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Induced Mutation by Colchicine Treatment of Somatic Embryos in 'Namwa' Banana (*Musa sp*. ABB)

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ABSTRACT

Hexaploids of the 'Namwa' banana (*Musa sp* ABB) were obtained by *in vitro* colchicine treatment of somatic embryos. Somatic embryos were induced on a medium containing MS medium supplemented with 8 mg/l picloram. Somatic embryos were treated with four different concentrations of colchicine (0, 0.3, 0.5, 1.0, %) in liquid MS medium supplemented with 0.22 mg/l zeatin, and shaken (60 rpm) at 25 0C in darkness for 48, 72 or 96 hours. Higher colchicine concentrations exhibited higher mortality rates ranging from 8–20%, 48–62% and 80–90% mortality on concentrations 0.3, 0.5, and 1.0 % colchicine respectively. Mortality rate generally increased with increased treatment time. Hexaploids were obtained at a frequency of 2 % with treatments 0.05 % colchicine for 96 hours, and 1 % colchicine for 48 hours as determined by flow cytometry.

1. Introduction

Bananas and plantains (*Musa* sp.*)* are the fourth most important food crop in the world (Moffat, 1999) cultivated in over 100 countries in the tropical and subtropical regions of the world where they constitute a major staple food crop for millions of people, as well as providing a valued source of income through local and international trade (Frison and Sharrock, 1999). However, banana production is hampered by many pests and diseases such as fungi, viruses, bacteria, insects, and nematodes (Jones, 2000). Genetic improvement to tackle these production

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bottlenecks through traditional breeding procedures has not been very successful. According to Novak *et al.* (1989), conventional breeding is complicated by the facts that only a few diploid clones produce viable pollen and that the germplasm of the most important commercial clones is both male- and female sterile. Hence, the major obstacles in banana breeding are high sterility, polyploidy and long generation times of most edible cultivars of most important banana and plantain varieties (Sagi *et al*., 1997; Sasson, 1997; Tenkouano and Swennen, 2004). The application of biotechnology for the improvement of this crop would be a useful tool to breeders for the manipulation of germplasm and introductions of traits of interest. Hence, nonconventional *in vitro* tools such as genetic engineering (Ságі *et al.*, 1995; Becker *et al.*, 2000), protoplast fusion techniques (Assani *et al*., 2005), mutagenesis (Jain, 2010), somaclonal variation (María and García, 2000), and *in vitro* chromosome doubling or (Hamill *et al.*, 1992) are becoming more and more important for genetic manipulation of such plants.

Vakili (1962) first induced polyploidisation in banana by immersion of M. balbisiana germinating seeds in a colchicines solution. The recovered plantlets showed to be tetraploid and octoploid, some tetraploids reverting to diploid level. Asif and Othman (2001) and Tesfaye (2005) later confirmed chromosome doubling on *Musa balbisiana* and various *Musa acuminata* subspecies and the species *Ensete ventricosum* respectively using seeds. Induction of autotetraploids was first reported by Hamill *et al.* (1992) in the SH-3362 diploid edible hybrid. Polyploidisation resulted from the *in vitro* application of colchicine on micropropagated shoot tips in liquid medium. Selection of induced tetraploids relied on gross morphology and stomatal characters. Stable autotetraploids have been established in the field; however reversions, formation of chimeras and aneuploids were common. In similar *in vitro* conditions, Van Duren *et al.* (1996) compared the efficiency of colchicine and oryzalin to induce polyploidization and found out that both anti mitotic agents varied in time and concentration in producing non chimerical tetraploids. The efficiency of colchicine in tetraploid induction was also reported by Ganga and Chezhiyan (2002). The induction of chromosome doubling depends on a large number of variables: media, antimitotic agents, explant types, exposure times and concentrations. Flow cytometry is the pre-eminent method for evaluation of the induced polyploidization. Alternative confirmation methods, such as chromosome counts and morphological observations are also used. However, In the study conducted on a wide range of mono- and interspecific diploid banana clones by Bakry *et al.* (2007), it was observed that chromosome counts led to distinguish diploid and tetraploid plants but did not detect chimeras while flow cytometry allowed an early screening of a larger number of plants leading to rapidly detect chimerical plants.

