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Full Length Research Paper

Colchicine and amiprophos-methyl (APM) in polyploidy induction in banana plant

Filipe Almendagna Rodrigues[1]*, Joyce Dória Rodrigues Soares[1], Roseneide Rocha dos Santos[1], Moacir Pasqual[1] and Sebastião de Oliveira e Silva[2]

[1]Federal University of Lavras (UFLA), Department of Agriculture, P. O. Box 3037, 37200-000, Lavras, Minas Gerais, Brazil.
[2]Embrapa Cassava and Tropical Fruit, 44380-000, Cruz das Almas, Bahia, Brazil.

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The objective was to assess the colchicine and amiprophos-methyl (APM) concentration and exposure period in the chromosome duplication of breed banana plants diploids. Banana stem tips were used from the following genotypes: breed diploids (1304-04 [Malaccensis x Madang (Musa acuminata spp. banksii)] and 8694-15 [0337-02 (Calcutta x Galeo) x SH32-63]). Colchicine was used at concentrations of 0 (control treatment), 1.25, 2.5 and 5.0 mM, while APM was used at 0 (control treatment), 40 and 80 µM, in solution under agitation (20 rpm), for 24 and 48 h periods. With the use of APM, 66.67% tetraploid plants were obtained in the 1304-04 genotype using 40 µM for 24 h and 18.18% in 80 µM for 48 h, while in the 8694-15 genotype using 40 and 80 µM colchicine for 48 h, 27.27 and 21.43% tetraploid plants were observed, respectively. For colchicine, in the 1304-04 genotype, only the 1.25 mM treatment for 48 h presented 25% tetraploid plants and in the 8694-15 genotype, the 5.0 mM concentration for 48 h produced 50% tetraploid plants. APM for 24 h enabled the tetraploid plant of the 1304-04 genotype to be obtained, while colchicine for 48 h resulted in tetraploid plants in the 8694-15 genotype.

Key words: *Musa acuminata*, antimitotic, flow cytometry, tissue culture.

INTRODUCTION

Banana plant cultivation is very important from both the social and economic points of view in more than 80 countries, especially on small farms (Silva et al., 2002). Diseases and pests affect the main varieties traditionally used in Brazil. One of the strategies used to solve these problems is the selection of new disease and pest resistant genotypes that have good agronomic characteristics which have been achieved in conventional banana plant breeding programs (Silva et al., 2000) or the chromosome duplication induced by antimitotic agents. Using colchicines in chromosome duplication in plants has been the most usual procedure (Doležel et al., 1994), but there are few reports regarding other antimitotic agents for introducing *in vitro* polyploid in banana plants (Ganga and Chezhiyan, 2002). According to Yahata et al. (2004), other antimitotic agents, such as amiprophos-methyl (APM) have demonstrated greater specificity for tubulin *in vitro* than colchicine.

Amiprophos-methyl (APM) is a type of phosphoric amide herbicide that directly interrupts the microtubule dynamic in plant cells (Morejohn and Fosket, 1984) and is used in agriculture throughout the world, because it is quickly degraded and leaves little residue for the environment (Brahma and Umesh, 1985). Even at low concentrations, it inhibits microtubule polymerization, preventing the formation of chromatic fuses and inducing separation of the metaphasic chromosomes.

The ploidy level in plants submitted to chromosome duplication can be determined directly by counting the chromosomes in mitotic and meiotic cells. According to Bakry et al. (2007), the chromosome count can be useful to identify polyploids plants when flow cytometry is not available. Omidbaigi et al. (2010) indicated that induced

*Corresponding author. E-mail: filipealmendagna @yahoo.com. br Tel: +55 35 3829 1323.

Abbreviation: APM, amiprophos-methyl.

tetraploid plants compared to diploid plants can be distinguished by flow cytometry that is a quick and easy method to identify the level of polyploidy in *Ocimum basilicum*. This method can be considered the most efficient to detect alterations in the polyploid level.

