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An Improved Method of Genome Size Estimation by Flow Cytometry in Five Mucilaginous Species of Hyacinthaceae

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Abstract

The family Hyacinthaceae constitutes about 900 species of bulbous geophytes usually characterized by high mucilage content. Taxonomic delimitation of Hyacinthaceae has been controversial since the time of Linnaeus due to the absence of reliable discriminating characters. Pattern of genome size variation can thus be considered as an added character to aid intergeneric and intrageneric relationship of the group. However, reports on genome size estimation by flow cytometric analysis of these plants are rare due to the presence of mucilage, which causes problem with nuclei sample preparation. To overcome this problem five reported nuclei isolation buffers were tested in *Drimia indica* of which Galbraith's buffer gave comparatively better results and was further modified by increasing pH, detergent concentration, and replacing sodium citrate by citric acid. The modified buffer enabled better sample preparation with increased yield, lesser debris, and improved DNA peak CV. The standardized buffer was used to estimate the 2C values of *Drimia indica, Drimia nagarjunae*, *Drimia wightii, Drimia coromandeliana,* and *Ledebouria revoluta* for the first time by flow cytometric analysis. This study also opens up the scope for further improvement in sample preparation for flow cytometric analysis of mucilaginous plants, which is otherwise problematic due to nuclei clumping and increased viscosity of sample.

Key terms

Hyacinthaceae; mucilage; flow cytometry; genome size; nuclei isolation; Galbraith's buffer; *Drimia indica*; *Drimia nagarjunae*; *Drimia wightii*; *Drimia coromandeliana*; *Ledebouria revoluta*

FLOW cytometry is a fast, easy, accurate system and the current preferred technique for genome size estimations and DNA ploidy analysis in plants (1). Although initially problematic due to the presence of rigid cell wall, thick tissues and interfering cytosolic components, flow cytometric analysis of plant nuclei took a leap after a rapid and convenient nuclei isolation method was established by Galbraith et al. (2) that included chopping of plant tissue in a lysis buffer. Since then many buffer formulas have been developed by different workers all containing some basic components required to maintain nuclei integrity, stability, and to reduce effect of interfering cytosolic compounds. However, even 30 years after the breakthrough paper of Galbraith et al. (2), sample preparation for DNA flow cytometry is still a problem in many recalcitrant plant species because of the presence of interfering cytosolic compounds like mucilages (3), tannic acid (4), etc.

Mucilages are polysaccharide compounds with sticky glue like texture, which make the sample viscous and attract isolated nuclei, thus result in clumping. Moreover, debris particles in the sample stick to nuclei surface (debris coatings) that result in decreased resolution of DNA content histograms. As a consequence, estimation of nuclear DNA content is seriously hampered. Thus, mucilages result in low nuclei yield and poor histograms with high coefficient of variations (CVs) of G_0/G_1 DNA

peaks in flow cytometric analysis. Loureiro et al. (4) showed that light scatter properties of nuclei were sensitive to the presence of tannic acid, a cytosolic compound in many plants and thus recommended the analysis of the same to verify the suitability of particular samples for plant DNA flow cytometry. However, the effect of mucilages on light scatter properties of nuclei is not yet known.

The family Hyacinthaceae constitutes ~900 species of deciduous bulbous geophytes distributed mainly in Mediterranean type habitats of Eurasia and sub-Saharan Africa (5–7). The taxonomic delimitation of this group has been controversial since the time of Linnaeus because of the absence of reliable discriminating characters. Hyacinthaceae was reduced to subfamily Scilloideae in the family Asparagaceae based on recent molecular phylogenetic studies (8,9) and was further subdivided into four tribes, that is, Hyacintheae, Ornithogaleae, Oziroeeae and Urgineeae based on chemotaxonomical, morphological, and molecular data (5,8,9). However, a satisfactory delimitation of genera within this group still remains doubtful (7–10).

Genome size or DNA C-value remains a key character in biology and biodiversity. C-values are increasingly useful in a phylogenetic study and genome size data in combination with other characters can contribute to intergeneric classification, taxa delimitation, or hybrid identification (11–13). Genome size shows correlations not only with different phenotypic characters at cell, tissue, and organism levels but also to a broad range of external ecological issues and environmental concerns (14–16). Thus pattern of genome size variation can be considered as an important parameter to aid intergeneric and intrageneric delimitation of the family Hyacinthaceae.

