

IN VITRO SHOOT REGENERATION OF INDONESIAN BANANAS (*Musa* spp.) cv. AMBON KUNING AND RAJA BULU, PLANTLET ACCLIMATIZATION AND FIELD PERFORMANCE

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ABSTRACT

The aim of this study was to observe responses of two banana cultivars 'Ambon' and 'Raja Bulu' on different BA concentrations and effects of different media and fertilizer on survival and growth of plantlets. Sterilized explants were cultured on initiation medium (MS with 1mg L⁻¹ BA) for 4 weeks, then subjected to media MS with 2.5, 5.0, or 7.5 mg L⁻¹ BA. Numbers of shoot buds, shoots and propagules were recorded after 4 consecutive passages with 4 weeks intervals. Rooted plantlets were acclimatized in three different media, then treated with or without NPK (32:10:10) fertilizer solution once a week. After 2 months, the survival and growth of plantlets were recorded. Cultures of banana 'Ambon Kuning' showed higher regenerative capacity compared to 'Raja Bulu', producing higher numbers of shoot buds, shoots and propagules. The best medium for propagule proliferation of both banana cultivars was MS+5 mg L⁻¹ BA, producing 40.7 propagules for 'Ambon Kuning', and 12.3 propagules for 'Raja Bulu' per explant. In all acclimatization media tested, 100% of plantlet survival was achieved. The best plantlet growth was found in sand: compost (1:1,v/v) with application of NPK solutions. The *in vitro*-derived plants were planted in the field and produced fruits of high quality.

Keywords: acclimatization, benzyladenine, fertilizer, genotypes, media mixtures

INTRODUCTION

Banana (*Musa* spp.) is the fourth most important crops in the world, belonging to the Musaceae family. Indonesia is the sixth top

banana producing country, which produced 6.19 millions MT, after India (in millions MT) (24.86), China (10.55), The Philippines (9.22), Ecuador (7.01) and Brazil (6.90) (Maps of World, 2014). In Indonesia, banana ranks first, becoming the most important fruit in term of production and harvested area. Two most popular bananas in Indonesia are 'Ambon Kuning' (AAA group) and 'Raja Bulu' (AAB group). Both cultivars are commonly consumed as fresh fruits or as the raw materials for banana powder. In Indonesia, banana cultivation is conducted mostly in small scales (0.5–5 ha), with minimum inputs, but the farmer involved in this cultivation is very large, i.e., up to 21,482,000 households (Indonesia Ministry of Agriculture, 2012).

Currently, the major constrains for banana production in Indonesia are banana wilt caused by *Fusarium oxysporum* f.sp. cubense (Foc), bacterial blood disease and moko disease caused by *Ralstonia solanacearum*. These diseases damage most of banana plantation in Indonesia, causing significant economic losses, i.e., a decline in production by 60-70% (Suyamto *et al.*, 2012). It was also reported that Black Sigatoka and Eumusae leaf spot disease infected most banana orchards in West and North Sumatera and Bengkulu, Indonesia at severity level of 15% to 62.3% (Sahlan and Soemargono, 2011). Monoculture cultivation of banana in a large area has met with difficulty for the availability of healthy and uniform suckers. Plant materials can be prepared from suckers or rhizome division (bit). However, those conventional propagation methods are prone to causing endogenous bacterial or fungal pathogen contamination of the planting materials. Plant tissue culture is a good alternative to suckers in

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providing disease-free and uniform plant materials of these cultivars in a large number and in a relatively short time. Established micro-propagation protocols for these genotypes are needed to overcome shortage of planting materials and to synchronize harvest time for local banana growers.

