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Development of the Flow Cytometric Protocol for Ploidy Analysis and Determination of Relative Nuclear DNA Content in Cashew (*Anacardium occidentale* Linn.)

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ABSTRACT

Flow cytometry method is capable of facilitating improvement in a long gestation crop like cashew through characterization and screening of parent plants, evaluation of population biodiversity, screening of hybrids and ploidy analysis of induced mutants. Unfortunately, report on the application of flow cytometry to cashew (*Anacardium occidentale*) in the literatures is rare thus limiting information on its genome. To develop a flow cytometric protocol amenable to the plant, five different buffer compositions, three sample sizes and five incubation periods of isolated nuclei were evaluated using six cashew accessions. Six plant species commonly used as internal reference standards were also screened to find most suitable reference for cashew. Data collected on the quality of nuclear suspension in terms of fluorescence intensity, background yield (%), nuclear yield (nuclei sec^{-1} mg^{-1}) and coefficient of variation (%) of G0/G1 peak were summarized and statistically analysed using analysis of variance and relevant descriptive statistics procedure. The results showed high background noise from the flow cytometric analysis of the isolated nuclei, an indication that cashew plant contains high content of pehnols and cytostil compounds. Among the buffer systems examined, cashew performed better with Otto buffer. Preparation from leaf tissue of about 70 mg incubated for about 10 min on cold ice in the dark gave satisfactory results. Tomato, *Solanum lycopersicum* cv. Stupicke with 1.96 pg/2C genome was found most ideal internal reference for determination of nuclear DNA content in cashew. The protocol reported here would be useful for ploidy analysis and estimation of relative nuclear DNA content and not absolute genome size in cashew.

Key words: Cashew (*Anacardium occidentale*), flow cytometry, genome size, lysis buffer, nuclear DNA content

INTRODUCTION

Cashew is a tropical evergreen perennial tree with dense foliage and can grow as high as 15 m or more. The tree flowers mostly at the end of wet season, although, preceded by the growth flush. The flowers are aggregated into a cymose inflorescence or helicoid panicle. Cashew panicles contain both staminate and hermaphrodite flowers in varying proportion and are produced at the periphery of the tree canopy. Flower pollination is predominantly by insects, although, some self-compatible and self-fertile genotypes had been reported (Northwood, 1966; Freitas and Paxton, 1996; Aliyu, 2008). Cashew fruit is a drupe (kidney shaped nut) attached to the basal portion of hypocarp commonly called apple. The nut is the main produce for the

growers and source of livelihood for significant percentage of rural households in the major producing countries in Asia and Africa (Topper *et al*., 2001). A highly nutritive kernel is derived from a healthy nut with about 25.3% crude protein, 26.8% carbohydrate and 29.4% fatty acids (Aremu *et al*., 2006). In addition, it contains mineral contents (mg/100 g) such as Na (22.8), K (38.2), Ca (21.9), Mg (36.4), P (18.0) Mn (1.6), Cu (0.4), Zn (0.8) and Fe (0.8) (Aremu *et al*., 2006). Because of this nutritive value, the kernel remains the premium product from cashew plant and its usage range from being eaten as dessert to supplements in confectioneries and livestock feeds (Ojewola *et al*., 2004; Ojewola and Ewa, 2005; Segun *et al*., 2009). The hypocarp i.e., apple is juicy, fibrous and sometimes astringent in taste. The apple colour at maturity range from fair yellow, deep yellow, orange to deep red and seldomly green. The mature apples are eaten fresh as a fruit like pawpaw, orange etc. and can be processed into juice, vinegars and wine (Lowor and Agyente-Badu, 2009).

Traditional cultivation of cashew as a commodity crop plant has been successful in Asia and Africa since its introduction about five century ago (Mitchell and Mori, 1987) but breeding programme for the development of improved cultivars has been hindered by lack of accurate data on systematics, evolution, cytology and molecular biology of the crop because the genus *Anacardium* has been scarcely studied (Al-Saghir, 2009). Classification in the past has been based on the hypocarp (apple) colour (Ohler, 1979) and till date the ploidy status has not been resolved. Few studies have been done on chromosome counting using a squash-staining technique and the plant has been described as a plant species with chromosome polymorphism (Mitchell and Mori, 1987). Cashew chromosome number reported in literatures ranged from 2n = 24 (Goldblatt, 1984; Khoslat *et al*., 1974), 2n = 30 (Machado, 1944) and 2n = 40 (Goldblatt, 1984; Simmonds, 1954) to 2n = 42 (Goldblatt, 1984; Khoslat *et al*., 1974; Darlington and Ammal, 1945; Purseglove, 1988; Aliyu and Awopetu, 2007). Significant variation in the chromosome number in cashew can result into wide variability in phenotypes and high cost of germplasm management. The squash-staining procedure for estimating nuclear content (chromosome) is slow, cumbersome, tissue-stage specific and difficult in tree species with characteristic small size and large number of chromosomes. Although, flow cytometry technique has long been used mainly for clinical research (Attallah *et al*., 2006; Hegazy *et al*., 2008) but with adaptation of the technique to plant research in the 1980s (Galbraith *et al*., 1983), it became possible to carry out estimation of nuclear DNA content in plant species without using tissues with dividing cells. Since then flow cytometry has contributed significantly to research in cyto-taxonomy, systematics, breeding and general crop production (Asif *et al*., 2000; Dolezel and Bartos, 2005; Shahriari-Ahmadi *et al*., 2008). It is a quick method for determining genome size and ploidy analysis. Interestingly, correlation between the nuclear DNA content and the ploidy level has been widely reported (Sharma *et al*., 1983; Galbraith, 1984; De Laat *et al*., 1987; Sree Ramulu and Dijkhuis, 1986). The flow cytometry method involves a simple mechanical homogenization of plant tissues in a nuclear isolation buffer (Galbraith *et al*., 1983) which facilitate release of nuclei into the liquid suspension medium and staining with fluorescence dye or fluorochromes (staining buffer). Flow cytometry method can facilitate plant breeding programme in a long gestation crop like cashew through rapid characterization and screening of parent plants, evaluation of population biodiversity (Meric *et al*., 2008), screening of offsprings after hybridization, screening of offtypes before transplantation and ploidy screening of induced mutants (haploidization and polyploidization) (Asif *et al*., 2000; Msogoya *et al*., 2008; Shahriari-Ahmadi *et al*., 2008).