These bulbous monocots of Hyacinthaceae are characterized by the presence of copious mucilage that interferes largely with nuclei isolation thus making flow cytometric analysis difficult. As a result, there are few genome size estimates made in Hyacinthaceae using flow cytometry (17–19), although there are many estimates reported in the genome size database (20), which have been estimated by Feulgen microdensitometry.

Considering the challenges in nuclei isolation from mucilaginous species, the objectives of the present study were to develop an optimized method for flow cytometric sample preparation in mucilaginous species of Hyacinthaceae and with the use of this method estimate the genome sizes of *Drimia indica* (Roxb.) Jessop (syn. *Urginea indica*), *Drimia nagarjunae* (Hemadri & Swahari) Anand Kumar (syn. *Urginea nagarjunae*), *Drimia wightii* (Wight) Lakshmin (syn. *Urginea congesta*), *Drimia coromandeliana* Wight (*Urginea coromandeliana*), and *Ledebouria revoluta* (L. f.) Jessop (syn. *Scilla indica*). Optimization of buffer system was initially done in *Drimia indica* and then the optimized buffer was used to estimate the nuclear DNA contents of these five species.

Materials and Methods

Plant Material

Bulbs of *Drimia indica*, *Drimia nagarjunae, Drimia wightii, Drimia coromandeliana,* and *Ledebouria revoluta* were collected from different parts of India (Table 2), herbarium vouchers were prepared for each species, identified by Prof. SR Yadav, Department of Botany, Shivaji University, Kolhapur and deposited to Shivaji University Herbarium, Kolhapur. Plants are grown and are maintained in the experimental garden of Department of Botany, University of Calcutta. The standard material *Allium cepa* cv. "Alice" was kindly provided by Prof. Jaroslav Doležel, Institute of Experimental Botany, Olomouc, Czech Republic with reported 2C value of 34.89 pg (21).

Chromosome Preparation

For mitotic chromosome preparation root tips were pretreated with 8-hydroxyquinoline (2 mM): colchicine (0.1%) [1:1] at 12°C for 4½ hrs, fixed overnight in 1:3 glacial acetic acid: methanol and stored at −20°C. Root tips were transferred to 45% acetic acid for 15 min, stained in mixture of 2% aceto-orcein: 1(N) HCl (9:1) for 2 h and squashed in 45% acetic acid. Chromosome counts for each species were based on 30 metaphases/5 root tips/plant.

Sample Preparation

Approximately 100 mg young root tissue (~1–3 cm long) from two year old bulbs of *D. indica* was used for sample preparation. Leaves, bulb scales, inflorescence axis as well as older roots (>3 cm in length) were found to be unsuitable for flow cytometric sample preparation because of high mucilage content. Nuclear suspensions were prepared according to the protocol of Galbraith et al. (2), with some modifications as mentioned here. About one milliliter of nuclear suspension was recovered and filtered through a 50 μm nylon mesh to remove cell fragments and large debris. Nuclei were stained with 50 μg ml^{-1} propidium iodide (PI; Sigma) and 50 μg ml^{-1} RNase A (Sigma) was added to it to prevent staining of double stranded RNA. Samples were incubated for 60 min in dark on ice before analysis. Five reported nuclei isolation buffers tested in preliminary analyses included Galbraith's buffer (2), LB01 buffer (22), Tris-$MgCl_2$ buffer (23), Tris-$MgCl_2$ buffer supplemented with 1% polyvinylpyrrolidone (PVP; 3) and Otto's buffer (24). Among these buffers, samples prepared with Galbraith's buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.10% v/v Triton X, pH 7.0) exhibited comparatively better results and was thus chosen for further modifications which included increasing the detergent concentration and pH and replacement of sodium citrate by citric acid. Two modified buffers tested were modified Galbraith's buffer [45 mM $MgCl_2$ (Merck, Germany), 30 mM sodium citrate (Sigma), 20 mM MOPS (Sigma), 0.15% v/v Triton X (Sigma, USA), pH 8.5] and Nath- Mallick's (NM) modified Galbraith's buffer [45 mM $MgCl_2$ (Merck, Germany), 50 mM citric acid (HIMEDIA, India), 20 mM MOPS (Sigma), 0.15% v/v Triton X (Sigma), pH 8.5].