The objective of this study was to adjust the colchicine and APM concentration and exposure period for chromosome duplication of banana plants diploids.

MATERIALS AND METHODS

This study was conducted in the Tissue Culture Laboratory in the Department of Agriculture of the Federal University of Lavras, Minas Gerais state, Brazil, using breed diploid banana plants developed by the genetic breeding program at Embrapa Cassava and Tropical Fruit.

Plant material

Banana plants stem tips of the following genotypes were used for the experiments: breed diploids (1304-04 [Malaccensis x Madang (*Musa acuminata* spp. *banksii*)] and 8694-15 [0337-02 (Calcutta x Galeo) x SH32-63]), which were established *in vitro*, multiplied in MS culture medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose and 4 mg/l 6-benzylaminopurine (BAP) for root proliferation and were submitted to the treatments with the antimitotics colchicine and APM. Two experiments were conducted, one for each antimitotic.

Experiment 1

Colchicine was used at concentrations of 0 (control treatment), 1.25, 2.5 and 5.0 mM, in solution under agitation (20 rpm) for 24 and 48 h periods.

After 24 and 48 h, the stem tips were removed from the antimitotic and washed three times in distilled water autoclaved. Shortly afterwards, they were transferred to test tubes containing 15 ml MS medium and then kept in a growth room with artificial light supplied by daylight special-type fluorescent light bulbs (OSRAM 20 W) with 42 W/m^2, mean irradiance, 16-h light period and 25±2°C.
A completely randomized design was used in a 4 x 2 factorial arrangement (four concentrations x two exposure periods). Sixteen plants were used per treatment, that is, four replications with four plants.

The survival rate of the plants was assessed after sixty days *in vitro* and the ploidy of the 1304-04 and 8694-15 genotypes was analyzed. The data were analyzed by statistical software SISVAR 4.3 (Ferreira, 2000) at 5% probability.

Experiment 2

APM was used at concentrations of 0 (control treatment), 40 and 80 µm, in a solution under agitation (20 rpm) for 24 and 48 h periods.

After 24 and 48 h, the stem tips were removed from the antimitotic and washed three times in distilled water autoclaved. Shortly afterwards, they were transferred to test tubes containing 15 ml MS medium and then kept in a growth room with artificial light supplied by daylight special-type fluorescent light bulbs (OSRAM 20 W) with 42 W/m^2, mean irradiance, 16-h light period and 25±2°C. A completely randomized design was used in a 4 x 2 factorial arrangement (four concentrations x two exposure periods). Sixteen plants were used per treatment, that is, four replications with four plants.

The survival rate of the plants was assessed after sixty days *in vitro* and the ploidy of the 1304-04 and 8694-15 genotypes was analyzed. The data were analyzed by statistical software SISVAR 4.3 (Ferreira, 2000) at 5% probability.

Flow cytometry

The flow cytometry analyses were carried out in a BD (Becton Dickinson) FACSCalibur^{TM} flow cytometer four color. Approximately, 70 mg young banana plant leaf tissue from each sample was fragmented with the help of a scalpel in 1 ml chilled LB01 extraction buffer to obtain nuclear suspension (Doležel et al., 1997). The squashed tissue was aspirated through two layers of gauze to remove excess leaf fragments. The nuclear suspension was then filtered through a 50 µM nylon mesh and 25 µl propidium iodide (1 mg/ml) were added to the nuclear suspension to stain the nuclei present in the sample and 25 µl RNase. The test tubes were placed at room temperature in the dark until analysis. At least 5000 nuclei were analyzed for each sample.

The histograms obtained in the cytometry were analyzed using the Cell Quest and WinMDI 2.9 softwares. The 1304-04 and 8694-15 genotypes that had not been treated with antimitotic were used as a standard reference and the polyploidy. From the position of the G1 peak formed, it was possible to estimate the ploidy of the genotypes in the histogram.

