RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2007.33.437

ScienceAsia 33 (2007): 437-442

Establishment of *in vitro* Culture of *Musa* AA Group 'Kluai Sa' and *Musa* AA Group 'Kluai Leb Mue Nang' and the Analysis of Ploidy Stability

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Received 13 Nov 2006
Accepted 1 Aug 2007

ABSTRACT: The use of shoot tip culture for 'Kluai Sa' (*Musa* AA group 'Kluai Sa') and 'Kluai Leb Mue Nang' (*Musa* AA group 'Kluai Leb Mue Nang') banana plant propagation is described. Excised shoot apices were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 3 mg/l BA (6-Benzyladenine) and then transferred to MS medium containing 100 mg/l casein hydrolysate, 15% CW (Coconut water) and various concentrations of BA. The maximum shoot numbers were obtained on the medium containing 5 mg/l BA with 4.9 shoots in 'Kluai Sa' and 5.5 shoots in 'Kluai Leb Mue Nang'. Root and callus formation were achieved when shoot buds were planted on MS media containing various concentrations of NAA (1-Naphthaleneacetic acid) in combination with 5 mg/l BA. Adventitious shoots were also regenerated from calli that were cultured on MS medium containing 5 mg/l BA and 0.1 mg/l NAA. All regenerated shoots rooted well on MS medium either supplemented with or without 0.2% activated charcoal. More than 95% of the surviving plantlets were acclimatized and successfully transferred into soil. Cytological and flow cytometric analyses of the mother plants and micropropagated plants derived from 2 years-old cultures of both cultivars showed no differences in ploidy level, they were all diploid (2n = 2x = 22) with a 2C peak.

KEYWORDS: Banana, *Musa* AA group, ploidy stability, flow cytometry, *in vitro* culture.

INTRODUCTION

Bananas (*Musa* sp.) are among the world's most important crops and one of the major export commodities of many developing countries. 'Kluai Sa' (*Musa* AA group 'Kluai Sa') and 'Kluai Leb Mue Nang' (*Musa* AA group 'Kluai Leb Mue Nang') are native bananas in southern Thailand that belong to the family Musaceae. They are seedless, tasty, and fragrant and are generally propagated by suckers. 'Kluai Leb Mue Nang' is well known as a local economic banana in southern Thailand, especially in Chumporn and Nakhon Si Thammarat Provinces. It 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. To date, much progress in the propagation of *Musa* sp. via tissue culture methods has been made.[1-2] It is well documented that the genome size of *in vitro* culture can be altered through changes either in the chromosome number or in the ploidy level. In *Musa* sp., occurrences of somaclonal variation was reported in dessert banana and plantain.[3] 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 *Musa* sp. is very difficult to assess using standard squash preparations since they are small (1-2 µm).[4] Recently, flow cytometry has been used to analyze DNA content in numerous plant species.[5] Flow cytometry offers an easy, rapid, accurate and convenient method for determining ploidy level, assessing DNA content and analyzing the cell cycle.[6] In addition, the method requires only a small amount of tissue and is therefore non-destructive, and has the ability to analyze large populations of cells where the possibility of mixoploidy or aneuploidy exists.[7]

In this study, we investigated an efficient protocol for mass propagation of 'Kluai Sa' and 'Kluai Leb Mue Nang'. Consequently, morphological traits, cytological study, and DNA content of the *in vitro* culture of both bananas were analyzed as well.

MATERIALS AND METHODS

Explants, Media Preparation and Condition of Cultures

Sword suckers of two banana cultivars, 'Kluai Sa' and 'Kluai Leb Mue Nang' were collected from a local farm in Ron Phi Bhun District, Nakhon Si Thammarat Province, Thailand. The suckers were cut down into pieces of 12 cm diameter and 25 cm long and washed

in tap water several times to release debris. They were trimmed down to 5-6 cm long until small buds were exposed. Tissue blocks containing buds were surface sterilized using 15% (v/v) Clorox™ (1.5% sodium hypochlorite, active ingredient) for 20 min, followed by 5 % (v/v) Clorox™ for 5 min, and then double rinsed with sterile distilled water to remove traces of disinfectant. Shoot buds were excised and precultured on agar-solidified MS[8] culture medium containing 3% sucrose and 3 mg/l BA for 30 days. The emerged shoot buds were then excised aseptically and split longitudinally in two parts prior to transfer to shoot multiplication 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 1 N NaOH or 1 N HCl prior to autoclaving at 1.05 kg/cm^2, 121°C for 20 min. All cultures were incubated at 25±1 °C air temperatures in a culture room with a 16-h photoperiod under an illumination of 20 μmolm^{-2}s^{-1} photosynthetic photon flux density provided by cool-white fluorescent light. Plant materials were stored in glass-capped culture jars (115 ml capacity) each containing 20 ml of medium.

