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Inducing and identifying artificially-induced polyploidy in bananas

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In the present work, polyploidy was induced in the diploid banana varieties 'Malbut', 'Gold', 'Lidi', and 'Thong Dok Mak' through the use of colchicine and oryzalin, and that condition was identified through stomatal analysis, flow cytometry, and chromosome counts. Shoots produced *in vitro* were treated with colchicine at concentrations of 0, 2.5, 7.5 and 12.5 mM for 24 and 48 h, and with oryzalin at 0, 10, 30 and 50 mM for 4 and 7 days. Young leaves were scanned by electron microscopy to determine their stomatal areas (polar diameter × equatorial diameter) and numbers for polyploid identification by stomatal analysis. Polyploid identification by way of flow cytometry analysis used samples of young leaves that were crushed to release their nuclei, with subsequent staining with propidium iodide; ten thousand nuclei were analyzed for each sample. For cytogenetic analyses, root tips were pretreated with 0.002 M 8-HQ for 3 h, fixed in Carnoy solution for 24 h, subjected to conventional squashing techniques, and stained with 10% Giemsa. We identified four tetraploid plants and six mixoploids using these three identification techniques.

Key words: Chromosomes duplication, *Musa acuminata,* tissue culture.

INTRODUCTION

Bananas (*Musa* sp.) are one of the principal fruits eaten in Brazil, providing a nutritious source of potassium and contributing to the regional economy by generating employment and income for farmers as well as for industries that process those fruits to make sweets, jams, yogurts (Silva et al., 2008). Banana breeding programs using chromosome duplication (through treatments with antimitotic agents such as colchicine and oryzalin) have been proposed to generate material from diploid hybrids that are resistance to pests and diseases in light of the reduced time and costs involved in using these treatments. Additionally, the high productivities of *Musa* banana strains are directly associated with their ploidy levels, as triploid and tetraploid varieties are more vigorous than diploids (Stover and Simmonds, 1987). Conventional methods of generating polyploid varieties

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that involve backcrossing three diploid hybrids obtained by crossing two diploid species are associated with lengthy development periods with high production costs (Simmonds and Dodds, 1949).

Chromosome doubling using antimitotic agents requires an efficient system of polyploid induction as well as an effective method for ploidy verification. Polyploidy induction has been undertaken with bananas (Ganga and Chezhiyan, 2002; Rodrigues et al., 2011) using the antimitotics oryzalin, amiprophos-methyl (AMP), and colchicine at different concentrations and for varying exposure times. Ganga and Chezhiyan (2002) produced 13% tetraploid plants in programs using colchicine, and 17% using oryzalin, and Rodrigues et al. (2011) reported that amiprophos-methyl was most efficient at inducing ploidy in some banana varieties at a concentration of 40 µM. The technique of exposing *in vitro* explants to colchicine has been investigated by a number of researchers (Sakhanokho et al., 2009; Wannakrairoj and Wondyifraw, 2013), but it is then necessary to check the suitability of each species or new variety generated, as their responses reflect their genetic constitutions and their reactions to the mitotic agents employed.

Chromosome counts and stomata morphology have been routinely used to identify polyploids (Campos et al., 2009), although the quickest and most efficient technique for ploidy analysis is flow cytometry of nuclear DNA content (Younis et al., 2013). This technique involves analyzing the optical properties (light scattering and fluorescence) of particles flowing in liquid suspension (Ochatt, 2008). As these particles pass an intersecting laser beam, they generate quantifiable light scattering, fluorescence, or emission signals. This principle can be used, for example, to measure the amount of DNA in a cell (Dolezel and Bartos, 2005).

The present study was part of the banana breeding program "*Embrapa Mandioca e Fruticultura Tropical"* at Cruz das Almas, Bahia State, Brazil, seeking to produce secondary triploid AAA varieties that produce palatable fruits by inducing chromosome doubling in diploid banana plants and subsequently crossing the autotetraploids obtained with diploid elite varieties to obtain new AAA varieties. We induced chromosome doubling using colchicine and oryzalin in four diploid banana varieties 'Malbut', 'Gold', 'Lidi', and 'Thong Dok Mak' and identified polyploids and verified their ploidy through stomatal measurements, flow cytometry analysis, and chromosome counts.

MATERIALS AND METHODS

Micropropagation of plant materials and their treatment with antimitotic agents

Diploid apical meristems from the banana varieties 'Malbut', 'Gold', 'Lidi', and 'Thong Dok Mak' were established *in vitro* and multiplied for two generations in MS medium (Murashige and Skoog, 1962) supplemented with 30 $g.L^{-1}$ sucrose and 4 $mg.L^{-1}$ benzylaminopurine (BAP) for shoot proliferation, and subsequently treated with the antimitotic agents colchicine and oryzalin. Colchicine was used at concentrations of 0, 2.5, 7.5 and 12.5 mM for 24 to 48 h and oryzalin at concentrations of 0, 10, 30 and 50 µM for 4 and 7 days in liquid medium with agitation (60 rpm).

