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Plant Regeneration from Callus Cultures of Diploid Bananas (*Musa acuminata*, AA Group) 'Kluai Sa' and 'Kluai Leb Mu Nang' and Analysis of Ploidy Stability Using Flow Cytometry

Korn Koarapatchaikul • Kamnoon Kanchanapoom*

Department of Biology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

Corresponding author: * **kamnoon_k@yahoo.co.th**

ABSTRACT

Plant regeneration of diploid bananas (*Musa acuminata*, AA group) 'Kluai Sa' and 'Kluai Leb Mu Nang' was achieved through organogenesis in callus cultures. Calluses were induced from *in vitro*-grown shoot tips of both cultivars on a modified Murashige and Skoog medium containing 100 mg L^{-1} malt extract, 50 mg L^{-1} proline, 50 mg L^{-1} cysteine, 100 mg L^{-1} glutamine, 1 mg L^{-1} biotin, 5-7 mg L^{-1} dicamba and 1-2 mg L^{-1} thidiazuron (TDZ). On these media, two morphologically distinct types of white and yellow compact calluses were produced. Green shoot buds were best induced after several transfers of the yellow compact calluses to MS medium supplemented with 1 mg L^{-1} α-naphthaleneacetic acid (NAA) and 3 mg L^{-1} benzyladenine (BA) with 19 shoots in both cultivars. Rooting in all regenerated shoots was promoted on MS medium devoid of plant growth regulators but containing 0.2% activated charcoal. All the resultant plants were shown to be morphologically normal. Acclimatization and survival when transferred to field conditions were shown to be 100% in the regenerated plants. Flow cytometric analysis of nuclear DNA content confirmed that they were all diploid with a 2C peak indicating that ploidy alteration did not occur.

Keywords: dessert bananas, nuclear DNA content, organogenesis, shoot tip culture
Abbreviations: BA, benzyladenine; **Dicamba**, 3, 6-dichloro-*O*-anisic acid; **NAA**, α-naphthaleneacetic acid; **TDZ**, phenyl-N^{1}-1, 2, 3,-thiadizol-5-ylurea (thidiazuron)

INTRODUCTION

Banana (*Musa* sp.) is a major food staple and plays a role as an economically important food crop in the world market. It provides export income for commodities of many developing countries. Traditionally, banana is propagated by suckers but this method is rather laborious and time consuming. The application of classical methods of breeding has met with only a limited success due to the high sterility and polyploidy of most economic bananas (Sasson 1997). In recent years, tissue culture has been used as a tool to overcome these impediments. In banana, plant regeneration via *in vitro* culture has been initiated from various sources of explants (Ko *et al.* 1991; Israeli *et al.* 1996). These reports have documented organogenesis without any intervening callus phase. *In vitro* regeneration via cell suspension cultures (Dhed'a *et al*. 1991; Côte *et al*. 1996) and somatic embryogenesis (Escalant *et al*. 1994; Khalil *et al.* 2002) are not only important for an integral part of genetic transformation but also a selection via somaclonal variation. At present there have been several reports on genetic transformation in banana (Tripathi 2003; Tripathi *et al.* 2007). Therefore, the present investigation was undertaken to optimize conditions for producing regenerative callus with the objective of developing a protocol for the regeneration of plants via somatic organogenesis in two important Thai banana cultivars, *Musa acuminata* 'Kluai Sa' and 'Kluai Leb Mu Nang'. Both bananas are native and well known as local economic bananas in southern Thailand. They are seedless, tasty, odors and highly priced. 'Kluai Leb Mu Nang' produces many suckers around the mother plants. In contrast, 'Kluai Sa' is a rare banana and expansion of production is limited by a shortage of suckers.

The current paper reports on the effect of TDZ and Dicamba on shoot-tip culture for regenerative callus induction; then NAA and BA were evaluated for plant regeneration. In addition, DNA content in mother plants and their regenerated plants were analyzed by flow cytometry to evaluate the occurrence of off-type plants during growth at the seedling stage in a nursery.

