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In vitro selection and characterization of water stress tolerant lines among ethyl methanesulphonate (EMS) induced variants of banana (*Musa* spp., with AAA genome)

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Abstract

Water stress enforces a serious threat to banana productivity. Therefore, the attempts to develop tolerant lines are of massive worth to increase cop productivity. To develop an efficient *in vitro* screening of banana for water stress tolerance, twelve EMS – induced variants derived from cultivars; 'Berangan Intan' and 'Berangan' involving shoot tips were subjected to a stepwise selection procedure contained three levels (10, 20 and 30 g L^{-1}) of PEG induced water stress. Water stress adversely affected fresh weight increase, shoot vigour and multiplication rate. However, L_{2-5} on the medium fortified with 30 g L^{-1} PEG, had significantly the greatest fresh weight increase (1.85 ± 0.18 g) while the parental lines (L_{1-1} and L_{2-1}) exhibited the lowest fresh weight increase (0.29 ± 0.08 and 0.28 ± 0.1 g, respectively). Higher proliferation rate was observed with L_{2-5} and L_{1-5} followed by L_{2-4}, L_{1-4} and L_{2-6}.at each stress level. Proline content was increased noticeably in L_{2-5} and L_{1-5} followed by L_{1-4}, L_{1-6}, L_{2-3}, L_{2-4} and L_{2-6}. A significant decrease in leaf water loss associated with the lessened levels of MDA and H_2O_2 were recorded with the water stress tolerant lines as compared to susceptible and non – mutated parental lines exposed to PEG. It was concluded that *in vitro* selection of banana could provide a method for distinguishing the variants for their morphological and physiological responses to water stress whereby L_{2-5}, L_{1-5}, L_{2-6}, L_{1-6}, L_{1-4} and L_{2-3} demonstrated better tolerance against water deficit than the rest and control plants.

Keywords: Banana, Ethyl methanesulphonate, *In vitro* selection, Mutation induction, Somaclonal variation, Water stress.
Abbreviations: BAP_ benzyl aminopurine, DMSO_ dimethyl sulphoxide, EMS_ ethyl methanesulphonate, LWL_ leaf water loss, MW_ molecular weight, MDA_ malondialdehyde, NAA_ naphthalene acetic acid, PEG (M.W. 6000) _ polyethylene glycol, ROS_ reactive oxygen species, TBA_ thiobarbituric acid, TCA_ trichloroacetic acid.

Introduction

Osmotic stress resulting from water stress is one of the important factors which endanger the worldwide productivity in crop plants as economic impact of water stress becomes even more noticeable by increasing of food demand for accelerated rising world populations (Altman, 2003; Ismail et al., 2004; Turner et al., 2007). The banana plant is very sensitive to water stress as no species is reported to be highly water stress tolerant although there is a considerable range of water stress resistance based on genome whereas B genome are more resistant than those based on A genome (Vosselen et al., 2005; Nelson, 2006; Turner et al., 2007; OGTR, 2008; Bakry et al., 2009; Robinson and Sauco, 2010). Regarding that a low tolerance to salinity and drought are the major limitations to growing crops and fruits, hence, the attempts to develop stress – tolerant plants are very important to enhance crop productivity (Predieri, 2001; Altman, 2003; Rai et al., 2011). Tissue culture as a possible technology for obtaining the desired characteristics of variants can lead the variation toward the expected outcomes while the probability to accomplish an *in vitro* selection depends on the accessibility of an effective regeneration system associated with an efficient selective agent (Jain, 2001; Predieri, 2001; Jain, 2002; Dita et al., 2006; predieri and Di Virgilio, 2007; Rai et al., 2011). Luan et al. (2007) succeeded to obtain salt tolerant cultivars of sweet potato from induced mutation caused by EMS. Successful *in vitro* selection for drought tolerance using polyethylene glycol (PEG), sorbitol, mannitol and agar as selection agents has been also applied to several crops such as coconut, banana, rice, sugarcane, potato, sweet potato, alfalfa and *Tagetes minuta* (Karunaratne et al., 1991; Dragiiska et al., 1996; Mohamed et al., 2000; Biswas et al., 2002; Ebrahim et al., 2006; Yadav et al., 2006; Gopal and Iwama, 2007; Luan et al., 2007; Gopal et al., 2008; Rai et al., 2011). Somaclonal variation was also employed in *Tagetes* to select drought tolerance (Mohamed et al., 2000). In that investigation, drought tolerant selected lines showed a greater growth when grown on medium with water stress pressure and when the lines were tested in the *in vivo* condition for drought, they also yielded a higher biomass than other clones, indicating that preparing *in vitro* cultures with water stress agents was efficient in selecting a drought tolerant plant. Tai et al. (2001) treated maize seedlings with PEG induced water stress. They concluded that PEG responsive membrane proteins are related to chloroplasts. *In vitro* assessment of banana cultivars for drought tolerance has been previously carried out (Chai et al., 2005; Ebrahim et al., 2006). However, regarding lack of reports on *in vitro* selection of banana for drought tolerance, regeneration of plants displaying an increased tolerance to water stress is significant propose for biotechnological improvement of bananas. Therefore, the morphological and physiological characteristics of proliferated shoots derived by growing mutated shoot tips on MS medium (Murashige and Skoog, 1962) without (unselected treatment) and with polyethylene glycol (selected treatment) was evaluated with the objective to develop *in vit*ro selection method for water stress tolerance in banana.

