:PI | Capparelli *et al.*, 2004 |
| *Citrus sinensis* | Rutaceae | 0·87 ± 0·003 | 425 | 4·02 | *S.l.* | 0·75 | FCM:PI | Ollitrault *et al.*, 1994*a* |
| | | | | | | 0·76, 0·85 | FCM:PI | Kayim *et al.*, 1998 |
| | | | | | | 0·76, 0·82 | FCM:PI | Arumuganathan and Earle, 1991*b* |
| | | | | | | 1·20 | Fe | Nagl *et al.*, 1983 |
| | | | | | | 1·24 | Fe | Guerra, 1984 |
| *Coriandrum sativum* | Apiaceae | 5·08 ± 0·10 | 2483 | 2·60 | *P.s.* | 4·10 | Fe | Olszewska and Osiecka, 1983 |
| | | | | | | 7·65, 9·55 | Fe | Das and Mallick, 1989 |
| | | | | | | 8·85, 9·45 | Fe | Chattopadhyay and Sharma, 1990 |
| *Diospyros kaki* | Ebenaceae | 5·08 ± 0·002 | 2482 | 2·27 | *P.s.* | N.D. | | |
| *Euphorbia peplus* | Euphorbiaceae | 0·69 ± 0·004 | 335 | 4·50 | *S.l.* | N.D. | | |
| *Ficus carica* | Moraceae | 0·73 ± 0·03 | 356 | 4·20 | *S.l.* | 1·41 | Fe | Ohri and Khoshoo, 1987 |
| *Forsythia* × *intermedia* | Oleaceae | 2·01 ± 0·01 | 985 | 3·22 | *G.m.* | N.D. | | |
| *Ginkgo biloba* | Ginkgoaceae | 22·85 ± 0·15 | 11172 | 2·48 | *V.f.* | 19·50 | FCM:EB | Marie and Brown, 1993 |
| | | | | | | 21·60 | FCM:PI | Barow and Meister, 2002 |
| | | | | | | 19·86 | Fe | Ohri and Khoshoo, 1986 |
| | | | | | | 19·76 | Fe | Greilhuber, 1988 |
| *Ilex aquifolium* | Aquifoliaceae | 1·93 ± 0·04 | 944 | 2·89 | *G.m.* | N.D. | | |
| *Laurus nobilis* | Lauraceae | 6·50 ± 0·09 | 3215 | 2·26 | *Z.m.* | 6·10 | FCM:PI | Zonneveld *et al.*, 2005 |
| *Magnolia* × *soulangiana* | Magnoliaceae | 9·83 ± 0·002 | 4806 | 2·43 | *Z.m.* | 11·95 | Fe | Nagl *et al.*, 1977 |
| | | | | | | 14·20 | Fe | Olszewska and Osiecka, 1983 |
| *Malus* × *domestica* | Rosaceae | 1·56 ± 0·02 | 765 | 3·39 | *S.l.* | 1·50–2·86[2] | FCM:PI | Dickson *et al.*, 1992 |
| | | | | | | 1·52–2·48[2] | FCM:PI | Tatum *et al.*, 2005 |
| *Olea europaea* ssp. *europaea* | Oleaceae | 3·24 ± 0·02 | 1583 | 3·80 | *P.s.* | 4·40, 4·52 | Fe | Rugini *et al.*, 1996 |
| | | | | | | 3·90–4·66 | Fe | Bitonti *et al.*, 1999 |
| | | | | | | 2·97–3·07 | FCM:PI | Loureiro *et al.*, 2007*b* |
| *Papaver rhoeas* | Papaveraceae | 11·00 ± 0·08 | 5378 | 1·95 | *Z.m.* | 5·20[2] | Fe | Nagl *et al.*, 1983 |
| | | | | | | 5·25[2] | Fe | Bennett and Smith, 1976 |
| | | | | | | 7·14[2] | Fe | Srivastava and Lavania, 1991 |
| *Pinus pinea* | Pinaceae | 56·09 ± 1·83 | 27429 | 3·34 | *V.f.* | 60·80 | FCM:PI | Grotkopp *et al.*, 2004 |
| *Prunus domestica* | Rosaceae | 0·66 ± 0·01 | 323 | 4·10 | *S.l.* | 0·61 | FCM:PI | Arumuganathan and Earle, 1991*b* |

*Continued*

TABLE 2. *Continued*

| Species | Family | Genome size | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | This work | | | | Previous reports | | |
| | | 2C (pg) | 1C (Mbp)[1] | Peak CV (%) | Stand. | 2C (pg) | Method | Reference |
| *Prunus persica* | Rosaceae | 0·62 ± 0·01 | 303 | 4·30 | *S.l.* | 0·54, 0·55 | FCM:PI | Arumuganathan and Earle, 1991*b* |
| | | | | | | 0·54, 0·55 | FCM:PI | Dickson *et al.*, 1992 |
| | | | | | | 0·57–0·64 | FCM:PI | Baird *et al.*, 1994 |
| *Pterospartum tridentatum* | Fabaceae | 4·64 ± 0·05 | 2269 | 2·92 | *Z.m.* | N.D. | | |
| *Pyrus communis* | Rosaceae | 1·24 ± 0·03 | 605 | 3·00 | *S.l.* | 1·03, 1·11 | FCM:PI | Arumuganathan and Earle, 1991*b* |
| | | | | | | 1·11 | FCM:PI | Dickson *et al.*, 1992 |
| *Quercus robur* | Fagaceae | 1·98 ± 0·06 | 968 | 2·88 | *G.m.* | 1·85 | FCM:EB | Favre and Brown, 1996 |
| | | | | | | 1·90 | FCM:EB | Zoldoš *et al.*, 1998 |
| *Rosa* sp. | Rosaceae | 2·46 ± 0·10 | 1204 | 2·89 | *Z.m.* | 0·78–3·04[2] | FCM:PI | Yokoya *et al.*, 2000 |
| | | | | | | 0·20–1·65[2] | FCM:PI | Dickson *et al.*, 1992 |
| | | | | | | 0·25–1·30[2] | Fe | Bennett and Smith, 1976 |
| | | | | | | 2·85[2] | Fe | Greilhuber, 1988 |
| *Saintpaulia ionantha* | Gesneriaceae | 1·50 ± 0·02 | 732 | 3·41 | *S.l.* | N.D. | | |
| *Salix babylonica* | Salicaceae | 1·61 ± 0·01 | 786 | 2·65 | *S.l.* | N.D. | | |
| *Tamarix africana* | Tamaricaceae | 3·30 ± 0·03 | 1612 | 2·66 | *Z.m.* | N.D. | | |
| *Vitis vinifera* | Vitaceae | 1·19 ± 0·02 | 583 | 2·86 | *S.l.* | 1·00 | FCM:PI | Arumuganathan and Earle, 1991*b* |
| | | | | | | 1·60 | FCM:HO | Faure and Nougarède, 1993 |
| | | | | | | 0·86–1·00 | FCM:PI | Lodhi and Reisch, 1995 |
| | | | | | | 1·17–1·26 | FCM:PI | Leal *et al.*, 2006 |

Values are given as mean and standard deviation of the mean genome size in mass values (2C, pg) and base pairs (1C, Mbp).