MATERIALS AND METHODS

Plant materials, callus induction and incubation condition

Sword suckers of two banana cultivars 'Kluai Sa' and 'Kluai Leb Mu Nang' were collected from Nakhon Si Thammarat Province, Thailand. Shoot-tips (3-5 mm long) were dissected and propagated aseptically on a solid Murashige and Skoog (1962, MS) medium supplemented with 100 mg L^{-1} casein hydrolysate, 15% coconut water and 5 mg L^{-1} BA. In order to increase the number of shoots needed for subsequent experiments, subculture at 4-week intervals was performed. Callus was initiated from 30 *in vitro*-grown shoot tips per cultivar cultured on MS medium with macro elements reduced to half and five organic addenda with (mg L^{-1}) malt extract 100; glutamine 100; proline 50; cysteine 50; and biotin 1 served as callus induction medium. TDZ (Fluka™) at concentrations of 0, 0.1, 1, 2 mg L^{-1} and Dicamba (Sigma-Aldrich) at concentrations of 0, 1, 3, 5, 7 mg L^{-1} were tested in various combinations. All media were solidified by 0.17% Gelrite™ and the pH was adjusted to 5.7 prior to autoclaving at 121°C, 1.1 kg cm^{-2} pressure for 20 min. The cultures were maintained at 25 ± 1°C with a 16-h photoperiod and 20 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density provided by cool-white fluorescent lamps. These environmentally controlled conditions were used in all experiments. Cultures were incubated for 8 weeks prior to evaluation. All calluses were transferred to a fresh medium and subcultured at 3-week intervals.

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Statistics

One explant was implanted per culture and all experiments were conducted on two different days with 15 replicates per treatment. The percentage of callus induction and number of regenerated shoots were monitored as growth parameters. The effects of NAA and BA for plant regeneration from calluses were determined by analysis of variance, which were followed by Sheffe's test.

Plant regeneration, root induction and acclimatization

Six-week-old calluses with 1 cm in diameter were selected and cultured on MS medium supplemented with different concentrations of NAA (0, 1, 2, 4 mg L^{-1}) and BA (0, 1, 3 mg L^{-1}) for plant regeneration. Forty-five days after inoculation, the number of callus forming buds was determined. A mass of slow growing buds was transferred to a MS medium supplemented with 3 mg L^{-1} BA, 3% sucrose and solidified by 0.75% agar to foster shoot elongation. Regenerated shoots (5-10 cm long) were excised individually and cultured on glass-capped test tube each containing 20 ml of MS plant growth regulator-free medium for 3 weeks. Agar was carefully washed from all regenerated plants and all leaves were clipped transversely in half. The plants were maintained with roots submerged in tap water for 1 week, and then transferred to black plastic bags filled with potting soil until root systems were well established. Acclimatized plants were maintained in a nursery under shading, natural photoperiod and high relative humidity for 2 months and normal looking banana plants were obtained before being planting in the field.

Flow cytometry analysis

For DNA content analysis, approximately 20-30 mg of young leaves of mother plants and regenerated plants were harvested and transferred to glass Petri dish containing 500 µl of Otto I buffer (Otto 1990) and 50 µg ml^{-1} RNase. The glass Petri dish was placed on top of ice in a bucket and nuclei were extracted by chopping leaf materials using a sharp razor blade. After chopping, the suspension was filtered through a 42 µm nylon mesh, centrifuged at 1000 rpm for 5 min. The supernatant was discarded then 1000 µl Otto II buffer and 50 µg l^{-1} propidium iodide was added. In every sample a minimum of 5000 propidium iodide-stained nuclei was analyzed with a FacScan cytometer (Becton Dickinson, Franklin Lakes, USA). Two samples of 5 plants from 'Kluai Sa' and 'Kluai Leb Mu Nang' were analyzed. Soybean (*Glycine max* cv. 'Polanka', 2C = 2.5 pg) was used as internal reference standard. The reference standard peak was set to show at channel 200 of relative fluorescence intensity. The obtained histograms were computerized by CellQuest software.

RESULTS

Effect of TDZ and Dicamba on callus formation

Callus could be initiated from shoot tip explants of 'Kluai Sa' and 'Kluai Leb Mu Nang' when cultured on MS medium supplemented with Dicamba singly or in combination with TDZ. No callus formation was observed on the media containing TDZ alone (0.1, 1, 2 mg L^{-1}). **Table 1** shows that different combinations of Dicamba and TDZ resulted in different responses of callus formation with respect to size, color and texture. Yellow friable calluses were observed on MS medium supplemented with 1-7 mg L^{-1} Dicamba without TDZ or 5, 7 mg L^{-1} Dicamba in combination with 1 mg l^{-1} TDZ. White friable callus was formed on MS medium supplemented with 1, 3 mg L^{-1} Dicamba in combination with 0.1-2 mg L^{-1} TDZ. The callus obtained on MS medium supplemented with 3-5 mg L^{-1} Dicamba in combination with 0.1-2 mg L^{-1} TDZ were white compact callus whereas MS medium fortified with 5-7 mg L^{-1} Dicamba in combination with 1-2 mg L^{-1} TDZ were yellow compact calluses. When all callus types were transferred to MS medium containing NAA and BA, only the yellow compact callus was able to produce regenerative callus and underwent shoot development. However, the yellow compact calluses were slow-growing thus took longer to proliferate. At the highest level of Dicamba (7 mg L^{-1}) and TDZ (2 mg L^{-1}) tested, the percentage of the yellow compact callus observed was 60% in 'Kluai Sa' and 55% in 'Kluai Leb Mu Nang'.