Results and discussion

Morphological responses of EMS – induced variants of banana to water stress

Until now, *in vitro* selection of mutated lines in banana cultivars for drought has received no attention, therefore, *in vitro* selection involving twelve ethyl methanesulphonate (EMS) – induced variants of banana through screening of shoot tips on media stressed with different levels (10, 20 and 30 g L^{-1}) of polyethylene glycol (PEG) was attempted to develop drought-tolerant lines (Fig 1). Likewise, Fuller et al. (2006) developed an efficient *in vitro* selection method for cauliflower to create chemical mutagens induced variants on stressed medium. The results presented here indicated that higher fresh weight increase was observed in the control set (unselected treatment, media without the addition of PEG treatment) than on water stressed medium (selected treatments) in all the fourteen lines as expected (Fig 2). Higher fresh weight increase was recorded by L_{2-5} and L_{1-5} on MS medium containing PEG at 10 and 20 g L^{-1} (selected treatments) as the difference between the latter two lines was not significant (Fig 2), but in the medium with PEG at 30 g L^{-1} (level 3) L_{2-5} exhibited significantly the greatest fresh weight increase (1.85 ± 0.18 g) among all variants (Fig 2). L_{1-3}, L_{1-4}, L_{1-6}, L_{2-3}, L_{2-4} and L_{2-6} displayed more fresh weight increase than those of L_{1-2}, L_{1-7}, L_{2-2} and L_{2-7} in the medium with 30 g L^{-1} PEG (selected), while the non – mutated parental lines (L_{1-1} and L_{2-1}) showed the lowest fresh weight increase as the latter two clones were at par (0.29 ± 0.08 and 0.28 ± 0.1 g, respectively) (Fig 2). Differential fresh weight response observed between water stress tolerant variants and the remaining lines investigated in the present study is in agreement with the finding of *Tagetes minuta* by Mohamed et al. (2000) who reported significant differences among clones under *in vitro* water stress condition induced by mannitol. In contrast to the present investigation, Mohamed et al. (2000) reported no significant differences in fresh weight among the lines of *Tagetes minata* under unstressed conditions. The comparison of shoot vigour in presence of PEG (selected treatments) and without the addition of treatment to media (unselected) revealed that growth was adversely affected by PEG, however, L_{2-5} and L_{1-5} showed higher shoot vigour rate by all doses of PEG (Fig 3). It is interesting to note that the rate of shoot vigour over the media without the addition of PEG treatment (unselected treatment) showed no substantial difference among the mutated and non - mutated parental lines (Fig 3). However, in the medium supplemented with various concentrations of PEG, it is obvious that the variants demonstrated a better growth status than the parental lines (Fig 3) as minimum shoot vigour was recorded in the non – mutated parental lines (L_{1-1} and L_{2-1}) by all PEG concentrations (Fig 3). Regarding that relative vigour has been proved to be an important component of tolerance to abiotic stresses (Munns, 1993; Gopal et al., 2008), it could be concluded that out of the fourteen lines, variants L_{2-5} and L_{1-5} could adapt best to water stress based on their shoot vigour responses (Fig 3). The lines were also tested for regeneration ability in response to PEG induced water stress in the media. It is clear from Fig 4 that variants L_{2-5} and L_{1-5} exhibited again superior performance followed by L_{2-4} and L_{1-4} and then L_{1-3} and L_{2-6}.at the highest level (30 g L^{-1}) of PEG. On all three PEG levels, the proliferation rate of water stress selected L_{2-5} and L_{1-5} (selected) were significantly lower ($P \leq 0.05$) than those of their respective controls (unselected) (Fig 4). Totally, the finding that the number of shoots per regenerating shoot tip subjected to the media with addition of varying concentrations (10, 20 and 30 g L^{-1}) of PEG (selected) was reduced compared to shoot tips on the media without the addition of PEG treatment (unselected) is similar to that reported by Biswas et al. (2002). However, PEG has an inhibitory effect on banana growth by declining the water potential of the medium, consequently growing shoot tips cannot absorb water and nutrients from the medium resulting in deficient performance among lines for multiplication rate, fresh weight and shoot vigour, which is similar to inferences of Mohamed et al. (2000). After exposure to all three levels of PEG, the non – mutated parental lines (L_{1-1} and L_{2-1}) exhibited significantly lower regeneration capacity than those of variants (Fig 4). Data presented in Fig 4 also showed that there were only slight differences in proliferation rate responses to increasing concentrations of PEG (from 10 to 30 g L^{-1}) among lines which could be due to the gradual adaptation of growing shoot tips for regeneration capacity during a long term exposure to different levels of PEG. When the dose of PEG was increased to 30 g L^{-1}, survival capacity was reduced for all the lines, but the inhibition was weaker in case of variants L_{2-5} and L_{1-5} than those of the rest, though on all three PEG levels (selected treatments), the survival capacity were lower than each respective control (unselected treatment) (Fig 5). Ebrahim et al. (2006) reported that although explant survival was adversely effected by increasing of PEG levels, the intensity of inhibition was also cultivar dependent, which supports our results. However, Sivritepe et al. (2008) reported no loss of survival capacity among explants of sweet cherry in response to water stress caused by PEG. Obviously, the injurious effect of water stress was more noticeable in parental lines than the tolerant variants (Fig 9) as L_{1-1} and L_{2-1} exhibited the lowest survival capacity by all levels of PEG (Fig 5). Variants as well as parental lines did not differ significantly in survival capacity when cultured under unselected treatment (media without the addition of PEG treatments) as all lines showed 100 % survival capacity on PEG free medium (Fig 5). Anyway, from the results and observations it could be concluded that percentage of survival should not be a good indicator of water stress as most shoot tips even in the medium containing the highest dose (30 g L^{-1}) of PEG, remained alive while showing no further growth or exhibiting noticeable abnormalities (Figs 9 I, J, K and L) which is consistent with similar inferences reported with other water stress agents such as mannitol (Mohamed et al., 2000).