Effect of BA and NAA on Shoot Bud Culture

For rapid shoot multiplication, different concentrations of BA (at 0, 1, 3, 5, 7 or 9 mg/l) and NAA (at 0, 0.1, 1, or 3 mg/l) in combination with 5 mg/l BA were supplemented into MS medium containing 15% coconut water and 100 mg/l casein hydrolysate. After 8 weeks of cultures the average number of shoot regeneration was recorded. They were routinely subcultured at eight-week-intervals and thirty regenerated plantlets of both cultivars were removed to plastic bags filled with sterilized soil after 6, 12, 18 and 24 months of culture in order to observe DNA contents, chromosome numbers and morphological characteristics.

Root Induction, Acclimatization and Transplantation

For root induction, each regenerated shoot (5-7 cm in length) was subcultured onto MS medium with or without 0.2% AC (activated charcoal). Emerging roots were recorded after 1 and 3 weeks of culture. Agar medium was gently removed from roots using running water. Leaves of rooted shoots were clipped in half and the roots submerged into water for one week. The acclimatized plants were transplanted in plastic bags filled with steriled soil and maintained in the nursery for 3 weeks to observe morphological characteristics.

Cytological Study and DNA Content Analysis

The chromosome numbers in root tips of mother plants and micropropagated plants were studied. Root tips (0.5 cm long) were taken at 9:00 a.m. (from preliminary studies) and fixed in a fresh solution of 3:1 (95% ethanol: glacial acetic acid) for 24 hr at room temperature. This treatment was followed by hydrolysis in 1 N HCl at 60 °C for 10-15 min. The root tips were washed in tap water stained for 4 hr and squashed in carbol-fuchsin and viewed under 1000x magnification with phase contrast microscopy.

For DNA content analysis, approximately 20-30 mg of young leaves of mother plants and regenerated plants were harvested and transferred to glass Petri dishes containing 500 μl of Otto I buffer [9] and 50 μg/ml RNase. Each glass Petri dish was placed on top of an ice-cold 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 and 50 μg/l propidium iodide was added. The internal reference standard (*Glycine max* Polanka, 2C = 2.5 pg) was kindly provided by Dr. Jaroslav Dolezel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Czech Republic. The fluorescence of 5000 propidium iodide-stained nuclei was estimated using a flow cytometer FACScalibur Becton Dickinson (Scientific Equipment Center, Prince of Songkla University). The reference standard peak was set to show at channel 200 of relative fluorescence intensity. The obtained histograms were computerized with CellQuest software.

Data Analysis

One explant was implanted per culture and 15 cultures were raised for each treatment. 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

Effect of BA and NAA on Shoot Bud Culture

After 30 days of culture, explant containing shoot buds (Fig 1A) precultured on MS medium supplemented with 3 mg/l BA swelled and grew vigorously up to 3-5 cm long (Fig 1B). Bulge buds were halved and transferred to tested media. The growth of buds cultured on a plant growth regulator-free medium remained white in color and only 40% of 'Kluai Sa' and 60% of 'Kluai Leb Mue Nang' survived (Table 1). These buds were able to continue their growth on this medium for only 6 weeks. On BA-containing media, they began to change from white cream to green color and proliferated considerably due to the development of leaf primordia.

