Responses of Soybean Mutant Lines to Aluminium under *In Vitro* and *In Vivo* Condition

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ABSTRACT

The main limited factors of soybean plants expansion in acid soil are Aluminium (Al) toxicity and low pH. The best approach to solve this problem is by using Al tolerance variety. *In vitro* or *in vivo* selections using selective media containing $AlCl_3$ and induced callus embryonic of mutant lines are reliable methods to develop a new variety. The objectives of this research are to evaluate response of soybean genotypes against AlCl3 under *in vitro* and *in vivo* condition. Addition of 15 part per million (ppm) $AlCl_3$ into *in vitro* and *in vivo* media severely affected plant growth. G3 soybean mutant line was identified as more tolerant than the control soybean cultivar Tanggamus. This mutant line was able to survive under more severe AlCl3 concentrations (15 ppm) under *in vitro* conditions. Under *in vivo* conditions, G1 and G4 mutants were also identified as more tolerant than Tanggamus since they produced more pods and higher dry seed weigh per plant. Moreover, G4 mutant line also produced more dry seed weight per plant than Tanggamus when they were grown on soil containing high Al concentration 8.1 me/100gr = 81 ppm. Al^{+3}.

INTRODUCTION

Soybean is the world's leading economic oilseed crop. Processed soybeans are also the largest source of vegetable oil and protein feed. In addition to being a source of macronutrients and minerals, soybeans contain secondary metabolites such as isoflavones [1]. Indonesia needs approximately 2.20 tons of soybeans per year. The domestic production only meets 35−40% of the demand and the remaining 60−65% are imported from foreign countries. It is difficult to meet the increased demand and hence approximately 50% productions. Therefore, through various programs, the government put strong efforts to increase soybean production toward self-sufficiency in 2010−2012 [2].

The production of soybean can be increased by intensification and extensification in Indonesia. Both of attempts need suitable varieties. Intensifying production by planting pattern and integrated cultivation technology has been done in paddy field and dry land in Java. However, the fertile soil in Java is decreasing approximately by 20,000 hectares every year for non agriculture purposes. Extensification by expansion of planted area can be performed outside Java which usually infertile. A total potential area for soybean plantation is 55.2 ha million in Sumatra, West Nusatenggara, East Nusatenggara and Papua separately. These areas are mostly dry and acid with PH < 5, an access of aluminium a deficiency in phosphate and poor buffering capacity.

The effort to increase soybean production through extensification program in Indonesia faces various soil problems, especially soil acidity and aluminium toxicity. Aluminium (Al) toxicity is a major constraint of crop production on acid soils. In view of the fact that 40% of the world's arable land is acidic [3]. Soybean is one of the most sensitive to Al toxicity

The complex inheritance of Al tolerance trait has so far been undermined by breeding efforts to develop Al-tolerant soybeans. Al toxicity remains as a major problem for increasing world food production especially in developing tropical and subtropical regions, where the increase in food production is much needed. Aluminium reduces crop yield through root growth inhibition and hinrance in nutrient and water uptake [4,5]. Al tolerance has been studied for many years in soybean. Since then, researchers have assessed genetic response in a number of test environments in

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cluding green house pots, sandy culture, nutrient solution and tissue culture [3]. Nowdays, however, only moderate levels of Al tolerance have been detected in soybean. All these factors may potentially useful the breeding of Al tolerant cultivars. In soybean, particularly information experimental work addressing the *in vitro* and *in vivo* of Al tolerance in roots and grain yield, are still limited.

The objectives of this research are to evaluate response of soybean genotypes against AlCl3 under *in vitro* and *in vivo* condition.

EXPERIMENTAL METHODS

Mutant lines soybean have been generated from soybean mutant H218 (selected lines derived from soybean cv. Muria and P143402) by Gamma irradiation treatment with the dose of 200 Gy. Breeding behavior and salient features of the mutants were studied through M3-M5 generations. In the M5 generations, five mutants with superior agronomic traits were evaluated for various quantitative characters in comparison with the parent. The purposes of generating those mutants (G1, G2, G3, G4 and G5) were to develop new promising soybean mutant lines that were high yield and Al tolerance. In these experiments, characterizations of their tolerance against AlCl3 toxicity were evaluated. Evaluations were conducted *under in vitro*, using hydroponic condition, and on soil containing high Al content. This research consist of 4 separate experiments: 1 in vitro selection of Callus soybean mutant lines; 2 in vitro selection of germination soybean mutant lines; 3 Hydroponic selection of soybean mutant lines; 4 Potted plants in soil acid selection. Plant material used in this experiment consist of Soybean genotype including Tanggamus (control of variety tolerant Al); Raja Basa (control of variety tolerant Al); Lumut (control of variety sensitive Al) and G1, G2, G3, G4 and G5 (unknown response).