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)	Regenerated somaclones (variants)	
	'Berangan Intan' (AAA)	'Berangan'(AAA)
Control	$L_{1\text{-}1}$	$L_{2\text{-}1}$
30 min/ 150 mM	$L_{1\text{-}2}$	$L_{2\text{-}2}$
60 min/ 150 mM	$L_{1\text{-}3}$	$L_{2\text{-}3}$
30 min/ 200 mM	$L_{1\text{-}4}$	$L_{2\text{-}4}$
60 min/ 200 mM	$L_{1\text{-}5}$	$L_{2\text{-}5}$
30 min/ 250 mM	$L_{1\text{-}6}$	$L_{2\text{-}6}$
60 min/ 250 mM	$L_{1\text{-}7}$	$L_{2\text{-}7}$

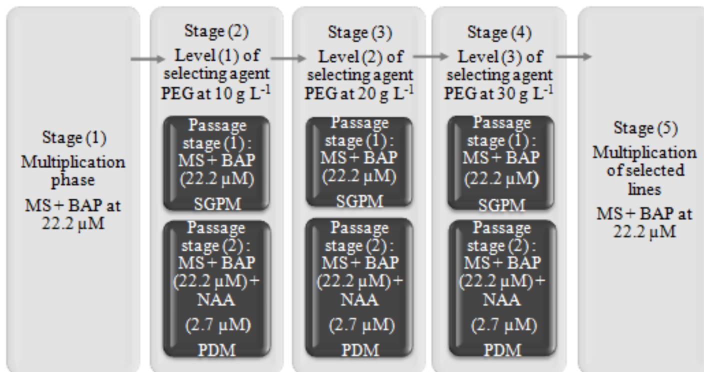


Fig 1. Protocol designed to select drought-tolerant mutated lines of banana cultivars 'Berangan Intan' and 'Berangan' via stepwise *in vitro* technique using PEG as a water stress agent. Every level of water stress agent comprised of two passage stages (1) and (2). Each passage stage (1) considered as shoot growth and proliferation medium (SGPM) was two months at the end of which shoots outgrowth from surviving shoot tips were subcultured on passage stage (2) considered as plantlet development medium (PDM) which lasted for one month. Multiplication phase including stages (1) and (5) lasted for six months to allow further proliferation of variants. Passage stage 1 (Shoot growth and proliferation medium; SGPM) = MS + 22.2 µM BAP with 10, 20 and 30 g L^{-1} PEG, passage stage 2 (plantlet development medium; PDM) = MS + 22.2 µM BAP + 2.7 µM NAA with 10, 20 and 30 g L^{-1} PEG.

Leaf water loss

Leaf water loss (LWL) of parental non-mutated lines ($L_{1\text{-}1}$ and $L_{2\text{-}1}$) was much higher than those of variants under water stressed conditions (Fig 6). PEG induced water stress decreased leaf water maintenance in all variants and parental lines of banana cultivars more than their respective lines under unstressed conditions and the reduction was more at higher water stress level (PEG at 30 g L^{-1}) in all lines which was similar to the observations of Valentovic et al. (2006) who observed the negative effect of drought stress on leaf water maintenance in maize cultivars. Variants $L_{2\text{-}5}$, $L_{1\text{-}5}$, $L_{1\text{-}6}$, $L_{2\text{-}3}$ and $L_{2\text{-}6}$ had lower leaf water loss than the remaining lines under all three levels of PEG induced water stressed conditions (Fig 6). Similarly Valentovic et al. (2006) reported that leaf water loss (LWL) of drought sensitive cultivars in maize was much higher than that of drought tolerant cultivars under drought stress conditions.

Effect of PEG induced water stress on proline accumulation

When shoot tips subjected to the media with addition of varying concentrations (10, 20 and 30 g L^{-1}) of PEG (selected), proline accumulation ranged from 0.49 ± 0.09 and 0.83 ± 0.06 µMoles g^{-1} FW in parental lines ($L_{1\text{-}1}$ and $L_{2\text{-}1}$, respectively) to 15.81 ± 0.91 and 14.90 ± 0.14 µMoles g^{-1} FW in variants $L_{2\text{-}5}$ and $L_{1\text{-}5}$, respectively (Fig 7). The accumulation of proline was slightly greater under PEG induced osmotic stress (selected) than those of the lines under unstressed condition (unselected) except for $L_{2\text{-}5}$ and $L_{1\text{-}5}$ which showed significantly higher proline content compared to their respective control (unselected treatment) followed by $L_{1\text{-}4}$, $L_{1\text{-}6}$, $L_{2\text{-}3}$, $L_{2\text{-}4}$ and $L_{2\text{-}6}$ (Fig 7). In the current study, increase in proline content in the case of susceptible variants under both conditions, without the addition of treatment to media (unselected) and with PEG addition to media (selected) was almost closed to each other but in contrast to susceptible lines, tolerant variants under water stressed conditions showed significantly higher proline content than their respective lines under unstressed conditions (Fig 7). Rai et al. (2011) stated that characterization of selected water stress tolerant plants could be based on accumulation of proline. It could, therefore, be postulated that enhanced production of proline might be a good indicator in selection for water stress tolerance as osmoregulatory role of proline for overcoming water stress conditions has been previously shown in apple and sweet cherry shoot tip cultures (Sotiropoulos, 2007; Sivritepe et al., 2008). Mohamed et al. (2000) reported that proline content did not increase significantly among *Tagetes* clones. This contrary result could be due to application of different water stress agent (mannitol) and plant (*Tagetes*) employed by the above-mentioned authors. In addition, variants $L_{2\text{-}5}$ and $L_{1\text{-}5}$ showed more increase in proline content compared to those of the rest under all concentration of PEG, whereas differences between

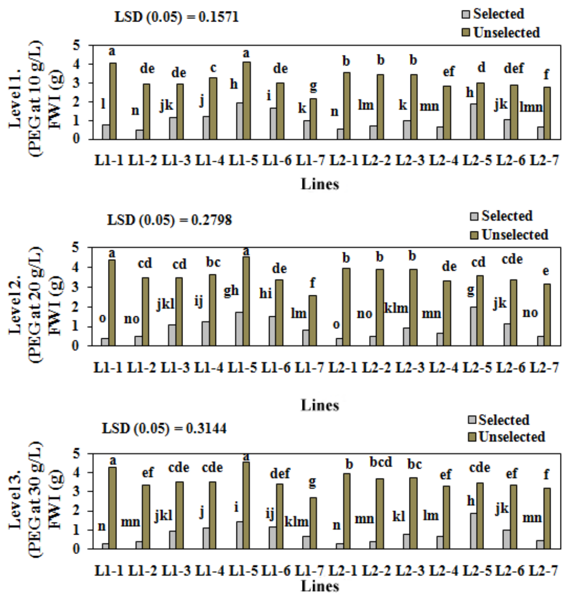


Fig 2. Mean values of shoot fresh weight increase (FWI) (g) in twelve mutated lines of banana L_{1-2}, L_{1-3}, L_{1-4}, L_{1-5}, L_{1-6}, L_{1-7}, L_{2-2}, L_{2-3}, L_{2-4}, L_{2-5}, L_{2-6}, L_{2-7} along with their parental cultivars: 'Berangan Intan' and 'Berangan' as lines L_{1-1} and L_{2-1}, respectively according to Table 1 without the addition of treatment to the media (unselected) and with PEG (10, 20 and 30 g L^{-1}) addition to the media (selected) for a period of 8 weeks in each level. Each value represents mean of four replicates. Different letters indicate values are significantly different at the 0.05 probability level according to the LSD test.