Survey of literatures has shown that application of flow cytometry to cashew research is rare and genome size of the plant has not been determined till date. In this study, a flow cytometric protocol for the analysis of ploidy and estimation of relative nuclear DNA content in cashew was developed by testing 5 buffer compositions, 3 leaf tissue sample sizes and 5 incubation periods for the isolated nuclei. This study also includes screening suitable internal reference standards since the genome size of cashew is yet to be determined. It is important to state that this trial was constrained to measuring relative genome size, because the available PAII Partec flow cytometer was only configured for 4',6-diamidino-2-phenylindole (DAPI) fluorochromes and limitation of DAPI fluorochromes to measure absolute DNA content has been well clocumented (Michael *et al*., 1991; Dolezel *et al*., 1992; Dolezel and Bartos, 2005).

MATERIALS AND METHODS

Planting materials: Matured nuts harvested from six cashew accessions were obtained from the Cocoa Research Institute of Nigeria, Ibadan, south western Nigeria (Latitude 07°10'N, Longitude 03°52'E) and raised in the greenhouse at Leibniz Institute for the Plant Genetics and Crop Research (IPK), Germany for this study. Although, five individual plants were raised per accession, only three healthy plants were eventually used for the analysis. The seedling plants were grown under controlled greenhouse-tropicalized conditions, with average temperature of 25°C, 16 h photoperiod and alternate day automated indoor-aerial-sprinkling-irrigation for 10-15 min. Sampling of the leaves for the analysis did not commence until 6 weeks after sowing when all the nuts had germinated and the plants are of 4-leaf stage.

Plant materials for the internal reference standard: Six plant species whose genome sizes were known and commonly used as internal reference standards (Dolezel and Bartos, 2005) (Table 1) were collected from Dr. Jaroslav Dolezel of the Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Sokolovska, Olomouc, Czech Republic, (http://olomouc.ueb.cas.cz/). The seeds were sowed and the seedling plants raised under greenhouse condition at the IPK, Germany. The genome sizes of the selected internal reference plants range from 1.11-26.90 pg/2C for *Raphanus sativus* cv. Saxa and *Vicia faba* cv. Inovec, respectively.

Different buffer systems screening on cashew: From the literatures, five buffers compositions commonly used were selected for screening on cashew plant (Table 2). The list include (1) Galbraith's buffer (Galbraith *et al*., 1983), (2) LB01 (Dolezel *et al*., 1989), (3) Otto's buffer (Otto, 1990, 1992; Dolezel and Gohde, 1995), (4) Tris-$MgCl_2$ (Pfosser *et al*., 1995) and (5) DAPI-MaVI commonly used in IPK's laboratory. The buffers were designated as G, L, O, T and M, respectively.

Table 1: List of six plant species with their nuclear DNA contents tested as internal reference standards for estimation of relative genome size in cashew, *A. occidentale*

Species	Family	Nuclear DNA content (pg per 2C)	References
Raphanus sativus cv. Saxa	Brassicaceae	1.110	Dolezel *et al*. (1992)
Solanum lycopersicum cv. Stupicke	Solanaceae	1.960	Dolezel *et al*. (1992)
Glycine max cv. Polanka	Fabaceae	2.500	Dolezel *et al*. (1994)
Zea mays CE-777	Poaceae	5.430	Dolezel *et al*. (1998)
Pisum sativum cv. Citrad	Fabaceae	9.090	Dolezel *et al*. (1998)
Vicia faba cv. Inovec	Fabaceae	26.90	Dolezel *et al*. (1992)

Table 2: List of five lysis buffer systems tested on cashew *A. occidentale* for the estimation of nuclear DNA content

Buffer	Chemical composition	References
*Galbraith	45 mM $MgCl_2$, 30 mM Sodium Citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100, pH 7.0	Galbraith *et al.* (1983)
*LB01	15 mM Tris, 2 mM Na_2 EDTA, 0.5 mM Spermine.4 HCl, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, pH 8.0	Dolezel *et al.* (1989)
*Otto	Otto I: 100 mM Citric acid, 0.5% (v/v) Tween 20 (pH 2-3)	Otto (1992),
	Otto II: 40 mM $Na_2PO_4.12H_2O$ (pH 8-9)	Dolezel and Gohde (1995)
*Tris-$MgCl_2$	200 mM Tris, 4 mM $MgCl_2.6H_2O$, 0.5% (v/v) Triton X-100, pH 7.5	Pfosser *et al.* (1995)
DAPI-MaVI	0.214 g $MgCl_2.6H_2O$, 1.0 g NaCl, 2.422 g Tris, 0.20 ml/0.1878 g Triton X-100, 1.8 g Sodium citrate, pH 7.0	Matzk (pers. comm)

*Source: Dolezel and Bartos (2005). MOPS: 4-morpholinepropane sulfonate, DDT: Dithiothreitol, TRIS: Tris (hydroxymethyl)-aminomethane, EDTA: Ethylenediamine tetra acetic acid, HEPS: 4-(hydroxymethyl) piperazine-1-ethanesulfonic acid

Data on the performance of each buffer on cashew was evaluated according to the quality of DNA nuclear suspension described by Loureiro *et al.* (2006a). The variables include background factor (%), fluorescence, coefficient of variation (%) and nuclear yield.

Leaf sample size for cashew and internal reference standard: Three categories of leaf sample sizes (40-50, 60-80 and 90-120 mg) from young cashew leaf tissue-(not youngest) were tested to determine suitable quantity of leaf sample size that will release sufficient number of nuclei into the lysis buffer with acceptable quality. Leaf sample of 40-50 mg was used for the internal reference plants as reported in literatures (Dolezel *et al.*, 1998; Loureiro *et al.*, 2006a).

