Research Paper

Development of Plant Regeneration Protocol through Male Bud Culture of *Kolikuttu* Banana (*Musa* spp.) Var. Agra

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Abstract

Developing efficient direct plant regeneration protocols using different explants are specially valuable for multiplying *Kolikuttu* banana (AAB) var. Agra which is known to be less responsive for micropropagation. In the present study, an alternative protocol was devised to regenerate true-to-type plantlets using immature hands of the male bud as explants. Two different hand sizes of male buds were cultured on 4 different solidified MS media combinations with 2 levels of 6-Benzylaminopurine (BAP), supplemented with and without coconut water. The results revealed that the number of responded hands significantly higher (65.5%) with <10 mm hand size than 10-20 mm size (48.5%) and cultured in the medium with 8 mg L^{-1} BAP without coconut water at the culture initiation step. Shoot bud formation was initiated in the 4th subculture cycle in the medium with 2 mg L^{-1} BAP, irrespective of the size of the hands. In the *in-vitro* rooting study, shoots that are longer than 5 mm were well rooted in the medium without rooting hormones, followed by the medium with 0.5 mg L^{-1} Indole-3-acetic acid (IAA). No significant variations were observed in the growth and yield parameters of regenerated plants during field evaluation. Fruit quality characteristics, including pH, ^{o}Brix, titratable acidity and flesh color were found to be comparable to control plants. These findings suggest that this technique can serve as a viable alternative for propagation, particularly in situations where an adequate supply of mother stock for shoot tip culture is not available.

Keywords: Immature hands, *In-vitro* propagation, Male bud, Silk banana

Introduction

The most extensively grown fruit crop, spanning 42715 ha in 2022 (AgStat, 2022), is the banana, which is also the most consumed fruit in Sri Lanka. Out of all cultivated banana, *Kolikuttu*, has high demand and fetches high price in the local market. Producing clean planting material of *Kolikuttu* bananas by *in-vitro* procedures is essential because the widely distributed soil-borne disease *Fusarium oxysporum f.sp. cubense*, which causes Fusarium wilt, poses a serious threat to *Kolikuttu* cultivations. Finding Fusarium wilt-free cultivations to collect sward suckers to start shoot tip culture may be also difficult in such circumstances.

Variety 'Agra' which has superior fruit quality characteristics is the recommended *Kolikuttu* (AAB) by the Department of Agriculture, Sri Lanka (DOA). The banana is probably the crop that is used in micropropagation the most, yet even within the silk banana subgroup, this specific variety multiplies poorly (Hirimburegama & Gamage, 1996; Department of Agriculture, 2012). Consequently, a number of researches have been conducted within the DOA in an effort to speed up the multiplication. In order to multiply about 1000 *in-vitro* shoots in six subculture cycles, an effective propagation technique using shoot tip culture was recently established (Shirani *et al.*, 2021). In order to maintain tissue-culture plant output, it would be beneficial to investigate alternative methods of producing clean planting materials in addition to developing efficient techniques for plant multiplication through shoot tip culture.

Banana can be *in-vitro* regenerated through direct and indirect methods via organogenesis and somatic embryogenesis using either shoot tip or male bud cultures. The meristems in the inflorescence apices of the male bud could be induced to form shoots (Krikorian *et al.*, 1993). This principle is applied to regenerate plants from male buds, male flowers or immature hands. Male bud culture is used to regenerate plants directly (Mahadev *et al.*, 2011) or, indirectly through callus phase (Sultan *et al.*, 2011) or through embryogenic cell suspension culture (Strosse *et al.*, 2003). The advantages of the male bud culture technique over the shoot tip culture are the low contamination percentage and have no or less economic value compared to the suckers. They offer an opportunity to select mother plants with desirable characteristics like a greater number of hands and fruits per bunch (Resmi & Nair 2007). This technique is particularly useful in situations where there is an insufficient supply of suckers for shoot tip culture. Direct regeneration protocols provide viable alternatives to shoot tip culture, and the absence of an unorganized callus phase during the *in-vitro* culture of male buds reduces the risk of somaclonal variations. There are evidences supporting the production of genetically identical plants through male bud culture (Harirah & Khalid, 2006; Sivakumar & Visalakshi, 2021). Furthermore, the potential for minimizing genetic variations can be enhanced by reducing cytokinin concentrations and limiting the number of

subculture cycles. Therefore, this study was conducted to develop a plant regeneration protocol using male flowers of the *Kolikuttu* banana variety Agra and to evaluate the regenerated plants for selected agronomic and fruit quality parameters to make certain the true-to-typeness.

