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MICROPROPAGATION OF *MUSA ACUMINATA* X *M. BALBISIANA* CV. PISANG AWAK (ABB GENOME) AND THREE OTHER CULTIVARS

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Abstract

A comparative study on three clonal propagation systems was carried out on banana cv. Pisang Awak for production of plantlets. The temporary immersion system (TIS) was found to be the most suitable method for *in vitro* propagation of Pisang Awak plantlets as compared to the gelled medium and the shake flask system. There was high shoot proliferation and more roots formation when they were cultured in MS medium supplemented with 5.0 mg/L BA in TIS. The plantlets produced from gelled medium system and TIS also acclimatized well in greenhouse except those derived from the shake flask system where none of the plantlet survived after transferred to the soil. The optimized TIS propagation protocol was found to be effective for mass propagation of other three banana cultivars, Pisang Raja, Pisang Rastali and Pisang Berangan.

Introduction

Banana (sweet bananas and plantains) represents the forth most important horticultural crops in tropical and subtropical regions of the world with current world production of more than 100 million tons per year (Anon., 2007). Pisang Awak (ABB genome) is one of the most vigorous and hardy cultivar of bananas of the Eumusa series. It is cultivated in Thailand, north-east India, Indonesia and Malaysia (Robinson, 1999). In Malaysia, Pisang Awak is cultivated at small scale for local consumption as a dessert fruit (Jamaluddin, 1994). The unripe fruits of Pisang Awak are used for making high fiber flour. It was reported that the partial substitution of wheat flour with Pisang Awak flour had higher total dietary fiber and caloric content (Chong & Noor Aziah, 2008).

Three other banana cultivars, Pisang Raja, Pisang Rastali and Pisang Berangan, are popularly and widely grown in Malaysia also as desert fruits. Pisang Raja and Pisang Rastali are grown normally by the villagers around the house compounds or along the paddy fields while Pisang Berangan is grown commercially for export purposes as in Puerto Rico, Jamaica. Due to the increasing demand of planting materials for these banana cultivars, the conventional tissue culture method using shake flask system and gelled medium have been used for the production of banana plantlets. However, the conventional gelled medium is generally labor intensive and slow and resulted in increased production cost. Micropropagation using liquid medium was found to be cheaper and more appropriate for scale up and mass production of plantlets but it resulted in asphyxia and hyperhydricity of the plantlets. As reported by Preil (2005), temporary immersion system (TIS) provides the *in vitro* plantlets better contact with the nutrients in the medium, ensure efficient gaseous exchange and resulted in better growth. Higher multiplication rate and better quality of banana plantlets had been reported by using TIS (Alvard *et al.,* 1993; Colmenares & Gimenez, 2003; Matsumoto & Brandao, 2002; Roels *et al.*, 2005). Since in TIS, the plantlets come in contact with the liquid medium only intermittently, it can hence be used to eliminate hyperhydricity and asphyxia in plantlets.

To our knowledge, the *in vitro* propagation of Malaysian banana cultivars using TIS has not been reported. The present study was carried out to compare the conventional gelled medium and shake flask system with TIS for clonal propagation of Pisang Awak. The effect of the three *in vitro* culture systems on the acclimatization process of the micropropagated Pisang Awak plantlets was also evaluated. The best culture system was then applied for mass production of four different Malaysian banana cultivars.

Materials and Methods

Plant material: Pisang Awak, Pisang Berangan, Pisang Rastali and Pisang Raja suckers were collected from the banana plantation at Sungai Petani, Kedah, Malaysia. Roots and outer leaves of the suckers were removed and the shoot tips embedded inside the pseudostems (approximately 1.0 – 2.0 cm) were used as explants. The shoot tips were washed thoroughly with mixture solution of detergent and 20% Clorox® (1:1) followed by rinsing under running tap water for 30 minutes. The cleansed shoot explants were then disinfected using double-stage surface-sterilization technique. In the first stage, the shoot explants were disinfected for 20 minutes with 20% (v/v) Clorox® plus 2 or 3 drops of Tween 20 followed by rinsing three times with sterile distilled water. Second stage surface sterilization was carried out by using 10% Clorox® with 2 or 3 drops of Tween 20 for 10 minutes followed by rinsing again with sterile distilled water (three times). The shoot explants were then inoculated on solid Murashige and Skoog medium (MS) (Murashige & Skoog, 1962) supplemented with 150 mg/L Kanamycin (Thai Meiji Pharmaceutical Co. Ltd.) for seven days. The aseptic and developing shoots were subcultured onto solid MS medium supplemented with 5.0 mg/L N^6 benzyladenine (BA) + 7.5 g/L agar (Algas, Chile), shoot proliferation medium, for four weeks for the production of multiple shoots.