RESULTS AND DISCUSSION

The treatments with the colchicine and APM antimitotics showed low toxicity in explants of genotype 8694-15 (Table 1) since it observed a high survival rate. Figure 1 shows the appearance of explants surviving the treatment with the antimitotic, toxic effect on plants and contaminated by fungi, respectively.

Table 1 shows the survival of the explants in 5.0 mM colchicine; in both 24 and 48 h exposure to the antimitotic and this was due to the fact that this antimitotic is toxic to plants at high concentrations. Opposite results were reported by Viehmannová et al. (2009), who studied yacon (*Smallanthus sonchifolius*) and observed a greater explants survival rate at concentrations of 5.0 mM colchicine for 24 h.

Colchicine is traditionally used to induce polyploidy in plants (Doležel et al., 1994), acting strictly on cells in division, inhibiting chromosome fusion so that the chromosomes are paralyzed in the metaphase, leading the cell to successive endomitoses and causing an increase in the polyploidy level (Jensen, 1974). Colchicine acts as a polyploidy inducer in plants *in vitro* but has limitations because it has a toxic effect at high concentrations, generating high mortality in the treated plants (Hamill et al., 1992). Therefore, for each species and type of material to be treated, the appropriate colchicine concentration should be determined, because tolerance to this antimitotic varies with the species (Wan et al., 1989).

Table 2 shows that plants of the 1304-04 genotype

Table 1. Percentage of surviving explants, dead and contaminated of the 8694-15 genotype banana plant treated with colchicine and APM (amiprophos-methyl) in different exposure periods, after sixty days *in vitro*.

Antimitotic	Concentration	Survival (%)		Death (%)		Contaminated (%)	
		24h	48h	24h	48h	24h	48h
Colchicine	Control	100^{aA}	94^{aA}	0^{aA}	0^{aA}	0^{aA}	6^{bB}
Colchicine	1.25 mM	100^{aA}	100^{aA}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
Colchicine	2.5 mM	100^{aA}	81^{bA}	0^{aA}	19^{bA}	0^{aA}	0^{aA}
Colchicine	5.0 mM	75^{bA}	75^{bA}	25^{bA}	25^{bA}	0^{aA}	0^{aA}
CV (%)		12.24		13.61		2.19	
APM	Control	100^{aA}	94^{aB}	0^{aA}	0^{aA}	0^{aA}	6^{bB}
APM	40 µM	100^{aA}	100^{bA}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
APM	80 µM	100^{aB}	94^{aB}	0^{aA}	6^{bB}	0^{aA}	0^{aA}
CV (%)		2.85		1.89		1.90	

Means followed by the same uppercase letter on the line and lowercase letter in the column do not differ by the Scott-Knott test at 5% probability.

