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The Influence of Temperature, Light and Cytokinins on DNA Content of *Musa acuminata* (AAA Group) 'Kluai hom thong' and *Musa balbisiana* (BBB Group) 'Kluai hin'

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Abstract

The changes in DNA content of triploid bananas *Musa acuminata* (AAA group) 'Kluai Hom Thong' and *Musa balbisiana* (BBB group) 'Kluai Hin' under different environmental conditions were investigated. The results revealed showed that different temperatures and light regimes had no effects on DNA content of both bananas cultivated on MS (Murashige and Skoog, 1962) medium supplemented with 5 mg/l BA (N^6-benzyladenine) and 15% coconut water. However, they had some influences on morphological characteristics. On the other hand, both bananas cultivated on MS medium containing different concentrations of TDZ (N-phenyl-1, 2, 3 thidiazol-5-ylurea; thidiazuron) and BA, differed in their DNA contents. Increase in TDZ and BA concentrations resulted in the decrease of DNA content in 'Kluai Hom Thong', while the DNA content of 'Kluai Hin' increased.

Keywords: DNA content, flow cytometry, *Musa acuminata, Musa balbisiana*, organogenesis, triploid banana

Introduction

Bananas (*Musa* spp.) are among the world's most important crops and one of the major export commodities of many developing countries. To date, much progress in the propagation of *Musa* spp. via tissue culture methods has been made (Ko et al., 1991; Panis et al., 1993; Eckstein and Robinson, 1995; Okole and Schulz, 1996; Navarro et al., 1997; Lee et al., 1997). It is well documented that genome size of *in vitro* culture can be altered through changes either in the chromosome number or in the ploidy level. In this context, information about DNA content would be essential by providing basic information to assess the significance of culture variation. Therefore extensive studies to ascertain plant genetic stability following micropropagation must be performed. The chromosome number of micropropagated plants of *Musa* spp. is very difficult to assess using microscope since most banana cultivars and all plantain (cooking banana) landraces have 33 chromosomes (2n = 3x) (Crouch et al., 1998). Recently, flow cytometry has been used to analyze DNA content in numerous plant species (Dolezel et al., 1998). Flow cytometry offers an easy, rapid, accurate and convenient method for determining ploidy level assessing DNA content and analyzing the cell cycle (Winkelmann et al., 1998). The present paper reports the effects of *in vitro* environment on DNA content of triploid bananas analyzed by flow cytometry.

Materials and methods

Plant materials

Young suckers of *Musa acuminat*a (AAA group) 'Kluai Hom Thong' and *Musa balbisiana* (BBB group) 'Kluai Hin' collected from a commercial plantation located in Hat-Yai District, Songkla Province and in Bannangsata District, Yala Province, southern Thailand, respectively were used in this study. Suckers were cut into pieces 20 cm diameter and 25 cm long and washed in running water to remove dirt. They were trimmed down to 5-6 cm long until lateral buds were exposed. Tissue blocks (2 cm^3) containing either lateral or apical buds were immersed in 70% ethanol for 30 sec then surface sterilized using a dilution of 20% (v/v) commercial Clorox containing 5.25% active chlorine and 1 drop of Tween 20 per 100 ml solution for 15 min, followed by 10% (v/v) Clorox for 10 min. After the surface decontamination was done, these explants were rinsed 3 times with sterile distilled water to remove traces of disinfectant.

Nutrient media and culture conditions

Apical and lateral buds were excised and cultured on a medium consisting of macro- and micronutrients and vitamins according to Murashige and Skoog (1962; MS). BA (N^6-benzyladenine) at the concentration of 5 mg/l either singly or in combination with 15% (v/v) coconut water (CW) were incorporated into the medium. All media were supplemented with 3% sucrose and solidified with 0.17%

Gelrite (Merck and Co., Kelco Div., NJ, USA). The pH of all media was adjusted to 5.7 with 0.1 N NaOH or 0.1 N HCl prior to autoclaving at 1.05 kg/cm^2, 121°C for 20 min. Regenerated shoots of each banana with 2-3 cm in length were used in the subsequent experiments.

Different temperature, light regimes and concentrations of BA and TDZ were used to study their effects on DNA content as follows:

1) Shoots cultured on MS medium supplemented with 5 mg/l BA and 15% CW were incubated at 25 ± 2°C and 35 ± 2°C with a 16-h photoperiod under an illumination of 20 μmolm^{-2}s^{-1} from white-fluorescent lamps.

2) Cultured shoots in the same condition as in 1 was incubated at 25 ± 2°C with a 16-h photoperiod under an illumination of 15, 1,500 and 2,500 lux.

3) Cultured shoots in the same condition as in 1 were transferred to MS media supplemented either with 1, 3 and 5 mg/l BA or 1, 3 and 5 mg/l TDZ in combination with 15% CW.

Plant material was stored in glass-capped culture jars (115 ml capacity) each containing 20 ml of medium. One explant was implanted per culture and 10 cultures were raised for each treatment. When an adequate shoots and roots were obtained, the plantlets were transferred to 330 ml screw-topped jars containing sterile vermiculite for 2 weeks for hardening.