Materials and Methods

Eight to ten weeks old male buds from healthy plants of *Kolikuttu* variety Agra were collected from the research field of Grain Legume and Oil crops Research and Development Centre (GLORDC), Angunakolapelessa and the size was reduced to 4-6 cm before surface sterilization by removing several bracts. The male buds were surface sterilized by immersing in 70% ethyl alcohol for 5 minutes. Two sizes of flower bunches (hands) (<10 mm and 10-20 mm) of male buds were carefully dissected and cultured *in-vitro* in four different combinations of MS (Murashige & Skoog, 1962) media as shown in Table 1.

Sucrose at the concentration of 30 g L^{-1} was used for the media and 3 g L^{-1} of gelrite® was added for solidification. Subculturing was done once in 4-5 week intervals up to the third subculture. To minimize the possible somaclonal variations, a high concentration of BAP was used only for the induction of bud proliferation. The number of subculture cycles was also limited to 5 cycles to minimize somaclonal variations. After induction of buds, the BAP levels were reduced to 2 mg L^{-1} (M_5: MS+2 mg L^{-1} BAP+30 g L^{-1} sugar) in the 4th subculture and 1 mg L^{-1} (M_6 medium) thereafter. The addition of coconut water to the medium can increase the cytokinins (Lazim *et al.*, 2015) and may be favourable for occurring variations. Therefore, coconut water was omitted from the 4th subculture cycle.

The experiment was laid as CRD with two factor factorial (Four media combinations and two hand sizes) and there were 25 replicates with two explants per replicate. The individual shoots formed in the presence of 1 mg L^{-1} BAP at the 5th subculture cycle were transferred to three different media for *in-vitro* root induction after categorizing them into three sizes (>5 mm, 3-5 mm and <3 mm). The three media tested for *in-vitro* root induction were M_0 (Hormone free MS medium), M_7 (MS+1 mg L^{-1} BAP+0.5 mg L^{-1} IAA and M_8 (MS+0.5 mg L^{-1} IAA). All the cultures were maintained at 25±1 ^oC temperature providing 16/8 D/N photoperiod and 2,500 lux light intensity in the culture room.

Table 1. Different MS media combinations in culture establishment and multiplication

Treatment	BAP (Sigma) (mg L^{-1})	NAA (Sigma) (mg L^{-1})	Ascorbic acid (Park Scientific) (mg L^{-1})	Coconut water (mL^{-1})
M_1	5	1	1	150
M_2	8	1	1	150
M_3	5	1	1	-
M_4	8	1	1	-

Data were collected on the number of responded hands and from the 3rd subculture onwards the number of hands that formed buds and in each vessel of the treatment. The number of rooted plantlets, number of multiplying explants, number of roots, shoot height and weight of plantlets were measured at the time of transferring for *ex-vitro* acclimatization. The rooted plantlets were transferred to coir pellets and then repotted in poly bags containing coir dust: sand 3:1 mixture.

Field valuation of regenerated plants

The regenerated plants from all 4 media (irrespective of the size of hands) were evaluated in the field along with control Agra for morphological variations and bunch yield during 2020 *Yala* and 2020/21 *Maha* seasons. The data on leaf characteristics were collected using the 3rd leaf from the base at flowering stage.