After treatment, the shoots were washed three times with distilled water and transferred to a proliferation medium for further culturing to reduce the frequency of mixoploids (plant material containing cells with chromosome number variations). The plants were then transferred to a rooting medium (MS supplemented with sucrose 30 $g. L^{-1}$ and solidified with 7 $g.L^{-1}$ agar). The explants were kept in a growth chamber with a light intensity of 36 micromol. $m^2.s^{-1}$, photoperiod of 16 h, and a temperature of 25 ± 2°C during their growth phase. The generation time was thirty days. For rooting, the plantlets were kept under the same conditions for 45 days.

The rooted plants were transferred to a greenhouse and placed in cultivation tubes (19 cm × 5 cm) with a substrate composed of vegetable substrate plantmax™ and coconut fiber (1:1), with 150 g PG MIX™ (14-16-18) and 150 g of Osmocote™ (6-19-10), and irrigated with an intermittent mist system under 50% shading. After 60 days, the plants were transplanted to 20 L plastic pots with the same substrate. Each treatment was replicated five times.

Calculations of stomatal areas and densities

Stomatal analyzes were performed on all plants one year after their acclimatization. We used fragments of the middle region of the abaxial side of leaf number 1 (youngest fully expanded leaf), without ribs. Fragments (0.5 mm^2) were fixed to metallic stubs with carbon tape, desiccated for 48 h, and subsequently coated with gold (20 nm) layer in a BAL-TEC, SCD- 050 sputter coater. The leaf material was examined in a LEO EVO-40 PVX scanning electron microscope (600x) and electromicrographed. To determine stomatal areas (polar equatorial diameter x diameter) and densities, five observation fields (five replicates) with the same area (1mm^2) were randomly chosen on the epidermis of the abaxial surface of each sample and five stomata of each were measured. Twenty-six Malbut varieties, 15 Gold, 52 Lidi, and 35 Thong Dok Mak varieties were treated with antimitotic agents and their survival rates calculated.

The criteria used to identify the tetraploid plants was that all plants that differed statistically in size and density from the stomata of diploid plants were considered possible tetraploids, being subsequently confirmed by flow cytometry analysis and chromosome counts. Analysis of variance (ANOVA) ($p<0.05$) was performed to evaluate the data; differences between the means were evaluated using the Scott-Knott test (<0.05).

Flow cytometric analysis

Nuclear DNA contents were determined by grinding approximately 20-30 mg portions of young banana plant and *Pisum sativum* leaves (the latter being the internal reference standard) in 1 ml of cold LB01 buffer to release the nuclei (Dolezel et al., 1998). The nuclei suspension was then aspirated through two layers of gauze using a plastic pipette, and filtered through a 50 micron mesh. The nuclei were stained by adding 25 µL of a 1 mg/1 ml propidium iodide solution with 5µL RNase to each sample. The samples were analyzed after storage in a refrigerator for 1-2 hours. At least 10 billion nuclei were analyzed for each sample. The analyses were performed using a FACSCalibur flow cytometer USA, 2010 (Becton Dickinson) using a logarithmic scale; the histograms were generated

Table 1. Anatomical characteristics of the epidermis of diploid Malbut variety banana plants subjected to treatment with colchicine.

Treatment	Anatomical characteristics			Flow cytometry	
	Area (µm^2)	Stomatal densities (n^o/mm^2)	Ploidy	DNA index (pg)	Ploidy
0	204.1^a	30.8^c	2x	-	-
0	242.6^b	27.2^b	2x	-	-
2.5 Mm/24 h	260.3^c	20.0^a	4x	-	-
2.5 Mm/24 h	286.1^d	25.8^b	4x	-	-
2.5 Mm/24 h	250.8^c	23.8^b	2x	1.05	2x
2.5 Mm/24 h	237.4^b	21.8^a	2x	-	-
7.5 Mm/24 h	255.5^c	30.4^c	2x	-	-
7.5 Mm/24 h	264.1^c	25.4^b	2x	1.34	2x
12.5 Mm/24 h	270.6^d	37.8^d	4x	1.34	2x

*Means followed by the same letter in the same column did not differ statistically by the Scott Knott (<0.05) probability level.

using Cell Quest software and statistically analyzed using WinMDI 2.8 software.

The nuclear DNA contents (pg) of the plants were estimated using the ratios of the fluorescence intensities of the G1 nuclei (nuclei in the G1 interphase stage) to the reference standard (*P. sativum*) and multiplying this ratio by the amount of DNA in the reference standard (9.09 pg).

Cytogenetic analyses

For ploidy analysis by chromosome counts, the roots of plants previously selected for stomatal analysis were pretreated with a solution of 0.002 M 8-hydroxyquinoline for 3 h and subsequently fixed in Carnoy solution (3:1) for 24 h. The roots were then washed three times for 10 min in de-ionized water and subsequently hydrolyzed in 1 N HCl at 60°C for 15 min.

