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RAPD Analysis of *Musa acuminata* cv. Berangan Plantlets in Nursery Stage from Long-term Subculture

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ABSTRACT

Banana *Musa acuminata* cv. Berangan is an important fruit crop in Malaysia. The use of tissue culture techniques can increase the number of planting materials for mass production of banana. However, the main problem in banana tissue culture is somaclonal variation, which is caused by many factors, such as long-term subcultures, which can reduce the production value and quality. In this study, the experiment focused on somaclonal variation caused by long-term subculture that had caused changes in their morphology which could be differentiated by RAPD pattern of micropropagated *Musa acuminata* cv. Berangan plantlets from long-term subcultures (15th subculture). Banana plantlets established from micropropagated banana using MS supplemented with 5 mgL^{-1} of BAP was maintained until 15th subculture before being hardened and acclimatized in commercial soils. In this experiment, banana seedlings were categorized into four groups based on their heights at 5 - 10 cm, 11 - 15 cm, 16 - 20 cm and 21 - 25 cm. Results showed that the tallest seedlings (21 - 25 cm) produced 9.56 ± 1.01 number of leaves, 218.88 ± 40.89 cm leaf area and 5.32 ± 0.78 cm girth of pseudostem whereby the shortest group (5 - 10 cm) produced 5.67 ± 0.98 leaves, 18.95 ± 12.37 cm of leaf area, and 1.63 ± 0.54 cm girth of pseudostem. RAPD analysis carried out using two primers, OPH09 and OPA15 showed variation between the tallest seedlings and the shortest seedlings. This study concluded that long-term subculture of banana cv. Berangan produced variation in the seedlings' growth thus may affect the quality of planting materials produced.

Keywords: Banana, Berangan, micropropagated, plant height, RAP

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INTRODUCTION

Banana is the first fruit crop to adopt tissue culture technique for mass propagation and is still actively used for commercial production compared to other fruit crops (Swennen et al., 2004). Conventional banana propagation using suckers has limitations for large-scale planting because only 5 to 10 suckers per clump can be produced per year (Rahman et al., 2002). Moreover, diseases can spread quickly through vegetative propagation as it may carry source of pathogen from soil, whereas tissue culture can rapidly produce healthy planting materials on a large scale (Kaçar & Faber, 2012). In the field, tissue cultured banana seedlings show better agronomical characteristic compared to suckers (Bairwa et al., 2015; Mensah et al., 2012). After going through the *in vitro* process for multiplication, plantlets must undergo acclimatization stage. Acclimatization takes place to allow plantlets to adapt to new environment which involve physiological adaptation due to changes in surrounding condition such as temperature, light and humidity in the nursery (Perez & Hooks, 2008; Scaranari et al., 2009). Acclimatization of banana tissue cultured plantlets in the soil is a critical stage as they are subjected to different stresses that may affect their survival as they need to adapt to new environment (Aragón et al., 2005; Elisama et al., 2013; Vasane & Kothari, 2006). Lower relative humidity and higher light exposure can cause wilting and necrosis of leaves which eventually may result into failure to survive during the acclimatization phase (Preece & Sutter, 1991).

Good management and planning of acclimatization phase are a combination of morphological, biochemical and physiological processes to produce better quality plantlets which can survive in the field (Bitar & Mohamed, 2009; Vasane & Kothari, 2008). In glasshouse, higher irradiance and lower humidity can affect the survival rate (Kaçar & Faber, 2012; Vasane & Kothari, 2006) as they usually need some weeks for acclimatization (Vasane & Kothari, 2008). Many seedlings die and wilting due to water loss in the leaves which is often not restricted during the first transplant to the soil as there are still poor conductivity of roots and root-stem connections. Therefore, there is a need to limit the water supply to reduce hydraulic conductivity between roots and stems (Kaçar & Faber, 2012). Reducing natural light by using shading is essential during the acclimatization process (Scaranari et al., 2009). Successful acclimatization of plantlets depends on the ability for high survival rates (Zaid & Hughes, 1995). Carbohydrates content such as starch and sucrose plays an important role in the acclimatization process (Aragón et al., 2005, 2006). Many studies on the acclimatization of banana tissue cultures to the soils have been reported (Choudhary et al., 2014; Elhory et al., 2009; Husin et al., 2014).

The profit gain in banana tissue culture industry depends on the multiplication rate

in every number of subculture (Chavan-Patil et al., 2010). However, Sheidai et al. (2008) reported that a high number of subculturing might produce a few percentages of somaclonal variation in the banana tissue culture. This was supported by Borse et al. (2011), who stated that higher multiplication rates could also produce more variation. Mohamed (2007) suggested that somaclonal variation rate was much higher after the 6th clonal generation. Martin et al. (2007) stated that deficiency of calcium due to necrosis would occur after the 7th subculture for the banana cv. Cavendish. Furthermore, better yield was produced from the 8th subculture as compared to the 15th subculture (Chavan-Patil et al., 2010). Seven months of culturing in solid medium media can trigger genetic changes (Aremu et al., 2013; El-Dougdoug et al., 2007) and variation can be increased with frequent number of subcultures (Rodrigues et al., 1998; Sheidai et al., 2008). However, Lakshmanan et al. (2007) reported that there were no genetic changes in a number of subcultures up to 150 times for banana cv. Nanjanagudu Rasabale.