The coefficient of variation (Peak CV,%) of sample $G_0/G_1$ peaks and the reference standard (Stand.) used to estimate the genome size in each species (*S.l.*, *Solanum lycopersicum* 'Stupické', 2C = 1·96 pg DNA, Doležel *et al.*, 1992; *G.m.*, *Glycine max* 'Polanka', 2C = 2·50 pg DNA, Doležel *et al.*, 1994; *Z.m.*, *Zea mays* 'CE-777', 2C = 5·43 pg DNA, Lysák and Doležel, 1998; *P.s.*, *Pisum sativum* 'Ctirad', 2C = 9·09 pg DNA, Doležel *et al.*, 1998; *V.f.*, *Vicia faba* 'Inovec', 2C = 26·90 pg DNA, Doležel *et al.*, 1992) are also given. For each species, previous genome size estimations together with the used methodology (Fe, Feulgen microdensitometry; FCM, flow cytometry; PI, propidium iodide; EB, ethidium bromide; HO, Hoechst 33342) and original reference are also provided.

N.D., not determined.

[1] 1 pg = 978 Mbp (Doležel *et al.*, 2003).

[2] These values may reflect differences in the ploidy level.

independently of the amount of sample tissue used. DF and YF were determined as follows (Loureiro *et al.*, 2006*a*):

$$\mathrm{DF}(\%) = \frac{\text{Total number of particles} - \text{Total number of intact nuclei}}{\text{Total number of particles}} \times 100 \quad (1)$$

$$\mathrm{YF}(\text{nuclei s}^{-1}\text{mg}^{-1}) = \frac{\text{Total number of intact nuclei}/\text{number of seconds of run (s)}}{\text{Weight of tissue (mg)}} \quad (2)$$

Histograms of FL obtained with each buffer were overlaid using WinMDI software (Trotter, 2000; Fig. 1). In each species, five replicates per buffer were performed on three different days. In each replicate at least 5000 nuclei were analysed.

For genome size estimations, three replicates on three different days were made using the buffer that performed better in a given species. The best buffer was usually characterized by higher FL and YF and lower CV and DF, with the main evaluating parameters being the FL and the CV. The nuclear DNA content of each species was calculated according to the formula:

$$\text{2C nuclear DNA content of sample (pg)} = \frac{\text{sample } G_0/G_1 \text{ mean FL}}{\text{reference standard } G_0/G_1 \text{ mean FL}} \times \text{2C nuclear DNA content of reference standard} \quad (3)$$

Conversion of mass values into numbers of base pairs was done according to the factor 1 pg = 978 Mbp (Doležel *et al.*, 2003).

### *Statistical analyses*

Differences between both buffers for each parameter were analysed using a *t*-test (SigmaStat for Windows Version 3·1, SPSS Inc., Richmond, CA, USA).

## RESULTS

### *Performance of the nuclear isolation buffers*

Testing the two new buffers with 37 plant species revealed pronounced differences (Table 3). Out of the seven species that were analysed by Loureiro *et al.* (2006*a*) (highlighted in Table 3), the use of either buffer resulted in good DNA content histograms in *Festuca rothmaleri*, *Oxalis pes-caprae* and *Sedum burrito*, and very good histograms in *Solanum lycopersicum*, *Pisum sativum* and *Vicia faba* (Fig. 1). The only exception was *Celtis australis* in which measurable samples were only obtained with WPB (Fig. 1). Out of the remaining 30 taxa, GPB yielded acceptable histograms with CVs below 5·0 % and no detectable 'tannic acid effect' (Loureiro *et al.*, 2006*b*) in only 15 of them (i.e. 50 % success rate), while WPB worked well with all 30 species. In most of the species where GPB failed, an effect similar to the 'the tannic acid effect' was observed. This effect was first described by Loureiro *et al.* (2006*b*) and involved the occurrence of two new populations of particles on cytograms of forward scatter *vs.* side scatter, and side scatter *vs.* fluorescence (arrows in Fig. 2). The tannic acid effect resulted in fluorescence histograms with higher DF, higher CVs of $G_0/G_1$ peaks, and lower nuclear fluorescence (Fig. 2).

Whereas the GPB performed better than WPB in 57·1 % of the original set of seven species (Loureiro *et al.*, 2006*a*), in the remaining 15 taxa where both buffers worked well, it was only better in *Allium triquetrum* and *Euphorbia peplus*. The better-performing buffer was usually characterized by higher FL and YF and lower CV and DF values (Table 3).

The yield factor was the parameter where more statistically significant differences were detected between both buffers (47·6 % of the species). With the exception of *Euphorbia peplus*, the differences observed were due to a higher yield observed with WPB. Also, when statistically significant differences were observed for FL (i.e. in 42·8 % of the cases), they were due to higher fluorescence of nuclei isolated with WPB than with GPB.

In 18 species, the CVs were lower than 3·0 %; in the remaining species, CVs ranged from 3·0 % to 5·0 %. The lowest CVs were observed after analysing *Allium triquetrum* nuclei isolated with WPB (mean CV = 1·79 %). Statistical analysis revealed that in contrast to YF and FL, CVs were more homogenous between buffers, with significant differences between both buffers being only detected in four species. Major differences in CVs were detected in *Ilex aquifolium* (2·57 % and 4·10 % for WPB and GPB, respectively), and *Vitis vinifera* (3·57 % and 4·77 % for WPB and GPB, respectively). Even if significant differences were detected between the two remaining species, *Olea europaea* and *Magnolia* × *soulangeana*, the CVs were low (<3 %) with any buffer.

When evaluating the DF, significant differences between the isolation buffers were only observed in five species, *Coriandrum sativum*, *Magnolia* × *soulangeana*, *Olea europaea*, *Pisum sativum* and *Vicia faba*. With the exception of *Magnolia* × *soulangeana*, samples isolated with GPB exhibited higher debris background. Although the DF differed in *Magnolia* × *soulangeana*, *Pisum sativum* and *Vicia faba*, they were among the lowest values obtained in this study. Contrarily, the species with the highest background debris were *Tamarix africana*, *Euphorbia peplus*, *Chamaecyparis lawsoniana* and *Salix babylonica*, with values usually higher than 30 %. In most of the other species, DF usually ranged between 10 % and 20 %.

Nuclei isolated with WPB and GPB differed more in FS than in SS. Out of the 21 species where both buffers worked well, FS values were significantly different in 11 species, while only in five species was this observed for SS. *Pterospartum tridentatum*, *Prunus domestica* and *Vicia faba* were the only species with statistically significantly differences between buffers, for both parameters.