Flow cytometric analysis

After 8 weeks of culture, approximately 50 mg each of 'Kluai Hom Thong' and 'Kluai Hin' young leaves was harvested and transferred to glass Petri dish containing 1 ml of LB01 modified lysis buffer (Dolezel et al., 1989) and 50 μg/ml RNase. Glass Petri dish was placed on top of ice-cold bucket and nuclei were extracted by chopping plant material using a sharp razor blade. ß-mercaptoethanol at a concentration of 500 μl was added to the isolation buffer immediately. After chopping, the suspension was filtered through a 42-μm nylon mesh and 20 μg/l propidium iodide was added. *Raphanus sativus* L. cv Saxa nova, 2C=1.11 pg DNA served as an internal reference standard. The fluorescence of 5000-10000 propidium iodide-stained nuclei was estimated from using a flow cytometer FACScalibur Becton Dickinson (Scientific Equipment Center, Prince of Songkla University). Each sample was analyzed twice in order to ensure calibration and accurate determination of DNA content. Histograms of DNA contents were analyzed by WinMDI version 2.8.

Data analysis

All experiments were conducted on two different days. Shoot numbers were submitted to analyze the variance and mean of shoot in each cultivar and the different response of explants to various plant growth regulators were compared using Sheffe's test at P≤0.05. The software used was SPSS for Windows XP Professional.

Results and discussion

At initial culture, browning of cultured buds was evident and this apparently inhibited the growth of the cultured tissue. This problem could be overcome by frequent subculture of the tissue to fresh medium hence increased the rate of proliferation. After 4-5 weeks on cytokinin containing MS media, apical and lateral buds of both cultivars showed slight variation in culture. They began to change from white cream to green color and swelled considerably due to the development of leaf primordia. The growth of lateral buds, however, was more vigorous than apical buds; therefore lateral buds were selected for further experiments. Bulge buds were halved and transferred to fresh media. Multiple shoots were obtained at the fourth week of culture. Both cultivars seemed to be influenced by the cytokinins incorporated in the multiplication medium. BA applied at a concentration of 5 mg/l and 15% CW induced an average of 5-6 shoots within 6 weeks of culture. By successive subculture, masses of proliferating shoot cultures have been established. Therefore we routinely use this protocol for multiplication of shoots for further experiments. These regenerated shoots could be rooted subsequently by transferring them to MS free medium. In most cases root formation took place within 2 weeks. All rooted plantlets survived after transplantation to soil.

The morphogenetic responses for two cultivars tested were in the same trend when grown in different culture temperatures and light intensities. Temperature and light did not affect *in vitro* multiplication significantly. They produced an average of 5-6 shoots at 25 ± 2°C which were larger and healthier than shoot obtained from 35 ± 2°C. Similarly, other than temperature, light intensities were investigated and results revealed that shoots maintained at constant 25 ± 2°C in a 16-h photoperiod with a light intensity of 2500 lux led to an increase in the number of multiple shoots (4-5 shoots). Fewer shoots (3-4 shoots) were produced at a light intensity of 1500 lux. Light intensity at 15 lux delayed shoot emergence and produced longer shoots with white pale color as compared to those grown in higher light intensities (Figure 1). The effects of BA and TDZ were further observed for a period of 8 weeks. Well-formed shoot were produced by two cultivars cultured on BA-containing medium. The cytokinin BA, at a concentration of 5 mg/l induced more shoots than shoot obtained from 1 and 3 mg/l BA. In contrast TDZ at all concentrations tested led to poor shoot bud formation with the concomitant suppression of the shoot elongation for both cultivars. The shoot proliferation in TDZ-containing medium was manifested by the appearance of a clump of several fleshy bulbous structures (Figure 2).

Initial flow cytometry data with banana leaves showed poorly defined peaks. The addition of ß-mercaptoethanol to the isolation buffer revealed sharp peaks and improved flow cytometry results markedly. The histograms obtained after analysis of the relative fluorescence intensity of the

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Figure 1 Banana shoot development on MS medium supplemented with 5 mg/l BA and 15% CW were incubated for 8 weeks at 25 ± 2oC in a 16-h photoperiod under an illumination of 15 lux (left) 1,500 (middle) and 2,500 lux (right)

Figure2 Fleshy bulbous shoot formation on MS medium containing 15% coconut water in combination with1 mg/l TDZ (left), 3 mg/l TDZ (middle), and 5 mg/l TDZ (right)

isolated nuclei in 'Kluai Hom Thong' and 'Kluai Hin' leaves from *in vitro* cultures contained one peak corresponding to 2C DNA content (Figure 3). The distribution of the nuclei extracted displayed a prominent peak at 2C indicating that they consisted of cells with G0/G1 phase of cell cycle. Table 1 presented the flow cytometric estimation of mean of DNA content from both cultivars grown in different culture environment. These data indicated that the nuclear DNA content of 'Kluai Hom Thong' sampled in the population was higher than 'Kluai Hin' (mean 2C DNA contents of 'Kluai Hom Thong' range from 1.30 to 2.07 pg and 'Kluai Hin' range from 1.37 to 1.80 pg). Temperature and light did not affect DNA content significantly ($P \geq 0.05$). Leaves of 'Kluai Hom Thong' exposed to elevated concentrations of BA or TDZ had lower DNA values and this was in contrast to what was observed in 'Kluai Hin'. There was significant difference between the two cytokinin concentrations ($P \leq 0.05$).

