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Field Evaluation of Aluminium Stress Response of EMS-Mutated Sugarcane Clones

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Abstract: Sugarcane is an important crop in Indonesia. Protocols were established to induce callus and treat with Ethyl Methane Sulphonate (EMS). Calli were induced and immersed in 0.1, 0.3 and 0.5% EMS solutions for 30, 60 and 120 min. PS 862 and VMC 7616 sugarcane varieties were used in this experiment. Mutant putative calli were selected on media with aluminium concentrations of 100, 200, 300, 400 and 500 ppm and then cultured on regeneration media. Sugarcane mutant plants were evaluated for its aluminum tolerance by planting them in acidic soil (pH of 4.1). This research showed that increasing EMS concentration reduced the regenerative capacity of sugarcane calli. Calli of PS 862 had a higher aluminium tolerance capacity compared to calli of VMC 7616. Mutant putative shoots could be induced to produce roots by treatment with 5 mg L^{-1} IBA. Field evaluation showed that mutant clones had higher agronomical characters value than mother plants which was due to the EMS-induced genetic mutation. Mutant clones had plant heights in the range of 2.8-4.3 m, the number of shoots 3-12, the number of nodes 9-16 and stem diameters 1.3-2.8 cm. Meanwhile, PS 862 and VMC 7616 (mother plants) had plant heights 2.9 m and 2.97 m, number of shoots 3.7 and 1.67; nodes number 8.14 and 7.33 and stem diameters 1.27 and 1.33 cm. The Brix values of mutant clones were in the range of 12-20% and fresh weights were 900-2610 gr while brix content of PS 862 and VMC 7616 (mother plants) were 16 and 15% and the fresh weight were 1419 and 1650 g.

Key words: Sugarcane, EMS, *in vitro* selection, acid soil, crop in Indonesia, EMS solutions

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is an important crop in Indonesia. The rapid increase of human population caused a higher demand of sugar. Production of sugarcane could be increased by utilizing dry lands which are available in a great amount in Indonesia and generally dominated by Red Yellow Podzolic acidic soil. In acidic soil, low crop productivity and soil fertility are mainly due to the combination of aluminium and manganese toxicities coupled with nutrient deficiencies (P, Ca, Mg and K) (Mulyani, 2006). Aluminium (Al) toxicity is a serious problem in an acidic soil which has low pH (<5.5) because, Al could be rapidly exchanged with Al^{3+} which is toxic to plants. The toxicity of Al and low soil pH could inhibit elongation of root cells which therefore reduce the rates of water and nutrient absorption and resulted in the thickening and shortening of roots (Zhang *et al*., 2007). The reclamation of soil's aluminium toxicity through the application of lime is an expensive method and also ineffective if applied in the subsoil. In some cases, heavy application of lime could otherwise destruct the soil structure. The best way to solve Al toxicity problem is by developing aluminium tolerant crop cultivars.

In vitro culture techniques in association with mutation can speed up the generation of many varieties of vegetatively propagated crops (Qosim *et al*., 2007; Jain, 2010; Suriyan *et al*., 2013). Ethyl Methane Sulphonate (EMS) is a chemical mutagen which belongs to the alkylating group and has been reported as the most effective and powerful mutagen because it is able to induce high frequency of gene mutations and low frequency of chromosome aberrations in plant's cells (Van Harten, 1998; Talebi *et al*., 2012). The combination of somaclonal variation and mutation will produce mutant plants which have random characters. Therefore, mutant plants need to be selected for obtaining clones which have the desired character. *In vitro* selection for obtaining new plant varieties which are resistant to acidic soil could be done by using acidic selection medium and supplementing it with $AlCl_3.6H_2O$ (Purnamaningsih and

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Mariska, 2005, 2008). Mutant plants should be evaluated in a field by planting them in the acidic soil for assessing the growth and potential of each mutant genotype clones. Field evaluation was done in acidic soil (pH 4.1) which has Al content 5.12 cmol kg^{-1} and base saturation level 14%. This research aimed to analyze the effect of EMS application and *in vitro* selection against the regenerative capacity of sugarcane calli and evaluate the growth and productivity of the obtained sugarcane mutant genotypes in the field.

MATERIALS AND METHODS

The plant materials used in this research were sugarcane varieties VMC 7616 and PS 862. Calli were induced from coiled sugarcane leaves by culturing them in Murashige Skoog (MS) media which were supplemented with 3 mg L^{-1} 2,4-D and 3 g L^{-1} casein hydrolysate. For increasing genetic diversity, calli were mutated by immersing them in 0, 0.3 and 0.5% EMS solution for 30, 60 and 120 min. Mutated calli were then subcultured on the same media to induce somaclonal variation and subcultured them once in every two weeks for two months.

