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Micropropagation of *M. balbisiana* and cultivated banana variety "Ambun", using immature male flowers

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ABSTRACT

Male inflorescences have potential to be used as explants of micropropagation of Musa sp. The male inflorescence of M. balbisiana and cultivated banana variety "Ambun" were cultured on MS medium (1962) supplemented with Benzyl Adenine (BA) as growth regulator, and different concentrations of BA and different sizes of male flower hand were tested for micropropagation. The 10 mg L^{-1} concentrations of BA showed the highest response in cultivated variety "Ambun" producing number of hands with white colour bodies (WCBs). However, in M. balbisiana produced WCBs in 12 mg L^{-1} concentrations of BA. But proliferation of shoots were could not be identified in this species. Medium and small sizes of explants produced highest number of hands which having WCBs. The highest number of male flower hands having shoots was observed in cultivated variety "Ambun" of medium size explants. In this variety, immature male flowers were more responsive in micropropagation than the immature male flowers of M. balbisiana.

Key words: *Musa balbisiana*, cv. "Ambun", Male inflorescence culture, Banana

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INTRODUCTION

Banana and plantains are the fourth most important food in the world today after rice, wheat and maize. Banana was grown in more than 100 countries over a harvested area of approximately 10 million hectares, with an annual fruit production of 88 million tones [1]. They can be grow in a wide range of environmental conditions. It may the reason for wide scale spreading.

Many pests and diseases have significantly affected banana and plantain cultivation. They have spread dramatically during the past years. As a consequence of these threats to banana cultivation, there has been renewed interest in banana breeding programmes. The wild species are important to produce disease resistant varieties in banana breeding programmes. Thus, micropropagation of these wild relatives is important for filed cultivation for breeding and can be preserving as for future use.

Micropropagation from immature male inflorescence of banana had been tested by several researchers in past few years for *Musa* cultivated varieties derived from wild *M. acminata.* In the present study, Present research effort to develop a tissue culture protocol for micropropagation using immature male flowers of *M. balbisiana* and cultivated banana variety in Sri Lanka called "Ambun". Therefore study investigated the possibility of using the male flower as explants of *M. balbisiana.*

MATERIALS AND METHODS

Plant material

Male buds of *M. balbisiana* that were required for micropropagation were obtain from plants that grew naturally along the roadside in Athweltota, Matugama as well as Warukandeniya in the Neluwa village, Sri Lanka. The male buds of the cultivated banana variety, "Ambun", used in micropropagation were collected from a home garden in Yatalamatta, Waduramba, Sri Lanka. Male buds were collected from inflorescences in which the female hands (fruits) had already emerged. The harvested buds were placed in polythene bags and transported to the laboratory. They were kept in the refrigerator until used the next day.

Basic MS medium [2] was used for the micropropagation of *Musa* male flowers. The medium was supplemented with the growth regulator BA at varying concentrations of 0, 4, 6, 8, 10, 12 mg L^{-1}. After adding agar and adjusting the pH, 25 ml of the medium was dispensed in to small jam jars. The jars were covered with polypropylene and autoclaved at 121 ^{0}C, 15 psi for 15 min. the medium was prepared one day before culturing.

Explants for culture were prepared by removing the outer bracts of the male bud of *M. balbisiana* and "Ambun", until the whitish inner bracts were exposed, and the length bud was approximately 4 cm. The peeled male buds were surface sterilized in the laminar air flow cabinet by dipping in 70% ethanol for 5 minutes. Then male buds were kept on sterilized paper until used.

The bracts of the sterilized male bud were removed one at a time using forceps. Then from the inside of each bract an entire dump of male flowers (hand) was carefully removed and placed in Petri dish with sterilized moist filter paper. Flower hands were grouped in to 3 size classes based on their length, as large (>10mm), medium (5-10mm) and small (<5mm). Male flowers of each size category were cultured in separate jam bottles (fig.1a), four hands in each bottle. Each treatment consisted of three replicate jars. Cultures were kept in the growth room under 16 h day light period. Cultures were sub cultured every month. Each and every step was mentioned above were done to the cultivated variety "Ambun" for the comparison with the development of wild species *M. balbisiana.*

The development stages of explant were subjected to analysis using statistical package SPSS version 16 in which one way ANOVA and Turky's Multiple Range test were performed at a significance level of ($p<0.05$) at 95% confidence limit to know the significant difference between the treatment.

