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INVESTIGATING THE BEST SUITABLE NUCLEAR ISOLATION BUFFER FOR POTATO FLOW CYTOMETRY

Hemant Balasaheb Kardile[1]*, Nirmal Kant Sharma[1], Rahul[1], Ankita Sharma[2], Salej Sood[1], Satya Narayan Jena[3], Som Dutt[1], Jagesh Kumar Tiwari[1], Vinay Bhardwaj[1], Satyender Yadav[4], Swarup Kumar Chakrabarti[1]

ABSTRACT: Potato harbors great amount of diversity in terms of its ploidy. The progress of breeding programme is often limited by the hybridization barriers confronted due to differences in ploidy. Hence, understanding the DNA ploidy is of prime importance. The DNA ploidy can be estimated using flow cytometry. Great choices of nuclear isolation buffers (NIB) are available for sample preparation in flow cytometry. Selecting the best suitable among them is critical and also govern the success of experiment. Here, we tested five modified NIBs to identify the best suitable one for potato sample preparation. Investigation reveals best suitability of modified HPI buffer for flow cytometry in potato. The same buffer was used to estimate the genome size and ploidy of randomly selected potato samples.

KEYWORDS: DNA ploidy, 2C DNA content, modified HPI buffer

INTRODUCTION

The cultivated potato is autotetraploid with basic chromosome number of 12 (X=12). It belongs to the genus *Solanum*, with 1,500–2,000 species and their ploidy ranges from diploid to hexaploid (Bradshaw *et al.,* 2006, Watanabe, 2015). Various reasons are attributed for enormous diversity, which has been summarized earlier by Machida-Hirano (2015). Historically, the available genetic diversity has been successfully exploited for futuristic breeding programmes. These wild relatives are valuable genetic resources for breeding the biotic and abiotic stress tolerant varieties. Their utility in breeding programme is often hampered by cross-hybridization barriers, generated due to the differences in endosperm balance number (EBN) and ploidy (Johnston *et al.,* 1980; Johnston and Hanneman, 1980). Hence, understanding the ploidy is of utmost important for breeder. Apart from this, genome size information is equally important in the era of next generation breeding. The DNA ploidy and genome size are estimated using flow cytometry. At present it is globally accepted and most widely used method for the genome size and DNA ploidy estimation (Dolezel *et al.,* 2007). The technology has potential applications in plant sciences for identification of aneuploids, somatic hybrids and mixoploidy (Kron *et al.,* 2007). Various sample preparation methodologies so far developed are based on the methodology given by the Galbraith *et al.,* (1983). They all share steps in common and involve isolation of intact nuclei followed by the staining of the nuclear DNA with fluorochromes. Among these steps, isolation of intact nuclei is critical and largely governed by the choice of nuclear isolation buffer (NIB). Number of NIBs are

[1]ICAR-Central Potato Research Institute, Shimla, 171001 Himachal Pradesh, India
[2]Dr YSR university of Hortticulture and Forestry, Nauni, Solan, HP, India
[3]CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow, India
[4]Potato Technology Center, Shamgarh, Karnal, Haryana, India
Corresponding author: kufrihemant@gmail.com

available and selecting the best suitable among them for potato leaf is empirical. With this objective, the present investigation was carried to identify the best suitable NIB for potato cytometry using the leaf tissues. We tested all the available NIBs in their modified form so as to use them in single step method.

MATERIALS AND METHODS

Plant Material and Equipment

Zea mays and *S. chacoense* was taken as external reference DNA standard for estimation of genome size and DNA ploidy respectively. The tetraploid potato variety, Kufri Girdhari was used to select the best suitable NIB. Seventeen randomly selected potato clones were used for estimation of genome size and DNA ploidy using the identified best suitable NIB. The analysis was performed using flow cytometer, BD FACS Canto II™.

Modified NIBs

The modified NIBs were prepared by adding the propidium iodide (PI) (50µg/ml) and RNase (10mg/ml) to the composition of NIB reported earlier in literature (Dolezel *et al.,* 2007; Krishan, 1975). Five NIBs namely modified LB01, modified Tris-$MgCl_2$, modified Galbraith's, modified HPI and modified Otto buffer were used in the study. The 50 ml modified HPI buffer was prepared in distilled water by adding trisodium citrate dihydrate (0.05 gm), triton X-100 (150µl), RNase (2.5 ml), PVP (0.5 gm). The solution was sterilized using filter sterilization, and β-mercaptoetanol (15 µl) and PI (1.25 ml) were added to the filtrate.