Fruit quality analysis

The fruit quality parameters; pH, total soluble solids, titratable acidity, and flesh colour, were assessed using samples from the fourth hand of three bunches from each treatment. These assessments were conducted at the food technology division of GLORDC, Angunakoapelessa, Sri Lanka. All experimental data were analyzed using the SAS 9.1 version. For mean separation, Duncan's Multiple Range Test (DMRT) or the Least Significant Difference (LSD) test was applied where appropriate.

Results and Discussion

The cultures of immature hands were successfully initiated without any contamination. The cultured hands exhibited swelling, and the bases of the hands turned into green colour 2-3 weeks after culture initiation. Table 2 presents the percentage of swollen explants in each medium, which were considered as responsive hands. The number of responded hands was significantly varied with different media and hand size (Table 3) independently.

Table 2. Percentage of responded hands in 4 different media 3 weeks after culture initiation

Medium	Responded hands (%)
M_1	51^{c}
M_2	43^{d}
M_3	61^{b}
M_4	73^{a}
CV% = 8.31	

**Means with different superscripts are significantly different at p=0.05 (DMRT)*

Table 3. Percentage of responded hands in 2 different sizes of explants 3 weeks after culture initiation

Size of hand	Responded hands (%)
<10 mm	65.5^{a}
10-20 mm	48.5^{b}
CV%=8.31	

**Means with different superscripts are significantly different at p=0.05 (DMRT)*

The position of immature hands is a critical factor influencing plant regeneration from male buds or explants derived from them. Strosse *et al.* (2003) recommended the use of male flowers located between positions 16 and 8 (with position 1 being the closest to the meristematic dome) to effectively induce embryogenic callus formation.

A higher percentage of responded hands would be favourable on increasing the number of regenerated shoots at the end of the multiplication phase. In this study, smaller hand size showed a better response than larger ones, supporting the findings of Strosse *et al.* (2003). The subsequent visual change in the immature hands was the formation of white-coloured bodies (WCBs), which later turned green and developed into shoot like structures within the first three subculture cycles. However, upon transferring to the next medium containing 2 mg L^{-1} of benzylaminopurine (BAP), these shoot-like structures did not develop into normal shoots. Instead, new buds were initiated and began to proliferate.

In the 4th subculture cycle, *i.e.* after 4-5 months from the culture initiation, normal shoot buds were formed in the explants. Statistical analysis showed that only the medium was significant. Though these buds were formed in the medium with a reduced BAP level (2 mg L^{-1}), the influential effect of BAP in the previous subculture cycles could still be seen. Therefore, the results are shown in Table 4 referring to the previous four media.

The influential effect of BAP and coconut water could be observed in the percentage of bud-producing hands (Table 4).

Not like the previous parameters, a high concentration of BAP along with coconut water resulted in the highest percentage (49.8%) of hands which formed the shoot buds. The necessity of higher levels of BAP has also been highlighted by Prabhath and De Silva (2014) on the male bud culture of *Amban* and *Musa bulbisiana*. However, the addition of coconut water with 5 mg L^{-1} BAP (M_1) did not show a significant difference when compared with the M_4 medium which is quite unusual and cannot be supported by the available literature. As reported by Mahadev *et al.* (2011) 5 mg L^{-1} BAP and 15% coconut water was the best medium for shoot initiation and multiplication of 2 varieties belonging to the AAB genomic group. The results obtained in this experiment might be due to the varietal difference and the variation in the male buds collected from different plants. *In-vitro* responses and multiplication can be varied with the mother plant even in shoot tip culture (Singh *et al.*, 2011; Shirani *et al.*, 2021). During the 4th subculture, the development of highly proliferating meristems which can be termed Cauliflower-like meristems (CLMs) was noticed in some of the hands. There were some hands with both shoot producing buds and proliferating meristems. In the fifth subculture cycle, vigorous multiplication of buds could be clearly observed but upon transferring the CLMs to the M_6 medium, the proliferating buds were unable to grow as normal shoots. A few more subcultures into M_6 medium may be required to develop shoots from the CLMs.

