Australian Journal of Crop Science

AJCS

AJCS 6(3):391-401 (2012) ISSN:1835-2707

Induced mutations for enhancing variability of banana (*Musa* spp.) shoot tip cultures using ethyl methanesulphonate (EMS)

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Abstract

The aim of this study was to induce variations in banana cultivars through *in vitro* mutagenesis by treating the shoot tips with varying doses (150, 200 and 250 mM) and time periods (30 and 60 min) of EMS and to apply RAPD analysis for discovery of genetic polymorphism among the variants. The results of analysis of variance indicated that the average number of shoots per explant, survival (%) and fresh weight of treated shoot tips were influenced by EMS doses and gave significant differences caused by time period of treatments. The highest percentage (31.04 ± 0.99) of dormant explants was observed in 'Rastali' with 200 mM EMS for 30 min treatment. Whilst, in 'Berangan Intan' and 'Berangan', 60 min/ 250 and 30 min/ 200 mM indicated the maximum increase in terms of dormant explants production. Based on the proliferation rate of 'Berangan Intan', 'Berangan' and 'Rastali', LD_{50} was estimated 189.8, 177.58 and 152.04 mM for 30 min duration treatment and 154.17, 148.21 and 141.83 mM for 60 min duration treatment respectively. After three months of culture, percentage of phenotypic variations derived from mutated shoot tips indicated an increase of 10 to 14 % with 60 min/ 200 mM and 30 min/ 250 mM of EMS treatments compared to those of controls. At the molecular level, the RAPD primers opc01, opc04, opa11, opa14, opd16, opk03 exhibited different amplification patterns in the variants. The results showed the variability among lines isolated through tissue culture and EMS mutagenesis, confirmed by using RAPD markers for banana.

Keywords: Banana, Ethyl methanesulphonate, Mutation induction, Proliferation rate, Somaclonal variation.
Abbreviations: BAP-benzyl aminopurine, DMSO-dimethyl sulphoxide, EMS-ethyl methanesulphonate, RAPD- randomly amplified polymorphic DNA, TBE-tris borate EDTA.

Introduction

The progress of banana improvement by conventional breeding methods has been relatively slow or even hindered due to narrow genetic variability resulting from the low female fertility (Silva et al., 2001; Pua, 2007; Bakry et al., 2009). Therefore, *in vitro* somaclonal variation caused by mutagenesis treatments to create new cultivars of banana where in genetic breeding is relatively difficult should be considered (Silva et al., 2001). Mutagenesis is delineated as the exposing of every type of biological substance to a physical or chemical mutagen to enhance the frequency of mutation above the normal rates (Kodym and Afza, 2003). Chemical mutagens (e.g. EMS) could be applied successfully where no irradiation facility is accessible. Furthermore, as compared with physical mutagens, chemicals may increase gene mutations (point mutation) rather than chromosomal changes (Van Harten, 1998; Predieri, 2001; Toker et al., 2007). The measurement of 50% reduction of growth and proliferation (LD_{50}) for chemicals is determined by altering the concentration and duration of treatment (Predieri, 2001; Kodym and Afza, 2003). Hofmann et al. (2004) reported that survival of soybean suspension cultures depended on the concentration of EMS. They reported that the percentage of survival ranged from 74% with 1 mM EMS to 43% with 30 mM EMS. Application of *in vitro* mutagenesis strategies systems especially among vegetative propagated crops such as banana, potato and *Gypsophyla paniculata* L. along with *in vitro* selection have significantly improved the efficiency of mutation techniques in breeding programs (Cassells, 2002; Cassells and Doyle, 2003; Kulkarni et al., 2007; Barakat and El - Sammak, 2011). *In vitro* mutagenesis has also been applied to isolate useful variants in banana and to induce further genetic variability in *Chrysanthemum* (Misra and Datta, 2007; Suprasanna et al., 2008). Among chemicals, ethyl methanesulphonate (EMS); belonging to the group of the alkylating agents has been reported as a very effective and efficient mutagen for creating of somaclonal variation in crop plants such as banana, grapevine, sweet potato, petunia, rose, soybean and chrysanthemum (Omar et al., 1989; Bhagwat and Duncan, 1997; Van Harten, 1998; Nonomura et al., 2001; Predieri, 2001; Kodym and Afza, 2003; Hofmann et al., 2004;

Latado et al., 2004; Luan et al., 2006; Khawale et al., 2007; Berenschot et al., 2008). Latado et al. (2004) obtained many new cultivars of chrysanthemum from induced mutation. Their results showed the efficiency of EMS to induce variation of chrysanthemum through *in vitro* culture. Mutation induction in banana has been carried out by subjecting of shoot tips to mutagen followed by regenerating of treated shoot tips (Bhagwat and Duncan, 1997; Predieri, 2001). Bhagwat and Duncan (1997) also used chemical mutagens in banana (*Musa* spp. AAA group) to produce variants displaying resistance against fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*). They determined 200 mM of EMS for 30 min as an optimal dose and duration treatment. *In vitro* mutation induction for salt tolerance using EMS was also reported in sweet potato (Luan et al., 2006). The genetic bases of variation in banana were previously confirmed by Martin et al. (2006) using the random amplified polymorphic DNA (RAPD). The RAPD – PCR analysis, using arbitrary primers has been applied to characterize the somaclonal variation derived *in vitro* induced mutation in banana (Vidal and Garcia, 2000), sugarcane (Yadav et al., 2006), *Chrysanthemum* (Barkat et al., 2010) and *Gypsophyla paniculata* L (Barakat and El – Sammak, 2011). The current study was carried out to enhance the chance of variation induced by ethyl methanesulphonate (EMS) in shoot tip cultures of banana associated with reduced lethal effect on explants by estimating the most suitable treatments based on *in vitro* growth and proliferation rate reduction by assessing the treatments that result in a 50% reduction of growth and proliferation (LD_{50}). In this investigation shoot tip cultures of banana cultivars were exposed to chemical mutagen, evaluated for survival and then RAPD markers were used to detect DNA polymorphisms in the subsequent regenerations obtained from EMS treated shoot tips.