The root meristems were excised with the aid of a stereoscopic microscope and then crushed in 45% acetic acid under a cover slip. The slides were air dried and the cover slips removed after immersion in liquid nitrogen (Barbosa et al., 2007), and the slides were subsequently immersed for 30 s in 45% acetic acid and stained with 10% Giemsa for 5 min.The observations and analyses of the slides were performed using a microscope (100 × oil immersion magnification) with digital camera image-capturing system.

RESULTS

One year after the end of the acclimatization period, banana seedlings survival was found to be influenced by the dosage levels and exposure times to colchicine and oryzalin. Higher doses and longer exposures to antimitotic agents had negative effects on seedling survival. The survival rate of the 'Lidi', 'Gold', 'Malbut', and 'Thong Dok Mak' varieties were 23.75, 33.75, 46.85 and 30.62% when treated with colchicine and 23.12, 15.625, 36.25 and 22.5% when treated with oryzalin respectively.

Cytogenetic analyses were only performed on mixoploid or tetraploid plants identified by flow cytometry analyses. Three of the diploid Malbut plants treated with colchicine showed larger and less frequent stomata as compared to control plants (Table 1), and were considered possible tetraploids, although cytometric analyses gave results typical of diploids. Two plants subjected to treatments with oryzalin showed larger stomatal areas and lower stomatal densities, but were likewise considered diploids by flow cytometry analysis (Table 2).

A Gold variety plant treated with colchicine was classified as tetraploid based on flow cytometry and stomatal analyses and had a DNA content of 2.18 pg, although chromosome counts classified it as a mixoploid composed of cells with different ploidy levels (Table 3). No polyploid plants were found after treatments with oryzalin (Table 4). Colchicine treatments were observed to efficiently promote polyploidization in five plants of the Lidi varieties (Table 5), as was confirmed by all three analyses.

The DNA index was approximately 2.18 pg for tetraploids, while mixoploids showed two peaks, one diploid and one tetraploid. A plant treated with oryzalin was classified as a tetraploid (based on chromosome counts and flow cytometry) as it had large stomata at high densities (Table 6).

Colchicine-treated plants of the Thong Dok Mak (TDM) variety (Table 7) were classified as tetraploids by both stomata analyses and chromosomal counts, but showed DNA contents of 1.78 pg by flow cytometry, and therefore must be considered mixoploids. These plants also showed high-density but small stomata. One oryzalin-treated plant was considered tetraploid in all three analyses, and another was classified as a tetraploid only by chromosome counts, as shown in Table 8.

The graphs of the positions of the peaks of diploid, tetraploid, and mixoploid plants are presented in Figure 1. Knowing the relative positions of the peaks of diploid

Table 2. Anatomical characteristics of the epidermis in diploid Malbut variety banana plants subjected to treatment with oryzalin.

Treatment	Anatomical characteristics			Flow cytometry	
	Areas (μm^2)	Stomatal densities (n^o/mm^2)	Ploidy	DNA index (pg)	Ploidy
0	246.4[a]	20.2[b]	2x	1.27	2x
0	210.6[a]	23.0[b]	2x	-	-
10 µM/4dias	285.9[a]	35.4[d]	2x	1.23	2x
30 µM/4dias	235.7[a]	37.8[e]	2x	1.23	2x
30 µM/4dias	292.2[a]	27.8[c]	2x	1.23	2x
30 µM/4dias	239.5[a]	21.4[b]	2x	-	-
30 µM/4dias	261.0[a]	39.4[e]	2x	1.19	2x
30 µM/4dias	266.4[a]	25.4[c]	2x	-	-
30 µM/4dias	229.9[a]	43.2[e]	2x	1.27	2x
30 µM/4dias	257.3[a]	28.2[c]	2x	-	-
10 µM/7dias	260.4[a]	23.0[b]	2x	1.27	2x
10 µM/7dias	239.5[a]	22.0[b]	2x	1.19	2x
10 µM/7dias	257.3[a]	21.2[b]	2x	1.16	2x
10 µM/7dias	265.4[a]	27.2[c]	2x	1.30	2x
10 µM/7dias	292.2[a]	32.0[d]	2x	1.27	2x
10 µM/7dias	446.7[b]	12.8[a]	4x	1.34	2x
10 µM/7dias	404.3[b]	17.8[a]	4x	1.34	2x

*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

Table 3. Anatomical features of the epidermis and cytogenetic analyses of diploid Gold variety banana plants subjected to treatment with colchicine.

Treatment	Anatomical characteristics			Flow cytometry		Cytogenetic analyses	
	Areas (μm^2)	Stomatal densities (n^o/mm^2)	Ploidy	DNA Index (pg)	Ploidy	Chromosome Numbers	Ploidy
0	260.8[a]	7.5[a]	2x	-	-	22	2x
0	242.1[a]	18.0[b]	2x	-	-	-	-
2.5 mM/24 h	260.9[a]	2.6[a]	2x	1.16	2x	-	-
2.5 mM/24 h	313.7[c]	15.0[b]	4x	2.18	4x	31	mixoploid
2.5 mM/24 h	285[b]	18.4[b]	2x	-	-	-	-
12.5 mM/24 h	289[b]	15.4[b]	2x	1.38	2x	-	-
2.5 mM/48 h	285.3[b]	16.0[b]	2x	1.16	2x	-	-
2.5 mM/48 h	353.3[c]	7.2[a]	4x	1.54	2x	-	-
2.5 mM/48 h	319.9[c]	13.8[b]	4x	1.27	2x	-	-
7.5 mM/48 h	289[b]	13.5[b]	2x	-	-	-	-