Success in tissue culture of banana from various genome groups has been reported by several researchers and it was well-documented that the regenerative capacity *in vitro* is highly genotype specific and greatly influenced by growth regulator amended in shoot-inducing medium (Karim *et al.*, 2009; Sipen and Davey, 2012; Rahman *et al.*, 2013; Iqbal *et al.*, 2013). Different genome groups, especially with *balbiana* (AAB, ABB or BBB groups) in their ploidy appeared to be more difficult to propagate than all *acuminata* (AAA) groups (Hirimburegama and Gamage, 1997; Saha-Roy *et al.*, 2010; Sipen and Davey, 2012). Previous procedures in micropropagation of various cultivars of banana have been reported (Kalimuthu *et al.*, 2007; Al-Amin *et al.*, 2009; Ali *et al.*, 2011; Hapsoro *et al.*, 2010); however, the best medium for shoot multiplication of each cultivar was different. The objective of the present study was to investigate the response of banana cultivars 'Ambon Kuning' (AAA group) and 'Raja Bulu' (AAB group) to different benzyladenine (BA) concentrations, to develop a reproducible method of plantlet acclimatization, and to evaluate performance of the tissue-derived plants in the field.

MATERIALS AND METHODS

This research was conducted in Plant Tissue Culture Laboratory, Department of Agrotechnology, College of Agriculture, University of Lampung from May 2012 to January 2014. Healthy shoot buds of bananas 'Ambon Kuning' and 'Raja Bulu' were taken from public collections in Lampung Province, Indonesia. Apical buds approximately 3 x 3 x 4 cm with rhizome parts at the base were excised from rhizomes, rinsed under tap water, and soaked in a solution containing 150 mg L⁻¹ ascorbic acid, 50 mg L⁻¹ citric acid and 2 g L⁻¹ of fungicide for 30 minutes before being surface sterilized in laminar air flow cabinet with 2.6% sodium hypochlorite (NaOCl) plus 2 drops of Tween-20 solution for 30 minutes. This was followed by aseptically trimming the buds to smaller size (approximately 1.5 x 1.5 x 2

cm) and shaking them in the 0.8% NaOCl solution for 10 minutes, followed by three-time rinsing with sterile distilled water. All explants were incubated on a preconditioning medium (Figure 3a) for 14 days to ensure aseptic condition of explants after sterilization and to induce early growth. The preconditioning medium was MS (Murashige and Skoog, 1962) basal salts, supplemented with 1 mg L⁻¹ N⁶-benzyladenine (BA) (Phyto Technology Laboratories®). Aseptic explants from the preconditioning medium were transferred into MS medium with different concentrations of BA (2.5, 5.0 or 7.5 mg L⁻¹). The basal media consisted of MS macro and micro salts supplemented with MS vitamins, 100 mg L⁻¹ myo-inositol and 3% (w/v) sucrose. The pH of medium was adjusted to 5.8 prior to being solidified with 0.8% (w/v) agar (Swallow Globe, Indonesia) and autoclaving. Subcultures into the same growth regulator-containing media were done every 4 weeks. All cultures were incubated in a culture room at 26 ± 2°C under continuous light from cool-white fluorescent lamps (Phillips) at 1500 lux, and 60% relative humidity.

The experiment was set up in a completely randomized design with three replicates. Each experimental unit consisted of four culture bottles each of which contained one explant. After 20 weeks of culture, the effects of different medium on axillary buds and shoot proliferation were observed by recording the percentage of explants forming multiple shoots, average number of shoot buds (≤ 0.5 cm), shoots (> 0.5 cm) and propagules (shoot buds + shoots) per explant. All data were subjected to analysis of variance (ANOVA) followed by mean separation test using least significant difference (LSD) at α 0.05.

Shoot clusters of 'Ambon Kuning' in roliferation medium (Figure 1c) were separated into individual shoot and transferred to MS + 1 mg L⁻¹ BA for 4 weeks for shoot elongation and rooting. Cultures with rooted shoots of 6-7cm in length (Figure 1b) were taken out from culture room and hardened off at room temperature with diffused sunshine for 1 week before being used for subsequent acclimatization experiment. The healthy rooted plantlets were selected for uniformity in size and were washed under tap water from the sticking media, then planted in plastic pots containing one of three different potting-mix media, namely: M1:rice husk:compost (1:1;v/v), M2:rice husk:sand (1:1;v/v), M3:rice

husk:compost:sand (1:1:1;v/v). The plantlets were covered with transparent polyethylene bags for a few (3-5) days to retain moisture and were gradually acclimatized to a shaded greenhouse.

