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Flow Cytometric Analysis for Ploidy and DNA Content of Banana Variants Induced By Gamma Irradiation

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***Abstract*: Nuclear DNA content of mutated banana plants was determined by using flow cytometric techniques. It is a powerful tool for large scale screening of ploidy levels. Nuclei were isolated from young leaves from (banana mutants & Glycine plants) supplemented with Propidium- iodide (PI) and RNAse. "Glycine max" used as internal reference standard for identifying the nuclear DNA content by FCM. For ploidy estimation DAPI was used. The results showed differences in DNA content between variants indicating the effect of gamma-irradiation on the genotype of these plants. Variants of short plant stature or stunted growth showed great differences in DNA content compared to control (non-irradiated). The phenotypic variations observed at high doses were likely due to changes in the DNA sequences at the chromosomal level. Nuclear DNA contents decreased with an increase of gamma-dose from 20 Gy to 60 Gy. However, there were no significant differences between DNA content at 20 Gy and 30 Gy and also between 40 Gy and 60 Gy, while they were differed significantly from the control. The results showed no significant differences in ploidy level between all samples used (3n); while all selected mutants (variants) showed differences in DNA content.**

***Keywords*: Flow cytometry , DAPI, Propidium- iodide , gamma-irradiation, Gy, Banana.**

I. INTRODUCTION

Bananas (*Musa spp.*) are the major export commodities of many developing countries and provide food for millions of people in the tropics and subtropics. The cultivated bananas are mostly triploid (2n=3x=33) and exhibit a marked degree of sterility. The progress of banana improvement by conventional breeding methods has been relatively slow or even hindered due to narrow genetic variability resulting from the low female fertility (Silva *et al.,* 2001; Pua, 2007; Bakry*et al.*, 2009). However, the current status of *Musa* biotechnology as reported by (INIBAP, 1992) is very close to making a significant contribution to the breeding of new cultivars. Mutation induction coupled with *in vitro* technique cause morphological changes and also increases variability in quantitative traits.

Physical mutagens such as gamma ray, are tools for enhancing and generating genetic variations by inducing mutations at the gene, chromosome and genome level, in nuclear and cytoplasmic organelle DNA (Mak*et al.* 1998; Larkin, *et al.*, 1998; Kodym and Afza, 2003; Wieczorck 2003). Somaclonal variation provides additional source of variation (Kaeppler*et al.,* 2000). FCM first developed for analysis of human cells has been adopted as a convenient tool for estimation of nuclear DNA content and ploidy level constitutions in plants (Dolezel, 1991& Dolezel, *et al.* 1994). At present, the technique is extensively used in basic and applied research (e.g. botany, zoology, microbiology, marine ecology, embryology, immunology, genetic and molecular biology) as well as in clinical diagnosis, medicinal practice and industry- including seed production and plant breeding (Valarik*, etal.,*2002) . The technique based on the use of DNA-specific fluorochromes and on analysis of the relative fluorescence intensity of stained nuclei (Gailbraith, *et al.* 1983 & Lister, 1990). During recent years, flow cytometry has established itself as a useful, quick novel method to determine efficiently, reproducibly and at a reduced cost per sample the relative nuclear DNA content and ploidy level of a large

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number of species (plants & animals), that has also been used to sort cells with different traits from a mixed cell population (e.g as when separating heterokaryons from parental protoplasts in protoplast fusion experiments for somatic hybrid production) and, more recently, for the analysis of the composition in various chemical components of different tissues (such as apoptotic markers bound to cell compartments) (Doleel, *et al.* 2000 & Sergio, 2006). The main advantage of flow cytometry assay are: rapidity, precision and convenience, no need for dividing cells, not destructive (requires small amount of tissue) and can be used for analysis of large populations of cells (detection of subpopulation–mixoploidy), in addition to computerized data processing.

The purpose of the present study was to use flow cytometry for determination of ploidy level and identification of nuclear DNA content variation in *Musa* (Berangan cv. Intan - AAA). Espcial attention was given to the occrrance of intraspecific changes in nuclear DNA content and genome level.