For genome size estimation, ~80 mg root tissue of sample was cochopped with 20 mg seedling of the reference standard *A. cepa* cv. "Alice" in NM modified Galbraith's buffer and the same protocol was followed. An external standard was also prepared using the same protocol.

Table 1. Flow cytometric parameters assessed for each buffer in *Drimia indica*

BUFFER	DF (%) MEAN ± SD	YF (NUCLEI S^{-1} MG^{-1}) MEAN ± SD	G_0/G_1 CV (%) MEAN ± SD	FL (CHANNEL UNITS) MEAN ± SD	FL CV (%) MEAN ± SD
LB01 buffer (19)	No peak	No peak	No peak	41.94 ± 6.42^{a}	86.77 ± 4.35^{a}
Tris-$MgCl_2$ buffer (21)	No peak	No peak	No peak	44.62 ± 3.58^{a}	$89.60 \pm 1.96^{a,b}$
Tris-$MgCl_2$ buffer with 1% PVP (22)	No peak	No peak	No peak	38.11 ± 2.05^{a}	102.82 ± 8.63^{b}
Otto's buffer (23)	78.34 ± 4.14^{b}	0.022 ± 0.008^{a}	12.46 ± 2.80^{b}	71.75 ± 3.70^{b}	$97.87 \pm 1.15^{a,b}$
Galbraith's buffer (15)	72.19 ± 7.45^{b}	0.090 ± 0.060^{a}	6.87 ± 1.56^{a}	72.50 ± 13.79^{b}	$90.26 \pm 6.42^{a,b}$
Modified Galbraith's buffer	$64.01 \pm 7.50^{a,b}$	$0.237 \pm 0.165^{a,b}$	5.67 ± 1.17^{a}	64.74 ± 9.02^{b}	84.37 ± 12.38^{a}
NM modified Galbraith's buffer	54.22 ± 11.26^{a}	0.364 ± 0.206^{b}	4.91 ± 0.72^{a}	60.17 ± 7.01^{b}	84.14 ± 11.80^{a}

Values are given as mean and standard deviation of the mean (SD) of debris factor (DF, %), nuclear yield factor (YF, nuclei s^{-1} mg^{-1}), coefficient of variation of the G_0/G_1 DNA peak (G_0/G_1 CV, %), relative fluorescence intensities of PI stained nuclei (FL, channel units) and coefficient of variation of the FL (FL-CV, %). Means followed by the same letters (a or b) are not statistically different according to Duncans Test at $P < 0.05$.

Flow Cytometric Analysis

Nuclei samples were analyzed using a BD FACS Verse flow cytometer with a 488 nm solid state laser (50 mW). Samples were run at a medium speed (60 µl min^{-1}) and data were acquired and analyzed using the BD FACS Suite software. A linear PI fluorescence area (PI-A) vs. PI fluorescence width (PI-W) plot was drawn to eliminate clumps and aggregates using qualitative gating. A PI fluorescence histogram (PI-A) was drawn to view nuclear DNA content. Amplification settings of the instrument were kept constant while comparing the buffers.

Parameters evaluated in each sample were- relative fluorescence intensities of PI stained nuclei (FL), coefficient of variation (CV) of the G_0/G_1 DNA peak (to estimate clumping, nuclei integrity, and staining variation), debris factor (DF, to assess background debris) and nuclear yield factor (YF, to compare the amount of nuclei in suspension independently of the amount of tissue used) (25).

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

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

Nuclei yield was obtained by adding the number of events in G_0/G_1 and G_2 peaks whose positions were ascertained by comparing the median PI fluorescence intensity values.

The analysis was performed on three different days. Five replicates were performed for each buffer and in each replicate at least 3000 nuclei were analysed. For nuclear genome size estimations at least three replicates were analysed, each replicate representing a separate individual. 2C value of sample was estimated according to the following formulae:

$$2\mathrm{C value\ of\ sample}\,(\mathrm{pg}) = \frac{\text{Sample } G_0/G_1 \text{peak MFI}}{\text{Standard } G_0/G_1 \text{peak MFI}} \times 2\mathrm{C value\ of\ Standard}\,(\mathrm{pg})$$

Conversion of mass value into base- pair numbers was done according to the factor 1 pg = 978 Mbp (26).