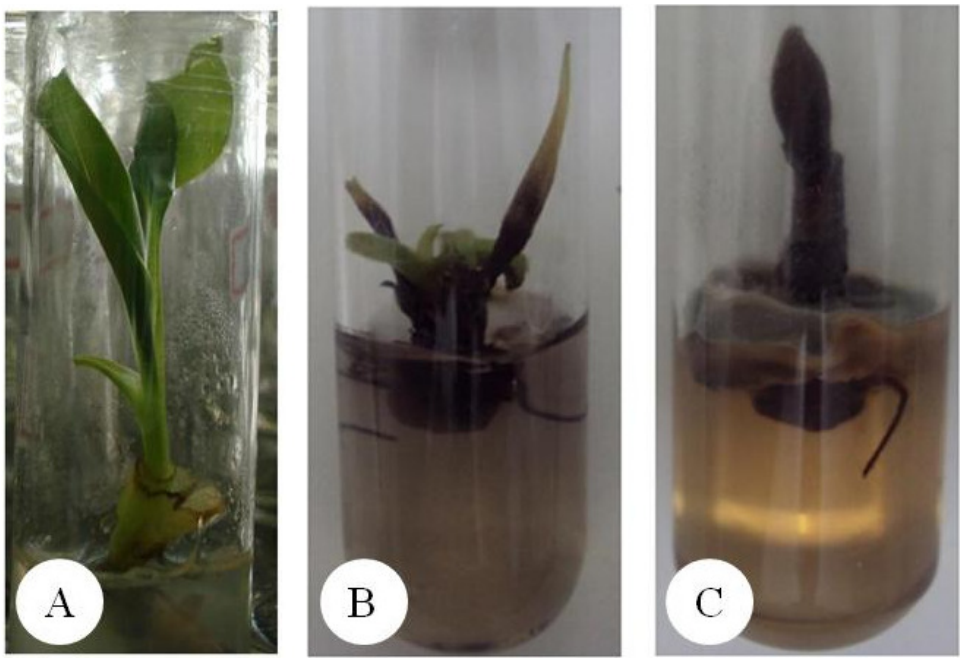

Figure 1. Aspect of banana explants treated with antimitotic. A) survived the treatment with the antimitotic; B) toxic effect on plants and C) contaminated

treated with colchicine presented a higher survival rate in the 24 h compared to 48 h exposure period, even with contamination and death in the treatments, a fact that might reduce the frequency of possible tetraploid plants. The survival of explants treated with colchicine depends on the concentration and duration of the treatment, but generally, high concentrations and long exposure periods to the inducing agent decrease plant survival (Thao et al., 2003). Lone et al. (2010) assessed the influence of colchicine concentration and exposure time on the survival and *in vitro* development of *Cattleya tigrina*. Protocorms were used as plant material, treated with 1.25 and 2.5 mM colchicine for 24, 48 and 72 h. Survival was assessed after 60 days culture, and the lowest survival rate was observed in the 2.5 mM concentration for 72 h.

Ishigaki et al. (2009) investigated the effect of different colchicine concentrations on multiple sprout survival. According to the authors, survival of multiple sprouts to colchicine depended on the concentration and treatment

Table 2. Percentage of surviving explants, dead and contaminated of the 1304-04 genotype banana plant treated with colchicine and APM (amiprophos-methyl) in different exposure periods, after sixty days *in vitro*.

Antimitotic	Concentration	Survival (%)		Death (%)		Contaminated (%)	
		24h	48h	24h	48h	24h	48h
Colchicine	Control	100^{aA}	94^{aA}	0 ^{aA}	6 ^{aA}	0^{aA}	0^{aA}
Colchicine	1.25 mM	50^{bA}	56^{aA}	50 ^{bA}	44^{aA}	0^{aA}	0^{aA}
Colchicine	2.5 mM	12^{bA}	37^{aA}	81 ^{bA}	56^{aA}	6^{aA}	6^{bA}
Colchicine	5.0 mM	69 ^{aA}	37^{aA}	31 ^{aA}	50 ^{aA}	0^{aB}	12^{bB}
CV (%)		49.41		65.01		12.24	
APM	Control	100^{aA}	81^{aB}	0 ^{aA}	0 ^{aA}	0^{aA}	19^{bB}
APM	40 µM	94^{aA}	87^{aA}	6 ^{aA}	12^{bA}	0^{aA}	0^{aA}
APM	80 µM	100^{aA}	87^{aB}	0 ^{aA}	12^{bB}	0^{aA}	0^{aA}
CV (%)		7.67		10.44		18.97	

Means followed by the same uppercase letter on the line and lowercase letter in the column do not differ by the Scott-Knott test at 5% probability.

Table 3. Percentage of surviving explants for the 1304-04 and 8694-15 genotypes banana plants treated with colchicine for different exposure periods, after sixty days *in vitro*.

Antimitotic	Genotype			
	1304-04		8694-15	
	Exposure period			
	24 h	48 h	24 h	48 h
APM	97.92^{aA}	85.42^{aB}	100.00^{aA}	95.83^{aB}
Colchicine	57.81^{bA}	56.25^{aA}	93.75^{aA}	87.50^{aA}
CV (%)	26.04		5.21	

Means followed by the same uppercase letter on the line and lowercase letter in the column do not differ by the Scott-Knott test at 5% probability.

duration. Furthermore, the treatment with colchicine at higher concentrations for more than 12 h resulted in a low survival rate. Treatments for 48 h resulted in low survival, regardless of the colchicine concentration.