Selection of best suitable NIBs

The external standard method of estimating the DNA ploidy and genome size was used to identify the best suitable NIB for potato leaves. The intact nuclei suspension of tetraploid potato cultivar, Kufri Girdhari was prepared for each buffer (test samples). The young leaf (20 mg) of test sample was taken in plastic petri plate and chopped with each modified NIB (800µl) using surgical blades. The samples were chopped on ice cold petri dish under dark conditions. The samples were then filtered through the 40 µm filters and the homogenate was incubated for 15 minutes on ice under dark condition with occasional shaking (**Fig. 1**). The samples were analyzed for estimation of DNA ploidy and genome size using diploid, *S. chacoense* and *Zea mays* as reference DNA standard respectively. The methodology for preparation of intact nuclei suspension for reference standard remains same as that of test samples. The test sample and reference standard were analyzed separately on flow cytometer with same instrument gain settings. The reference standard were first analysed in the flow cytometer and positioned the G_1 peak on the absicca. Similarly, Kufri Girdhari (test samples) was analyzed and positioned the G_1 peak without changing the instrument gain settings. The DNA ploidy and genome size was calculated as per the formula: Sample ploidy (integer) = Reference ploidy × mean position of the G_1 sample peak/Mean position of the G_1 reference peak.

The best performing NIB was used to estimate the ploidy and genome size of randomly selected potato clones using same methodology. It only differs for reference standard used in analysis. *A Zea may was* used as the reference DNA standard for genome size estimation. The genome size of test samples was estimated using formula (Dolezel *et al.,* 2007): Sample 2C value (DNA pg or Mbp) = Reference 2C value × sample 2C mean peak position/ Reference 2C mean peak position. The 2C DNA content in pg can be converted into the Mbp and vice-versa using the formula, genome size (bp) = (0.978

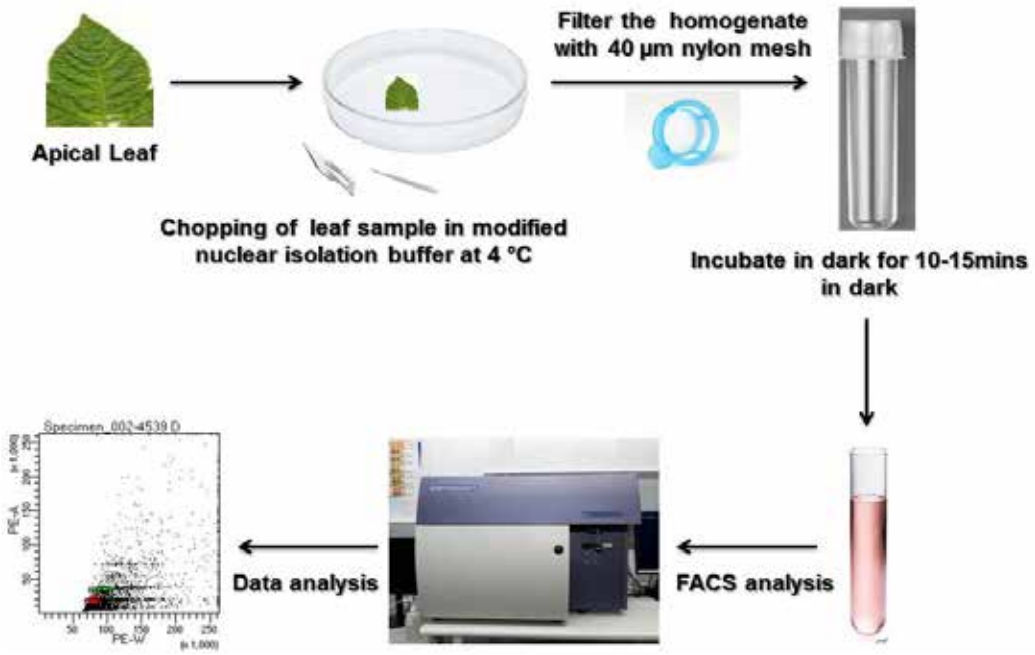

Fig 1. Methodology for flow cytometry analysis in potato for estimation of DNA ploidy and genome size.

x 10^9) x DNA content (pg), DNA content (pg) = genome size (bp) / (0.978 x 10^9), 1 pg = 978 Mbp.

Statistical analysis

Anova was carried out as per the CRD design for statistical differences among the treatments using SAS software in Indian NARS statistical computing portal.

RESULTS AND DISCUSSION

Examining the best suitable NIBs

The significant differences were observed for ploidy in five different modified NIBs. Among them, modified HPI buffer gave the best results with average ploidy of 3.90, which was near to expected ploidy (approximately equal to four) (**Fig. 2** and **Table 1**). The population of G_0/G_1 and G_2 cells were well separated (**Fig. 3A** and **B**) and the average mean position of G_2 (63025.33) was approximately double than that of G_0/G_1 (32516.33) peaks (**Fig. 3C**). Apart from this, samples tested with Galbraith's buffer also performed well with average ploidy of 3.8 but the separation of population on absisica was not good and Per cent CV was high. The samples tested with modified Otto buffer did not perform well as samples prepared with this buffer failed to record the events. The intact nuclei suspension prepared with the modified HPI buffer for

Table 1. Average ploidy estimated with five modified NIB in potato cv. Kufri Girdhari.

