Micropropagation of *Musa* (AA) 'Kluai Khai' Using Temporary Immersion Bioreactor System (TIBs)

Duangporn Premjet[1, 3], Wimonmas Kunkeaw[1,3] , Anuphan Kongbungkerd[2,3], and Siripong Premjet [2,3*]

[1]Department of Agricultural Science, Faculty of Agriculture, Natural Resources, and Environment, Naresuan University, Muang, Phitsanulok ,65000, Thailand
[2]Department of Biology, Faculty of Sciences, Naresuan University, Muang, Phitsanulok, 65000, Thailand
[3]Center of Excellence in Research for Agricultural Biotechnology, Muang, Phitsanulok, 65000, Thailand

*Corresponding author. E-mail: siripongp@nu.ac.th

ABSTRACT

The factors affected on micro-propagated banana, *Musa* (AA) 'Kluai Khai' using temporary immersion bioreactor system (TIBs) were investigated. Suckers of good quality mother plants were obtained from the banana conservation farm. *Musa* (AA) 'Kluai Khai' is a geographic indication plant in Kamphaeng Phet Province, Thailand. Excised shoot tips were used to initiate shoot on semi-solid MS medium supplemented with 3 mg/L of BA. Shoot multiplication was done through subcultures every month at intervals. Small shoot buds of the fourth subculture were transferred to liquid MS medium supplemented with 5 mg/L of BA and cultivated for 5 weeks. Optimization conditions for the TIB production system was determined by varying shoot density, media volume, time and frequency of immersion. The experimental group was divided into 16 treatments. Data was collected over the course of 35 days in a 2000 ml TIBs glass vessel. The optimized conditions for cultivation of Kluai Khai in TIBs was found when using 10 shoots per reactor, immersion time of 10 minutes for every 12 hours a day, and medium volume of 600 ml. The maximum plantlets (4.19 ± 0.68/explant) with 91.41% survival rate were obtained. Assessment of genetic fidelity was performed using a flow cytometer. Results revealed that both the mother plant and TIBs's plantlets were diploid (2n).

Keywords: *Musa* (AA) *'Kluai Khai', geographic indication, temporary immersion bioreactor, flow cytometry, ploidy*

INTRODUCTION

The first *in vitro* clonal propagation of banana was reported by Ma and Shii in Taiwan (Ma & Shii, 1972). The high incidence of *Fusarium* wilt (Thangavelu et

Article history:
Received 26 September 2022; Received in revised from 29 September 2022;
Accepted 29 September 2022; Available online 31 December 2022.

al., 2021) and *Odoiporus longicollis* attacking (Krishnan et al, 2015), replanting in large areas annually necessitated the use of clean planting material. Banana propagation in Thailand with the current tissue culture method commonly used semi-solid media system, which is the method to obtain a plant that has the same genetic characteristics as the mother plant (cloning) in a short time and large quantities, but the technology of plant production with this system still has limitations (Zou et al., 2013). Shoot tip culture provides a low multiplication rate. Many factors, such as having high production costs because it must use a lot of glass or plastic containers, and labor requirement for sub-culturing plants into new media every 6-8 weeks. Production cost is considered an important factor in limiting the commercial use of such methods. Labor costs are generally considered to be approximately 40-60% of the cost of production. In addition, the main cost is also the loss of seedlings through succulent planting and roots and during the seedling process (Lyam et al., 2012). The use of liquid media systems has therefore been adopted in plant micro-propagation to reduce the use of labor and consumables such as agar, which is an expensive filler material. The ultimate in liquid media systems makes work more automated (Etienne and Berthouly, 2002). As a result, the production is stable, which improves the efficiency of work and reduces the total cost of production. However, the liquid media system still has technical problems such as lack of air and hyperhydricity. The TIB system has been developed for culturing plants and is the aeration of the solution through the air system (pneumatic system) that has passed microbial filtration with a filter set with a fine filter of 0.2 μm to the plant parts for the duration and frequency that can be set with a timer which works as a semi-automatic (Bukar, et al., 2017). At present, this principle has been widely used by researchers in many countries. The limitations and advantages of plant tissue culture with the TIB system are to avoid continuous drowning, which will cause abnormal growth and shape, and to supply and replace enough oxygen. Reduce the lack of oxygen in plants because there is aeration of the vial through a sterilization unit to the cultured plant parts, and provide a period of feeding (duration time or flush time): the time during which the plant encounters media and the frequency of feeding appropriately, ensuring that the plant parts are supplied with sufficient nutrients. This will cause a thin film of liquid media to envelop the plant parts, which can prevent drying of plant parts, use pressure limited to no more than 1 bar, able to provide automatic periodic feeding by relying on the compressed air system to dissolve the media and out of culturing. It also reduces the accumulation of carbon dioxide and ethylene gas because it is a closed system, causing the rate to increase the quantity of plants, reduce the cost of production in terms of the cost of labor that requires subculture (Ahmadian et al., 2017). Easy to change media and uses less space than semisolid system. Many plant species have been successfully reported in production commercially using TIBs (Kunakhonnuruk et al., 2019). The objective of this research was to determine the optimum conditions of the TIBs system for production of good quality material of *Musa* (AA) 'Kluai Khai' as well as assess ploidy level variation using a flow cytometer.