*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

material in relation to a reference standard can be used to detect polyploid material. Most of the plants classified as tetraploid by stomatal testing were confirmed as such by flow cytometry analysis, and other characteristics of tetraploid mixoploids were likewise confirmed. In all, six plants were classified by flow cytometry as tetraploid and three as mixoploids. No plant identified as diploid by stomatal analyses was found to be tetraploid by flow cytometry, demonstrating and confirming the ability of flow cytometry to identify and separate plants into two groups: diploids and mixoploids versus tetraploids.

The chromosome counts described here were performed on 15 plants (5 diploids and 10 polyploids) (Figure 1). A summary of the results of all analyzes are

Table 4. Anatomical characteristics of the epidermis of diploid Gold variety banana plants subjected to treatment with oryzalin.

Treatment	Anatomical characteristics			Flow cytometry	
	Areas (μm^2)	Stomatal densities (n^o/mm^2)	Ploidy	DNA Index (pg)	Ploidy
0	277.7[b]	12.8[a]	2x	-	-
10 µM/7 dias	280.0[b]	14.8[a]	2x	1.30	2x
10 µM/7 dias	337[c]	16.6[a]	2x	1.30	2x
30 µM/7 dias	324.3[c]	15.6[a]	2x	1.30	2x
30 µM/7 dias	219.3[a]	21[b]	4x	-	-

*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

Table 5. Anatomical features of the epidermis and cytogenetic analyses of diploid Lidi variety banana plants subjected to treatment with colchicines.

Treatment	Anatomical characteristics			Flow cytometry		Cytogenetic analyses	
	Areas (μm^2)	Stomatal densities (n^o/mm^2)	Ploidy	DNA Index (pg)	Ploidy	Chromosome Numbers	Ploidy
0	161.1[b]	60.6[e]	2x	-	-	22	2x
0	160.3[b]	49.2[d]	2x	-	-	-	-
0	177.0[b]	45.8[d]	2x	-	-	-	-
0	161.5[b]	38.2[c]	2x	-	-	-	-
2.5 mM/24 h	184.1[c]	52.8[e]	2x	1.02	2x	-	-
2.5 mM/24 h	220.6[d]	43.8[d]	2x	0.98	2x	-	-
2.5 mM/24 h	193.3[c]	32.8[b]	4x	1.13	2x	-	-
2.5 mM/24 h	162.8[b]	49.8[d]	2x	1.09	2x	-	-
2.5 mM/24 h	167.7[b]	47.4[d]	2x	1.20	2x	-	-
7.5 mM/24 h	229.3[e]	40.2[c]	4x	1.05	2x	-	-
7.5 mM/24 h	175.8[b]	45.8[d]	2x	1.09	2x	-	-
7.5 mM/24 h	162.1[b]	39.8[c]	2x	1.05	2x	-	-
7.5 mM/24 h	175.1[b]	54.0[e]	2x	1.20	2x	-	-
12.5 mM/24 h	256.6[f]	19.8[a]	4x	2.14	4x	44	4x
12.5 mM/24h	190.2[c]	32.6 [b]	4x	2.18	4x	31	mixoploid
12.5 mM/24h	210.8[d]	33.4[b]	4x	1.16 e 2.18	mixoploid	33	mixoploid
2.5 mM/48 h	194.3[c]	43.6[c]	2x	0.98	2x	-	-
2.5 mM/48 h	171.5[b]	43.4[c]	2x	-	-	-	-
2.5 mM/48 h	180.5[c]	37.2[c]	2x	1.13	2x	-	-
2.5 mM/48 h	170.6[b]	50.8[d]	2x	1.16	2x	-	-
2.5 mM/48 h	183.0[c]	43.2[c]	2x	-	-	-	-
7.5 mM/48 h	230.5[e]	39.2[c]	4x	2.18	4x	44	4x
7.5 mM/48 h	232.9[e]	40.8[c]	4x	1.09 e 2.18	mixoploid	44	4x
7.5 mM/48 h	181.2[c]	55.0[e]	2x	1.20	2x	-	-
7.5 mM/48 h	195.4[c]	41.4[c]	2x	-	-	-	-
7.5 mM/48 h	177.0[b]	53.4[e]	2x	1.13	2x	-	-
7.5 mM/48 h	202.3[d]	38.6[c]	2x	-	-	-	-
12.5 mM/48 h	104.1[a]	39.6[c]	2x	1.09	2x	-	-

*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

presented in Table 9, and this data shows that there were variations in ploidy determinations among the three different methods used. Only plants that showed positive results in all three analyses were considered tetraploids,

Table 6. Anatomical features of the epidermis and cytogenetic analyses of diploid Lidi variety banana plants subjected to treatment with oryzalin.

