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Polyploid Induction in a Local Wild Banana (*Musa acuminata ssp. malaccensis*)

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Abstract: Flow cytometry was successfully used for screening ploidy levels in a large population of *in vitro* induced autopolyploids of the *Musa acuminata ssp. malaccensis* a wild banana. Use of zygotic embryo culture for large production of banana seed progenies and improved media compositions has facilitated the process of polyploidy induction. Treating shoot apices with 0.5% colchicine in combination with 2% DMSO for 2 h successfully produced tetraploids in Musa. Tetraploids produced were further confirmed through chromosomal counts.

Keywords: Musa - Colchicine - DMSO (dimethyl sulfoxide) - FCM (Flow cytometry) - Autotetraploid - BAP (6-bezylaminopurine) - IAA (indole, 3-acetic acid) - 2,4-D (2,4- dichlorophenoxy acetic acid).

Introduction

Banana and plantains are important staple food crops for many people in developing countries. Frequently, national economies depend largely on dessert bananas as an export crop. Dessert bananas are mainly triploid Cavendish types (*Musa acuminata*) which are increasingly threatened by Fusarium wilt (*Fusarium oxysporum f.sp. cubense* races 1 and 4) and Black Sigatoka (*Mycosphaerella fijiensis var. difformis*). To sustain adequate yields, the chemical control of these diseases requires significant financial management and investment that can only be afforded by larger plantations. Moreover, the use of pesticides is environmentally unfriendly and becoming politically unacceptable. In nature polyploids are formed by the union of a haploid pollen gamete (n = 11) with restituted polyploid egg cell (Dodds and Simmonds, 1946). The edible banana of commercial importance are triploids which have descended from parthenocarpic diploids and seeded species by means of hybridization, mutation and selection by man throughout centuries past (Simmonds, 1955). The main goal of the major banana and plantain improvement programs is to create new varieties with increased resistance or tolerance to the major diseases (Persley and Langhe, 1987). The future of the banana industry is therefore going to be largely dependent on the ability of plant breeders.

However the breeding of most commercially acceptable banana is made difficult by their parthenocarpic nature and triploidy, so producing hardly any seeds. Since there is no cross - incompatibility between Musa species, it may be possible to use hybridization in breeding programs (Novak, 1992; Vuylsteke *et al.*, 1993). Banana and plantain hybridization nevertheless complicated by the combination of different ploidy levels and by female restitution associated with the formation of unreduced female gametes. Secondly triploids may be produced at a very low efficiency by crossing primary triploids with improved diploids. An alternative procedure to obtain synthetic triploids would be to induce chromosome doubling of promising diploids and then crossing them with diploids (Novak, 1992). Production of triploids by crossing autotetraploids with improved diseased resistant diploids may be promising way to increase productivity (Hamill *et al.*, 1992).

In addition to an effective system for induction of polyploidy mass production of autotetraploids requires an effective method for ploidy screening. Classically this is done using chromosome counting (Hamil *et al.*, 1992). However, this procedure is not suitable for mass screening. Ploidy estimation based on stomata number, length and density (Speckmann *et al.*, 1965; Sreenivasan *et al.*, 1992; Blanke *et al.*, 1994), and recently Tenkouano *et al.*, (1998) reported ploidy determination based on pollen and chloroplast characteristics, is easier, however the method is not always reliable due to environmental effects. Flow cytometric analysis of nuclear DNA content is being increasingly used for large-scale ploidy screening (De Laat *et al.*, 1987; Dolezel *et al.*, 1989; Dolezel, 1991).

Flow cytometry is the measurement of cells in a flow system, which has been designed to deliver particles in single file past a point of measurement. The power of flow cytometry lies in the ability to measure several parameters on terms of thousands of individual cells within a few minutes. The method can therefore be used to define and to enumerate accurately sub - population. Once identified, such populations can be sorted physically for further study.

