

Full Length Research Paper

Micropropagation and *in vitro* germplasm conservation of endangered *Musa balbisiana* 'Kluai Hin' (BBB group)

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Multiple shoot formation of edible bananas (*Musa balbisiana*, BBB group) 'Kluai Hin' was achieved through organogenesis in bud culture. Excised apical and lateral buds were cultured on a modified Murashige and Skoog (MS) medium supplemented with 22 μ M BA and 15% (v/v) coconut water (CW). For mass multiplication, the proliferated shoots were subcultured to MS media containing several concentrations of BA and TDZ. The highest shoot numbers was 21.2 shoots per explant when subcultured to MS medium supplemented with 44 μ M BA. Shoot produced roots within 7 days on MS basal medium without plant growth regulator. Microshoots of 'Kluai Hin' were stored in 4 different conditions and on three types of carbon sources (including, sucrose, glucose and sorbitol) at the concentrations of 1, 3 or 5%. Results revealed that sucrose was the suitable carbon source for storing microshoots of 'Kluai Hin' at 25°C under a 16 h photoperiod for 6 months. Alginate encapsulation of microshoots for conservation of 'Kluai Hin' was established as well. Microshoots excised from multiple shoot cultures were encapsulated in 3% sodium alginate prepared in MS medium and complexed with 50 mM calcium chloride. Maximum conversion frequency of 73% was noted from encapsulated microshoots cultured on MS medium supplemented with 22 μ M BA. Plantlets developed from encapsulation were successfully transferred to field conditions and all the resultant plants were shown to be morphologically normal.

Key words: Encapsulation 'Kluai Hin', micropropagation, *Musa balbisiana*, slow growth.

INTRODUCTION

Dessert bananas and plantains (cooking bananas) are important staple food crop in tropical and subtropical countries. Edible bananas evolved from two wild species- *Musa acuminata* Colla and *Musa balbisiana* Colla (Simmonds and Shepherd, 1956). Banana fruit production is limited by many diseases, pests and traditional propagation methods. *M. balbisiana* 'Kluai Hin' is so far known only in the lower south of Thailand. It is found to have a sporadic distribution along the Pattani to the collapse of the bank. It is propagated vegetative by

suckers and not sufficient to overcome the treat of extinction.

Recent advances in biotechnology for crop improvement have had a great impact on plant cultivation. The development of tissue culture applications can be utilized for banana breeding program and to satisfy the demand for planting materials. Different *in vitro* conservation methods such as slow growth, alginate encapsulation and cryopreservation have been developed in recent time. *In vitro* storage of plant germplasm is an alternative way to maintain a gene bank of plants at treat and readily available for use. Slow growth has been widely employed in many crop species including bananas to reduce growth and prolong subculture intervals (Van den Houwe et al., 1995; Tyagi et al., 2009; Ahmed and Anjum, 2010). Alginate encapsulation is a technique that can be used in

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conjunction with micropropagation for *in vitro* conservation. Encapsulation of shoot tips in calcium alginate beads offers another space saving option for storage (Lisek and Orlikowska, 2004). Artificially encapsulated shoots or other explants can be sown under *in vitro* or *ex vitro* conditions, producing uniform genotypes (Aitken-Christie et al., 1995; Shii et al., 2004). Encapsulation of axillary buds or shoot tips has been reported in many plant species, for example, banana (Ganapathi et al., 1992), lilac (Refouvet et al., 1998), tropical forest trees (Maruyama et al., 1997), strawberry and raspberry (Lisek and Orlikowska, 2004).

In this study, we described the usual *in vitro* strategies for propagation of *M. balbisiana* 'Kluai Hin' by shoot regeneration from buds, storage using slow growth conditions and alginate encapsulation for conservation.

MATERIALS AND METHODS

Source of explant materials

The suckers of *M. balbisiana* 'Kluai Hin' (BBB group) were used in the experiments. Suckers were cut into pieces (20 cm in diameter and 25 cm long) and washed in running water to remove dirt. Suckers were excised by removing the outer layers of tissue until the lateral buds were exposed. Tissue blocks (2 cm²) containing lateral buds were cut off and suckers were further trimmed down. Under aseptic conditions, tissue blocks and a portion containing apical bud were immersed in 70% ethanol for 30 s. Then, the explants were surface sterilized by successive immersion using a dilution of 10% (v/v) CloroxTM and 2 drops of Tween 20 per 100 ml solution for 15 min, followed by 5% (v/v) CloroxTM for 10 min. After sterilization, these explants were rinsed 3 times with sterilized distilled water to remove traces of disinfectant. Leaf sheaths were discarded until the diameters of the explants were about 1.5 cm and then transferred to culture medium.