RESULTS

Micropropagation of cultivated variety "Ambun" using immature male flowers.

Out of a total of 216 male flower-hands that were cultured on MS media 100 explants (nearly 46%) produced an in vitro response. The remaining explants turned brown and were unresponsive.

In the responsive cultures four different stages of growth and regeneration could be identified over a period of 6 months (fig. 1 b-e). The characteristic features of each stage were on follows; Stage I -Explants swelled and turned green after 2-3 weeks. Stage II-Explants produced white colour bodies after one month. Stage III-Tiny shoots appear from white colour bodies after three to four months. Stage IV- Formation of shoot after four months.

The number of responsive explants in different size classes (i.e- small, medium and large) that grew on MS medium supplemented with different BA concentrations and their stage of in vitro growth and regeneration, recovered over a period of 6 months given in table 1. Considering statistical analyzes, swelling of male flower hands may significantly different with the concentrations of BA in the medium ($p < 0.05$). Concentrations of 0 and 4 mg L^{-1} BA showed significant different with other concentration. Different concentration of BA was effect in different manner on explants. According to the statistical analyzes explants that response to 6, 8, 10, and 12 mg L^{-1} concentrations of BA, did not show significant different between these concentrations. Browning is also significantly different with the concentrations of 0 and 4 mg L^{-1} BA with the other concentrations ($p < 0.05$).

Considering the size of the explants, large size explants were less responsive to regeneration. During the first month of culture highest amount swelling and turn in to green shown in small size male flower hand. According to the statistical analyzes size of the explants did not significantly different with the swelling of the flower hand except in 10 and 12 mg L^{-1} BA concentrations (Table 2).