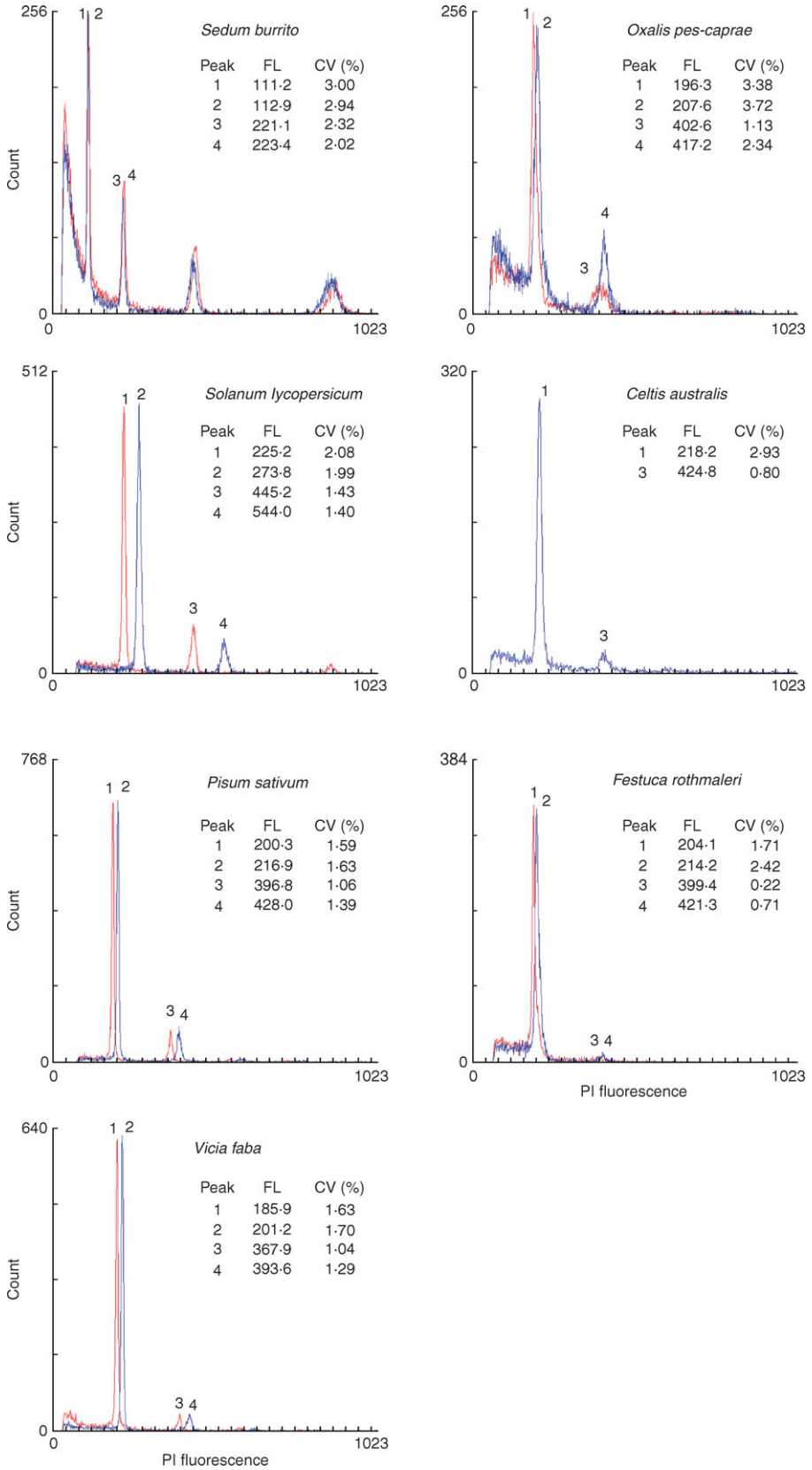


FIG. 1. Histograms of relative fluorescence intensities (PI fluorescence, channel numbers) with overlays of distributions obtained with the general purpose buffer (GPB, red) and the woody plant buffer (WPB, blue). Mean channel numbers (FL) and coefficients of variation (CV,%) of $G_0/G_1$ (peaks 1 and 2) and $G_2$ peaks (peaks 3 and 4) are given.