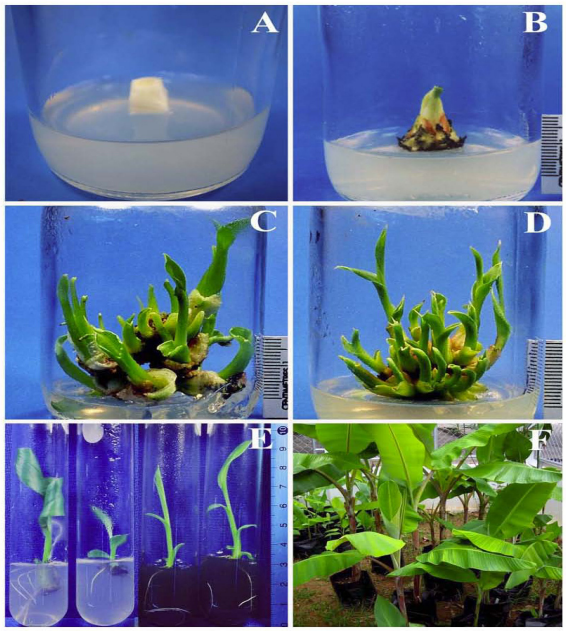

Fig 1. Plant regeneration from shoot-tip cultures in 'Kluai Sa' and 'Kluai Leb Mue Nang'. (A) Explant embedded shoot tip, (B) Swelled explant 30 days after precultured, (C) Multiple shoot formation in 'Kluai Sa', (D) Multiple shoot formation in 'Kluai Leb Mue Nang', (E) Root formation on MS medium supplemented either with (left) or without (right) activated charcoal, (F) Eight-month-old plantlets.

Table 1. Effect of various concentrations of BA on shoot induction of 'Kluai Sa' and 'Kluai Leb Mue Nang' after culture for 8 weeks.

BA (mg/l)	'Kluai Sa'		'Kluai Leb Mue Nang'	
	Survival (%)	No. of shoot	Survival (%)	No. of shoot
0	40	0 ± 0.00^{e}	60	0 ± 0.00^{e}
1	100	1.4 ± 0.17^{d}	100	1.5 ± 0.20^{d}
3	100	2.8 ± 0.25^{bc}	100	2.7 ± 0.33^{bc}
5	100	4.9 ±0.43^{a}	100	5.5 ± 0.41^{a}
7	100	3.3 ± 0.26^{b}	100	3.4 ±0.33^{b}
9	100	2.3 ± 0.22^{c}	100	2.4 ± 0.24^{bc}

The different letters within columns show significant differences of shoot number (Mean ± SE.), as analyzed by Sheff's test at p<0.05.

The typical outgrowth of pale green, conical-shaped foliage was apparent 2 weeks after inoculation. Multiple shoot formation was visible at the fourth week of culture. BA dramatically influenced survival (increasing it to 100%) and axillary shoot production in both bananas. The data in Table 1 revealed that BA promoted shoot regeneration with a correlation to the concentrations used in the medium. At a low concentration of BA (1 mg/l), a single shoot was regenerated, while increasing BA level progressively increased shoot number. The culture showing number of shoots was highest on MS medium fortified with 5 mg/l BA. On this medium, cultures showed average shoot numbers of 4.9 (P<0.05 level) in 'Kluai Sa' and 5.5 (P<0.05 level) in 'Kluai Leb Mue Nang' (Table 1 and Fig 1C, D). It was also noticed that both cultivars appeared to have the same optimum BA concentration to achieve a maximum shoot production. At higher concentrations of BA (7, 9 mg/l), shoot number was decreased in both bananas. By successive subculture on MS medium containing 5 mg/l BA, masses of proliferating shoot cultures were established. Experiments were conducted to determine in different combinations of NAA and BA. An interaction of NAA and BA was in the same trend as compared to BA alone. The average shoot number at 5.1 (P<0.05 level) in 'Kluai Sa' and 5.6 (P<0.05 level) in 'Kluai Leb Mue Nang' was recorded on MS medium supplemented with 5 mg/l BA and devoid of NAA (Table 2). The morphogenetic responses of the cultured buds seemed to be influenced by the concentrations of NAA and the genomic configuration of the cultivars. Thus, the number of axillary shoots per explant was greater for NAA at low concentration (0.1, 1 mg/l) compared to NAA at high concentration (3 mg/l) in 'Kluai Sa' (Table 2). This is in contrast to 'Kluai Leb Mue Nang', for which the number of shoots on MS media supplemented with NAA did not vary significantly (Table 2). Furthermore NAA-containing media effectively induced both adventitious roots and callus in both cultivars. The root formation percentage was increased according to NAA concentrations. Shoots cultured on medium without NAA also rooted, but the frequency and growth were suboptimal compared to NAA treatments. This trend was similar in both bananas

Table 2. Effect of various concentrations of NAA (0, 0.1, 1 or 3 mg/l) in combination with 5 mg/l BA on shoot, root and callus induction of 'Kluai Sa' (Sa) and 'Kluai Leb Mue Nang' (LMN) after culture for 8 weeks.