Modified IB/replicates	Modified LB01	Modified Galbraith's	Modified Tris.$MgCl_2$	Modified HPI	Modified Otto
KG1	3.87	4.69	1.65	3.94	11.32
KG2	3.34	1.92	3.49	4.02	0
KG3	3.39	5.02	1.35	3.76	9.12
Avg. Ploidy	3.53	3.87	2.16	3.90	6.81

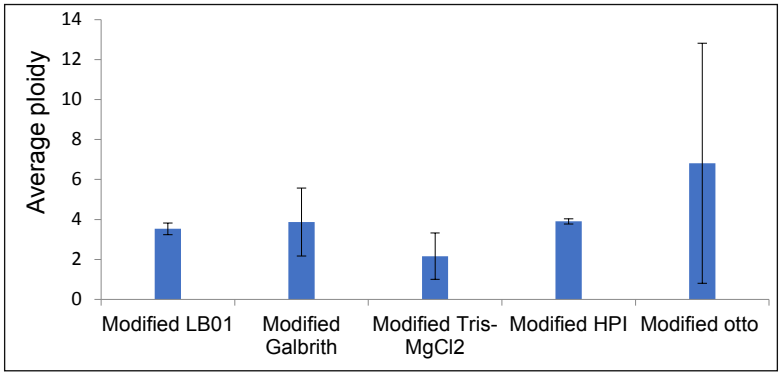

Fig 2. Estimated Average ploidy with five modified NIBs.