Materials and Methods

Explant collection, surface sterilization, and semi-solid cultures systems

The explant, *Musa* (AA) 'Kluai Khai' disease free sword suckers, which is a geographical indication (GI) variety that is grown in Kamphaeng Phet province, northern Thailand, was used for the initiation of shoot buds *in vitro*. The collected suckers were washed with running tap water to remove adhering soil residues. For 15 minutes, a 10 cm long trimmed sucker was immersed in a 0.1% Metalaxyl (fungicide) solution. The samples were then soaked for 15 minutes in 1.8 and 0.90% (v/v) sodium hypochlorite (6% active ingredient Haiter®). Transferred all explants into laminar flow. Rinsed the explants in sterilized distilled water thrice. The explants were cut into size 1 cm^3 and placed in 8-ounce bottle glass containing initiation semi-solid medium, MS (Murashige and Skoog, 1962) supplemented with 3 mg/L of BA (Benzyladenine) and 3% (wt./v) sucrose. The culture medium was adjusted to pH 5.6 with 0.1 N NaOH and 0.1 N HCl and 8 g/L of agar was added as a gelling agent before the medium was sterilized in an autoclave at 120 ^oC and 15 Psi for 20 minutes. Culture conditions were set at 25 ± 2 ^oC, light intensity at 40 µmol/m^2/s, and warm-white LED light was provided 12 hours a day. Multiplication of shoot buds was done by sub-culturing once a month in the MS supplemented with 5 mg/L of BA.

Twin-flasks Bioreactor system

The twin-flasks bioreactor, TIB, has been invented using 24 pairs of flasks (SCHOTT DURAN, 2000 ml We used two translucent glass containers with a capacity of 2 liters, timers, pumps (ETOP OIL Free), an Air Compressor® model no. XH-25-30, a silicone tube 1/4″ high temperature resistant with micropore filters of Poly Tetrafluoroethylene 0.20 m (Midisart 2000), and two solenoid valves Air TAC® model: 2W-025-08. The required power for performing the immersion is produced by the pumps. With pumps, air was distributed through silicone tubes. The culture system (TIBs) was placed on aluminium shelves. Aluminium shelves were equipped with white fluorescent lamps to provide the systems with 40 µmol /m^2 /s PPFD, and other environmental conditions were the same as for semi-solid cultures. Our designed TIB was equipped with timers, which allow the adjustment of the number of immersions in a day in each container. When one of the solenoid valves becomes open, air pushes culture medium from its container to the plant material container, which was controlled via a timer based on its program. Then, the second solenoid valve is opened to make the culture pass back to the medium container. The experiment was set to 16 treatments by varying factors: immersion duration (5, 10 min) and frequency (2, 4 times/day), media volume (600 ml, 1200 ml) and explant density (10, 20 buds/flask) as shown in Table 1. The buds 1.5 cm long obtaining from the third sub culturing of semi-solid cultures system were used as inoculum of TIB and cultures system. The liquid MS supplemented with 5 mg/L was used for shoot buds' multiplication for a duration of 5 weeks. The number of buds, number of shoots, number of roots, number of leaves were recorded. Liquid MS containing 0.1

mg/L NAA was used as a rooting medium and roots were obtained within 2 weeks. The acclimatization was done in the same manner as that of semi-solid.

