

TECHNICAL ARTICLE

Novel nuclei isolation buffer for flow cytometric genome size estimation of Zingiberaceae: a comparison with common isolation buffers

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• **Background and Aims** Cytological parameters such as chromosome numbers and genome sizes of plants are used routinely for studying evolutionary aspects of polyploid plants. Members of Zingiberaceae show a wide range of inter- and intrageneric variation in their reproductive habits and ploidy levels. Conventional cytological study in this group of plants is severely hampered by the presence of diverse secondary metabolites, which also affect their genome size estimation using flow cytometry. None of the several nuclei isolation buffers used in flow cytometry could be used very successfully for members of Zingiberaceae to isolate good quality nuclei from both shoot and root tissues.

• **Methods** The competency of eight nuclei isolation buffers was compared with a newly formulated buffer, MB01, in six different genera of Zingiberaceae based on the fluorescence intensity of propidium iodide-stained nuclei using flow cytometric parameters, namely coefficient of variation of the G₀/G₁ peak, debris factor and nuclei yield factor. Isolated nuclei were studied using fluorescence microscopy and bio-scanning electron microscopy to analyse stain–nuclei interaction and nuclei topology, respectively. Genome contents of 21 species belonging to these six genera were determined using MB01.

• **Key Results** Flow cytometric parameters showed significant differences among the analysed buffers. MB01 exhibited the best combination of analysed parameters; photomicrographs obtained from fluorescence and electron microscopy supported the superiority of MB01 buffer over other buffers. Among the 21 species studied, nuclear DNA contents of 14 species are reported for the first time.

• **Conclusions** Results of the present study substantiate the enhanced efficacy of MB01, compared to other buffers tested, in the generation of acceptable cytograms from all species of Zingiberaceae studied. Our study facilitates new ways of sample preparation for further flow cytometric analysis of genome size of other members belonging to this highly complex polyploid family.

Key words: bio-SEM, chromosome number, flow cytometry, fluorescence microscopy, genome size, nuclei isolation buffer, UPGMA-dendrogram, Zingiberaceae

INTRODUCTION

Since the inception of flow cytometry (FCM) and its use in plant science in 1973 by Friedrich Otto Heller (Heller, 1973), assessment of nuclear DNA content, determination of DNA ploidy level and cell cycle analysis have emerged as its most popular applications (Galbraith, 2004; Bennett and Leitch, 2005). However, this technique has been plagued by a myriad of problems, such as the presence of cell walls and thick tissues, which compromised its utility, until the pioneering work of David W. Galbraith, whose innovative homogenization technique facilitated rapid nuclei isolation (Galbraith *et al.*, 1983). Galbraith's nuclei isolation method was fast, easy and accurate, with a higher degree of resolution, and had distinct advantages over the enzymatic method of Heller (1973) and the hypotonic lysis of protoplast method (Ulrich and Ulrich, 1986; Bergounioux *et al.*, 1988, 1992; Ulrich *et al.*, 1988).

A distinct disadvantage of FCM in plants is that the diversity of plant tissues and their chemical composition necessitates the use of specific buffers for each type of plant tissue under study. Although the quest for the most effective nuclei isolation buffer

began nearly three decades ago, even today, successful preparation of nuclei suspension remains problematic in many plant species. Terpenoids, essential oils, alkaloids, phenolics, flavonoids, tannins, glycosides and saponins are the most commonly present secondary compounds in plants (Kabera *et al.*, 2014). These compounds aggravate the problem of nuclei isolation by mixing with the isolated nuclei, thus affecting sample quality and causing stoichiometric errors in DNA staining (Noirot *et al.*, 2000, 2003; Pinto *et al.*, 2004; Loureiro *et al.*, 2006a; Walker *et al.*, 2006). This contention was emphasized when Loureiro *et al.* (2006a) showed that the light scattering property of nuclei changed in *Pisum sativum* and *Zea mays* in the presence of a common phenolic compound, tannic acid.

Zingiberaceae, the pantropical family of aromatic rhizomatous perennial herbs, comprises 53 genera and over 1200 species worldwide (Kress *et al.*, 2002). Many taxonomic problems are associated with this huge group of plants. Many authors argue that the characters governing the classification and definition of the four subfamilies and six tribes are often variable and conflicting (Kress *et al.*, 2002). As the members are mostly rhizomatous with a fleshy inflorescence, it is extremely difficult to

preserve them and the lack of a type specimen frequently leads to ambiguous assignment of names and usage. The very short flowering period and the predominance of vegetative propagation have added to the confusion in the taxonomic circumscription of Zingiberaceae.

C-values and genome size (= size of the monoploid chromosome set) are the most important markers of biodiversity, which can help in infrageneric classification, species delimitation and identification of hybrids (Keller *et al.*, 1996; Buitendijk *et al.*, 1997; Bare *et al.*, 1998; Morgan *et al.*, 1998; Ohri, 1998; Thibault, 1998; Thalmann *et al.*, 2000; Zonneveld, 2001; Šiško *et al.*, 2003). Genome size also reveals plant phenology (Grime and Mowforth, 1982; Baranyi and Greilhuber, 1999) and life history (Bennett, 1972; Price and Bachmann, 1975; Nandini *et al.*, 1997) and can be correlated with environmental factors, climatic variation and geographical plant distribution (Bennett, 1976; Levin and Funderburg, 1979; Ohri and Khoshoo, 1986; Poggio *et al.*, 1989, 1998; Wakamiya *et al.*, 1993; MacGillivray and Grime, 1995; Bottini *et al.*, 2000; Hall *et al.*, 2000; Knight and Ackerly, 2002). Therefore, genome size estimation of the members of Zingiberaceae may be used as a vital parameter in resolving its taxonomic problems. It is pertinent to mention here that members of the family show huge variability in somatic chromosome numbers and variable ploidy ranges. Natural hybridization and subsequent neutralization of these crosses have also been reported in this family (Škorničková and Sabu, 2005; Škorničková *et al.*, 2007), indicating a potential role of chromosomal events in the evolution of Zingiberaceae.

Zingiberaceae is well known for the presence of different types of bioactive metabolites, namely flavonoids, phenolic acids, essential oils, oleoresin, etc. (Conell, 1970; Chan *et al.*, 2008; Santos *et al.*, 2012; Nag *et al.*, 2013; Yusuf *et al.*, 2013; Taheri *et al.*, 2014). This perhaps explains why DNA content estimates of only five genera, *Alpinia*, *Curcuma*, *Hitchenia*, *Kaempferia* and *Stahlianthus* (Bharathan *et al.*, 1994; Leong-Škorničková *et al.*, 2007), could be reported using FCM (FC:PI) while the DNA content of only three genera, *Amomum*, *Curcuma* and *Zingiber*, were reported using feulgen microdensitometry (FE) (Rai *et al.*, 1997; Das *et al.*, 1999). A review of all available literature revealed the lack of uniformity in buffer compositions in the protocols reported for nuclei extraction from different members of Zingiberaceae and that no single reported buffer could successfully isolate nuclei from both shoot and root tissues of the same species.

According to the FLOWer database (<http://flower.web.ua.pt/>; Loureiro *et al.*, 2007a) only eight of the 28 lysis buffers listed are commonly used. The buffers devised by different authors share certain common characteristics that facilitate efficient nuclei release from intact cells, ensure nuclei stability throughout the experiment, provide protection to the DNA from degradation and promote stoichiometric staining. Although the chemical constitution of the buffers varies, each nuclei isolation buffer primarily includes organic pH-stabilizing buffers (e.g. MOPS, Tris, HEPES), chromatin stabilizers (e.g. MgCl₂, MgSO₄, Spermine); divalent cation binding metal chelators (e.g. EDTA, sodium citrate) to bind divalent cations that serve as a nuclease cofactor; inorganic salts (e.g. KCl, NaCl) to achieve proper ionic strength; and non-ionic detergents (e.g. Triton X-100, Tween 20) to release nuclei, disrupt chloroplasts and

remove debris from the surface of nuclei (Coba de la Peña and Brown, 2001; Doležel and Bartoš, 2005).