TABLE 3. *Flow cytometric parameters assessed in each species*

| Taxa | G.t. | Buffer | FS (channel units) | | SS (channel units) | | FL (channel units) | | CV (%) | | DF (%) | | YF (nuclei $s^{-1}$ $mg^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| *Acer negundo* L. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 29·16 | 3·05 | 26·14 | 10·24 | 234·3 | 17·2 | 3·24 | 0·35 | 16·45 | 1·62 | 0·79 | 0·27 |
| *Actinidia deliciosa* (A Chev.) C.F. Liang & A.R. Ferguson | H | GPB | 5·85[a] | 2·53 | 10·56[a] | 2·29 | 197·1[a] | 10·3 | 3·02[a] | 0·19 | 14·00[a] | 4·15 | 0·45[a] | 0·17 |
| | | **WPB** | 16·60[b] | 3·93 | 13·74[a] | 3·02 | 210·2[a] | 8·4 | 2·76[a] | 0·36 | 12·22[a] | 0·82 | 0·84[b] | 0·56 |
| *Allium triquetrum* L. | H | **GPB** | 9·02[a] | 1·49 | 5·12[a] | 1·75 | 194·9[a] | 2·6 | 1·79[a] | 0·38 | 12·47[a] | 2·39 | 0·22[a] | 0·08 |
| | | WPB | 12·88[b] | 1·53 | 4·77[a] | 1·19 | 194·2[a] | 3·2 | 2·14[a] | 0·29 | 10·51[a] | 2·08 | 0·35[b] | 0·07 |
| *Aloysia triphylla* (L'Hér.) Britton | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 10·13 | 1·13 | 6·88 | 0·78 | 203·2 | 11·6 | 2·93 | 0·37 | 25·68 | 6·13 | 0·63 | 0·11 |
| *Chamaecyparis lawsoniana* (Murr.) Parl. | W | GPB | 24·72[a] | 7·40 | 33·82[a] | 20·37 | 193·1[a] | 4·8 | 2·56[a] | 0·25 | 36·18[a] | 5·53 | 0·10[a] | 0·04 |
| | | **WPB** | 14·88[b] | 1·47 | 16·82[a] | 0·64 | 192·8[a] | 3·1 | 2·48[a] | 0·17 | 35·26[a] | 1·37 | 0·13[a] | 0·05 |
| *Celtis australis* L. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 54·25 | 16·27 | 9·59 | 3·95 | 208·0 | 13·8 | 2·99 | 0·38 | 21·31 | 4·76 | 0·30 | 0·20 |
| *Citrus limon* (L.) Burm. f. | W | GPB | 2·29[a] | 1·86 | 3·34[a] | 0·56 | 171·3[a] | 5·4 | 3·62[a] | 0·26 | 16·50[a] | 3·86 | 1·00[a] | 0·39 |
| | | **WPB** | 4·15[a] | 1·89 | 3·45[a] | 0·690 | 170·6[a] | 8·1 | 3·75[a] | 0·30 | 12·09[a] | 2·66 | 1·13[a] | 0·30 |
| *Citrus sinensis* (L.) Osbeck | W | GPB | 1·44[a] | 2·05 | 6·00[a] | 3·90 | 174·9[a] | 7·0 | 3·75[a] | 0·34 | 18·00[a] | 6·44 | 0·89[a] | 0·26 |
| | | **WPB** | 4·36[a] | 2·14 | 10·83[a] | 3·13 | 192·1[b] | 14·6 | 4·29[a] | 1·25 | 17·28[a] | 6·57 | 1·30[b] | 0·24 |
| *Coriandrum sativum* L. | H | GPB | 19·52[a] | 2·58 | 18·90[a] | 4·26 | 206·1[a] | 4·3 | 2·69[a] | 0·70 | 26·56[a] | 9·43 | 0·89[a] | 0·40 |
| | | **WPB** | 18·92[a] | 6·04 | 29·58[b] | 7·99 | 199·8[a] | 7·0 | 2·13[a] | 0·12 | 12·84[b] | 1·48 | 2·16[b] | 0·72 |
| *Diospyros kaki* L. f. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 21·28 | 6·42 | 13·96 | 6·41 | 205·7 | 4·8 | 2·09 | 0·20 | 26·36 | 3·92 | 0·68 | 0·24 |
| *Euphorbia peplus* L. | H | **GPB** | 15·10[a] | 3·49 | 15·36[a] | 6·50 | 202·2[a] | 3·0 | 3·66[a] | 0·13 | 36·12[a] | 4·50 | 2·74[a] | 0·76 |
| | | WPB | 9·72[b] | 0·93 | 12·26[a] | 1·95 | 221·1[b] | 4·9 | 4·00[a] | 0·58 | 38·80[a] | 2·43 | 2·11[b] | 0·41 |
| *Festuca rothmaleri* (Litard.) Markgr.-Dann. | H | **GPB** | 12·76[a] | 2·20 | 7·91[a] | 1·02 | 205·4[a] | 14·7 | 2·59[a] | 0·60 | 9·67[a] | 1·97 | 0·21[a] | 0·15 |
| | | WPB | 15·52[a] | 6·12 | 15·03[b] | 3·50 | 209·5[a] | 8·7 | 3·25[a] | 0·75 | 10·33[a] | 5·46 | 0·59[b] | 0·35 |
| *Ficus carica* L. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 20·60 | 5·00 | 8·77 | 3·49 | 214·9 | 4·6 | 4·16 | 0·31 | 26·98 | 2·16 | 0·46 | 0·11 |
| *Forsythia* × *intermedia* Zabel | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 44·42 | 2·31 | 26·62 | 8·78 | 198·2 | 10·7 | 2·70 | 0·36 | 10·00 | 1·01 | 1·07 | 0·34 |
| *Ginkgo biloba* L. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 10·84 | 2·14 | 20·42 | 4·35 | 196·6 | 8·8 | 2·35 | 0·39 | 19·98 | 5·06 | 0·32 | 0·22 |
| *Ilex aquifolium* L. | W | GPB | 15·03[a] | 1·56 | 20·70[a] | 6·29 | 194·0[a] | 19·9 | 4·10[a] | 0·91 | 18·48[a] | 4·64 | 1·01[a] | 0·72 |
| | | **WPB** | 9·50[b] | 1·65 | 22·32[a] | 15·58 | 271·4[b] | 13·6 | 2·57[b] | 0·34 | 19·16[a] | 2·62 | 1·20[a] | 0·63 |
| *Laurus nobilis* L. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 19·88 | 7·22 | 3·65 | 1·44 | 235·5 | 5·4 | 2·35 | 0·60 | 25·78 | 4·96 | 0·84 | 0·56 |
| *Magnolia* × *soulangeana* Soul.-Bod. | W | GPB | 28·60[a] | 2·33 | 22·13[a] | 3·92 | 141·3[a] | 9·5 | 2·90[a] | 0·81 | 4·13[a] | 1·25 | 0·90[a] | 0·18 |
| | | **WPB** | 28·58[a] | 2·35 | 24·60[a] | 2·13 | 199·5[b] | 4·0 | 1·80[b] | 0·12 | 9·26[b] | 2·38 | 0·80[a] | 0·22 |
| *Malus* × *domestica* (Borkh.) Borkh. | W | GPB | 8·83[a] | 2·45 | 26·94[a] | 13·23 | 202·8[a] | 2·3 | 3·70[a] | 0·66 | 17·98[a] | 0·34 | 1·10[a] | 0·38 |
| | | **WPB** | 9·84[a] | 2·07 | 15·14[a] | 2·30 | 214·2[b] | 5·1 | 3·24[a] | 0·46 | 16·82[a] | 3·80 | 1·89[a] | 0·82 |
| *Olea europaea* L. ssp. *europaea* | W | GPB | 16·56[a] | 4·37 | 11·26[a] | 4·03 | 178·1[a] | 10·5 | 2·97[a] | 0·56 | 23·72[a] | 3·02 | 0·16[a] | 0·06 |
| | | **WPB** | 12·38[a] | 0·97 | 12·22[a] | 1·66 | 210·3[b] | 5·0 | 2·18[b] | 0·26 | 17·28[b] | 1·30 | 0·33[b] | 0·10 |
| *Oxalis pes-caprae* L. | H | **GPB** | 54·50[a] | 19·49 | 9·00[a] | 5·10 | 198·7[a] | 11·7 | 3·29[a] | 0·29 | 8·94[a] | 2·48 | 0·68[a] | 0·16 |
| | | WPB | 66·19[a] | 34·88 | 11·34[a] | 5·47 | 206·3[a] | 5·0 | 3·80[a] | 1·06 | 11·94[a] | 3·99 | 0·64[a] | 0·15 |