Nutrient media preparation

MS (Murashige and Skoog, 1962) salts and vitamins supplemented with 3% sucrose were used as the culture medium. MermaidTM agar (0.8%) was used as a gelling agent. Apical and lateral buds were aseptically cultured on MS medium supplemented with 22 µM BA and 15% (v/v) CW. For rapid shoot multiplication 4.4, 22, 44 µM BA or 0.1, 0.5, 1, 5 and 10 µM TDZ in combination with 15% (v/v) CW were used as the plant growth regulators either singly or in combination. The pH of all media was adjusted to 5.8 with 1 N NaOH or 1 N HCl prior to autoclaving at 1.05 kg/cm², 121°C for 20 min.

Culture conditions

All plant materials were cultured in 330 ml screw-topped jars each containing 30 ml of medium. Cultures were incubated at 25°C air temperature in a culture room with a 16 h photoperiod under an illumination of 20 µmol m⁻² s⁻¹ photosynthetic photon flux intensity provided by white-fluorescent lamps (Philips 36W/54 daylight).

These environmentally controlled conditions were used in all experiments. Cultures were incubated for 9 weeks prior to evaluation. All regenerated shoots were transferred to a fresh medium and subcultured at 3 week intervals.

Root induction and acclimatization of regenerated plants

Regenerated shoots with 2 to 3 leaves from multiple shoot experiments were excised individually and transferred to MS plant growth regulator-free medium for root induction for 3 weeks. When rooted plantlets reached 4 to 5 cm in height, all regenerated plants were carefully washed free of agar and then transferred to black plastic bags containing sterile vermiculite. Plants were acclimatized at 25°C in a culture room. After 2 weeks of acclimatization, these plantlets were moved to a nursery under shading, natural photoperiod and high relative humidity for 2 months. Normal looking banana plantlets were obtained before being planted in the field.

Slow growth treatments

The effect of osmoticums and low temperature were investigated. Proliferating shoots from the previous shoot multiplication experiments were dissected and inoculated in 330 ml screw-topped jars containing MS medium and absorbent cotton submerged with 20 ml of sucrose, glucose or sorbitol at the concentrations of 1, 3 or 5%. A parafilm M was wrapped around the jar lid to minimize desiccation. The jars were stored under the following conditions: 4°C and in the dark; 16 h photoperiod at 4°C and 20 µmol m⁻² s⁻¹; 25°C and in the dark; 16 h photoperiod at 25°C and 20 µmol m⁻² s⁻¹.

Encapsulation in alginate beads

Four-millimeter-long microshoots obtained from the multiple shoot production were immersed in 2 or 3% sodium alginate (Sigma, USA) dissolved in either sterile distilled water or MS medium. These microshoots were then picked up with 1 cm glass tube and dropped individually into 50 mM calcium chloride. The beads containing the entrapped microshoots were left in calcium chloride solution for 20 min for complexation. Thereafter, the calcium chloride solution was decanted and the calcium alginate beads, each containing one microshoot, were rinsed three times with sterilized distilled water. The beads were placed on sterile absorbent cotton moistened with ¼ MS liquid medium at 25°C either in the dark or at 16 h photoperiod for 0, 15 and 30 days.

Regrowth and establishment of plantlets

After 6 months storage without subculture, stored shoots under slow growth conditions were transferred to MS medium supplemented with 22 µM BA and cultured for 8 weeks. Shoots from all treatments were taken from the culture vessels and planted in potted soil hereafter. For subsequent plant regrowth after encapsulation in calcium alginate bead, the effects of alginate bead compositions, duration of time in storage and light conditions on regrowth of encapsulated shoot tips were studied for a period of one month. Encapsulated microshoots were transferred to either MS medium supplemented with 22 µM BA or mixture of vermiculite and soil (1:1). Plant conversion was calculated after 15 days of culture as the percentage of encapsulated microshoots that were able to grow on several substrates into healthy plantlets.

Data collection and statistical analysis

One explant was implanted per culture and all experiments were conducted on three different days with 20 replicates per treatment. Data were submitted to ANOVA and the difference between the means was compared using Duncan's multiple range test (DMRT).