Incubation period for the isolated nuclei: Five incubation periods ranging from 0, 10, 20, 40-60 min after nuclei isolation were tested in this study. The incubation was done on cold ice in the dark box.

Flow cytometric procedure for the isolation of nuclei: Extraction of nuclei was done by either chopping/co-chopping of leaf sample from cashew/or cashew and internal reference standard with a sharp razor for 30 sec in a Petri dish containing about 1.0 mL extraction buffer solution (Galbraith *et al.*, 1983). About 0.8 mL of nuclei suspension recovered was filtered through a 30 μm Partec single tube filter to remove cell fragments and large debris. Isolated nuclei in lysis buffer were stained with 4 μg mL^{-1} of 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Germany) and **β**-mercaptoethanol (2 μL mL^{-1}) was added to reduce polyphenolic oxidation (Dolezel and Bartos, 2005). For Otto's buffer, staining was done in ratio of 1 part of nuclear suspension in Otto I extraction buffer to 4 parts of the Otto II staining buffer. Isolated stained nuclear suspensions were incubated for 0 (i.e., without incubation), 10, 20, 40 and 60 min (for different treatments) on cold ice in the dark before cytometric analysis. The nuclear suspension was analyzed on a Partec CyFlow® Ploidy Analyser II (Partec, Münster, Germany) equipped with laser excitation at 488 nm as well as UV LED by a mercury arc lamp.

Experimental design and data collection: The experimental layout is a spilt-spilt plot design of three treatment factors (5 buffer systems i.e., Galbraith, LB01, Otto, Tris-$MgCl_2$ and DAPI-MaVI; 3 leaf sample sizes i.e., 40-50, 60-80 and 90-120 mg and 5 incubation periods i.e., 0, 10, 20, 40 and 60 min). The treatment factors were applied to the six accessions. In addition to three individual plants used for the investigation, the analysis was repeated on three different days (Dolezel *et al.*, 2003, 2007). Data on quality of nuclear suspension in terms of fluorescence intensity (FL),

coefficient of variation (CV) of G0/G1 peak (a measure of nuclear integrity and variation in DNA staining), background factor (BF) (a measure of a sample quality) and nuclear yield factor (YF) (a proportion of nuclei in suspension in respect to the sample size) were collected using a Partec Flowmax Software, Windows 2.5 version (Loureiro *et al*., 2006a). The computation analyses of background factor and nuclear yield followed (Loureiro *et al*., 2007) i.e.:

$$BF(\%) = \frac{N-n}{N} \times 100$$

where, N is total number of particles and n is total number of intact nuclei and YF (nuclei sec^{-1} mg^{-1}) = (n/t)/w, where n is total number of intact nuclei, t is number of run time in second and w is leaf sample size in weight (mg).

Data and statistical analyses: The data collected on the four nuclear suspension variables, i.e., fluorescence intensity, coefficient of variation (%), background factor (%) and nuclear yield (nuclei sec^{-1} mg^{-1}) were summarized and statistically analyzed using a split-spilt plot analysis of variance method of the Genstat software Discovery Edition version 3.0 (http://www.vsni.co.uk/). The treatment means were ranked using a Duncan multiple range test (DMRT) at LSD p<0.05. The degree of difference in the performance of buffers was plotted into hierarchical dendogram using a group average linkage Euclidean distance method.

RESULTS

The analysis of variance (ANOVA) (Table 3) of the four nuclear suspension quality parameters (fluorescence intensity, background factor, nuclear yield and coefficient of variation of G0/G1 peak), showed significant difference (p<0.001) in the performances of the 5 buffers, 3 sample sizes and 5 incubation periods tested. All combination interactions of these three factors i.e., 'buffer x sample size', 'buffer x incubation period', sample size x incubation period' and 'buffer x sample size x incubation period' were statistically significant. In the overall, the fluorescence intensity varies between 70 and 136 while the background noise of the nuclear suspension was between 26 and 55%. Moreover, the nuclear yield for the cashew sample was between 0.70 and 1.75 with average

Table 3: Analysis of variance for the quality of nuclear suspension (background noise, fluorescence intensity, coefficient of variation and nuclear yield) of cashew as affected by buffer composition, leaf tissue size and incubation period of isolated nuclei

		Nuclear suspension characteristics			
Source of variation	Degree of freedom	Background factor (%)	Fluorescence	Coefficient of variation (%)	Nuclear yield factor (nuclei sec^{-1} mg^{-1})
Buffer	4	396.09***	7636.11***	15.14***	1.55***
Error	8	1.89	4.06	0.03	0.001
Sample size	2	756.28***	2195.54***	1.74***	0.46***
Sample size×buffer	8	14.40**	66.03**	0.50***	0.06***
Error	20	3.59	14.27	0.02	0.00
Incubation period	4	440.92***	528.25***	1.80***	0.26***
Incubation period×buffer	16	10.48***	14.35ns	0.10***	0.02***
Incubation period×sample size	8	28.72***	24.35*	0.25***	0.03***
Incubation period×sample size×buffer	32	6.02*	13.16ns	0.11***	0.01***
Error	120	3.36	10.03	0.03	0.001

, and * mean statistically significant at p<0.001, p<0.01 and p<0.05, respectively, ns: Not significant