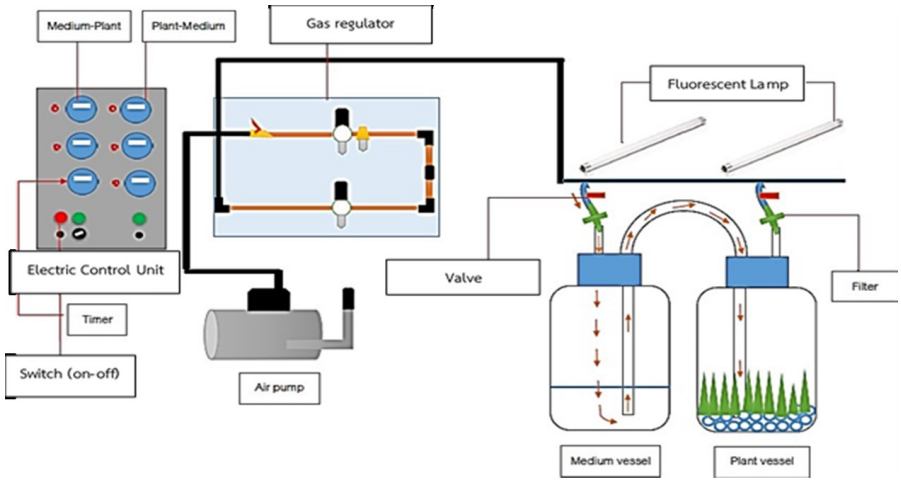

Figure 1. The bioreactor is a simple piece of equipment consisting of a 2-L culture vessel and a liquid media vessel with a fully automated control system, with air inflow and outflow systems.

Table 1. Factors affected *M. acuminata* shoot bud multiplication in twin flask TIB system

Treatments	Medium volume (ml/reactor)	Immersion time (min)	Feeding medium Frequency (time/day)	Number inoculated shoots
1	600	5	4	10
2	600	5	4	20
3	1200	5	4	10
4	1200	5	4	20
5	600	10	2	10
6	600	10	2	20
7	1200	10	2	10
8	1200	10	2	20
9	600	5	4	10
10	600	5	4	20
11	1200	5	4	10
12	1200	5	4	20
13	600	10	2	10
14	600	10	2	20
15	1200	10	2	10
16	1200	10	2	20

Acclimatization

Young shoot buds were transferred to rooting in MS supplemented with 0.1 mg/L NAA for 2 weeks, to obtain the banana plantlets at a size of 4-5 cm. long. Following that, the plantlets were transferred to a first acclimatization period of 2-4 weeks in peat moss (Klassmann®) with 70-90 percent humidity. Later, the plantlets were further acclimated in peat moss and soil (1:1) in a greenhouse with low light intensity and high humidity for a month. For the third acclimatization, plantlets were grown in soil bags for a month.

Flow cytometry analysis

Plantlets were further confirmed for polyploidy level by a flow cytometry assay. A leaf sample (100 mg) was chopped with a razor blade in a petri dish containing 1 mL nuclei extraction buffer (200 mM Tris, 4 mM MgCl2.6H2O, 0.5% (v/v) Triton X-100, pH 7.5) according to the method of Pfosser et al. (1995). The nuclei suspension was filtered through a 40 µm nylon net (Merck Millipore Ltd., Germany) and stained using the Muse™ Cell Cycle Kit (Merck KGaA, Germany). The polyploidy level was analyzed on a Guava® easyCyte Flow Cytometer with InCyte™ software version 2.7 (Merck KGaA, Germany).

