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Original research article

Increasing Al-Tolerance of Sugarcane Using Ethyl Methane Sulphonate and *In Vitro* Selection in the Low pH Media

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ABSTRACT

Increased production of sugarcane in Indonesia can be done with extensification sugarcane plantations which largely dominated by acidic upland red-yellow podzolic soil. High aluminium (Al) content and low pH of the soil can inhibit plant growth and development. Tolerant sugarcane in acid soil is the most efficient way, but the adaptive variety is still limited. *In vitro* culture technique can increase genetic variability to assemble new varieties through somaclonal variation combined with mutation using ethyl methane sulphonate (EMS). The new characters was directed by in vitro selection using $AlCl_3.6H_2O$ with pH = 4 as a component of selection for resistance to high aluminium. VMC 7616 and PS 862 varieties were used as materials. Mutation induced using EMS at concentrations of 0.1%, 0.3%, and 0.5% for 30, 60 and 120 minutes. Plantlets mutant obtained through callus formation, immersion callus in EMS, in vitro selection, and regeneration of callus. Result of study showed that the long immersion in the EMS solution caused greater damage to the cells, as indicated by the change in callus colour. Callus immersion time in EMS gave greater influence to regeneration compared to concentration of EMS. PS 862 had higher Al tolerance than VMC 7616. Rooting of shoot induced using indole-3-butyric acid (IBA) 3 mg/L.

1. Introduction

Sugarcane (*Saccharum officinarum* L.) is an important crop of high economic value, especially Indonesia. The need for increasing sugar always leads Indonesia to import sugar every year. Increased production of sugarcane can be done by using the dry land which is generally dominated by red-yellow podzolic acid soils. In acidic soils, poor crop productivity and low soil fertility are mainly due to the combination of aluminium (Al) and manganese toxicities coupled with nutrient deficiencies (P, Ca, Mg and K) (Mulyani 2006). Among these problems, Al toxicity has been identified as a major growth limiting factor in acidic soils. Al toxicity is a serious problem in low pH acidic soils (<5.5). Because Al can be exchanged, Al_{dd} is relatively high so that it becomes toxic for plants. Low pH and Al toxicity caused short root thickening and inhibited cell elongation process that decreases the absorption of water and nutrients (Marschner 1995). The reclamation of Al toxicity through application of lime is an expensive method, ineffective in the subsoil and in some cases heavy application may have a deleterious effect on the soil structure. Developing cultivars with improved tolerance to acid soil stress is a solution to address this problem, but sugarcane variety which is tolerant to Al toxicity is very limited.

Tissue culture techniques through mutation induction can be used to increase the speed or efficiency of breeding programs to get a new diversity of germplasm (Jain 2010). Mutation induction can be done by using chemical mutagens in combination with the use of growth regulators which have high activity, such as 2,4-D. Ethyl methane sulphonate (EMS), a chemical mutagen of the alkylating group, has been reported to be the most effective and powerful mutagen and usually causes high frequency of gene mutations and low frequency of chromosome aberrations in plants (Van Harten 1998). Because of its potency and ease with which it can be used, EMS is the most commonly used chemical mutagen in plants. Chemicals induce mainly point mutations, and are thus ideal for producing missense and nonsense mutations, which would provide a series of change-of-function mutations (Talebi *et al.* 2012).

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Chemical mutagens were also used by treating banana shoot tips to produce variants for tolerance to Fusarium wilt (Jain 2010). EMS has been successfully used on chrysanthemum, yielding a frequency of 5.2% mutants. A wide range of variations in petal colour (pink-salmon, light-pink, bronze, white, yellow and salmon colour) have been recorded. Luan *et al.* (2007) treated sweet potato (*Ipomoea batatas* L.) and callus with EMS and obtained salt tolerant lines. Cha-um *et al.* (2013) selected six mutant lines of sugarcane which were conducted from gamma irradiation and EMS and resulting in two cultivars namely AE-11 and KK3 that were identified as salt tolerant.

Changes of character that occur due to somaclonal variation and mutation happen in random. To direct the change of characters that occurred, *in vitro* selection by using chemicals as agent of selection can be done. This method is more effective and efficient because it made more directional selecting properties. *In vitro* selection to obtain new varieties which are resistant to acid soil can be done using $AlCl_3.6H_2O$ and low acidity (pH = 4) as a component selection (Purnamaningsih and Mariska 2005; Purnamaningsih and Mariska 2008; Jain 2010). The evaluation of Al tolerance in tissue culture may be more useful for breeding programs, because selection is earlier and faster in tissue culture than in the field. Moreover, the selection by tissue culture can be applied to identify Al-tolerant plants in segregating populations. Several studies have indicated that Al-tolerant plants can be identified by comparing growth of callus in an acidic medium with and without added Al. This suggests that similar mechanisms of Al tolerance are active in both cell cultures and whole plants (Singh *et al.* 2011). The aim of the research was to produce sugarcane mutant genotypes which are tolerant to Al stress using *in vitro* mutagenesis and *in vitro* selection method.