Mutated calli were subcultured to selection media, i.e., MS+3 mg L^{-1} 2,4-D+3 g L^{-1} casein hydrolysate (pH 4.0) which were supplemented by 0, 100, 200, 300, 400 and 500 mg L^{-1} $AlCl_3.6H_2O$ (repeated 20 times each). Viable calli on selection media were subcultured to regeneration media, i.e., MS supplemented with 0.5 mg L^{-1} BA+0.5 mg L^{-1} GA_3. Roots were induced in MS media supplemented with 3, 5 and 7 mg L^{-1} of IBA or NAA. Parameters observed were the number of shoots, plant height, number of roots, root length and root morphology.

Plantlets were acclimatized in polybags which contain soil and organic fertilizer. Growth and productivity of mutant genotypes were evaluated by planting them in acidic soil (pH of 4.1) which contains a high amount of aluminium (5.12 cmol kg^{-1}) and has a low base saturation (14%). Parameters observed were the plant height, number of shoot, number of nodes, stem diameter, shoot fresh weight and brix content.

RESULTS AND DISCUSSION

Mutation induction with EMS: Calli which had been immersed in EMS solution showed various growth responses which were determined by the immersion time and EMS concentration. Initial growth response could be analyzed by observing the changing of calli's color. Generally, calli of VMC 7616 variety were very sensitive to EMS. EMS could damage the cells, so their color will become chocolate or black and be unable to regenerate. The regenerative capacity of mutated VMC 7616 calli was lower than that of PS 862. Cells which could survive in the aluminium stress and low pH condition will be able to regenerate and produce shoots in the regeneration media (Table 1 and 2). Regenerative capacity depends on the tolerance mechanism of each cell to survive living in the selection medium which contains a high amount of Al (Purnamaningsih and Mariska, 2005). The higher the aluminium concentration, the lesser the shoots produced.

Table 1 and 2 show that the numbers of shoots regenerated from calli of sugarcane varieties VMC 7616 and PS 862 were different to each other. This was due to the different types genetic mutations among mutated cells. Longer immersion time and higher aluminium concentration decreased the regenerative capacity of shoots. However, calli which were not immersed in EMS solution could not regenerate and did not produced shoots in aluminium selection media. The ability of calli to regenerate in media which contain a high amount of aluminium was due to the mutation of cells. Mutated calli have undergone genetic expression modification, including activation of genes which confer aluminium's toxicity tolerance ability. Due to this genes activation, calli were able to regenerate in selection media which contain a high amount of aluminium.

Not all of the regenerated calli in selection media could differentiate to become a structurally complete plant. Calli of sugarcane VMC 7617 were very sensitive to EMS so they produced lesser shoots than calli of PS 862. Mutation induction is one of the ways to increase genetic diversity for obtaining superior plant varieties (Singh *et al*., 2011). The mutation possibility of a plant depends on the plant explant's type, age, part and growth phase. One of the most effective chemical mutagens is EMS which has also been used for inducing mutation of a wide variety of plants (Talebi *et al*., 2012; Bashir *et al*., 2013). EMS is very beneficial for plant breeding because it could induce point mutations but few chromosome aberrations. EMS is commonly used because of its availability, cheap price and non-toxicity (Van Harten, 1998). According to Svetleva (2005), mutation induction combined with *in vitro* culture is very effective to generate many new plant varieties. Technically, *in vitro* culture method is able to produce somaclonal varieties and the variation could be further increased by applying mutagen.

Mutagen usually diminishes cell viability and therefore, reduce the regenerative capacity of mutant putative cells. The regenerative capacity of each cell is determined by their sensitivity to a given stress

Table 1: Number of shoots and shoot length produced from mutated calli of VMC 7616 which have been immersed in EMS solution at various concentrations of aluminium (parameters were observed at 8th week)

EMS immersion time (min)	EMS concentration (%)	Aluminium concentration (mg L^{-1})	Number of shoots/calli	Shoot length (cm)
30	0.5	0	6.00^{b}	1.0^{b}
		100	4.00^{cd}	0.7^{c}
		300	3.00^{de}	0.7^{c}
		400	9.00^{a}	0.6^{cd}
		500	5.00^{bc}	0.5^{d}
60	0.3	0	5.00^{bc}	0.7^{c}
		300	5.00^{bc}	0.5^{d}
		400	6.00^{b}	2.0^{a}
		500	2.00^{e}	1.0^{b}
60	0.5	100	9.00^{a}	1.0^{b}
120	0.1	100	2.00^{e}	0.5^{d}
		200	1.00^{fe}	1.0^{b}
		300	4.33^{c}	1.0^{b}
		500	3.00^{de}	1.0^{b}