Statistical Analysis

Statistical analyses were performed using a one way Analysis of Variance (ANOVA) to detect significant differences, if any among the buffers and means were compared using Duncans test at a 5% probability level. Data analysis was performed using SPSS v 16.0 software.

Results

High mucilage content of chosen five species led to nuclei clumping and increased sample viscosity, which hindered filtration preventing nuclei isolation and subsequent flow cytometric analysis. In an attempt to overcome the problem of mucilage, five reported nuclei isolation buffers were tested to find out optimum sample preparation conditions for mucilaginous species of Hyacinthaceae. The selection criteria were based on the best combination of high YF and FL values and the low DF and G_0/G_1 peak CV values (Table 1). Although there are previous reports on the effect of cytosolic compounds on light scattering properties (4), no such effect of mucilages are still known. Among the five buffers initially tested, G_0/G_1 and G_2 peaks with intact nuclei were observed only in Galbraith's and Otto's buffer (Fig. 1). Visible clumps were observed in other three buffers resulting in low yield after filtration and no appreciable DNA peaks were observed upon flow cytometric analysis. Among Otto's and Galbraith's buffer the latter enabled better results with highest YF

Table 2. Estimation of nuclear DNA content

SPECIES	SITE OF COLLECTION	SOMATIC CHROMOSOME NUMBER	NUCLEAR DNA CONTENT		G_0/G_1 CV (%)	PREVIOUS REPORTS		
			PG/2C MEAN ± SD	MBP/2C MEAN ± SD	MEAN ± SD	2C (PG)	METHOD	REFERENCE
Drimia indica (Roxb.) Jessop	Fonda Ghat, Maharashtra	20	23.83 ± 0.90	23306 ± 879	4.79 ± 0.33	Nil	Nil	Nil
Drimia nagarjunae (Hemadri and Swahari) Anand Kumar	Tamil Nadu	20	23.96 ± 0.86	23433 ± 840	5.67 ± 0.45	Nil	Nil	Nil
Drimia wightii (Wight) Lakshmin	Halkarni, Maharashtra	20	23.80 ± 0.16	23276 ± 155	6.05 ± 1.42	Nil	Nil	Nil
Drimia coromandeliana Wight	Kagal, Kolhapur, Maharashtra	40	40.81 ± 0.88	39912 ± 866	3.77 ± 0.40	Nil	Nil	Nil
Ledebouria revoluta (L. f.) Jessop	Bangalore, Karnataka	30	7.89 ± 0.28	7716 ± 278	7.49 ± 0.53	11.70	Feulgen densitometry	Chakravarty and Sen 35)

Values are given as mean and standard deviation of the mean (SD) of nuclear DNA content (2C) in pictograms (pg), mega base- pairs (Mbp), and coefficient of variation of the G_0/G_1 DNA peak (G_0/G_1 CV, %).