In bananas shoot-tip cultures are traditionally used for mutagenesis. The main problem with this type of culture is the presence of chimerism. In contrast, somatic embryogenesis has been shown to be an effective method for eliminating chimeras since embryos are presumed to regenerate from single cells in several crops (Van Harten, 1998; Liu *et al.*, 1992; Toonen *et al.*, 1994; Roux *et al.*, 2004). This phenomenon made somatic embryos especially preferable for genetic manipulation such as genetic transformation (Sagi *et al.*, 1995; Becker *et al.*, 2000), protoplast fusion (Xiao *et al.*, 2009) and chromosome doubling (Roux *et al.*, 2004; Yang *et al.*, 2006). In this study, the response of somatic embryos obtained in a liquid medium to colchicine treatment was investigated.

2. Materials and method

2.1 Source and preparation of explant

Explants were prepared following the method used by Filippi *et al.* (2001) from *in vitro* plants of the Thai banana variety 'Namwa' (*Musa sp*. ABB) provided by the Starch Biosynthesis Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC). Plants of approximately similar height, diameter, and growth vigor were used for this purpose. Roots and leaves were removed from the plants and reduced to approximately 1.5 cm height and 0.5 cm diameter. These were cut into two by dissecting longitudinally in the middle to expose the meristem tissue thereby producing two explants from a single *in vitro* plant.

2.2 Induction of embryogenic callus

Explants were placed with the meristem (cut side) in contact to a medium containing MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 8 picloram and 30 g/l sucrose and was solidified with 2 g/l of gelrite. The medium was adjusted to pH 5.8 with KOH (1N) and HCl (1N) prior to autoclaving and poured onto 9 cm diameter sterile plastic petridishes. Plates were sealed with Parafilm and maintained in culture room at 28°C in darkness.

2.3 Establishment of embryogenic cell suspensions

Cell suspension cultures were initiated by placing embryogenic callus in 150 ml Erlenmeyer flasks dispensed with 20 ml liquid multiplication medium (half strength MS medium, MS vitamins, 10 mg/l ascorbic acid, 0.22 mg/l zeatin, 30 g/l sucrose) supplemented with 8 mg/L picloram and sealed with aluminium foil and Parafilm. Suspensions were

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subsequently cultivated on a rotary shaker at 100 rpm under darkness and 26 ± 1ºC for three months. The culture medium was refreshed every week for the first month and then every two weeks there after by keeping 10–20% of the old 'preconditioned' medium.

2.4 Colchicine treatment

An experiment containing a control and three concentrations of colchicine (0, 0.3, 0.5, 1.0 %) and three durations of treatment (48, 72, or 96 hours) was conducted. The explants selected for colchicine treatment were vigorously growing small size somatic embryos of approximately 2mm taken from embryogenic cell suspensions known to have retained their capacity to regenerate and from which shoots were regenerated into whole plantlets. Colchicine dissolved in 1% dimethylsulphoxide (DMSO) was filter sterilized and added to autoclaved liquid MS medium supplemented with 0.22 mg/l zeatin. Aliquots of 20 ml liquid medium containing the various colchicine concentrations were placed into 100 ml Erlenmeyer flasks. Fifty vigorously growing globular embryos were immediately transferred into each flask. Flasks were agitated on a gyratory shaker at 60 rpm during treatment.

2.5 Conversion of somatic embryo and plant regeneration

Following the colchicine treatment, all treated embryos were washed three times with sterile water and transferred to a regeneration medium containing MS medium supplemented with 5 mg/l BAP, 1 mg/l NAA, 20 mg/l Maltose, 25 g/l glucose, 25 g/l sucrose 50 mg/l Inositol, and 500 mg/l casamino acid. The number of surviving embryos and normal green shoots regenerated from somatic embryos were recorded.

2.6 Ploidy detection

Ploidy detection was performed using a Partec PAS II flow cytometer (FCM). Samples were prepared according to Dolezel *et al.* (1994, 1997). Approximately 20-30 mg of fresh leaf samples from *in vitro* grown plants (control+sample) were chopped with a sharp scalpel blade in a glass petri dish containing 2ml of LB01 lysis buffer and 2µg/ml DAPI (3,5-dinitro-N4, N-dipropylsulphate). This sample preparation was filtered through a nylon mesh (50 µm pore size). The relative DNA content of the sample was then determined using FCM analysis.

3. Results

3.1 Somatic embryogenesis

Yellow loose callus developed in the 6th week and globular somatic embryos started to develop starting the 7th week of culturing while steadily increasing in number and size into the

8th and 9th weeks (Figure 1). To establish embryogenic suspension, calli were excised from the explants and cultured on a liquid medium containing half strength MS medium, MS vitamins, 10 mg/L ascorbic acid, 0.22mg/L zeatin, 30g/L sucrose and 8mg/l picloram.