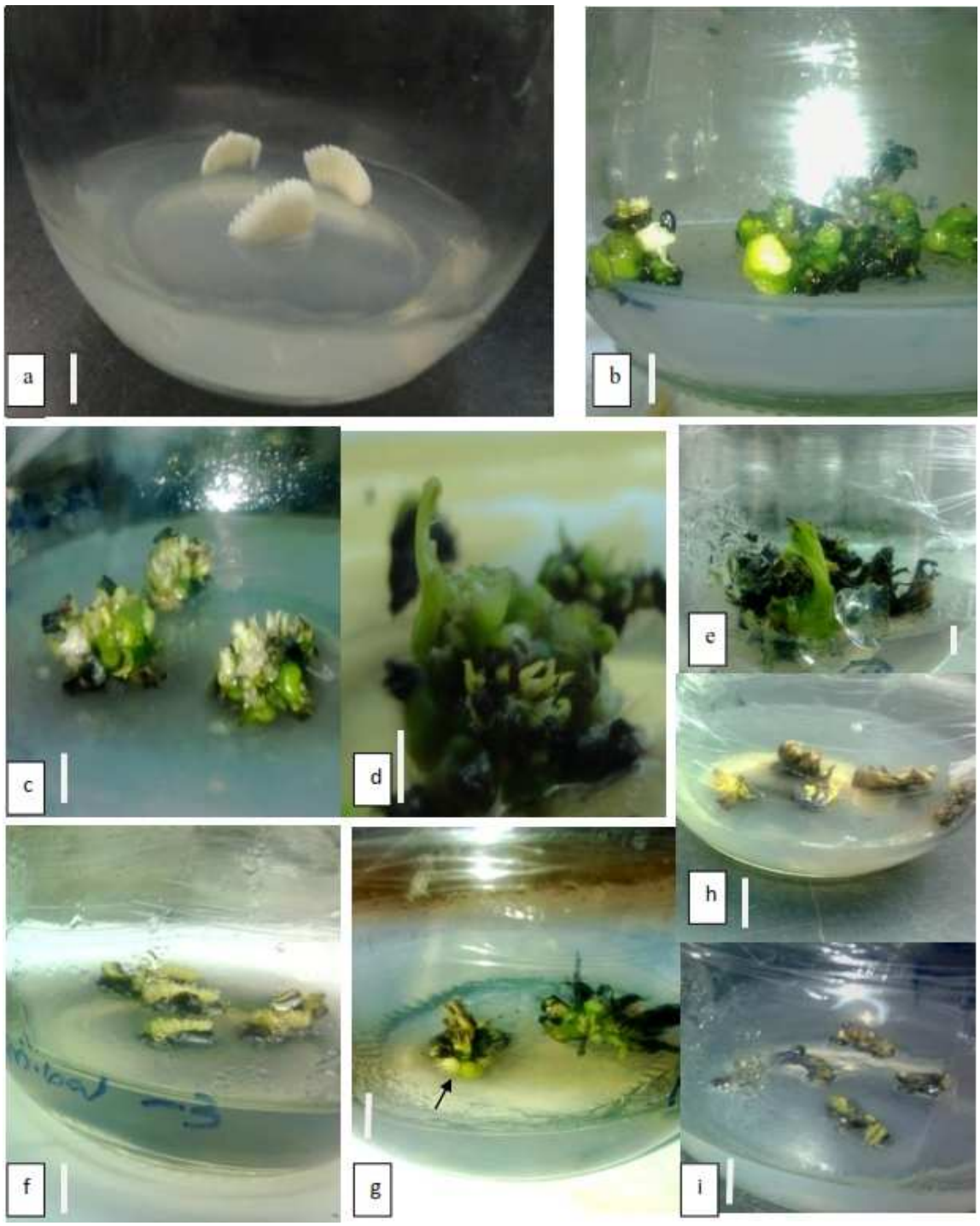

Figure 1. Development stages of male flower culture of cv."Ambun" (b-e) in MS medium supplemented with 10 mg L^{-1} BA. (a) Initial explant, (b) Stage I- swelling of male flower hand and turned in to green colour after 2-3 weeks, (c) Stage II- white colour bodies appear after month, (d) Stage III- tiny shoots appear from white colour bodies after three to four months, (e) Stage IV-formation of shoot after four months, *M. balbisiana* (f-i) (f) Stage I- explants remained white or light green after 2-3 weeks, (g) Stage II- explants produced white colour bodies (WCB) after 3 weeks arrow is pointing 'WCB', (h) and (i) explants became brown after a month and end of second month explants towards to black colour; Bar=5mm

Table 1. Development of the male inflorescence on MS medium with different BA concentrations in culture of cv. " Ambun" and *M. balbisiana* (only responsive male inflorescence were shown)

Size of the explants (mm)	BA concentration (mg L^{-1})	Banana variety	No. of explants cultured	No. of explants at different stages of development			
				Stage I	Stage II	Stage III*	Stage IV*
Large (>10)	0	cv."Ambun"	12				
		M. bal.	12				
	4	cv."Ambun"	12	2^{ab}			
		M. bal.	12				
	6	cv."Ambun"	12	5^{bc}	1^{ab}		
		M. bal.	12	1^{d}			
	8	cv."Ambun"	12	7^{c}	5^{c}		
		M. bal.	12	2^{d}			
	10	cv."Ambun"	12	6^{c}	4^{bc}		
		M. bal.	12	2^{d}			
	12	cv."Ambun"	12	4^{bc}	2^{abc}		
		M. bal.	12	1^{d}			
Medium (5-10)	0	cv."Ambun"	12				
		M. bal.	12				
	4	cv."Ambun"	12				
		M. bal.	12				
	6	cv."Ambun"	12	8^{a}	2^{ab}		
		M. bal.	12	5^{b}			
	8	cv."Ambun"	12	9^{a}	4^{cb}	2^{a}	1^{a}
		M. bal.	12	4^{b}	2^{ef}		
	10	cv."Ambun"	12	10^{a}	7^{c}	1^{a}	2^{a}
		M. bal.	12	6^{b}	2^{ef}		
	12	cv."Ambun"	12	9^{a}	2^{ab}	1^{a}	
		M. bal.	12	6^{b}	3^{f}		
Small (<5)	0	cv."Ambun"	12	1^{a}			
		M. bal.	12				
	4	cv."Ambun"	12	1^{a}			
		M. bal.	12				
	6	cv."Ambun"	12	7^{b}	1^{ab}		
		M. bal.	12	6^{d}			
	8	cv."Ambun"	12	10^{cb}	3^{bc}	1^{a}	
		M. bal.	12	6^{d}	1^{e}		
	10	cv."Ambun"	12	11^{b}	6^{d}	2^{a}	
		M. bal.	12	6^{d}	2^{e}		
	12	cv."Ambun"	12	10^{cb}	4^{bd}		
		M. bal.	12	5^{d}	4^{f}		

