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MICROPROPAGATION OF *MUSA ACUMINATA* VAR. *ZEBRINA* (VAN HOUTTE *EX* PLANCH.) NASUTION, AN ORNAMENTAL PLANT FOR THE HORTICULTURE INDUSTRY

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Abstract

Micropropagation protocol for an ornamental banana *Musa acuminata* var. *zebrina* (Van Houtte *ex* Planch.) Nasution. was established by using shoot tip explants. The effect of different concentrations (0, 10, 15, 20 and 30 µM) of BAP with and without 1 µM NAA supplemented to Murashige and Skoog (MS) medium was evaluated for *in vitro* shoot multiplication. The results revealed that 20 µM BAP without NAA as the best supplement to induce multiple shoots. Further, this treatment resulted in 202 multiple shoots after 3 cycles of subculture compared to only two shoots in control. MS medium supplemented with 1.0 µM IBA induced better rooting with the maximum number (10.2 ± 0.2) of elongated roots (5.5 ± 0.3 cm). The well rooted plantlets were successfully acclimatized in the glasshouse condition. Among different potting media, peatmoss : perlite : vermiculite (3 : 2 : 1) showed better plant growth characteristics. About 86% of plantlets survived under field condition.

Introduction

The genus *Musa* includes several evergreen herbaceous perennial plants. They are recognized for food, fiber, and ornamental purposes. Some species of this genus are prominent in horticulture industry as an ornamental plant because of their attractive leaves and the inflorescence (Ploetz *et al.* 2007). One such popular ornamental banana species is *Musa acuminata* var. *zebrina* (Van Houtte *ex* Planch.) Nasution. also known as a red banana tree or variegated blood banana has a huge demand in horticulture industries for its unique form of broad green with burgundy-brown patches on its leaves (http://homeguides.sfgate.com/ plant-zebrina-rojo-64346.html). *M. acuminata var. zebrina* is planted outdoors in the landscape or indoor as a houseplant. It is suitable as garden center piece and tropical concept near water bodies (http://stokestropicals.plants.com/Musa-sumatrana-Rojo-P403.aspx). This species is usually propagated by conventional method using the suckers. This method is easy, but can only provide a small number of planting material at a time and may carry pathogens and diseases to the next generation. In addition, they exhibit slow growth characteristics. Consequently, the well-known micropropagation technique to produce abundant planting materials is important to substitute the conventional method for this species. Moreover, for commercialization, a consistent supply of good planting material is essential. Thus, as practiced in edible banana, establishment of a simple micropropagation protocol using shoot tip explants can be a good option for producing high quality ornamental banana planting materials (Arias 1992, Ahmed *et al.* 2014a).

Micropropagation of the edible banana is well established using meristem and shoot tip cultures, especially for large scale multiplication (Navarro *et al.* 1997, Lee *et al.* 1997, Madhulatha *et al.* 2004). To obtain good quality clones, optimization of culture media and its components are

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very essential. The optimization of *in vitro* culture system using plant growth regulators (PGRs), medium strength and other culture conditions is very necessary to achieve a successful regenerative protocol in banana species (Munguatosha *et al.* 2013, Ahmed *et al.* 2014a). MS (Murashige and Skoog 1962) media is widely used for the micropropagation of *Musa* species by different researchers. The success of micropropagation depends on the use of optimized concentrations of PGRs (Sudipta *et al*. 2011). Precisely, cytokinin and auxin are employed either individually or in combinations in the media for establishing *in vitro* growth and development of the plant cells (Ikram *et al.* 2007, Munguatosha *et al.* 2013). Usually, adenine-based cytokinin, N^6-benzylaminopurine (BAP) is widely used in the micropropagation of *Musa* genus. The optimal concentration of BAP is the main factor that affect multiplication rate in banana, especially to induce multiple shoots. Auxin such as naphatalene acetic acid (NAA) has been reported to promote plant rooting *in vitro* (Hussein 2012). The management of auxin and cytokinin level in the media can positively alter the growth performance of plant cell cultures. The concentration and the combination of cytokinin and auxin can determine the success rate of plant regeneration in micropropagation (North *et al.* 2010, Kumara *et al.* 2010, Kaushik *et al.* 2015).