*Continued*

TABLE 3. *Continued*

| Taxa | G.t. | Buffer | FS (channel units) Mean | FS (channel units) SD | SS (channel units) Mean | SS (channel units) SD | FL (channel units) Mean | FL (channel units) SD | CV (%) Mean | CV (%) SD | DF (%) Mean | DF (%) SD | YF (nuclei $s^{-1}$ $mg^{-1}$) Mean | YF (nuclei $s^{-1}$ $mg^{-1}$) SD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Papaver rhoeas* L. | H | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 19·98 | 3·33 | 20·28 | 6·26 | 199·2 | 10·1 | 2·65 | 0·53 | 23·00 | 14·39 | 0·24 | 0·10 |
| *Pinus pinea* L. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 57·58 | 10·57 | 98·14 | 28·47 | 185·6 | 12·4 | 3·09 | 0·30 | 22·88 | 5·23 | 0·03 | 0·02 |
| *Pisum sativum* L. | H | **GPB** | 12·59[a] | 2·43 | 4·50[a] | 2·43 | 185·1[a] | 4·2 | 1·79[a] | 0·23 | 11·98[a] | 2·62 | 0·62[a] | 0·23 |
| | | WPB | 32·34[b] | 2·95 | 6·49[a] | 2·95 | 195·5[b] | 5·7 | 1·92[a] | 0·18 | 7·39[b] | 1·21 | 1·15[b] | 0·28 |
| *Prunus domestica* L. | W | GPB | 4·25[a] | 0·98 | 3·76[a] | 0·52 | 189·9[a] | 1·7 | 4·35[a] | 0·46 | 21·98[a] | 3·13 | 1·64[a] | 0·38 |
| | | **WPB** | 10·45[b] | 2·27 | 6·04[b] | 1·38 | 204·6[a] | 5·0 | 4·24[a] | 0·53 | 22·98[a] | 4·76 | 1·82[a] | 0·42 |
| *Prunus persica* (L.) Batsch | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 8·87 | 2·12 | 8·38 | 2·16 | 219·0 | 8·1 | 4·91 | 0·70 | 21·22 | 4·98 | 1·31 | 0·46 |
| *Pterospartum tridentatum* (L.) Willk. | H | GPB | 31·84[a] | 12·80 | 45·86[a] | 15·99 | 196·8[a] | 6·9 | 3·25[a] | 0·76 | 29·40[a] | 2·18 | 1·18[a] | 0·78 |
| | | **WPB** | 12·38[b] | 2·66 | 13·82[b] | 1·63 | 201·5[a] | 5·9 | 2·71[a] | 0·32 | 29·38[a] | 3·31 | 0·87[a] | 0·64 |
| *Pyrus communis* L. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 8·26 | 2·98 | 14·59 | 3·96 | 203·0 | 5·1 | 3·20 | 0·36 | 18·26 | 3·24 | 1·66 | 0·23 |
| *Quercus robur* L. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 13·96 | 1·75 | 19·86 | 0·90 | 212·7 | 8·3 | 2·76 | 0·75 | 26·64 | 5·19 | 0·56 | 0·41 |
| *Rosa* L. sp. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 20·44 | 3·99 | 12·17 | 4·41 | 200·1 | 5·5 | 2·46 | 0·35 | 18·98 | 2·49 | 1·52 | 0·58 |
| *Saintpaulia ionantha* Wendl. | H | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 12·25 | 1·76 | 22·76 | 3·89 | 204·7 | 5·2 | 3·42 | 0·31 | 22·32 | 1·99 | 0·47 | 0·17 |
| *Salix babylonica* L. | W | GPB | 10·80[a] | 3·91 | 8·62[a] | 4·98 | 192·9[a] | 9·0 | 3·45[a] | 0·18 | 32·52[a] | 3·86 | 1·30[a] | 0·46 |
| | | **WPB** | 6·53[b] | 0·54 | 4·24[a] | 1·91 | 194·0[a] | 7·6 | 3·17[a] | 0·43 | 26·24[a] | 5·26 | 1·53[a] | 0·88 |
| *Sedum burrito* R. Moran | S | **GPB** | 8·34[a] | 1·90 | 0·55[a] | 0·34 | 114·0[a] | 3·2 | 3·00[a] | 0·50 | 54·64[a] | 6·94 | 0·10[a] | 0·03 |
| | | WPB | 12·81[b] | 0·92 | 0·58[a] | 0·16 | 113·1[a] | 4·2 | 3·24[a] | 0·32 | 49·96[a] | 11·97 | 0·09[a] | 0·04 |
| *Solanum lycopersicum* L. | H | GPB | 7·61[a] | 1·10 | 1·14[a] | 0·40 | 232·9[a] | 6·7 | 2·31[a] | 0·49 | 15·16[a] | 1·85 | 1·00[a] | 0·46 |
| | | **WPB** | 11·53[b] | 1·30 | 1·72[a] | 0·78 | 264·8[b] | 7·9 | 2·23[a] | 0·14 | 14·36[a] | 1·50 | 1·33[a] | 0·40 |
| *Tamarix africana* Poir. | W | GPB | – | – | – | – | – | – | – | – | – | – | – | – |
| | | **WPB** | 26·60 | 3·11 | 22·88 | 4·91 | 208·8 | 5·5 | 2·75 | 0·28 | 39·00 | 4·46 | 0·96 | 0·31 |
| *Vicia faba* L. | H | GPB | 36·84[a] | 7·26 | 4·46[a] | 1·04 | 202·3[a] | 4·7 | 1·60[a] | 0·23 | 7·25[a] | 2·44 | 0·21[a] | 0·12 |
| | | **WPB** | 74·35[b] | 11·33 | 6·49[b] | 1·33 | 212·3[b] | 5·2 | 1·72[a] | 0·18 | 6·45[b] | 1·25 | 1·03[b] | 0·35 |
| *Vitis vinifera* L. | W | GPB | 7·33[a] | 3·09 | 3·99[a] | 1·95 | 206·6[a] | 6·2 | 4·77[a] | 0·42 | 27·46[a] | 8·23 | 0·65[a] | 0·21 |
| | | **WPB** | 4·32[a] | 0·90 | 2·67[a] | 1·44 | 213·1[a] | 8·2 | 3·57[b] | 0·20 | 21·86[a] | 6·92 | 1·29[b] | 0·49 |

Values are given as mean and standard deviation of the mean (SD) of forward scatter (FS, channel units), side scatter (SS, channel units), fluorescence (FL, channel units), coefficient of variation of $G_0/G_1$ DNA peak (CV,%), debris background factor (DF,%) and yield factor (YF,%).

Means for the same species followed by the same letter (a or b) are not statistically different according to a *t*-test at $P \leq 0{\cdot}05$.

The buffer chosen for the genome size estimations in each species is shown in bold type.

G.t., Growth type; W, woody; H, herbaceous; S, succulent; GPB, general purpose buffer; WPB, woody plant buffer.