In the present study, we propose a novel nuclei isolation buffer, Modified Buffer 01 (MB01) that is capable of isolating good quality nuclei in various species of Zingiberaceae that are rich in phenolic compounds and essential oils. The efficiency of MB01 was assessed against eight widely used nuclei isolation buffers: Arumuganathan and Earle (A&E), Galbraith's buffer (GB), general purpose buffer (GPB), LB01, Marie's nuclear isolation buffer (MNB), Otto buffers (OB), Tris-MgCl₂ buffer (TMB) and Tris-MgCl₂ buffer with 1 % PVP (TMP). An array of FCM parameters, namely relative fluorescence intensity of propidium iodide (PI)-stained nuclei (FL), half peak coefficient of variation (CV) of the G₀/G₁ peak, debris factor (DF, i.e. background debris) and nuclei yield factor (YF, i.e. amount of nuclei in the suspension independently of the amount of tissue used), were analysed in six species belonging to *Alpinia*, *Curcuma*, *Globba*, *Hedychium*, *Kaempferia* and *Zingiber* to evaluate the efficacy of MB01. Furthermore, MB01 was used to isolate nuclei from 21 Zingiberaceae species that were analysed using FCM for genome size estimation. Genome sizes of 14 such species are reported here for the first time. Our results affirm the proficiency of the novel buffer over conventionally used buffers in terms of the above critical FCM determinants in members of Zingiberaceae.

MATERIAL AND METHODS

Plant material

Plant rhizomes collected from different areas of India (Table 1) were cultivated for at least one year under homogeneous conditions in the Experimental Garden of the Department of Botany, University of Calcutta, West Bengal, India. Plants were identified and herbarium sheets were submitted to the University of North Bengal, West Bengal, India. For FCM estimation of nuclear DNA, *Raphanus sativus* 'Saxa', *Lycopersicon esculentum* 'Stupicke polni tyckove rane' and *Glycine max* 'Polanka' were used as internal standards, kindly supplied by Dr Jaroslav Doležel (Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Czech Republic). The 2C DNA contents of these plants are 1.11, 1.96 and 2.5 pg, respectively (Doležel *et al.*, 1992, 1994, 1998).

Chromosomal study

Mitotic chromosome numbers of these plants were studied in young root tips according to the protocol of Bhadra and Bandyopadhyay (2015, 2016). Somatic chromosome numbers of each species were confirmed by examination of at least 50 independent plates from at least five different root tips.

Sample preparation for flow cytometry

Approximately, 50 mg of young leaves ~5–10 mm long were selected from each plant. Nuclei suspensions were prepared by mechanically chopping the tissue using a sharp razor blade according to Galbraith *et al.* (1983) in a Petri dish with

TABLE 1. Place of collection and herbarium accession numbers of the plants analysed

Sl. no.	Plant	Place of collection	Accession no.
1	<i>Alpinia calcarata</i> (Haw.) Roscoe	North Bengal University, Siliguri, West Bengal	MB-LAB-A14*
2	<i>Alpinia galanga</i> (L.) Willd.	North Bengal University, Siliguri, West Bengal	NBU-09697
3	<i>Alpinia malaccensis</i> (Burm.f.) Roscoe	Botanical Survey of India, Eastern Regional Center, Umiam, Shillong, Meghalaya	MB-LAB-A13*
4	<i>Alpinia zerumbet</i> (Pers.) B.L. Burt & R.M.Sm.	Botanical Survey of India, Eastern Regional Center, Umiam, Shillong, Meghalaya	NBU-09713
5	<i>Curcuma amada</i> Roxb.	Kolkata, West Bengal	NBU-09701
6	<i>Curcuma aurantiaca</i> Zipp	Calicut University, Kozhikode, Kerala	NBU-09705
7	<i>Curcuma caesia</i> Roxb.	Experimental Garden, Dept of Botany, CU, Kolkata, West Bengal	NBU-09709
8	<i>Curcuma inodora</i> Blatt.	Calicut University, Kozhikode, Kerala	NBU-09704
9	<i>Curcuma longa</i> L.	Ram Krishna Mission, Medicinal Plant Garden, Narendrapur, Kolkata, West Bengal	NBU-09703
10	<i>Curcuma rubescens</i> Roxb.	Kalimpong, West Bengal	NBU-09699
11	<i>Globba marantina</i> L.	Experimental Garden, Dept of Botany, CU, Kolkata, West Bengal	NBU-09706
12	<i>Globba sessiliflora</i> Sims	Calicut University, Kozhikode, Kerala	NBU-09707
13	<i>Globba schomburgkii</i> Hook.f.	Calicut University, Kozhikode, Kerala	NBU-09708
14	<i>Hedychium spicatum</i> Sm.	National Bureau of Plant Genomic Resources, Bhowali Research Station, Uttarakhand	NBU-09696
15	<i>Hedychium coronarium</i> J.Koenig	Agrihorticultural Garden, Kolkata, West Bengal	MB-LAB-He6*
16	<i>Kaempferia angustifolia</i> Roscoe	North Bengal University, Siliguri, West Bengal	MB-LAB-Ka4*
17	<i>Kaempferia elegans</i> (Wall.) Baker	Kolkata, West Bengal	NBU-09711
18	<i>Kaempferia galanga</i> L.	North Bengal University, Siliguri, West Bengal	MB-LAB-Ka2a*
19	<i>Kaempferia rotunda</i> L.	National Bureau of Plant Genomic Resources, Umiam, Shillong, Meghalaya	NBU-09700
20	<i>Zingiber officinale</i> Roscoe	Ram Krishna Mission, Medicinal Plant Garden, Narendrapur, Kolkata, West Bengal	NBU-09702
21	<i>Zingiber zerumbet</i> (L.) Roscoe ex Sm.	National Bureau of Plant Genomic Resources, Bhowali Research Station, Uttarakhand	NBU-09716

*Live collection, Department of Botany, University of Calcutta.

chilled nuclei isolation buffers. Eight widely used nuclei isolation buffers were tested along with the prepared buffer (Table 2). In total, 500 μL of each nuclear suspension was taken in a 2-mL centrifuge tube and, to prevent staining of double-stranded RNA, 50 $\mu\text{g mL}^{-1}$ RNase A was added. The suspension was then filtered through a 50- μm nylon mesh to remove cell fragments and large debris.

To minimize the pipetting error and ensure uniform dye distribution, PI was added to each buffer and the PI-containing buffer solutions were added to the filtered nuclear suspension making the final volume up to 1 mL. The final concentration of PI in the suspension was 50 $\mu\text{g mL}^{-1}$. After addition of PI, the samples were incubated on ice in the dark and analysed within 10 min.

The FCM parameters were analysed in shoots of all six genera using all nine lysis buffers and three best working buffers were chosen for analysis in roots of the two most abundantly available plants, *C. amada* and *G. sessiliflora*.

To estimate the genome size, shoots of the reference standards were co-chopped with sample shoots of unknown genome size using MB01 buffer, following the same protocol. External standard nuclei suspensions were also prepared separately using the same isolation method.

Flow cytometric analysis

A BD FACSVerser (Becton Dickinson, Franklin Lakes, NJ, USA) laser flow cytometer was used to analyse PI-stained nuclei. A blue laser operating at 488 nm of 20 mW power was used for excitation. Fluorescence emission of PI (>615 nm) reaches the photomultiplier through a 560-nm long pass dichroic mirror (560 LP) and a 586/42 band pass filter. Prior to sample run, the instrument was set up with BD DNA QC Particles

(Becton Dickinson) for DNA analysis and linearity checking. Sample analysis was performed according to the method of Doležel *et al.* (2007). A medium flow rate (60 $\mu\text{L min}^{-1}$) was used and at least 5000 nuclei were analysed from each sample. The flow rate and amplification settings were kept constant throughout the experiment to compare the buffers.

A PI fluorescence area (PI-A) vs. PI fluorescence width (PI-W) dot plot was drawn on a linear scale to eliminate clumps and aggregates using qualitative gating. A PI-A histogram was drawn to view nuclear DNA content. The data were recorded and analysed using BD FACSuite software version 1.0.5.3841 (Becton Dickinson).

The relative fluorescence intensity of PI-stained nuclei (FL), half peak coefficient of variation (CV) of the G_0/G_1 peak (to evaluate nuclei integrity and staining variation), debris factor (DF, i.e. background debris) and nuclei yield factor (YF, i.e. amount of nuclei in the suspension independently of the amount of tissue used) were assessed for each sample (Loureiro *et al.*, 2006b).