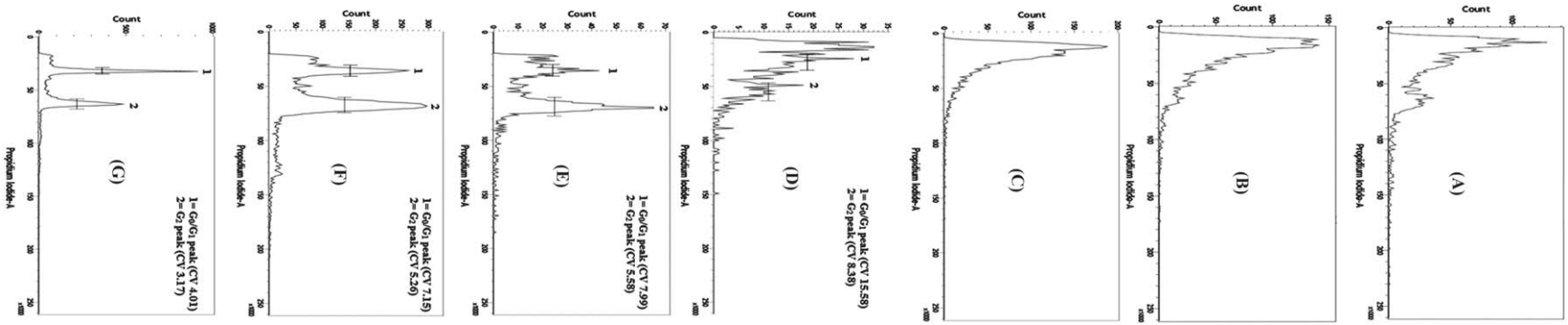

Figure 1. Flow cytometric analyses of homogenates prepared from *Drimia indica* root tissue in different buffers. **A–G**: PI-A histograms in LB01 buffer, Tris-$MgCl_2$ buffer, Tris-$MgCl_2$ buffer with 1% PVP, Otto's buffer, Galbraith's buffer, modified Galbraith's buffer and NM modified Galbraith's buffer respectively. Abbreviations: PI-A (propidium iodide fluorescence intensity area).