Table 2: Number of shoots and shoot length produced from PS 862 mutated calli which have been immersed in EMS solution at various aluminium concentrations (parameters were observed at 8th week)

EMS immersion time (min)	EMS concentration (%)	Aluminium concentration (mg L^{-1})	Number of shoots/calli	Shoot length (cm)
30	0.1	0	$10^{defghijk}$	2.0^{de}
		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}
120	0.3	500	6.0^{ghijkl}	0.37^{hij}
120	0.5	0	5.0^{hijkl}	2.17^{cde}

[a-j]Numbers followed by the same letter at the same column are not significant to each other based on Duncan test at 5% significance level

(Gadakh *et al*., 2015) such as aluminium stress. In this research, calli of PS 862 had a higher regenerative capacity compared to those of VMC 7616. The presence of aluminium in the growth media inhibited the absorption of nutrient for plant's cell growth. The ability of a cell to regenerate and become shoot depends largely on its

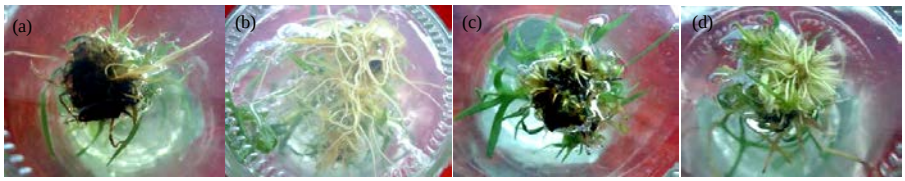

Fig. 1: Root induction of sugarcane *in vitro* plant by IBA and NAA a = IBA 3 mg L^{-1}; b = IBA 5 mg L^{-1}; c = NAA 3 mg L^{-1}; d = NAA 7 mg L^{-1}

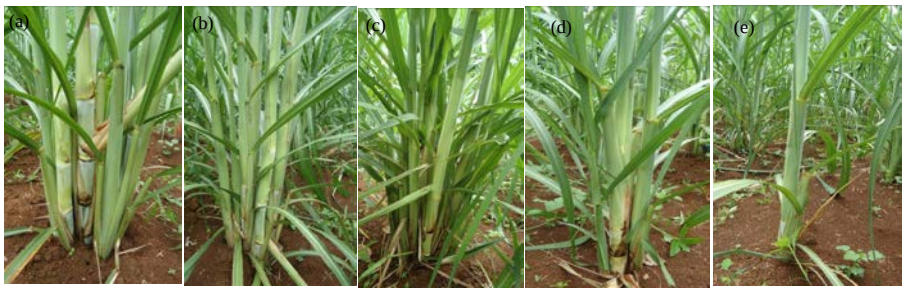

Fig. 2: Growth of mother plants and MV1 somaclonal mutant clones in Jasinga (4th month) a-c = somaclonal mutant clones, d = PS 862 (control), e = VMC 7616 (control)

Table 3: Root induction of mutant putative shoots by using different types and concentrations of auxin

Media formulation (mg L^{-1})	No. of roots	Root length (cm)
MS+IB A 3	9.6	0.63
MS+IBA 5	13.0	0.85
MS+IBA 7	8.5	0.61
MS+NAA 3	8.5	0.80
MS+NAA 5	11.2	0.31
MS+NAA 7	20.0	0.15

aluminium tolerance mechanism which determined its survival in the selection medium which contains high aluminium concentration (Purnamaningsih and Mariska, 2005).

Roots induction and plantlet acclimatization in greenhouse: Mutant putative shoots should had a fully functional root system in order to grow maximally by the time they were acclimatized in greenhouse. A good root system will support the growth of the plant in soil. Mutant putative shoots had a different root-producing capacity. Most of the mutated shoots could not produce roots in the regeneration media, so they should be induced by adding auxin, i.e., IBA or NAA. The rate of root growth depends on the type and concentration of auxin. Application of 7 mg L^{-1} NAA resulted in the most number of roots. However, roots produced in this media were short and thick. NAA is a synthetic auxin-type plant growth regulator which has a strong activity due to its long-term availability in the medium (Wattimena, 1988). According to Yan *et al.* (2014), NAA is commonly used at a relatively low dose to induce cell division.

Table 3 showed that the higher the concentration of IBA or NAA, the shorter the produced roots. This phenomenon was probably due to the high amount of auxin which exerted a negative effect on root elongation. IBA induced production of normal roots while NAA resulted in thicker roots (Fig. 1) which was probably due to the stronger activity of NAA than IBA. A plant with perfect root system will be able to survive growing in the greenhouse when acclimatized.