Materials and Methods

For ploidy induction seed progenies from wild species of *Musa acuminata* were raised through in vitro zygotic embryo culture. Fruits were thoroughly washed with distilled water and then soaked in 20% Clorox for 30 minutes. Fruits skin was peeled off, and seeds were removed inside the laminar flow. These seeds were again soaked in 20% Clorox for 10 minutes followed by quick washing with 70% ethanol. Embryos in *Musa acuminata* are present just beneath the micropylar end. Embryos were exposed by breaking apart the seed coat and then removed with the help of a needle. The excised embryos were cultured in the glass jars with each jar containing 10 embryos.

The culture medium consists of MS salts supplemented with Nicotinic acid (0.125mg/l), Ascorbic acid (0.2mg/l), Thiamine HCl (0.5mg/l), Pyridoxine HCl (0.125mg/l), Myo-ionositol (2.5mg/l), Glutamine (150mg/l), and Sucrose 5%. Media pH was adjusted to 5.8 before autoclaving. Media was autoclaved at 121°C for 30 min.

Ploidy induction: One-month-old seedlings grown from zygotic embryo culture were used. Plantlets were cut few mm above the corm and all roots were also trimmed off. Colchicine was used at a concentration of 0.5 % dissolved in 2% DMSO (dimethyl sulfoxide , Sigma) and then final volume was adjusted with sterile distilled water (Azhar *et. al*, 1998). Colchicine solution was then autoclaved at 121C° for 30 min. Solution flask was wrapped in aluminum foil to protect from light. Corm pieces were then treated with colchicine solution for 2 hr while shaking on the shaker. Corm pieces were then washed with distilled autoclaved water, dried on filter paper. Corms pieces were then cultured on Modified MS media supplemented with 1μM/l of IAA and 10μM/l of BAP (P5) for one subculture followed by 1μM/l IAA and 100μM/l of BAP (P4) for two subcultures. Fourth subculture was made in media with 1 mg/l of 2-4-D to induce roots. Cultures were maintained at 28 ± 2C° with 16 h light and 8 h dark.

Leaf samples: For ploidy detection fresh young leaf samples were taken from the tissue culture grown plants. Leaf discs were placed on a moist filter paper in a petri dish.

Sample preparation: The specimen to be analyzed by FCM must be in the form of a single cell suspension. The problems associated with low penetration of DNA – specific flourochromes can be avoided if cell nuclei were isolated prior to staining. This approach also eliminates the effect of cytoplasmic DNA stains and the problem of non-specific chlorophyll fluorescence as individual chloroplast exhibited low levels of fluorescence in comparison with stained nuclei. Galbriath *et al.* (1983) described a simple and rapid method for the direct isolation of nuclei from plant tissues. The tissue (approx. 100mg) was chopped with a razor blade in a glass petri dish containing buffered hypotonic solution with non-ionic detergent (lysis buffer), named LB01 lysis buffer. 200μl DAPI (3,5-dinitro-N4, N-dipropylsulphate) was added to LB01 solution before chopping of the tissue. Tissue was chopped in 1ml of lysis buffer. Then the homogenate was filtered through a nylon filter (50μm pore size), to remove the leaf tissue debris.

Flow cytometer analysis of ploidy: Fluorescent intensity of DAPI stained nuclei was measured by using a Partec CCA flow cytometer (Partec GmbH, Munster, Germany), that used high-pressure mercury arc lamp as an excitation light source. For the analysis of DAPI stained nuclei the following optical filters were used. KGI, BG38 AND VG1 for excitation, TK420 as dichromic mirror and GG435 as a barrier filter. Where as, as a sheath fluid deionized water was used. The instrument was aligned by using suitable particles (e.g.; fish erythrocyte nuclei provided by Partec) for maximal signal amplitude and minimal coefficient of variation (CV).

Sample analysis: For the calibration of the instrument an external standard of untreated diploid *Musa acuminata ssp. malaccensis* was used. Ploidy of the samples was then determined as if, peak appeared at channel 50 sample was a diploid and if peak was observed on channel 75 or 100 those samples were triploid and tetraploids respectively. Flow speed was adjusted to 20-50 nuclei per second. Next step was to adjust the gain so that the peak of nuclei in G1 phase of the cell cycle should appear at channel 50 everytime when a diploid sample was run. Once the gain was fixed it was not changed which otherwise could result in shifting of the channel. When necessary, histograms of DNA content were evaluated using the Partec software package. The precision of the analysis was based on the estimation of the co-efficient of variability (CV) of the G1 peak. So smaller the CV, more precise would be the measurements.

