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Rapid detection of aneuploidy in *Musa* using flow cytometry

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Abstract We report a procedure for the rapid and convenient detection of aneuploidy in triploid *Musa* using DNA flow cytometry. From a population of plants derived from gamma-irradiated shoot tips, plants were selected based on aberrant morphology and their chromosome numbers were counted. Aneuploids plants with chromosome numbers 2n=31 or 32 were found as well as the expected triploid plants (2n=3x=33). At the same time, the nuclear DNA content of all plants was measured using flow cytometry. The flow cytometric assay involved the use of nuclei isolated from chicken red blood cells (CRBC), which served as an internal reference standard. The relative DNA content of individual plants was expressed as a ratio of DNA content of CRBC and *Musa* (DNA index). In order to estimate the chromosome number using flow cytometry, the relative DNA content of plants with unknown ploidy was expressed as a percentage of the DNA content of triploid plants. The classification based on flow cytometry fully agreed with the results obtained by chromosome counting. The results indicated that flow cytometry is a convenient and rapid method for the detection of aneuploidy in *Musa*.

Keywords Banana · Flow cytometry · Nuclear DNA content · Gamma irradiation

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Introduction

Bananas (*Musa* spp.) are a staple food for hundreds of millions of people in the tropics, with world production reaching around 95 million tons per year (FAO 2000). In the developing world, they rank fourth among food crops (after rice, wheat and maize). In contrast to their socio-economic importance, bananas have largely been neglected in terms of research and breeding (Ortiz 1995), which has largely been due to inherent difficulties with *Musa* genetics and a lack of investment in research. Conventional breeding in *Musa* is difficult due to triploidy and parthenocarpy (Rowe 1984).

Mutation induction techniques appear to be a logical approach for improving seed-sterile *Musa* species, which lack sexual reproduction for generating genetic variation (De Langhe 1969, 1987; Krikorian and Cronauer 1984). De Guzman et al. (1982) reported a low efficiency and a low yield of mutants after in vivo irradiation of suckers and the subsequent in vitro culture of meristem tips isolated from the suckers. A combination of mutation induction and in vitro culture (also called in vitro mutagenesis) has been more effective for the induction and selection of induced somatic mutations. Methods of in vitro mutagenesis have been pioneered by the International Atomic Agency Laboratories (Novak et al. 1990). Nevertheless, the selection of desirable mutants is hampered by the occurrence of multiple mutational events in individual genomes (Cassells 1998). Therefore, the breeder must select desirable mutants immediately after inducing genetic variation.

Aneuploidy, which involves an under- or over-representation of one or more chromosomes, is a frequent type of mutation in *Musa*. Shepherd et al. (1996, 1999) reported a surprisingly high level of chromosome number variation in plants propagated with suckers as well as by in vitro techniques. Reuveni et al. (1986) characterized the 'mosaic type' variant by a high percentage of aneuploid cells. Aneuploid *Musa* plants were also generated from 3x × 2x sexual crosses (Osuji et al. 1997). Recently, Shepherd and Bakry (2000) evaluated the impact

of gamma irradiation on chromosome loss in the cultivar Maçà (AAB).

Aneuploidy in *Musa* has traditionally been detected by chromosome counting (Sandoval 1996; Shepherd et al. 1996), which is a time-consuming and laborious procedure. The method is not practical for large-scale screening (Fahleson et al. 1988) and does not reflect the ploidy of all three histological layers (LI, LII and LIII), since only root-tip meristems (representing the LIII layer) are usually used for chromosomal analysis. Measurement of the nuclear DNA content by flow cytometry has been suggested as an alternative (Dolezel 1998). The method is being increasingly used for large-scale ploidy screening and has already been well-established in *Musa* spp. (Dolezel et al. 1994, 1997). Bashir et al. (1993) showed that flow cytometry is quite sensitive in detecting the presence of a pair of rye chromosomes in wheat-rye addition lines. Pfosser et al. (1995) demonstrated that this method might detect the presence of a pair of rye chromosome arms added to the wheat-rye addition lines. Flow cytometry has an advantage over the traditional chromosome counting technique in that it can be used to screen many plants in a short time and can be applied to any plant tissue.

The aim of the investigation reported here was to develop an efficient system for the rapid detection of aneuploidy in *Musa*. We used in vitro mutagenesis to generate aneuploid plants, which were subsequently characterized both by chromosome counting and by DNA flow cytometry. The results of both analyses were correlated and indicated that flow cytometry is a suitable method for the rapid detection of aneuploidy in *Musa*.