Statistical Analysis

The experiment was designed as a CRD (completed randomized design). The differences of mean was evaluated by ANOVA (Analysis of Variance) and DMRT ($p \leq 0.05$) using SPSS.

Results

In the present investigation, the experiment was set to 16 treatments by varying factors: immersion duration (5, 10 min) and frequency (2, 4 times/day), media volume (600 ml, 1200 ml) and explant density (10, 20 buds/flask) as shown in Table 1. The results are shown in table 2. The number of shoots, number of leaves, and number of roots per explants and the survival rate of plantlets were observed at 35 days after cultured in the TIB system.

Effect of treatments on shoot number

Results showed that treatments 5, 6, and 13 produced shoot numbers of 4.19±0.60, 3.28±0.60, 2.89±0.60, and 3.0 ±0.84, respectively, and the data is not statistically significant. The treatments that contained a higher volume of medium (1200 ml) tended to decrease the number of shoot buds. Treatment 5 produced the most shoots (media volume of 600 ml, immersion time of 10 minutes, feeding frequency of 2 times per day, number of inoculated shoots=10).

Effect of treatments on leaf number

After 35 days of culture in TIBs, the number of leaves was counted. Treatment 6, 7, 8 gave number of leaves ranged from 5.11±0.98-7.61±1.30. However, the treatment 6 (media volume 600 ml, immersion time of 10 minutes,

feeding frequency of 2 times per day, number of inoculated shoots=20) provided the highest number of leaves at 7.61±1.30 leaves/shoots.

Effect of treatments on number of roots

Although the experiment was designed to get the optimum conditions for shoot production, the root was also observed. The average root number was low in MS supplemented with 5 mg/L BA. The rooting rate in all TIB system treatments ranged from 0-0.43 ±0.05 root/shoot. BA was a supplement for shoot proliferation. Rooting medium (MS + 0.1 mg/L NAA) was feeding to the bioreactor after finished shoot production and left for 15 days. Root length 3-10 centimeter were obtained (Figure 3).

Effect of treatments on banana Biomass production

Figure 2 shows the results of biomass production for the TIB system. The fresh weight (g) of the initial inoculum and the fresh weight of the shoot at harvest were recorded. The fresh weight of starting inoculum was 0.39-0.56 g/shoot after 35 days in the TIB system. The fresh weight at harvest of all treatments was 0.62±0.02-2.49±0.27g/shoot. All treatments of the TIB system showed an increase in fresh weight, with the exception of treatments 11, 12, 15, and 16, which showed very low biomass at harvest and the shoot cultures exhibited hyperhydricity features. Treatment 5 gave the maximum biomass after cultured in the TIB system for 35 days with a fresh weight gain of 2.49 ±0.27 g/shoot which was four times the initial fresh weight.

Effect of treatments on survival rate

The survival rate (%) was observed after acclimatization. The healthy young plants at 30 cm height were counted. The lowest survival rate was found in T16 (50± 5%) and the highest was found in treatment 3(97± 5.77%) (Table 2). The survival rate of 88-97 % was acceptable.

Assessment of ploidy variation

Somaclonal variation is a phenomenon that might occurs during plant tissue cultures and leads to genetic variation that can be observed from morphological and biochemical characteristics. Several chemical and physical factors have been used in the technique, causing genetic variation (Nwauzoma and Jaja, 2013). The ploidy uniformity of the *Musa* (AA) 'Kluai Khai' was evaluated using a flow cytometer. Figure 4 displays the chromatogram of diploid nucleus (2n) at channel 100 for both the mother plant and the TIB system's plants. Results confirmed that the TIB system produces genetically stable *Musa* (AA) 'Kluai Khai' plants.

Table 2. Effects of media volume, immersion duration, feeding frequency, and explant density on the growth of *Musa* (AA) 'Kluai Khai' in the TIB system in 35 days.