Treatment	Anatomical characteristics			Flow cytometry			Cytogenetic analyses	
	Areas (μm²)	Stomatal densities (nº/mm²)	Ploidy	DNA Index (pg)		Ploidy	Chromosome Numbers	Ploidy
0	128.3a	28.8b	2x	1.09		2x	22	2x
0	182.7c	28.8b	2x	1.20		2x	-	-
0	197.1c	23 a	2x	-		-	-	-
0	213.8c	24.2a	2x	-		-	-	-
10 μM/4dias	163.3b	26.2b	2x	1.09		2x	-	-
10 μM/4dias	284.5e	19.8a	2x	1.05		2x	-	-
10 μM/4dias	234.5d	43.4d	4x	1.05		2x	-	-
10 μM/4dias	201.9c	53.4e	2x	1.13		2x	-	-
50 μM/4dias	156.6b	31b	2x	-		-	-	-
50 μM/4dias	281.2e	27.2b	2x	-		-	-	-
10 μM/7dias	202.2c	22.4a	2x	1.05		2x	-	-
10 μM/7dias	182.4c	38c	2x	1.13		2x	-	-
10 μM/7dias	161.4b	43d	4x	1.05		2x	-	-
10 μM/7dias	194.8c	37c	2x	1.09		2x	-	-
10 μM/7dias	187.8c	44d	2x	1.20		2x	-	-
10 μM/7dias	185.3c	43.2d	2x	1.13		2x	-	-
10 μM/7dias	214.6c	22.8a	2x	1.20		2x	-	-
10 μM/7dias	193.8c	37.8c	4x	1.16		2x	-	-
10 μM/7dias	217.3c	42.2d	4x	1.23		2x	-	-
10 μM/7dias	195.8c	37.6c	2x	1.16		2x	-	-
10 μM/7dias	233.5d	30.8b	2x	1.23		2x	-	-
10 μM/7dias	199.4c	36c	4x	1.23		2x	-	-
10 μM/7dias	237.7d	45d	4x	2.22		4x	44	4x
50 μM/7dias	188.7c	22.4a	2x	0.98		2x	-	-

*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05)probability level.

Table 7. Anatomical features of the epidermis and cytogenetic analyses of diploid Thong Dok Mak variety banana plants subjected to treatment with colchicines.

Treatment	Anatomical characteristics			Flow cytometry		Cytogenetic analyses	
	Areas (μm²)	Stomatal densities (nº/mm²)	Ploidy	DNA Index (pg)	Ploidy	Chromosome Numbers	Ploidy
0	282.5^{c}	12.4^{a}	2x	-	-	22	2x
0	258.9^{b}	17.8^{b}	2x	-	-	-	-
2.5 mM/24 h	214.4^{a}	29.6^{d}	4x	1.05	2x	-	-
2.5 mM/24 h	267.7^{b}	25.2^{c}	2x	1.31	2x	-	-
2.5 mM/24 h	252.5^{b}	26.6^{c}	2x	1.13	2x	-	-
2.5 mM/24 h	264.6^{b}	25.0^{c}	2x	1.27	2x	-	-
2.5 mM/24 h	236.8^{a}	29.8^{d}	4x	1.31	2x	-	-
2.5 mM/24 h	230.4^{a}	29.8^{d}	4x	1.49	2x	-	-
2.5 mM/24 h	226.0^{a}	29.0^{d}	4x	1.38	2x	-	-
7.5 mM/24 h	346.6^{d}	11.6^{a}	2x	-	-	-	-
7.5 mM/24 h	220.4^{a}	23.4^{c}	2x	-	-	-	-
2.5 mM/48 h	263.4^{b}	30.4^{d}	2x	-	-	-	-
2.5 mM/48 h	270.2^{b}	17.0^{b}	2x	-	-	-	-

Table 7. Contd.

7.5 mM/48 h	326.1[d]	14.6[a]	2x	1.31	2x	-	-
7.5 mM/48 h	237.3[a]	37.8[e]	4x	1.78	mixoploid	44	4x
7.5 mM/48 h	198.4[a]	27.8[d]	4x	-	-	-	-
7.5 mM/48 h	241.0[a]	26.0[c]	4x	-	-	-	-
12.5 mM/48 h	283.6[c]	19.8[b]	2x	1.27	2x	-	-

*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

Table 8. Anatomical features of the epidermis and cytogenetic analyses of diploid Thong Dok Mak variety banana plants subjected to treatment with oryzalin.