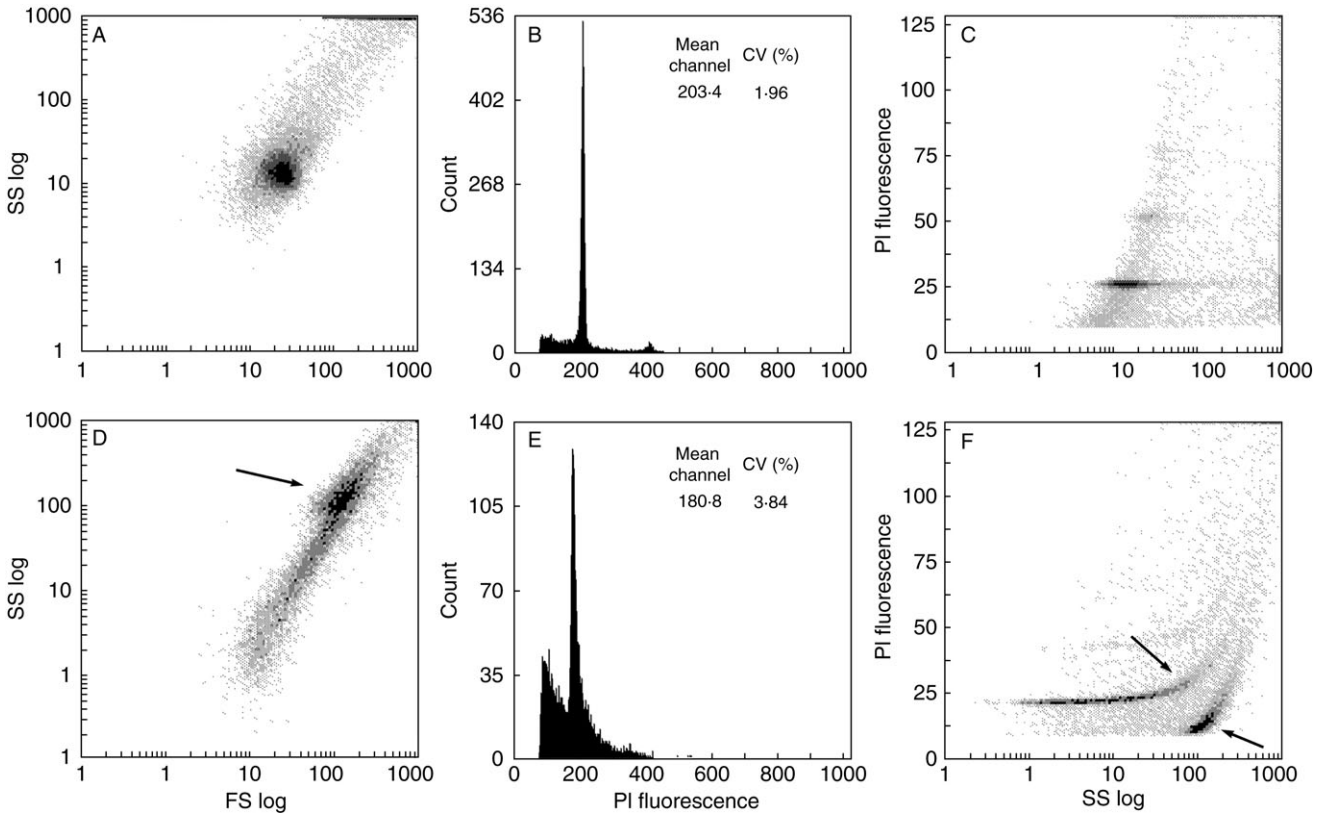


FIG. 2. Cytograms of forward scatter (logarithmic scale, FS log) *vs.* side scatter (logarithmic scale, SS log) (A, D), histograms of PI fluorescence intensity (PI fluorescence, channel numbers) (B, E), and cytograms of SS log *vs.* PI fluorescence (C, F) of nuclear suspensions of *Rosa* sp. obtained with WPB (A–C) and GPB (D–F). An effect similar to the 'tannic acid effect' (Loureiro *et al.*, 2006*b*) was observed in nuclear suspensions obtained with GPB. Arrows indicate two additional populations of particles. The first population comprises nuclei to which weakly fluorescent particles were attached (higher SS and FL values). The second population consists of clumps of weakly fluorescent particles (higher SS and lower FL values). Mean channel numbers (Mean channel) and coefficients of variation (CV,%) of $G_0/G_1$ peaks are given.

*Estimation of nuclear genome size*

Table 2 lists C-values for 30 species as determined in this study, five of which are first estimates using flow cytometry and ten are new estimates. The buffer that performed better with each species was selected to estimate its genome size.

As expected, mean CVs of DNA peaks (Table 2 and Fig. 3) were generally within the range of values obtained in the first part of the study (Table 3). Also, the standard deviations were low, with values higher than 4 % in only one species (*Rosa* sp., 4·06 %), indicating that the three replicates per species on three different days yielded homogenous estimates of nuclear DNA amount.

Plant species used in this work have a wide range of genome size, ranging from 0·62 pg/2C DNA in *Prunus persica* to 56·09 pg/2C DNA in *Pinus pinea*. Following the genome size classes (in C-values) of Soltis *et al.* (2003), most of the species studied in this work (80·0 %) belong to the 'very small' ($\leq$1·4 pg) or 'small' ($>$1·4 to $\leq$3·5 pg) genome size categories. In four species (13·3 %) 'intermediate' ($>$3·5 to $\leq$14·0 pg) genome sizes were found and only two species (6·7 %) are characterized by 'large' ($>$14·0 to $\leq$35·0 pg) or 'very large' ($>$35·0 pg) genomes. While in some species our assessments were in close agreement with previous reports, considerable differences were observed in other cases with most of the discrepancies concerning the results obtained with Feulgen microdensitometry (Table 2).

## DISCUSSION

Our recent studies (Loureiro *et al.*, 2006*a*,*b*) provided quantitative data on performance of the most popular nuclear isolation buffers and showed that none of them worked well with all species that represented different types of leaf tissues and different nuclear genome sizes. It was also clear that the chemical composition was important to cope with the negative effect of cytosolic compounds such as tannic acid. The results of these studies prompted us to develop improved buffers.

The popular nuclear isolation buffers are based on organic buffers such as MOPS (Galbraith *et al.*, 1983), Tris (Doležel *et al.*, 1989; Pfosser *et al.*, 1995) and 4-(hydroxymethyl) piperazine-1-ethanesulfonic acid (HEPES) (de Laat *et al.*, 1987; Arumuganathan and Earle, 1991*a*) that stabilize pH of the solution and keep nuclei in an intact or even sub-vital state (Greilhuber *et al.*, 2007). Non-ionic detergents, such as Triton X-100 and Tween 20, are used to facilitate the release of nuclei from cells and prevent nuclei clumping and attachment of debris, while the nuclear chromatin is stabilized by $Mg^{2+}$ (Galbraith *et al.*, 1983; Arumuganathan