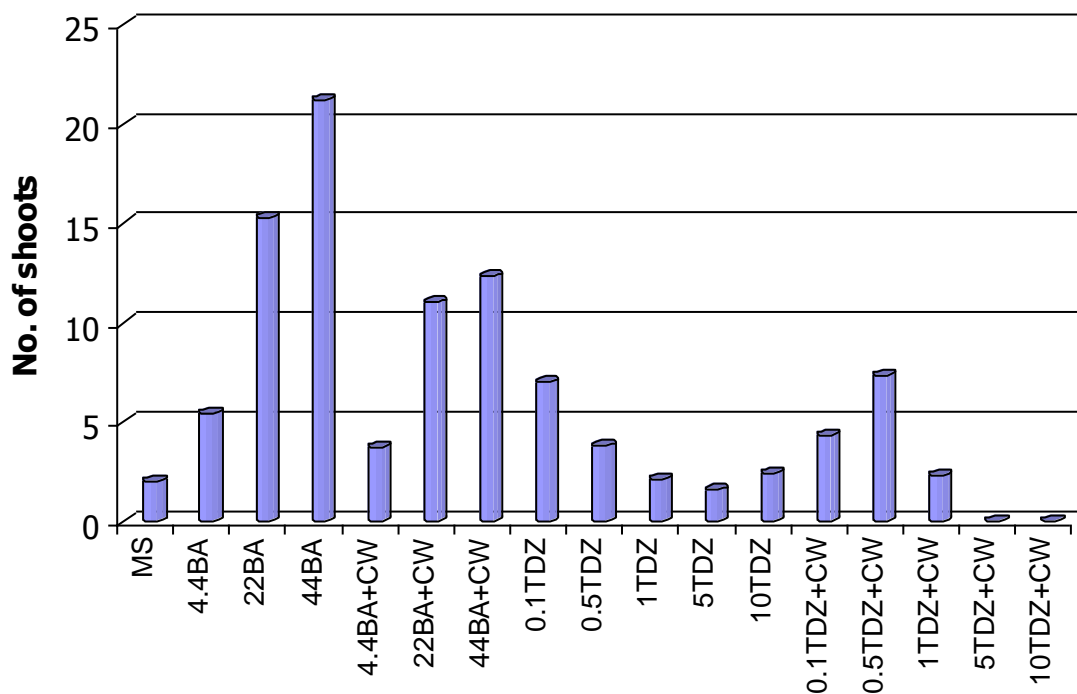


Figure 1. Effect of different combinations of BA or TDZ and 15% CW on shoot multiplication in *M. balbisiana* 'Kluai Hin' bud explants cultured on MS medium.

RESULTS

Micropropagation by adventitious shoot induction was obtained on both lateral and apical buds. All buds showed the same morphogenetic responses and seemed to grow fast and vigorously. After 3 to 4 weeks on MS medium supplemented with 22 μM BA and 15% CW bulge buds were halved, transferred to fresh media and serial subculture was made every 3 weeks. By successive subculture, masses of proliferating shoot cultures have been established. The cytokinin types and concentrations in the culture media dramatically influenced axillary shoot production after 12 weeks of culture. The data are presented in Figure 1. Each tested cytokinin promoted shoot regeneration with the concomitant of the concentrations used in the medium. Within concentrations tested, the number of shoots was highest at 44 μM (21.2) and the number was lowest at 4.4 μM (5.4). Although, BA at 44 μM gave the highest number of shoot production, it was not significantly better than 22 μM BA ($p \leq 0.05$). Experiments were conducted in different combinations of BA and 15% CW. The results revealed that CW in combination with BA did not enhance axillary shoot production when compared with BA alone since an average of 12.3 shoots was recorded at 44 μM BA and 15% CW. However, the number of shoots obtained on MS medium supplemented with CW and BA was larger than BA alone. From TDZ experiments, the number of axillary shoots per explant was greater for TDZ at low concentration (0.1 μM) compared with TDZ at

concentrations higher than 0.1 μM ($p \leq 0.05$). An increase in TDZ level progressively decreased shoot number and retarded shoot elongation. When CW was incorporated into the TDZ containing medium, explants showed poor shoot multiplication except in treatment with 0.5 μM TDZ, whereas no shoots were produced at 5 and 10 μM TDZ.

The multiple shoots thus obtained from shoot experiments either on hormone-free or cytokinin supplemented media developed roots *per se*. Roots originating from shoots were thick, long and fibrous and easy to handle. All plants derived from tissue culture survived and grew extremely vigorously and reached a 3 to 4 m height after 4 months cultivation in the field.