Molecular marker technology such as random amplified polymorphism DNA (RAPD) is a very useful technique to discover polymorphism in the DNA sequence for analysis of genetic variations, such as somaclonal variations (Chinmayee et al., 2012). The study was conducted to identify morphology and genetic changes in the long-term micropropagated banana cv. Berangan plantlets using RAPD.

MATERIALS AND METHODS

Plant Material

Banana cv. Berangan plantlets raised *in vitro* up to 15th of subculture using MS medium supplemented with 5 mgL^{-1} of BAP in plant tissue culture laboratory of Universiti Malaysia Kelantan were acclimatized using commercial soil in a nursery. After 3 months of acclimatization, the performance of each seedlings was recorded. Morphological parameters observed were plant height (measurement was taken from the point above the surface, up to the point at the last intersection of two new leaves), number of leaves, leaf length (determined by measuring of the second leaf), leaf width (determined by measuring the second leaf) and girth of pseudostem (measured at 1 cm above ground level using a tape-meter). Measurement for these parameters were as shown in Figure 1. Leaf area was calculated

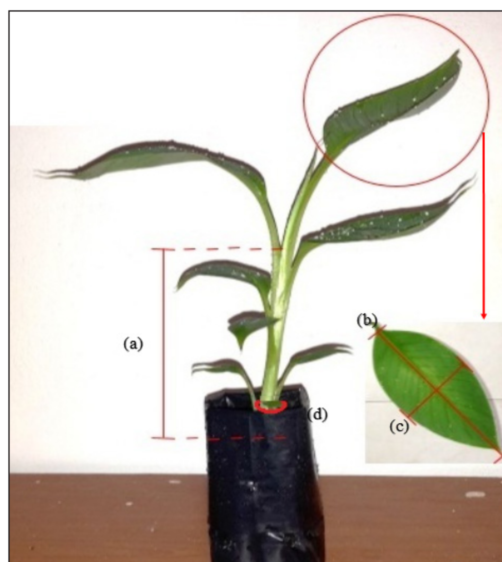

Figure 1. Photo showing measurement for (a) plant height, (b) leaf length of the 2nd leaf, (c) leaf width of the 2nd leaf, and (d) girth of the pseudostem

using equation following Turner and Lahav (1983). DNA extracted from banana leaves of different treatments were subjected to RAPD analysis.

DNA Extraction

Total genomic DNA was extracted using CTAB method from young leaf samples following the protocol by Doyle and Doyle (1987). DNA qualification and quantification were determined and samples were diluted to a concentration of 25 ng μL^{-1}.

Random Amplified Polymorphism DNA Analysis

In this experiment, DNA from different groups was pooled from 10 replications. The experimental design for this study consisted of only two groups of the highest and lowest plants heights (groups 1 and 4) which were subjected to RAPD analysis. RAPD analysis was performed following the method proposed by Weising et al. (1995). DNA amplification reactions were prepared for 25 µl reaction containing 50 ng μl^{-1} of banana genomic DNA, 1× PCR buffer, 200 µM of dNTPs, 2.5 mM $MgCl_2$, 10 pmoles of 7 random decamer oligonucleotide primers from different sets (OPA19, OPJ04, OPA06, OPH09, OPA01, OPA02 and OPA15) and 1.25 U *Taq* DNA polymerase. Amplification reactions were carried out using an Eppendorf Thermal Cycler at 95ºC for 3 min for initial denaturation. This was followed by 40 cycles at 30 sec denaturation kept at 95ºC, primer annealing at 36ºC for 30 secs, primer extension for 2 min at 72ºC, and a final extension at 72ºC for 5 min. This was then held at 4ºC. 100 bp DNA ladders was used as standard markers and PCR products were subjected to electrophoresis using 1X TBE buffer consisting of tris base at 10.8 gL^{-1}, boric acid (H_3BO_3) at 5.5 gL^{-1}, EDTA at 0.74 gL^{-1} and pH 8 in 1.4% (w/v) agarose gel. Gels were stained with 0.0001% ethidium bromide and visualized under UV and photographed.