The 4 weeks old multiple shoots were seperated into individual shoots. Each of the shoot (1.5 cm) was cut longitudinally into 2 half shoots and used as explants for subsequent studies. All the culture media used were autoclaved (Tomy SS-325, Japan) at 121^0C for 11 minutes and the pH of the medium was adjusted to 5.75 before autoclaving. All the cultures were maintained at 25 ± 2ºC in a culture room with continuous light provided by cool white fluorescent tubes at an intensity of 32.5 µE m^{-2} s^{-1}.

Comparison of different clonal propagation system: The 4 weeks old half shoot explants were inoculated into 250 ml Erlenmeyer flasks containing 30 ml shoot proliferation medium, MS supplemented with 5.0 mg/l BA. The gelled system was added with 7.5 g/L agar (Algas, Chile). The shake flask system consisted of liquid medium and the cultures were placed on the rotary shaker at 120 rpm. For TIS, the shoot explants were inoculated into one of the 250 ml Erlenmeyer flasks while the other flask contains the same shoot proliferation medium. The atmospheric air was supplied into the system using the pneumatic pump (GAST, USA) via 0.2 µm non-pyrogenic hydrophilic syringe filters (Sartorius). In order to exchange the medium between the flasks, pressure was applied by the pneumatic pump which operated automatically using a digital timer. Two half shoot explants were inoculated into each flask and 10 flasks were used for each culture system. Fresh shoot biomass (g), shoot height (cm), shoot diameter (mm), the number of shoots and number of roots produced by each shoot explant of Pisang Awak were compared under 3 different culture systems after 5 weeks of culture.

Effect of different culture system on acclimatization of in vitro plantlets: Shoots of approximately 2.0 cm in height that did not produce any root were randomly selected from the gelled medium, shake flask system and TIS. They were inoculated into 300 ml glass jar containing 30 ml gelled basic MS medium for rooting. After 2 weeks, all the rooted plantlets were transplanted into plastic trays containing soil mixture of top soil : organic soil : sand (1:1:1) and placed in the growth chambers with relative humidity of 70 - 90% and temperature of 25 ± 3ºC for 2 weeks without direct sunlight. The survived plantlets (10 to 12 plantlets) were then transferred to polyethylene bags with same soil mixture under lower relative humidity (50 - 70%) and higher temperature (28 ± 3ºC) in shaded greenhouse for six weeks secondary hardening process. Survival rate (%) of the plantlets was recorded after 2 weeks for primary hardening process and every week until 6 weeks for secondary hardening process. The experiment was repeated 3 times.

Application of TIS for different banana cultivars: For each banana cultivar, 8 half shoot explants were inoculated into 250 ml Erlenmeyer flasks with another flask containing 100 ml liquid MS medium supplemented with 5.0 mg/l BA and 30 g/l sucrose with pH adjusted to 5.7 (Fig. 1). The explants were immersed in the shoot proliferation medium every 24 hours for 10 minutes under continuous light provided by cool white fluorescent tubes at an intensity of 32.5 µE m^{-2} s^{-1}. Four banana cultivars (Pisang Awak, Pisang Berangan, Pisang Raja and Pisang Rastali) were then compared for their shoot proliferation rate in TIS after 5 weeks of culture. Four culture vessels were used for each banana cultivars.