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

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

The PMT voltages for forward light scatter (FS), side light scatter (SS) and fluorescence intensity of PI-stained nuclei (FL) were adjusted. A threshold was given to cut low-channel signals of cell debris or autofluorescent compounds. The number of

TABLE 2. Nuclei isolation buffers and their compositions

Buffer	Composition
Arumuganathan and Earle (Arumuganathan and Earle, 1991)	9.53 mM MgSO ₄ ·7H ₂ O; 47.67 mM KCl; 4.77 mM HEPES; 6.48 mM DTT; 0.25 % (v/v) Triton X-100; pH 8.0
Galbraith's buffer (Galbraith et al., 1983)	45 mM MgCl ₂ ; 30 mM sodium citrate; 20 mM MOPS; 0.1 % (v/v) Triton X-100; pH 7.0
GPB (Loureiro et al., 2007b)	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
LB01 (Doležel et al., 1989)	15 mM Tris; 2 mM Na ₂ EDTA; 0.5 mM spermine.4HCl; 80 mM KCl; 20 mM NaCl; 15 mM β-mercaptoethanol; 0.1 % (v/v) Triton X-100; pH 7.5
Marie's nuclear isolation buffer (Marie and Brown, 1993)	50 mM glucose; 15 mM KCl; 15 mM NaCl; 5 mM Na ₂ EDTA; 50 mM sodium citrate; 0.5 % (v/v) Tween 20; 50 mM HEPES; 0.5 % (v/v) β-mercaptoethanol; pH 7.2
MB01	20 mM MOPS; 2.5 mM Na ₂ EDTA; 0.7 mM spermine.4HCl; 80 mM KCl; 20 mM NaCl; 1 % (w/v) PVP; 0.5 % (v/v) β-mercaptoethanol; 0.2 % (v/v) Triton X-100; pH 7.4
Otto buffers*	Otto I buffer (Otto, 1990): 100 mM citric acid; 0.5 % (v/v) Tween 20 (pH approx. 2.3) Otto II buffer (Doležel and Göhde, 1995): 400 mM Na ₂ HPO ₄ ·12H ₂ O (pH approx. 8.9)
Tris-MgCl ₂ buffer (Pfosser et al., 1995)	200 mM Tris; 4 mM MgCl ₂ ·6H ₂ O; 0.5 % (v/v) Triton X-100; pH 7.5
Tris-MgCl ₂ buffer with 1 % PVP (Doležel et al., 1989)	200 mM Tris; 4 mM MgCl ₂ ·6H ₂ O; 0.5 % (v/v) Triton X-100; 1 % (w/v) PVP; pH 7.5

*The pH of these buffers was not adjusted. The nuclei isolation was done in Otto I buffer; fluorochrome was added in a mixture of Otto I and Otto II buffers (1:2)

events in G₀/G₁ and G₂ peaks as verified by the median PI fluorescence intensity value was added to obtain the nuclei yield. All experiments were repeated three times, and each sample was analysed five times in each buffer. For genome size estimation five replicates from different individuals were analysed and the 2C values of the unknown samples were calculated using the following formula:

$$\text{Sample 2C DNA content} = \frac{\text{Sample G}_0/\text{G}_1 \text{ peak mean}}{\text{Standard G}_0/\text{G}_1 \text{ peak mean}} \times \text{Standard 2C DNA content}$$

The mass values were converted to base-pair numbers by the factor 1 pg = 978 Mbp (Doležel et al., 2003).

Physical quality of isolated nuclei

To visualize the quality of isolated nuclei, the parameters shape, agglomeration property, stain–nuclei interaction and nuclear topology were analysed using fluorescence microscopy and bio-scanning electron microscopy (bio-SEM).

Fluorescence microscopy of the nuclei

Nuclei from *A. zerumbet* shoot tissue were scanned under a Leica DM IL LED (Leica, Wetzlar, Germany) fluorescence microscope to check the quality of nuclei. Nuclei were isolated from 50 mg of tissue according to the method described above using each of the nine buffers stained by PI (50 µg mL⁻¹). After incubation for 10 min, nuclei suspensions were passed through a 50-µm nylon mesh to remove cellular debris. To remove excess PI, nuclei were centrifuged at 3000 r.p.m. for 5 min and resuspended in 500 µL of the respective buffers. Nuclei were analysed in 35-mm Petri dishes and photomicrographs were taken using a Leica DFC 450C camera (Leica) using Leica Application Suite V.4.7.1 software (Leica).

bio-SEM of the nuclei

As chemical constituents of the buffers can alter the nuclei structure, nuclei isolated from a representative species, *A. zerumbet*, with each of the nine buffers were investigated by SEM. To evaluate the nuclei topology, 10 µL of each nuclei suspension isolated by the method described above was drop-casted on grease-free glass coverslips and analysed in a Zeiss EVO-MA 18 special edition (Zeiss, Oberkochen, Germany), under variable pressure (75 Pa; for biological samples) using VPSE G3 detectors and 20 kV EHT voltage.

Statistical analysis

Statistical analyses of the mean values were carried out using SigmaPlot 12.1 software. One-way analysis of variance (ANOVA) was performed to detect significant statistical differences among the buffers. The means were compared using the Holm–Sidak multiple comparison test for pairwise comparison at a 5 % probability level. Hierarchical cluster analysis was done using FL, G₀/G₁ CV, DF and YF parameters of the isolated nuclei as multistate data. A simple matching coefficient (Sneath and Sokal, 1973) was calculated using the program SIMQUAL of NTSYSpc 2.02 (Rohlf, 1999). Utilizing this data matrix, a UPGMA (unweighted pair group method with arithmetic mean) dendrogram was generated using the SAHN (sequential, agglomerative, hierarchical and nested clustering methods) module of NTSYSpc. All data are expressed as mean ± SD.

RESULTS

Comparative analysis of flow cytometric parameters

The efficiency of the buffers was evaluated depending on the best combination of high YF and FL values and low DF and G₀/G₁ peak CV values. In this study, species belonging to six different genera of Zingiberaceae were chosen to compare the nuclei isolation ability of the buffers given that each genus exhibits unique phytochemical background. With the exception of MB01, which was able to isolate a reasonable number of nuclei

TABLE 3. Flow cytometric parameters (FL, CV, DF, YF) analysed in each species from shoot tissue