Values with same superscript did not show significant difference (P≥0.05)

*In "Ambun" Stage I -Explants swelled and turned green after 2-3 weeks. Stage II-Explants produced white colour bodies after one month. Stage III-Tiny shoots appears from white colour bodies after three to four months. Stage IV- Formation of shoot after four months (*Stage III and IV were seen only in "Ambun") In M. balbisiana Stage I -Explants remained white or turned light green after 2-3 weeks. Stage II-Explants produced white colour bodies after 3 weeks.*

Micropropagation of *M. balbisiana* using immature male flowers

Out of a total of 216 male flower- hands that were cultured on MS media only 50 explants (nearly 23%) produced an in vitro response within the first month. The remaining explants turned brown and were unresponsive. All explants were become black during the second month.

In the responsive cultures, two different stages of growth and regeneration could be identified in first month (fig. 1 f-g, Table 1). Further development had not identified after stage II and cultures failed to grow beyond stage II (fig. h-i). The characteristic features of each stage were on follows; Stage I -Explants remained white or turned light green after 2-3 weeks. Stage II-Explants produced white colour bodies after 3 weeks.

The number of responsive explants in different size classes (*i.e*- small, medium and large) (Table 2) that grew on MS medium supplemented with different BA concentrations (Table 1) and their stage of in vitro growth and regeneration, recovered at end of the experiment.

Table 2. Development of the male inflorescence on MS medium with different sizes of explants in culture of cv. " Ambun" and *M. balbisiana* (only responsive male inflorescence were shown)

Size of the explants (mm)	BA concentration (mg L^{-1})	Banana variety	No. of explants cultured	No. of explants at different stages of development			
				Stage I	Stage II	Stage III*	Stage IV*
Large (>10)	0	cv."Ambun"	12				
		M. bal.	12				
Medium (5-10)	0	cv."Ambun"	12				
		M. bal.	12				
Small (<5)	0	cv."Ambun"	12	1^{a}			
		M. bal.	12				
Large (>10)	4	cv."Ambun"	12	2^{a}			
		M. bal.	12				
Medium (5-10)	4	cv."Ambun"	12				
		M. bal.	12				
Small (<5)	4	cv."Ambun"	12	1^{a}			
		M. bal.	12				
Large (>10)	6	cv."Ambun"	12	5^{a}	1^{a}		
		M. bal.	12	1^{b}			
Medium (5-10)	6	cv."Ambun"	12	8^{a}	2^{a}		
		M. bal.	12	5^{c}			
Small (<5)	6	cv."Ambun"	12	7^{a}	1^{a}		
		M. bal.	12	6^{c}			
Large (>10)	8	cv."Ambun"	12	7^{a}	5^{a}		
		M. bal.	12	2^{b}			
Medium (5-10)	8	cv."Ambun"	12	9^{a}	4^{a}	2^{a}	1^{a}
		M. bal.	12	$4b^{c}$	2^{b}		
Small (<5)	8	cv."Ambun"	12	10^{a}	3^{a}	1^{a}	
		M. bal.	12	6^{c}	1^{b}		
Large (>10)	10	cv."Ambun"	12	6^{a}	4^{a}		
		M. bal.	12	2^{c}			
Medium (5-10)	10	cv."Ambun"	12	10^{b}	7^{a}	1^{a}	2^{a}
		M. bal.	12	6^{d}	2^{b}		
Small (<5)	10	cv."Ambun"	12	11^{b}	6^{a}	2^{a}	
		M. bal.	12	6^{d}	2^{b}		
Large (>10)	12	cv."Ambun"	12	4^{a}	2^{a}		
		M. bal.	12	1^{c}			
Medium (5-10)	12	cv."Ambun"	12	9^{b}	2^{a}	1^{a}	
		M. bal.	12	6^{d}	3^{b}		
Small (<5)	12	cv."Ambun"	12	10^{b}	4^{a}		
		M. bal.	12	5^{d}	4^{b}		