Results

Phenotypic outcomes of EMS on treated shoot tips and regenerated shoots

Different concentrations of EMS and time of incubation treatments were applied to investigate their effects on proliferating shoot tips and to evaluate their potential in creating variation among banana cultivars; 'Berangan Intan', 'Berangan' and 'Rastali'. Significant decrease was observed in the survival of shoot tip cultures as the dose of EMS increased. The percentages of surviving explants ranged from 98.89 to 37.78% in 'Berangan Intan', 100 to 34.44% in 'Berangan' and 100 to 31.03% in 'Rastali' (Fig 2). Solutions with the highest dose (250 mM) and 60 min time period treatment of EMS caused reductions of around 60 to 70% in the survival capacity in shoot tips when compared with those obtained from the treatment of immersion in water or buffer (controls) (Fig 2). The results also showed that the shoot tips of banana immersed for 60 min in EMS solutions caused a significant reduction in the survival capacity when compared with those from the 30 min treatment (Fig 2). Nevertheless, not all surviving shoot tips gave rise to shoot and a large number of them remained dormant as they represented no further growth even after six months of culture following EMS treatments (Fig 1). Dormant shoot tips had taken place in all EMS treatments except the controls (Table 2). The occurrence of dormant shoot tips was observed to be cultivar dependent responses to different concentrations and duration treatments of EMS, as in 'Rastali' 200 mM concentration for 30 min treatment caused the highest percentage of dormant explants (31.04 ± 0.99) but in 'Berangan Intan' and 'Berangan', EMS at 250mM/ 60 min and 200 mM/ 30 min gave the highest percentage of dormant explants production (Table 2). There were also significant differences in the percentage of shoot tips regenerating shoot with altering the concentration and duration of EMS as around thirteen weeks after treatments with EMS, the percentage of shoot tips regenerating shoots ranged from 78.00 ± 2.19, 75.67 ± 2.34 and 74.00 ± 1.79 with 150 mM of EMS solution for 30 min treatment to 30.63 ± 1.05, 33.33 ± 1.63 and 23.50 ± 1.76 with 60 min/ 250 mM treatment for 'Berangan Intan', 'Berangan' and 'Rastali', respectively. Anyway, the control treatments (buffer and water soaked) caused more than 90% of shoot tips to regenerate shoot (Fig 3). 'Berangan Intan' and 'Berangan' (AAA genome) did not show significant difference in their performance for shoot regeneration capacity (2.41 ± 0.1 and 2.36 ± 0.05, respectively) at lower concentrations and time period treatment of EMS (150 mM for 30 min) but 'Rastali' exhibited significantly higher multiplication rate (3.08 ± 0.07) (Table 2). However, significant differences were observed in the proliferation rate of shoot tips subjected to the higher concentrations and time period of EMS treatments for all cultivars tested in this investigation (Table 2). The average number of shoots per explant declined significantly from the controls to the highest dose (250 mM) of EMS, this decrease being similar at both treatment periods (Table 2), however, cultivar 'Rastali' showed the highest potential of shoot regeneration in all EMS treatments (Table 2). Statistical analysis of the weights of shoot tips 45 days after treatments revealed significant differences between the controls and all EMS treatments (Table 2). Significant differences among shoot tip fresh weights depended on the mutagen concentration as well as duration treatment which are presented in Table 2. Regarding the weight of shoot tips, the lowest values (1.68 ± 0.05 g, 1.38 ± 0.09 g and 1.89 ± 0.17 g for 'Berangan Intan', 'Berangan' and 'Rastali', respectively) were recorded at the highest (250 mM) concentration for 60 min incubation of EMS. A similar trend was found in shoot tips immersed for 30 min treatment (Table 2).

Chemo sensitivity assessment on in vitro growth and multiplication rate from shoot tips

The LD_{50} for mutagenesis of banana shoot tips with EMS was estimated through linear regression based on proliferation rate and *in vitro* growth (Tables 3 and 4). Based on the proliferation rate of 'Berangan Intan', 'Berangan' and 'Rastali', LD_{50} was estimated 189.8, 177.58 and 152.04 mM for 30 min duration treatment and 154.17, 148.21 and 141.83 mM for 60 min duration treatment respectively (Table 3). In the case of *in vitro* growth reduction, LD_{50} was estimated 302.30, 334.64 and 217.16 mM for 30 min duration treatment and 175.77, 166.99 and 167.79 mM for 60 min duration treatment in 'Beragan Intan', 'Berangan' and 'Rastali' respectively (Table 4). After the estimation of the dose inducing $LD_{50,}$ the most useful dose for treatment could be picked out, as doses lower than LD_{50} support plant recovery after treatment, while application of higher doses enhances the chance to induce mutations regardless of negative effects caused by mutagen.