2. Materials and Methods

2.1. Mutation induction

Research was conducted in Tissue Culture Laboratory of ICA-BIOGRAD in 2013. Materials used were sugarcane varieties VMC 7616 and PS 862. Callus was induced from sugarcane leaves which still curled using MS medium added with 2,4-D 3 mg/L and casein hydrolyzate (CH) 3 g/L. To increase the genetic variability of callus, cell population were mutation-induced using EMS at concentrations 0.1%, 0.3%, and 0.5% for 30, 60, and 120 minutes by immersion. Fifty callus were used in each treatment. Mutated callus was cultured back on MS medium + 2,4-D 3 mg/L + CH 3 g/L for 3 weeks to induce somaclonal variability. Sub-cultures were performed every 2 weeks until the age of 2 months. Percentage of live callus and callus colour were observed.

2.2. *In vitro* selection

Callus mutant were transferred to callus induction media added with $AlCl_3.6H_2O$ as a component selection and low pH (4.0) at five level concentrations of 0, 100, 200, 300, 400, and 500 ppm with 20 replications. To bring out the toxicity of Al in the selection media, macroelements in MS medium were modified: NH_4NO_3 was increased from 1650 to 2400 mg/L, $CaCl_2.2H_2O$ was decreased from 440 to 15 mg/L and KH_2PO_4 was decreased from 170 to 13 mg/L. $FeSO_4$ 28 mg/L was used as a source of Fe. The medium used for selection was MS + 2,4-D 3 mg/L + CH 3 g/L + component selection.

2.3. Regeneration of callus after mutation and *in vitro* selection

The live callus on the selection medium were transferred to regeneration media using MS medium added with BA 0.5 mg/L + GA3 0.5 mg/L. Root induction was done by adding IBA 3 mg/L in the MS basic medium. Randomized block design was used in this experiment with 10 replications. Percentage of viable callus, number of callus regeneration, number of shoots, shoot height and root were observed visually.

3. Results

Callus which was soaked in EMS solution showed different growth responses depending on the time of immersion and concentration used. Initial response showed growth of callus discoloration. In generally callus from the VMC 7616 was very sensitive to EMS treatment. The callus colour changed into brown or black within 4 weeks ranged between 30% and 100% (Table 1). Percentage of viable callus of VMC 7616 was lower than that of PS 862 which had higher tolerance to EMS treatment. Callus was still viable after it was soaked in 0.1% EMS solution for 60 minutes, and changed to brown if time and EMS concentration increased (Table 2). Callus without EMS treatment (control) cannot live after growing in media with Al. The callus becomes brown and black (not viable) because of high Al content in the media. These indicate that callus of VMC 7616 and PS 862 varieties were sensitive to Al.

Callus putative mutant PS 862 or VMC 7616 which were able to withstand the stress of Al and low pH could regenerate to form shoots after being transferred to the regeneration medium (Tables 3 and 4). The ability of callus to regenerate depends on the mechanism of tolerance of each cell to cope with high Al availability on selection media. Immersion time in EMS was more influential to callus regeneration than the concentration of Al used. Increasing Al concentration resulted in reduction of shoot growth. Among callus which could survive on selection media, few could not differentiate (Figures 1 and 2). From this study, there were 206 shoots of putative mutant derived from PS 862 variety (Table 5).

Shoots putative mutants should have a good root system in order to grow well at the time of acclimatization in the greenhouse. Nearly 50% of the shoots of putative mutants were unable to form roots when cultured on regeneration medium, therefore shoots were grown on media rooting induction which used IBA 3 mg/L. Speed of root formation depends on the growth ability of each plant.

Table 1. The colour of callus after immersion in EMS solution

Time of immersion in EMS solution	Colour of callus
VMC 7616	
Without immersion	White
30 min, 0.1%	White
30 min, 0.3%	White
30 min, 0.5%	Brownish white
60 min, 0.1%	Brownish white
60 min, 0.3%	White
60 min, 0.5%	Brownish white
120 min, 0.1%	Brownish white
120 min, 0.3%	Brown, black
120 min, 0.5%	Brown, black
PS 862	
Without immersion	White
30 min, 0.1%	White
30 min, 0.3%	White
30 min, 0.5%	White
60 min, 0.1%	White
60 min, 0.3%	Brownish white
60 min, 0.5%	Brownish white
120 min, 0.1%	Brownish white
120 min, 0.3%	Brown
120 min, 0.5%	Brown, black

EMS = ethyl methane sulphonate.