Material and methods

Plant material

In vitro plantlets of the triploid (2n=3x=33) clone 'Grande Naine' (ITC.1256) were obtained from the Musa Germplasm Transit Center, International Network for the Improvement of Banana and Plantain (INIBAP), Katholieke University of Leuven, Belgium.

Propagation method

The shoot tips were multiplied according to Novák et al. (1990). Shoot-tips (5–10 mm long) consisting of a meristematic dome with two to five leaf primordia were cultured on MS minimal inorganic medium (Murashige and Skoog 1962) supplemented with 1 mg l^{-1} thiamine, 20 µ*M* 6-benzylaminopurine (BA), 40 mg l^{-1} cysteine and 40 g l^{-1} sucrose. The pH of the medium was adjusted to 5.8 with NaOH prior to autoclaving at 121 °C and 100 kPa for 20 min. The cultures were grown in Magenta boxes (Magenta, Chicago, USA) containing 50 ml multiplication medium solidified with 2 g l^{-1} Gelrite (Kelco, San Diego, USA). Cultures were grown in a growth chamber at 27±1°C under a 16/8-h (day/night) photoperiod with a light intensity at the surface of 60 µmol m^{-2} s^{-1}. Five proliferating plantlets were multiplied until a minimum of hundred shoot tips was obtained after three subcultures.

Mutagenesis

After 30 days of growth, meristem tips containing two to three pairs of leaf primordia were excised from in vitro-growing shoots for the gamma irradiation experiments. Excised explants were transferred to sterile Petri dishes 5 cm in diameter (ten explants/dish) containing a few drops of sterile water and the plates sealed with Parafilm. The shoot tips were irradiated in a gamma cell with a ^{60}Co source at 35 Gy at a dose rate of 35 Gy min^{-1}. Immediately after irradiation, the explants were placed onto fresh multiplication culture medium. One hundred explants were irradiated. Each growing shoot was separately multiplied in vitro to generation M_1V_4, i.e. by three subcultures in the multiplication medium. Roots were induced on full-strength MS basal medium supplemented with 1 µM BA, 1 µ*M* indole acetic acid (IAA), 230 mg l^{-1} potassium phosphate, 100 mg l^{-1}ascorbic acid and 30 g l^{-1}sucrose solidified with 2 g l^{-1}Gelrite. The rooted plantlets were acclimatized in Jiffy pots containing a composted soil mixture. Twenty-five plants derived from irradiated shoot tips showing altered morphology were selected at the six-leaf stage and compared with ten control plants regenerated from non-irradiated cultures.

Chromosome counting

Slides for chromosome counting were prepared according to Dolezel et al. (1998). Meristems of actively growing roots were treated with 0.05% 8-hydroxyquinoline for 3 h at room temperature and fixed in a fixative (ethanol: acetic acid, 3:1) overnight. The roots were stored in 70% ethanol at 4°C for up to several months. Meristem tips of 5–15 roots per plant were digested for 60 min at 30°C in an enzyme mixture consisting of 1% pectinase (Sigma P-2401, St. Louis, USA), 0.5% pectolyase (Sigma P-3026) and 0.5% cellulase (Serva 16419) made in 0.1 *M* citrate buffer (pH 4.7). The suspension of released protoplasts was filtered through a 150 µm nylon mesh and washed in 75 m*M* KCl and 7.5 m*M* EDTA (pH 4). The protoplasts were then stored in 70% ethanol at –20°C for up to several months.

For slide preparation, 7 µl of protoplast suspension in 70% ethanol was dropped onto an ice-cold slide. The suspension was allowed to spread out and air-dry. Just before it completely dried up, 7 µl ice-cold fixative (ethanol: acetic acid, 3:1) was added to the drop to induce cell bursting. Again just before the suspension completely dried out, the slide was briefly rinsed in 100% ethanol and air-dried at room temperature. Chromosomal DNA was stained with 100 µl 4,6-diamidino-2-phenylindole (DAPI) (2 µg ml^{-1}) on the slides for 10 min. The stain was removed by washing the slide with 2× SSC and the slides mounted in fade solution (Vectashield, Vector, Burlingame, USA) and observed under a fluorescence microscope (Nikon, Eclipse E-800). The chromosomes were counted under a 100X/1.35 oil immersion objective. Images were captured using a CCD camera and processed using image analysis software (Lucia, version 4.21). At least ten metaphase cells showing well-scattered and contracted chromosomes were counted for each plant.