Species	Buffer	FL (channel unit)	CV (%)	DF (%)	YF (nuclei s ⁻¹ mg ⁻¹)
<i>Alpinia zerumbet</i>	Arumugnathan and Earle	63.57 ^a ± 3.732	4.36 ± 0.044	77.79 ± 0.286	0.61 ^a ± 0.062
	Galbraith's buffer	61.50 ^a ± 2.436	8.20 ± 0.315	79.83 ± 0.490	0.62 ^a ± 0.046
	GPB	—	—	—	—
	LB01	96.24 ^b ± 1.138	4.64 ± 0.128	52.84 ± 0.715	1.61 ^b ± 0.208
	Marie's nuclear isolation buffer	—	—	—	—
	MB01	103.10 ± 2.443	3.87 ± 0.157	22.00 ± 0.509	6.63 ± 0.897
	Otto buffers	90.41 ± 0.584	3.06 ± 0.077	33.04 ± 2.650	2.68 ± 0.266
	Tris-MgCl ₂ buffer	—	—	—	—
	Tris-MgCl ₂ buffer with 1 % PVP	94.34 ^b ± 1.456	3.41 ± 0.073	48.20 ± 1.978	1.15 ^{a,b} ± 0.075
	<i>Curcuma amada</i>	Arumugnathan and Earle	—	—	—
Galbraith's buffer		81.77 ^a ± 1.159	5.35 ^a ± 0.098	64.47 ^a ± 1.034	1.01 ^a ± 0.180
GPB		69.70 ± 1.091	6.36 ± 0.054	80.06 ± 0.356	0.54 ± 0.020
LB01		—	—	—	—
Marie's nuclear isolation buffer		81.38 ^a ± 1.760	7.43 ± 0.071	60.62 ^b ± 0.792	1.31 ^a ± 0.080
MB01		96.95 ± 1.517	3.91 ^b ± 0.114	20.23 ± 2.336	18.43 ± 5.400
Otto buffers		92.56 ± 1.228	4.07 ^b ± 0.384	27.65 ± 0.744	11.99 ± 2.810
Tris-MgCl ₂ buffer		77.80 ± 1.128	5.48 ^a ± 0.131	62.65 ^{a,b} ± 0.937	6.69 ± 1.580
Tris-MgCl ₂ buffer with 1 % PVP		74.09 ± 2.808	6.71 ± 0.170	51.67 ± 2.357	0.61 ^a ± 0.100
<i>Globba sessiliflora</i>		Arumugnathan and Earle	146.49 ± 2.756	3.71 ^a ± 0.024	71.54 ± 1.357
	Galbraith's buffer	—	—	—	—
	GPB	—	—	—	—
	LB01	125.80 ± 0.346	3.67 ^a ± 0.032	42.89 ± 2.530	1.74 ± 0.105
	Marie's nuclear isolation buffer	—	—	—	—
	MB01	168.45 ± 1.645	3.78 ^a ± 0.182	28.70 ± 0.301	7.21 ± 0.282
	Otto buffers	96.25 ± 2.294	3.89 ^a ± 0.157	75.50 ± 0.915	1.51 ± 0.094
	Tris-MgCl ₂ buffer	139.29 ^a ± 4.419	4.85 ± 0.084	92.22 ± 0.381	0.79 ^a ± 0.041
	Tris-MgCl ₂ buffer with 1 % PVP	138.16 ^a ± 1.995	4.15 ± 0.171	89.85 ± 0.455	0.85 ^a ± 0.055
	<i>Hedychium spicatum</i>	Arumugnathan and Earle	58.64 ± 0.444	3.41 ^a ± 0.092	19.10 ± 0.503
Galbraith's buffer		—	—	—	—
GPB		49.57 ^b ± 0.697	5.64 ± 0.254	37.65 ± 0.654	3.74 ± 0.354
LB01		52.31 ^a ± 0.673	3.28 ^a ± 0.087	17.57 ± 0.572	4.34 ± 0.195
Marie's nuclear isolation buffer		43.04 ± 0.351	4.11 ^b ± 0.048	40.56 ± 0.870	2.89 ± 0.069
MB01		51.78 ^{a,b} ± 0.382	2.97 ± 0.115	11.35 ± 0.284	7.52 ± 0.172
Otto buffers		53.03 ± 0.410	4.13 ^b ± 0.076	16.05 ± 0.464	7.00 ± 0.039
Tris-MgCl ₂ buffer		—	—	—	—
Tris-MgCl ₂ buffer with 1 % PVP		4.02 ± 0.464	5.43 ^b ± 0.375	99.41 ± 0.215	0.04 ± 0.011
<i>Kaempferia elegans</i>		Arumugnathan and Earle	41.14 ± 0.698	3.19 ± 0.287	54.50 ^a ± 10.109
	Galbraith's buffer	22.49 ^a ± 11.444	4.14 ^a ± 0.105	86.68 ^b ± 1.083	0.35 ^a ± 0.225
	GPB	20.54 ^a ± 3.578	5.89 ^b ± 0.457	88.38 ± 3.677	0.31 ^a ± 0.379
	LB01	19.21 ^a ± 0.893	3.80 ± 0.429	86.14 ^b ± 10.371	0.77 ^a ± 0.114
	Marie's nuclear isolation buffer	27.14 ^a ± 0.273	5.13 ^b ± 0.705	52.92 ^a ± 14.074	3.41 ± 0.071
	MB01	70.52 ± 0.809	2.90 ± 0.400	8.62 ± 14.193	4.26 ± 0.914
	Otto buffers	55.87 ± 8.121	4.18 ^a ± 0.031	54.87 ^a ± 3.374	0.17 ^a ± 0.139
	Tris-MgCl ₂ buffer	—	—	—	—
	Tris-MgCl ₂ buffer with 1 % PVP	—	—	—	—
	<i>Zingiber zerumbet</i>	Arumugnathan and Earle	—	—	—
Galbraith's buffer		—	—	—	—
GPB		—	—	—	—
LB01		—	—	—	—
Marie's nuclear isolation buffer		—	—	—	—
MB01		83.08 ± 1.903	3.40 ± 0.181	21.00 ± 0.750	8.43 ± 0.517
Otto buffers		—	—	—	—
Tris-MgCl ₂ buffer		—	—	—	—
Tris-MgCl ₂ buffer with 1 % PVP		—	—	—	—

Values are given as mean and standard deviation of the mean (SD) of fluorescence (FL, channel units); coefficient of variation of G₀/G₁ DNA peak (CV, %); debris factor (DF, %) and nuclear yield factor (YF, nuclei s⁻¹ mg⁻¹). Means followed by the same letter (a or b) are not statistically different according to the multiple comparison Holm-Sidak test at *P* < 0.05. —, buffer failed to generate acceptable results.

(4.26–18.43 nuclei s⁻¹ mg⁻¹ of tissue) and secure well-defined histograms with DNA peaks demonstrating acceptable CV values (<5 %; Galbraith *et al.*, 2002; Table 3), no other buffer was able to isolate a good quality of nuclei from all the samples studied.

Alpinia zerumbet. GPB, MNB and TMB failed to generate acceptable results for all parameters studied (Fig. 1C, E, H). The highest FL value was observed in nuclei isolated by MB01 (Table 3), while GB yielded nuclei with the lowest FL value, which was statistically similar to A&E nuclei. The lowest G₀/

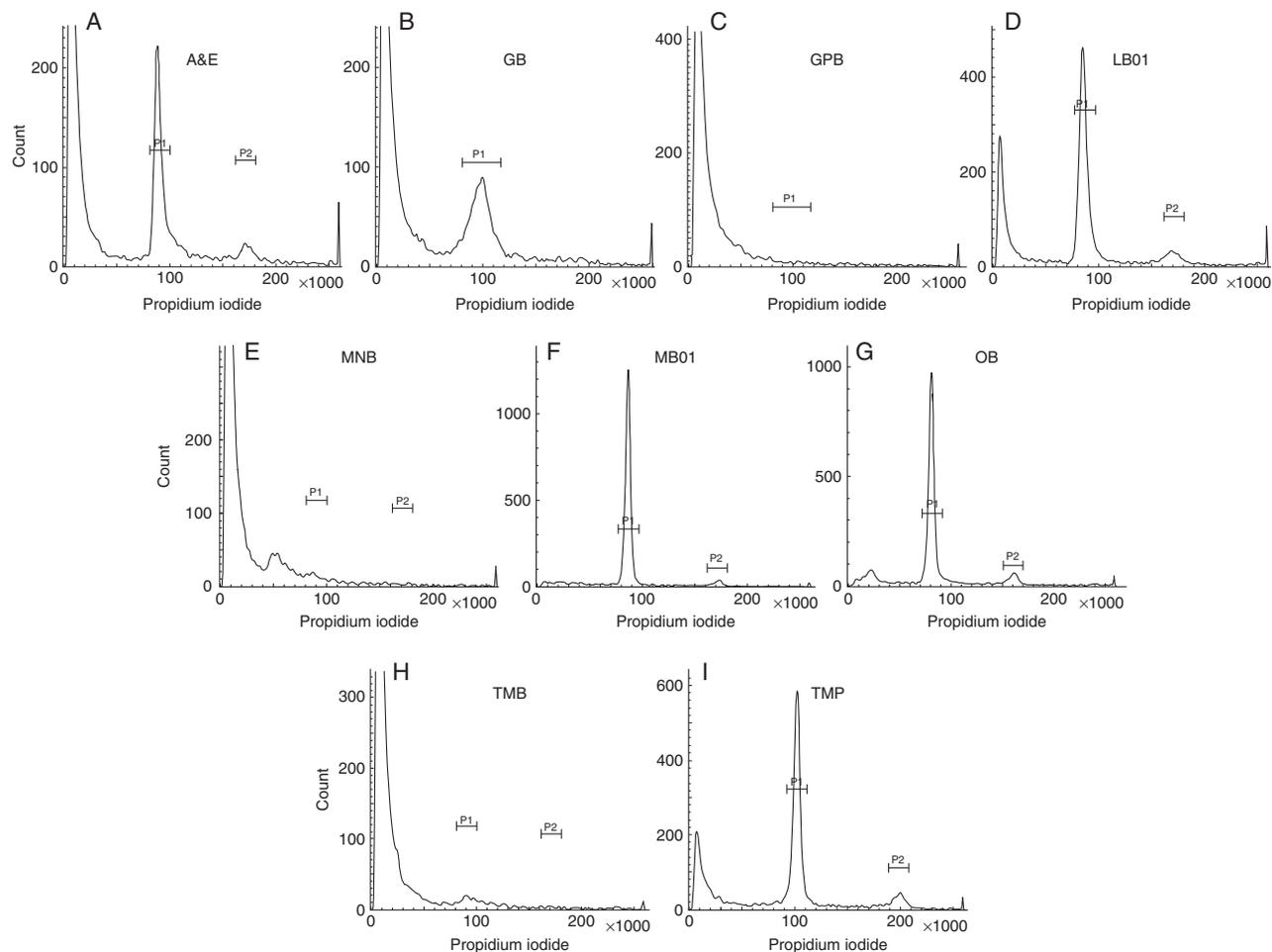


Fig. 1. FCM histograms of nuclei suspensions prepared from shoot tissues of *A. zerumbet* in different buffers. (A) Arumuganathan and Earle buffer, (B) Galbraith's buffer, (C) General purpose buffer, (D) LB01 buffer, (E) Marie's nuclear isolation buffer, (F) MB01 buffer, (G) Otto buffers, (H) Tris-MgCl₂ buffer, (I) Tris-MgCl₂ buffer with 1 % PVP.