Table 2. Percentage of viable callus putative mutant of VMC 7616 and PS 862 after selection with five aluminium concentrations at 1 week

Time of immersion in EMS/EMS concentration	Life of callus (%)				
	Al concentration (mg/L)				
	100	200	300	400	500
VMC 7616					
Without immersion	0	0	0	0	0
30 min, 0.1%	0	0	0	0	0
30 min, 0.3%	0	0	0	0	0
30 min, 0.5%	100	0	100	50	0
60 min, 0.1%	0	0	0	0	0
60 min, 0.3%	0	0	30	30	50
60 min, 0.5%	0	0	0	0	0
120 min, 0.1%	100	50	30	0	100
120 min, 0.3%	0	0	50	30	0
120 min, 0.5%	0	0	0	0	0
PS 862					
Without immersion	0	0	0	0	0
30 min, 0.1%	100	100	100	100	100
30 min, 0.3%	100	100	100	66.7	33.3
30 min, 0.5%	50	100	100	50	50
60 min, 0.1%	100	100	100	100	100
60 min, 0.3%	100	66.7	66.7	100	33.3
60 min, 0.5%	50	50	100	66.7	33.3
120 min, 0.1%	33.3	33.3	66.7	33.3	66.7
120 min, 0.3%	0	0	0	0	0
120 min, 0.5%	0	0	0	0	0

EMS = ethyl methane sulphonate.

4. Discussion

Mutation is one way to improve genetic variability in complement to plant breeding (Singh *et al.* 2011). The chances of a mutation depend on the number, age, and the growth stages of plant which are used as plant material (explant). The use of callus as plant material for mutation induction is very effective because the callus is population of unorganized mass of cells that have not undergone differentiation and divide continuously (Jain 2001; Mattjik 2005). Callus was also very sensitive to mutagen because the cells are actively dividing so that the chance of a mutation is very large (Patade and Suprasanna 2008).

One of the most effective chemical mutagen and has been used on various types of plants is EMS (Talebi *et al.* 2012; Bashir *et al.* 2013; Soeranto 2003). EMS can cause point mutations and a little damage to the chromosomes that are very favourable for breeding activities. EMS is often used because it is easy to get, cheap and does not leave toxins after hydrolyzate (Van Harten 1998; Medina *et al.* 2005). According to Svetleva and Crino (2005) induction of mutations combined with *in vitro* culture is a favourable method because it can increase the frequency of formation of new variations. *In vitro* culture technique can produce somaclonal variation and this variation can be improved by physical or chemical mutagen.

The use of mutagen often causes cell damage and thus affects the ability of the putative mutant cell regeneration (Nikam *et al.* 2015). The ability of each cell to regenerate shoots depends on the level of sensitivity of each cell. Our research showed that callus putative mutants from PS 862 had the ability to regenerate higher than callus from the VMC 7616. Other factors was the ability of the cells to withstand the stress of the selection agent used, in this case were Al and low pH. Media containing Al with pH arranged at 4 will cause macro nutrient contained in the media was not available and could not be used for the growth of these cells. Regeneration ability of cells to form shoots depends on the mechanism of tolerance of each cell to cope with high Al availability on selection media (Purnamaningsih and Mariska 2005).

Table 3. Shoots formation from VMC 7616 viable callus after EMS and aluminium treatments at 8 weeks

Time of immersion in EMS (min)	EMS concentration (%)	Aluminium concentration (mg/L)	Average number of shoot	Shoot height (cm)
30	0.5	0	6 b	1.0 b
		100	4 cd	0.7 c
		300	3.0 de	0.7 c
		400	9 a	0.6 cd
		500	5 bc	0.5 d
60	0.3	0	5 bc	0.7 c
		300	5 bc	0.5 d
		400	6 b	2.0 a
		500	2 e	1.0 b
	0.5	100	9 a	1.0 b
120	0.1	100	2.0 e	0.5 d
		200	1.0 fe	1.0 b
		300	4.33 c	1.0 b
		500	3.0 de	1.0 b

EMS = ethyl methane sulphonate.
Numbers followed by the same letter in the same column do not differ according to the Duncan test at 5% level.