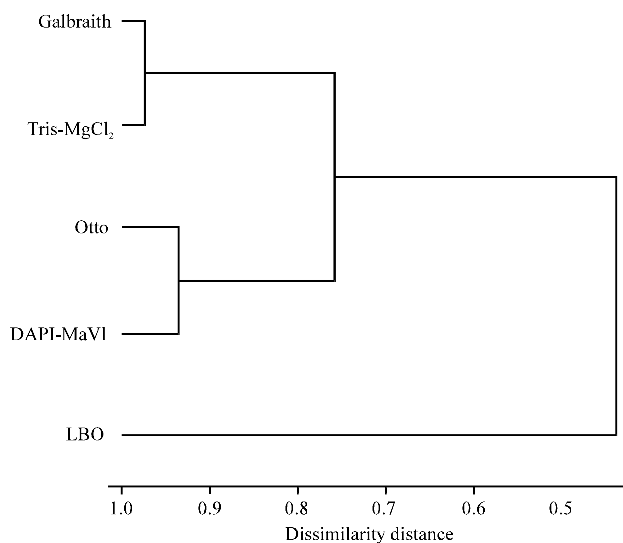

Fig. 1: Group average linkage dendogram showing grouping of the five 5 lysis buffers based on the degree of similarity in their capability to extract nuclei for the estimation of relative nuclear DNA content and ploidy analysis in cashew. Data were derived from four parameters for grading the quality of the nuclear suspension (see materials and methods)

of 1.02 (data not shown). The coefficient of variation of the histogram peaks averaged 4.4% with a range between 3.02 and 6.02% (data not shown). The overview of descriptive statistics of the quality of nuclear suspension from cashew suggests presence of high content of secondary metabolites characteristic of woody tree plants. Among the five buffers screened a two-step Otto's buffer system gave the best results in terms of coefficient of variation of G0/G1 peak, nuclear yield, fluorescence intensity and background noise (Fig. 2). By contrast, nuclei isolated with LB01 buffer consistently recorded the least quality. Based on the quality of nuclear suspension the five buffers grouped into two categories (Fig. 1) i.e., (a) Otto, DAPI-MaVI, Tris-$MgCl_2$ and Galbraith that are comparatively efficient in extracting nuclei as they fused at about 80% similarity index against less than 50% in LB01. Difference between the quality of nuclear suspensions derived from Galbraith and Tris-$MgCl_2$ buffer was indiscernible (less than 10% dissimilarity index). Similar performance was recorded between Otto and DAPI-MaVI buffers. This implies that either of each of the pair can be used for nuclei isolation for ploidy analysis. The unique characteristics of each of these lysis buffers have been documented (Dolezel *et al*., 1992, 1998; Dolezel and Bartos, 2005).

For the sample size, leaf tissue of about 70 mg (i.e., 60-80 mg) gave best quality in terms of sufficient nuclei (yield), better fluorescence signal, least background noise and lowest coefficient of variation of histogram G0/G1 peak for cashew (Fig. 2a-d). The interaction effect between buffer and amount of leaf tissue deed show that the efficiency (buffering capacity) of each buffer differs with increasing leaf sample size but quite obvious was that excess amount of leaf tissues compromise the quality of nuclear suspensions (Fig. 2a-d). For example, significant decrease in background noise was recorded for all the buffers when sample size was increased from 40-50 to 60-80 mg but further increase to 90-120 mg resulted to an increase in background noise significantly (Fig. 2a), indicating the sensitivity of lysis buffers to quantity of leaf sample size. However, the response tends to be buffer-specific for nuclei yield, fluorescence intensity and coefficient of variation of G0/G1 i.e., each buffer responded at different rate to changes in quantity