Phenotypic variations in regenerated shoots

Data collected for phenotypic variations monitored among regenerated shoots three months after treatment of shoot tips with EMS are presented in Table 5. The phenotypic variations caused by EMS were observed as leaf colour change, short interval among leaves as dwarfism,

Table 1. Regenerated somaclones comprised of variants caused by different EMS treatments as well as non mutated parental lines in banana cultivars 'Berangan Intan' and 'Berangan'.

EMS treatments (Duration/ Concentration)	Variants and parental lines	
	'Berangan Intan'	'Berangan'
Control	L_{1-1}	L_{2-1}
30 min/ 150 mM	L_{1-2}	L_{2-2}
60 min/ 150 mM	L_{1-3}	L_{2-3}
30 min/ 200 mM	L_{1-4}	L_{2-4}
60 min/200 mM	L_{1-5}	L_{2-5}
30 min/250 mM	L_{1-6}	L_{2-6}
60 min/250 mM	L_{1-7}	L_{2-7}

Fig 1 A, B, C, D, E and F. Dormant shoot tips of banana cultivar 'Berangan Intan' caused by EMS at 150 mM for 60 min treatment (represented in Fig A), 250 mM EMS for 60 min treatment (shown in Fig B), 200 Mm EMS for 60 min treatment (shown in Fig C), EMS at 250 mM for 30 min treatment (represented in Fig D), 200 mM EMS for 30 min (shown in Fig E) and 150 mM EMS for 30 min (represented in Fig F). Bar = 5mm.

hyperhydricity and aberrant morphology production (Fig 4). A noticeable effect was that all leaf colour changes which were observed as a narrow violet stripe on the leaf blades (Figs 4 A and B). Total percentage of phenotypic variations among regenerated shoots ranged from 2.90, 1.87 and 3.66 with 150 mM EMS for 30 min treatment to 10.74 with 60 min/ 200 mM of EMS, 12.42 with 60 min/ 200 mM of EMS and 14.78 with 30 min/ 250 mM of EMS for 'Berangan Intan', 'Berangan' and 'Rastali' respectively (Table 5). The highest phenotypic variation was obtained with 250 mM EMS for 30 min treatment in 'Rastali' and 200 mM EMS for 60 min treatment in 'Berangan Intan' and 'Berangan' (Table 5).

Molecular characterization of EMS induced variants derived from cultivars 'Berangan Intan' and 'Berangan' (AAA genome) using RAPD markers

The genetic variability of twelve lines of banana cultivars caused by EMS treatments along with their parental lines ('Berangan Intan' and 'Berangan') as controls was assessed using RAPD markers technique. Therefore, sixteen random primers were applied to identify the mutagenic modifications of the variants and among them, six primers namely opc01, opc04, opa11, opa14, opd16, opk03 showing polymorphic fragments among shoot tips caused by treatments with the chemical mutagen EMS exhibited different amplification patterns as presented in Table 6 and Fig 5. Primer opc01 revealed a 600 bp fragment in the variant L_{2-3}. This fragment was not detected in the other lines (Fig 5). Also the RAPD profile generated by primer opc01 showed a loss of 1900 bp band in the lines L_{1-6} and L_{2-6} (Fig 5). Of the fragments amplified with primer opa11 (Fig 5) two bands (2300 and 3600 bp) were indentified in the variants L_{1-5} and L_{2-5}, respectively. These fragments were not observed in the other lines. Three bands of 1470, 1400 and 1240 bp in size were also identified when using primer opa11 in the variant L_{2-3}, not being found in the other lines (Fig 5). L_{1-6} and L_{2-6} did not monitor 1900 bp fragment with primer opc01. Primer opc04 distinguished the lines L_{1-4} and L_{2-6} with absence of 1180 and 2100 bp fragments, respectively (Fig 5). The primer opa14 showed a band 1870 bp in size that was absent in L_{1-5}, L_{1-7}, L_{2-4}, L_{2-5}, L_{2-6} and L_{2-7} (Fig 5). The polymorphism generated by opd16 in L_{1-4} displayed a fragment 860 bp in size. The bands that were absent in L_{2-3} was also generated by the primer opd16 (Fig 5). The primer opk03 generated polymorphic fragments in the variants caused by EMS treatments from both cultivars 'Berangan Intan' and 'Berangan' (Table 6).

Discussion

The results of this study showed that different EMS treatments revealed a gradual reduction in survival, growth and regeneration capacity of the treated shoot tips in all cultivars as the concentration and duration treatment of EMS increased. These results are in agreement with previous findings in banana, chrysanthemum, petunia and soybean (Omar et al., 1989; Bhagwat and Duncan, 1997; Latado et al., 2004; Hofmann et al., 2004; Berenschot et al., 2008). Omar et al. (1989) reported an obvious reduction in the number of regenerated shoots from treated explants with increasing concentration of EMS. Furthermore, their results also showed the essential role of DMSO as a carrier agent to accelerate the

Table 2. Fresh weight of proliferating shoot tips, taken 45 days after treatment with ethyl methanesulphonate. The percentage of dormant shoot tips and the average number of shoots per explant, recorded after the second period of 45 days (second subculture) in response to the treatments with ethyl methanesulphonate (EMS) in banana cultivars; 'Berangan Intan', 'Berangan' and 'Rastali'.