NAA(mg/l)	No. of shoot/explant		Root formation (%)		Callus formation (%)	
	Sa	Leb Mu Nang	Sa	Leb Mu Nang	Sa	Leb Mu Nang
0	5.1 ±0.53 ^{a}	5.6 ±0.43^{a}	13.3	20.0	0	0
0.1	3.0 ±0.50 ^{b}	3.2 ±0.38^{b}	20.0	26.7	27.7	40.0
1	3.3 ±0.45 ^{b}	2.9 ±0.26^{b}	53.3	73.3	13.3	6.7
3	2.9 ±0.28 ^{c}	2.3 ±0.24^{b}	100.0	100.0	6.7	13.3

The different letters within columns show significant differences of shoot number (Mean ± SE), as analyzed by Sheff's test at p<0.05.

(Table 2). Callus initiation was observed 8 weeks after culturing. Shoot-derived callus was either friable or compact and ranged from yellowish to brown in color (Fig 2A). The frequency of callus formation was higher in 'Kluai Leb Mue Nang' than in 'Kluai Sa' (Table 2). After completion of frequent subculture, the hard compact callus that was yellowish was directly capable of forming shoots via organogenesis with the presence of green and opaque structures (Fig 2B, C). Differentiation of these shoots was evidenced and they further developed to normal plants when they were transferred to MS medium containing lower BA concentration (3 mg/l) (Fig 2D).

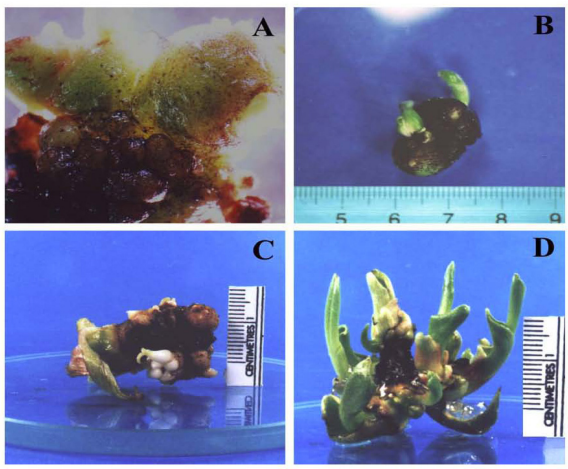

Fig 2. Plant regeneration from shoot derived callus cultured on MS medium containing 0.1 mg/l NAA and 5 mg/l BA, 100 mg/l casein hydrolysate and 15% coconut water. (A) Compact callus, (B) Development of green meristemoid from callus, (C) Development of shoot meristemoid emerged from the base of explant, (D) Shoot regeneration on a medium containing a low concentration of BA.

Root Induction, Acclimatization and Transplantation

Healthy green shoots thus obtained from shoot experiments either on BA alone or NAA in combination with BA developed roots *per se* (Table 2). At higher NAA concentrations, better root formation was obtained and in most cases root induction took place within 2 months. However, MS medium devoid of growth regulators facilitated better rooting, elongation and healthy growth of the roots. We have observed one instance that MS medium supplemented either with or without activated charcoal had no effect on root induction in both cultivars (Fig 1E). Several methods of acclimatization and transplantation were tried in order to get the best survival of the micropropagated plants (Data not shown). When these regenerated shoots reached 4 to 5 cm in height, the leaves were clipped in half and the roots were submerged in water for one week. This resulted in 100% survival in 'Kluai Sa' and 96.67% in 'Kluai Leb Mue Nang'. The plantlets were transferred to polyethylene bags containing a mixture of manure and soil (1:1), and kept in the greenhouse for acclimatization. Micropropagated bananas grew vigorously and normal-looking banana plants were obtained (Fig 1F). Phenotypes of mother plants and micropropagated plants did not differ i.e. leaf blades had the same thickness and width, and flowers were similar in size. All plants reached a 3 to 4 m height after 4 months of cultivation in the glasshouse. The total time from sucker excision to field establishment was about 5-6 months.