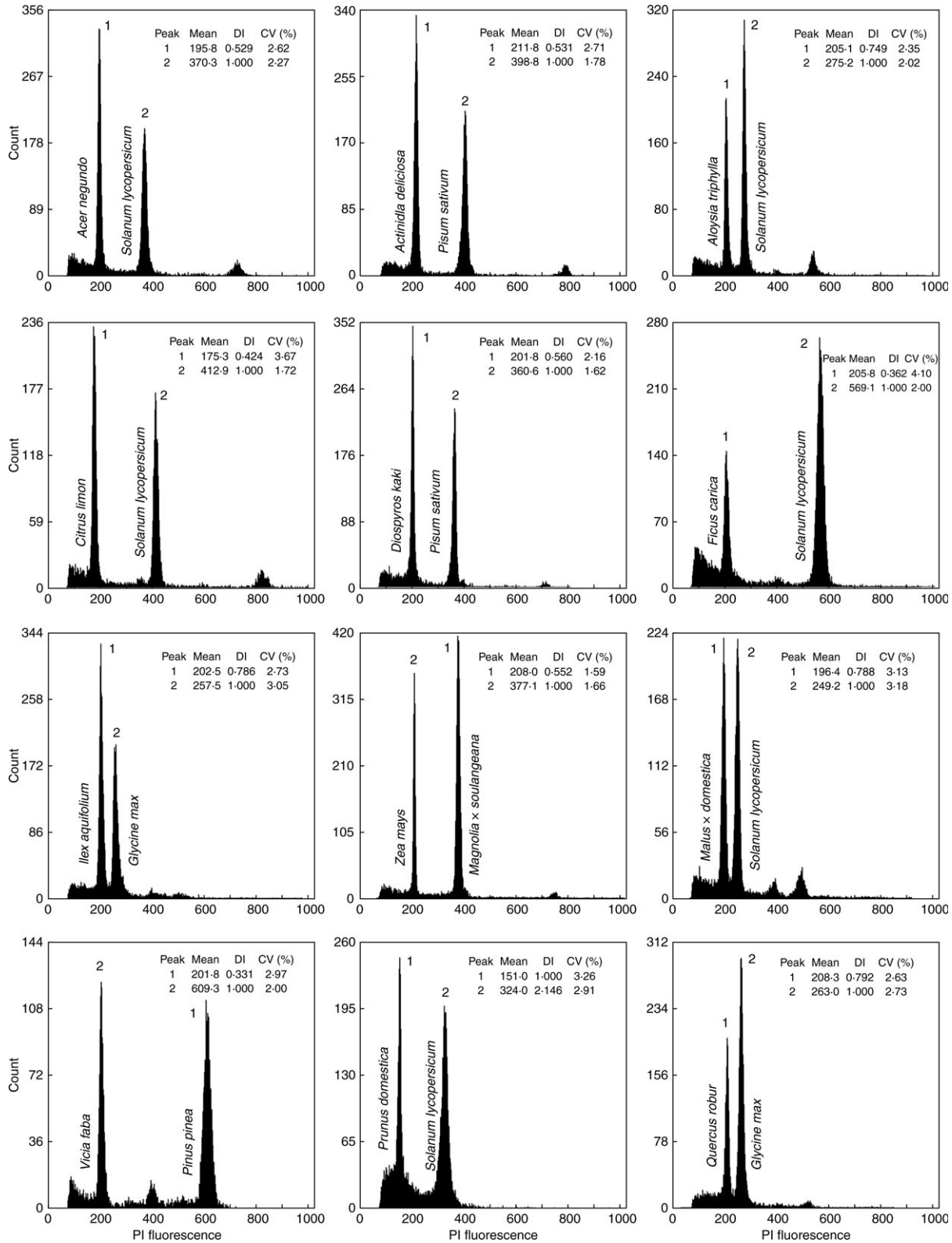


FIG. 3. Histograms of relative fluorescence intensities (PI fluorescence, channel numbers) obtained after simultaneous analysis of nuclei isolated from sample (peak 1) and internal reference standard (peak 2) using the buffer that performed better (see Table 3). The following reference standards were used: *Solanum lycopersicum* 'Stupické' (2C = 1·96 pg DNA) (A, C, D, F, I, K); *Glycine max* 'Polanka' (2C = 2·50 pg DNA) (G, L); *Zea mays* 'CE-777' (2C = 5·43 pg DNA) (H); *Pisum sativum* 'Ctirad' (2C = 9·09 pg DNA) (B, E); *Vicia faba*'Inovec' (2C = 26·90 pg DNA) (J). Mean channel number (Mean), DNA index (DI = mean channel number of sample/mean channel number of internal reference standard), and coefficients of variation (CV,%) of $G_0/G_1$ peaks are given.

and Earle, 1991*a*) or spermine (Doležel *et al.*, 1989). In some buffers, chelating agents (e.g. EDTA, sodium citrate) are added to bind divalent cations, which serve as cofactors of DNases; inorganic salts (e.g. KCl, NaCl) are used to achieve proper ionic strength (Doležel and Bartoš, 2005). Some buffers are supplemented with reducing agents such as β-mercaptoethanol, metabisulfite and dithiothreitol to prevent the action of phenolic compounds, while PVP is added to bind the phenolics kept in a reduced state (Greilhuber *et al.*, 2007).

GPB was developed considering the results of Loureiro *et al.* (2006*a*) and its chemical composition is based on that of LB01, the buffer that performed best in that study. As MOPS was shown to be a better buffer than Tris, this component was used in GPB instead of Tris at the same concentration as in the Galbraith's buffer. Moreover, the concentration of Triton X-100 in GPB was raised to 0·5 % which helped to keep isolated nuclei free from attached debris (Loureiro *et al.*, 2006*a*, *b*). The composition of WPB is based on the Tris.$MgCl_2$ buffer, which counteracts the negative effects of tannic acid better than other buffers (Loureiro *et al.*, 2006*b*). The WPB formula includes a chelating agent and inorganic salt (both from LB01 buffer) and Triton X-100 at 1·0 % (the highest concentration reported in the literature). Although a simultaneous inclusion of $MgCl_2$ and EDTA has been proposed to be counterproductive (Greilhuber *et al.*, 2007), preliminary tests did not reveal any negative effect on nuclei quality and stability, possibly due to a higher affinity of EDTA to other metals and to a sufficient concentration of free $Mg^{2+}$ in the solution necessary to stabilize the chromatin structure. Sodium metabisulfite (a reducing agent) and PVP-10 (a phenol competitor) were added to make WPB suitable for use in recalcitrant species such as woody plants with tissues rich in phenols and other secondary metabolites.

The main goal of this work was to develop new formulas for nuclei isolation buffers based on the experience with existing ones, generally using their components at the same concentrations. Systematic evaluation of the effects of different concentrations of each component was beyond the scope of this study. However, future efforts on the improvement of nuclei isolation buffers should consider this aspect.

Both buffers described in this work provided good results in many of the 37 species. However, while good samples of isolated nuclei could be prepared from any species using WPB, GPB failed in most woody plants. On the other hand, in unproblematic species GPB resulted in samples of similar or higher quality than those obtained with WPB.

Woody plants are considered recalcitrant for DNA flow cytometry as their tissues often contain cytosolic compounds that interfere with fluorescent staining of nuclear DNA (Noirot *et al.*, 2000, 2005; Loureiro *et al.*, 2006*b*). This was the case in most of the species where GPB failed and where the tannic acid effect was observed. The addition of sodium metabisulfite and PVP-10 to WPB seemed essential for its success in species where GPB failed and for the overall good performance of WPB. Sodium metabisulfite, PVP, and other compounds with similar properties (e.g. β-mercaptoethanol, ascorbic acid) had been used previously to counteract the negative effect of cytosolic compounds on nuclear fluorescence in oak (Zoldoš *et al.*, 1998), rose (Yokoya *et al.*, 2000) and olive (Loureiro *et al.*, 2007*b*). Antioxidants keep phenolics in a reduced state, enabling the reversibility of the free hydrogen bonds and its resolution by an added competitor (usually PVP-10 or PVP-40) (Greilhuber *et al.*, 2007).

Generally, GPB and WPB yielded better results than the four popular buffers evaluated by Loureiro *et al.* (2006*a*). This was evident for the CV of DNA peaks, as in most species an improvement in peak resolution was achieved. Improved nuclear fluorescence and less debris background were also observed with the new buffers. Unexpectedly, in *Celtis australis* measurable samples were only obtained with WPB. Although GPB has the same concentration of Triton X-100 as the Tris.$MgCl_2$ buffer (the best buffer for this species in Loureiro *et al.*, 2006*a*), it failed to surpass the negative effect of mucilaginous compounds. Interestingly, both GPB and WPB seem to exhibit good buffering capacity, as they were suitable for isolation of nuclei from leaf tissues of *Oxalis pes-caprae* with highly acidic cell sap (Loureiro *et al.*, 2006*a*; Castro *et al.*, 2007). The only apparent drawback of GPB and WPB was that for some species (especially in the unproblematic ones) rather low YF was observed. This was surprising as the concentration of Triton X-100 in both buffers was increased as compared with LB01 and Galbraith buffers. However, this drawback can be compensated by using a higher amount of sample tissue.