Furthermore, optimization of acclimatization protocol will further boost the success rate of plant survival and performance after transplanting in the field. Some of the difficulties observed during *in vitro* culture establishment include difficult to remove endophytic microbes during surface sterilization, low shoot regenerative capability, and difficult to root the *in vitro* derived micro shoots. Overall, these factors affect the explant survival and plantlet survival rate during the acclimatization stage (Gutiérrez *et al*. 2011). So far, plant tissue culture approach has been successfully employed in the large-scale propagation of edible banana plants, but there are no reports on tissue culture studies of *M. acuminata* var. *zebrina*. Hence, the aim of this present study is to establish an efficient and rapid *in vitro* plant regeneration protocol for *M. acuminata* var. *zebrina*.

Materials and Methods

The sword suckers of *Musa acuminata* var. *zebrina* (2 - 3 months old) were separated from a healthy mother plant growing in the field at UPM Agriculture farm, Serdang, Selangor. The soil that adhered to the suckers was removed and the roots and leaves were trimmed and brought to the lab for surface sterilization. The suckers were carefully washed under running tap water and soaked in water containing Tween-20 (Hi-Media, India) for 20 min. Following which, the suckers were again thoroughly washed for 40 min under running tap water before being moved inside the laminar air flow (LAF). Inside the LAF, shoot meristems were isolated from the suckers to obtain the size of 4 cm × 3 cm. Then, they were soaked in a beaker containing 95% alcohol for 30 seconds. After that, they were soaked in 10 and 20% (v/v) commercial bleach containing a few drops of Tween-20 and left for 25 min in each concentration. Then, shoot meristems were washed three times by using sterilized distilled water and were subjected to trimming by removing outer parts that were damaged due to the sterilization process. Finally, shoot tip explant of 2 cm × 1 cm was obtained.

MS basal medium comprising 3% (w/v) sucrose and different concentration of BAP (Sigma-Aldrich Co., St. Louis, USA) with or without NAA (Sigma-Aldrich Co., St. Louis, USA) was used. All media were adjusted to pH 5.7 ± 0.1 prior to the addition of 0.8% agar (Algas, Chile) and autoclaved at 121ºC and 15 lbs pressure for 20 min. All culture bottles after inoculation were incubated in a clean growth chamber at 25 ± 1°C under a 16/8 (light/dark) photoperiod with a light intensity of 3000 lux supplied by cool-white fluorescent lamps and relative humidity of over 70%.

The shoot tips were inoculated into the MS medium without PGRs for two days. After that, the shoot tips were taken out from the medium, black parts of the explants were removed, and they were cut into half vertically. Then, the shoot tips were implanted onto the solid MS medium with different PGRs concentration. The experiment consisted of 2 (types of PGRs) × 5 (concentrations of PGRs) treatments, namely (0 µM BAP + 0 µM NAA, 10 µM BAP + 0 µM NAA, 15 µM BAP + 0 µM NAA, 20 µM BAP + 0 µM NAA and 30 µM BAP + 0 µM NAA) and (0 µM BAP + 1 µM NAA, 10 µM BAP + 1 µM NAA, 15 µM BAP + 1 µM NAA, 20 µM BAP + 1 µM NAA and 30 µM BAP + 1 µM NAA). The shoot tips were subcultured every four weeks up to four times. Three replicates were used in the study and each replicate consisted of 5 explants. The data on number of multiple shoots produced and shoot length (cm) were recorded at each subculture cycle.

After fourth subculture, shoot clumps were separated to obtain a single microshoot and inoculated into full and half strength MS medium supplemented with varied levels (0.0, 0.5, 1.0 and 2.0 µM) of indole butyric acid (IBA; Sigma-Aldrich Co., St. Louis, USA) for *in vitro* rooting. The data on the number of roots, the longest and average root length (cm) were recorded using root analyzer. The roots of the plantlets were gently washed by using water to remove the agar from the rooting media.

The *in vitro* rooted plantlets were carefully removed from the culture vessels and the roots were gently washed under running tap water to remove agar attached to the roots. Later, the plantlets were transferred to peatmoss contained glass pots (5 cm × 12 cm) and kept in the culture room by covering with a plastic cover to maintain humidity. After 2 weeks, the plantlets were moved to greenhouse and planted in pots (12 cm × 12 cm) containing different potting media, such as sand : perlite : vermiculite (3 : 2 : 1), peatmoss : perlite : vermiculite (3 : 2 : 1), and sand : perlite (2 : 1) and sprayed with water around the plantlets at every 24 hrs. Foliar spray was applied every week once. The parameters such as plant height (cm), number of leaves and survival percentage were recorded after 8 weeks. The acclimatized plantlets were finally transferred to the field condition and their survival percentage was recorded after 4 months.