G₁ CV was obtained from nuclei extracted using OB, which was approx. 20 % less than MB01. It was evident that MB01 significantly reduced the background factors (Table 3) and increased YF compared with other buffers (Table 3; Fig. 1F).

Curcuma amada. Although Leong-Škorničková *et al.* (2007) indicated the efficiency of OB in yielding an acceptable amount of nuclei from this species, use of MB01 gave a higher FL value and YF as well as lower G₀/G₁ CV and DF, when compared to OB, as well as all the other buffers (Table 3). Neither A&E- nor LB01-extracted nuclei generated an evaluable histogram, indicating failure of these buffers in providing the requisite amount of nuclei. GB and MNB gave statistically similar results for the FL value, while the CV of G₀/G₁ MB01-extracted nuclei was not significantly different from that of OB. MB01 showed the highest nuclei YF in all the species among the nine buffers analysed and hence indicated its efficiency.

Globba sessiliflora. Acceptable results for this species were obtained from all the buffers except GB, GPB and MNB. The lowest FL value was demonstrated by OB and the highest by

MB01 (Table 3). The nuclei suspension prepared using MB01 showed significantly low DF, i.e. 28.70 %, while LB01, OB and TMB showed DF values of 42.89, 75.50 and 92.22 %, respectively. The YF value was also higher in samples prepared with MB01 (>5 nuclei s⁻¹ mg⁻¹) compared with the other buffers used.

Hedychium spicatum. All buffers used showed mixed results except GB and TMB. Nuclei isolated with A&E showed the highest FL value, although LB01 and MB01 nuclei showed significantly homogenous results. Nuclei isolated using MB01 showed the lowest CV of G₀/G₁ (Table 3), with lowest background DF, and thus YF value was higher in MB01 (>7 nuclei s⁻¹ mg⁻¹ of tissue) compared with the other buffers.

Kaempferia elegans. Nuclei extracted using MB01 showed the best combination of parameters analysed here, whereas nuclei extracted with TMB and TMP failed to generate any estimable histogram. With regard to FL and YF values, MB01 showed acceptable results that were higher than for the other buffers, along with the lowest G₀/G₁ CV and DF values among all the

species and buffers analysed. In terms of DF value, A&E, MNB, GB and LB01 buffers showed similar results, while values for GB, LB01 and OB were similar.

Zingiber zerumbet. Only MB01 was able to isolate nuclei from this species. None of the other buffers could generate any evaluable histogram when scanned in the flow cytometer. FL and YF values were fairly high with low CV and DF values (Table 3).

Three of the best-performing buffers, i.e. MB01, OB and LB01, were also analysed in root tissues of *C. amada* and *G. sessiliflora* (Supplementary Data Fig. S1). In *C. amada*, the FL and YF values were significantly higher for MB01 than for OB and LB01, while G_0/G_1 CV and DF values were lower for MB01 than for OB and LB01. Although in *G. sessiliflora* the FL value was higher in OB-isolated nuclei than MB01 nuclei, the other parameters investigated supported the use of MB01 with a higher YF value, and lower G_0/G_1 CV and DF values than for OB buffer, while LB01 did not generate any evaluable cytogram (Fig. S1).

Quality of isolated nuclei

Both FM and bio-SEM analysis showed similar observations for each of the buffers in *A. zerumbet*, congruent with the FCM data. As GPB, MNB and TMB failed to yield a legitimate number of nuclei for generation of histograms in FCM (Fig. 1C, E, H), nothing other than cellular debris was found under the FM (Fig. 2C, E, H). SEM photomicrographs showed only debris along with salt depositions for the above buffers (Fig. 3C, E and H). Large clumps were observed in GB-isolated nuclei suspension (Fig. 2B), whereas small clumps were formed in LB01-isolated nuclei (Fig. 2D), which resulted in poor-quality FCM histograms (Fig. 1B, D). Nuclear deformities, due to inadequate ionic strength of the buffer and lack of stabilizing agents, were confirmed by SEM in GB-extracted nuclei (Fig. 3B), which impaired stain–nuclei interactions, leading to a high DF and higher G_0/G_1 CV of nuclei. A&E, MB01, OB and TMP nuclei were clearly visible under the FM. As YF values were lower and not significantly different in A&E and TMP suspensions with higher DF (Table 3), fewer nuclei were seen under FM (Fig. 2A, I). SEM images revealed deposition of salt crystals along with a few nuclei in some buffers (Fig. 3A, C and I). Although OB was a potent competitor to MB01, the number of nuclei was higher in MB01 (Table 3; Figs 1F, G and 2F, G). Nuclei isolated by MB01 also lacked deformities (Fig. 3F) and thus proper stain–nuclei interaction helped in the estimation of genome size.

Hierarchical cluster analysis

Analysis of FCM data clustered the buffers according to their effectiveness and chemical composition. The UPGMA dendrogram, generated using hierarchical cluster analysis of the buffers based on FCM parameters and nuclei isolation ability in all six species, separated MB01 buffer from the other buffer in the dendrogram, again confirming its effectiveness among the buffers analysed (Fig. 4). OB segregated next to MB01, away from the remaining buffers, which formed two sub-clusters. In the

first sub-cluster, LB01 was separate from the A&E and TMP clade, indicating its difference from these buffers and its nuclei isolation ability. In the next sub-cluster, GB, GPB and MNB yielding nearly identical results, separate from TMB.

2C nuclear DNA contents

Table 4 shows the DNA values (2C) of the species investigated, reconfirming the efficiency of MB01 buffer. Relative 2C nuclear DNA contents were estimated by comparing them with plant DNA standards (Fig. 5). In *Kaempferia* the genome content ranged from 3.43 to 8.61 pg with highest intrageneric variation of 86.04 % among the species studied. No statistical differences were found among nuclear DNA contents in different individuals of the same species. 2C nuclear DNA content varied from 1.59 pg (*C. amada*) to 8.61 pg (*K. galanga*). Plants from different genera showed variable somatic chromosome numbers, according to which the 1Cx value varied from 0.265 pg (*C. amada*, $2n = 42$, $x = 6$) to 1.945 pg (*Z. zerumbet*, $2n = 22$, $x = 2$).

DISCUSSION

The estimation of absolute DNA content depends on the preparation of a suspension of intact nuclei, while the quality of nuclei depends on the chemical constituents of nuclei isolation buffers. The aim of this study was to standardize a single nuclei isolation buffer that would be capable of isolating nuclei from all members of Zingiberaceae in comparison with eight widely used nuclei isolation buffers.

Members of Zingiberaceae contain a wide variety of secondary metabolites, which can potentially interfere with the isolated nuclei in the cell-free cytoplasmic lysate. Among them, different types of essential oils, viscous hydrophobic compounds and phenols are predominant. Moreover, nuclei suspensions are prepared by mechanical chopping, thus containing cell-wall remnants (cellulose, hemicellulose) as well as Ca^{2+} -pectate gel residues, a major component of the sticky middle lamella (Buren, 1991; Vincken *et al.*, 2003). Pectin is made up of galacturonans (homogalacturonan, rhamnogalacturonan, xylogalacturonan) mainly consisting of glucuronic acid, galacturonic acid and rhamnose (Ovodov, 1998). These components promote isolated nuclei to aggregate, and such clumping is enhanced in the presence of the sticky essential oils, making the samples unstable for FCM estimation. This agglomeration is enhanced by the hydrophobic layer that forms around nuclei and induces them to stick together.