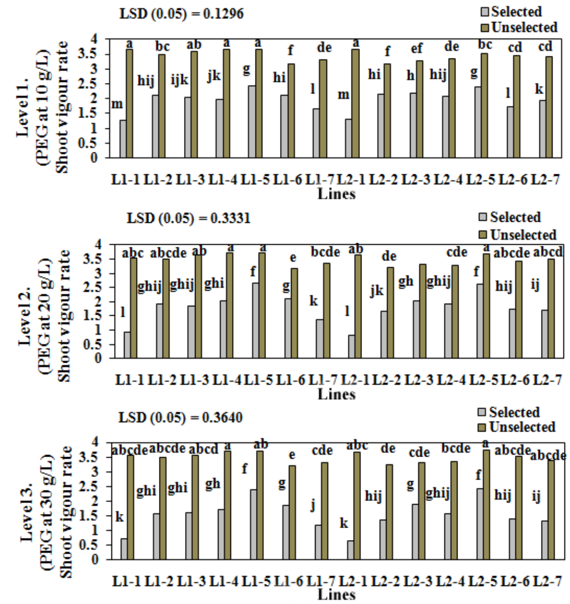


Fig 3. Shoot vigour rate of twelve EMS induced variants of banana L_{1-2}, L_{1-3}, L_{1-4}, L_{1-5}, L_{1-6}, L_{1-7}, L_{2-2}, L_{2-3}, L_{2-4}, L_{2-5}, L_{2-6}, L_{2-7} as well as their parental cultivars: 'Berangan Intan' and 'Berangan' as lines L_{1-1} and L_{2-1}, respectively according to Table 1 in presence of 10, 20 and 30 g L^{-1} PEG (selected treatments) and without the addition of treatment to the media (unselected) for a period of 8 weeks in each level. Each value symbolizes mean of four replicates. Different letters indicate values are significantly different at the 0.05 probability level according to the LSD test.

the latter two lines were not significant and they were at par with each other (Fig 7). Fuller et al. (2006) stated that all of the selected variants of cauliflower which demonstrated good resistance to stress, all had high proline contents though lines S42, S65 and S81 were all resistance to salt and frost but had comparatively lower proline. Thus, they hypothesized that enhanced proline is not essential for improved resistance to abiotic stress in cauliflower. Anyway, during all three levels of PEG induced osmotic treatments, parental non-mutated lines (L_{1-1} and L_{2-1}) exhibited the lowest proline content even compared to their respective controls on the media without the addition of PEG treatment to media (unselected) (Fig 7). Therefore, on the basis of our results it could be concluded that proline accumulation is a sign of stress tolerance in banana.

Lipid peroxidation (malondialdehyde accumulation) and hydrogen peroxide (H_2O_2) content

Oxidative stress caused by various abiotic stresses results in the formation of reactive oxygen species (ROS) such as H_2O_2 which can lead to the formation of harmful free radicals that cause lipid peroxidation and denaturing protein (Wang, 1999; Jaleel et al., 2009). Therefore, measurement of the level of malondialdehyde (MDA) in the leaves of twelve variants of banana 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}, L_{2-7} along with their parental cultivars: 'Berangan Intan' and 'Berangan' as lines L_{1-1} and L_{2-1}, respectively was applied as an index of lipid peroxidation. The formation of MDA was significantly greater under PEG induced water stress than those of the lines under unstressed condition (Fig 8). In the present study, increase in MDA formation of susceptible variants under water stress condition was almost closed to each other but tolerant variants L_{2-5}, L_{1-5}, L_{1-6} and L_{2-6} showed significantly lower MDA content (48.26 ± 4.66, 50.50 ± 6.50, 51.57 ± 8.87 and 55.06 ± 3.96 nmol g^{-1} FW, respectively) and the highest amount of MDA was recorded with parental lines L_{2-1} and L_{1-1} (89.60 ± 7.72 and 84.57 ± 12.49 nmol g^{-1} FW, respectively) under water stressed condition (Fig 8). In the media without the addition of PEG treatment (unselected treatment), MDA content ranged from 29.36 ± 7.49 and 26.77 ± 4.88 nmoles g^{-1} FW in parental lines (L_{1-1} and L_{2-1}, respectively) to 14.20 ± 1.41 and 15.10 ± 2.77 nmoles g^{-1} FW in the variants L_{2-5} and L_{1-5}, respectively (Fig 8). Studying the oxidative damages on banana cultivar 'Berangan', Chai et al. (2005) reported that the concentration of MDA was 13.75 nmol g^{-1} FW under control treatment (PEG at 0 g L^{-1}) which was close to the findings reported in the present study, but under PEG induced water stress, the noticeable increment in MDA concentration (89.60 ± 7.72 nmol g^{-1} FW) obtained in the current investigation compared to that reported by those authors (54.59 nmol g^{-1} FW), could be due to the long term exposure of shoot tips to different levels of PEG treatment. In agreement with our finding, the previous authors also indicated that PEG treatment resulted in more MDA accumulation in micropropagated banana