Experimental design and statistical analysis: All the studies were carried out using complete randomized design. The data collected for each parameter were analyzed using one-way analysis of variance (ANOVA) followed by Duncan multiple range tests at p=0.05 using the statistical program SPSS version 12.0.

Fig. 1. The modified TIS apparatus used in the experiments.

Results and Discussion

TIS produced significantly higher number of multiple shoot (5.6 shoot/explant) and roots (1.2 root/explant) for Pisang Awak as compared to gelled medium and shake flask system (Table 1). The higher shoot proliferation and more roots formation in TIS indicated that TIS was a better micropropagation method for *in vitro* propagation of Pisang Awak as compared to gelled medium and shake flask system. Our results were consistent with the result of Alvard *et al.,* (1993) who found that TIS showed highest multiple shoots formation (>5 shoots/explant) for banana cv. Grande Naine as compared to liquid and solid medium. Similiar result was obtained for plantain in which the number of shoots formation was significantly higher in TIS as compared to the semi-solid medium (Roels *et al.,* 2005; Memon *et al*., 2010). Matsumoto & Brandao (2002) also reported that TIS produced more shoots as compared to permanent immersion system and semi-solid system for *Musa* spp. TIS was found to induce significantly higher shoot multiplication of banana cv. Williams and Horn Plantain than solid medium (Colmenares & Gimenez, 2003).

The shoots produced in the shake flask system were bigger, in term of biomass, height and shoot diameter as compared to the TIS and gelled medium system (Table 1). This could be due to the shoots immersed for a long duration in the liquid medium and able to absorb more nutrients and rapidly. The same result was obtained for East African highland bananas (Sadik *et al.,* 2007) and *Spathiphyllum cannifolium* (Dewir *et al.,* 2005) in which the shoot biomass was significantly higher in the liquid shake flask system with continuous immersion in the culture medium as compared to TIS. TIS was also found to be effective in stimulating higher shoot formation and increased fresh weight for other plant species such as pineapple (*Ananas comosus*) (Escalona *et al.,* 1999) and sugarcane (Lorenzo *et al.,* 1998).

No hyperhydrated shoots were observed in all the three tested culture systems. This indicated that Pisang Awak was not sensitive to hyperhydricity. The multiple shoots formation of Pisang awak in TIS was normal and healthy (Fig. 2). However, Alvard *et al.,* (1993) reported that banana cv. Grande Naine plantlets continuously aerated by bubbling in liquid medium showed hyperhydricity. Roels *et al.,* (2005) explained that the renewal of the headspace with every immersion in TIS resulted in supply of O_2 and prohibited the accumulation of CO_2 and C_2H_4. This had a positive effect for producing better quality of plantain plantlets.

Table 1. Effect of different micropropagation methods on shoot proliferation and growth of Pisang Awak after five weeks of culture.

Culture system	No. of shoot/ explant ± se	Fresh biomass ± se (g)	Shoot height ± se (cm)	Shoot diameter ± se (mm)	Number of roots/explant ± s.e
Gelled medium	3.4 ± 0.2 a	1.06 ± 0.06 c	2.3 ± 0.1 f	5.4 ± 0.2 h	0.4 ± 0.2 j
Shake flask	2.9 ± 0.3 a	2.94 ± 0.23 d	4.1 ± 0.2 g	7.6 ± 0.3 i	0 ± 0 j
TIS	5.6 ± 0.2 b	2.13 ± 0.12 e	2.6 ± 0.1 f	5.9 ± 0.2 h	1.2 ± 0.2 k

Mean values within the same column with different alphabets were significantly different according to DMRT, $p \leq 0.05$