Cytological Study and DNA Content Analysis

The regeneration capability of the cultures persisted up to two years of repeated subcultures. Under these conditions, the morphogenetic responses for 'Kluai Sa' and 'Kluai Leb Mue Nang' did not show any phenotypic variation, since morphology, size and number of stomata of both bananas did not differ. Variegated leaves were observed in some plants in the cultures of more than 15 months and disappeared after the plants were planted in plastic bags and kept in a nursery for 4 months. The cytological study of root tips at metaphase of long term cultures revealed complete accurate counts of 22 chromosomes in 'Kluai Sa' and 'Kluai Leb Mue Nang' (Fig 3).

The DNA contents of 'Kluai Sa' and 'Kluai Leb Mue Nang' were analyzed using soybean (*Glycine max* cv. Polanka) as an internal reference plant. The standard peak of soybean was set at channel 200 and determined on each measuring date. The distribution of the nuclei extracted from both mother plants and regenerated plants of 'Kluai Sa' and 'Kluai Leb Mue Nang' displayed a prominent peak at 2C, indicating that they consisted of cells in the G0/G1 phase of cell cycle, thus no ploidy variation occurred (Fig 4, 5).

Discussion

The results described here showed that BA strongly enhanced shoot multiplication of 'Kluai Sa' and 'Kluai Leb Mue Nang', as it did in other bananas.[10-11] Shoot multiplication was not observed on plant growth regulator-free medium suggesting that the endogenous

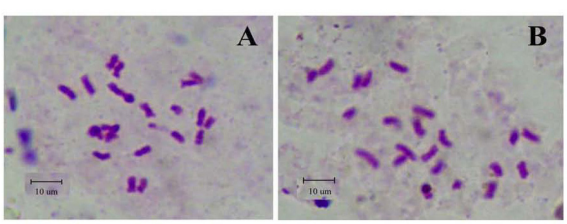

Fig 3. Mitotic metaphase of root tips showing 2n = 22 chromosomes in (A) 'Kluai Sa' and (B) 'Kluai Leb Mue Nang'.

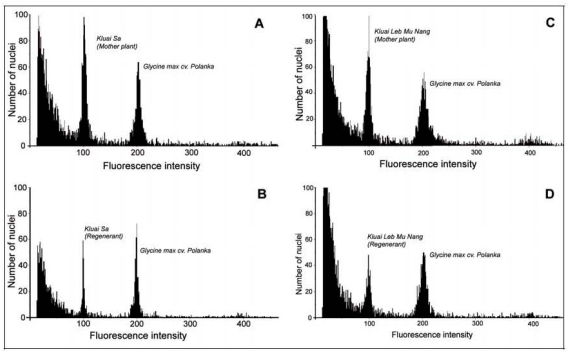

Fig 4. Histograms of flow cytometric analysis of 'Kluai Sa' (A, B) and 'Kluai Leb Mue Nang' (C, D). The ploidy levels of mother plant (top row, A, C) were compared to 2 years-old cultures of shoot-tips derived plants (below, B, D).

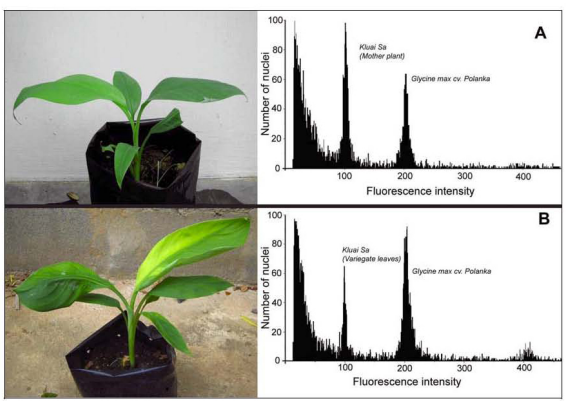

Fig 5. Shoot morphology and ploidy histograms of 'Kluai Sa' (A) mother plant from sucker, (B) tissue cultured-derived plant with variegated leaves.