In vitro selection of Callus

Callus were induced from those plant material were made by culturing immature cotyledons on callus inducing medium MS media MS salts [6], B5 vitamins [7], 3% sucrose, 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2% Gelrite, pH 5.8. Sample were surface-sterilized in 70% ethanol followed by 1.05% sodium hypochlorite, and rinses in sterile distilled water. Immature seeds, 3-5mm in length, were aseptically removed from the pods, and the embryonic axis was excised and removed using a scalpel blade. Cotyledons were placed abaxial side down on MS media MS containing of 2,4-D. Twelve cotyledon pairs were placed onto disposable petri dish containing 25 ml of medium. The explants of were cultured at 25°C with a 24-h photoperiod. Cotyledons were assessed for initiation of callus after 1 month. Callus quality was determined based on both morphology and color. These callus were used for Al selection under *in vitro* condition.

Embryonic callus of plant material were culture on half-strength (1/2) Murashige and Scoog (MS) liquid media with an addition at 0, 7.5, 10, 12.5, or 15 part per million (ppm) of AlCl3 and their growths were monitor. The media PH was adjusted at 4.0. Five hundred embryonic callus were evaluated during the selection. Each culture consists of five embryonic callus. Cultures were incubated at 26^{o}C in the light.

Callus observation was made by measuring the fresh weight of control and Al-adapted callus was at beginning (W0) and 4 weeks after the experiment (Wf). Relative fresh weight growth rate (RFWG) of callus ware calculated with the formula as follows:

$$RFWG= (Wf - W0)/W0$$

In vitro selection of germination

Seeds of the plant materials were germinated for 3 days. The seedlings were grown on *in vitro* half-strength MS liquid medium containing $AlCl_3$ at 0, 7.5, 10, 12.5, or 15 ppm and were monitored for their growth, pH media was adjusted at 4.0. The length of roots and weight of dried roots were measured after 4 weeks.

Hydroponic selection

The plant materials were planted in plastic pots containing mixture coconut fiber, sand and thinned plant was watered over 15 days old. At 16 days old the other half was treated exposing Al by watering daily with 1/2 MS media containing Al 15 ppm (pH was adjusted at 4.0). The other half was treated under optimal condition by watering media without Al. Al treatment was given for 70 days when plants reached 85 days old (Fig. 3). The average number of pods and grain dry weight per plant were also measured.

Potted plants in soil acid selection

Soybean seeds the plant materials were planted in plastic pots containing acid soil taken from Jasinga (West Java) [notorious for their high Al-toxicity (81 me kg^{-1}) characters], and PH 4.3 and maintained in the green house until harvest. Soils taken from Bogor Cimanggu (without Al and PH 6.7) were used as control treatment. After harvesting, the average number of pods and dried weight of seeds were per plant were measured.

Al tolerance was measured using sensitivity index (S) [8]. Following this formula S = (1-Ys/Yp)/(1-X/Xp), where (Y) average callus dry weight, root dry weight, number of pot, seed dry weight of Al-treated genotype; (Yp) = average of variables of optimally grown genotype; X = variable value of all Al treated genotypes; and (Xp) = variables of optimally grown genotype. Plants were classified as tolerant if having sensitivity Index of <0.5; midly tolerant if 0.5≤S≤1; sensitive if S>1. Experiments were conducted in randomize design with 3 replication. Data were analyzed using SAS system for Windows version 9.0 (SAS Institute Cary, NC) software.

RESULTS AND DISCUSSION

In vitro selection of Callus

Although it is generally recognized that Al is a major factor limiting plant root growth, some reports did show beneficial effect of Al on plant growth. The effects of Al on growth of seedling roots and callus, dry weight of callus and dry weight roots of seedling were investigated. The relative root elongation of eight genotypes in the presence of Al was significantly different with Al^{+3} -tolerant (Data not shown).