Table 2 shows the effect caused by APM on the 1304-04 genotype. There was greater survival of plant in the 24 h compared to the 48 h exposure period because of the lower plant loss, whether by contamination or death also observed in this period.

The present study showed lower toxicity of APM compared to colchicine (Table 3), which was also reported by Sri Ramulu et al. (1991) and Yahata et al. (2004), who observed that APM was more efficient in inducing chromosome duplication and less cytotoxic than colchicine. Thus, APM can be considered as an efficient substitute for colchicine (Sri Ramulu et al., 1991).

Polyploidy inducing with colchicine is the most used procedure but there are few reports regarding other antimitotic agents for *in vitro* polyploidy induction in banana plants (Hamill et al., 1992; Van Duren et al., 1996; Ganga and Chezhiyan, 2002).

Analyzing the 8694-15 and 1304-04 genotypes for the percentage of polyploid plants (Table 4) obtained, in relation to the antimitotic concentration, a large number of tetraploid plants were observed. With the use of APM, 66.67% tetraploid plants were obtained in the 1304-04 genotype at the 40 µM concentration for 24 h and 18.18% were obtained in 80 µM for 48 h. For the concentrations 40 and 80 µM for 48 h, 27.27 and 21.43% tetraploid plants were observed, respectively. Regarding colchicine, in the 1304-04 diploid, only the 1.25 mM concentration for 24 h resulted in 6.67% tetraploid plants, 1.25 mM for 48 h in 18.19% tetraploid plants, 2.5 mM for 48 h 50% tetraploid plants and the concentration of 5.0 mM for 48 h produced 50% tetraploid plants. Similar results were reported by Van Duren et al. (1996) who obtained 23.1% induced *Musa acuminata* auto tetraploid plants by using 5.0 mM colchicine for 48h.

There are few reports of polyploidy inducing in the banana plant in the literature, although, Ganga and Chezhiyan (2002) assessed colchicine concentrations in banana plants with different treatment periods to obtain tetraploid plants. The chromosome duplication capacity of the substance was 13% tetraploid plants. Polyploid

Table 4. Percentage of polyploidy plants obtained for the 1304-04 and 8694-15 genotypes banana plants treated with APM (amiprophos-methyl) and colchicines for different exposure periods, after sixty days *in vitro*.

Antimitotic	Concentration	Genotype			
		1304-04		8694-15	
		Exposure period			
		24 h	48 h	24 h	48 h
APM	Control	0	0	0	0
	40 µM	66.67	0	0	27.27
	80 µM	0	18.18	0	21.43
Colchicine	Control	0	0	0	0
	1.25 mM	0	25	6.67	18.19
	2.5 mM	0	0	0	50
	5.0 mM	0	0	0	50