RESULTS AND DISCUSSION

After three months of acclimatization (Figures 2 and 3), the plant height increased significantly in all four different groups on the plant height at 6.71 ± 2.01 cm, 13.55 ± 1.18 cm, 17.13 ± 1.11 cm and 21.74 ± 1.69 cm, respectively (Table 1). The number of leaves of different groups also

Table 1
Plant height, number of leaves, leaf area and girth of pseudostem for Musa acuminata *cv. Berangan after 3 months being acclimatized in the nursery*

Group of seedling (cm)	Plant height (cm)	No. of leaves	Leaf area (cm^2)	Girth of pseudostem (cm)
5-10	6.71 ± 2.01^d	5.67 ± 0.98^c	18.95 ± 12.37^d	1.63 ± 0.54^d
11-15	13.55 ± 1.18^c	8.57 ± 0.90^b	87.01 ± 31.29^c	3.56 ± 0.60^c
16-20	17.13 ± 1.11^b	9.38 ± 0.79^a	156.98 ± 37.94^b	$4.40 \pm 0. 35^b$
21-25	21.74 ± 1.69^a	9.56 ± 1.01^a	218.88 ± 40.89^a	$5.32 \pm 0. 78^a$

Note. Different letters indicate values are significantly different (P ≤ 0.05) by Duncan's multiple range test; Values are mean ± standard deviations based on at least ten replicates

increased at 5.67 ± 0.98 cm, 8.57 ± 0.90 cm, 9.38 ± 0.79 cm and 9.56 ± 1.01 cm, respectively (Table 1). However, only two groups showed a significant increment in the number of leaves at 5.67 ± 0.98 cm for the 5 - 10 cm group and 8.57 ± 0.90 cm for the 11 – 15 cm group while the remaining two groups were not showing significant result. Meanwhile the leaf area for the 2nd leaf was highly significant at 18.95 ± 12.37 cm, 87.01 ± 31.29 cm, 156.98 ± 37.94 cm and 218.88 ± 40.89 cm, respectively. The girth of pseudostem of the banana seedlings also showed significant increment at 1.63 ± 0.54 cm, 3.56 ± 0.60 cm, 4.40 ± 0.35 cm and 5.32 ± 0.78 cm, respectively.

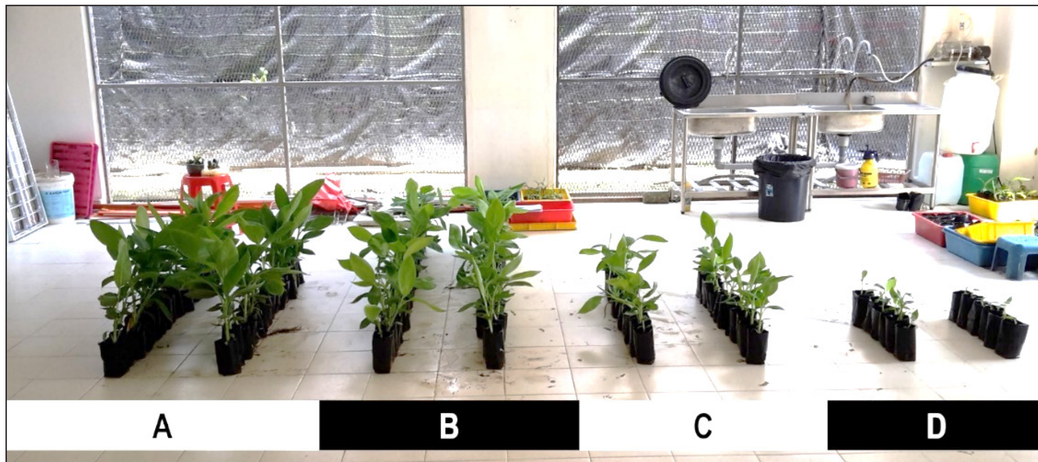

Figure 2. Four groups of long-term micropropagation of banana cv. Berangan plantlets based on different plant height observed after three months of acclimatization. A: 20 - 25 cm, B: 16 – 20 cm, C: 11 - 15 cm, and D: 6 – 10 cm

Figure 3. Morphology of banana cv. Berangan seedlings across different groups based on plant height (1) 20 - 25 cm, (2) 16 – 20 cm, (3) 11 - 15 cm, and (4) 6 – 10 cm after 3 months being acclimatized in the nursery