Cultivar	EMS concentration (mM) and duration treatment	Fresh weight (g)	Percentage of dormant shoot tips	Shoots per explant (average)
Berangan Intan	*30 – min treatment*			
	150	5.02 ± 0.09 h	11.44 ± 1.40 gh	2.41 ± 0.1 h
	200	4.28 ± 0.06 j	18.89 ± 0.54 c	1.58 ± 0.06 k
	250	3.31 ± 0.04 n	12.64 ± 1.08 f	0.71 ± 0.03 p
	60 – min treatment			
	150	3.86 ± 0.05 kl	12.46 ± 0.84 fg	2.06 ± 0.1 i
	200	2.43 ± 0.03 q	14.37 ± 1.79 e	1.25 ± 0.04 m
	250	1.68 ± 0.05 s	18.95 ± 1.32 c	0.42 ± 0.04 q
	Untreated controls			
	Water soaked	6.12 ± 0.08 d	0.00 ± 0.00 k	3.49 ± 0.14 e
	Buffer soaked	5.56 ± 0.09 f	0.00 ± 0.00 k	2.92 ± 0.08 g
Berangan	*30 – min treatment*			
	150	5.05 ± 0.09 h	7.19 ± 0.69 i	2.36 ± 0.05 h
	200	4.68 ± 0.05 i	19.31 ± 1.26 c	1.67 ± 0.04 k
	250	3.76 ± 0.1 l	8.54 ± 0.56 i	1.08 ± 0.08 n
	60 – min treatment			
	150	3.62 ± 0.06 m	14.71 ± 0.57 e	1.83 ± 0.06 j
	200	2.68 ± 0.05 p	10.92 ± 1.01 h	1.42 ± 0.05 l
	250	1.38 ± 0.09 t	19.13 ± 1.30 c	0.6 ± 0.03 p
	Untreated controls			
	Water soaked	6.35 ± 0.07 c	0.00 ± 0.00 k	4.25 ± 0.05 c
	Buffer soaked	5.78 ± 0.21 e	0.00 ± 0.00 k	3.69 ± 0.12 d
Rastali	*30 – min treatment*			
	150	5.40 ± 0.11 g	21.03 ± 0.50 b	3.08 ± 0.07 f
	200	4.63 ± 0.22 i	31.04 ± 0.99 a	2.29 ± 0.13 h
	250	2.89 ± 0.19 o	16.47 ± 1.41 d	1.44 ± 0.13 l
	60 – min treatment			
	150	3.99 ± 0.13 k	9.56 ± 1.02 i	2.80 ± 0.07 g
	200	3.60 ± 0.13 m	6.18 ± 1.04 j	1.88 ± 0.14 j
	250	1.89 ± 0.17 r	9.31 ± 0.70 i	0.89 ± 0.03 o
	Untreated controls			
	Water soaked	7.85 ± 0.06 a	0.00 ± 0.00 k	6.99 ± 0.04 a
	Buffer soaked	7.05 ± 0.24 b	0.25 ± 0.03 k	4.70 ± 0.04 b
	$LSD_{(0.05)}$	**0.1353**	**1.054**	**0.1303**

Values (means ± SD) within columns with the same letter are not significantly different at the 0.05 Probability level according the LSD test. Each treatment was replicated six times with each replicate having six explants.

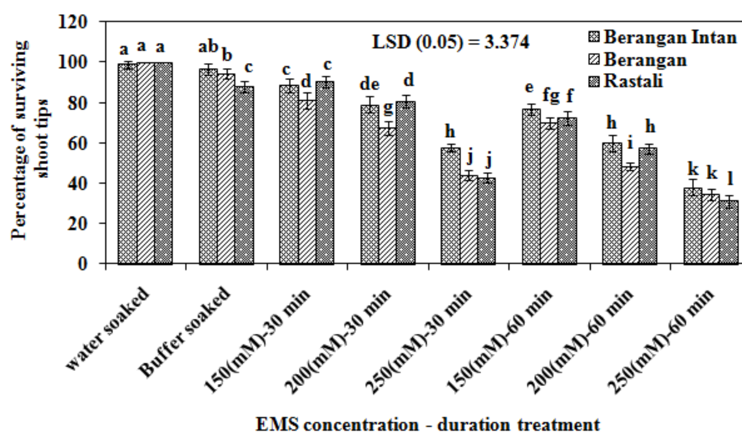

Fig 2. Effect of dose and time duration treatments of ethyl methanesulphonate (EMS) on survival (%) of proliferating shoot tip cultures of banana cultivars. Bars showing the same latter are not significantly different at p ≤ 0.05 as determined by least significant difference (LSD) test. Each treatment was replicated six times with each replicate having six explants. Each value represents the mean ± SD (*n = 6).*

uptake of EMS into the explants. Regarding their offer based on lower concentrations of DMSO to prevent browning of explants and enhance absorption to present better results, we applied just 1mM of dimethylsulphoxide (DMSO) as a carrier agent in mutagen solution treatments. Using radiation induced variation in *Chrysanthemum*, Misra and Datta (2007) reported a reduction in the percentage of responding cultures and number of shoots per explant with the increase in radiation dose. The results of the current investigation obviously indicate that mutation frequency increases with increasing of dosage and time duration of EMS treatment which is in agreement with the findings reported by Bhagwat and Duncan (1997), but regarding with the significant reduction of survival and regeneration capacity of treated shoot tips, the efficiency of mutagen also decreases. Previously, Hofmann et al. (2004) reported a reduction in survival of soybean cell cultures with increasing dose of EMS. Khawale et al. (2007) also reported a decline in the regeneration capacity of shoots in grapevine as the dose of mutagenic agent increased.