growth regulator level was not conductive to bud proliferation. The optimal addition of 5 mg/l BA in the medium was similar to the optimal concentration for multiple shoot formation in dessert banana clones ('Philippine Lacatan' and 'Grande Naine') and cooking type clones ('Saba' and 'Pelipita').[12]

The selection of BA and NAA in the medium was based on earlier reports which revealed that adventitious shoots and callus formation was obtained in the banana cultivar 'Williams' and two plantain cultivars 'Horn' and 'Cachaco'.[1] It was noted 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 Mue Nang'. In addition, organogenesis from callus culture and subsequent plantlet formation was found in BA-NAA interactions, which suggested a synergistic effect. Such a synergistic effect of NAA and BA has been reported earlier in other bananas.[1-2, 13-14] This is not surprising since the synergistic effect of BA and NAA is quite normal in plant tissue culture. Besides BA and NAA, plant regeneration in *Musa* sp. was obtained from embryogenic cultures induced by several plant growth regulators, such as BA and IAA [15], and IAA, 2, 4-D and NAA [16]. In our experiments, the plausible role of BA and NAA through callus growth was characterized. The BA-NAA balance is one of the factors determining the pattern of organogenesis and causes interactions that result in altered morphogenetic responses. It can be inferred from our results that the combination of BA in high amount (5mg/l) and NAA in low amount (0.1 or 1 mg/l) is effective for callus formation and its further redifferentiation. This suggested that NAA was effective in callus induction and BA promoted shoot primordia in 'Kluai Sa' and 'Kluai Leb Mue Nang'. It is interesting to note that the use of casein hydrolysate in the culture medium helped maintain the amount of shoot multiplication in the subsequent subculture in both bananas. Without casein hydrolysate added to the medium, the number of shoots generated decreased markedly.

The success of *in vitro* propagation relies on both rooting and the survival of plantlets under field conditions. The stimulatory effect of auxin types such as IBA [17-18], IBA and IAA[19] in the root formation employed in bananas were also reported. In the present result, however, the addition of activated charcoal did not enhance the root formation. Development of roots on each shoots *per se* when cultured on growth-regulator-free medium is beneficial at the commercial level and regenerated shoots were obviously rooted readily, thereby providing for rapid clonal propagation. Each mother shoot explant cultured on MS medium containing 5 mg/l BA, 100 mg/l casein hydrolysate and 15% coconut water in both bananas produced about 5 shoots. The subculture interval was 2 months, and each subculture could produce 25 shoots in 2 months, for a total production of 625 plantlets per explant per 6 months.

Ploidy variations were not evidenced during subculture of more than 20 cycles in 2 years *in vitro* as detected by cytological study and flow cytometry. Combination of cytological study and flow cytometry achieved better results in terms of both accuracy and rapidity. The chromosome number in both cultivars was 22 which is in agreement with the previous report .[4] The occurrence of chromosome changes has often been observed during application of tissue culture, especially in plants, calli or cells that were maintained for long-term cultures *in vitro*.[20] Applying flow cytometry has several advantages in this banana experiment, since both diploid bananas have the small chromosome sizes. At present, flow cytometry is a rapid and efficient method for estimating the DNA content of large populations of cells.[21-22] It was interesting to note that alteration of DNA content in

'Kluai Sa' and 'Kluai Leb Mue Nang' were not observed among micropropagated plants compared to the mother plants. It is axiomatic that plants regenerated from well-developed meristematic tissue, showed the lowest tendency of genetic variation.[23] Ploidy changes are considered to be a possible cause for somaclonal variation in tissue cultures, but how polyploidy is generated during tissue culture is unclear.[24] 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, we demonstrated the establishment of rapid *in vitro* plant production of 'Kluai Sa' (*Musa* AA group 'Kluai Sa') and 'Kluai Leb Mue Nang' (*Musa* AA group 'Kluai Leb Mue Nang'). This report revealed that a simple cytological study and flow cytometric method can be used to monitor the ploidy stability of long term *in vitro* grown plants.

Acknowledgements

This research was financially supported by the Thailand Research Fund through the Royal Golden Jubilee Ph. D. Program (RGJ) Grant No. PHD 4BPS45B1. This work was also supported in part by Ministry of University Affairs, and the Graduate School, Prince of Songkla University.

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