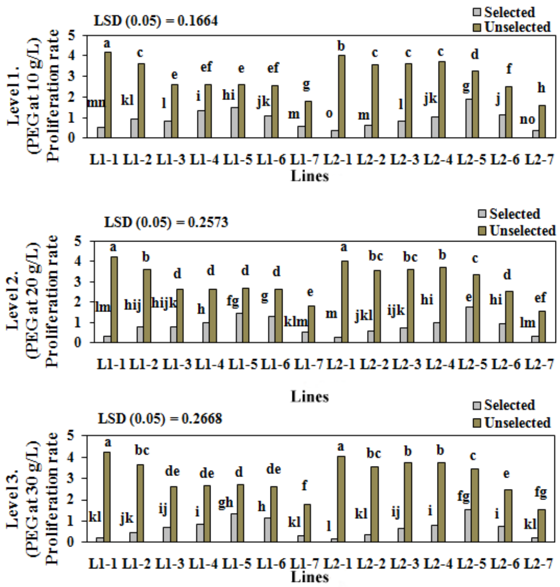


Fig 4. Proliferation rate responses of twelve EMS induced variants of banana $L_{1\text{-}2}$, $L_{1\text{-}3}$, $L_{1\text{-}4}$, $L_{1\text{-}5}$, $L_{1\text{-}6}$, $L_{1\text{-}7}$, $L_{2\text{-}2}$, $L_{2\text{-}3}$, $L_{2\text{-}4}$, $L_{2\text{-}5}$, $L_{2\text{-}6}$, $L_{2\text{-}7}$ as well as their parental cultivars; (‘Berangan Intan’ and ‘Berangan’) according to Table 1 following exposure to the media without the addition of treatment (unselected) and the media with addition of varying concentrations (10, 20 and 30 g L^{-1}) of PEG (selected) for a period of 8 weeks in each level. Each value represents mean of four replicates Different letters indicate values are significantly different at the 0.05 probability level according to the LSD test.

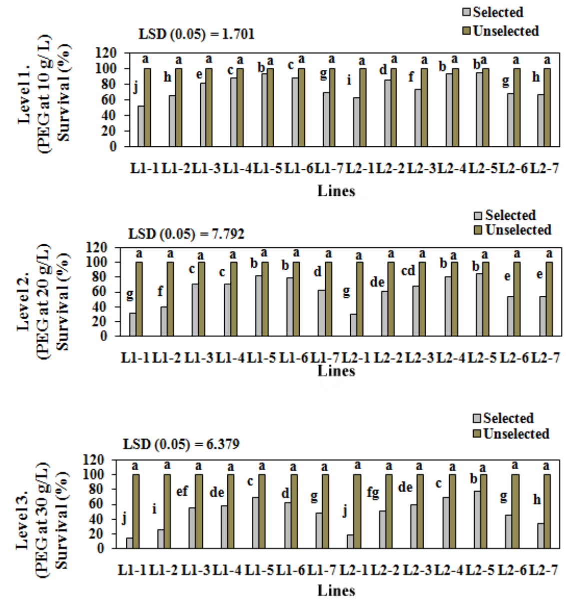


Fig 5. Survival capacity (%) of twelve EMS induced variants of banana $L_{1\text{-}2}$, $L_{1\text{-}3}$, $L_{1\text{-}4}$, $L_{1\text{-}5}$, $L_{1\text{-}6}$, $L_{1\text{-}7}$, $L_{2\text{-}2}$, $L_{2\text{-}3}$, $L_{2\text{-}4}$, $L_{2\text{-}5}$, $L_{2\text{-}6}$, $L_{2\text{-}7}$ as well as their parental banana cultivars (‘Berangan Intan’ and ‘Berangan’) as lines $L_{1\text{-}1}$ and $L_{2\text{-}1}$, respectively according to Table 1 from excised shoot tips subjected to the media without addition of PEG (unselected treatment) and with PEG (10, 20 and 30 g L^{-1}) addition to the media (selected treatment) for a period of 8 weeks in each level. Each value symbolizes mean of four replicates. Different letters indicate values are significantly different at the 0.05 probability level according to the LSD test.

cultivars than their respective cultivars under unstressed condition (Chai et al., 2005). Although the content of MDA was almost the same in both non-mutated parental lines and variants in PEG free medium, $L_{1\text{-}5}$ and $L_{2\text{-}5}$ exhibited the lower amount of MDA even under unstressed condition (Fig 8). Our results showed a significant decrease in the level of MDA in the water stress tolerant lines compared to susceptible and non – mutated parental clones exposed to PEG. The results of lipid peroxidation in the present study are in agreement with the observations of many other authors in the case of banana, rice, pea and wheat responses to different types of stresses (Alexieva et al., 2001; Chai et al., 2005; Wang et al., 2009). When variants and parental lines of banana cultivars were subjected to PEG induced water stress, hydrogen peroxide (H_2O_2) was increased significantly in comparison with unstressed treatment (Fig 8). However tolerant variants $L_{2\text{-}5}$, $L_{1\text{-}5}$, $L_{1\text{-}6}$ and $L_{2\text{-}6}$ showed significantly lower H_2O_2 content (17.04 ± 3.47, 20.52 ± 1.90, 23.98 ± 3.11 and 25.01 ± 2.62 µmol g^{-1} FW, respectively) and the highest level of H_2O_2 was recorded with parental lines $L_{2\text{-}1}$ and $L_{1\text{-}1}$ (47.04 ± 5.01 and 46.03 ± 6.11 µmol g^{-1} FW, respectively) under water stressed condition (Fig 8). Although both non-mutated parental clones and variants did not show significant difference in the content of H_2O_2 under PEG free medium (unselected treatment), $L_{1\text{-}5}$ and $L_{2\text{-}5}$ exhibited the lower H_2O_2 level even under unstressed condition (Fig 8). Increment of H_2O_2 level in response to water stress has been also reported in wheat (Sairam et al., 1998). Our results showed an increase in the levels of MDA and H_2O_2 in banana lines exposed to polyethylene glycol induced water stress, indicating that water stress induces oxidative stress in banana. What is clear from the results it that the MDA content exhibited a positive correlation with the level of H_2O_2 among variants and parental lines in banana (Fig 8).