Therefore, an optimum dose of EMS should be chosen based on some factors such as percentage of survival and proliferation rate as well as reduction of regeneration capacity by 50% (LD_{50}). Bhagwat and Duncan (1997) pointed out that a 50% growth reduction was not necessarily the best criterion for determining optimum treatment conditions for chemical mutagens which in the case of 50% *in vitro* growth reduction we are also agree with them, due to occurrence of dormant shoot tips caused by EMS, but regarding with the huge necessity of recovery and highly speediness of cloning variants after mutation induction, we concluded that expression of LD_{50} based on the proliferation rate should be an important criterion for determining of the optimum dose of chemical mutagen treatment. Likewise, Latado et al. (2004) expressed the LD_{50} for the mutagenesis of chrysanthemum with EMS. The appearance of shoot tips displaying no further growth and remaining dormant even after six months of culture could be due to the inhibitory effect of EMS according to previous reports (Bhagwat and Duncan, 1997). However, frequency of this *in vitro* response to EMS concentrations and duration treatments was not the same among three cultivars tested in this study. Comparatively, the higher frequency of morphological variations (10 to 14 %) observed among regenerated shoots from all cultivars tested in this investigation comprise of leaf color changes, aberrant morphology and dwarfism than that obtained by Bhagwat and Duncan (1997) who reported 5.8% as the highest frequency of phenotypic variation among regenerated shoots, enable us to assume EMS as an efficient treatment to create variability in banana cultivars. Suprasanna et al. (2009) expressed morphological variations resulted from mutagenesis in banana by some agronomic characters in the field such as the pseudostem height and leaf shape. They isolated the dwarf types of banana plants derived from tissue culture and mutagenesis. In the current investigation, all leaf color changes caused by mutagenesis were observed as narrow violet strips on the leaf blades which have never been reported before. Given that the cultivars used in this investigation possessed different genome including 'Berangan Intan' and 'Berangan' with AAA and 'Rastali' with AAB genome, to observe and distinguish the obvious genetic variations caused by EMS, comparison was achieved only among variants derived from the same genomes (AAA group) where in any detectable different amplification could be arisen through the mutation without intervening genomic differences. RAPD technique has been successfully applied to examine variability obtained from mutagenesis in sugarcane (Khan et al., 2009), *Chrysanthemum* (Barakat et al., 2010) and *Gypsophila* (Barakat and El – Sammak, 2011). Analysis of RAPD in *Gypsophila* indicated 105 different amplification products identified as polymorphisms (Barakat and El – Sammak, 2011). In the present investigation, the technique of RAPD markers resulted in the genetic variation and polymorphism among the EMS treated lines L_{1-2}, L_{1-3}, L_{1-4}, L_{1-5}, L_{1-6}, L_{1-7}, L_{2-2}, L_{2-3}, L_{2-4}, L_{2-5}, L_{2-6},and L_{2-7} in comparison to their donor parents 'Berangan Intan' and 'Berangan' (L_{1-1}, L_{2-1}, respectively). However, it is not distinguished whether the desired traits have been arisen from these variants. Therefore, employment of *in vitro* methods through an appropriate selecting agent in the culture media is needed to detect variants with desirable agronomic characteristics. Regarding our results, the RAPD profiles generated by primers opc01, opc04, opa11, opa14, opd16 and opk03 indicated polymorphic fragments among EMS induced variants of banana and the amplified products ranged from 600 bp to 2100 bp. Using a SCAR – based molecular technique to characterize of radiation induced dwarf types in banana, Suprasanna et al. (2008) reported a variation in some characters such as height compared to control.

They reported the amplified product of 500 bp in dwarf types. Using RAPD markers, Hofmann et al. (2004) reported that soybean cultures subjected to EMS showed different amplification which further supports our results in banana. They also assumed RAPD markers as a useful technique in detecting polymorphisms in soybean cultures subjected to chemical mutagenesis. Similarly Kurup et al. (2009) reported that RAPD primer opc02 presented different amplified DNA bands to distinguish cultivars in date palm, which supports our results in banana. Ochatt et al. (1999) employing 70 RAPD primers as proper markers to distinguish lines of potato, reported that only primer opb13 revealed a loss of a DNA fragment in the variants compared to the control line. Out of six primers used by Yadav et al. (2006), only two primers detected polymorphism among variants of sugarcane.

Materials and methods

Plant materials and media preparation

Micropropagated cultures of banana cultivars; 'Berangan Intan', 'Berangan' (AAA group) and 'Rastali' (AAB group) were used as the source of materials for the excised shoot tips. Micropropagation medium consisted of the MS medium (Murashige and Skoog, 1962) supplemented with 22.2 µM benzyl aminopurine (BAP). After three months of culture to allow further proliferation, the shoot tips were separated from shoot clumps according to Strosse et al (2008) by cutting of pseudo stem tissue at about 5 mm above the apical meristems and then the sheathing leaves were gradually removed from the shoot tips as the explants consisted of apical meristems enveloped by three to five leaves and 2 – 3 mm of corm tissue so that the final size of initial explants was as uniform as possible (about 6 to 8 mm).

Ethyl methanesulphonate (EMS) treatments of banana shoot tips

Aqueous solution containing 1 M of ethyl methanesulphonate (EMS) along with 1% v/v dimethylsulphoxide (DMSO) as a

Table 3. $LD_{(50)}$ calculation on the proliferation rate response of shoot tips to different EMS treatments.