Table 4. Shoots formation from PS 862 variety at various concentrations of aluminium, soaked in EMS for 8 weeks

Time of immersion in EMS (min)	EMS concentration (%)	Aluminium concentration (mg/L)	Average number of shoot	Shoot height (cm)
30	0.1	0	10 defghijk	2.0de
		100	24 ab	2.5 bcd
		200	23 abc	1.7 e
		300	16.33 cde	3.0 b
		400	16.3 cde	2.0 de
		500	5 hijkl	1.0 fg
30	0.3	0	6 ghijkl	1.0 fg
		100	21.67 abc	0.2 j
		200	14.33 def	4.0 a
		300	14.33 def	0.7 fghij
		400	11.3 defghij	1.0 fg
		500	4.0 ijkl	1.0 fg
30	0.5	100	7.33 fghijkl	0.3 ij
		200	12.0 defghi	0.2 j
		300	13.0 defgh	2.0 de
		400	9.0 efghijkl	0.5 ghij
		500	7.0 fghijkl	1.2 f
60	0.1	0	10.0 defghijk	0.9 fgh
		100	17.0 bcd	1.8 e
		200	11.67 defghi	2.2 cde
		300	8 .0 fghijkl	3.0 b
		400	12.67 defgh	4.3 a
		500	4.0 ijkl	2.6 bc
60	0.3	100	13.67 defg	2.0 de
		200	24.67 a	0.8 fghi
		300	17.5 defghi	3.1 b
		400	12.0 defghi	2.17 cde
		500	8.0 fghijkl	0.4 hij
60	0.5	100	9.0 defghijkl	0.7 fghij
		200	8.0 fghijkl	0.3 ij
		300	1.67 l	0.3 ij
		400	14.5 defghijk	2.5 bcd
		500	3.0 kl	1 fg
120	0.1	0	7.0 fghijkl	1.8 e
		100	3.67 jkl	0.5 ghij
		200	6.0 hijkl	0.5 ghij
		300	7.0 fghijkl	0.4 hji
		400	2.67 kl	0.6 ghij
		500	7.0 fghijkl	0.6 ghij
	0.3	500	6.0 ghijkl	0.37 hij
	0.5	0	5.0 hijkl	2.17 cde

EMS = ethyl methane sulphonate.
Numbers followed by the same letter in the same column do not differ according to the Duncan test at 5% level.

There was a tendency that the higher concentration of Al, caused fewer number of shoots produced. Similar result was obtained by Talebi *et al.* (2012) in the induction of mutations in Malaysian Rice (cv. MR219). Callus which can regenerate after selection with high Al can indicate genetic changes within its cells. EMS treatment activated certain genes that function in the mechanism of tolerance to Al toxicity. According to Singh and Chauhan (2011) one of the tolerance mechanisms of plant to Al is by releasing the organic acids that are localized in root tips to detoxify Al toxicity. The same mechanism occurs in sugarcane variant which

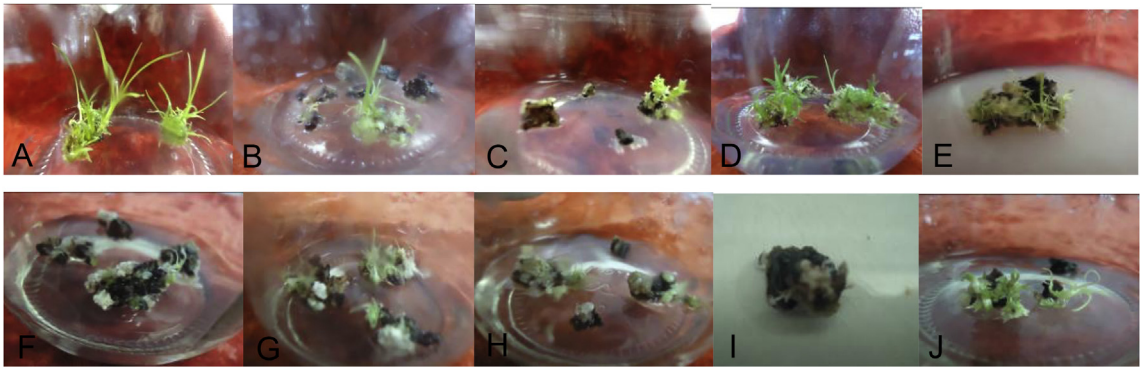

Figure 1. Putative mutant shoots produced from callus of VMC 7616 on selection media with the addition of aluminium. (A) 30 minutes, 0.5% Al 0 mg/L. (B) 30 minutes, 0.5% Al 300 mg/L. (C) 30 minutes, 0.5% Al 400 mg/L. (D) 30 minutes, 0.5% Al 500 mg/L. (E) 60 minutes, 0.5% Al 100 mg/L. (F) 60 minutes, 0.3% Al 300 mg/L. (G) 120 minutes, 0.1% Al 100 mg/L. (H) 120 minutes, 0.1% Al 200 mg/L. (I) 120 minutes, 0.1% Al 300 mg/L. (J) 120 minutes, 0.1% Al 500 mg/L.