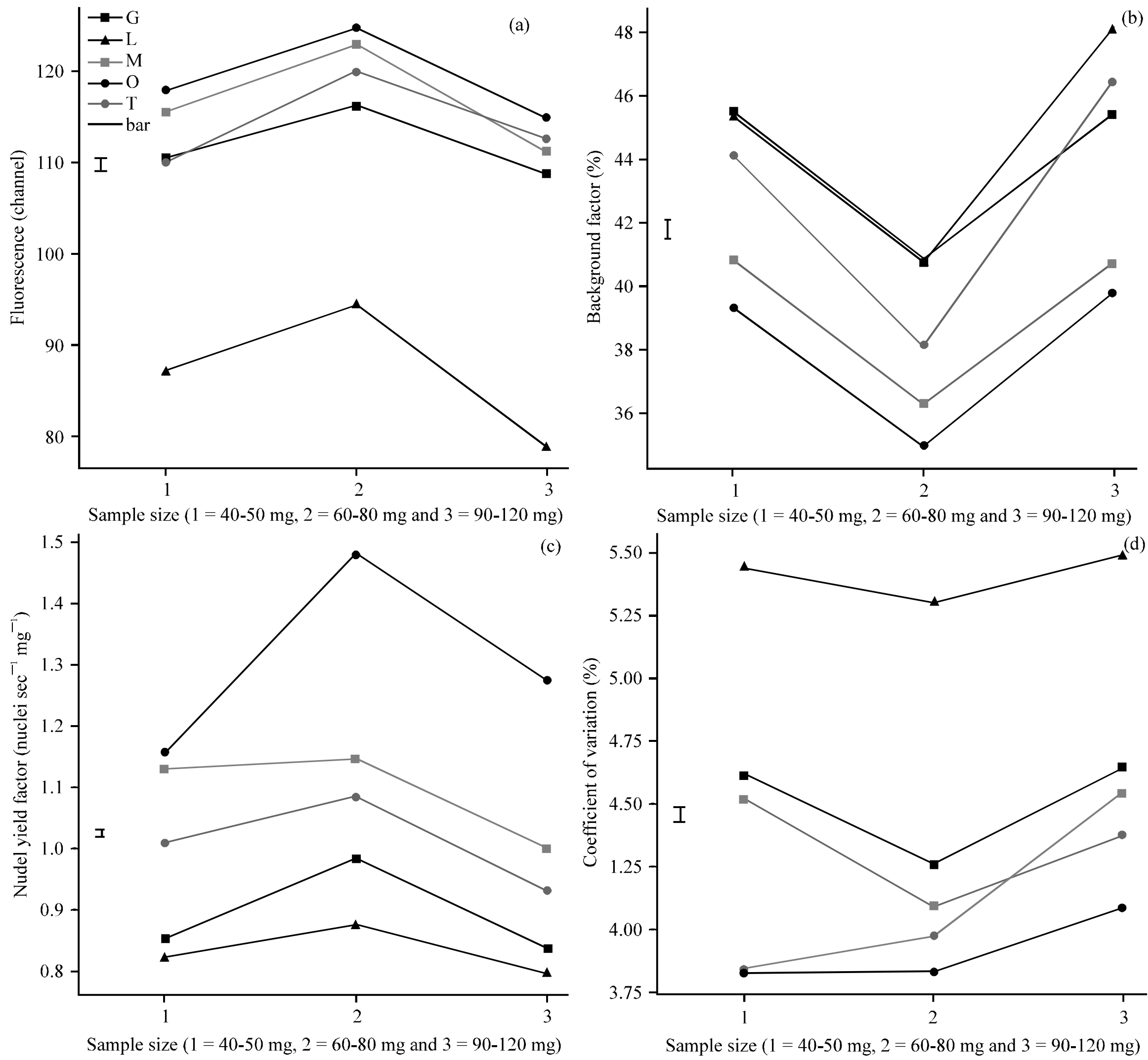

Fig. 2(a-d): Interaction effects of the quantity of leaf tissue sample and buffer composition on the quality of nuclear suspension (a) Fluorescence intensity; (b) Background factor (%); (c) Nuclear yield factor and (d) Coefficient of variation (%)) in cashew. Legend: Buffer: O: -Otto, L: LB01, M: DAPI-MaVI, G: Galbraith and T: Tris-$MgCl_2$ Sample size: -1 = 40-50 mg, 2 = 60-80 mg and 3 = 90-120 mg)

of leaf sample. Otto's buffer was most sensitive (positive or negative) to sample size in terms of nuclear yield (Fig. 2c), LB01 for fluorescence intensity (Fig. 2a) and Galbraith and Tris-$MgCl_2$'s buffers for coefficient of variation G0/G1 (Fig. 2d).

The quality of nuclear suspension was found to improve with incubation as extracted nuclei incubated for 10 min on cold ice in the dark was better than control (0 min i.e., analysed immediately) across the buffer system. However, interaction effect of buffer and incubation time presented in Fig. 3a-d show that the optimum incubation window for efficient DNA staining of isolated nuclei from cashew would be between 10 and 15 min. The interaction effect further showed that each buffer responded differently with increased incubation period until plateau is reached and subsequent decline in quality (Fig. 3a-d).