Flow cytometric analysis

Flow cytometric analysis was performed with a PA-I flow cytometer (Partec, Münster, Germany). Samples were prepared according to Dolezel et al. (1994, 1997). Between 20 and 30 mg of freshly cut midrib of the first open leaf was chopped with a sharp razor blade in a plastic Petri dish containing 0.5 ml OTTO I buffer consisting of 0.1 *M* citric acid and 0.5% Tween 20 (Dolezel and Göhde1995). The sample was filtered through a 50-µm nylon mesh. Chicken red blood cell nuclei (CRBC) were prepared according to Galbraith et al. (1998) and added to the suspension of released nuclei as an internal reference standard. To stain the nuclear DNA, we added 2 ml OTTO II buffer containing 0.4 *M* sodium hydrogen phosphate and 5 µ*M* DAPI (Dolezel and Göhde

1995) to the suspension of released nuclei. The gain of the instrument was adjusted so that the G_o/G_1 peak of the CRBC nuclei was positioned approximately at channel 100. The relative DNA content of *Musa* was then determined by comparing the peak positions of CRBC nuclei and nuclei of the sample. In each sample, 5,000–10,000 nuclei were analyzed. At least six measurements were made for each plant (two different days, each with three replicates).

Theoretical considerations

The probability of detecting an aneuploid plant by flow cytometry depends on the precision of the measurement, which is characterized by the coefficient of variation of DNA peaks on the histograms of nuclear DNA content, and on the difference in DNA content between aneuploid and euploid plants (Dolezel 1997). In triploid *Musa*, an average chromosome represents approximately 3% of the total DNA content. When nuclei isolated from aneuploid and triploid plants are analyzed simultaneously, only analyses resulting in coefficients of variation of the DNA peaks that are lower than half of the difference in DNA content (i.e. 1.5% for triploid *Musa*) enable discrimination of the peak representing aneuploid G_1 nuclei. In practice, it is very difficult to achieve regularly a coefficient of variation less then 1.5%.

Another possibility for detecting aneuploidy is by comparing the DNA content of a sample with that of an internal reference standard. The standard should have a DNA content similar but not equal to that of the sample. The 2C DNA content of triploid *Musa acuminata* var. 'Grande Naine' is 1.905 pg DNA (Lysak et al. 1999). According to Galbraith et al. (1983), the 2C DNA content of chicken (*Gallus domesticus*) is 2.33 pg. Therefore, CRBC nuclei seem to be a suitable internal standard for triploid *Musa* (Fig. 3). A further advantage of using CRBC nuclei is the ease of preparation and stability of isolated nuclei during long-term storage (Galbraith et al. 1998). In this study, CRBC nuclei were used as the internal reference standard. Relative DNA content of individual plants was expressed using a DNA index (DI) calculated according to the formula:

$$\mathrm{DI} = \frac{\text{Mean of the relative DNA content of the } G_0/G_1 \text{ nuclei of the sample}}{\text{Mean of the relative DNA content of the } G_0/G_1 \text{ of CRBC nuclei}}$$

Results

Among the banana plants regenerated after irradiation, 25 individuals were selected on the basis of their abnormal morphology. Using the descriptor for banana (*Musa* spp.) and the Musalogue (IPGRI-INIBAP/CIRAD1996), we characterized the plants for height, leaf shape and color and pseudostem color (Table 1). Examples of a control plant and two off-type plants are shown in Fig. 1, A1, B1, C1.

Table 1 List of 25 plants selected for altered morphology at the M_1V_4 generation from irradiated shoot tips and one plant var. 'Grande Naine' (control) with a normal phenotype selected from non-irradiated cultures. The terms were used according to the descriptor for banana (*Musa* s*pp*.) and the Musalogue (IPGRI-INIBAP/CIRAD 1996)

Plant no.	Variant characteristics:			
	Height	Leaves	Pseudostem	Other
Control	Intermediate	Dark-green with red-purple blotches	Green-red	
1				Abnormal phyllotaxy
2				Abnormal phyllotaxy
3		Narrow, elongated		
4	Dwarf	Erected,black streaks on limb and petiole	Black streaks	
5		Deformed and drooping, black streaks	Black streaks	
6		Narrow, elongated		
7	Short internodes			Abnormal phyllotaxy
8	Giant	Narrow, elongated	Black streaks	
9	Dwarf	Narrow, elongated and drooping		
10	Dwarf, short internodes	Mosaic (yellow-green)	Light-green	
11	Giant	Narrow, elongated	Long petioles, with dark-red margins	
12		Elongated		Abnormal phyllotaxy
13	Dwarf	Narrow, elongated and drooping		
14		Variegated or deformed leaves ("Massada type")		
15	Dwarf, short internodes			
16	Dwarf			Abnormal phyllotaxy
17		Redish midrib	Petiole with dark-red margins	
18	Dwarf	Red ventral and dorsal surface	Dark-red	
19	Giant, long internodes	Narrow elongated		
20	Dwarf	Black streaks on limb and midrib	Black streaks	
21	Dwarf	Petiole with red edges		
22		Deformed, mosaic type	Black streak	
23	Dwarf		Dark-green	
24		Larger leaves without red blotches		
25			Black blotches	