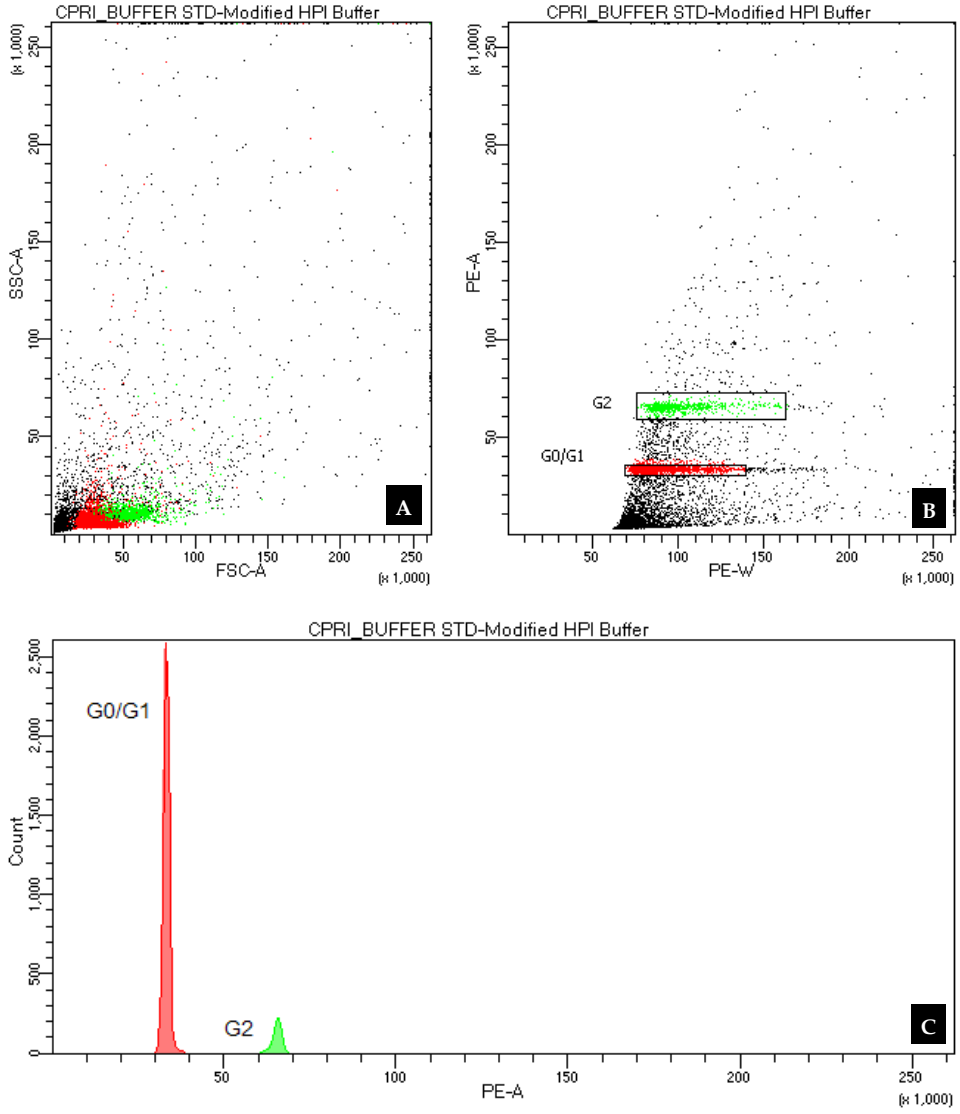

Fig. 3. A. Dot plot showing the forward and side scattered pattern for population potato cv. Kufri Girdhari prepared with modified HPI buffer. B. G_0/G_1 and G_2 population plotted on the dot plot of PE-W Vs PE-A. C. Histograms showing the G_0/G_1 and G_2 peak.

genome size estimation as performed well. The estimated genome size for the tetraploid potato clone Kufri Girdhari was 3.51 and in terms of base pairs it is 3436.60 Mbp. The results were in line with the earlier reports on genome size of monoploid potato. The genome size of monoploid potato was estimated to be is 840 Mbp and it expected to be 3360 Mbp for tetraploids (Xu *et al.,* 2011). Each buffer performed differently for ploidy value estimation as observed from the analysis of variance. Modified HPI buffer was the most suitable in terms of the closeness of the observed values for the ploidy. The same buffer has also showed expected genome size for tetraploid potato cultivar Kufri Girdhari. Next to the modified HPI, the modified Galbraith buffer performed well with the potato leaf samples. The same buffer in its original composition has been used previously for flow cytometry using the leaf and tuber samples (Uijtewaal, 1987; Laimbeer *et al.,* 2017). The other NIBs did not find suitable for intact nuclei isolation from potato leaf. It may be because of chemical composition of the NIBs, and their interaction with secondary metabolites of potato released during the isolation process. These metabolites may interact differently with type of buffer, as the composition of each buffer is different. This may have increased background noise and coefficient of variation as recorded in modified Otto buffer. Apart from this, phenolic compounds like tannins directly interact with the nuclear DNA and or the DNA binding dyes, thereby causes the stoichiometric errors (Loureiro *et al.,* 2006; Loureiro *et al.,* 2007). It is also reported that plant inhibitors also hinder DNA fluorochrome interaction and thereby decreases the observed fluorescence of isolated nuclei (Price *et al.,* 2000). Studies have shown that potato contain a high content of glycoalkaloids (α-solanine and α-chaconine) (Korpan *et al.,* 2004) but its effect on the DNA and its interaction of fluorochrome binding is yet to be studied.

Modified HPI buffer best suitable for potato cytometry

The analysis revealed modified HPI is best suitable for the potato cytometry. Hence the same was used for preparation intact nuclei suspension of 17 randomly selected potato clones. The analysis revealed that 50 Per cent potato clones were diploid (Avg. ploidy =2.03) and rest were tetraploid (Avg. ploidy=3.98) with average genome size of 1.83 and 3.59, respectively (**Table 2**). The observed average per cent CV was less than three, and the G_1 and G_2 populations were well separated. For diploid potato clones the ploidy value ranges 1.93 to 2.14 whereas it remained in the range of 3.80 to 4.13 for tetraploid clones. The genome size

Table 2. Potato clones with their ploidy and genome size estimated with modified HPI buffer.

Potato clones	Ploidy		Genome size (2C)	Mbp
C13	2.14	Diploid	1.93	1888.763
SS2595	2.09	Diploid	1.88	1846.487
SS1725	2.01	Diploid	1.81	1772.611
SS1763	2.01	Diploid	1.81	1776.944
SS2332	1.99	Diploid	1.79	1751.79
SS2295	1.93	Diploid	1.73	1701.271
SS1767-0	4.06	Tetraploid	3.66	3580.839
SS1773-04	4.13	Tetraploid	3.72	3642.984
SS1773-01	3.87	Tetraploid	3.48	3411.314
SS-2289-0	3.80	Tetraploid	3.42	3351.494
SS1794-07	4.09	Tetraploid	3.68	3605.993
SS4540	1.99	Diploid	1.79	1760.14
SS4542	2.03	Diploid	1.82	1789.521
SS4541	2.11	Diploid	1.90	1860.121
SS1764-19	3.88	Tetraploid	3.49	3419.452
SS1770-06	4.05	Tetraploid	3.64	3566.254
SS4539	2.04	Diploid	1.84	1803.895

for diploid and tetraploid clones was in the range of 1.73 to 1.93Mbp and 3.42 to 3.72 Mbp, respectively.

CONCLUSION

Estimation of ploidy and genome size is important in a crop like potato where large amount of diversity is present in terms of ploidy. We tested five modified NIBs and found modified HPI buffer for best suitable for potato ploidy and genome size estimation using flow cytometry.

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