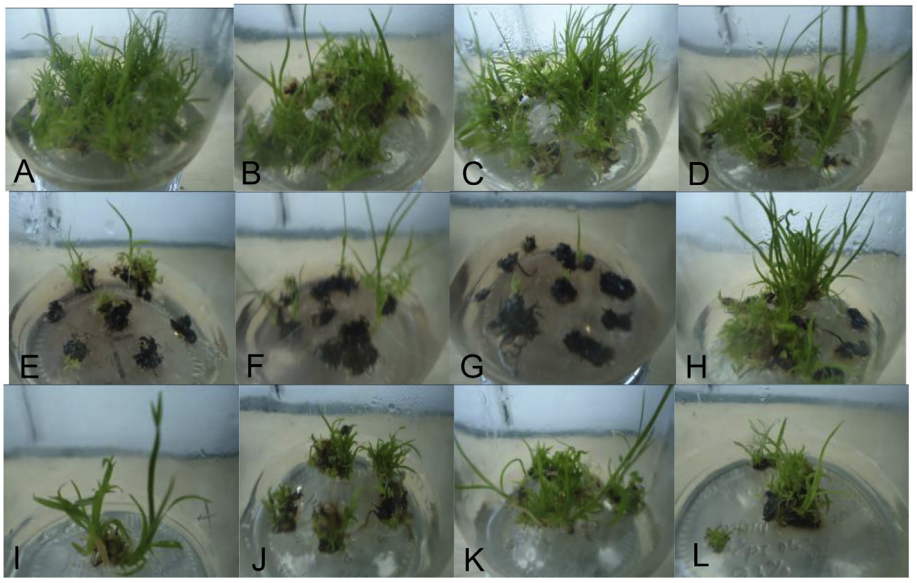

Figure 2. Putative mutant shoots produced from callus of PS 862 on selection media with the addition of aluminium. (A) 30 minutes, 0.1% Al 100 mg/L. (B) 30 minutes, 0.1% Al 200mg/L. (C) 30 minutes, 0.1% Al 300 mg/L. (D) 30 minutes, 0.1% Al 400 mg/L. (E) 30 minutes, 0.5% Al 100 mg/L. (F) 30 minutes, 0.5% Al 100 mg/L. (G) 30 minutes, 0.5% Al 300 mg/L. (H) 30 minutes, 0.5% Al 400 mg/L. (I) 120 minutes, 0.3% Al 100 mg/L. (J) 120 minutes, 0.3% Al 200 mg/L. (K) 120 minutes, 0.3% Al 300 mg/L. (L) 120 minutes, 0.3% Al 500 mg/L.

Table 5. Number of shoots putative mutants of sugarcane after EMS treatment and *in vitro* selection

Variety of callus/time of immersion of EMS	Number of regeneration of callus	Number of shoot	Average of shoot/callus
VMC 7616			
30 min	50	83	1.66
60 min	50	81	1.62
120 min	22	42	1.90
PS 862			
30 min	170	644	3.78
60 min	160	495	3.09
120 min	48	136	2.83

EMS = ethyl methane sulphonate.

is tolerant to saline soils which can minimize the transport of Cl^- from roots to shoots that do not inhibit the growth of plants, thus probably the plant has genetic potential to avoid harmful ions accumulation (Shomelli *et al.* 2011).

Among callus which could survive on selection media, few could not differentiate. Callus derived from VMC 7616 varieties were very sensitive to the treatment of immersion in the EMS and only few shoots can be obtained, both in treatment selection with Al or unselected. In contrasts, callus derived from PS 862 variety had better ability to regenerate. Those callus could regenerate to form shoot on selection medium with various concentrations of Al level, except the EMS immersion for 120 minutes with EMS concentrations of 0.3% and 0.5%.

Mutation *and in vitro* selection using Al reduced the ability of callus to forming shoots and root. Yasmin *et al.* (2011) showed that sugarcane callus treatment with 30Gy and 40Gy radiation exhibited negative impact on the tillering potential of the plant and potential of shoot to form roots. The maximum root length (10.3 cm) was obtained in control at 2 mg/L IBA. Similar result was obtained from our research. Root induction of putative mutants was very difficult and it formed after induced with IBA 3 mg/L. IBA is one type of auxin that has a strong activity because has a long persistence power (Nandagopal and Kumari 2007). The roots which were formed was thick and long with white colour.

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