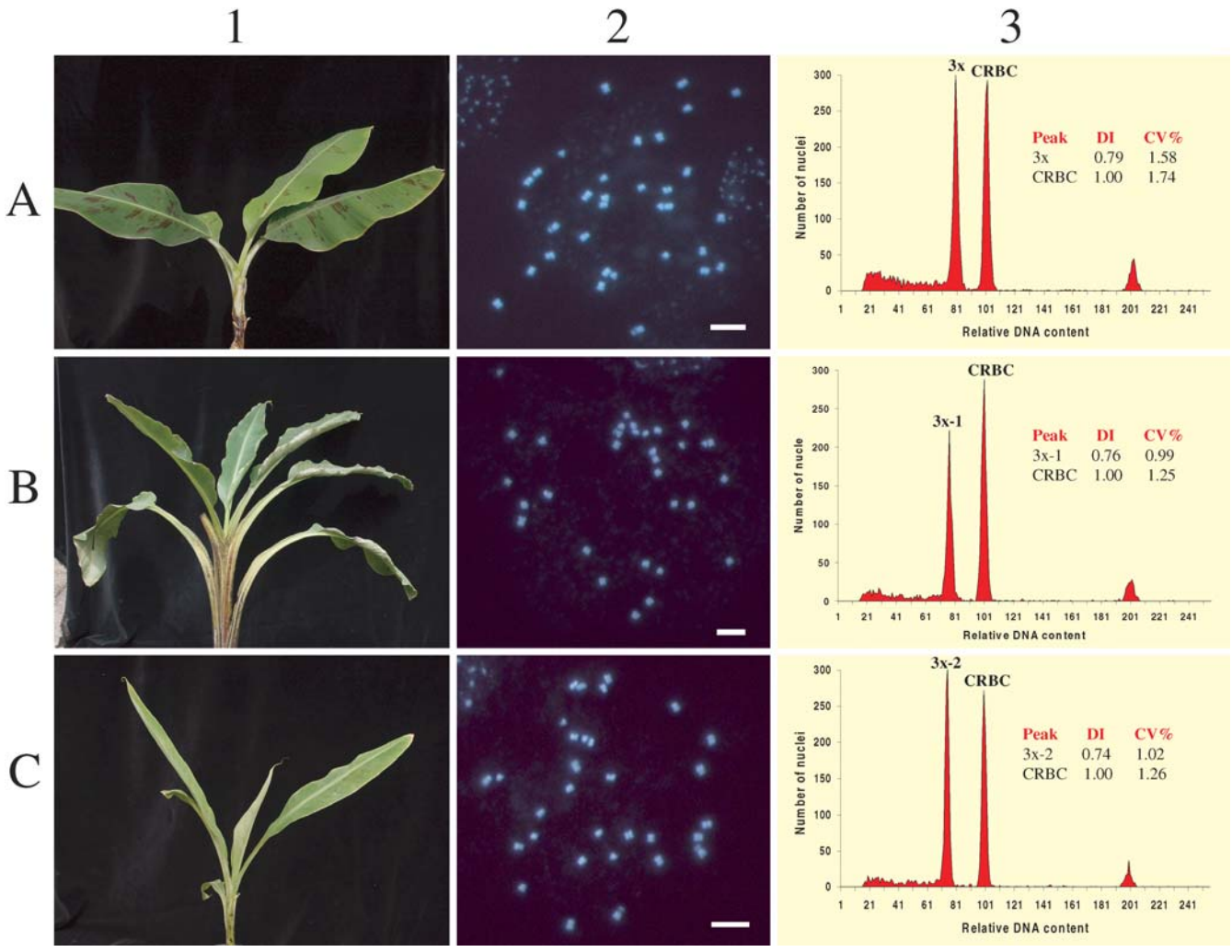

Fig. 1 A1, Control 'Grande Naine' plant (2n=3x=33). A2, A3, two selected aberrant plants: A2, plant no. 5 (2n=3x−1=32) with deformed and dropping leaves and black streaks on pseudostem and midrib, A3, plant no. 6 (2n=3x−2=31) with narrow and elongated leaves. B1–B3, Mitotic metaphase plates. B1, Control 'Grande Naine' plant (2n=3x=33); B2, B3, two selected aberrant plants: B2, plant no. 5 (2n=3x−1=32) with deformed and dropping leaves and black streaks on pseudostem and midrib, B3, plant no. 6 (2n=3x−2=31) with narrow and elongated leaves. *Bar*= 5 µm. C1–C3, Histograms of relative nuclear DNA content obtained after simultaneous analysis of nuclei isolated from *Musa* leaf tissues and chicken red blood cell nuclei (*CRBC*), the latter serving as an internal reference standard. The flow cytometer was adjusted so that the peak representing CRBC nuclei was localized at channel 100. C1, Control 'Grande Naine' plant (2n=3x=33), C2, plant no. 5 (2n=3x−1=32) with deformed and dropping leaves and black streaks on pseudostem and midrib, C3, Plant no. 6 (2n=3x−2=31) with narrow and elongated leaves

To avoid any problems in the chromosome counting of small chromosomes on squash preparations (Dolezel et al.1998), we used a protoplast dropping technique, which yielded well-spread metaphases suitable for chromosome counting. It was