For chromosomes counting roots from in vitro grown plants were harvested between 9-10 a.m and then treated with 0.02% of colchicine solution for 4 h. Roots were then fixed in a fixative glacial acetic acid ; alcohol and few drops of choloroform were added to it. Chromosomes were stained with acetocarmine and then viewed under microscope.

Results and Discussion

Shoot apices treated with 0.5 % Colchicine and 2% DMSO for 2 h successfully induced tetraploids in *Musa*. Hamil *et al.*, (1992) obtained 31.5 % tetraploids from a diploid *Musa acuminata* clone SH-3362 after treatment with 0.5 % w/v colchcine for 2 h. A number of mixoploids particularly (2n + 4n) and a few (4n + 8n) were also obtained. The low concentration of colchicine and DMSO in the medium did not effect the development of new shoots and growth of the plants, a negative effect of DMSO was greater in the case when oryzalin was used than colchicine - treated explants (van Duren *et al.*, 1996). However a marked

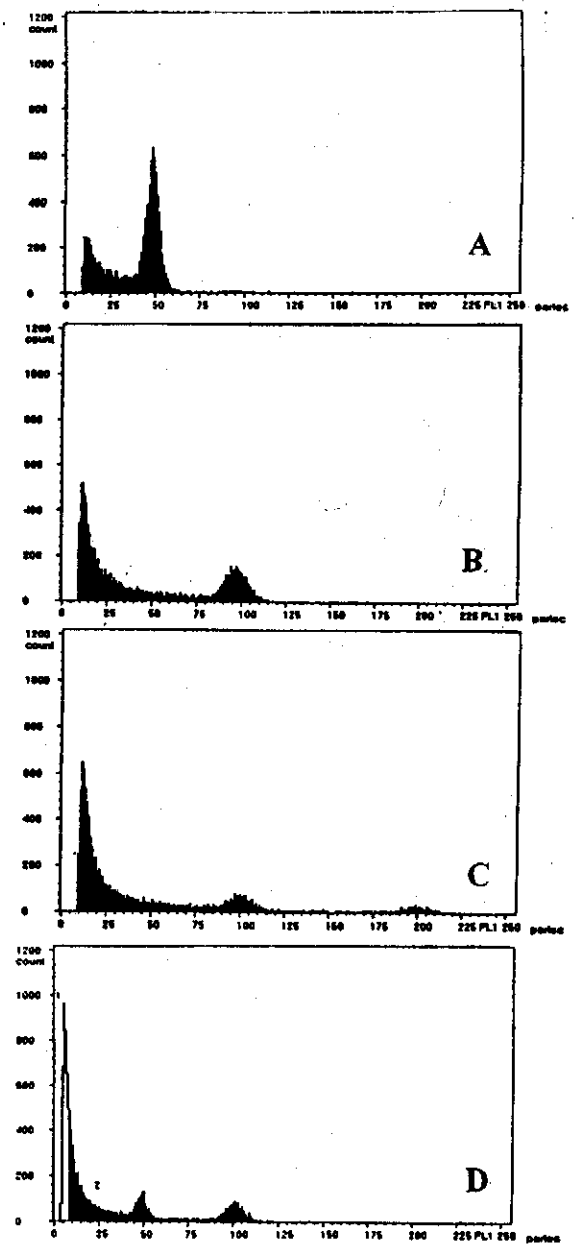

Fig. 1: Histograms showing different ploidy levels in *Musa acuminata ssp. malacensis* (a) Standard diploid (2n = 22) (b) Tetraploid, Mixoploids (c) 2n + 4n (d) 4n + 8n.