Table 1 Effect of TDZ and Dicamba on the callogenesis from 30 shoot tips each of 'Kluai Sa' (Sa) and 'Kluai Leb Mu Nang' (LMN)

Plant growth regulators (mg L^{-1})		Explants forming callus (%)		Morphology of the callus
TDZ	Dicamba	Sa	LMN	
0	0	0	0	No callus
0	1	100	100	Yellow friable
0	3	100	100	Yellow friable
0	5	85	65	Yellow friable
0	7	35	25	Yellow friable
0.1	0	0	0	No callus
0.1	1	100	90	White friable
0.1	3	100	100	White friable
0.1	5	40	35	White compact
0.1	7	20	20	White compact
1	0	0	0	No callus
1	1	40	35	White friable
1	3	30	30	White compact
1	5	25	15	Yellow compact
1	7	55	25	Yellow compact
2	0	0	0	No callus
2	1	25	10	White friable
2	3	10	15	White compact
2	5	55	45	Yellow compact
2	7	60	55	Yellow compact

Table 2 Effect of various combinations of NAA and BA on callus and shoot induction of 'Kluai Sa' (Sa) and 'Kluai Leb Mu Nang' (LMN)

Treatments (mg L^{-1})		Callusing (%)		No. of shoots per callus	
NAA	BA	Sa	LMN	Sa	LMN
0	0	0	0	0.00 e	0.00 e
1	0	0	0	0.00 e	0.00 e
1	1	25	30	1.30 d	1.70 d
1	3	100	100	19.10 a	18.80 a
2	0	0	0	0.00 e	0.00 e
2	1	25	35	1.00 d	1.60 d
2	3	60	45	4.70 b	3.00 b
4	0	0	0	0.00 e	0.00 e
4	1	15	30	1.00 d	1.70 d
4	3	25	40	1.60 c	2.80 c

The different letters within column show significant difference of shoot number (Mean ± SE.) analyzed by Sheffe's test at $p<0.05$.

Effect of BA and NAA on shoot regeneration

After completion of the callusing phase, the yellow compact callus was subcultured on MS medium containing BA (1, 3 mg L^{-1}) or NAA (1, 2, 4 mg L^{-1}) plus BA (1, 3 mg L^{-1}). These calluses formed green shoot buds within 5 weeks of culture (**Fig. 1A**). The average of regenerated shoots per callus was approximately 19 shoots for both 'Kluai Sa' and 'Kluai Leb Mu Nang' on MS medium supplemented with 1 mg L^{-1} NAA and 3 mg L^{-1} BA (**Table 2**). Shoots elongated on these media (**Fig. 1B**) and plantlets regenerated from callus culture through organogenesis were allowed to grow for another 5 weeks (**Fig. 1C**). The healthy green shoots were excised from each other and transferred to MS plant growth regulator-free medium for 3 weeks (**Fig. 1D**). However, the two cultivars exhibited different morphological characteristics viz. longer and broader leaf in 'Kluai Sa' (**Fig. 2A**), and brown red streaks on the upper leaf surface in 'Kluai Leb Mu Nang' (**Fig. 2B**). The differences were the same as found in mother plants grown in the field. The total duration of the cycle through complete plantlets was 21