possible to detect around ten cells in metaphase per slide, of which at least five could be selected with well-scattered and contracted chromosomes. Among the 25 off-type plants of 'Grande Naine' obtained after gamma irradiation, five were classified as being euploid with 33 chromosomes, 13 were classified as aneuploid with 32 chromosomes and seven were classified as aneuploid with 31 chromosomes (Fig. 1, A2, B2, C2).

Flow cytometric analysis of relative DNA content resulted in histograms with two dominant peaks representing the G_1 nuclei of *Musa* and CRBC (Fig. 1, A3, B3, C3). The G_1 peaks were narrow with a coefficient of variation (CV) equal to 2.24%±0.10 (mean of 270 measurements ± standard deviation). CVs lower than 1% were occasionally obtained (Fig. 1, A3, B3, C3). The DNA index estimated for triploid 'Grande Naine' plants (2n=3x=33) was equal to 0.797 (mean of 20 measurements – four times on five different days). The DNA content of euploid and aneuploid plants, expressed as the DNA index, was highly correlated with chromosome number (R^2=0.90). However, while there was no overlap between the DNA indices of triploid and aneuploid plants with 32 chromosomes, DNA indices of plants with 32 and 31 chromosomes were partially overlapping (Fig. 2).

In order to estimate chromosome number, the relative DNA content of aberrant plants was expressed as a per-