The callus growth of five mutant lines variety at 7.5 ppm $AlCl_3$ concentrations was significantly stimulated after 4 weeks Al treatment. The callus growth for lumut was inhibited after exposure to 7.5 ppm $AlCl_3$ for 4 weeks, while that for five mutant lines were still significantly enhanced (Table 1). Addition of 15 ppm $AlCl_3$ into *in vitro* and *in vivo* media severely affected plant growth. Our data demonstrated that dry weight of callus of Al tolerant G3 mutant line (440 mg) and Tanggamus (450 mg) in the presence of high Al concentrations were higher than those in Al sensitive Lumut (25 mg). Based on dry weight callus G3 soybean mutant line was identified as more tolerant than the control soybean cultivar Tanggamus, suggesting that G3 soybean mutant line was more tolerant to Aluminum than Tanggamus at tissue level (Table 1 & Fig. 1). Al treatment caused dry weight decrease in callus (Fig. 1). The callus of Lumut in the presence 15 ppm Al showed lowerst than all genotype. These result confirmed that Al induced an increase in super oxide dismutase activity in both seedling roots and calluses of Al-tolerant PI and sensitive Young, which was dependent on the time of exposure and Al concentration [9]. Aluminum stress was reported to decrease of number regenerated of plants and inhibit growth and development cell [10]. Our results are agreement the previous observations addition of PEG to the culture media decreased the water potential of the media, thereby inducing water stress that adversely affected the callus growth and *in vitro* regeneration capacity of the tomato cultivars. Callus growing in the presence of increasing PEG concentrations increased their percent dry matter content and reduced relativ growth rate in all tomato cultivars [11,5].

Table 1. Effects addition of various concentrations of AlCl3 onto callus inducing medium on dry weight of callus of soybean mutant lines and three varities (mg).

Soybean Lines	AlCl3 Concentration (ppm)				
	0	7.5	10	12.5	15
Mutant G1	480b	470a	470a	430a	430a
Mutant G2	450bc	450ab	400ab	400ab	360ab
Mutant G3	460bc	450ab	450ab	440a	**440a**
Mutant G4	500b	490a	470a	460a	400ab
Mutant G5	600ab	460ab	430ab	400ab	410ab
Raja Basa	450bc	440ab	430sb	360ab	340ab
Tanggamus	690a	450ab	430ab	360ab	450a
Lumut	420b	380b	320abc	290abc	**25b**

Notes: Values followed by same letters within a row are not significantly different by Duncan Multiple Range Test (P<0.05).

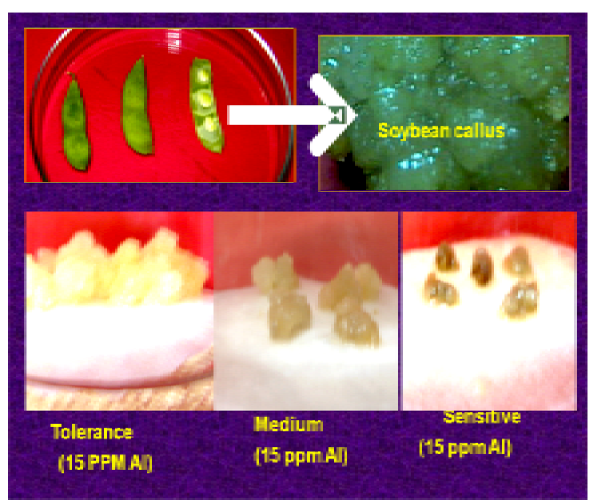

Fig. 1. Response of soybean callus on medium with AlCl.

In vitro selection of germination

The results revealed that 15 ppm Al produced the largest difference in root growth between the eight genotypes (Fig. 2). The difference between Al tolerant and sensitive genotypes disappeared and at 15 ppm Al the root growth of all genotypes was nearly completely inhibited. Previous studies have observed a large difference in root growth between PI 416937 and Young at 1.5 μM Al activity in similar culture medium [12]. Analysis of variance showed that main effects of treatment and genotype were significant ($p < 0.05$), means were therefore presented for those effects (Table 2). Five mutant lines were significantly higher dry weigh root under aluminum at 15 ppm compared to control tolerant Al variety (Tanggamus). G3 mutant line showed highest dry weigh root (0.29 mg) and lowest was shown by Lumut (0.07 mg).