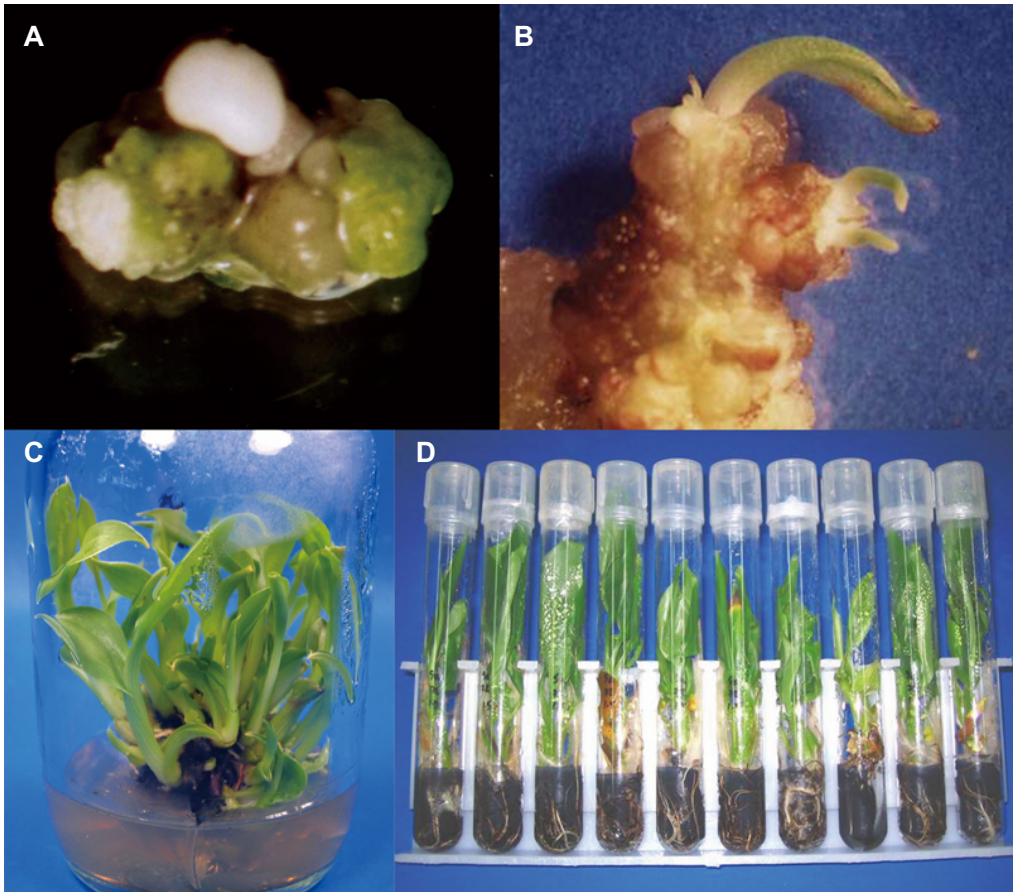

Fig. 1 Plant regeneration from callus culture in 'Kluai Sa'. (**A**) Compact callus mass cultured on MS medium containing 1 mg L^{-1} NAA and 3 mg L^{-1} BA, 3 weeks after culture. (**B**) Small shoot buds with young leaves emerged from callus cultured. (**C**) Multiple shoots formation in 'Kluai Sa'. (**D**) Root formation of excised single shoot on MS medium supplemented with 0.2% activated charcoal, 3 weeks after culture.