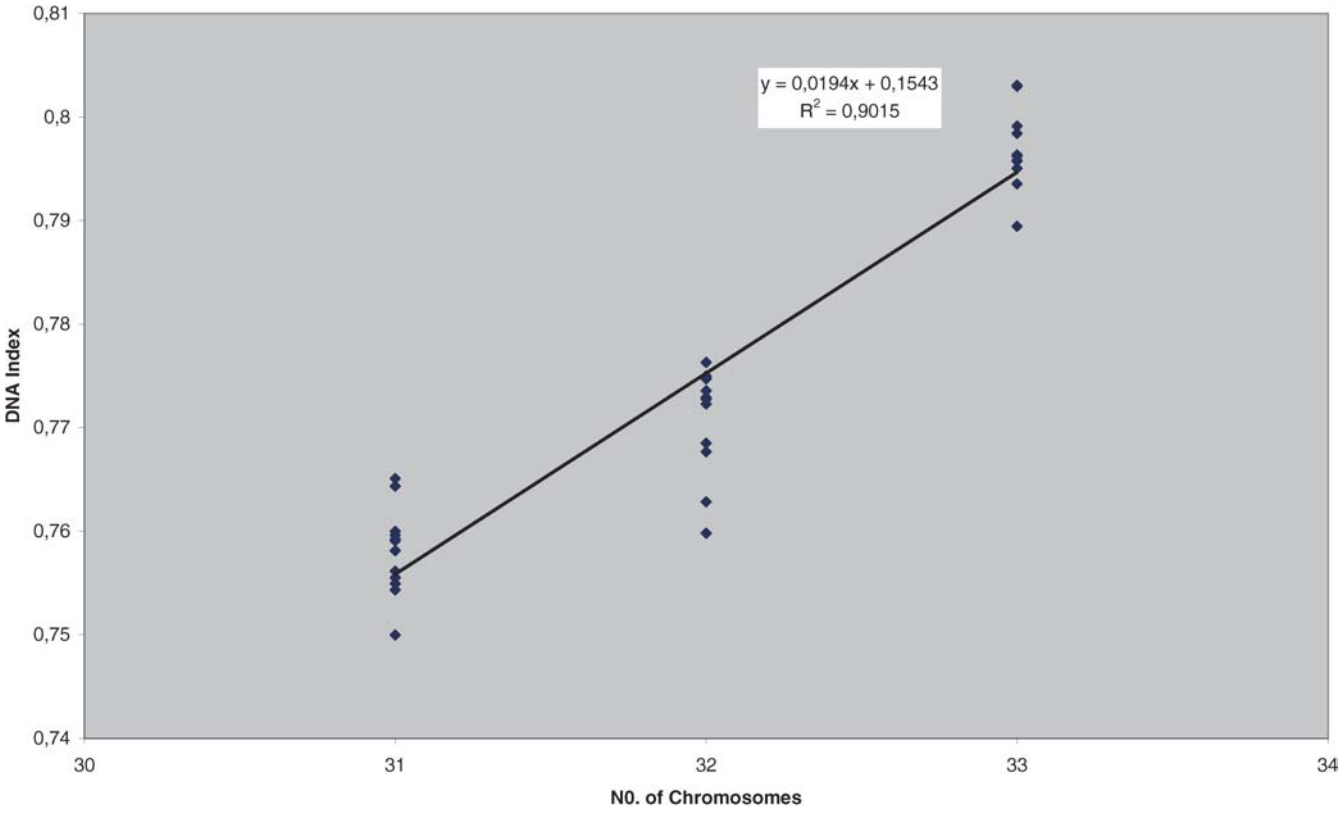

Fig. 2 Relationship between the DNA index and chromosome number

centage of the relative DNA content of the control plants according to a formula: 0.797/DI of aberrant plant × 100. To avoid the risk of error due to instrument drift, we measured a control plant with 33 chromosomes each day at least three times before and after measuring the off-type plants. Each of the 25 off-type plants was measured six times (three times on two different days). The largest difference observed between the six measurements of the same plant was less than 1.5%. The chromosome number in the off-type plants was estimated on the basis of relative DNA content taking into account the fact that an average chromosome in a triploid represents 3% of the genome (Fig. 3). Five off-type plants were classified as euploid with 33 chromosomes, 13 off-types were classified as aneuploid with 32 chromosomes and seven off-types were classified as aneuploid with 31 chromosomes. For all of the plants, the classification based on flow cytometry fully agreed with the results obtained by chromosome counting.

The distribution of relative DNA contents (expressed as a percentage of relative DNA content of control plants) within the three defined chromosome sub-groups (31, 32 and 33) was tested using the Kolmogorov-Smirnov test and found to be normal at the 99% confidence level. Based on *F*-test (comparison of variances) and Student *t*-test (comparison of means) analyses, there was a statistically significant difference between the means of the three sub-groups corresponding to different chromosome numbers at the 99% confidence level. These data indicate that the relative DNA content of euploid plants will lie within the 98.43%–101.61% interval at the 99% probability level. This corresponds to our theoretical assumption for 33-chromosome sub-group (Fig. 3). Subsequently, at this level of confidence, all results detected within defined theoretical ranges for both of the chromosome sub-groups (31 and 32) correspond to two statistically different groups of data.

Based on the current results, the following procedure is recommended to determine if a plant is aneuploid. A control plant with a known chromosome number (determined by chromosome counting) is measured three times at the beginning of the day, three times after each five samples and three times at the end of the day. This procedure is repeated at least on two different days. Each day the DI of each sample is determined and expressed in percentage of the control plant measured on the same day. Thus a plant with unknown ploidy is measured at least six times (three replicates × two days) before it is classified as shown in Fig. 3.

Discussion

The application of low doses of X-rays, gamma rays and fast neutrons to seeds, flower organs or pollen grains is known to generate monosomics in the M_1 or later generations (Khush 1973). The exact mechanism by which radiation treatment induces monosomy is not fully under-