Growth of sugarcane somaclonal mutants in acidic soil: Plant growth is strongly determined by the availability of nutrients. Therefore, plants which can not absorb nutrients will not be able to grow maximally, as observed from low values of plant height, number of shoots and nodes. Most of MV1 somaclonal mutant clones were able to grow, but they produced a different number of shoots and nodes (Table 4 and Fig. 2). Mutant clones had plant heights in the range of 2.8-4.3 m, number of shoots 3-12, number of nodes 9-16 and stem diameter 1.3-2.8 cm. Meanwhile, PS 862 and VMC 7616 (mother plants) had plant heights 2.97 and 2.9 m, shoots number 3.7 and 1.67 nodes number 8.14 and 7.33 and stem diameters 1.27 and 1.33 cm. Brix values of mutant clones were in the range of 12-20% while the fresh weights were in the range of 900-2610 g (Table 4).

Table 4: Growth characters and Brix values of MV1 sugarcane mutant genotypes

Mutant clone	Plant height	Number of shoots	Number of nodes	Stem diameter (cm)	Plant's fresh weight (g)	Brix value (%)
84-2	4.3	5	13	2	2550	12
39-1	2.9	5	12	1.5	1180	13
41-2	3.7	10	10	1.3	900	14
27-3	3.6	6	12	2	1650	14
74-4	3.7	6	13	2.1	1800	14
24-1	3.6	5	13	2	1100	15
72-7	3.8	6	12	1.6	1300	15
72-5	3.7	3	16	2.4	2610	15
27-2	2.8	6	11	1.8	1360	15
23-3	3.7	7	15	1.5	1410	15
84-1	4.2	8	13	1.8	1450	15
41-1	3.3	12	10	1.6	1460	15
48-3	4.3	7	15	2.8	2710	15
48-1	3.7	3	13	2	1720	16
24-2	3.7	4	14	2	1350	16
79-2	3.9	3	12	2	2090	16
31-3	3.7	9	9	2	1010	16
82-1	4.1	6	10	2	1530	16
38-2	3.7	6	11	2.3	1700	16
39-3	3.7	9	12	1.4	1140	16
24-3	3.8	6	14	1.7	1770	16
47-2	3.5	8	15	1.6	1400	16
60-3	3.6	6	13	1.8	2040	16
29-4	3.7	7	13	1.7	1880	16
74-6	3.7	7	15	2.2	2300	16
48-2	3.7	7	14	2.3	2300	16
29-2	3.5	3	10	1.6	1410	17
23-2	3.7	5	13	2	1250	17
47-3	3.7	8	12	1.7	1150	17
27-1	3.8	5	13	1.5	1990	17
74-3	3.6	8	12	2.1	1310	17
60-1	3.3	6	13	2	1850	17
70-4	3.8	7	12	1.6	1780	17
79-4	4.1	5	12	2.4	2540	17
72-6	4.3	7	15	2	1880	17
26-5	3.8	8	13	2.2	2190	17
26-1	3.7	4	15	2.1	1740	18
82-4	3.8	6	10	1.5	1190	18
A-1	3.8	5	13	2.4	1650	18
60-2	3.8	7	12	2	1250	18
74-5	3.7	7	13	2.1	1300	18
B-1	3.8	6	14	2.3	1720	18
76-1	3.0	3	9	2	1210	19
81-1	3.8	4	12	2.1	2090	19
31-1	3.7	7	11	1.7	1620	19
87-2	3.8	8	12	2	1500	19
40-2	3.8	7	11	2.4	2300	19
89-2	3.8	8	13	2	2250	19
18-1	3.2	3	13	1.6	1650	20
42-4	3.7	7	13	2	1820	20
Mother plants						
PS 862	2.9	3.7	8.14	1.27	1417	16
VMC 7616	2.97	1.67	7.33	1.33	1650	15

CONCLUSION

The process for obtaining mutant plantlets was as follows: calli induction, calli immersion in EMS solution, *in vitro* selection by using $AlCl_3.6\ H_2O$ and regeneration of mutated calli. Longer immersion time and higher EMS concentration will further destruct cells. The higher the aluminium concentration, the lower the regeneration capacity of calli. PS 862 had a higher aluminium tolerance capacity than VMC 7616. Roots of mutant putative shoots could be induced by supplementing MS media with 5 mg L^{-1} IBA. Field evaluation showed increasing value of agronomic character of mutants, which was caused by genetic mutation of EMS-treated calli. Mutant clones had plant heights in the range of 2.8-4.3 m, number of shoots 3-12, nodes 9-16 and stem diameter 1.3-2.8 m. Meanwhile, PS 862 and VMC 7616 (mother plants) had plant heights 2.9 m and 2.97 m, number of shoots 3.7 and 1.67; nodes number 8.14 and 7.33; and stem diameters 1.27 and 1.33 cm. The Brix values of mutant clones were in the

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