Morphological disorders caused by in vitro water stress mediated through PEG

The leaves of some growing shoots started to curl with prolonged exposure to PEG induced water stress (Figs 9 F and G). This reaction could be due to a possible mechanism for tolerance against water stress. Tissue browning and leaf rosetting observed among some variants; particularly susceptible and parental lines (Figs 9 C and D) could be the consequence of water loss resulting from exposure of lines to osmotic stress caused by PEG. In addition, water stress caused some morphological disorders such as abnormalities in growing shoot tips and severity of these injuries increased with enhanced concentrations of PEG, especially in non – mutated parental lines (Figs 9 I and J). PEG also resulted in callusing of tissues (Figs 9 K and L) which have been reported as a sign of abnormality (Al-Maarri and Al-Ghamdi, 1996; Joyce et al., 2003). The injurious effect of PEG induced water stress was observed to be more arresting in non-mutated parental lines (Fig 9 C). As reviewed by Joyce et al. (2003) and Hazarika (2006), morphological aberrations and abnormalities in tissue culture could be influenced by water stress due to a lack of chlorophyll. Gaspar et al. (1998) also showed that increasing frequency of abnormality during tissue culture process could be due to high osmotic pressure of culture medium.

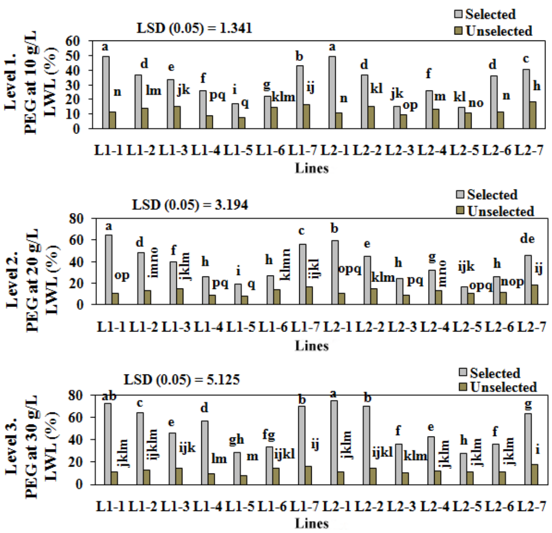


Fig 6. Leaf water loss (LWL%) of twelve EMS induced variants L_{1-2}, L_{1-3}, L_{1-4}, L_{1-5}, L_{1-6}, L_{1-7}, L_{2-2}, L_{2-3}, L_{2-4}, L_{2-5}, L_{2-6}, L_{2-7} accompanied by their parental cultivars; 'Berangan Intan' and 'Berangan' as lines L_{1-1} and L_{2-1} respectively according to Table 1 in presence of 10, 20 and 30 g L^{-1} PEG (selected treatments) and without the addition of treatment to the media (unselected) for a period of 4 weeks in each level. Each value represents mean of four replicates. Different letters indicate values are significantly different at the 0.05 probability level according to the LSD test.

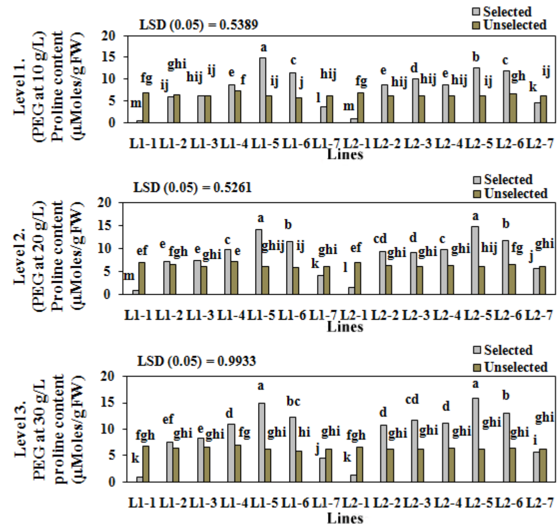


Fig 7. The performance of twelve EMS induced variants of banana L_{1-2}, L_{1-3}, L_{1-4}, L_{1-5}, L_{1-6}, L_{1-7}, L_{2-2}, L_{2-3}, L_{2-4}, L_{2-5}, L_{2-6}, L_{2-7} as well as their parental banana cultivars ('Berangan Intan' and 'Berangan') as lines L_{1-1} and L_{2-1}, respectively according to Table 1 for changes in proline content (µMoles/ g of fresh weight) in presence of 10, 20 and 30 g L^{-1} PEG (selected treatments) and without the addition of treatment to the media (unselected) for a period of 4 weeks in each level. Each value represents mean of four replicates. Different letters indicate values are significantly different at the 0.05 probability level according to the LSD test.

Materials and methods

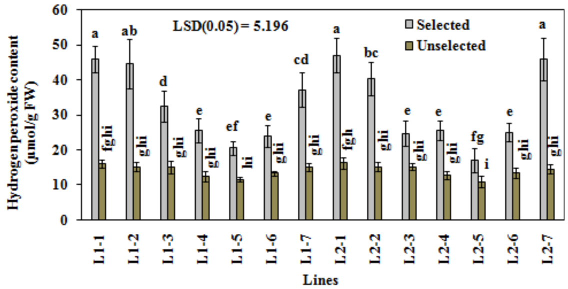
Plant materials, media preparation, EMS treatment of shoot tips and isolation of variants

Micropropagated cultures of banana cultivars; 'Berangan Intan' and 'Berangan' (AAA genome) 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 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 leaves (pseudo stem tissue) at about 5 mm above the apical meristems and then the inclosing tissues (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 (true stem, straight below the apical meristems) so that the final size of initial explants was as uniform as possible (about 6 to 8 mm). Mutagenesis treatments using ethyl methanesulphonate (EMS) (Sigma – Aldrich, Inc) at 150, 200 and 250 mM for 30 and 60 min was carried out after preparing shoot tips. Water based solution containing 1 M of EMS along with 1% v/v dimethyl sulphoxide (DMSO) (Sigma – Aldrich, Inc) as a carrier agent was prepared in the deionized water and then diluted with 0.1 M phosphate buffer (pH 7) (Sigma – Aldrich, Inc) using filter - sterilization under aseptic conditions. The excised shoot tips were submerged in mutagen solutions (EMS) for different periods of 30 and 60 min for each concentration (150, 200 and 250 mM). Subsequently, mutagen was removed by decanting and washing the shoot tips three times with sterile distilled water and then allowed to be dry for 10 min.