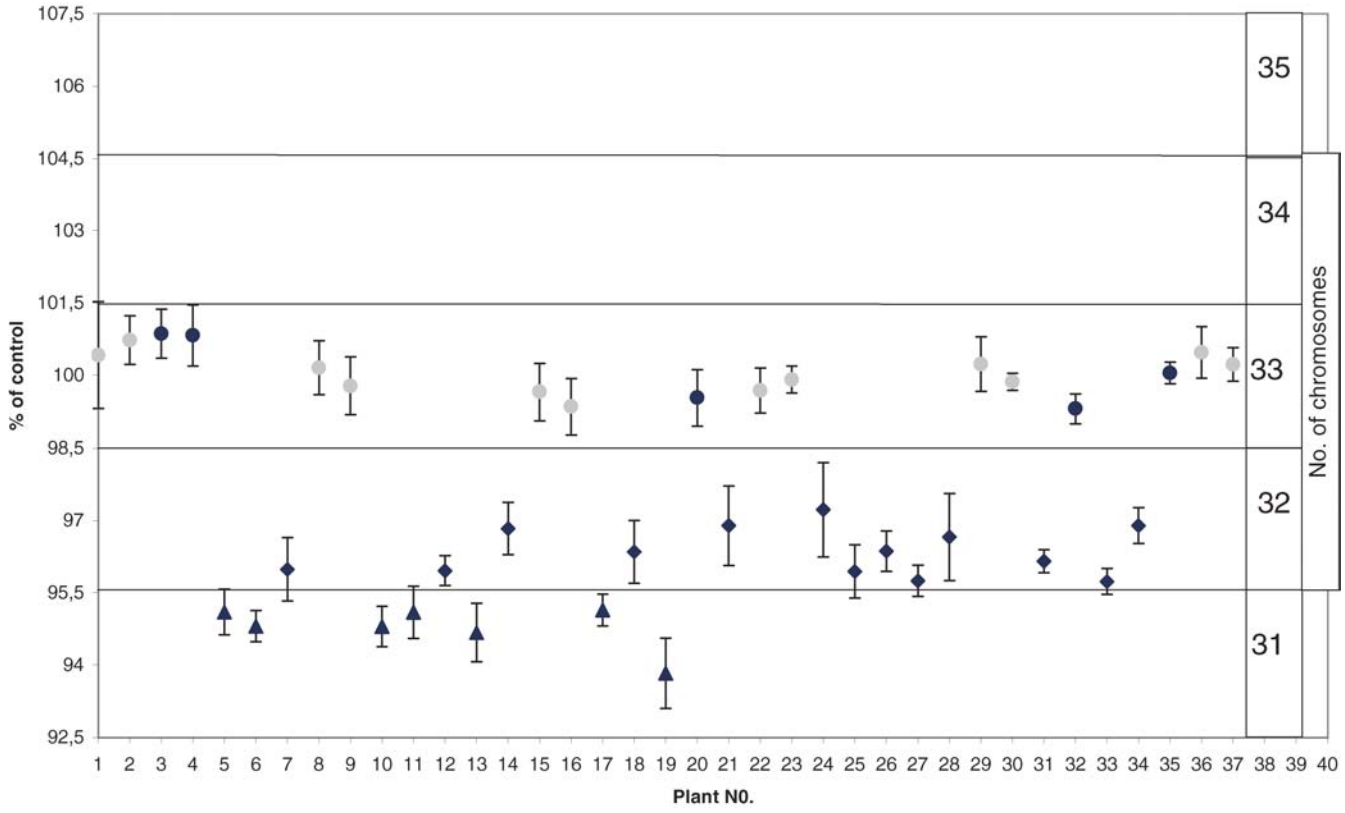

Fig. 3 Relative DNA content of banana plants expressed as the percentage of the DNA index established for control plants. *Open circle* Control plants, *filled circle* abnormal plants with 33 chromosomes, *filled diamond* abnormal plants with 32 chromosomes, *filled triangle* abnormal plants with 31 chromosomes. Each plant was measured three times on two different days. *Bars*: standard deviations

stood. Khush and Rick (1966) suggested that perhaps a chromosome is broken or damaged in the centromere region so that it is unable to show normal centromeric activity and is subsequently eliminated. Costa-Rodriguez (1954) assumed that the radiation causes a break in the two chromatids, with the subsequent formation of a dicentric chromosome. The dicentric chromosome presumably goes through a breakage-fusion-bridge cycle during early cell divisions of the embryo and is ultimately lost.

Although chromosome number is a simple and basic characteristic of a karyotype, it is of considerable importance in taxonomy, karyotype analysis and karyotype evolution. In addition, it may provide important information to the plant breeder with respect to barriers to the introduction of genes from related or more distant species (Sybenga 1992). Chromosome counting is best performed at the mitotic metaphase stage when the chromosomes are fully contracted. This prerequisite limits the choice of material for chromosome counting to tissues with a high mitotic activity. Because of accessibility and ease of preparation, root tips are the tissue of choice when available. However, *Musa* spp. have small and numerous chromosomes, and their counting is laborious. Furthermore, this method cannot be used to establish the ploidy of non-dividing cells in differentiated tissues, such as leaves (Roux et al. 2001). Alternative methods for ploidy screening based on stomata size, density and size of pollen have been found to be slow and unreliable (Adniya and Ardian 1994).