In this study, OB and LB01 buffers performed best as they showed higher FL and YF values and lower G_0/G_1 CV and DF values than the other buffers investigated. However, their failure in *Z. zerumbet* and *C. amada* led to the formulation of MB01. Although, citric acid (the chief constituent of OB) is known to be a polymer stabilizer forming ester or amide bonds (Wing, 1996; Gaffar, 2002), also acting as a reducing agent (Robertson *et al.*, 1940; Hale, 1944), it causes increasing nuclei agglomeration in suspension with time (data is not shown) perhaps due to its pH, which is also supported by the findings of Nath *et al.* (2014). By contrast, MB01 showed better results than OB and LB01. This buffer provided better stain–nuclei interactions than other

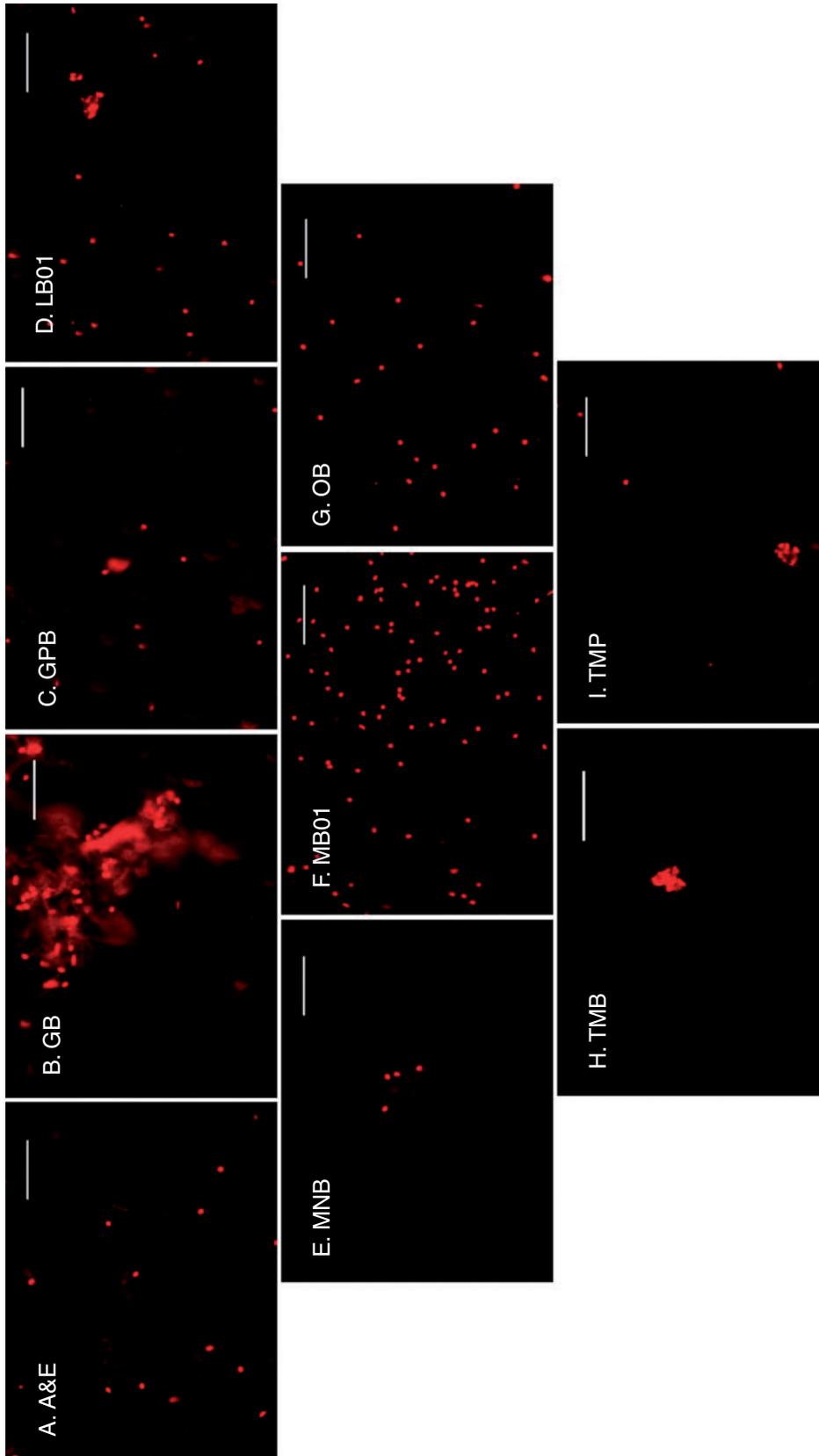


FIG. 2. *Alpinia zerumbet* shoot nuclear suspension under fluorescence microscopy in different buffers. (A) Arumuganathan and Earle buffer, (B) Galbraith's buffer, (C) General purpose buffer, (D) LB01 buffer, (E) Marie's nuclear isolation buffer, (F) MB01 buffer, (G) Otto buffers, (H) Tris-MgCl₂ buffer with 1 % PVP, (I) Tris-MgCl₂ buffer with 1 % PVP. Scale bars = 50 μ m.

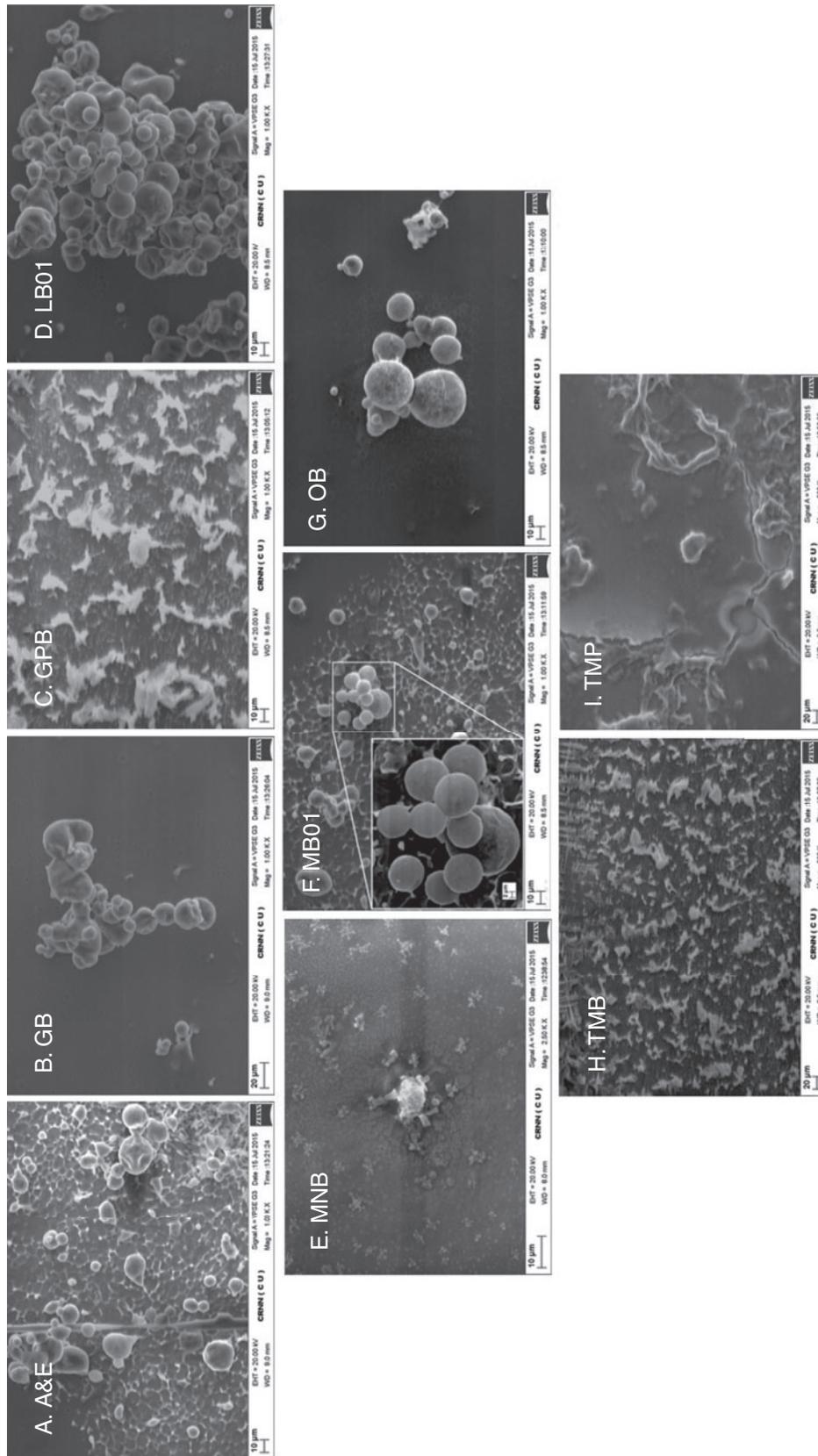


FIG. 3. Photomicrographs obtained from bio-SEM analyses of *A. zerumbet* shoot nuclear suspensions using different isolation buffers. (A) Arumuganathan and Earle buffer, (B) Galbraith's buffer, (C) General purpose buffer, (D) LB01 buffer, (E) Marie's nuclear isolation buffer, (F) MB01 buffer, (G) Otto buffers, (H) Tris-MgCl₂ buffer, (I) Tris-MgCl₂ buffer with 1 % PVP.