Table 4. Formation of shoot buds in the 4th subculture cycle as influenced by the medium

Treatment	Mean % of bud-producing hands
M_1: MS+5 mg L^{-1} BAP+150 mll^{-1} CW	37.50^b
M_2: MS+8 mg L^{-1} BAP+150 mll^{-1} CW	49.80^a
M_3: MS+5 mg L^{-1}BAP	30.68^c
M_4: MS+8 mg L^{-1} BAP	38.39^b
CV% = 15.4	

**Means with different superscripts are significantly different at p=0.05 (DMRT)*

At the end of the 4th subculture, the number of shoot buds per hand was counted and transferred to a medium with 1 mg L^{-1} BAP (M_6). Table 5 shows significant difference in the rate of multiplication and smaller hands produced more number of shoot buds per hand and this result agreed with the findings of Stross *et al.* (2003).

Another structural change in the cultured hands was the formation of flower-like structures from the 2nd subculture cycle and continued up to the end of multiplication in all the treatment combinations (data not shown). While subculturing, those parts were dissected and discarded.

Table 5. Number of shoot buds multiplied per hand at 5th subculture cycle

Treatment	Mean rate of multiplication per hand
10-20 mm hands	3.18^a
<10 mm hands	2.57^b
CV% = 19.6	

**Means with a different superscript are significantly different at p=0.05 (DMRT)*

Shoots with varying sizes resulted at the end of the 5th subculture. Therefore, those shoots and buds were categorized into three groups according to height (large:>0.5 mm, medium: 3-5 mm and small:<3 mm) and cultured into 3 media to achieve both growth of the shoots and roots.

Figure 1 shows the percentages of rooted plantlets and multiplying explants on M_0 (a), M_7 (b) and M_8 (c) media, respectively. In hormone-free MS medium (M_0) 92% of the largest buds converted into rooted plantlets whereas 8% of the buds tend to multiply. The Figure clearly shows that the size of the buds is a determining factor in the formation of rooted plantlets. Smaller-sized shoots tend to proliferate even in the absence of BAP in the medium. Very small shoots were hardly rooted in the M_0 medium.

However, in the M_7 medium with BAP and IAA, only 35% of the large-sized shoots produced rooted plantlets while the other two-third was proliferated in to shoots. Medium sized shoot buds also tend to multiply in the presence of BAP while the multiplication of the smallest buds remained less than 5%.