Cultivar	EMS concentration(mM) and duration of treatment	Proliferation rate	Model	$LD_{(50)}$* (mM)	CV%**
'Berangan intan'	*30 min – treatment*		Y= -0.010x+3.648	189.80	16.702
	0	3.49			
	150	2.41			
	200	1.58			
	250	0.71			
	60 min – treatment		Y= -0.012x+3.600	154.17	13.250
	0	3.49			
	150	2.06			
	200	1.25			
	250	0.42			
'Berangan'	*30 min- treatment*		Y= -0.012x+4.251	177.58	1.241
	0	4.25			
	150	2.36			
	200	1.67			
	250	1.08			
	60 min – treatment		Y= -0.014x+4.195	148.21	8.23
	0	4.25			
	150	1.83			
	200	1.42			
	250	0.6			
'Rastali'	*30 min- treatment*		Y= -0.022x+6.835	152.04	9.674
	0	6.99			
	150	3.08			
	200	2.29			
	250	1.44			
	60 min – treatment		Y= -0.024x+6.894	141.83	9.70
	0	6.99			
	150	2.8			
	200	1.88			
	250	0.89			

*The dose of EMS that resulted in a 50% reduction of proliferation (LD_{50}) was assessed based on the number of regenerated shoots recorded in the second subculture. The EMS dose inducing LD_{50} was calculated with the equation by substituting (y) with the value of 50% of control (0 Mm of EMS). **Coefficient of variation%.

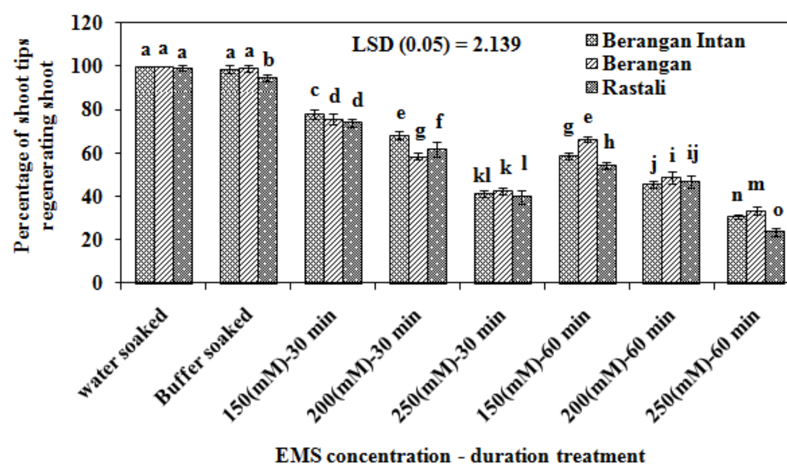

Fig 3. Percentage of shoot tips regenerating shoot 90 days after treated shoot tips with varying doses and time durations treatments of ethyl methanesulphonate (EMS). Bars showing the same latter are not significantly different at $p \leq 0.05$ as determined by least significant difference (LSD) test. Each treatment was replicated six times with each replicate having six explants. Each value represents the mean ± SD ($n = 6$).

Table 4. LD $_{(50)}$ calculation on *in vitro* growth response (%) of shoot tips to different EMS treatments.

Cultivar	EMS concentration(mM) and duration of treatment	*In vitro* growth%	Model	$LD_{(50)}$ * (mM)	CV% **
'Berangan intan'	*30 min – treatment*		Y= -0.174x+102.6	302.30	7.57
	0	100			
	150	82.03			
	200	69.93			
	250	54.08			
	60 min – treatment		Y= -0.293x+101.5	175.77	8.21
	0	100			
	150	63.07			
	200	39.54			
	250	27.45			
'Berangan'	*30 min- treatment*		Y= -0.153x+101.2	334.64	4.61
	0	100			
	150	79.65			
	200	73.66			
	250	59.31			
	60 min – treatment		Y= -0.306+101.1	166.99	5.49
	0	100			
	150	57.1			
	200	42.27			
	250	21.77			
'Rastali'	*30 min- treatment*		Y= -0.239x+101.9	217.16	8.47
	0	100			
	150	68.67			
	200	58.98			
	250	36.82			
	60 min – treatment		Y= -0.294+99.33	167.79	9.17
	0	100			
	150	50.83			
	200	45.86			
	250	24.08			

*The dose of EMS that resulted in a 50% reduction of growth (LD_{50}) was assessed based on the *in vitro* growth reduction calculated in the first subculture after treatment. The EMS dose inducing LD_{50} was calculated with the equation, by substituting (y) with the value of 50% of control (0 Mm of EMS). **Coefficient of variation%.

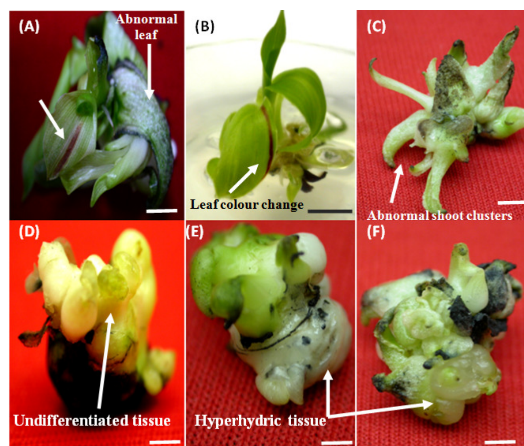

Fig 4. Morphological variability caused by EMS treatments among proliferated shoot tips of banana cultivars. (A) and (B) represent leaf color changes caused by EMS at 200 mM for 60 min in 'Berangan Intan' and 'Berangan', respectively. Arrows show narrow violet strips on leaf blade. Bar = 5 mm. (C) shows abnormal shoot regeneration from shoot tip of cultivar 'Berangan' in response to EMS at 200 mM for 60 min. Bar = 5 mm. (D) shows undifferentiated tissue produced from shoot tip of 'Rastali' with EMS at 200 Mm for 60 min duration treatment. Bar = 4 mm. (E) and (F) represent the hyperhydric tissues caused by EMS at 150 mM for 60 min in shoot tips of cultivars 'Berangan' and 'Berangan Intan', respectively. Bar = 4 mm.