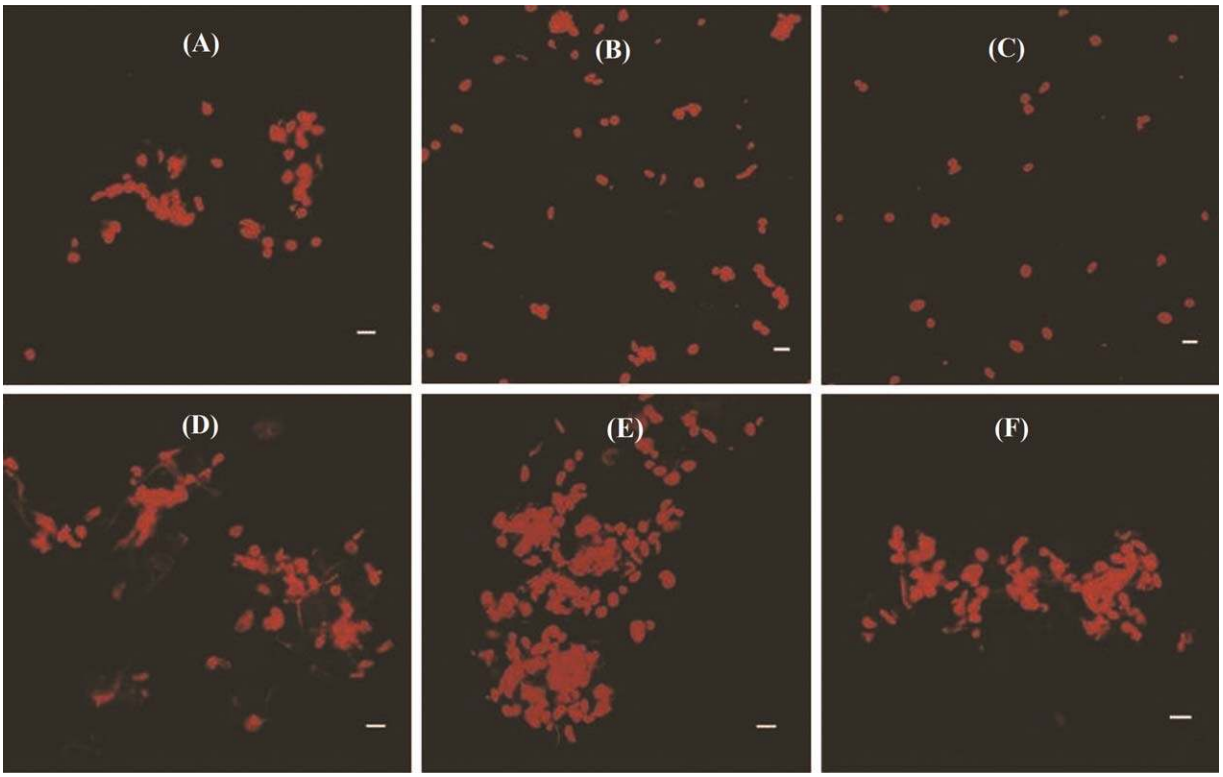

Figure 2. Photomicrographs of nuclei suspensions prepared from *Drimia indica* root tissue in different buffers. **A**: Galbraith's buffer. **B**: Modified Galbraith's buffer. **C**: NM modified Galbraith's buffer. **D**: LB01 buffer. **E**: Otto's buffer. **F**: Tris-$MgCl_2$ buffer with 1% PVP. Bar = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(0.090 ± 0.060) and FL (72.50 ± 13.79) and lowest DF (72.19 ± 7.45) and significant lowering of G_0/G_1 peak CV (6.87 ± 1.56; Table 1). Microscopic analysis also showed similar observations, large clumps being observed in LB01, Tris-$MgCl_2$, Tris-$MgCl_2$ (1% PVP), and Otto's buffers while clumps were lesser and smaller in Galbraith's buffer (Fig. 2). Thus, Galbraith's buffer was chosen for further modifications such as increasing the detergent concentration, pH and replacement of sodium citrate by citric acid.

Increasing pH and detergent concentration improved sample quality as compared to Galbraith's buffer, decreasing viscosity, and nuclei clumping as evident in microscopic analysis (Fig. 2). Modified Galbraith's buffer with 0.15% Triton X and pH 8.5 gave comparatively lower G_0/G_1 peak CV (5.67 ± 1.17) and DF (64.01 ± 7.50) and higher YF (0.237 ± 0.165) as compared with Galbraith's buffer (Table 1, Fig. 1). The NM modified Galbraith's buffer with sodium citrate being replaced by citric acid (0.05M), increased pH (8.5) and detergent concentration (0.15%) resulted in lower G_0/G_1 peak CV (4.91 ± 0.72) and DF (54.22 ± 11.26) and higher YF (0.364 ± 0.206) as compared to both Galbraith's buffer and modified Galbraith's buffer (Table 1, Fig. 1). Thus with NM modified Galbraith's buffer, YF and DF were significantly improved as compared with Galbraith's buffer. Although G_0/G_1 peak CV was not significantly improved over Galbraith's buffer, it was lowest in NM modified Galbraith's buffer. No significant difference in FL was observed among the Galbraith's buffer and the modified buffers but FL-CV was significantly lower in the modified buffers. Thus the NM modified Galbraith's buffer reduced the difficulties associated with high mucilage content and can be used for flow cytometric analysis of mucilaginous plants as employed in this study to estimate the nuclear DNA content of five Hyacinthaceae species for the first time by flow cytometric analysis. However even NM modified buffer failed to improve the sample quality when differentiated tissues like leaves, bulb scales and inflorescence axis were used because of their higher mucilage content.

The nuclear DNA contents of *D. indica*, *D. nagarjunae, D. wightii, D. coromandeliana,* and *L. revoluta* are presented in Table 2 (Fig. 3). Statistical analysis did not reveal significant differences in nuclear DNA content among individuals of the same species. Somatic chromosome number of $2n = 20$ were observed in *D. indica, D. nagarjunae,* and *D. wightii* while *D. coromandeliana* exhibited $2n = 40$ chromosomes and *L. revoluta* showed 2 $n = 30$ chromosomes (Table 2). *D. indica* (23.83 ± 0.90 pg/2C), *D. nagarjunae* (23.96 ± 0.86 pg/2C), and *D. wightii* (23.80 ± 0.16 pg/2C) are diploids with $2n = 20$ chromosomes and no significant difference in nuclear DNA contents were observed among them (Table 2). *D. coromandeliana* (40.81 ± 0.88 pg/2C) is a tetraploid with 40 chromosomes and its nuclear DNA content was lesser than the putative value as inferred from doubling the genome size of diploid species studied here.