Present study also indicated that TIS induced higher number of root as compared to gelled and shake flask systems. The higher number of roots formed could be beneficial in terms of survival rate during the acclimatization process in greenhouse. This result was similar with the result obtained by Murch *et al.,* (2004). They reported that plantlets immersed temporary in TIS produced significantly more roots than those in solid and liquid medium culture. In this study, the plantlets produced using TIS showed the highest survival rate (100%), followed by those produced from the gelled medium (92%), while only 30% of the plantlets produced from the shake flask system survived after two weeks of primary hardening process. Those plantlets derived from gelled medium maintained at 92% survival until the 6th week while the survival of those derived from TIS reduced from 100% to 89% at 2nd week and maintained until the 6th week during secondary hardening process. However, all plantlets from shake flask system died at the 3rd week of secondary hardening process (Table 2). All the Pisang Awak plantlets produced from the gelled medium and TIS grow normally during the acclimatization process (Fig. 3). Aragón *et al.* (2009) reported that *in vitro* plantain plantlets derived from TIS had functional stomata and trichome structures that were more identical to *ex vitro* plants and ultimately gave better acclimatization survival rate.

Since TIS was found to induce better shoot proliferation and enable production of normal healthy plantlets of Pisang Awak, it was applied to three other Malaysian banana cultivars, Pisang Raja, Pisang Berangan and Pisang Rastali. Results revealed that TIS enabled Pisang Awak (5.6 shoots/explant) and Pisang Raja (5.5 shoots/explant) produced significantly higher number of multiple shoots as compared to Pisang Berangan (3.8 shoots/explant) and Pisang Rastali (3.1 shoots/explant). However, other parameters sush as fresh biomass, shoot height, shoot diameter and number of roots produced, did not show any significant difference among the different cultivars (Table 3). This indicated that the optimized TIS protocol for the mass production of Pisang Awak was also suitable for mass production of Pisang Raja, Pisang Rastali and Pisang Berangan even though each cultivar responded differently to TIS for production of *in vitro* plantlets. Similar results were reported by Lorenzo *et al*., (1998) in which three sugarcane varieties (C-91-301, C-120-78 and C-323-68) have been micropropagated by using the TIS protocol for sugarcane cv. C-1051-73 and they found that each variety responded differently in terms of shoot multiplication rate. Mordocco *et al.,* (2008) also reported that the frequency of shoot regeneration was highly variable among the seven cultivars of sugarcane that were propagated in TIS. However, Hanihneva *et al.,* (2005) reported that the regeneration frequencies of the five cultivars of strawberry tested in TIS was not significantly different.

Fig. 2. Multiple shoot formation of Pisang Awak in TIS after five weeks of culture.

Fig. 3. Acclimatized six weeks old *In vitro* Pisang Awak plantlets.

Table 2. Effect of different micropropagation system on the survival of Pisang Awak plantlets during acclimatization

Micropropagation system	Survival rate (%)						
	2 weeks Primary hardening	Secondary hardening (weeks)					
		1	2	3	4	5	6
Gelled medium	92	92	92	92	92	92	92
Shake flask	30	10	10	0	0	0	0
TIS	100	100	89	89	89	89	89

Table 3. Application of TIS for shoot growth and propagation of four banana cultivars

Banana cultivars	No. of shoot/ explant ± s.e	Fresh biomass ± s.e (g)	Shoot height ± s.e (cm)	Shoot diameter ± s.e (mm)	Number of roots/ explant ± s.e
Pisang Awak	5.6 ± 0.3 a	2.11 ± 0.17 c	2.6 ± 0 d	6.0 ± 0.3 a	1.1 ± 0.3 a
Pisang Berangan	3.8 ± 0.4 b	2.10 ± 0.10 c	2.8 ± 0.2 a	6.5 ± 0.5 a	1.0 ± 0.2 a
Pisang Raja	5.5 ± 0.3 a	2.50 ± 0.24 c	2.3 ± 0.1 a	6.2 ± 0.4 a	1.1 ± 0.2 a
Pisang Rastali	3.1 ± 0.2 b	1.98 ± 0.09 c	2.9 ± 0.2 a	6.8 ± 0.5 a	1.9 ± 0.3 a

Mean values within the same column with different alphabets were significantly different according to DMRT, $p \leq 0.05$

Conclusion

Temporary immersion system was found to be the best method for producing *in vitro* Pisang Awak plantlets and the plantlets produced from the gelled medium and TIS could acclimatize well in green house. The temporary immersion system could be used for clonal propagation of other bananas cultivars.

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