carrier agent was prepared in the deionized water and kept in 4°C. This solution was diluted with 0.1 M phosphate buffer (pH 7) using filter - sterilization under aseptic condition to give working solutions (150, 200 and 250 mM) of EMS. Sterile deionized water and sterile phosphate buffer were also prepared as controls. Excised shoot tips were submerged in mutagen solutions and controls as 1ml/ shoot tip for 30 and 60 min for each concentration. In the case of control solutions only 60 min period was considered. Each treatment replicated 6 times including 6 shoot tips. Therefore, 36 shoot tips was used for each treatment. All treatments were carried out at 25°C under room temperature. Following EMS treatment, shoot tips were rinsed three times with sterile distilled water and allowed to be dry for 10 min.

Tissue culture, isolation of variants and data analysis

Subsequently, EMS treated explants were inoculated in 300 ml capacity jars (6 treated shoot tips/ jar) consisting of 60 ml MS basal salts and vitamins and sucrose (30 g L^{-1}), solidified with 2.8 g L^{-1} gelrite supplemented with 22.2 µM BAP. Cultures were kept under a controlled environment at 28 ± 2°C with 16 h photoperiod and cool white florescent light for six months to allow further proliferation and during this period subcultures were carried out at 45 days interval. After the first period of 45 days, the fresh weights of proliferating shoot tips for each replicated treatment were recorded and then subculture was carried out to the same proliferation medium as above for a further 45 days. After the second period of 45 days, data were collected from all replicated treatments to document the percentage of surviving, the percentage of shoot tips regenerating shoots, the average number of shoots per treated explant and the percentage of shoot tips which showed no further growth but were alive which in this study called as dormant shoot tips.

Chemo sensitivity assessment on in vitro growth and multiplication rate from shoot tips

When explants are used for chemical mutagenesis, a protocol will be needed to support their *in vitro* growth and to produce the maximum proliferation of shoots after mutation induction treatment. Therefore, the percentage of *in vitro* growth of EMS treated shoot tips was calculated using the following formula according to Musoke et al. (1999):

In vitro growth (%) = FWT/ FWU × 100

Where, FWT and FWU represent fresh weight of treated shoot tips (g) after 45 days and fresh weight of untreated shoot tips (g) after 45 days, respectively. Since the fresh weight of the initial explants need to be as steady as possible, The mean fresh weight of 6 EMS treated explants for each treatment was considered 0.8 (g) at the starting point, therefore, the fresh weight of proliferating shoot tips was taken after 45 days (First period of subculture). Then the LD_{50} was calculated as the dose of EMS which reduces the percentage of *in vitro* growth among treated shoot tips to 50% of untreated control shoot tips based on the linear regression. LD_{50} calculation on the capacity of the proliferation rate was also worked out as the dose of EMS that reduces the number of shoots regenerated per treated shoot tips to 50% of untreated explants based on the linear regression according to Predieri and Virgilio (2007). The dose of EMS that resulted in a 50% reduction of proliferation and growth (LD_{50}) was assessed based on the number of regenerated shoots recorded in the second subculture (Table 3) and based on the *in vitro* growth reduction (Table 4) calculated in the first subculture after treatment. The EMS dose inducing LD_{50} was calculated with the equations (Tables.3 and 4), by substituting (y) with the value of 50% of control (0 Mm of EMS).

Phenotypic variations

After three months of culture, data were recorded for phenotypic variations including: leaf color changes, dwarfism, undifferentiated tissues and aberrant morphology production (Figs 4) such as; hyperhydricity and abnormality observed among total shoots regenerated from treated and untreated shoot tips, then the percentage of phenotypic variations among all shoots regenerated from treated shoot tips compared to the untreated control shoot tips was assumed as a criterion for efficiency of the different EMS treatments to induce mutation. The percentage of phenotypic variation was calculated according to Bhagwat and Duncan (1997) with modification as fallow:

Phenotypic variation (%) = TV/ TS × 100

Where, TV and TS represent total number of variations and total number of shoots regenerated after 3 months, respectively.

Statistical analysis

The experiments were arranged in a completely randomized design with six replicates and the data collected and calculated were analysed using SAS and MSTAT-C computer programs and comparison of means were tested for significance, using least significant difference (LSD) test, at 0.05 level of probability.

DNA extraction and electrophoresis

The multiplication phase of EMS - treated shoot tips lasted for six months and after this period, twelve mutated lines derived from different concentrations and duration treatments of EMS, which are listed in Table 1, were evaluated for their genetic variability using RAPD molecular markers technique. Genomic DNA was extracted from 200 mg of leaf samples by using DNeasy DNA extraction kit (Qiagen, Research Biolabs SDN. BHD) and was diluted to concentration of 50 ng μl^{-1}. DNA amplification was prepared in volumes of 25 µl consisted of 50 ng/ µl of banana genomic DNA, 10×PCR buffer (Fermentas), 200 µM of dNTPs (Fermentas), 2.5 mM $MgCl_2$ (Fermentas), 10 pmoles of sixteen random decamer oligonucleotide primers (AccuOligo, Bioneer) from different sets (OPA, OPB, OPC, OPD, OPK, OPM and OPN) and 1.25 unit of Taq DNA polymerase (Fermentas). All amplification reactions were carried out using a thermocycler (TECHNE, TC - 512) programmed for initial denaturation of three min at 95°C followed by 40 cycles of 30 sec denaturation at 95°C,

Table 5. Percentage of phenotypic variations based on observation among regenerated shoots from EMS treated shoot tips of banana cultivars.