blackening and stunted abnormal seedling growth was observed at high colchicine concentrations and longer treatment time (Azhar *et al.*, 1998). The histograms of different ploidy levels of the plants are shown in Fig.2. The initial peak represents the debris background. Nuclei released form a diploid plantlet appeared at a channel 50 for G1 phase as shows in Fig. 1a. Plantlets with double chromosomes level spots the G1 peak at channel 100, Fig. 1b. Correspondingly the ploidy level of these clones would be a tetraploid banana. Similarly, in case of mixoploids two peaks were obtained in case 2n + 4n are appeared at channels 50 and 100 respectively, Fig. 1c. Where as in case of 4n + 8n histogram obtained appeared on channels 100 and 200 Fig. 1d. The tetraploids had drooping, thick leaves with short petioles but appeared sturdier than the diploids. Tetraploids growth observed to be very slow as compared to diploids (Fig 2.). Ploidy determination through chromosomal counting did not seem

Fig. 2: Growth comparison of (a) diploid (b) mixoploid (2n + 4n) (c) Tetraploid seedlings.

Fig. 3: Chromosomal counting using root tips in a tetraploid *Musa acuminata ssp. malaccensis.*

Fig. 4: Shoot proliferation observed on media P5 and P4.

efficient as problems like poor spreading and small size of banana chromosomes made difficult to count the number of chromosomes (Fig. 3.). Use of P5 and P4 media combinations resulted in an increase shoot proliferation rate, increasing the frequency of tetraploids (Fig. 4.).

Use of zygotic embryo culture for the production of seed progenies proved useful for the induction of a polyploid in *Musa*, as it resulted in efficient and high percentage of germination compared to treating intact seeds. Vakili (1962) reported that unsoaked intact seeds were less affected by colchicine treatment than seeds in early stages of germination. This was most likely due to the presence of the thick seed coat and quiescent state of the embryos. Flow cytometry analysis proved to be easy and efficient and since DNA contents are not influenced by the external factors such as light intensity, leaf blade development and water contents of the plant tissues. Flow cytometry analysis resulted in the selection of solid tetraploid as compared to other screening techniques as it screened 10,000 nuclei in every sample being screened. Ploidy detection through chromosomal counting is more tedious and as it is difficult to get good smears for the counting of the chromosomes. Similarly use of stomata size and number has been reported to be inefficient as it is difficult to differentiate between a mixoploid and a tetraploid (Azhar *et al.*, 1998). In case of flow - cytometer ploidy of many more plants (several hundreds) can be measured per day, as DNA content is not influenced by external factors such as light intensity, leaf blade development, and water content of the leaf tissue. The chimeric plants were present at low concetration and shorter time which could be due to the strong toxic effects of colchicine as it is a stron killing agent even at millimolar concentrations (Morejohn, *et al.*, 1984). The chimerism would probably be inherited or revert to diploid gradually through continuous micropropagation. The chimeras can be reduced only to a certain extent by several cycles of vegetative propagation but still 30-40 % of plant will remain mixoploid (Azhar, *et al.*, 1998). Similar distribution of mixoploid plants was also recorded in the vegetative clones of the chimeric Mulberry mutants (Vijayan *et al.*, 1998).

Chromosome counts using root tips in banana has long been known as valuable tool for plant breeders and cytogeneticists (Vakili, 1966; Vakili, 1967; Hamill, 1992 and Osuji *et al.*, 1996). It was agreed that chromosome isolation is a difficult task in banana since the chromosome are extremely small, idistinguishable and frequently a few cell divisions are visible in a single root tip. Dropping technique by pre-treatment with 8-hydroxyquinoline gave the most condensed and can enhance the visibility of the chromoses (Azhar *et al.*, 1998). However this technique is very laborious and its use for the screening of polyploids would be limited. Perhaps this is one of the reasons why cytological research on Musa sp. is limited. Strong influence of genotype, further complicated with mixoploids showed that these techniques were not reproducible and cannot be recommended for reliable ploidy estimation in Musa sp. (Hilde, *et al.*, 1995, van Duren, *et al.*, 1996). Therefore FCM technique is preferred as rapid, precise and simple method of choice for the screening of ploidy levels.

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