Values with same superscript did not show significant difference (P≥0.05)

DISCUSSION

Results show that BA is necessary for male flower regeneration. Darvari et al. [3] found that explants expand and they became green around 10 to 15 days in *Musa* AAA, AAB and ABB. Wirakarnain et al. [4] research indicate, after being cultured for 10 days in 10 mM of BA,1 mM IAA medium the explants started to swell up and turned in to green colour in *M. acuminta* cv."Pisang Mas'(AA). In the present experiment 0 mg L^{-1} BA concentration did not induce the development of male flower hands. In the absence of BA, 99% of the explants became brown colour within a month. Concentration of 4 mg L^{-1} BA also did not induce male flowers and 98% of male flower hands became brown colour within a month. Comparable results have been reported for *Musa* AAA cultivated variety, Cavendish inflorescences which had not shown any organogenesis response or proliferation in MS basal medium lacking any cytokinins [5]. The most number of male flower hands became swollen and turned to green in concentrations of 8 and 10 mg L^{-1} BA in the medium. Explants turning brown colour became less with the increase in the concentration of BA up to 10 mg L^{-1}. Browning becomes increasing due to high BA concentration at 12 mg L^{-1}. Wirakarnain et al. [4] found that, when the BA concentration was increased to more than 100 mM the explants did not show any response and instead were covered by a thick black layer. Rashid et al. [6] found out that prolific multiple shoot formation and elongation were obtained from the suckers and male flower buds cultured on MS basal medium with 0.014 mg L^{-1} BAP. Luxuriant proliferation and high frequency induction (97.0%) of callus was noticed from the accessory floral part of the explant at 7.0 mg L^{-1} 2, 4-D and 1.0 mg L^{-1} BAP, later it preceded towards the gynoecium[7]. They also revealed 2,4 –D can be used as hormone for callus induction from floral explants. The better response (20%) was recorded in MS medium for callus formation, containing 2.0 2, 4-D + 0.5 NAA + 0.5

IAA (Indole-3- acetic acid) mg L^{-1} recorded by Sultan et al. [8] in *Musa* male flower culture. The MS media supplemented with BAP showed that the number of bud formation in shoot cultures of *M. acuminata* cv. Berangan during the initiation stage increased proportionately with the concentrations used as 11, 22 and 33 µM [9]. Thus, BA had ability to bulk shoot formation from the explants. Mahadev et al.[10] tested MS medium supplemented with 5 mg L^{-1} BAP and coconut water (15%) and reported the most efficient media for shoot initiation and multiple shoot formation.