Cultivar	EMS concentration(mM) and duration treatment	Total shoot regenerated after three months	Phenotypic variations among regenerated shoots			Total %
			Leaf color changes	Dwarfism	Aberrant morphology	
BI*						
	30 – min treatment					
	150	241	3	2	2	2.90
	200	183	7	1	3	4.56
	250	114	4	3	4	9.65
	60– min treatment					
	150	121	5	3	6	7.65
	200	153	7	1	5	10.74
	250	90	3	1	2	6.67
	Untreated controls					
	Water soaked	510	0	0	0	0.00
	Buffer soaked	360	0	0	0	0.00
B**						
	30 – min treatment					
	150	267	3	1	3	1.87
	200	187	6	2	4	6.42
	250	125	4	1	4	7.20
	60– min treatment					
	150	210	5	4	5	6.67
	200	153	9	3	7	12.42
	250	95	2	1	3	6.31
	Untreated controls					
	Water soaked	470	0	0	0	0.00
	Buffer soaked	387	0	0	0	0.00
R***						
	30 – min treatment					
	150	273	8	0	2	3.66
	200	220	9	2	5	7.27
	250	115	8	4	5	14.78
	60– min treatment					
	150	191	6	1	2	4.71
	200	167	11	4	7	13.17
	250	87	5	1	4	11.49
	Untreated controls					
	Water soaked	542	0	0	0	0.00
riations observed among regenerated shoots from EMS *	*Buffer soaked*	434	0	0	0	0.00

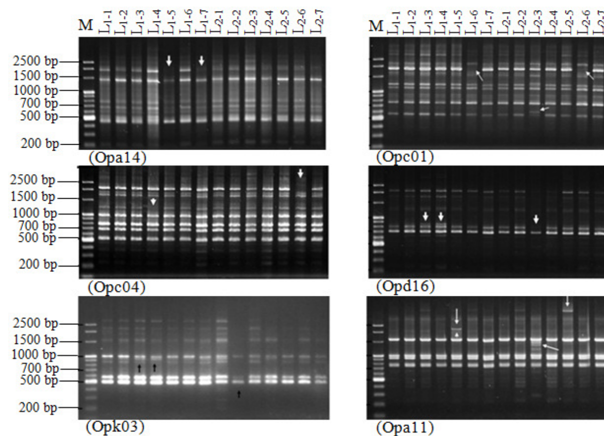

Fig 5. Gel electrophoresis pattern of randomly amplified polymorphic DNA (RAPD) amplification using different primers obtained from mother plants ('Berangan Intan' and 'Berangan' as L_{1-1} and L_{2-1}, respectively and EMS treated lines including L_{1-2}, L_{1-3}, L_{1-4}, L_{1-5}, L_{1-6}, L_{1-7}, L_{2-2}, L_{2-3}, L_{2-4}, L_{2-5}, L_{2-6} and L_{2-7} according to Table 1. Arrows show different amplification in each regenerated line caused by EMS – induced variation.

Table 6. Primers which produced RAPD polymorphisms in shoot tip cultures of banana cultivars; 'Berangan Intan' and 'Berangan' treated with EMS. Remaining primers included opa19, opa20, opb18, opc02, op05, opc08, opk01, opm16, opn03, opn09 generated only monomophic bands.

Primer	Sequence (5' to 3')	Number of bands		
		Total loci	Polymorphic bands	
			'Berangan Intan'	'Berangan'
Opa14	CTCGTGCTGG	5	2	2
Opc01	TTCGAGCCAG	9	3	4
Opc04	CCGCATCTAC	8	2	2
Opd16	AGGGCGTAAG	6	3	4
Opk03	CCCTACCGAC	8	4	6
Opa11	CAATCGCCGT	9	2	4

primer annealing at 36°C for 30 sec, primer extension for two min at 72°C with a final extension at 72°C for five min and then held at 4°C. Along with the PCR products, 100 bp DNA ladders (Eurx) as standard markers were subjected to electrophoresis in 1.4% agarose gel with 5 ×TBE buffer consist of tris base ($C_{14}H_{11}NO_3$) at 54 g L^{-1}, boric acid (H_3Bo_3) at 27.5 g L^{-1}, EDTA ($C_{10}H_{11}N_2O_8Na_2.2H_2O$) at 3.7 g L^{-1} and pH 8, according to Muchugi et al. (2008). The gels were stained with 0.0001% ethidium bromide and visualized with UV transilluminator and photographed.

Conclusion

Subjecting of shoot tips cultures of banana to EMS treatments could provide an alternative strategy for inducing variants as the phenotypic variations ranged from around 2 to 14% which were visible three months after culture. What is clear from the results of RAPD markers-assisted characterization of variants in banana cultivars tested in the present study it that, RAPD markers could be assumed as an efficient tool for estimation of genetic variability among EMS treated lines in banana. Consequently, this incoming can be employed to detect and select variants with requested agronomic attributes using an efficient *in vitro* selection procedure.

Acknowledgments

This work was supported by grants received from RUGS – UPM, University Putra Malaysia.

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