Treatment	Number of shoot / explant	Number of leave / plant	Number of root / plant	Survival rate (%)
1	1.64 ± 0.88^{cd}	4.84 ± 1.33^{bcd}	0.29 ± 0.07^{abcd}	90 ± 10.00^{a}
2	3.24 ± 0.76^{abc}	4.98 ± 0.78^{abcd}	0.16 ± 0.07^{cdef}	92 ± 7.64^{a}
3	1.85 ± 0.61^{cd}	4.98 ± 0.73^{abcd}	0.15 ± 0.05^{cdef}	97 ± 5.77^{a}
4	1.79 ± 0.47^{cd}	4.62 ± 0.62^{bcd}	0.43 ± 0.05^{a}	93 ± 7.64^{a}
5	4.19 ± 0.60^{a}	4.30 ± 0.68^{bcd}	0.31 ± 0.07^{abc}	93 ± 5.77^{a}
6	3.28 ± 0.46^{abc}	7.61 ± 1.30^{a}	0.21 ± 0.07^{bcde}	88 ± 12.58^{ab}
7	1.92 ± 0.58^{cd}	5.11 ± 0.98^{abc}	0.38 ± 0.05^{ab}	97 ± 5.77^{a}
8	2.89 ± 0.66^{abcd}	5.85 ± 0.88^{ab}	0.21 ± 0.08^{bcde}	92 ± 7.64^{a}
9	3.00 ± 0.84^{abcd}	3.44 ± 1.37^{bcde}	0.14 ± 0.05^{cdef}	93 ± 5.77^{a}
10	2.63 ± 0.45^{abcd}	3.20 ± 0.80^{bcde}	0.35 ± 0.07^{ab}	87 ± 18.93^{ab}
11	1.20 ± 0.52^{d}	3.04 ± 0.76^{cde}	0.10 ± 0.08^{ef}	80 ± 10.00^{abc}
12	1.51 ± 0.46^{cd}	2.49 ± 0.66^{cde}	0.13 ± 0.08^{cdef}	88 ± 12.50^{ab}
13	3.95 ± 0.65^{ab}	3.80 ± 0.70^{bcde}	0.10 ± 0.05^{def}	75 ± 25.00^{abc}
14	2.28 ± 0.71^{bcd}	3.20 ± 0.63^{bcde}	0.11 ± 0.06^{def}	57 ± 7.64^{bc}
15	1.23 ± 0.56^{d}	1.58 ± 0.70^{e}	0.42 ± 0.08^{a}	80 ± 0.00^{abc}
16	1.79 ± 0.52^{cd}	2.35 ± 0.64^{de}	0.00 ± 0.00^{f}	50 ± 5.00^{c}

Means followed by different letters, in the column, statistically different by DMRT

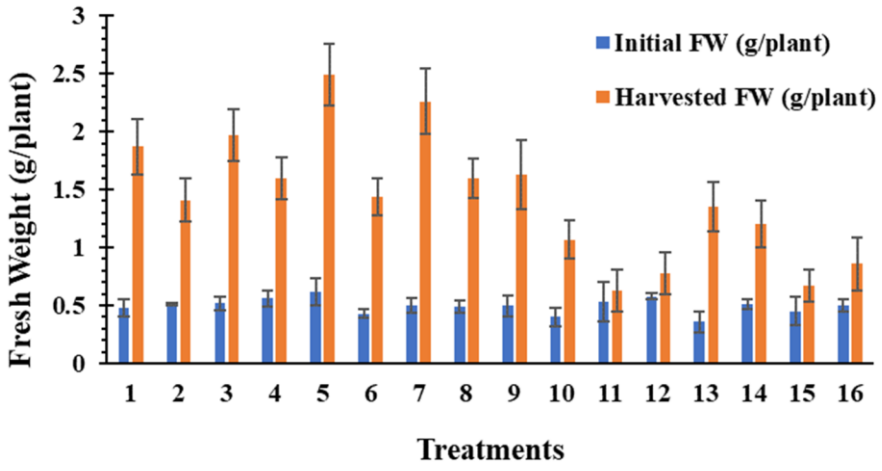

Figure 2. Biomass as fresh weight (g/plant) obtained from 16 treatments in the TIB system