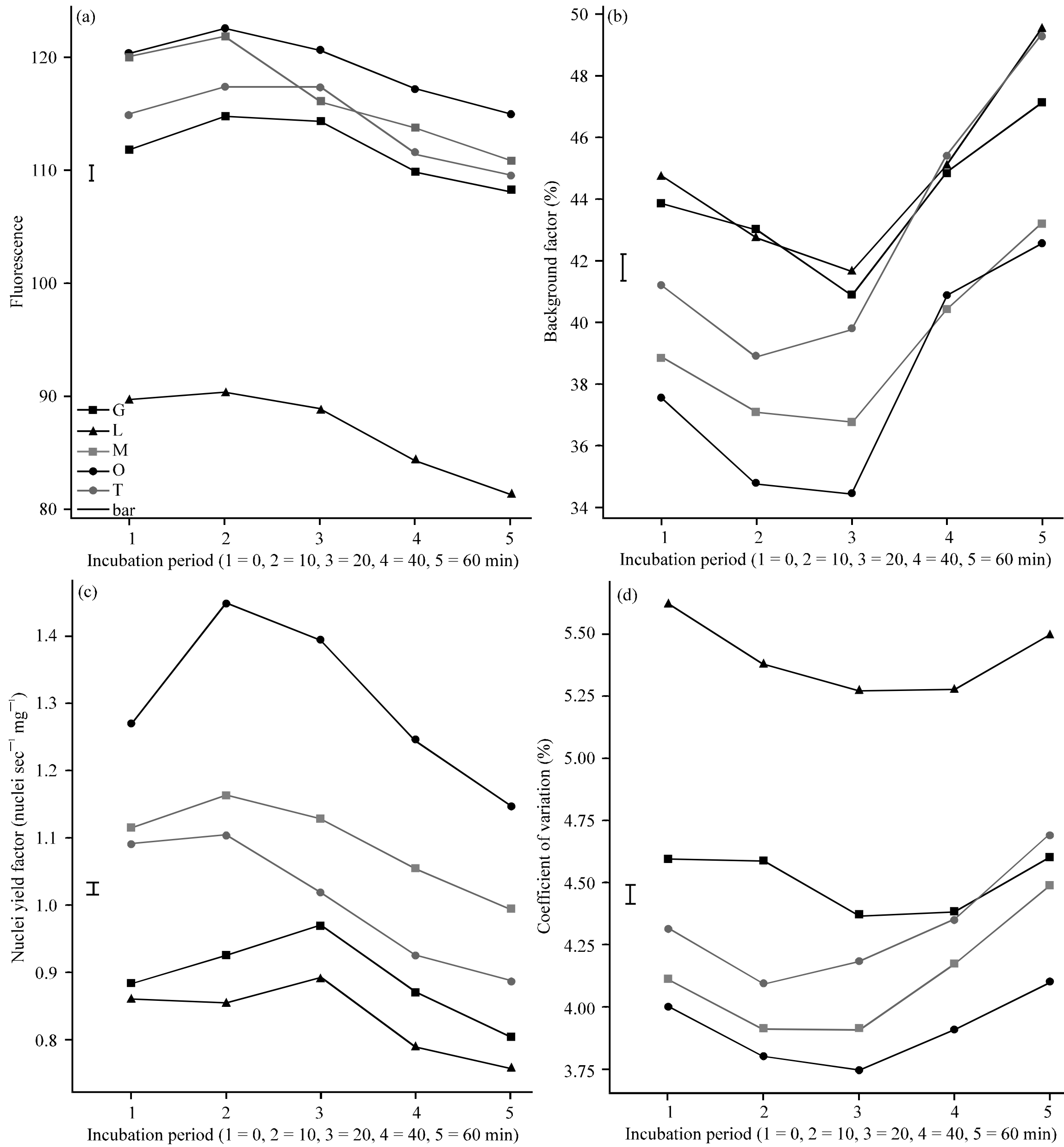

Fig. 3(a-d): Interaction effect of period of incubation (min) and buffer composition on the quality of DNA nuclear suspension; (a) Fluorescence intensity; (b) Background factor (%); (c) Nuclear yield factor and (d) Coefficient of variation (%) of G0/G1 peak) in cashew. Legend: Buffer:- O: Otto, L: LB01, M: DAPI-MaVI, G: Galbraith and T: Tris-$MgCl_2$ tested Incubation period:- 1 = 0 min, 2 = 10 min, 3 = 20 min, 4 = 40 min and 5 = 60 min)

Internal reference standards for flow cytometry in cashew: Without attempting to reinvent the will, the five buffers were not tested on the reference standards as numerous reports on their flow cytometry are available in the literatures (Dolezel and Bartos, 2005). However, Otto buffer

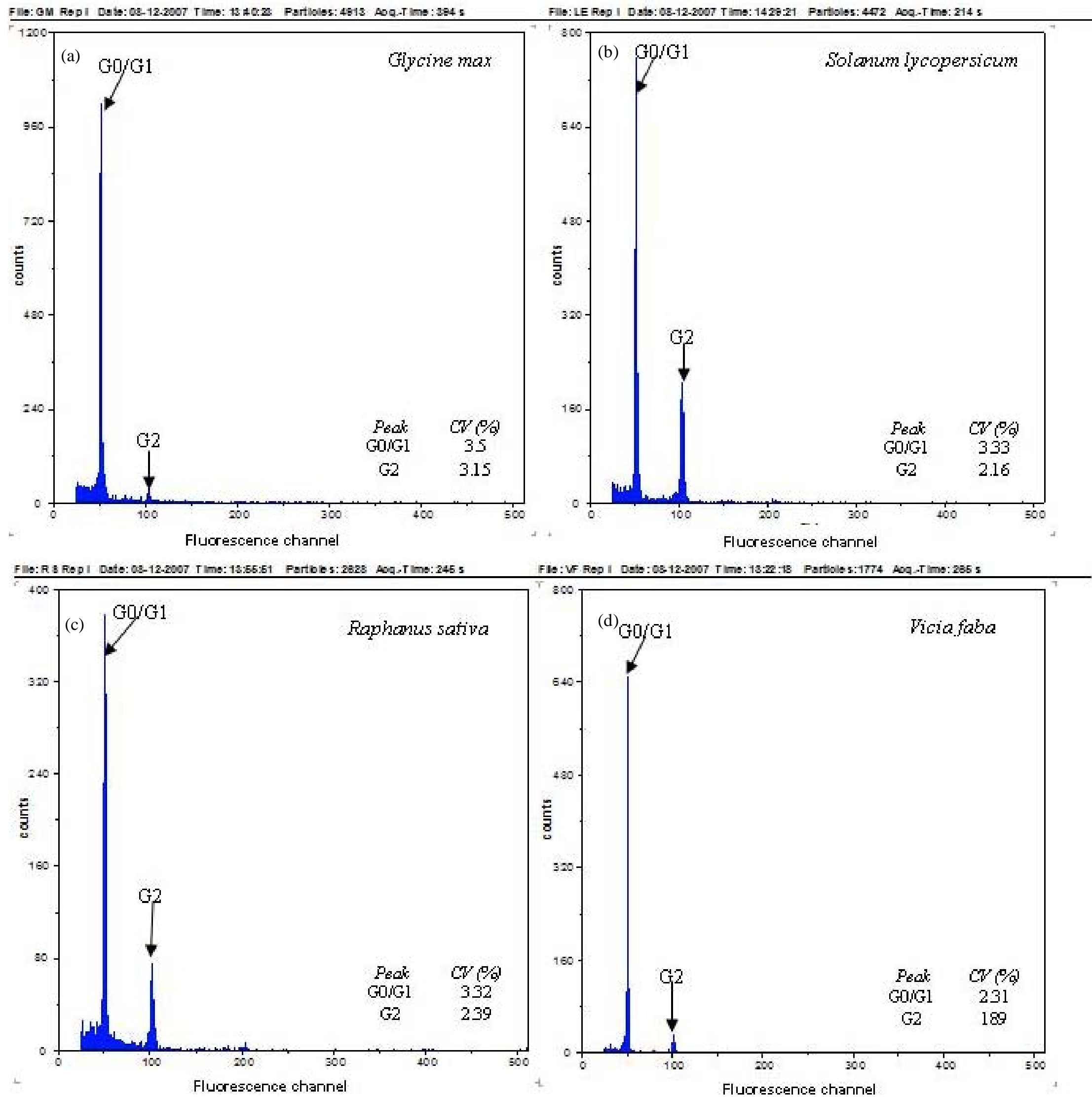

Fig. 4(a-d): Histograms showing relative fluorescence of nuclei at G0/G1 and G2 histogram peaks of (a) *Glycine max*; (b) *S. lycopersicum*; (c) *Raphanus sativus* and (d) *Vicia faba* using Otto's buffer (Table 2)

that shown better performance on cashew was used for the determination of suitable reference standard. Interestingly, the buffer gave higher resolution G0/G1 peak for all the reference standards (Fig. 4). Histogram profiles of isolated nuclei from cashew leaf sample co-chopped with each of the six reference plants show that *Raphanus sativus* cv. Saxa, *Glycine max* cv. Polanka and *S. lycopersicum* cv. Stupicke produced G0/G1 peaks within the same range of fluorescence channels (Gains) with cashew, suggesting a relative small genome size i.e., less than 2.0 pg/2C in this plant species. Among the 3 close reference plants mentioned above, histogram G0/G1 peak of *S. lycopersicum* cv. Stupicke was about 2 folds to that of cashew and shown to be the most suitable