After 4 weeks, white colour bodies (WCBs) were produced on the explant. Darvari et al. [3] reported that the highest number of cauliflower-like bodies (CLBs) found on second month in some Malaysian banana and plantain cultivars using male flowers. During the second month concentration of 10 mg L^{-1} BA show the higher amount explant that produced WCBs than the other concentrations in the present experiment. Darvari et al. [3] found that after 60 days, the MS medium, which was supplemented with Thidiazuron (TDZ) and BA, produced average of 4.5 and 3.9 cauliflower-like bodies (CLBs) clusters in *Musa* cv. AAA, AAB and ABB. In the results of [3] MS medium supplemented with 8 mg L^{-1} of BA induced the highest 'CLBs' cluster in all the cultivars. The number of 'CLBs' cluster significantly varied with different concentrations of BA. Same results can be identified in [11], culture after 2 weeks, whitish bud-like structures (WBLS) were obtained and MS media supplemented with 70.0 µM BAP was observed as the best media for the growth of WBLS in male inflorescence derived plant *M. acuminata* cv. Berangan. In the present experiment 6 mg L^{-1} of BA concentration produced less number of 'WCBs', none was produced at 0, 4 mg L^{-1} of BA. Similar results were obtained by Darvari et al. [3] that 'CLBs' clusters produced were less in quantity at BA concentration lower than 6 mg L^{-1}. Considering the size of the explants, higher number of medium size flower hands that produced 'WCBs' compared to other two sizes. Similar result had been found in [3] maximum number of 'CLBs' produced medium size (20mm) male flower hand in *Musa* cv. AAA,AAB and ABB. Within the third month initiate the conversion of 'WCBs' to tiny shoot like structures. It can be found first in 8 mg L^{-1} followed by 10 and 12 mg L^{-1} BA. It not happened in 6 mg L^{-1} BA. Similar result found in [3] that showed, after second subculture (in third month) the 'CLB' clusters converted to shoots, that also found average 9.5 shoots per explant at 8 mg L^{-1} BA concentration in *Musa* cv. AAA, AAB and ABB. Comparable result found in [11], after 3 months, shoot-like structures emerged and MS media supplemented with 31.0 µM BAP gave a large number of shoot formation. When considering both development stages I and II, 'WCB' formation was favored at a higher concentration (10 mg L^{-1}) of BA, and shoot initiation was favored at a lower concentration (8 mg L^{-1}) of BA. Similar result was discussed in [11], whitish bud like structures (WBLS) were obtained and MS media supplemented with 70.0 µM BAP was observed as the best media for the growth of WBLS and shoot like structures emerged on MS media supplemented with 31.0 µM BAP gave a large number of shoot formation. Considering size of the explants, higher number of medium size flower hands that produced tiny shoot like structure compared to other two sizes. It can be say that survival rate of the large male flower hand that became less than the other two sizes. After 4 to 6 months tiny shoot like structure converted to little shoots. Shoots were first formed in 10 mg L^{-1} BA concentration and the medium size male flower hands. After six month shoots can be clearly identified. Explants which supplement with 0 and 4 mg L^{-1} concentrations of BA did not induce and explants turned brown colour within a week. BA concentration of 6 to 12 mg L^{-1} were induced the explants in different manner in different concentration. According to the statistical analyzed explant turn in to light green may significantly different with the concentration of BA ($p<0.05$). A few explants (5%) produced 'WCBs'. Higher number of 'WCB' produced explants was shown in 12 mg L^{-1} BA concentration. Medium and small size explants having more survival rate than the large size explants. Large size explants were turned in to brown within a week. At least one explant of large size that did not produced 'WCB". When it comes to second month all explants were became black colour. This may happen due to concentration of phenolic compound and the toxic substance, released by the explants. After stage II explants did not show further development.

Proliferation of the explants may depend with the genotype of *Musa*. Comparable result found in [3] and revealed several cultivated varieties such as 'Berangan'(AAA), 'Rastali'(AAB), 'Nangka'(AAB), 'Abu'(ABB) which having several genome types in *Musa*. Among them 'Abu' was shown less number of 'CBLs' formation and shoot formation. Regarding its genotype, triploid genome contain with double BB (*balbisiana*) genome, which were containing bulk part of BB genome cause to less proliferation of explant. The diploid cultivar (Sanna- chenkadali, AA) induced a maximum number of multiple shoots in 8.9 l M BA whereas the triploid cultivar (Red banana, AAA) exhibited maximum multiplication in 22.2 lM BA [12]. In the present experiment *M. balbisiana* explants, only had BB genome. Thus *M. balbisiana* did not potent to developed shoots from the explant. Random amplified polymorphic DNA (RAPD) was carried out to determine the clonal fidelity on in vitro *M. acuminata* cv. Berangan by Harirah & Khalid [11] and reveled the micropropagated from male inflorescence derived from the same mother

plant. Although Plants regenerated through somatic embryogenesis showed minor variation when assessed by randomly amplified polymorphic DNA (RAPD) and sequence characterized amplified regions (SCAR) markers revealed by Meenakshi et al. [13] . Thus, direct regeneration through male inflorescence having less or minimum capability of variations happened in progeny.