II. MATERAILS AND METHODS

A. Plant Materials and Sample Preparation:

Berangan cv. Intan (AAA) was the chosen variety in this study. Samples of different variants induced by gamma-irradiation were selected for ploidy analysis and DNA content. The shoot-tip meristem pieces were aseptically excised from micropropagated plantlets of Berangan. The meristem pieces were irradiated in a gamma cell with a Cobalt-60 (^{60}Co) source (GC 400A, 10 Kci). The doses included 0 (control), 20, 30, 40 & 60 Gy at a dose rate of 0.54 Gy/sec. The variants chosen for DNA analysis include those with short plant stature (stunted growth), late to flowering (or late in maturity) and abnormal bunch characteristic.For determination of ploidy level and identification of nuclear DNA content by FCM, nuclei were isolated from young leaves following the method developed by (Gailbraith, *et al.* 1983). Approximately 50 mg of young leaves from each sample (mutated Berangan& *Glycine* plants) were chopped up together with a sharp scalpel blade in a glass petri dish containing 1 ml LBOI lysis (Dolezel, 1991), supplemented with 50 ug/ml Propidium-iodide (PI) and 50 ug/ml RNAse. *Glycine max* was used as internal reference standard for identifying the nuclear DNA content by FCM. An intact interphase nucleus was released from the cut surface directly into 2-ml Lysis buffer supplemented with 0.1 mg/ml of 4,6-Diamidino-2-phenylindole (DAPI). The homogenate-stained nuclei were filtered through 50 µm nylon mesh into the analysis tube and its fluorescence was analyzed by FCM.

B. Sample analysis :

About 2 ml of the homogenate nuclei was analyzed in the Cell Counter Analyzer Cytometer (Model Partec CCA-II). The fluorescence was measured with a 100 W high-pressure mercury arc lamp as a light source filtered as mentioned above. The gain of the instrument was adjusted so that the G_0/ G_1 peak of standard was approximately on channel 100. The DNA genome size of the sample was then estimated using the ratio of G_1 peaks.

C. Nuclear DNA content in (pq) of Musa was estimated as:

Sample 2C nuclear DNA content [pg] = 2.5/ fluorescence ratio

Symbol C is used for DNA content of haploid set of chromosomes. *Glycine max* has

2C DNA content = 2.5 pg (Tiersch, *et al.* 1989).

Analysis of variance and statisticalanalysis for variants (at different irradiation doses) were employed to analyze the variation in DNA content.

III. RESULTS AND DISCUSSION

Determination of ploidy and DNA content:

Analysis of the relative fluorescent intensity of propidium-iodide stained nuclei yielded a histogram (Fig. 1) showing two dominant peaks corresponding to G_1 and G_2 nuclei of *Musa* and *Glycine max* cv. *Palmetto*, respectively. Estimation of ploidy level was done by external standardization procedure comparing the position of the G_1 peak on a histogram to that of a reference plant with known ploidy.

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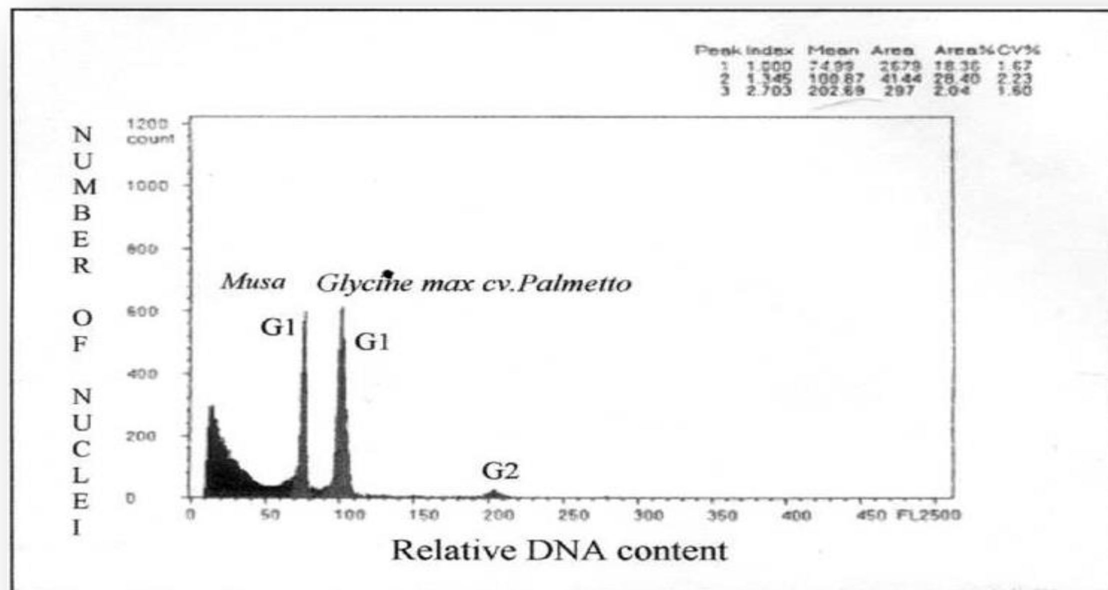

Fig. 1:Histogram of relative nuclear DNA content. Genomic DNA content were obtained after simultaneous analysis of Musa & Glycine max. Two peaks were obtained for Glycine max i.e. G1 & G2 compared to only one observed in Musa (Berangan) at channel 75. The relative DNA content for Musa was determined from the ratios of the G1 peaks of the reference standard and the samples.