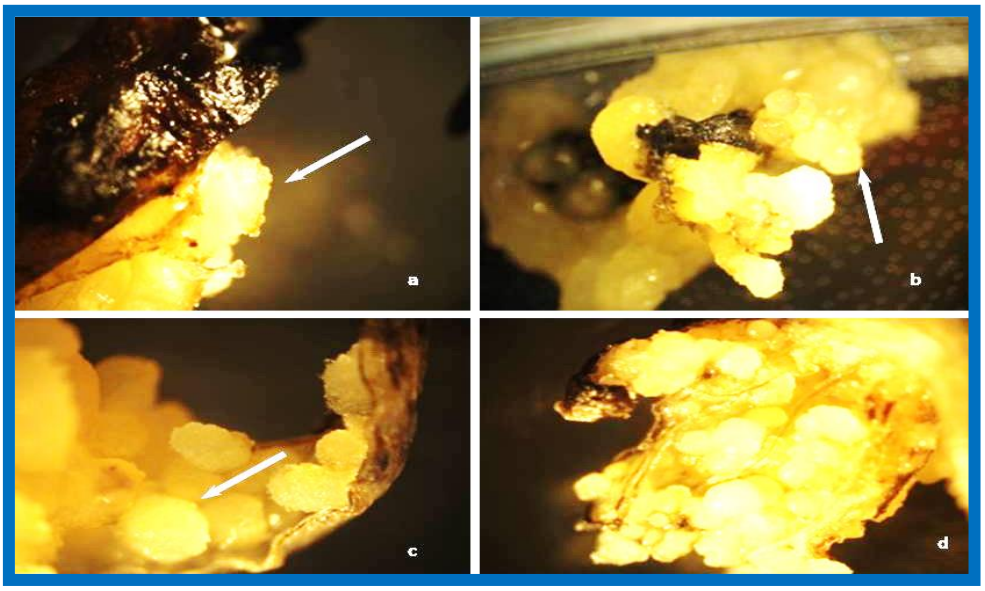

Figure 1: Picloram induced somatic embryos. a, b, yellow loose callus developed starting the 6th week from culturing and c, d. globular somatic embryos started to develop starting the 7th week of culturing while steadily increasing in number and size into the 8th and 9th weeks.

Table 1: Effects of concentrations and durations of colchicine treatment on survival and doubling efficiency of somatic embryos from suspension cultures of 'Namwa' banana (Musa sp. ABB)

Colchicine conc. (%)	Duration (h)	No. of Treated Embryos	Percentage of ploidy plants (%)		Mortality (% ± SD*)
			3x	6x	
0	48	50	100	0	0
	72	50	92	0	8
	96	50	90	0	10
0.1	48	50	100	0	0
	72	50	92	0	8
	96	50	92	0	8
0.3	48	50	92	0	8
	72	50	88	0	12
	96	50	80	0	20
0.5	48	50	52	0	48
	72	50	40	0	60
	96	50	36	2 (1 plantlet)	62
1.0	48	50	18	2 (1 plantlet)	80
	72	50	10	0	90
	96	50	12	0	88

* Standard Deviation = 33.9

3.2 Effects of colchicine on somatic embryos

The survival and germination of the somatic embryos after colchicine treatment were dependent on colchicine concentration and the treatment duration. In general, higher concentration and longer duration tended to reduce survival and germination of the embryos

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(Table 1). Embryos treated with colchicine either grew slowly or died within 2 weeks of transfer onto conversion medium. The number of surviving embryos after treatment decreased with increasing colchicine concentration. Although the 0.1% concentration of colchicine and the control (no colchicine) had similar mortality rates of embryos (8 - 10 %), higher colchicine concentrations exhibited higher mortality ranging from 8–20 %, 48–62 % and 80–90 % mortality for concentrations of 0.3, 0.5, and 1.0 % colchicine respectively. The mortality rate generally increased with increased treatment time. The effect of colchicine on polyploidy induction was very low. Only somatic embryos treated with 0.5 colchicine for 96 hours and those treated with 1% colchicine for 48 hours were able to produce one (2 %) hexaploid plant each out of 50 embryos which exhibited 62 and 80 % mortality respectively.