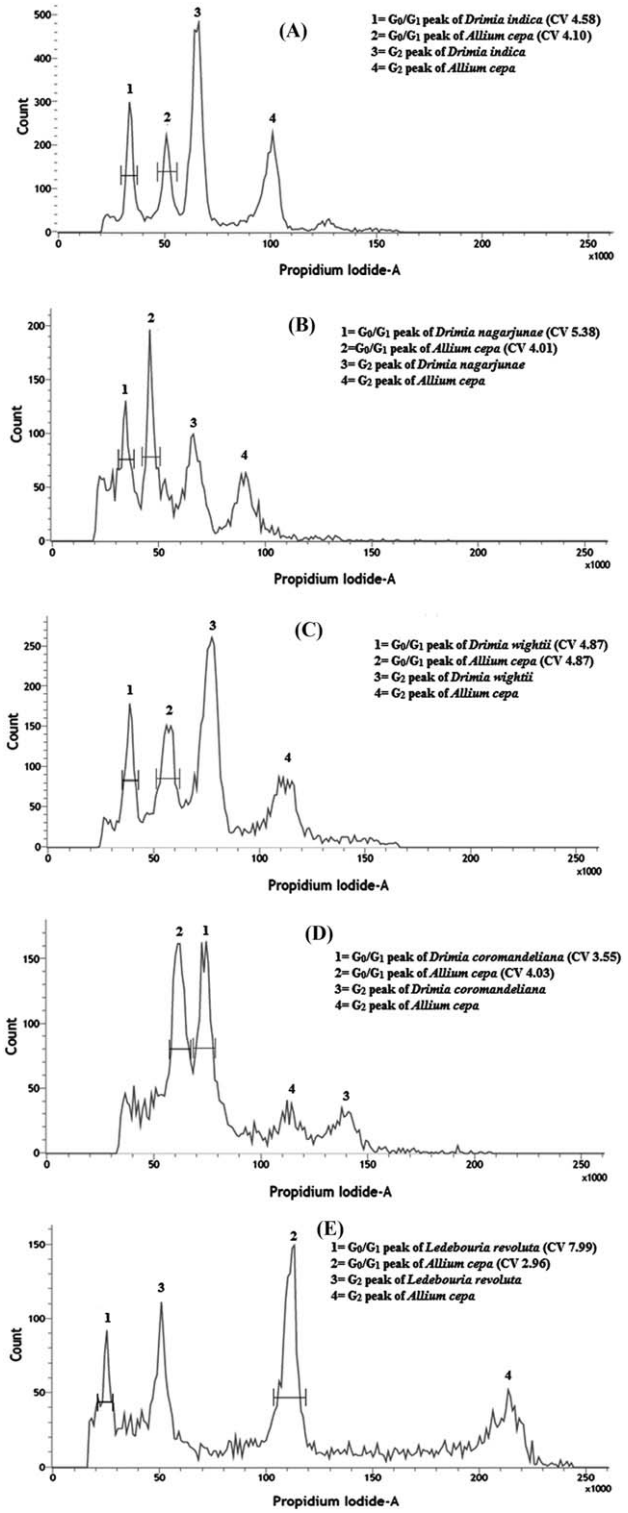

Figure 3. Propidium iodide fluorescence intensity-area (PI-A) histograms of (**A**) *Drimia indica* with internal standard *Allium cepa* cv. Alice (**B**) *Drimia nagarjunae* with internal standard *Allium cepa* cv. Alice (**C**) *Drimia wightii* with internal standard *Allium cepa* cv. Alice (**D**) *Drimia coromandeliana* with internal standard *Allium cepa* cv. Alice (**E**) *Ledebouria revoluta* with internal standard *Allium cepa* cv. Alice.

Discussion

Mucilages are complex polysaccharide compounds (rhamnogalacturonans and arabinogalactans) constituting mainly of glucuronic acid, galacturonic acid, rhamnose, arabinose, and galactose in varying proportions along with some proteins and minerals (27). Mucilages have viscous glue like texture, thus results in nuclei aggregation making the samples unsuitable for flow cytometric analysis. Viscosity of polysaccharides is attributed to cross-linking between the entangled polymer chains as well as its interaction with proteins.

Among the five reported buffers tested here Galbraith's buffer allowed better flow cytomeric analysis of nuclei samples. This may be explained by the fact that all components of Galbraith's buffer, that is, $MgCl_2$, sodium citrate and Triton X are reported to reduce the viscosity of polysaccharides, the main hindrance in sample preparation. Presence of divalent cations like Mg^{2+}, Ca^{2+} etc is reported to decrease viscosity of okra and baobab mucilage (28). This is probably related to the ability of these cations to interact with the adjacent polymer chains and prevent crosslinking. Citrate derivatives of polysaccharides are reported to exhibit reduced crosslinking and viscosity (29,30).