The results showed no significant differences in ploidy level between all samples used (3n), indicating that irradiation at the doses used did not have an effect on ploidy. Dolezel, *et al.* (1995) and Valarik, *et al.* (2002), reported similar findings in his analysis of a large populations of nuclei (5-2000) of mutated African plantations in which the occurrence of even small subpopulations differing in ploidy level (mixoploidy) can be detected.The nuclei isolated from *Glycine max cv. Palmetto* (2n = 2.50 pg) were used as an internal reference standard. Most of the nuclei were in G_1 phase of the cell cycle. The first peak represented G_1 nuclei of *Musa,* where the second peak represented G_1 nuclei of*Glycine* (Fig. 1). However, G_1 peak of Berangan (cv. Intan) was positioned on channel 75, and the other peak of *Glycine max* was on channel 100. A third peak also appeared at channel 200 reflecting the G_2 phase of the reference internal standard nuclei. The majority of cells in full-grown plants do not participate in cell division and reside in a so-called G_0 (G is gap) stage of the cell cycle. At this stage the nuclear DNA content reflect the ploidy state of the plant. Cells which are involved in divisions start from a comparable so-called G_1 state and subsequently pass through S (= DNA-synthesis), G_2 (= an interphase nuclear stage with a doubled DNA content preceding the actual nuclear division). Accordingly nuclear DNA content in absolute units (genome size in picogram-pg DNA) was adopted for all samples used. Such estimation requires comparison with a reference standard having a known DNA content. In this study, *Glycine max* was as an internal standard because its genome size is relatively constant (Dolezel, *et al.* 1994; Vindelov, *et al.* 1983) and to avoid bias due to staining and instrumental changes when estimating nuclear DNA ploidy by flow cytometric analysis.

For the estimation of DNA content in *Musa*, young leaves (cigar leaf) were preferably used. Dolezel, *et al.* (1994) reported that optimum results (low CV of G_1 peaks and low background) could only be obtained when young leaf tissues were used. Furthermore the use of the LBOI buffer that contained mercaptoethanol as a reducing agent. It was found to be important to preserve browning of suspensions of nuclei due to phenolic compounds. For DNA content propidium iodide was used as DNA stain compared to DAPI for ploidy estimation. Propidium iodide quantitatively intercalates to DNA without base specifically (Crissman, *et al.* 1990).

DNA content of different types of variants induced by gamma-irradiation (mutants):

All selected mutants (short stature, late flowering and bunch characteristics) showed variation in DNA content (Fig.2). However, short plant variants displayed a wide range of DNA content (1.0% - 8.0%) as compared with control. The

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variants showing lateness to flowering showed moderate changes in DNA content compared to short stature variants, while bunch abnormalities variants showed slight to moderate changes. An effect of irradiation appears to be reflected by an increase in variation of nuclear DNA content (increase of coefficient of variation of G_1 and G_2 peaks).

The mean of the distribution of G_0/ G_1 nuclei represents a 2C (presynthetic level) DNA amount in relative units. The ratio of G_0/ G_1 peak means (*Musa/Glycine*) was calculated and DNA content of *Musa* was estimated according to the formula:

DNA content (Pg) = 2.5/peak ratio (Dolezel, et al. 1994)

Flow cytometric estimation of nuclear DNA content in absolute units (genome size in picogram-pg DNA) or Mbp was achieved by simultaneous measurement of Berangan samples and reference standard (nuclei) having a known DNA content (*Glycine max*, 2C = 2.50 pg DNA). The ratio of G1 peak means for non-irradiated control plants was *Glycine/Musa* i.e. G_1/ G_2, 100/75 = 1.333. The 2C DNA content of Berangan (AAA) was calculated using the ratio of G_1 peak means: 2.5/1.333=1.875 pg DNA or 1.875 ±0.02 pg. The estimated value of peak ratio of these plants did not differ from the mean value obtained by FCM analysis (1.85 pg). DNA content for non-irradiated and gamma-irradiated samples were highly significant different ($p \leq 0.01$) as shown by ANOVA. Also statistical analysis of results for estimation of nuclear DNA content showed high variations (Table 1). Whereas, the non-irradiated showed lowest mean values ranging between 1.823 to 1.872 with an average mean of 1.8460, indicating that DNA content for these plants are similar to the standard value of *Musa.* The effect of gamma treatment was clearly observed in the mutated plants especially at high doses.However, the difference in DNA content from control was 1 -2 fold as shown in (Table 1, Table 2 & Fig 3). However, the DNA content at 20 & 30 Gy differed from control plants by 2.5% and 2.8% respectively. While the other variants showed more changes (4.7% and 4.5%) for 40 Gy and 60Gy treatments respectively.