After 2 weeks in potting media, the plantlets were treated with or without fertilizer NPK (32-10-10) solution at 2 g L^{-1} weekly, 10 ml per pot. Data of the percentage of acclimatized plantlets, plant height, number of leaves, root length, and plant fresh weight were recorded at 10 weeks after acclimatization. This experiment was also set up in a completely randomized design with three replicates, each of which consisted of 10 plantlets.

Acclimatized plantlets were then transferred to bigger polyethylene bags (5 kg capacity) with soil-mix media consisting of top soil: compost (2:1,v/v). Some of these planting materials were planted in the field, maintained and grown until fruiting, and harvested after 12 months in the field.

RESULTS AND DISCUSSION

Since the early culture establishment, there was a big challenge with 'Raja Bulu' (AAB group)

due to high rate of contamination and blackening of explants. In contrast, establishment of 'Ambon Kuning' (AAA group) cultures was much easier with lighter blackening. In fact, our preliminary research showed that all 'Raja Bulu' explants cultured in MS medium without growth regulator underwent severe blackening and eventually died. Therefore, to avoid the dead of 'Raja Bulu' explants, the MS without growth regulator as the control treatment was not assigned in this experiment.

In the first two passages of cultures, most 'Raja Bulu' explants produced severe black exudation compared to light blackening of 'Ambon Kuning' explants (Figure 1a, 1b). The severe blackening exudation in 'Raja Bulu' cultures remained high for up to three passages of cultures, and this apparently played an important role in inhibition of shoot bud initiation. Blackening or browning of explants in tissue culture is widely known as the oxidation of phenolic compounds resulting in the formation of quinines which are highly reactive and toxic to plant tissue (Titov *et al.*, 2006; Ko *et al.*, 2009; Ahmad *et al.*, 2013).

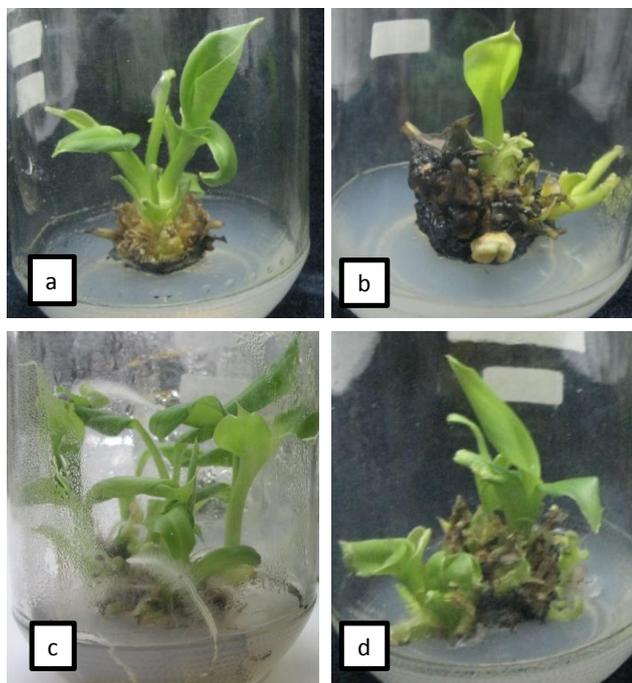


Figure 1. (a) 'Ambon Kuning' explants underwent less blackening; (b) severe blackening occurred in 'Raja Bulu' explants in the second passage; (c) Cultures of 'Ambon Kuning' with normal shoots in MS + 5 mg L^{-1} BA; (d) A few shoots and many buds that failed to elongate in 'Raja Bulu' culture at the fourth passage