To detect aneuploidy using flow cytometry, it is necessary to eliminate the variation between individual measurements and to establish the number of observations required to reach reproducible classification. Our results (Fig. 2) indicate a correlation between the chromosome number and relative nuclear DNA content in *Musa*. However, the estimation of chromosome number appeared to become less precise with an increase in the difference in DNA contents between the standard and the sample. This could be explained by the fact that the higher the DNA index, the more prone it is to larger variation—for example, due to instrument drift and/or zero offset error (Vindeløv et al. 1983). Thus, in contrast to chromosome counting, the flow cytometric assay incorporating an internal reference standard seems to be a more precise technique for detecting changes involving smaller numbers of chromosomes.

In addition to a minor variation in the estimation of relative DNA content, the flow cytometric detection of aneuploids following irradiation may be compromised by structural chromosome changes induced by irradiation, such as deletions. Furthermore, the flow cytometric detection of aneuploidy is based on a simplified assumption the all chromosomes have the same DNA content. However, this is not the case in *Musa*, where chromosome size ranges from about 1 μm to 2 μm (Dolezel et al. 1998).

Even though repeated vegetative propagation to dissociate chimeras was carried out after mutagenic treatment, in vitro mutagenesis of multicellular meristems of *Musa* spp. may lead to chimeras (Roux et al. 2001). To be able to detect chimeric tissue containing cells with different chromosome number, it is important to perform precise flow cytometric analysis.

The accuracy of flow cytometric detection of small differences in nuclear DNA content generally requires three conditions to be met (Benson and Braylan 1994): (1) the lowest possible CV of DNA peaks, (2) an equal proportion of cells within each peak (standard and sample), (3) a small difference in the DNA contents between the standard and the sample (small DNA index). The procedure described here resulted in low CVs of DNA peaks. The use of CRBC nuclei—whose DNA content is close to that of triploid *Musa*—as an internal standard resulted in a high reproducibility and precision of the analysis. It is probable that CRBC could also be useful as an internal reference standard for flow cytometric detection of aneuploidy in other species with DNA content similar to that of triploid *Musa*.

Chromosome numbers estimated using flow cytometry in the *Musa* off-types obtained after irradiation fully agreed with the results obtained by chromosome counting. The proportion of aneuploid plants among the aberrant plants observed in this study was surprisingly high, with 80% of plants being aneuploid. Hypotriploids were detected with 2n=32 or 31. The fact that some off-type plants were euploid indicates that abnormal plant height, color or leaf shape induced by irradiation could be the consequence of phenomena other than aneuploidy (e.g. structural chromosome changes involving small chromosome segments, DNA methylation or retrotransposon activation).

Although the causes of chromosome instability are poorly understood, chromosome instability itself is believed to be one of the most common causes of tissue culture-induced variation (Phillips et al. l994). Morphological features (for example, dropping leaf habit) may indicate the tetraploid or aneuploid status of *Musa* regenerants (Reuveni and Israeli 1990; Vuylsteke et al. 1988). The presence of dwarf off-types among tissue culture regenerants that have passed through five subcultures demonstrate that this character, usually caused by a single gene mutation, is a relatively stable genetic trait induced by tissue culture (Smith and Drew 1990). Thus, in our experiment, it is not impossible that the abnormal morphological characters obtained after irradiation were also the result of somaclonal variation rather than gamma irradiation, given that the plants were previously cultured for long periods of time in vitro in the International Transit Center. A possibility for detecting aneuploids rapidly at an early stage (at the greenhouse level or even earlier) would improve the quality of plants to be grown in the field and consequently limit the appearance of somaclonal variants due to chromosome number instability.

Considerable morphological variation is observed among regenerated plants following mutagenic treatment. The frequency of phenotypical variation ranges from 3% to 40% of the tested M_1V_4 plants, depending on genotype and irradiation dose (Novak et al. 1990). By using flow cytometry, mutants with undesirable agromorphological traits (e.g. aneuploids) may be rejected. This will increase the efficiency of induced mutagenesis as a genetic improvement technique since screening could then be limited to lower number of plants. However, aneuploids may also be used for genetic studies. Here we show a fast and efficient method to select aneuploids for further molecular-cytogenetic studies.

The present results illustrate the usefulness of flow cytometry for the rapid detection of aneuploidy in *Musa*. Compared to conventional chromosome counting, flow cytometry is more rapid and convenient.

While the occurrence of aneuploidy is undesirable during vegetative propagation, aneuploids may be useful for gene mapping and isolation. In this study we have shown that aneuploid stocks can be easily induced and detected. These stocks could be maintained in vitro and would provide a considerable resource for fundamental and applied *Musa* research.

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