The variation in DNA content means of different variants is shown in Table 2. Variants of short plant stature or stunted growth showed higher DNA content compared to control followed by late maturing variants. DNA content of late maturing variants that showed moderate changes (approximately 1.5%) from the control. Whereas, variants of bunch-abnormalities showed only slight changes in DNA content compared to the control. The analysis showed that phenotypic variation due to mutagensis is reflected in the DNA content of plants. Moreover, the difference in DNA content indicated the effect of gamma-irradiation on the genotype.

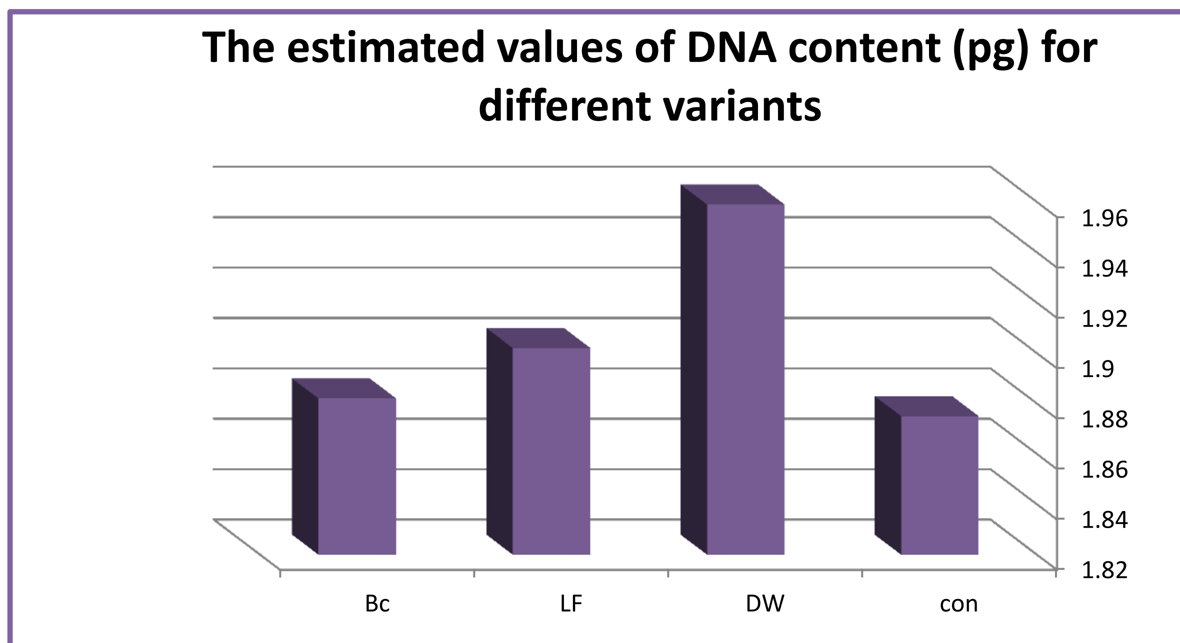

Fig. 2: The statistical analysis of results obtained by flow cytometric estimation of nuclear DNA content in Musa (Berangan) for different types of selected variants.

1 Pg = 965 Mbp (Bennett & Smith, 1976) ;Con : control (SE = 0.014) ; DW : dwarf variant (SE = 0.029) ; LF : Late to flowering (SE = 0.022) ;BC : Bunch characters (SE = 0.039)

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Table 1: Summary and statistical analysis of results obtained by flow cytometric estimation of nuclear DNA content in *Musa* (Berangan, AAA) at different Gamma-doses.

Irradiation doses (Gy)	2CDNA content values (pg)	1CDNA content values (Mbp)	Differences (%) from control	± SE
0 (Control)	1.8460	890.70	-	0.0018
20	1.8913	912.60	21.9 (2.5%)	0.0045
30	1.8980	915.80	25.1 (2.8%)	0.0050
40	1.9327	932.50	41.8 (4.7%)	0.0054
60	1.9296	931.03	40.3 (4.5%)	0.0093

** 1Pg = 965 Mbp (Bennett and Smith, 1976).*

S.E for the Total = 3.232E-03

Van Harten (1998), stated that at least 90% of the radiation-induced mutations refers to deletions. However, as shown in this study, the high phenotypic changes at 40 Gy and 60 Gy treatments might be due to broken segments lost or become attached somewhere on a chromosome (translocation).The summation of these findings makes of flow cytometry an interesting tool for the early prediction of both the regeneration competence from undifferentiated tissues and also of the further fertility of the regenerants obtained. These uses add to the better known utilization of flow cytometry for the characterization of plants, tissues and regenerants in terms of ploidy level, of nuclear DNA content and of division frequency (through the detailed analysis of the cell cycles).