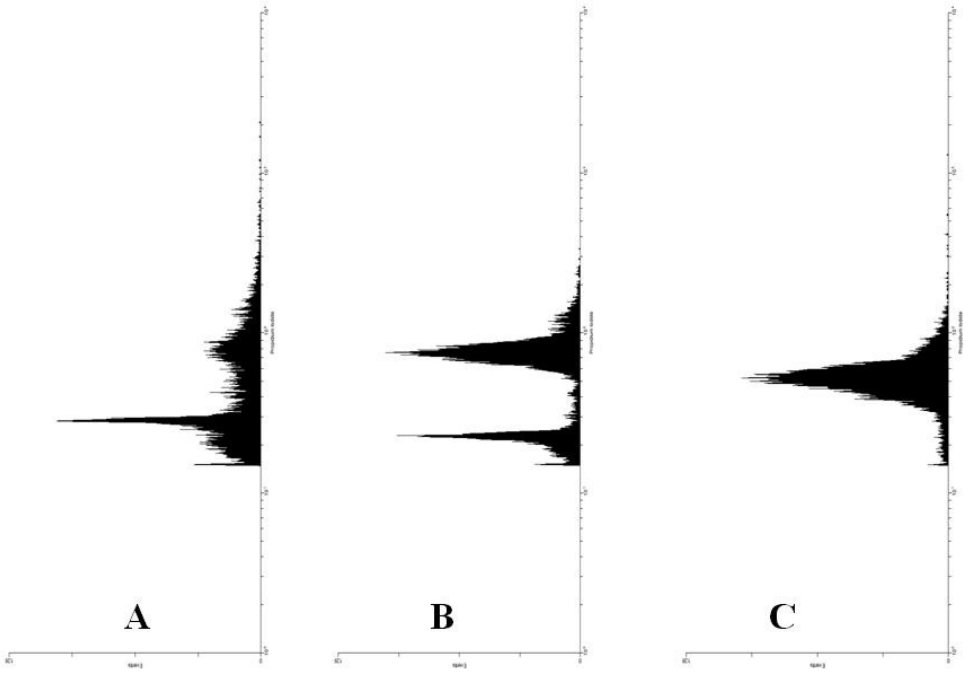

Figure 2. Ploidy levels in banana plants: A) Diploid (2x); B) Mixoploid (2x+4x) and C) Tetraploid (4x).

induction also was studied in wild banana plant (*M. acuminata* ssp. *malaccensis*) by Asif et al. (2000), and allowed the successful production of tetraploid plants in treating shoot apices with 12.5 mM colchicine.

Figure 2 shows the histograms obtained in flow cytometry, showing the differences that occurred among the ploidy levels (diploid, mixoploid and tetraploid). Figure 2A shows the histograms of diploid (2x) banana plants with the formation of two peaks (G1, greatest peak and G2, smaller peak). The G2 peak presents double the nuclear DNA of the G1 peak. Figure 2B is the mixoploid (2x+4x) which represents a material with different ploidy levels (both diploid and tetraploid cells) in the same sample that is not interesting in a genetic breeding program because the diploid cells multiply at higher rates than tetraploid cells. Figure 2C shows the formation of only one peak exactly in the position where the G2 peak is formed in the diploid material, thus, it corresponds to a tetraploid material (4x).

Banana plants not having a very high ploidy level (up to

6x) are usually fertile in development. This is because, although, the triploid and tetraploid plants are more vigorous than the normal diploid plants, there is a “dose effect” of the mapped genes, in that up to a determined moment, they gain vigor and earliness, but after this limit they spend a lot of energy and time on duplication of their own DNA. These delay the vital cycles that are expressed in less biomass production per time unit (Dressler, 1993).

The main problem of using antimitotic agents results from the fact that these substances only act efficiently on the cells that are not dividable. Thus, polyploidization generally does not reach all the cells of the treated material and the appearance of mixoploid plants is common (variation in the chromosome number). Therefore, the choice of cultivars to be used and the variation in the concentration, exposure time and forms of application of these antimitotic agents are indispensable requirements in any genetic breeding program for chromosome duplication.

Vakili (1967) used colchicine to induce polyploidy in banana plants and observed that the antimitotic agent increased mortality, delaying plant growth, but induced the duplication of the chromosome number. According to the same authors, the tetraploids plants were higher and more robust than the diploid plants but grew more slowly with more inclined leaves and less developed root system.

Thus, new studies should be carried out to really define which antimitotic and concentration to use to promote polyploidization in banana plants.

Conclusion

APM had a less toxic effect as compared to colchicines. The 8694-15 genotype is suitable for use in chromosome duplication, in that 40 µM APM for 24 h in the 1304-04 genotype resulted in tetraploids plants and 2.5 mM colchicine for 48 h resulted in tetraploids in the 8694-15 genotype.

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