CONCLUSION

Considering the results micropropagation of *M. balbisiana* and cultivated variety "Ambun" using immature male flowers, "Ambun" was more success than *M. balbisiana.* Thus it may be say proliferation of male flower of banana related with *M. acumnata* (AA) genome type rather than the *M. balbisiana* (BB) genome type. Also conclude that, small and medium size flower hand more responsive than the large size male flower hand in both "Ambun" and *M. balbisiana*. Proliferation of male flower hand was induced only when BA was in the medium. More effective concentration of BA was 8 and 10 mg L^{-1} for "Ambun". In *M. balbisiana* it was 10 and 12 mg L^{-1}. Less than 6 mg L^{-1} BA concentration was not initiating the proliferation in both "Ambun" and *M. balbisiana*. More than 10 mg L^{-1} of BA concentration cause for blacking of male flower hand and decreased the proliferation rate in "Ambun".

For cultivated variety "Ambun" described protocol can be developed to use as direct regeneration from male inflorescence is a rapid and simple method for clonal and mass propagation. Harirah and Khalid [11] analysis plants that producing this technique and has proven these regenerated plants were clonal in nature. This method is cost effective and has an advantage of the absence of latent contamination occasionally faced by meristem cultures [14]. Thus, it can be used to develop as commercial scale. In *M. balbisiana* this protocol was not success to regenerate plants, only a few development stages were initiated in less quantity. Then hands were become black and stop the further development. It must be happened due to phenolic and toxic substances. Thus, some alternative procedures can be tested. One can be suggested that alternative cytokinins must be tested instead of BA. Comparable results can be identified in [3] test for several cultivated varieties such as 'Berangan'(AAA), 'Rastali'(AAB), 'Nangka'(AAB), 'Abu'(ABB) which having several genome types in *Musa*. Among them 'Abu' was shown high number of 'CBLs' formation and shoot formation, Thidiazuron contain as cytokinin in the medium. Thus Thidiazuron can be tested micropropagation of *M. balbisiana* male flower hands. Liquid medium or semisolid medium can be tested instead of solidified medium. Silayoi [15] tested medium solidification effect of the proliferation of male flower hand. Culturing of the explants in liquid medium being shaken enabled the entire tissue to obtain nutrient resulting in fast growth as well as reduced the concentration of phenolic compound, the toxic substance, released by the explants. In addition, shaking of liquid medium also provided more aeration, thus increasing O_2 and in turn enhanced metabolism of the explants of immature male flower hand. According to the results of [15], inflorescences cultured on liquid medium, supplemented with 5ppm BAP produced the first shoot at 52nd day, and also inflorescences, on semisolid medium supplemented with 5ppm BAP were found to have shoot at 130th day. Thus, in liquid medium shoot proliferation were faster than semi solid medium. Thus it can be tested for micropropagation of *M. balbisiana* male flower hands. In this research sub culturing was done in every month in both "Ambun" and *M. balbisiana.* Frequent sub culturing can reduce the accumulation of penolic and toxic substance. Thus frequent sub culturing can be tested for present experiment to reduce the browning of explants. These suggestions can be tested for microprpagation of *M. balbisiana* and "Ambun" using immature male flower hands to overcome the barrier that was found in present experiment.

In "Ambun" Stage I -Explants swelled and turned green after 2-3 weeks. Stage II-Explants produced white colour bodies after one month. Stage III-Tiny shoots appears from white colour bodies after three to four months. Stage IV-Formation of shoot after four months (*Stage III and IV were seen only in "Ambun") In M. balbisiana Stage I -Explants remained white or turned light green after 2-3 weeks. Stage II-Explants produced white colour bodies after 3 weeks.

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