Table 2: Summary and statistical analysis of results obtained by flow cytometric estimation of nuclear DNA content in *Musa* (Berangan) for different types of selected variants

Variants	2C DNA Content (pg)	1C DNA Content (Mbp)	SE	Differences (%) from control
• Control	1.8750	904.6	0.140	
• Dwarf &stunted growth	1.9595	945.5*	0.029	40.9(4.5%)
• Late to flowering	1.9024	917.9*	0.022	13.3(1.5%)
• Abnormalities in bunch character	1.8828	908.5	0.039	3.9(0.43%)

1pg = 965 Mbp (Bennett & Smith, 1976)

**Significant at $p<0.05$*

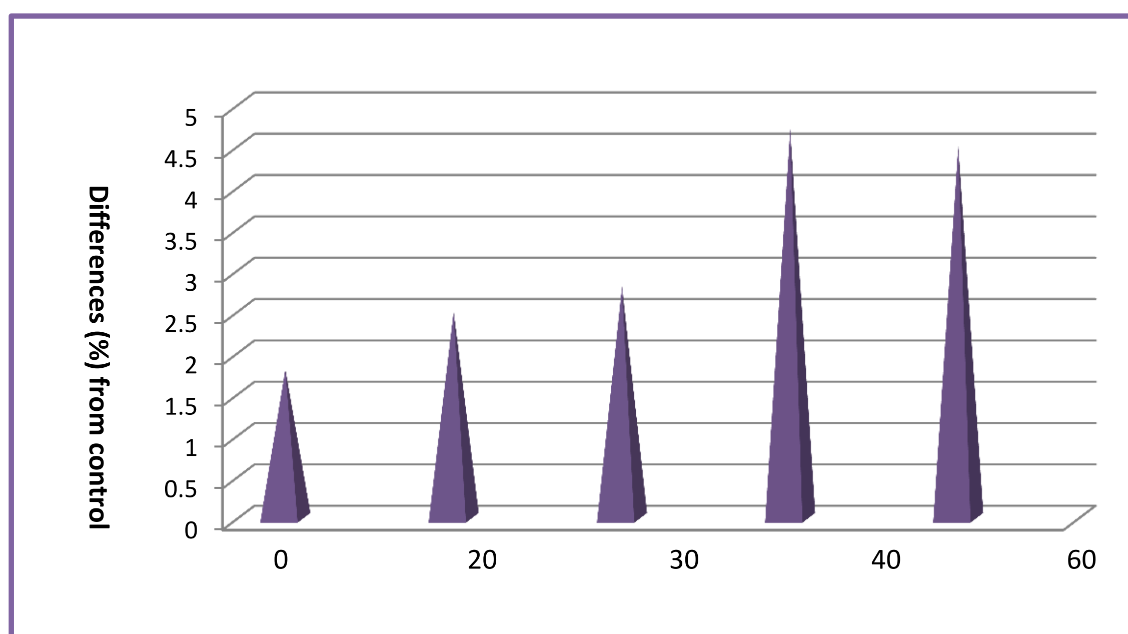

Fig. 3: Statistical analysis of results obtained by FCM estimation of nuclear DNA content in (Berangan) at different Gamma doses.

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IV. CONCLUSION

Flow cytometry has become a powerful tool for the study of plant genomes with applications ranging from basic research to industrial uses. It may be expected that the number of practical applications will increase and flow cytometry will be even more extensively used by plant breeders and seed companies. For breeding of Berangan, the technique has immediate application for selection of potentially useful mutants. Monitoring of mutagen-induced DNA damage remains an unexplored possibility. Acording to the recent results, it may be expected that flow cytometric sorting of chromosomes will play an increasingly important role in the analysis of plant genome structure and function.

Abbreviations:

DAPI : 4,6- Diamidino-2- phenylindole

FCM :Flow cytometry

Gy : Gray- unit of radiation rate

INIBAP: International Network for the Improvement of Banana and plantain

Mbp : Mega base pair

Pg : picogram

PI : propidium Iodide

µg : microgram

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