Eventually, EMS treated explants were inoculated in 300 ml capacity jars (6 apices/ jar) consisting of 60 ml MS basal salts and vitamins, sucrose at 30 g L^{-1}, solidified with 2.8 g L^{-1} gelrite supplemented with 22.2 µM benzylaminopurine (BAP). Cultures were kept under a controlled environment at 28 ± 2^{o}C with 16 h photoperiod supplemented with cool white florescent light of 1500 Lux for 6 months to allow further proliferation and during this period the subculture of growing EMS - treated shoot tips were achieved at 45 days interval in order to obtain sufficient number of regenerated shoots (variants). The multiplication phase lasted for 6 months and after this period, twelve regenerate lines derived from different dose and duration treatments of EMS, which are listed in Table 1, was used for *in vitro* selection. Water soaked and buffer soaked without the addition of EMS were also served as controls and all shoots regenerated from both treatments was considered as L_{1-1} and L_{2-1} (non mutated parental lines of 'Berangan Intan' and 'Berangan', respectively) according to Table 1

Protocol designed for in vitro selection of tolerant lines against water stress

After *in vitro* mutagenesis and multiplication phase, twelve EMS induced variants of banana 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}, L_{2-7} caused by different dose and duration of EMS treatments as a chemical mutagen as well as their parental lines ('Berangan Intan' and 'Berangan') as L_{1-1} and L_{2-1}, respectively according to Table 1 were obtained. In order to isolate the tolerant lines against water stress, a selection method as a stepwise procedure was designed using three levels of PEG (Sigma – Aldrich, Inc) as a water stress



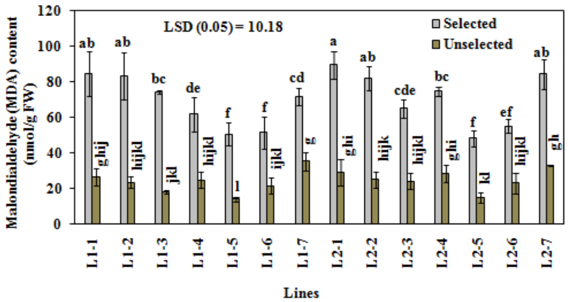


Fig 8. Contents of hydrogen peroxide (H_2O_2) and Lipid peroxidation (measured as malondialdehyde accumulation) in twelve EMS induced variants of banana $L_{1\text{-}2}$, $L_{1\text{-}3}$, $L_{1\text{-}4}$, $L_{1\text{-}5}$, $L_{1\text{-}6}$, $L_{1\text{-}7}$, $L_{2\text{-}2}$, $L_{2\text{-}3}$, $L_{2\text{-}4}$, $L_{2\text{-}5}$, $L_{2\text{-}6}$, $L_{2\text{-}7}$ accompanied by their parental banana cultivars ('Berangan Intan' and 'Berangan') as lines $L_{1\text{-}1}$ and $L_{2\text{-}1}$, respectively according to Table 1 in presence of 30 g L^{-1} PEG (selected treatments) and without the addition of treatment to the media (unselected) for a period of 4 weeks. Each value symbolizes mean of four replicates. Different letters indicate values are significantly different at the 0.05 probability level according to the LSD test.

agent (Fig 1). Fourteen lines were subjected to the stepwise selection contained three levels (10, 20 and 30 g L^{-1}) of PEG, as every level was comprised of two passage stages (Fig 1). At the end of every first passage stage from each level containing shoot growth and proliferation (SGPM) medium, (Fig 1) which lasted for two months, some tissue cultural (morphological) parameters such as the percentage of surviving shoot tips, the number of shoots produced per explant (determined by counting all shoots/ explant), shoot vigour and increase of fresh weight were recorded. This SGPM medium consisted of MS salts and vitamins, 30 g L^{-1} sucrose, 22.2 µM BAP and varying concentrations (10, 20 and 30 g L^{-1}) of PEG solidified with 2.8 g L^{-1} gelrite. Shoot vigour was calculated according to Ebrahim et al. (2006) by taking a rating scale: 1 = no growth, 2 = below average, 3 = average, 4 = above average, 5 = excellent. Since the fresh weight of the initial explants used for every steps of *in vitro* selection need to be as steady as possible, the mean fresh weight of 6 shoot tips derived from mutagenesis experiment for each replication was considered 0.8 (g) at the starting point, then the fresh weight of the proliferated shoots including newly formed leaves after 60 days of culture on shoot growth and proliferation medium (SGPM) minus shoot tips fresh weight at the starting point was served as increase of fresh weight. Shoots out growth from surviving proliferated shoot tips from each first passage stage were isolated and transferred to the second passage stage which considered as plantlet development medium (PDM) containing MS basal salts, vitamins and sucrose at 30 g L^{-1} supplemented with 22.2 µM benzylaminopurine (BAP), 2.7 µM naphthalene acetic acid (NAA) and varying concentrations (10, 20 and 30 g L^{-1}) of PEG solidified with 2.8 g L^{-1} gelrite (Fig 1). At the end of every second passage stage (PDM medium) from each level of water stress agent which lasted for one month, some physiological factors such as proline accumulation, percentage of leaf water loss (LWL%) and ROS production were measured.