Despite their commonness and/or economical interest, until now DNA content has not been analysed by flow cytometry in 15 out of the 37 species used in this study. Moreover, in *Chamaecyparis lawsoniana* (Hizume *et al.*, 2001), *Ginkgo biloba* (Marie and Brown, 1993; Barow and Meister, 2002), *Laurus nobilis* (Zonneveld *et al.*, 2005) and *Prunus domestica* (Arumuganathan and Earle, 1991*b*), the published reports do not include DNA content histograms and data on CV, making any comparison of buffer performance impossible. For the remaining species only indirect comparisons can be made as the experimental conditions in each work are unlike the ones followed here. However, judging from published CVs and DNA content histograms, with the exception of *Pinus pinea*, the buffers described in the present work provided better (e.g. *Quercus robur*, *Malus* × *domestica*, *Diospyros kaki*) or similar (e.g. *Olea europaea*, *Vitis vinifera*) results. Particularly interesting are the high-resolution histograms obtained in *Quercus robur* using WPB. Leaves of this and other species from this genus contain phenolic compounds that interfere with fluorescent staining of nuclear DNA (Zoldoš *et al.*, 1998; Loureiro *et al.*, 2005). In order to estimate genome size in seven *Quercus* species, including *Quercus robur*, Zoldoš *et al.* (1998) modified Galbraith's buffer by adding metabisulfite. In their study, CVs ranged from 4·2 % to 6·9 % for *Quercus robur*, while in our work mean CVs below 3 % and low DF values (<20 %) were achieved. In *Pinus pinea*, GPB and WPB resulted in CVs around 3 %, i.e. higher than those obtained by Grotkopp *et al.* (2004) who used a

modified Galbraith buffer to obtain CVs typically below 2 %. It should be noted, however, that we used fine needles to prepare nuclear suspensions, while Grotkopp *et al.* (2004) used a megagametophyte, from which it is easier to prepare nuclear suspensions.

In addition to the comparison of two new nuclear isolation buffers, this work provides data on nuclear DNA content in 30 plant species. It was noted that samples prepared from species with small genome sizes (< 1·0 pg/2C DNA) exhibited higher CVs. Even in unproblematic species, a negative relationship between genome size and DF was observed (e.g. *Sedum burrito* and *Euphorbia peplus*). This was clearly due to the presence of particles other than intact nuclei in the samples (Galbraith *et al.*, 2002). These include autofluorescent chlorophyll, nuclei fragments and non-specifically stained cellular debris, which contribute to the background distribution over which nuclear DNA content distribution is superimposed. Debris attached to isolated nuclei then increases the variation in nuclei fluorescence intensity (Loureiro *et al.*, 2006*b*).

For the 20 species whose genome size had been estimated before, better agreement was observed for previous results that were obtained by flow cytometry as compared with those obtained by Feulgen microdensitometry. This was the case of *Coriandum sativum*, where our estimate of 5·08 pg DNA (2C) differs from earlier estimates using the Feulgen technique that ranged from 7·65 pg to 9·55 pg (Das and Mallick, 1989; Chattopadhyay and Sharma, 1990). Our estimates of C-values are also lower than Feulgen-based estimates for *Magnolia* × *soulangeana* and *Chamaecyparis lawsoniana* (Nagl *et al.*, 1977; Olszewska and Osiecka 1983; Ohri and Khoshoo 1986). However, our estimate for the latter species is similar to that of Hizume *et al.* (2001) who used flow cytometry. Another noteworthy difference concerns *Ficus carica* (Moraceae), in which our estimate of 2C value is only half of that determined by Feulgen microspectrophotometry (Ohri and Khoshoo, 1987). On the other hand, we determined 2C = 11·00 pg DNA for *Papaver rhoeas* (Papaveraceae), which is double that obtained by Nagl *et al.* (1983), Bennett and Smith (1976) and Srivastava and Lavania (1991) using the Feulgen procedure. In this species the differences in genome size may be explained by the occurrence of minority cytotypes (Albers and Pröbsting, 1998), with our individuals being probably tetraploid.

The differences between flow cytometry and Feulgen densitometry are rather unexpected as Doležel *et al.* (1998) showed a close agreement between both methods. However, as noted by these authors, there are many critical points of the Feulgen procedure (e.g. fixation, slide preparation and storage, acid hydrolysis) which determine its precision. Moreover, stoichiometry of the Feulgen procedure can be negatively affected by various components of cytosol (Greilhuber, 1988). Some differences between flow cytometry estimates of genome sizes in different laboratories may be explained by the use of different reference standards, sample preparation and staining protocols, and flow cytometers (Doležel *et al.*, 1998; Doležel and Bartoš, 2005).

This work reports the first estimates of genome size in ten plant species. Most of the families to which these species belong are poorly represented at the genus or species level in the plant DNA C-values database (Bennett and Leitch, 2005). The estimates for *Acer negundo* (Aceraceae, 0·75–4·05 pg/2C), *Aloysia triphylla* (Verbenaceae, 0·95–5·51 pg/2C), *Forsythia* × *intermedia* (Oleaceae, 1·95–4·66 pg/2C), *Pterospartum tridentatum* (Fabaceae, 1·03–26·50 pg/2C) and *Saintpaulia ionantha* (Gesneriaceae, 1·35–2·80 pg/2C) are at the lower limit of the known range of genome size for each family. Contrarily, our 2C-value for *Salix babylonica* is near the upper limit of the known range of 2C-values in *Salix* sp. (0·70–0·96 pg/2C for diploids and 1·62–1·72 pg/2C for tetraploids). Our estimates for *Ilex aquifolium* and *Euphorbia peplus* are the lowest so far in Aquifoliaceae (2·25–4·25 pg/2C) and in the *Euphorbia* genus (1·30–28·70 pg/2C), respectively. By contrast, our genome size estimation for *Diospyros kaki* is the highest among the three species of *Diospyros* already analysed (2·40–3·30 pg/2C). Finally, our 2C-value for *Tamarix africana* is close to that of Zonneveld *et al.* (2005) for *Tamarix tetrandra* (3·10 pg/2C), which was until now the only species analysed in Tamaricaceae.

In conclusion, the present results show that in species relatively free of cytosolic compounds, GPB provides similar and, in some cases, better results than WPB, and may be preferred. With problematic tissues, GPB usually performs less well than WPB, which is more suitable for the recalcitrant samples characterized, among other, by the presence of phenolics and mucilaginous compounds. When compared with other nuclear isolation buffers, the use of WPB results in improved histogram quality. Therefore it is recommended as the first choice when problematic tissues/species are to be analysed for DNA content using flow cytometry.

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