Treatment	Anatomical characteristics			Flow cytometry		Cytogenetic analyses	
	Areas (μm^2)	Stomatal densities (n^o/mm^2)	Ploidy	DNA index (pg)	Ploidy	Chromosome Numbers	Ploidy
0	273.2[e]	32.4[b]	2x	-	-	22	2x
0	202.3[c]	27.6[b]	2x	-	-	-	-
0	209.1[c]	35.2[c]	2x	-	-	-	-
0	372[g]	15.2[a]	2x	-	-	-	-
10 µM/4dias	235.9[d]	27.6[b]	2x	1.23	2x	-	-
10 µM/4dias	302.8[f]	27.2[b]	2x	1.31	2x	-	-
10 µM/4dias	215.1[c]	30.4[b]	2x	1.09	2x	-	-
10 µM/4dias	258.0[d]	31.8[b]	2x	-	-	-	-
10 µM/4dias	253.8[d]	31.4[b]	2x	1.13	2x	44	4x
10 µM/4dias	206.8[c]	30.0[b]	2x	-	-	-	-
10 µM/4dias	241.3[d]	30.0[b]	2x	1.16	2x	-	-
10 µM/4dias	172.4[b]	38.2[c]	4x	-	-	-	-
10 µM/4dias	127.9[a]	58.0[d]	4x	2.18	4x	44	4x
30 µM/7dias	244.0[d]	29.0[b]	2x	1.09	-	-	-
30 µM/7dias	242.6[d]	19.2[a]	2x	-	-	-	-
30 µM/7dias	228.4[c]	36.4[c]	2x	1.23	2x	-	-
50 µM/7dias	226.3[c]	18.2[a]	2x	1.34	2x	-	-

*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

the others were considered tetraploid mixoploids.

DISCUSSION

The action of antimitotic agents is related to their specific effects on chromosome duplication. High concentrations or long periods of exposure to these substances are usually observed to cause phytotoxic effects - generating high mortality among treated seedlings- and tolerance to colchicine and oryzalin varies among species and varieties, depending upon their genetic make up (Vakili, 1962; Ganga and Chezhyan, 2002).

Colchicine toxicity at high dosages was reported by Lone et al. (2010), who found lower survival rates of the protocorms of *Cattleya tigrina* (32%) that had been treated with 1 $g.L^{-1}$ colchicine for 72 h. Regeneration rates of 27.26 % after colchicine exposure were observed in *Tagetes erecta* (Sajjad et al., 2013). Regeneration rates of 37 and 67% were observed in explants of *Aframomum corrorima* treated for 7 days with colchicine and oryzalin respectively (Wannakrairoj and Wondyifraw, 2013).

Both oryzalin and colchicine caused tissue oxidation that resulted in explant death, especially those treated with colchicine for 48 h or oryzalin for seven days. Similar results were observed with *Musa* varieties treated with colchicine for 48 h (Rodrigues et al., 2011) and with *Catharanthus roseus* seeds treated with colchicine for 48 h (Xing et al., 2011). Regenerated plants subjected to these treatments showed slower growth and thicker and smaller leaves when compared with controls - indicating