One way to control blackening of *in vitro*-cultured banana explants was by treating the explants with 1.2 g L⁻¹ ascorbic acid, followed by addition of 100 mg L⁻¹ ascorbic acid in the medium (Ngomuo *et al.*, 2014). Treatments of explants with potassium citrate and citrate (K-C:C) as an antioxidant during explants preparation were also reported to overcome browning in plantain cultures (Onuoha *et al.*, 2011). In this experiment, explants were soaked for 30 minutes in 150 mg L⁻¹ ascorbic acid and 50 mg L⁻¹ citric acid prior to sterilization, and it appeared that this treatment was effective for 'Ambon Kuning', but less effective for 'Raja Bulu'.

The results of this experiment showed that numbers of shoot buds, shoots and propagules (buds + shoots) per explant after 16 weeks in 'Ambon Kuning' cultures in each concentration of BA being tested were much higher than those in 'Raja Bulu' cultures (Table 1). The blackening of 'Raja Bulu' cultures in BA-containing media seemed to decrease along with further subcultures to fresh culture media. As seen in Table 1, all (100%) of 'Ambon Kuning' explants produced multiple shoots on media with various concentrations of BA tested (2.5, 5.0, and 7.5 mg L⁻¹), whereas the 'Raja Bulu' explants produced 100% multiple shoots only on media with 5 mg L⁻¹ or 7.5 mg L⁻¹ BA. Only 50% of 'Raja Bulu' explants produced multiple shoots on media with 2.5 mg L⁻¹ BA. These data showed significant genotype effects on regenerative capacity of 'Ambon Kuning' vs 'Raja Bulu' cultivars. The number of shoots and propagules were significantly higher in 'Ambon Kuning' compared to 'Raja Bulu'.

Explants from both cultivars produced maximum number of shoots and propagules (25.6

shoots and 40.7 propagules per explants in 'Ambon Kuning', and 1.9 shoots and 12.3 propagules per explant for 'Raja Bulu') in MS amended with 5 mg L⁻¹ BA. Almost similar result was reported by Saha-Roy *et al.* (2010), that the highest shoot proliferation among different BA concentrations tested for 'Malbhog' (AAB group) was BA 5 mg L⁻¹ + 1 mg L⁻¹ IAA. However, these results were different from other results reported previously. The highest shoot proliferation of 'BARI-1' banana was obtained in MS + BA 7.5 mg L⁻¹ + NAA 0.5 mg L⁻¹ (Al-Amin *et al.*, 2009), whereas the best medium for maximum multiplication of 'Agnishwar' banana was MS + BA 4 mg L⁻¹ (Rahman *et al.*, 2013). Hapsoro *et al.* (2010) reported that the bud regenerative capacity of 'Tanduk' (horn type AAB group) was higher than that of 'Ambon Kuning' (AAA group), and the maximum shoots of 'Tanduk' was obtained in MS + 2 mg L⁻¹ BA, while that of 'Ambon Kuning' was obtained in MS + 5 mg L⁻¹ BA. In addition, it was shown from the figures with respect of the proportion of shoot buds over the propagules produced, that the propagules of 'Raja Bulu' were predominated by shoot buds that failed to elongate into shoots, whereas 'Ambon Kuning' produced more normal shoots. The average length of shoots of 'Ambon Kuning' was higher than that of 'Raja Bulu', especially in lower (2.5-5.0 mg L⁻¹) BA concentrations. Therefore, the subsequent acclimatization experiment was conducted only with 'Ambon Kuning' plantlets. The culture performance of 'Ambon Kuning' vs 'Raja Bulu' in media with 5 mg L⁻¹ BA is presented in Figure 1c and 1d, respectively.