Plantlets produced *in vitro* must adapt to nursery condition before being transplanted into the field (Aragón et al., 2006). This is a critical stage whereby the banana plantlets need to undergo acclimatization process as they will be subjected to different environments as well as physiological and biochemical changes during this period (Pati et al., 2013). The use of polybags helps to deliver the greatest development of tissue-cultured banana plantlets as it provides the best draining system that does not allow roots to drown with over supply of water (Perez & Hooks, 2008). Banana cv. Grand Naine, Amritasagar and Sabri plantlets had in the past successfully survived after acclimatization using micropropagated banana from MS medium supplemented with 5 mgL^{-1} of BAP in the nursery (Hossain et al., 2016). Plant height of the cultivated banana seedlings obtained after acclimatization depends on variety and their environments. Study by Manjula et al. (2014) showed that maximum shoot height observed for banana cv. Rajapuri was 16.17 cm from plantlets cultured in MS medium supplemented with 5 mgL^{-1} of BAP and 2 mgL^{-1} of NAA after 75 days acclimatized in the nursery. They noticed that 20 % of plantlets cultured in MS medium supplemented with 0.2 mgL^{-1} TDZ produced dwarfism. However, the highest plant height obtained for banana cv. Grand Naine in the nursery after 45 days of acclimatization was 23.6 cm (Vasane & Kothari, 2008) and 24.3 cm (Vasane & Kothari, 2006). Banana seedlings with the height of around 20 cm were suitable for transferring in the field (Perez & Hooks, 2008). Banana seedling height of 15.7 cm for cv. Grand Naine produced 8.66 number of leaves after 45 days of acclimatization in the nursery (Ahmed et al., 2014). In addition, banana seedlings of 13.89 cm in height produced 4.7 number of leaves for cv. Nanjanagud Rasabale after 45 days in the nursery (Waman et al., 2014) while banana cv. Shima produced 7.5 number of leaves at 50 cm height after two months in the nursery (Buah et al., 2000).

Genetic differences between groups 1 and 4 were evaluated using RAPD analysis (Figure 4). In this study, two of seven RAPD primers (OPH09 and OPA02) showed loss of bands when compared between the two groups of different plant heights described in Figure 4. Primer OPH09 produced four bands in group 1 while five bands were observed in group 4. However, primer OPA15 lost 3 bands in the tallest group of plantlets as compared to the shortest group. Figure 4 also showed that primers OPH09 and OPA02 also displayed polymorphism. Primer OPH09 showed a unique 1800 bp fragment in group 4 which was absent in group 1. In addition, the RAPD profile produced by primer OPA02 had a missing band at 1700 bp in group 1 but present in group 4. Long-term subculture was the main focus in this study in order to detect genetic differences using the RAPD analysis. Results showed that RAPD primers could distinguish between groups of 1 and 4. The banana seedlings morphology of group 1 was considered as normal plant height as compared to group 4, which was depicted by a dwarf morphology. This morphology

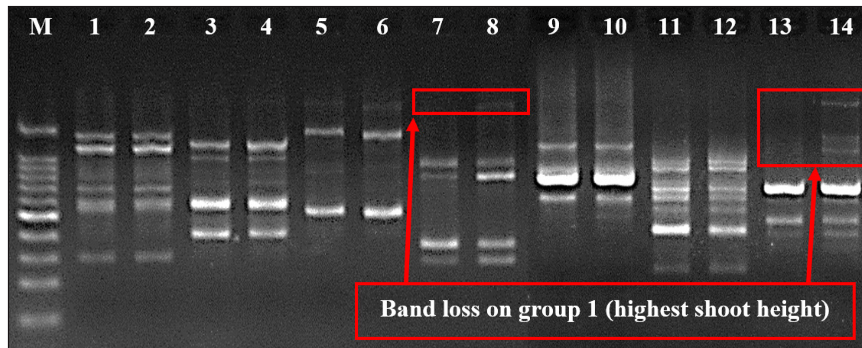

Figure 4. RAPD profiles of different groups of banana cv. Berangan seedlings after 3 months acclimatized in nursery conditions based on their height (groups 1 and 4) of in (lanes 1, 3, 5, 7, 9, 11 and 13) tallest group of plant height and (lanes 2, 4, 6, 8, 10, 12 and 14) group 4 with shortest plant height [RAPD profiles using decamer primers (lanes 1 and 2) OPA19, (lanes 3 and 4) OPJ04, (lanes 5 and 6) OPA06, (lanes 7 and 8) OPH09, (lanes 9 and 10) OPA01, (lanes 11 and 12) OPA02 and (lanes 13 and 14) OPA15. M: 100kb DNA ladder]

resulted from the effects of long-term exposure to *in vitro* condition (up to 15th subculture) which had led to genetic changes that could be determined by using RAPD analysis.

The use of RAPD analysis for nursery studies had been conducted by several researchers for detection of somaclonal variations in banana tissue cultures (Chinmayee et al., 2012; Ganapathi et al., 2008). However, study by Chinmayee et al. (2012) showed that clones of different traits consisting of normal, dwarf and giant at nursery stage showed different banding patterns when analyzed using RAPD.

CONCLUSION

Micropropagated banana cv. Berangan from long-term subculture produced different plant heights ranging from 5 to 25 cm which was not uniform. This phenomenon is related to the genetic changes which can be proven by using RAPD analysis. RAPD analysis showed that banana cv. Berangan plantlet height of 20 – 25 cm depicted variation as compared to 6 – 10 cm plantlets.

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