Sucrose, glucose and sorbitol solutions at 1, 3 or 5% were used to test for their ability in slow-growth of tissue culture-derive shoots. The results showed that storage of shoots over absorbent cotton saturated with sucrose could extend the survival time without subcultures for 6 months. The survival of shoots incubated on 1% sucrose was 25% and developed into plantlets, whereas shoots incubated on glucose and sorbitol at all concentrations eventually died. Our results also showed that 25°C and a 16 h photoperiod were better than 4°C and in the dark (Table 1). The shoots survived the slow-growth storage had longer shoot height than those not exposed to slow-growth. The leaves curled, became yellow and dried during the storage period. After 6 months, these shoots were transferred to fresh MS medium supplemented with 22 μM BA and cultured for 8 weeks. Then the new shoots were transferred to MS medium without

Table 1. Effect of various *in vitro* storage conditions on microshoots of *M. balbisiana* 'Kluai Hin' for 6 months.

Slow growth condition (temperature, °C/light, h)	Survival (%) at different concentration of sucrose (%)			
	0	1	3	5
4/0	0	0	0	0
4/16	0	0	0	0
25/0	0	0	0	0
25/16	0	25	0	0

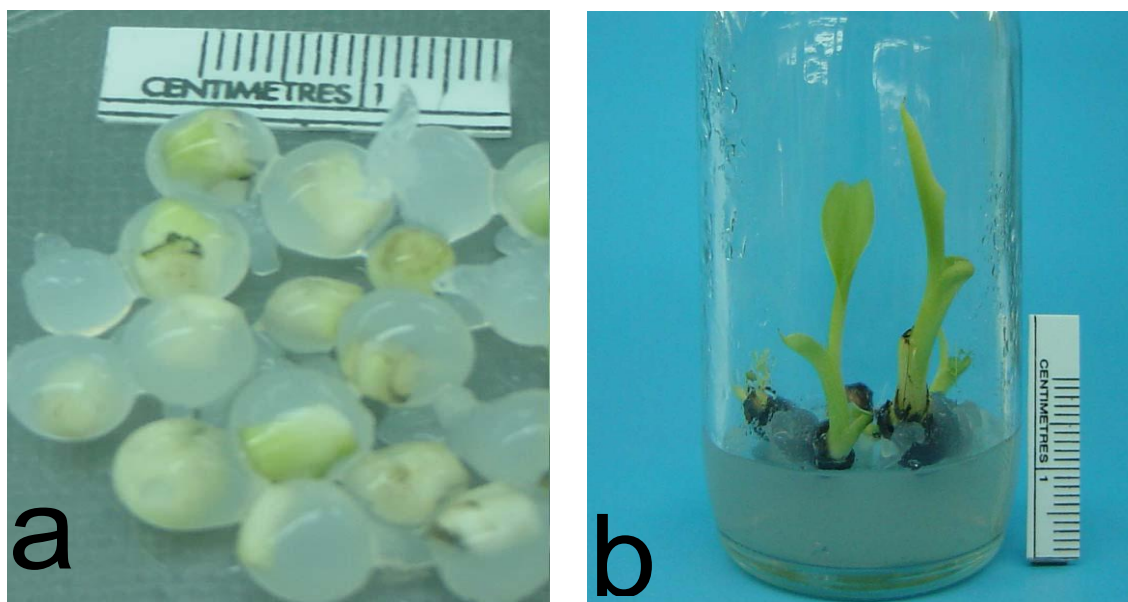


Figure 2. Encapsulation and regrowth of *M. balbisiana* 'Kluai Hin' microshoots. (a) Encapsulated in 3% sodium alginate dissolved in MS liquid medium; (b) healthy green shoots protruded from the beads on MS medium containing 22 μM BA.

plant growth regulators and they rooted normally. Sodium alginate concentration at 3% was found to be superior to 2% sodium alginate. It coated the microshoots very well and the beads formed were clear, shiny and solid and firm (Figure 2a). Non-capsulated microshoots (control) permitted microshoots to survive (50%) better than the alginate beads prepared in water (34.2%). Encapsulated microshoots with MS medium survived the best (73.3%) compared with the control treatment and the encapsulation with water (Table 2) and this method was used in the subsequent experiment. The duration of time in storage significantly affected the regrowth of encapsulated microshoots. As storage time increased, the regrowth of shoot tip decreased significantly ($p \leq 0.05$). Dark and light treatment had no significant effect on the regrowth (Table 2). Shoots and roots were able to protrude from the beads upon germination. When transferred, these beads to germinate in vermiculite and soil (1:1) all microshoots could not survive; however, microshoots could develop into whole plantlets when they

were placed on MS medium supplemented with 22 μM BA. Single vigorous green shoots with good emergence from the beads were obtained in less than two weeks (Figure 2b).