In a preliminary test, it was found that two-step surface sterilization gave better result than one-step procedure. In our previous work, the problem of phenolic compounds exudated to the medium could be overcome by the use of antioxidant. However, in this work we did not utilize antioxidant but frequent subculture to new fresh medium instead. Banerjee and Langhe (1985) reported that the problem of blackening of the culture of seven *Musa* cultivars could be overcome by a combined effect of the addition of ascorbic acid and frequent transfer of the tissue to fresh medium.

Table 1 Nuclear 2C DNA content (pg) of leaves of triploid bananas *Musa acuminat*a 'Kluai Hom Thong' and *M. acuminata* 'Kluai Hin' under different environmental conditions

DNA content (X ± SE)		
Treatment		
	'Kluai Hom Thong'	'Kluai Hin'
Temperatures (°C)		
25	1.65 ± 0.05 a	1.46 ± 0.05a
35	1.91 ± 0.09 a	1.57± 0.06 a
Light intensities (Lux)		
15	1.64 ± 0.07 a	1.37 ± 0.10 a
1,500	1.53 ± 0.07 a	1.53 ± 0.06 a
Plant growth regulators (mg/l		
TDZ (1)	2.07 ± 0.15 a	1.72 ± 0.08 ab
TDZ (3)	1.75± 0.21 ab	1.44 ± 0.14 ab
TDZ (5)	1.58± 0.09 ab	1.80 ± 0.10 a
BA (1)	1.80 ± 0.09 ab	1.35 ± 0.03 b
BA (3)	1.44 ± 0.17 ab	1.50 ± 0.11ab
BA (5)	1.30 ± 0.11b	1.46 ± 0.05 ab

The different letters within column show significant difference analyzed by Sheffe's test at $p<0.05$.

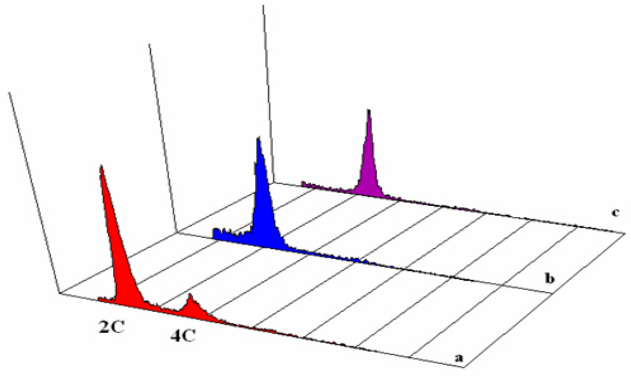

Figure 3 Histogram of propidium iodide fluorescence intensity of nuclei isolated from young leaves of *Raphanus sativus* L. cv Saxa nova (a), Kluai Hin (b) and Kluai Hom Thong (c). The fluorescence histograms were resolved into 2C and 4C cell cycle compartments with a peak-reflect algorithm using Win MDI version 2.8. X-axis represents DNA content; Y-axis represents event of cell counts.

Cytokinin commonly used for *Musa* spp. micropropagation is BA. We previously reported the use of TDZ, a substituted phenylurea (N-phenyl-1, 2, 3 thidiazol-5-ylurea), on triploid banana 'Gros Michel', AAA group (Srangsam and Kanchanapoom, 2003). In this experiment TDZ did not promote adventitious shoot formation and this may be due to the high concentrations applied. TDZ is among the most active cytokinin for tissue culture of woody plant species and at low concentration induces axillary shoot proliferation but may inhibit shoot elongation (Ledbetter and Preece, 2004).

A nuclei isolation method was optimized for use with the leaves of 'Kluai Hom Thong' and 'Kluai Hin'. ß-mercaptoethanol was used in this study due to the presence of phenolic compounds which probably resulted from tissue damage during the explant preparations. Noirot et al. (2000) stated that phenols released from plants could affect dye accessibility to DNA and caused a stoichiometric error in DNA content estimation. An antioxidant such as PVP-10 at the concentration of 1% was also routinely used in other reports (O'Brien et al., 1996; Meng and Finn, 1999).

In our experiments, BA and TDZ are most likely to affect DNA content since nuclear DNA variation was observed. The genomic changes in plantlets obtained from tissue culture may result from medium composition, plant hormones and environmental factors thus causes chromosome instability and nuclear DNA variability. Chromosome instability and nuclear DNA variation were also observed in other *in vitro*-derived plants of sugar beet and cucumber (Thiem and Sliwinska, 2003). Our flow cytometric analyses revealed that both cultivars were characterized by the same ploidy level as internal standard plant. Estimated nuclear DNA content in 'Kluai Hom Thong' was slightly higher than nuclear DNA content in 'Kluai Hin' which was in support the finding that the A and B genome of *Musa* differ in size, the B genome being smaller by 12% on average (Lysak et al., 1999).

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