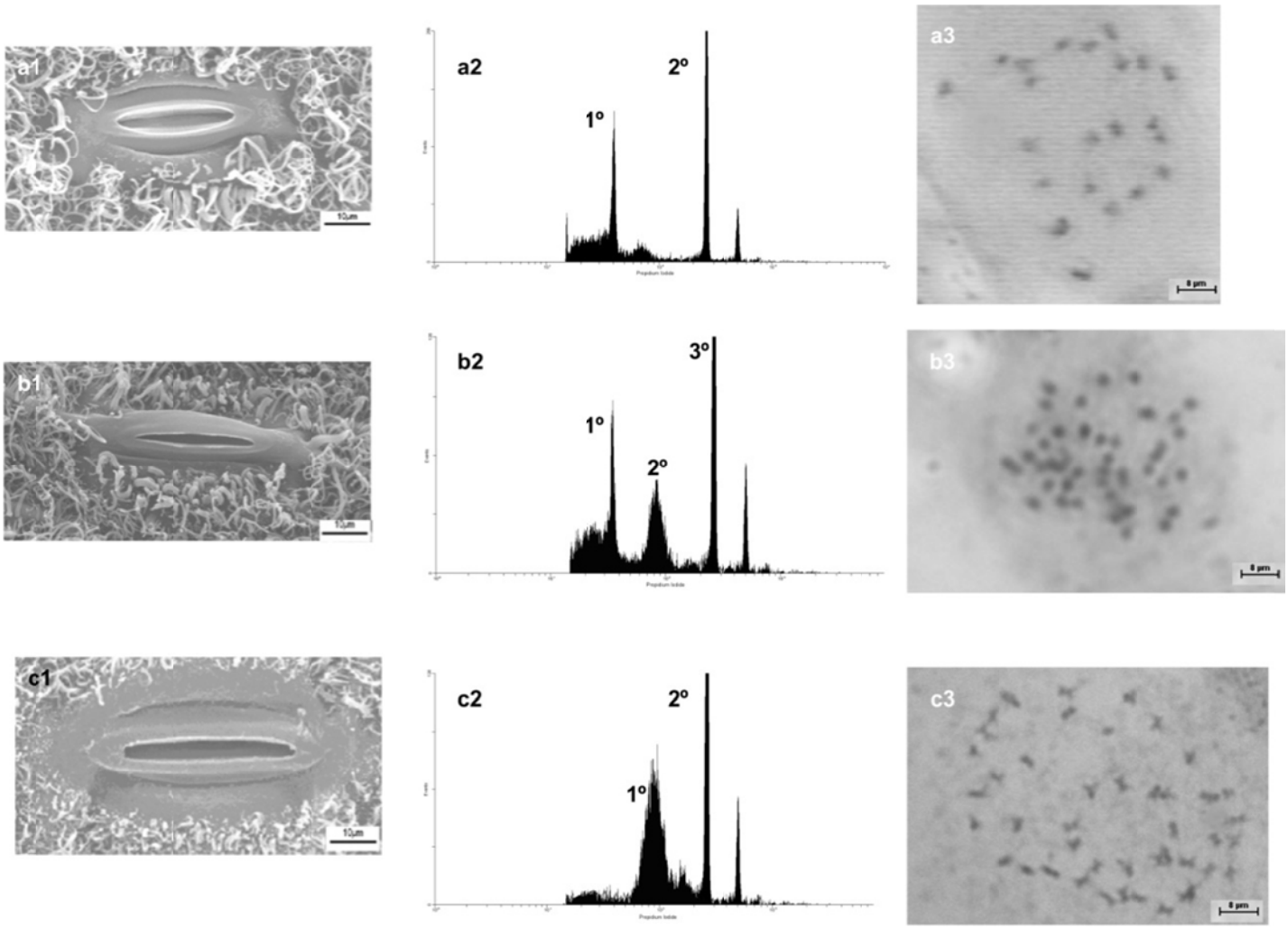

Figure 1. A, Diploid material (a1, stomata; b1, Histogram of flow cytometry results with the first and second peaks of the sample and the reference standard; a3, metaphase). **B,** Mixoploid material (b1, stomata; b2, histogram of flow cytometry results with the first and second peaks of the sample and 3rd peak of the reference standard and b3: metaphase). **C,** Tetraploid material (c1, stoma; c2, flow cytometric histogram with the first and second peaks of the sample and the reference standard; and c3, metaphase).

the presence of tetraploid plants. Sajjad et al. (2013) found that colchicine application resulted in the formation of tetraploid individuals of *Tagetes erecta* that showed slower initial growth rates and smaller leaves. Ganga and Chezhiyan (2002) tested different concentrations of colchicine and oryzalin and different exposure periods in attempts to obtain tetraploid bananas, and found that chromosomal replication was similar after exposure to either substance, although the required colchicine concentrations were 125 to 200 times greater than with oryzalin.

Slower initial growth rates of seedlings treated with colchicine have been noted when using this anti-mitotic agent to increase ploidy levels, and its effects on growth are apparently due to reductions in cell division rates (Sajjad et al., 2013). Colchicine inhibits the formation of linear structures of microtubules in plant cells (polymers of tubulin involved in various cellular functions), thus interfering with the cell cycle (Ade and Rai, 2010). Most of the plants classified as tetraploid in our study had been treated with colchicine. Although a number of authors have reported oryzalin as being efficient at inducing polyploidy in some plants, it is necessary to check these antimitotic ages with each plant type, as their responses can vary as a function of their genetic makeup and the developmental stages of their tissues.

Flow cytometry analysis can process large numbers of plants (several hundred) per day with reliable results, as DNA content is not influenced by external factors such as light intensity, leaf blade development, or plant tissue content water; the analyses are also rapid, and numerous plants can be evaluated in a very short time (Xing et al.,

Table 9. Cytogenetic analysis and stomatal analyses of diploid Thong Dok Mak. Gold. and Lidi banana varieties subjected to treatment with colchicine and oryzalin.

Treatment	Stomatal analyses	Flow cytometry analysis	Cytogenetic analysis
TDM - colchicine - control	Diploid	Diploid	Diploid
TDM - oryzalin - control	Diploid	Diploid	Diploid
Lidi - colchicine - control	Diploid	Diploid	Diploid
Lidi - oryzalin - control	Diploid	Diploid	Diploid
Gold - colchicine - control	Diploid	Diploid	Diploid
TDM - colchicine - 7.5 mM/48 h	Tetraploid	Mixoploid	Mixoploid
Lidi - colchicine - 12.5 mM/24 h	Tetraploid	Mixoploid	Mixoploid
Gold - colchicine - 2.5 mM/24 h	Tetraploid	Tetraploid	Mixoploid
Lidi - colchicine - 12.5 mM/24 h	Tetraploid	Tetraploid	Mixoploid
Lidi - colchicine - 7.5 mM/48 h	Tetraploid	Mixoploid	Tetraploid
TDM - oryzalin - 10 µM/4 dias	Diploid	Diploid	Tetraploid
Lidi- colchicine - 12.5 mM/24 h	Tetraploid	Tetraploid	Tetraploid
Lidi - colchicine - 7.5 mM/48 h	Tetraploid	Tetraploid	Tetraploid
Lidi - oryzalin - 10 µM/4 dias	Tetraploid	Tetraploid	Tetraploid
TDM - oryzalin - 10 µM/4 dias	Tetraploid	Tetraploid	Tetraploid

2011). Nuclear DNA content as determined by flow cytometry has been studied in various plants of economic importance, such as *Brachiaria* (Pinheiro et al., 2000), *Triticum* (Kubalakova et al., 2002), *Malus* (Höfer and Meister, 2010), and *Jatropha* (Kaewpoo and Te-chato, 2010). Van Duren et al. (1996) used flow cytometry to identify autotetraploid banana plants induced through chromosomal doubling of diploid material. The phenol-menon of mixoploidy was also commonly encountered in this work, and flow cytometry was efficient at detecting and confirming these cases. The use of flow cytometry to identify chromosome duplication has been described in several studies (Costich et al., 2010; Xing et al., 2011; Nemorin et al., 2013).