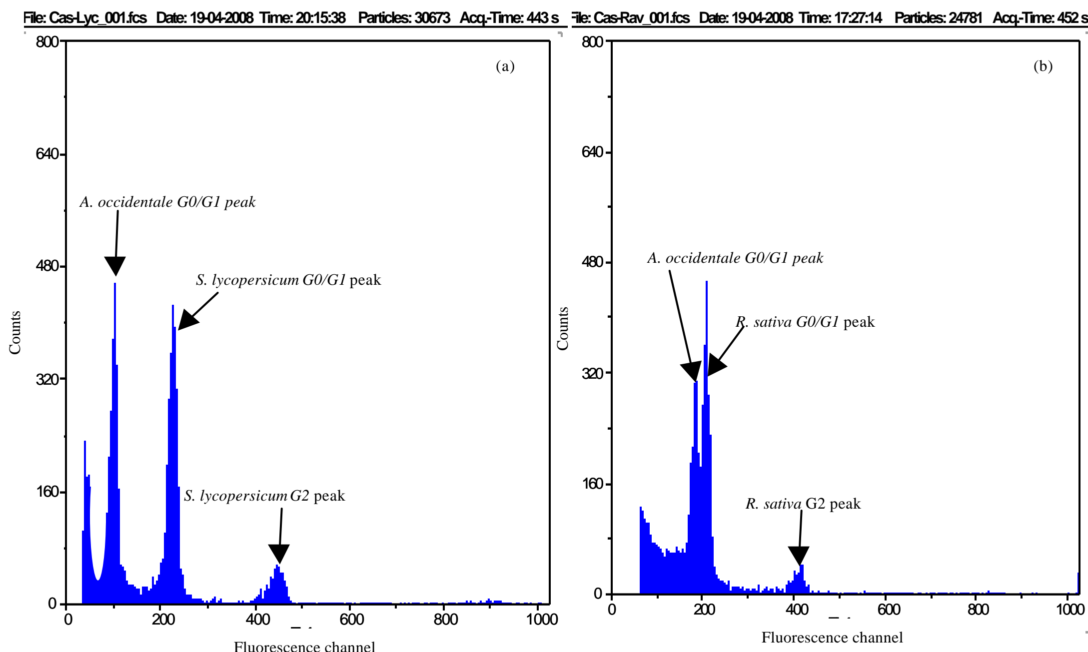

Fig. 5(a-b): Flow cytometric histograms showing relative fluorescence intensity of nuclear suspensions obtained from (a) Cashew, *A. occidentale* with *Raphanus sativus* as an internal reference and (b) Cashew, *A. occidentale* with *S. lycopersicum* as an internal reference using Otto's buffer (Table 2). The coefficient of variations of *A. occidentale* G0/G1 peak = 3.56% and *S. lycopersicum* G0/G1 peak = 2.85% in Fig. 5a

internal reference standard plant for the estimation of relative nuclear DNA content (genome size) in cashew (Fig. 5a). On one hand, the G0/G1 histogram peaks of *Raphanus sativus* cv. Saxa and cashew nearly overlap (Fig. 5b), an indication that the genome sizes of these two plant species are very close and that *R. sativus* may not be suitable as an internal reference standard for estimation of nuclear DNA content or genome size in cashew (Dolezel and Bartos, 2005). On the other, the histogram G0/G1 peaks of *Glycine max* and cashew were about 2.5 folds apart (data not shown) which can also be used as reference standard in the absence of *S. lycopersicum*.

DISCUSSION

Because of dearth of information on the application of flow cytometry to cashew, investigation into the buffer systems, sample size and internal reference standards as a preliminary measure to develop a flow cytometric protocol suitable for the isolation of nuclear suspension for the cashew plant species was carried out. Preparation of suspension of intact nuclei for estimation of relative or absolute DNA amount has been universally performed following the method of Galbraith *et al.* (1983). This flow cytometric method involves mechanical homogenization of small quantity of fresh tissue (leaf, pollen, root etc.) in the lysis buffer for the extraction of nuclei for fluorescence analysis on a Flow Cytometer. Critical components in this process are buffer and the nature of sample. Plants are known to contain many chemical compounds that can interfere with the process of nuclei release during extraction. Dolezel and Bartos (2005) remarked that the composition of the buffer

is important in order to optimally isolate sufficient nuclei free of cytoplasm and secondary compounds that are capable of confounding DNA staining.

Given the different chemical compositions of the five buffers (Galbraith LB01, Otto, Tris-$MgCl_2$ and DAPI-MaVI) tested, each performed differently with Otto buffer being the most satisfactory in terms of quality of nuclear suspension obtained. Notwithstanding, it will too hasty to draw conclusion on the non-suitability of other buffers as the results might have been exacerbated by the presence of cytosolic compounds released during nuclei isolation that are capable of increasing background noise and give histogram with high coefficient of variation as recorded in LB01. Studies have shown evidence of high cytosolic compounds in woody plants. Particularly, cashew plant is known to contain a high content o f phenolic compounds and tannins (Lowor and Agyente-Badu, 2009). Such compounds are known to interact with nuclear DNA and/or the fluorochrome, thereby causing stoichiometric errors (Loureiro *et al.*, 2006b, 2007) during flow cytometric analysis. In this investigation, Otto's buffer with a 4', 6-diamidino-2-phenylindole (DAPI) fluorochrome has shown to be most appropriate lysis medium for ploidy analysis and estimation of relative nuclear DNA content of cashew, because it gave consistent results with very low coefficient of variation of G0/G1 peak (Fig. 2d, 3d). Loureiro *et al.* (2006b) opined that a reliable estimate of nuclear DNA content would require a balance between the digitized fluorescence signal and the DNA content and such hypothesis would depend on the stoichiometry of dye binding to DNA, accessibility of DNA to the fluorochrome, fluorescence absorption and linearity of the instrument amplification (Bagwell *et al.*, 1989). Plant inhibitors are other important secondary compounds that hinder DNA staining and decrease dye fluorescence of isolated nuclei (Price *et al.*, 2000).