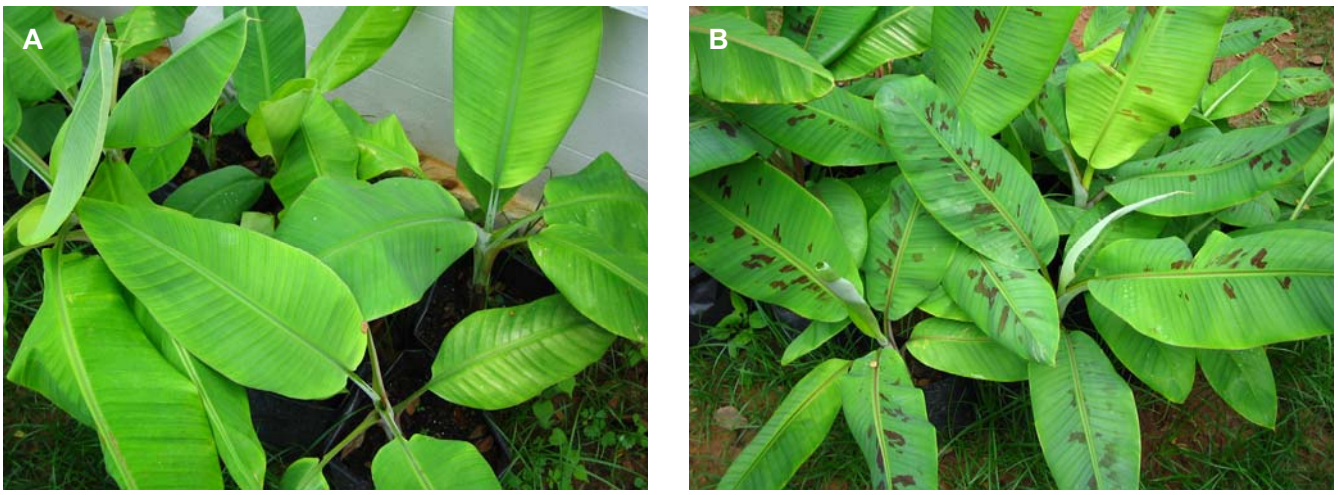

Fig. 2 Hardened plants established in plastic bags 4 months after transplantation in the glasshouse. (**A**) 'Kluai Sa' and (**B**) 'Kluai Leb Mu Nang'.

weeks; 8 weeks for callus induction, 10 weeks for shoot growth and development and 3 weeks for rooting.

Flow cytometry analysis

The conformity of the mother plant and regenerated plants of 'Kluai Sa' and 'Kluai Leb Mu Nang' in terms of their ploidy level and relative nuclear DNA content was carried out by flow cytometry. Flow cytometric results indicated that all regenerated plants were true to type with similar intensities to those obtained for the mother plant. The distribution of the nuclei extracted was at the G_0/G_1 phase of the cell cycle having 2C DNA content, with their peak on channel 100 for both cultivars and channel 200 for *Glycine max* cv. 'Polanka' (internal standard) (**Fig. 3**). No changes in relative peak positions were observed during the study, indicating the absence of variation in DNA ploidy levels. In addition, the regenerated plants of both cultivars during growth over a long period of time (~9 months) did not show any abnormalities.

DISCUSSION

Musa sp. is one of the world's most important cultivated fruits of the tropics and subtropics. Production is limited by viral, fungal diseases and insect pest problem (Sagi *et al.* 1998). Moreover; propagation is necessarily via asexual means. For these reasons there is a considerable focus on the development of *in vitro* culture together with genetic engineering technology to improve this species. Plant rege-

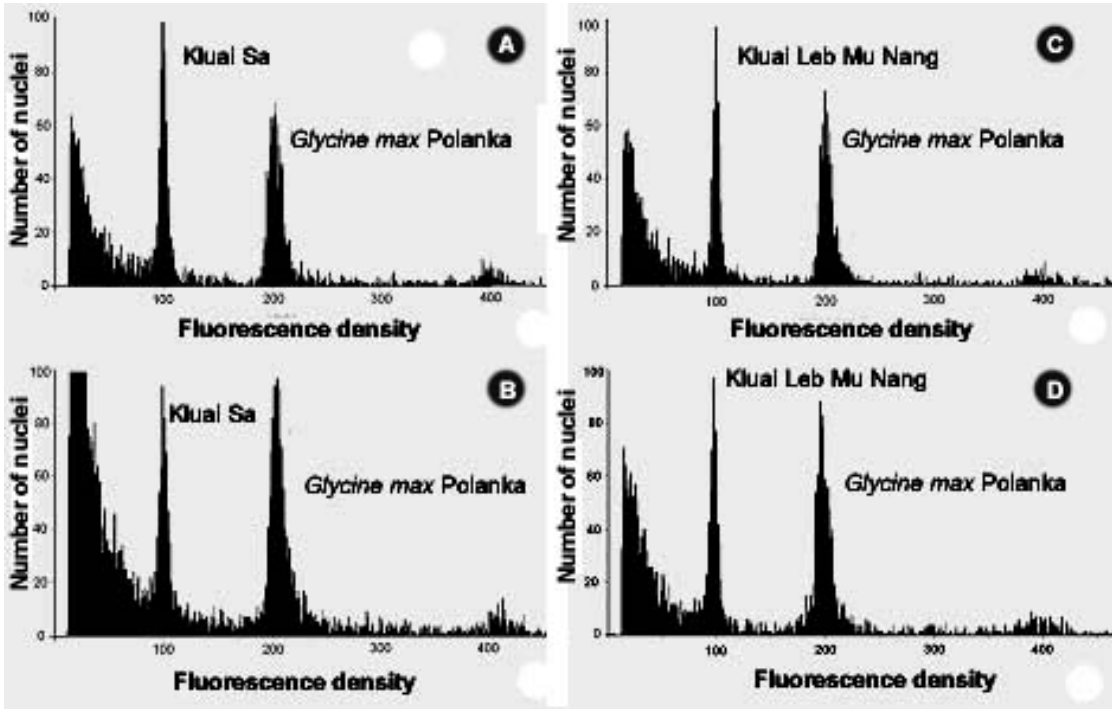

Fig. 3 Histograms of relative nuclear DNA content (in channel numbers) obtained after flow cytometric analysis of 'Kluai Sa'. (**A, B**) and 'Kluai Leb Mu Nang' (**C, D**). *Glycine max* 'Polanka' was used as internal reference standard and set at channel 200. Note that most nuclei are in G_0/G_1 phase with 2C DNA content.