Pre-checking based on stomatal characteristics was found to be very helpful in identifying populations of tetra-ploid mixoploids, thus reducing the numbers of plants that had to be examined after *in vitro* cultivation, highlighting the need for care when selecting the morphological characterisitics used to comfirm plant ploidy levels. The safest method is to compare the material in at least three steps, as performed in the present work.

Motonobu et al. (1997) and Kim and Kim (2003) reported that the numbers of stomata on *Chrysanthemum* and *Cymbidium* were directly proportional to their ploidy levels, but stomata size did not vary among plants with different ploidy levels. Vichiato (2004) found that tetraploid plants of *Dendrobium nobile* showed higher stomatal frequencies because their epidermal cells were smaller than those of diploids.

Thus, while morphological descriptors can be very useful, they should not be used alone for checking ploidy levels as they are subject to genetic effects, so that the isolated analysis of only certain variables may lead to classification errors for certain genotypes (Souza and Queiroz, 2004; Madon et al., 2005). Lacerda et al. (2008) examined the morphological features of the stomata of Silver Dwarf cultivars obtained from three *in vitro* plant variants, and found that only one showed low stomata densities; they did not determine if this variant was mixoploide or not, however. Studies of chromosome doubling in *Hedychium muluense* by treatments with colchicine and oryzalin demonstrated that ploidy analysis by flow cytometry, coupled with chromosome counting, was more reliable than comparing stomata sizes (Sakhanokho et al., 2009).

Flow cytometery had an important role in identifying mixoploids in the present work as they have been erroneously classified as triploids and tetraploides in other studies. One hypothesis that could explain the occurrence of mixoploidy in the present work is chromosome elimination. Abreu et al. (2006) and Barbosa (2004), working with chromosomal duplication induction in triploid hybrid elephant grass and millet, observed mixoploidy in most of the plants analyzed. According to these authors, there were great variations in chromosome numbers in the metaphases analyzed, and they concluded that these variations were due to chromosome eliminations. Certain mitotic alterations are characteristic of chromosome elimination - such as the lack of chromosome orientation during metaphase, late anaphase segregation, chromatin degradation, chromosome fragmentation, and micronuclei formation (Singh, 2002).

Mixoploids may arise because antimitotic agents may not always reach all of the meristems on a plant (or those

that are actively dividing) (Carvalho et al., 2005). Several authors (Poutaraud and Girardin, 2005; Campos et al., 2009; Wannakrairoj and Wondyifraw, 2013) have reported different effects of antimitotic agents when different tissues are targeted or different treatment periods are used. As cytogenetic analyses were performed using root meristems, while stomata and flow cytometric analyses were performed using young leaves, it would not be unexpected if these distinctly different tissues in distinct localities demonstrated different ploidy levels after treatment with antimitotic agents.

Thus, the possibility of inducing autotetraploidy in banana plants should have a significant impact on breeding programs as this will reduce the time needed, manpower expenditures, and costs involved in obtaining tetraploids as compared to conventional methods. Some types of tetraploids may be impossible to obtain using conventional methods, making antimitotic agents of great importance to breeding programs. The autotetraploids obtained in this study cannot necessarily be recommended as cultivars, as tetraploids generally have arched leaves and their fruits easily separate and fall, so it will be necessary to obtain triploid plants from them. To that end, future studies will be conducted with the tetraploid plants generated here to cross them with diploid elite varieties in the germplasm bank at Embrapa Cassava. It is expected that crossing tetraploids with diploids will generate triploids that, after agronomic evaluation, can be distributed by Embrapa as new and improved varieties.

Bananas are of significant importance in the Brazilian diet, mainly among low-income families who lack economic resources to purchase non-tropical fruits. The climate in Brazil also favors banana cultivation throughout the year, which ensures an abundant supply of these fruits at low cost. Therefore, research efforts to generate more resistant, productive, nutritious, and good-tasting bananas must be continued.

Additionally, there is a need to promote more balanced and sustainable agriculture techniques that use less agro-defensive products that - and tetraploid induction using antimitotic agents presents the possibility of inducing disease resistance through the generation of duplicated genetic material, as was observed using disease-resistant Cavendish triploids that had previously been extremely difficult to obtain through conventional breeding. The next step will be to evaluate the agronomic characteristics of plants generated through chromosome duplication to certify their taste, vigor, and resistance to the fungal diseases and pests that are quite common and destructive in Brazilian plantations.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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