3.3 Ploidy level determination by flow cytometry

Flow cytometry performed on regenerated plant was employed to give an accurate estimation of nuclear DNA content. Figure 2 shows the result of flow cytometry measurement with two types of histograms. Controls containing 3C DNA showed peak 1 that had been determined by analyzing the standards with known ploidy. Hexaploids with 6C DNA showed histogram with peak 2 (Figure 2).

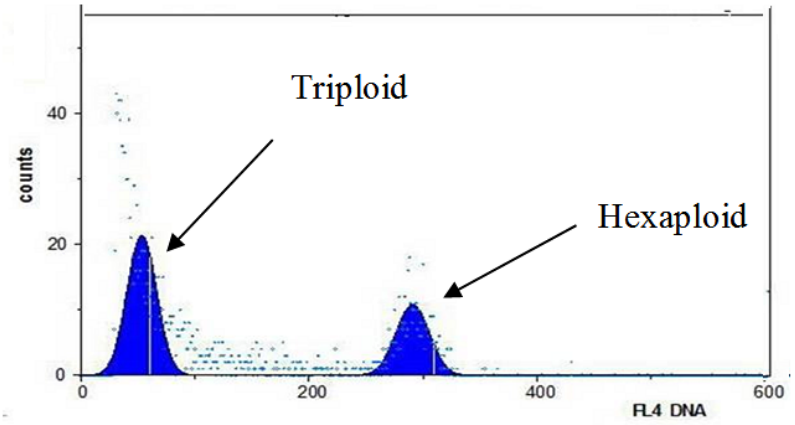

Figure 2: Histogram of a control triploid and a hexaploid from colchicine treatment of control and colchicine treated 'Namwa' banana (*Musa sp*. ABB).

4. Discussion

With regard to the induction of somatic embryogenesis, Filippi *et al.* (2001) found similar results to this study where they were able to obtain embryogenic response from shoot apex explants of *Musa* spp. cv 'Nanicão' cultured in picloram. In agreement with this finding Smitha and Nair (2011) and Huang and Chi (1988), reported similar results from *Musa acuminata* (diploid) cv 'Njalipoovan' (AB) and *Musa sapientum* L. respectively. Picloram was also used

to induce embryogenic callus from bract (Divakaran and Nair, 2011) and immature male flowers (IMF) (Kulkarni *et.al.*, 2002; Wei *et al.*, 2007; and Houllou-Kido *et al.*, 2005). Differential response of genotypes to auxins type and concentration is reported by Sidha *et al.* (2007).

Low frequency (5.3 %) of chromosome doubling was reported by Roux *et al.* (2004) from somatic embryos on the variety Williams (AAA Group) treated with colchicine. They speculated that the low frequency of regeneration of hexaploid plants may be due to that triploid cells have an advantage over hexaploid cells during culture. Ganga and Chezhiyan (2002) also reported that anti mitotic agents and especially colchicine had a negative effect on the *in vitro* regeneration cultivars reflected in terms of delay in regeneration, reduced multiple shoot regeneration rates, regeneration of smaller microshoots with lower fresh weights, and reduced response to rhizogenesis. Low frequency of chromosome doubling and regeneration of colchicine treated somatic embryos is also reported in other plant species (Wu and Mooney, 2002; Yang *et al.*, 2006; Samala and Te-chato, 2012).

The results obtained when screening using flow cytometry indicated that no chimeras with both 3C and 6C nuclei was produced from colchicine-treated somatic embryos in all the analyzed samples. This further confirmed that somatic embryogenesis, unlike organogenesis, decreased considerably the possibility of chimerical plant production in inducing chromosome doubling. Flow cytometry is used routinely for ploidy analyses and it is regarded as the most accurate tool for ploidy determination (Dolezel *et al.*, 1994).

5. Conclusion

Colchicine treatment of somatic embryos of the Thai banana cultivar 'Namwa' (Musa sp. ABB group) maintained on embryogenic suspension culture resulted in chromosome doubling. Treatment of somatic embryos with colchicines at 0.5 % for 96 hours and 1 % for 48 hours resulted in hexaploid plants (one plantlet each). This finding indicates that chromosome doubling could be achieved by treating somatic embryos. Since the origin of somatic embryos is widely believed to be single cell, this method could significantly reduce the problem of chimerism and mixploidy whish are major problems in chromosome doubling results from colchicine treated tissues or organs. The absence of mixploids and chimeras in this study strengthens the usefulness of using somatic embryos for chromosome doubling. In this study,

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flowcytometry proved to be an effective tool in assessing the ploidy level of regenerated plants early and allows working easily on a large number of plants.

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