Data were subjected to Analysis of Variance (ANOVA) using Statistical Analysis System (SAS) version 9.1 (SAS Institute INC., Cary, NC, USA). The means were compared by the Least Significant Different (LSD) at significance level of 0.05.

Results and Discussion

This study describes the effects of BAP on shoot differentiation of *Musa acuminata* var. *zebrina* with or without NAA to induce multiple shoots. The results revealed the existence of significant differences ($p \leq 0.05$) on *in vitro* shoot morphogenesis among different concentration of BAP when used alone or in combination with NAA (Table 1). Multiple shoot induction was better evidenced on the PGRs supplemented media. Likewise, it has been stated that PGRs and their appropriate concentrations are necessary for breaking dormancy and to induce *in vitro* multiple shoot production in *Musa* species (Jafari *et al.* 2011).

The highest multiple shoots (3.0 ± 1.0) were established on media fortified with 1 µM NAA and 15 µM BAP after 4 weeks, however it was not statistically significant with 15 µM BAP (2.9 ± 0.6) and 20 µM BAP (2.0 ± 1.2) (Fig. 1A, B). Among BAP concentrations, 15 µM was found to induce better shoot morphogenesis with 1.5 ± 0.2 cm shoot length when used without NAA. Similarly, it has been reported that higher concentrations of cytokinins might result in an adverse effect on the shoot multiplication rate as well as culture morphology (Strosse *et al.* 2004, Jafari *et al.* 2011, Kumara Swamy *et al.* 2010). An increased concentration of BAP above 20 µM was found to be detrimental to multiple shoot formation and shoot elongation. Likewise, it has been reported that increased exposure of BAP leads to reduced shoot multiplication with abnormality

and exhibits mutagenic effect yielding off type plants in most of *Musa* cultivars (Buah *et al.* 2010, Munguatosha *et al.* 2013). Moreover, the shoot numbers increased with subculture cycle (Table 2 and Fig. 1C).

Table 1. Effect of different concentrations of NAA and BAP on *in vitro* shoot proliferation in *M. acuminata* var. *zebrina* after 4 weeks of inoculation.

NAA (µM)	BAP (µM)	Shoot number	Shoot length (cm)
0	0	0.0 ± 0.0 b	0.0 ± 0.0 e
0	10	0.0 ± 0.0 b	0.0 ± 0.0 e
0	15	2.9 ± 0.6 a	1.5 ± 0.2 ab
0	20	2.0 ± 1.2 a	0.7 ± 0.4 bcde
0	30	1.7 ± 1.7 ab	0.2 ± 0.2 de
1	0	1.0 ± 0.0 ab	2.3 ± 0.2 a
1	10	0.7 ± 0.3 ab	1.3 ± 0.7 bc
1	15	3.0 ± 1.0 a	0.9 ± 0.2 bcd
1	20	1.3 ± 0.7 ab	0.5 ± 0.4 cde
1	30	1.3 ± 0.3 a	1.2 ± 0.2 bc

Means within column followed by different letters are significantly different according to LSD at $p \leq 0.05$.