Further modifications of Galbraith's buffer done in this study were based on previous reports on effects of chemicals and pH on mucilage viscosity. Increasing detergent concentration for combating mucilage interference has been suggested by many authors (3,21). Certainly, the slightly higher detergent concentration used in the modified buffers (0.15% rather than 0.10%) reported here yielded improved results. However, since detergent concentrations >0.15% were shown to affect nuclei morphology, these were not tested further. Mucilage viscosity in many plants is reported to decrease with increase of pH from acidic to basic (28,31,32). pH probably alters the charges on —COOH group of the polysaccharide units and hence their interaction with adjacent polymer molecules or proteins. In this study, pH <8 resulted in clumping while pH 8.5 was optimized to give better results. Further increasing of pH did not improve the results in the preliminary studies and thus were not tested. In addition, microscopic analysis of nuclei sample prepared in Otto's I buffer (pH 2–3) resulted in large clumps, which indicates that low pH increases clumping.

The previous report of nuclear DNA content estimation in *Drimia* (19) used Otto's buffer. The chief constituent of Otto's buffer is citric acid, which is reported to decrease the viscosity of polysaccharides, the main component of mucilage. Thus, citric acid was chosen to replace sodium citrate in Galbraith's buffer. Citric acid is a polymer stabilizer, forms ester or amide bond in basic conditions, involving the carboxyl functions of citric acid and the hydroxyl or amino functions present on polysaccharide. This results in reduced cross linking and viscosity (29,30). Citric acid also acts as a reducing agent and polysaccharides viscosity is reported to decrease by treatment with reducing agents (33,34). It was suggested that the change of viscosity might be due to a dephosphorylation of the viscous polysaccharide. The replacement of sodium

citrate by citric acid in NM modified Galbraith's buffer improved G_0/G_1 peak CV probably by decreasing nuclei clumping. Statistical analysis using a one way ANOVA also showed significant improvement of NM modified Galbraith's buffer over Galbraith's buffer.

Thus, the modifications used in the present study improved the sample quality for flow cytometric analysis of the chosen mucilaginous plants, that is, DNA peaks with relatively acceptable G_0/G_1 peak CV values, higher yield, and lower background debris. This allowed flow cytometric analysis of the five chosen mucilaginous species for the first time. There are no previous reports on genome size estimations of *Drimia* species studied here. However there are previous reports of genome size estimation in two other species of *Drimia* (syn. *Urginea*) listed as belonging to *Urginea-U. hesperia* with 21.04 pg/2C (19,20) and *U. maritima* with 27.10 pg/2C (18,20). Also the mean 2C value of the genus *Drimia* (syn. *Urginea*) as mentioned in the Kew online database for C-value of angiosperms based on these two species is 24.06 pg/2C (20). There is only one report on genome size estimation for *L. revoluta* by Feulgen microdensitometry (20,35). Among *Drimia* species statistical analysis did not reveal significant differences in nuclear DNA content between *D. indica* ($2n = 20$, 23.83 ± 0.90 pg/2C), *D. nagarjunae* ($2n = 20$, 23.96 ± 0.86 pg/2C), and *D. wightii* ($2n = 20$, 23.80 ± 0.16 pg/2C). In addition, the estimated 2C values of these three species of *Drimia* are comparable to previous reports of related species. *D. coromandeliana* ($2n = 40$) has 40.81 ± 0.88 pg/2C, which is lesser than the putative value as inferred from doubling the genome size of diploid species studied here. Although reduction of genome size is a common trend among polyploids (36) the probable ancestors of this species are not known yet. Thus, it is possible that the genome sizes of the diploid parental donors are actually lower than any of the species analyzed so far. In addition, so few species have been analysed that the reported mean given may not be representative of the diploids. Genome size of *L. revoluta* ($2n = 30$) was estimated as 7.89 ± 0.28 pg/2C, which is lesser than the previously reported value of 11.70 pg/2C as estimated by Chakravarty and Sen (35) by Feulgen densitometry.

Thus, the present approach can improve sample quality for flow cytometric analysis in members of Hyacinthaceae as well as other mucilaginous species, providing opportunities for the future application of the technique in other aspects. However more extensive studies on the effect of each buffer component on mucilage interference are required for further improvement of the process. This study thus opens up the scope for improvement of sample preparation for flow cytometric analysis in mucilaginous plants.

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