DISCUSSION

TDZ, a synthetic phenyl urea derivative, increases shoot formation of several woody plant species more efficiently than adenine derivatives. TDZ at low concentration (<1 μM) can induce greater axillary proliferation than many other cytokinins (Huetteman and Preece, 1993). Our study indicated that BA, an adenine derivative, induces more shoot multiplication than TDZ suggesting that BA is more suitable for shoot proliferation of *M. balbisiana* 'Kluai Hin' than TDZ. This result agrees with our previous findings with *M. balbisiana* 'Kluai Hom Thong' (Kanchanapoom and Chanadang, 2000). TDZ may inhibit shoot elongation due to an apical dominance release that

Table 2. Effect of various encapsulation conditions on microshoots of *M. balbisiana* 'Kluai Hin'.

Treatment	Regrowth (Mean \pm SE)
Encapsulation	
Non encapsulated (control)	50.0 \pm 9.8 ^{ab}
Encapsulated with MS medium	73.3 \pm 7.4 ^a
Encapsulated with water	34.2 \pm 8.5 ^b
Storage duration of encapsulation with MS medium (day)	
0	86.7 \pm 4.9 ^a
15	49.2 \pm 9.2 ^b
30	21.7 \pm 7.7 ^c
Light condition of encapsulation with MS medium	
In the dark	53.3 \pm 7.3 ^a
Under light	51.7 \pm 7.7 ^a

Each value represents the mean of twenty replicates. Means within a column followed by different letters show significant difference as analyzed by DMRT at $p \leq 0.05$.

accelerate axillary bud formation and stunts the length of explants (Huetteman and Preece, 1993) and these events were also observed in this study.

Using slow growth treatments, the subculture interval of *M. balbisiana* 'Kluai Hin' was prolonged up to 6 months. Survival and growth was recovered from *in vitro* microshoots storage in the treatment of 1% sucrose. This indicates that sucrose allowed the slow growth of stored materials and maintained their viability during storage and subsequent micropropagation. Light regimes did not improve the capacity to the regrowth of *in vitro* microshoots. Similarly, microshoots cultures did not tolerate to cold (4°C) treatment during the storage period. These results are in agreement with Banerjee and de Langhe (1985) who were able to keep 25% of the meristem tips of 'Cavendish' banana at reduced temperature and low light intensity. Surviving cultures at 25°C exhibited no carry-over effect of slow growth storage similar to control in subsequent subcultures. The consistently reduced survival at cold storage suggests that *M. balbisiana* 'Kluai Hin', a tropical origin, is chilling sensitive and to suffer physical dysfunction when stored below 25°C and temperature may play a critical role in storage duration.

Studies on *in vitro* germplasm conservation using calcium alginate bead techniques have been reported for many plant species; however, there has been one report on the encapsulation of banana shoot tips (Ganapathi et al., 1992). According to this report, the presence of 0.1% activated charcoal in the beads enables better survival of storage explants from browning. In this study, the addition of activated charcoal was not necessary, probably due to the frequent subculture prior to encapsulation. Sodium alginate at 2 to 3% was found to facilitate the handling of beads while still maintaining

microshoots integrity. However, 2% sodium alginate beads started deteriorating due to rapid loss of water and probable that nutritional leakage occurred as the alginate beads dried. The percentage of the development of microshoots regrowth from encapsulated beads prepared in MS medium was better than with distilled water. These similar observations have been made in *Morus* spp. (Pattnaik et al., 1995), *Plumbago zeylanica* L. (Rout et al., 2001), and *Ananas comosus* L. Merr. (Soneji et al., 2003).

In conclusion, an effective micropropagation system for *M. balbisiana* 'Kluai Hin' has been worked out utilizing bud explants. Our studies also provide an efficient protocol for storage of *M. balbisiana* 'Kluai Hin' microshoots under slow growth conditions at 25°C under a 16 h photoperiod for 6 months with no maintenance. In addition, germplasm conservation utilizing encapsulation technique was attempted using 3% sodium alginate dissolved in MS medium and 50 mM calcium chloride for complexation. Recovery plants were rooted and established in soil successfully.

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