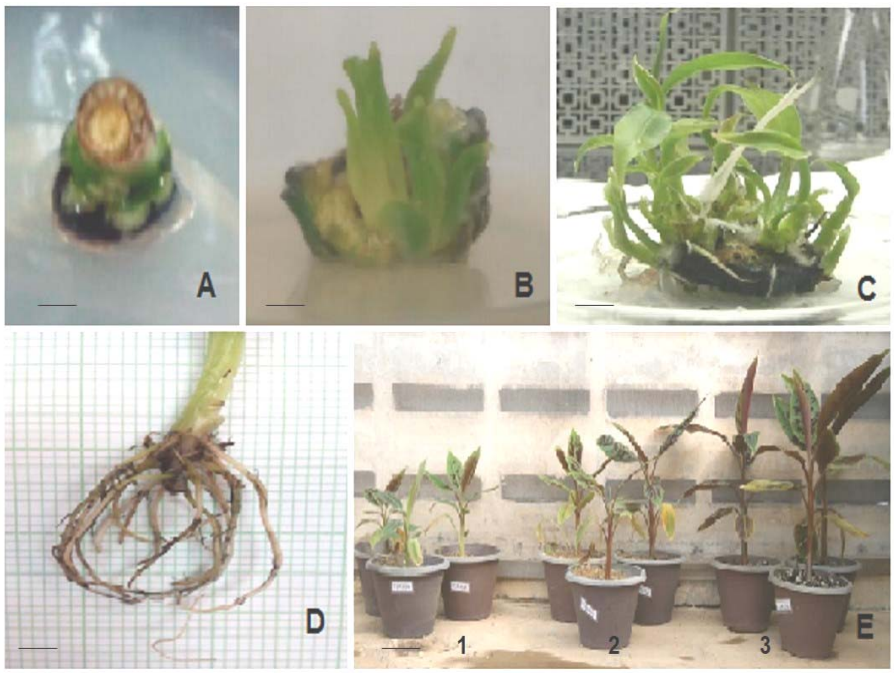

Fig. 1. Various micropropagation stages of *Musa acuminata* var. *zebrina*. (A and B) Microshoots induction from shoot tip (Scale bar = 5 mm), (C) multiple shoots formation on MS medium + 20 µM BAP (Scale bar = 5 mm), (D) well rooted shoots on MS medium + 1 µM IBA (Scale bar= 8 mm), growth performance of regenerated plantlets on (E1) sand : perlite : vermiculite (3 : 2 : 1), (E2) sand : perlite (2 : 1) and (E3) peatmoss : perlite : vermiculite (3 : 2 : 1) after 8 weeks of acclimatization in shade house. Scale bar = 40 mm).

The highest number of shoots per single explant was observed after third subculture on 20 µM BAP containing media (202.0 ± 1.7), but was not statistically significant with 30 µM BAP supplemented media (155.3 ± 1.9). Shoot length was found to increase with subculture cycles. In all the subculture cycles, the use of NAA showed a high significant difference on shoot length, but failed to induce multiple shoots. Interestingly, the shoot length and multiple shoot formation were found to increase after each subculture cycle irrespective of the treatments. Furthermore, it is evident that for shoot elongation there is no requirement of PGRs during subculture cycles for *M. acuminata* var. *zebrina*. Hence, this can reduce the cost of the culture media as PGRs are very expensive. Although, shoot proliferation rate in banana depends on the cultivars (Arinaitwe *et al.* 2000), previous shoot tip culture studies have reported the use of BAP in the range of 11 to 22 µM as ideal for obtaining high proliferation rate (Venkatachalam *et al.* 2007, Jafari *et al.* 2011).

Table 2. Effect of subculture cycle on *in vitro* shoot proliferation in *M. acuminata* var. *zebrina*.

Hormones		Subculture cycle					
NAA (µM)	BAP µM)	First		Second		Third	
		Length (cm)	Number*	Length (cm)	Number*	Length (cm)	Number*
0	15	3.1 b	16.3d	4.4b	27.8d	4.9b	45.9d
0	20	1.5cd	64.3a	2.4de	144.7a	3.4d	202.0a
0	30	0.9d	36.0b	1.8e	88.0b	3.6cd	155.3b
1	0	7.2a	1.3f	8.0a	1.3g	8.0a	1.3g
1	10	2.8b	9.3e	3.2cd	15.3f	4.6bc	24.0f
1	15	2.4bc	19.7c	3.6bc	23.7e	5.1b	27.7f
1	20	2.5bc	16.0d	3.6bcd	26.0de	5.0b	32.7e
1	30	2.1bc	20.3 c	2.7cde	63.7c	4.0bcd	120.7c

Means within column followed by different letters are significantly different according to LSD at $p \leq 0.05$.
Total number of shoots formed per single explant inoculated initially.

For most species, it is necessary to transfer a single shoot to a suitable medium to induce better roots. In this study, *in vitro* rooting was found to be significantly different among the treatments (Table 3). Full strength MS medium added with 1 µM IBA induced better rooting compared to any other treatments (Fig. 1D). Similarly, it has been reported that auxins are necessary for *in vitro* root induction in banana species (Raut and Lokhande 1989). In contrast, studies have shown that supplementation of auxins to half strength MS medium induces better rooting (Molla *et al.* 2004). Likewise, Rahman *et al*. (2013) found that the usage of half strength MS medium containing 1.0 mg/l IBA was effective in inducing *in vitro* roots in *Musa* sp. cv. Agnishwar's. In this study, though roots were induced in half strength MS medium, the rooting frequency was observed to be poor. Interestingly, full strength MS medium also witnessed better rooting frequency and increased the root length.