neration through shoot multiplication is not appropriate for genetic transformation since many chimeric plants are produced (Khalil *et al.* 2002). In these accounts somatic embryogenesis from cell cultures (Strosse *et al.* 2006; Pérez-Hernández and Rosell-García 2008; Ghosh *et al.* 2009) and protoplast cultures (Assani *et al.* 2005; Assani *et al.* 2006) in *Musa* sp. already exist. Genetic transformation of *Musa* sp. was first achieved by Sagi *et al*. (1994). Since then some successes on *Musa* transgenic plants were carried out (Khanna *et al.* 2004; Tripathi *et al.* 2004; Tripathi *et al.* 2005; Acereto-Escoffié *et al.* 2005; Sreeramanan *et al.* 2006; Tripathi *et al.* 2008; Ghosh *et al.* 2009). In the present study, a system for routine regeneration from callus induced from shoot tip explants with the potential to explore genetic modification is described.

When the yellow compact callus which formed on Dicamba (5-7 mg L^{-1}) and TDZ (1-2 mg L^{-1}) supplemented medium was further subcultured to MS medium containing 1 mg L^{-1} NAA and 3 mg L^{-1} BA, shoot bud induction was achieved. It can be inferred that combination of Dicamba with TDZ induced callus formation with organogenic competence. This is in contrast to our previous finding with *Musa acuminata* 'Gros Michel' since embryogenic callus was induced when subcultured compact callus to half-MS medium in the presence of TDZ (Srangsam and Kanchanapoom 2003). One plausible explanation could be that the genome of explants was different. However, the present finding is in accordance with Novak *et al.* (1989) who reported that proembryogenic calli were initiated from basal leaf sheath and rhizome tissue of dessert and cooking bananas on SH medium with 6.6 mg L^{-1} Dicamba and 1.1 mg L^{-1} TDZ. The results described here showed that BA in combination with NAA was effective in causing multiple shoots indicating that both may play an important role in hormonal control of induction of adventitious bud outgrowth in 'Kluai Sa' and 'Kluai Leb Mu Nang' as it did in other bananas (Okole and Schulz 1996; Arinaitwa *et al.* 2000; Côte *et al.* 2000; Khalil *et al.* 2002). The shoots obtained from callus were different depending on the combination of NAA and BA. In addition, the organogenesis from callus culture and subsequent plantlet formation was found in BA-NAA interactions suggested the synergistic effect. This is not surprising since the synergistic effect of BA and NAA is quite normal in plant tissue culture. Beside BA and NAA, plant regeneration in *Musa* sp. was obtained from embryogenic cultures induced by several plant growth regulators such as BA and IAA (Ganapathi *et al.* 1999) and IAA, 2, 4-D and NAA (Grapin *et al.* 2000). In our experiments the plausible role of BA and NAA through callus growth is characterized. The BA-NAA balance is one of the factors determining pattern of organogenesis and causes interactions that resulted in altered morphogenetic responses.

Ploidy variations were not evidenced during subculture of more than 20 cycles in 2 years *in vitro* as detected by flow cytometry. Applying flow cytometry has several advantages on banana experiment since both diploid bananas have the small size of the chromosomes. It was interesting to note that alteration of DNA content in 'Kluai Sa' and 'Kluai Leb Mu Nang' were not observed among micropropagated plants compared to the mother plants. Our results show that flow cytometry offers an easy, rapid, accurate and convenient method for determining ploidy level assessing DNA content for both regenerated plants and mother plants. In this context, information about DNA content would be essential by providing basic information to assess the significance of culture variation. The absence of ploidy variation and off-type plants in our protocol make it well-suited for large scale propagation and conservation of rare bananas in Thailand.

In conclusion, the successful method of regenerating true-to-type plants from two Thai diploid banana cultivars was established. The present results clearly demonstrated the best medium for callus induction, organogenesis and regeneration of stable diploids especially for the cultivar 'Kluai Sa' that is extremely difficult to propagate. The information given here will be valuable for future research in genetic transformation for an improvement of these two bananas.

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