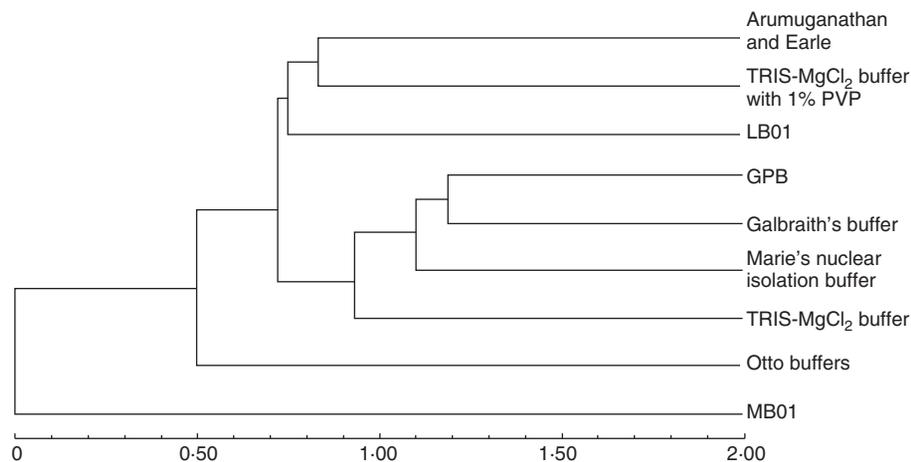


FIG. 4. Dendrogram obtained after hierarchical cluster analysis of FCM parameters in all species for all buffers studied.

TABLE 4. Somatic chromosome number ($2n$), ploidy level (x), $2C$ nuclear DNA content with standard deviation, monoploid genome size (Cx -value, determined as $2C$ DNA amount/ploidy level) expressed in DNA picograms and megabase pairs, for 21 Indian plants belonging to six genera of Zingiberaceae and previous reports; using flow cytometry (FC:PI) and Feulgen microdensitometry (FE)

Sl. no.	Taxon	Present report					Previous reports				
		$2n$	Ploidy level (x)	$2C$ DNA content (pg) \pm SD	$1Cx$ -value (pg)	$1Cx$ -value (Mbp)	$2n$	Ploidy level (x)	$2C$ DNA content (pg)	Method	Reference
1	<i>Alpinia calcarata</i>	48	4	4.43 \pm 0.015	1.108	1083.62	–	–	–	–	–
2	<i>Alpinia galanga</i>	48	4	3.86 \pm 0.018	0.965	943.77	–	–	–	–	–
3	<i>Alpinia malaccensis</i>	48	4	4.38 \pm 0.012	1.095	1070.91	–	–	–	–	–
4	<i>Alpinia zerumbet</i>	52	4	3.95 \pm 0.014	0.988	966.26	–	–	–	–	–
5	<i>Curcuma amada</i>	42	6	1.59 \pm 0.042	0.265	259.17	42	6	1.86	FC:PI	Leong-Skornickova <i>et al.</i> (2007)
							40	–	1.56	FE	Das <i>et al.</i> (1999)
6	<i>Curcuma aurantiaca</i>	42	6	2.84 \pm 0.083	0.473	462.59	42	6	2.2	FC:PI	Leong-Skornickova <i>et al.</i> (2007)
7	<i>Curcuma caesia</i>	63	9	2.52 \pm 0.013	0.280	273.84	63	9	2.82	FC:PI	Leong-Skornickova <i>et al.</i> (2007)
8	<i>Curcuma inodora</i>	42	6	2.03 \pm 0.009	0.338	330.56	42	6	2.29	FC:PI	Leong-Skornickova <i>et al.</i> (2007)
9	<i>Curcuma longa</i>	63	9	2.40 \pm 0.010	0.267	261.13	63	9	2.71	FC:PI	Leong-Skornickova <i>et al.</i> (2007)
10	<i>Curcuma rubescens</i>	63	9	2.71 \pm 0.027	0.301	294.38	42	6	1.87	FC:PI	Leong-Skornickova <i>et al.</i> (2007)
11	<i>Globba marantina</i>	52	4	3.63 \pm 0.139	0.908	888.02	–	–	–	–	–
12	<i>Globba sessiliflora</i>	52	4	3.05 \pm 0.134	0.763	746.21	–	–	–	–	–
13	<i>Globba schomburgkii</i>	48	4	3.00 \pm 0.053	0.750	733.50	–	–	–	–	–
14	<i>Hedychium spicatum</i>	68	4	2.71 \pm 0.048	0.678	663.08	–	–	–	–	–
15	<i>Hedychium coronarium</i>	34	2	2.14 \pm 0.011	1.070	1046.46	–	–	–	–	–
16	<i>Kaempferia angustifolia</i>	36	3	5.21 \pm 0.040	1.370	1339.86	–	–	–	–	–
17	<i>Kaempferia elegans</i>	22	2	3.43 \pm 0.004	1.715	1677.27	–	–	–	–	–
18	<i>Kaempferia galanga</i>	54	5	8.61 \pm 0.055	1.722	1684.12	–	–	–	–	–
19	<i>Kaempferia rotunda</i>	44	4	7.45 \pm 0.163	1.863	1822.01	–	–	–	–	–
20	<i>Zingiber officinale</i>	22	2	3.60 \pm 0.045	1.800	1760.40	22	–	12.05	FE	Rai <i>et al.</i> (1997)
21	<i>Zingiber zerumbet</i>	22	2	3.89 \pm 0.036	1.945	1902.21	–	–	–	–	–

–, no previous genome size report.

buffers, which was reflected by a higher FL value. Only in *H. spicatum* was the FL value highest in A&E nuclei. Although G_0/G_1 CV was lower in OB-isolated nuclei of *A. zerumbet* and in LB01-isolated nuclei of *G. sessiliflora* (not statistically significant) than in MB01-isolated nuclei, the MB01 values were acceptable according to the recommendations of Galbraith *et al.* (2002). A perfect nuclei suspension should not contain any cellular debris and autofluorescent compounds, as they increase background noise. High background noise is usually the cause of high CV values (Emshwiller, 2002) and lower yield of nuclei. In all genera, the chemical makeup of MB01 helped to minimize

background noise, i.e. DF, and consequently increased the overall nuclei count, i.e. YF.

Because the original composition of the buffers did not work well, the formulation of MB01 was carried out based on previous reports (Doležel *et al.*, 1989; Nath *et al.*, 2014) taking into account the properties of the constituent chemicals and the effect of pH. The pH of MB01 buffer was fixed based on Shen *et al.*'s (2013) observation that while many cellular organelles possess high pH, nuclei remain viable at pH 7.2. As MOPS has a better buffering capacity (pKa of 7.2) than Tris (pKa of 8.1) (Loureiro *et al.*, 2006b), the former was favoured over the latter