Ex vitro survival of tissue culture derived plantlet decides the success of any micropropagation protocol. Some of the factors such as substrate choice for hardening and nutrients for the plantlets are known to affect the acclimatization process and thus require optimization of these factors (Waman *et al.* 2015). This hardening is important since the *in vitro* plantlets are quite delicate and hence lose water rapidly when transferred to *ex vitro* conditions (Ahmed *et al.* 2014b). Therefore, *in vitro* derived plantlets must undergo a period of

acclimatization to obtain higher survival rate. Further, the establishment of micropropagated plantlets mainly depends on the chemical, physical and biological properties of potting mixtures. In our study, there was a significant difference among the potting medium treatments on the plantlets survival in the glass house (Fig. 1E). The treatment of peatmoss : perlite : vermiculite (3 : 2 : 1) gave the highest number of leaves (11.0 ± 0.6), increased the plant height (31.7 ± 0.9 cm) and showed 100% survival rate compared to other treatments (Table 4). In general, *M. acuminata* var. *zebrina* is a hardy plant and does not require extensive care during acclimatization, however the use of organic matter in the potting medium allowed vigorous growth. Likewise, it has been reported that different potting mixtures are known to influence the plantlet growth and survival rate (Ahmed *et al.* 2014b).

Table 3. The effect of MS media strengths and IBA concentrations on *in vitro* rooting of *Musa acuminata* var. *zebrina* after 4 weeks.

MS strength + IBA (µM)	Root number	Average root length (cm)	Longest root (cm)
Full strength MS + 0.0	6.9 ± 0.1 cd	3.6 ± 0.1 c	4.6 ± 0.2 cd
Full strength MS + 0.5	8.9 ± 0.1 b	4.8 ± 0.1 b	6.6 ± 0.1 b
Full strength MS + 1.0	10.2 ± 0.2 a	5.5 ± 0.3 a	7.7 ± 0.1 a
Full strength MS + 2.0	9.6 ± 0.1 ab	3.5 ± 0.1 c	4.8 ± 0.0 c
Half strength MS + 0.0	6.0 ± 0.2 d	2.6 ± 0.1 d	4.2 ± 0.1 de
Half strength MS + 0.5	7.5 ± 0.8 c	2.6 ± 0.3 d	3.6 ± 0.3 f
Half strength MS + 1.0	8.8 ± 0.1 b	3.1 ± 0.0 cd	4.1 ± 0.0 ef
Half strength MS + 2.0	10.0 ± 0.7 ab	2.7 ± 0.1 d	3.7 ± 0.1 ef

Means followed by same letter within a column are not different at p = 0.05 based on LSD.

Table 4. The effect of potting media on number of leaves and height of *Musa acuminata* var. *zebrina* after 8 weeks of transplantation.

Treatments	Number of leaves	Plant height (cm)	Plant survival (%)
Sand : perlite : vermiculite (3 : 2 : 1)	9.11 ± 1.0	21.7 ± 1.1 [c]	90 ± 0.1
Peatmoss : perlite : vermiculite (3 : 2 : 1)	11.0 ± 0.6	31.7 ± 0.9 [a]	100 ± 0.0
Sand : perlite (2 : 1)	9.6 ± 0.4	26.6 ± 0.9 [b]	95 ± 0.1
LSD at 0.05	-	1.8868	0.1
F value	4.56	83.69	0.2

Means followed by same letter within a column are not different at p = 0.05 based on LSD.

In contrast, Rahman *et al*. (2005) and Ali *et al.* (2011) have reported the better growth and survival rate of banana plantlets when potting mixture of soil : sand : farmyard manure (2 : 1 : 1) was used. This difference could be attributed to the unique plant traits specific to each species of *Musa* genus. When the plants were transferred to the field condition, a relatively good percentage of survival (86) was noticed after 4 months.

The shoot multiplication rate can be better induced on MS media supplemented with 20µM BAP. The study clearly suggests that combination of BAP and NAA is not necessary for *in vitro* propagation of *M. acuminata* var. *zebrina*. Further, the subculture cycles improve the shoot

morphogenetic response and the higher shoot length and shoot numbers can be achieved after third subculture. Full strength MS medium in combination of 1 µM IBA was found to be optimal for *in vitro* rooting. The use of peatmoss : perlite : vermiculite (3 : 2 : 1) exhibited better plant growth responses under the glasshouse condition.

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