Table 1. Effects of benzyladenine (BA) concentrations on bud, shoot and propagule proliferation of banana 'Ambon Kuning' and 'Raja Bulu' after 20 weeks of culture

Banana cultivars	BA concentrations (mg L ⁻¹)	% of explants forming multiple shoots	Mean number of shoot buds per explant	Mean number of shoots per explant	Mean number of propagules per explant	Mean length of shoots (cm)
'Ambon Kuning' (AAA)	2.5	100	4.70 c	7.70 b	12.30 c	5.10 a
	5.0	100	15.10 ab	25.60 a	40.70 a	4.70 a
	7.5	100	18.90 a	11.90 b	30.80 b	2.60 b
'Raja Bulu' (AAB)	2.5	50	5.30 c	1.10 c	6.40 c	2.80 b
	5.0	100	10.40 bc	1.90 c	12.30 c	2.70 b
	7.5	100	5.80 c	1.30 c	7.10 c	2.10 b
LSD 0.05	-	-	7.75	4.77	7.19	1.46

Remarks: Means in the same column followed by different letters differ significantly at P ≤ 0.05 according to LSD multiple range test. All values are an average of 12 explants

These findings and the supportive literatures discussed above confirmed that responses of *in vitro* shoot regeneration in banana were highly influenced by genotypes as well as cytokinin concentrations. Hirimburegama and Gamage (1997) reported that bananas having genome with *balbisiana* (AAB, ABB or BBB groups) have poorer regenerative capacity than those of the pure *acuminata* (AAA group) as shown by lower axillary shoot numbers or propagule multiplication. Sipen and Davey (2012) also found that for banana 'Nangka' (AAA group), the maximum number of shoots was obtained in MS medium with 5 mg L⁻¹ BA, whereas for 'Mas' (AA) and 'Berangan' (AAA) with 6 mg L⁻¹ BA and for 'Awak' (AAB) with 7 mg L⁻¹ BA. In their experiment, it was found that at the same concentration of growth regulator, namely MS + 6 mg L⁻¹ BA, the shoot number of 'Awak' (AAB) was less than those obtained in 'Nangka' (AAA), 'Mas' (AA) and 'Berangan' (AAA). Apparently, this was due to the stronger apical dominance in the AAB type compared to the AAA group as suggested by Ortiz and Vuylsteke (1994) and discussed by Saha-Roy *et al.* (2010). The fact that the horn type banana 'Tanduk' (AAB group) showed higher shoot bud regeneration capacity than 'Ambon Kuning' (AAA group) found by Hapsoro *et al.* (2010) suggests that difficulties in shoot bud

multiplication might not be solely influenced by the involvement of *balbisiana* (B group) in the genome. Rather, it might also be influenced by the variation of ability to re-generate among the *balbisiana* types. Furthermore, the difficulties to regenerate buds, shoots or propagules seem to be closely related with the blackening of explants caused by exudation of phenolic compounds.

For acclimatization experiment, 'Ambon Kuning' shoots were cultured individually in MS containing 1 mg L⁻¹ BA and 3% sucrose for further growth and rooting. After 6 weeks, when the shoots reach 6-7 cm in length all of the shoots showed satisfactory root formation (Figure 3b). These cultures of rooted shoots were transferred to a room with diffused sunshine at room temperature (28^o-29^oC) for 1 week to harden-off the shoots. After this period of hardening-off, healthy plantlets with relatively uniform size and roots were obtained and could be used for the subsequent acclimatization experiment (Figure 3c).

The results showed that after 8 weeks in *ex vitro* condition, all plantlets (100%) from all treatments were fully acclimatized (Figure 2) and ready to be transplanted to bigger polyethylene bags for full exposure to full sunshine. It was observed that both the media composition and fertilizer treatments affected growth of the plantlets.