Proline determination

Proline content was assessed according to method of Bates et al. (1973). Approximately 0.5 g of leaf samples was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and then the homogenate filtered through whatman filter paper. Two ml of filtrate was reacted with 2 ml acid – ninhydrin and 2 ml of glacial acetic acid in the test tubes for 1 hour under water bath condition at 100°C. Then, the reaction was terminated in the ice bath for 10 minutes. The reaction mixtures were extracted with 4 ml toluene and mixed vigorously for 30 sec. The chromophore containing toluene was aspirated from the aqueous phase and the absorbance was read at 520 nm using toluene as a blank. The standard curve for proline was prepared by dissolving proline in distilled water with covering the concentration range 1 – 10 µg/ml.

Leaf water loss

Percentage of leaf water loss (LWL %) was evaluated according to the method of Xing et al. (2004). Leaf samples were weighed (as fresh weight, W1) immediately after detaching from *in vitro* developing shoots, then the leaves were left to evaporate under room condition for 2 hours and reweighed (W2). LWL was calculated by follow formula:

$$LWL\ (\%) = (W1 - W2)/W1 \times 100$$

Determination of ROS production

The concentration of malondialdehyde (MDA) which is a product of lipid peroxidation was measured by the thiobarbituric acid (TBA) according to Wang et al. (2009). Water stress selected and non water stress selected variants were tested for MDA determination with three replicate plants at the end of Level (3) of selecting agent (PEG at 30 g L^{-1}), as 1 g of fresh leaves were detached from *in vitro* regenerating shoots, placed in a mortar contained 5 ml 0.6% TBA in 10% trichloroacetic acid (TCA) and ground with a pestle. The mixture was heated at 100°C for 15 min. Samples were cooled on ice for 5 min; afterwards, the mixtures were centrifuged at 5000 rpm for 10 min. The absorbance of the supernatant at 450, 532 and 600 nm wavelengths were recorded and MDA content was calculated on a fresh weight by (nmol MDA g^{-1} FW) = 6.45 (OD_{532} – OD_{600}) – 0.56 (OD_{450}) × 1000. Hydrogen peroxide was assessed spectrophotometrically after reaction with potassium iodide (KI), according to method of Velikova and Loreto (2005). Leaf tissues (1 g) were ground and homogenized in a mortar contained 10 ml 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 12000 × g for 15 min. Afterwards, 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml reagent (1 M KI in fresh double distilled water) and then the absorbance of the supernatant was read at 390 nm. The blank

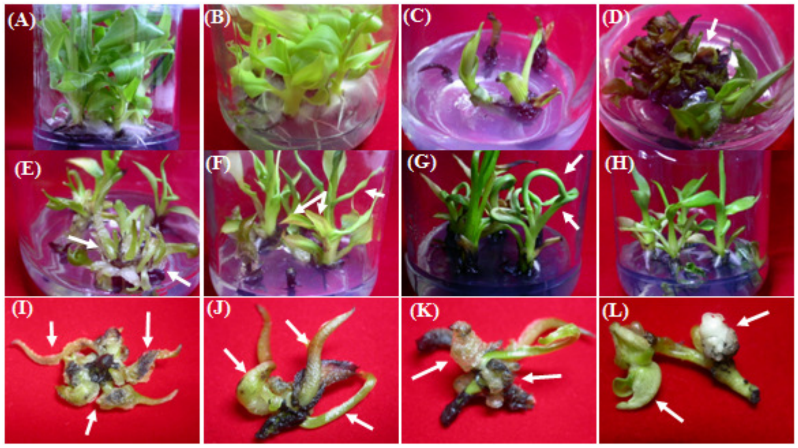


Fig 9. Different developmental patterns distinguished two months after transfer of mutated shoot tips (variants) of banana on shoot growth and proliferation medium (SGPM) supplemented with 30 g L^{-1} PEG (stage 4 according to Fig 1). (A), (B): growing shoot tips of banana cultivar 'Berangan' (parental line L_{2-1}) on the media without the addition of PEG (unselected treatment). (C): parental line L_{2-1} on the medium with addition of 30 g L^{-1} PEG (selected treatment). (D): the line L_{2-7} on the medium with 30 g L^{-1} PEG. Arrow indicates leaf rosetting and tissue browning. (E): clusters of thick, rigid and easily breakable shoots developing at the base of the inoculated variant L_{2-6} (indicated by white arrows) on the medium with addition of 30 g L^{-1} PEG (selected). (F), (G), and (H): variants L_{1-4}, L_{1-5} and L_{2-5}, respectively, growing on the SGPM medium with the addition of 30 g L^{-1} PEG. The folding of the leaves associated with pale green to yellow leaf colour (indicated by white arrows) could be signs of water stress and a possible escaping mechanism in response to water stress. (I), (J), (K), and (L): morphological and physiological disorders (abnormalities) caused by 30 g L^{-1} PEG in the parental line 'Berangan Intan' (L_{1-1}). (I) and (J): cluster of abnormal, hyperhydric and easily breakable shoots developing at the base of the inoculated shoot tips. (K) and (L): callusing of tissues and production of undifferentiated tissues (indicated by white arrows) and deformation of shoot tip in line 'Berangan Intan' (L_{1-1}).

probe consisted of 0.1% TCA in the absence of leaf extract. The content of H_2O_2 was calculated applying a standard curve prepared with identified concentrations of hydrogen peroxide.

Data analysis

Differences in morphological and physiological parameters measured in every step among water stress selected and non-water stress selected lines were examined by analysis of variance (ANOVA) using SAS and MSTAT-C computer programs. Means were separated at 5% significance using the least significant difference (LSD).

Conclusion

This is the first study whereby shoot tip cultures of banana have been subjected to mutagenesis and then selected for water stress tolerance. Therefore, based on our studies including morphological and physiological analysis, we suggest that *in vitro* screening with the induction of chemical drought using PEG (M.W. 6000) to examine water stress tolerance could be a proper track to develop drought-tolerant lines in banana. At the molecular level, further research is needed in this area to inspect whether genetic modifications could be arisen through mutation caused by EMS. Moreover, the promising tolerant lines recognized by this selection method however should be allowed to continue to grow into fully developed plants and then evaluated for tolerance against water stress in the field to confirm the genetic stability of selected lines. The procedure is proportionately easy and inexpensive as it may become a noticeable supply in banana stress tolerance breeding programs.

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