Figure 3. Temporary immersion bioreactors and *Musa* (AA) 'Kluai Khai' plantlets

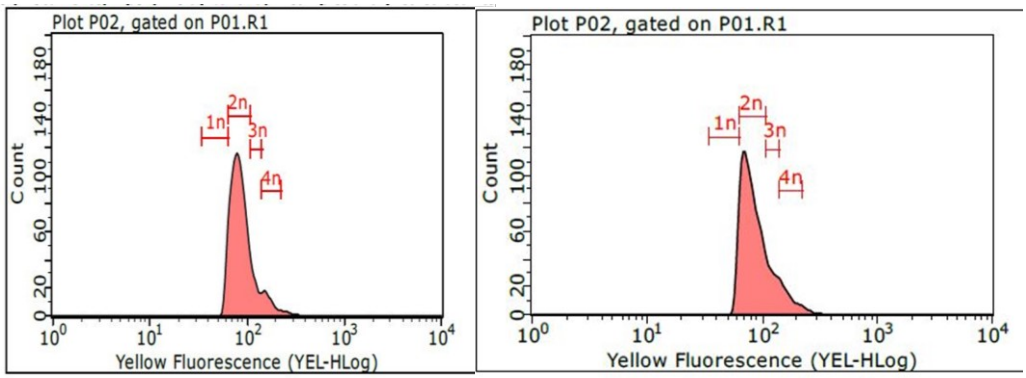

Figure 4. Histograms from ploidy analysis of *Musa* (AA) 'Kluai Khai'. The figure shows a peak at channel 100 representing diploid level at both donor plants (left) and TIBs plantlets (right).

Discussion

According to the results of this research, it was found that the optimum conditions for enhancing shoot proliferation of *Musa* (AA) 'Kluai Khai' in the TIB system were volume medium (600 ml), number of starting inoculum (10 shoots), duration of immersion of plant in medium (10 min), and the number of daily feeding times (twice/day). *Eucalyptus* shoots' fresh weight and dry weight increased in the TIB system (Gonzalez et al., 2011). Many buds and shoots form due to well gas exchange during plant growth in this condition. Each time the plant is filled with medium solution, a thin film of the solution is coated on top of the shoots, which the shoots will utilize during the absence of medium until the next feeding. But too frequent feeding caused the coated medium on plant surfaces to not completely

evaporate, so the plant parts were always covered with a film of medium solution. As a result, gas exchange was lower, resulting in less growth as well. An ornamental plant, *Gerbera jamesonii* Bolus ex Hooker f., also produced low shoots in the TIB system when feeding medium frequency was more than 3 times a day (Frometa et al., 2017). In addition, oil palm (*Elaeis guineensis* Jacq.) produced more embryogenic callus when the interval of immersion was every 3 hours for 3 minutes (Marbun et al., 2015). Data obtained indicated that a large-scale production of *Musa* (AA) 'Kluai Khai' using the TIB system should be concerned with genetic uniformity and confirmed that it is a suitable method for gaining good quality plant materials.

Conclusion

The explants cultured in twin-flasks temporary immersion bioreactor (TIB) produced a high number of shoots, leaves, shoot length, fresh weight, and dry weight when compared to *Musa* (AA) 'Kluai Khai' produced in the semi-solid medium system. The study found that the morphological characteristics of *Musa* (AA) 'Kluai Khai' produced by the twin-flasks temporary immersion bioreactor (TIB) were not different when compared to the donor plant. The Temporary Immersion Bioreactor system produced 78-96% similarity when compared to the donor plants. The technology of the Temporary Immersion Bioreactor system is genetically stable as far as the micropropagation of *Musa* (AA) 'Kluai Khai' is concerned.

ACKNOWLEDGMENTS

This research was funded by Naresuan University (R2561B058) and the thesis grant for master's degree students from the National Research Council of Thailand (486639) in fiscal year 2019.

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