Another compound that may reduce quality of nuclear suspension is tannin, a common phenolic compound mostly found in various tissues of woody plants. Studies have established correlation between tannins and stoichiometric errors in the estimation of genome size in woody plants (Greilhuber, 1986, 2005; Favre and Brown, 1996; Loureiro *et al.*, 2005). Incidentally, high content of tannins has been reported for cashew plant (Michodjehoun-Mestres *et al.*, 2009). Moreover, the choice of young leaf for nuclei isolation in this investigation in addition to incorporation of **β**-mercaptoethanol into the buffer compositions was to minimize the effect of secondary compounds like tannins. However, Loureiro *et al.* (2006b) studying the effect of tannic acid on plant nuclei and nuclear DNA content had established that different nuclear isolation buffers gave variable resistance to the negative effect of tannins and concluded that the basis for such phenomenon has not been proven. Based on this buffer resistance-hypothesis, it can be adduced that least quality of nuclear suspension derived from LB01 buffer can be attributed to less resistance to confounding effect of secondary compounds like tannins. Furthermore, the improved quality of nuclear suspension from Otto and DAPI-MaVI buffer may be due to the presence of citric acid in their compositions. Dolezel and Bartos (2005) opined that citric acid has the capacity to enhance better chromatin accessibility and improve staining of the DNA in nuclei isolation medium. Corroborating the result obtained in this investigation, Emshwiller (2002) reported Otto buffer as most suitable for ploidy analysis of *Oxalis*, a highly acidic plant like cashew. The author however, remarked that the failure of Galbraith and LB01 buffers can probably be attributed to the fact that the buffering capacity of these buffers had been exceeded.

Two-step nature in the application of Otto's buffer i.e., isolation of in lysis Otto I buffer before staining the isolated nuclei in Otto II may confer advantage on it over other buffers, as concentration of intact nuclei available for DNA staining will be higher in comparison to others.

Excellent performance of Otto buffer has been widely reported for many species especially those with lower nuclear DNA content (genome size) (Dolezel *et al.*, 1992, 1998; Dolezel and Bartos, 2005; Loureiro *et al.*, 2006a, b; Loureiro *et al.*, 2007). Although, the coefficient of variation (i.e.,<5%) of G0/G peak recorded for the Otto buffer in this investigation is acceptable for the woody plant species like cashew, further improvement is still possible to achieve better results i.e., lower CV with less background noise. Addition of compound like polvinylpyrrolidone would further reduce negative effect of cytosolic and phenolic compounds (Blondon *et al.*, 1994; Yokoya, 2000; Thiem and Sliwinska, 2003) and improve the quality of the nuclear suspension.

In addition to the composition of buffer, size of leaf tissue was found to also influence the quality of nuclear suspension derived from cashew. Leaf tissue of about 70 mg (60-80 mg) was found ideal for the recovery of sufficient nuclei with minimal background noise. It was obvious that further increase in sample size compromised the quality of the nuclear suspension in all ramifications. Increasing cashew leaf tissue (a highly acidic plant with tannins) will amount to increasing cytosolic compounds in the isolation medium and the effect on the quality of nuclear suspension will be dire when the buffering capacity of the buffer is exceeded. This probably explains the trend observed in this study. Arumuganathan and Earle (1991) recorded similar effect of large sample size on quality of nuclear suspension in pea, papaya and citrus. Although, only leaf tissue was used during this investigation, it is however, not impossible that other tissues especially reproductive parts that may contain less phenolic compounds may be more ideal for flow cytometric analysis in cashew. The incubation window for achieving quality nuclear suspension in cashew revolves around 10 and 15 min as obtained in this study. Delay in the cytometric analysis beyond 20 min is not advisable because of the rapid degradation of intact nuclei in the suspension after the incubation time has been exceeded. Such prolonged incubation could cause a reduction in fluorescence intensity and poor-quality measurements, especially in a buffer system where lysis and staining components are not separated as in Otto buffer (Dolezel and Bartos, 2005).

Vindelov *et al.* (1983) and Bagwell *et al.* (1989) hypothesized that an ideal DNA reference standard should have a genome size close to the target species in order to avoid risks termed as nonlinearity and offset errors. In this study, of the six plant species screened as reference standards for the estimation of relative nuclear DNA content in cashew, *Raphanus sativus* cv. Saxa-, *Glycine max* cv. Polanka and *S. lycopersicum* cv. Stupicke, with genome sizes of 1.11, 1.96 and 2.50 pg/2C, respectively, gave G0/G1 histogram peaks in the range of 1 to 2.5 folds to G0/G1 histogram peak of cashew, an indication of relative closeness between genome size of cashew and genome sizes of these three species. Although, *Raphanus sativus* was the closest to cashew in terms of G0/G1 histogram peak but the two peaks almost overlap (Fig. 5b), as such, it may not be an ideal reference standard for the estimation of nuclear DNA content in this plant species. Hence, for a reliable estimation of relative nuclear DNA content of cashew, *S. lycopersicum* cv. Stupicke with G0/G1 peak at almost 2 folds to cashew (Fig. 5a) would be the most ideal internal reference standard for this tropical tree species. The genome size of this tomato plant is known to be constant and the plants are genetically stable (Dolezel and Bartos, 2005).

The improved performance recorded for Otto's buffer with DAPI fluorochrome in this study makes it a suitable flow cytometric buffer system for the ploidy analysis and estimation of relative nuclear DNA content in cashew. In addition, the leaf tissue should not exceed 70 mg and isolated nuclei could be incubated for maximum of 15 min. It is important to mention that the Otto buffer and DAPI fluorochrome are not suitable for the determination of absolute genome size. For absolute genome size determination, Galbraith buffer with Propidium Iodide (PI) fluorochome has been reported as most suitable for many plant species.

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