Figure 2. Eight week-old plantlets acclimatized under different media mixture and applicants of NPK solution. M1: rice husk:compost (1:1,v/v), M2: sand:compost (1:1;v/v), M3: rice husk:compost:sand (1:1:1;v/v); P0: without NPK fertilizer, P1: with application of NPK fertilizer solution



Figure 3. (a) Primary explants; (b) micro shoots of banana 'Ambon Kuning' on medium for shoot elongation and rooting, i.e MS with 1.0 mg L^{-1} BA; (c) plantlet acclimatization and (d) three-month-old plantlets ready to be transferred to the field



Figure 4. (a) Field performance of *in vitro*-derived banana cv. Ambon Kuning, 10 months, (b,c) 11 months in the field, (d) fruits at harvest time, and (e) ripe 'Ambon Kuning' fruits

In general, application of NPK (32:10:10) solution in acclimatized banana plantlets increased growth as shown by the increase in plant height and fresh weight. In addition, acclimatization media consisting a mixture of sand : compost (1:1,v/v) proved to be the best media for banana acclimatization since it produced the highest value of plant height, number of leaves and fresh weight, in both fertilized and without fertilizer treatment (Table 2). This finding was consistent with the previous

results reported in acclimatization of *Sansevieria trifasciata* cv. Lorentii and cv. Hahnii that were successfully *ex-vitro* rooted and acclimatized with 95.9%-100% survival in sand:compost (1:1,v/v) (Yusnita *et al*, 2011). Figure 2 shows performance of acclimatized plantlets in different media and fertilizer treatment after 8 weeks in *ex vitro* condition, while Figure 3d shows performance of tissue cultured plants in polyethylene bags under full sunshine after 12 weeks.

Table 2. Effects of media mixture and NPK fertilizer solution on growth of banana cv. 'Ambon Kuning' plantlets after 8 weeks in *ex vitro* condition

Fertilizer NPK (32:10:10)2 g L ⁻¹	Media mixture	Plant height (cm)	Number of leaves	Length of roots (cm)	Plant fresh weight (g)
Without Fertilizer	M1=Rice Husk Charcoal : Compost (1:1.v/v)	31.00 d	3.00 b	24.10 a	10.88 c
	M2=Sand : Compost (1:1,v/v)	40.00 b	3.20 b	25.90 a	15.45 bc
	M3=Rice Husk Charcoal: Sand:Compost (1:1:1,v/v)	33.80 c	3.10 b	24.00 a	11.27 c
With Fertilizer	M1=Rice Husk Charcoal : Compost (1:1.v/v)	41.40 b	3.30 b	20.60 a	21.30 a
	M2=Sand : Compost (1:1,v/v)	45.70 a	3.70 a	22.30 a	25.63 a
	M3=Rice Husk Charcoal: Sand:Compost (1:1:1,v/v)	44.60 a	3.30 b	19.70 a	20.85 ab
LSD 0.05		2.45	0.35	ns	5.69

Remarks: Means in the same column followed by different letters differ significantly at $P \leq 0.05$ according to LSD multiple range test. All values are an average of 30 plantlets

When these *in vitro* derived 'Ambon Kuning' plants were planted in the field and maintained under farmer procedures of banana cultivation for 12-13 months or until harvesting time, vigorous growth of plants with 8-16 suckers were observed among tissue cultured banana plants in the field as shown in Figure 4a-c. Most plants produced flowers at 8-8.5 months after planting in the field and produced fruit bunches with 8-12 hands per bunch and 12-17 fingers/hands. The green and yellow fruits of 'Ambon Kuning' (Figure 4d, 4e) were of high quality.

CONCLUSIONS AND SUGGESTIONS

Banana 'Ambon Kuning' showed higher shoot and propagule multiplication capacity than 'Raja Bulu'. The best shoot and propagule proliferation medium for both banana cultivars were MS + 5 mg L⁻¹ BA. All acclimatization media tested caused 100% plantlet survival, and the mixture of sand:compost (1:1;v/v) was the best medium for plant growth. The *in vitro*-derived plants were planted in the field and produced fruits of high quality. Further research is needed to overcome severe blackening in early passages of 'Raja Bulu' cultures, and to increase its multiplication rate using stronger type (s) of cytokinin (s).

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