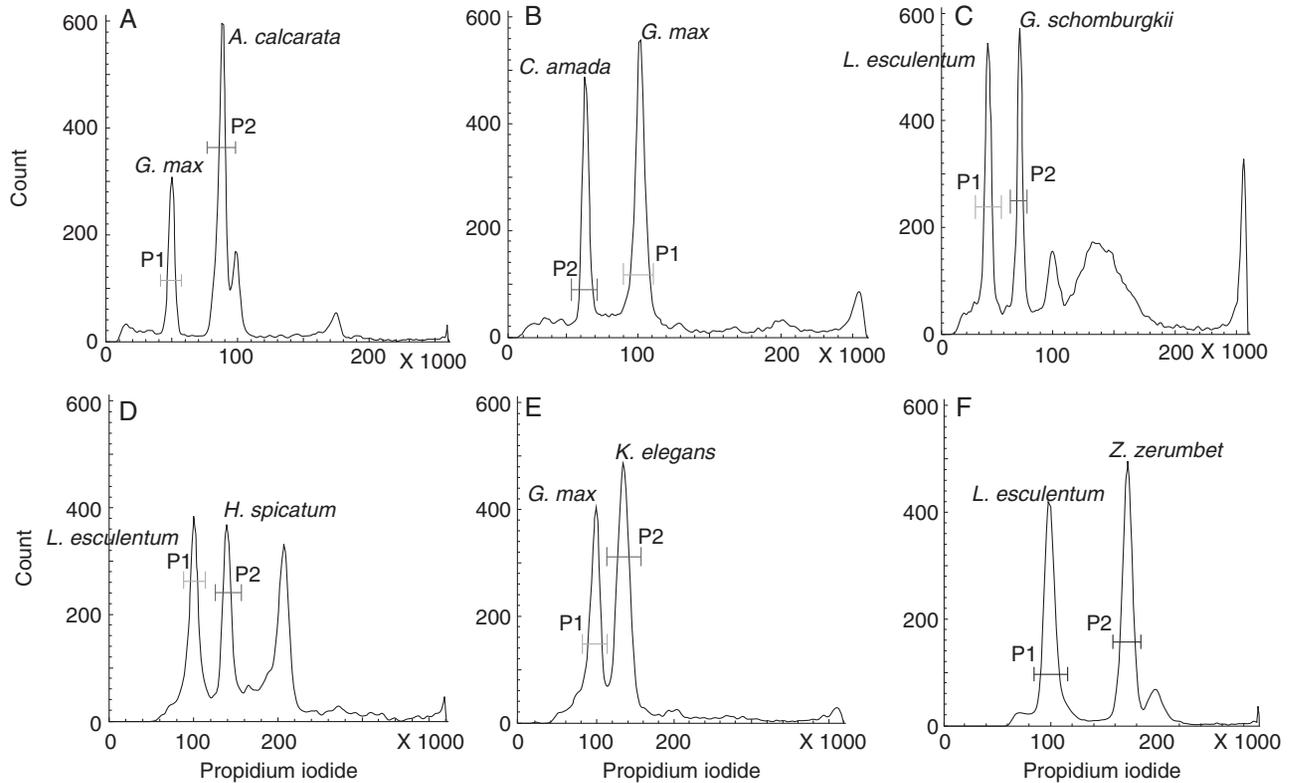


Fig. 5. Representative FCM histograms documenting genome size determination in six genera evaluated. (A) The P1 peak representing the G_0/G_1 peak of internal standard *G. max* and P2 peak representing the G_0/G_1 peak of unknown sample *A. calcarata*. (B) The P1 peak representing the G_0/G_1 peak of internal standard *G. max* and P2 peak representing the G_0/G_1 peak of unknown sample *C. amada*. (C) The P1 peak representing the G_0/G_1 peak of internal standard *L. esculentum* and P2 peak representing the G_0/G_1 peak of unknown sample *G. schomburgkii*. (D) The P1 peak representing the G_0/G_1 peak of internal standard *L. esculentum* and P2 peak representing the G_0/G_1 peak of unknown sample *H. spicatum*. (E) The P1 peak representing the G_0/G_1 peak of internal standard *G. max* and P2 peak representing the G_0/G_1 peak of unknown sample *K. elegans*. (F) The P1 peak representing the G_0/G_1 peak of internal standard *L. esculentum* and P2 peak representing the G_0/G_1 peak of unknown sample *Z. zerumbet*.

during the formulation of MB01. To increase nucleic acid stability, magnesium ions in magnesium chloride buffers (Galbraith *et al.*, 1983; Pfosser *et al.*, 1995), magnesium sulfate buffers (Arumuganathan and Earle, 1991) and glucose in MNB (Marie and Brown, 1993) was replaced with 0.7 mM spermine in MB01 at a concentration higher than in LB01 buffer (Doležel *et al.*, 1989). KCl and NaCl concentrations were kept unchanged. To protect nucleic acids from nuclease activity, EDTA was used (Doležel *et al.*, 1989; Marie and Brown, 1993) as a chelating agent for divalent cations, which acted as a nuclease cofactor (Doležel *et al.*, 1989). Previous reports had shown that PVP decreased the effect of polyphenols by changing their conformational structure, forming hydrogen bonds, and maintaining them in a reduced state (Doyle and Doyle, 1987; Greilhuber *et al.*, 2007; Loureiro *et al.*, 2007b). A higher concentration of the non-ionic detergent Triton X-100 in MB01 buffer facilitated the higher chloroplast lysis, and resulted in a decreased number of fluorescent debris particles (Coba de la Peña and Brown, 2001). β -ME, being a strong reducing agent, breaks the hydrophobic interactions and checks the activity of phenolic compounds in the presence of another competitor (e.g. PVP) (Greilhuber *et al.*, 2007). Some buffers are supplemented with sodium metabisulfite (Loureiro *et al.*, 2007b), which acts as a reducing agent. However, sodium metabisulfite is also

known to have genotoxic properties (Rencüzoğullari, 2001), which can result in erroneous genome evaluation and thus, this compound and buffers containing it were avoided in this study. Therefore, the addition of PVP with increased concentrations of β -ME and Triton X-100 in MB01 buffer was perhaps instrumental in yielding higher YF and lower DF than the other reported buffers.

The photomicrographs (Figs 2 and 3) also depicted buffer-specific nuclei quality. While MB01 showed the highest number of well-dispersed nuclei (Fig. 2F) of proper shape and size (Fig. 3F), GB showed clumped nuclei (Fig. 2B) of deformed shape (Fig. 3B). Thus, MB01 buffer provided improved nuclei quality for FCM analysis, with higher fluorescence level and yield, lower G_0/G_1 peak CV values and background debris factors. These favourable properties allowed the estimation of the genome size of the Zingiberaceae species studied here. The UPGMA dendrogram provided insight into the efficacy of the buffers by comparing them on the basis of the above parameters, which can be correlated with the respective buffer compositions. In the dendrogram, MB01 buffer separated from the remaining buffers as it performed best with its unique chemical constitution (Fig. 4).

Therefore, the formulation of MB01 buffer improved sample quality for FCM analysis of the chosen plants, and facilitated

the estimation of DNA contents of 14 new species among the investigated taxa. Although there are previous reports of the genome sizes of six species of *Curcuma*, using FC:PI (Leong-Škorničková *et al.*, 2007) and FE (Rai *et al.*, 1997), the results obtained by MB01 are comparable. Moreover, the effects of OB in other genera were largely unknown. 2C content of *Zingiber officinale* ($2n = 22$) by Rai *et al.* (1997) was reported as $12.05 \text{ pg}/2C$, which is higher than the putative value as inferred from doubling the genome size of the diploid species studied here ($2n = 22$; $3.60 \pm 0.045 \text{ pg}$). Analysis of the non-replicated monoploid genome (1Cx-value) reaffirmed the reduction in genome size as a common trend among polyploids (Leitch and Bennett, 2004). The mean 2C value of the family Zingiberaceae as given in the Kew online database (Bennett and Leitch, 2012) for C-value of angiosperms based on 39 species (one each of *Alpinia*, *Hitchenia*, *Kaempferia*, *Paracattleya*, *Stahlianthus* and *Zingiber*, and 33 *Curcuma*), is $2.79 \pm 1.7 \text{ pg}/2C$. Our estimation of novel 2C nuclear genome sizes in this study has increased the average genome size of the family to $3.18 \pm 1.8 \text{ pg}/2C$, an increase of 13.06 %.

Thus, this is the first study in which eight widely used nuclei isolation buffers were compared with a newly formulated buffer, MB01, for Zingiberaceae plant flow cytometry. The results revealed that MB01 yielded superior quality of nuclei from plants of different genera of the family from both shoot and root tissues. This buffer might be used to improve nuclei quality in other plant families rich in secondary metabolites. Nevertheless, further studies are required to gain thorough knowledge of the interaction between buffers and cellular components. Thus, using this study as a platform, it will be possible to unlock new ways of FCM sample preparation in plants to decrypt the correlation among chromosome number, ploidy and genome size.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Figure S1: FCM histograms, of nuclei suspensions prepared from root tissues of *G. sessiliflora* in different buffers. (A) LB01 buffer, (B) MB01 buffer, (C) Otto buffers.

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