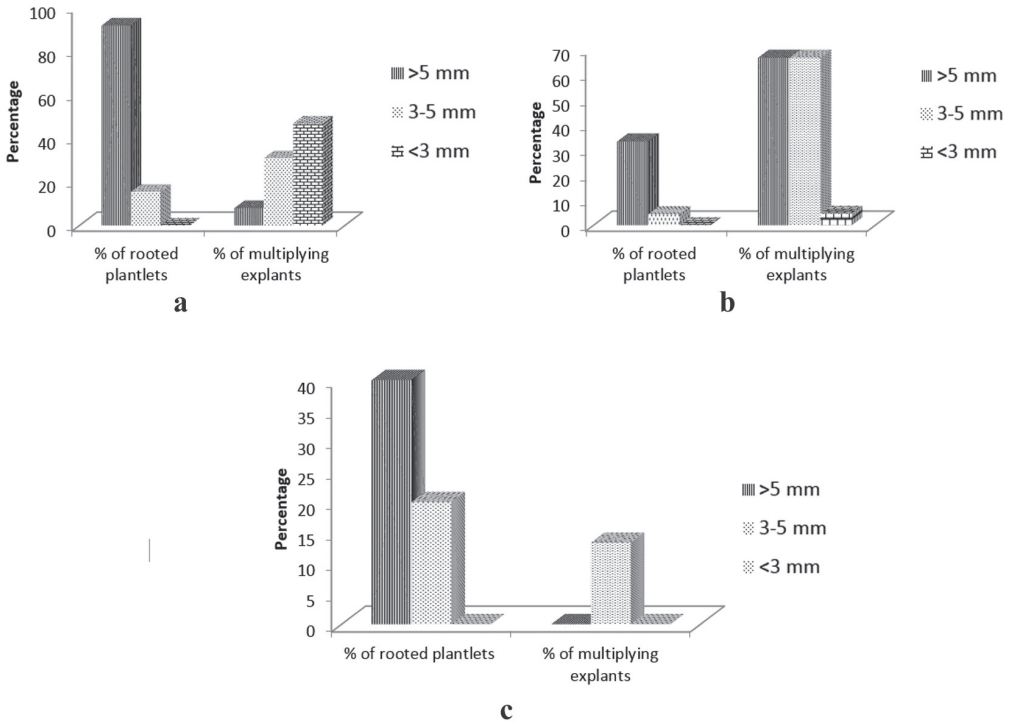

Figure 1. Effect of media on percentages of rootedplantlets and multiplying explants (a). M_0 (MS+ hormone free medium), (b) M_7 (MS+1 mg L^{-1} BAP+0.5 IAA mg L^{-1}) and (c) M^8 (MS+0.5 IAA mg L^{-1})

IAA was included in this medium along with BAP to grow the shoots bigger as found by Qamar *et al.* (2015) and Ngomuo *et al.* (2013). However, it seemed that very small shoot buds do not respond to multiplication and rooting in the present study. They may require a growing medium or shoot elongation medium before transferring into rooting.

IAA favours inducing roots of many species both *in-vitro* (Iqbal *et al.*, 2013) and *in-vivo* (Sevik & Guny, 2013). However, in the presence of IAA at 0.5 mg L^{-1}, only 40% of the largest shoots were rooted which is quite unusual and difficult to explain. The medium-sized shoots were rooted at a more or less similar percentage as with the M_0 medium. The smallest buds were neither rooted nor multiplied in the medium containing IAA at 0.5 mg L^{-1}. Plant growth in M_0, M_7 and M_8 was measured through shoot height and weight of the plantlet at the time of transferring plantlets (deflasking) for hardening.

Variations in the mean number of roots, mean height of shoot and mean weight of plantlets grown in M_0, M_7 and M_8 media are shown in Figure 2. Large-size (Figure 2a) and medium-size (Figure 2b) shoot buds behave in a more or less similar manner in different media. Medium without added hormones (M_0) and M_8 medium which contained 0.5 mg L^{-1} IAA were equally performed on the measured parameters.

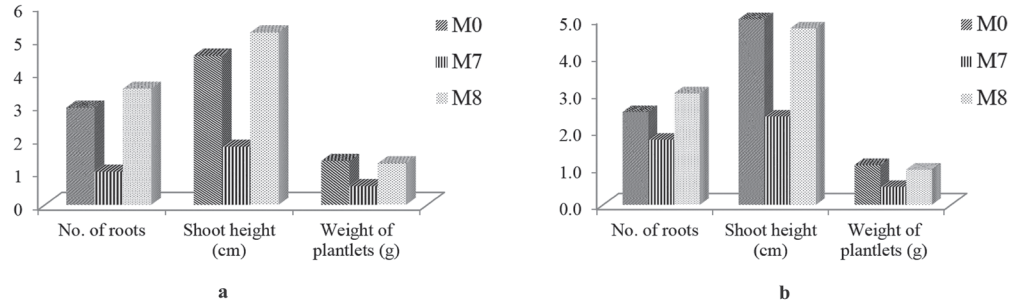

Figure 2. Growth parameters of plantlets in 3 different rooting media at the time of transferring for acclimatization a. Shoot buds longer than 5 mm, b. Shoot buds 3-5 mm long

This study suggests that shoots longer than 5 mm are better for *in-vitro* rooting and no added hormones are required for root induction. The small shoot buds may require a shoot elongation medium before the root induction or they must be discarded. The bigger shoots required no rooting hormones as found in similar studies (Mahadev *et al.*, 2011) and they rooted well in medium with no hormones. Plantlets hardened in coir pellets showed 100% survival.

Evaluation of tissue-cultured plants regenerated from the male buds

The morphological parameters observed at flowering and yield parameters of plants grouped according to the media used in the tissue culture showed non-significant variations (Tables 6 and 7). Though the regenerated plants were at same age at the time of planting, variations in days to flowering among the plants irrespective of the treatments were noticed. These minor variations could be attributed to the differences in the soil conditions rather than the variations at the genetic level. Harirah and Khalid (2006) have also found no variations at a detectable level among the plants produced from the floral meristems.

The quality attributes of ripened banana fingers, as presented in Table 8, exhibited minor variations that were statistically non-significant. These variations may be attributed to the environmental factors, such as weather conditions, and the maturity stage at the time of harvesting. As indicated by Kheng *et al.* (2011), the eating qualities of bananas, particularly sweetness reflected by the ^{o}Brix value and the sugar acid ratio are influenced by the harvesting age. Their study demonstrated that 'Rasthali' (AAB) bananas harvested 12 weeks after the opening of the first hand had a higher sweetness level compared to those harvested at 11 weeks.

Table 6. Morphological parameters of male bud-derived tissue cultured plants at flowering grown at Agriculture Research Station, Weerawila

Treatment	Leaf length (cm)	Leaf breadth (cm)	Petiole length (cm)	Pseudostem	
				Girth (cm)	Height (cm)
M_1	2.21	51.0	58.7	64.5	267.6
M_2	2.04	46.5	59.0	62.3	258.3
M_3	2.24	53.6	59.0	62.6	257.6
M_4	2.18	62.5	60.5	65.2	264.0
Control	2.26	52.5	61.2	63.0	287.0
CV%	11.0	20.5	5.9	7.2	7.47
	ns	ns	ns	ns	ns

Note: ns – Not significant at p =0.05 (LSD test)

Table 7. Yield parameters (mean values) of male bud-derived tissue cultured plants

Medium	Days to Flower	Days to harvest	Bunch weight (kg)	No. of hands	Total No. of fingers	Weight of rachis (kg)
M_1	335	428.4	14.98	6.4	76.3	1.96
M_2	312	415.0	10.87	7.0	70.5	1.41
M_3	327	423.3	11.05	6.3	80.4	1.44
M_4	307	415.6	12.32	7.5	94.3	1.91
Control	313	398.2	12.31	6.9	82.6	1.76
CV%	23.4	21.5	31.2	18.9	22.3	12.3
	ns	ns	ns	ns	ns	ns

Table 8. Fruit quality parameters (mean values) of male bud-derived tissue cultured plants

Sample	pH	°Brix	Titratable Acidity	Sugar/ acid ratio	Chroma	Hue	Lightness
M_1	3.47	26.57	0.57	47.07	25.38	81.61	75.02
M_2	3.59	23.83	0.61	38.95	24.16	82.76	75.56
M_3	3.56	24.14	0.61	39.93	24.31	82.46	75.83
M_4	3.76	25.53	0.73	35.13	27.64	82.68	75.82
Control	3.67	23.83	0.56	46.05	30.31	80.42	72.07
CV %	8.9	6.5	12.5	18.3	8.5	5.4	4.7
	ns	ns	ns	ns	ns	ns	ns

Note: ns - Not significant at p=0.05 (LSD test)

Conclusion

A tissue culture media protocol was developed for plant regeneration from the immature hands of the male bud of *Kolikuttu* Var. 'Agra'. The hands which were less than 10 mm size performed better than the 10-20 mm sized hands in tissue culture response at initial stage. The medium with 8 mg L^{-1} BAP plus 150 mL coconut water significantly increased the percentage of bud-producing explants. Shoots exceeding 5 mm in size were successfully rooted in a MS medium without the addition of exogenous rooting hormones. The regenerated plants demonstrated no significant differences in bunch yield and fruit quality characteristics compared to the mother plants. Consequently, male flowers or immature hands of the male bud can be utilized as alternative explants in cases where an adequate supply of suckers is unavailable for micropropagation *via* shoot tip culture. It is recommended that this protocol be applied to male buds collected from a selected mother plant to ascertain the true-to-typeness of the micropropagated plants.

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