Mutant lines and cultivars were decreased with increasing $AlCl_3$ concentrations (Table 2). Root dry weight of wheat could be the best criterion for selecting Al tolerant varieties [13]. The mutant line seedlings generally showed remarkable tolerance to. Aluminium affected seedling of different mutant lines and varieties of soybean and the inhibitory effect increased with the increase of Al^{3+} concentration (Fig. 2) and (Table 2).

Table 2. Effects of addition of various concentrations of $AlCl_3$ onto germination medium on dry weight of roots of seedling of soybean mutant lines and three varieties under *in vitro* condition.

Soybean Lines	$AlCl_3$ Concentration (ppm)				
	0	7.5	10	12.5	15
	Dry weight of root (mg)				
Mutant G1	0.30a	0.20a	0.18abc	0.22a	0.16b
Mutant G2	0.24ab	0.17ab	0.20ab	0.16ab	**0.27a**
Mutant G3	0.28a	0.20a	0.18abc	0.19a	**0.29a**
Mutant G4	0.21ab	0.19a	0.26a	0.15abc	0.25ab
Mutant G5	0.17b	0.21a	0.23a	0.17ab	0.19abc
Raja Basa	0.26a	0.24a	0.27a	0.13abc	0.14bc
Tanggamus	0.16bc	0.20a	0.22a	0.15abc	**0.15bc**
Lumut	0.22ab	0.16abc	0.21ab	0.08b	**0.07c**

Notes: Values followed by same letters within a row are not significantly different by Duncan Multiple Range Test (P<0.05).

The inhibitory sequence of different varieties at 15 ppm Al was: Lumut > Raja Basa > Tanggamus > G2 > G4 > G5 > G1 > G3. Lumut had the lowest of root dry weight. G3 mutant lines showed better seed germination growth on medium containing 15 ppm AlCl3 than that of Tanggamus. Aluminum treatment decreased dry weigh root in all soybean genotypes. This decreased was much more in Al sensitive (Lumut variety). Similarly, it was shown that the taproots of all genotypes soybean were inhibited by Aluminum addition and inhibition was more severe with increasing Al activities [14]. The higher accumulation of genotype Al in roots of sensitive Lumut was caused by a higher penetration of Al into their roots as compared to mutant lines and Al-tolerant Tanggamus. In the present study, a lower accumulation of Al in roots of Aluminum tolerant G3 mutant line and Tanggamus are likely associated with protein accumulation in roots which was induced by Al stress). G3 mutant line has been identified as potential Al tolerant mutant under *in vitro* selection.

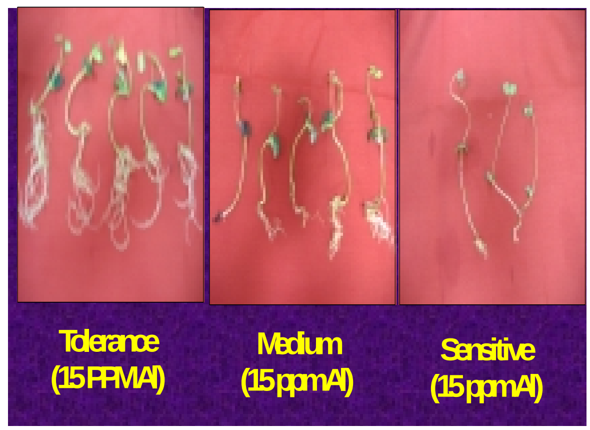

Fig. 2. Response of soybean seedlings on germination medium with addition of $AlCl_3$.

Hydroponic selection

Plants grown at 0 and 15 ppm Al levels of aluminium toxicity were characterized by number of pods per plant and dry weigh seed. Consequently, data were available for these concentrations. Mean squares for number of pods per plan and dry weigh seed traits of 8 soybean genotypes grown at two levels of aluminium activity are summarized in (Table 3).

The results showed a markedly difference between eight of soybean genotypes in their tolerance to Al stress as indicated by pods dry weigh of seed per plant (Table 3).Tolerant genotype (Tanggamus) had higher number of pods and dry weigh of seed per plant under Aluminum condition as compared to sensitive plants (Lumut). In the study we could revealed that mutant of grain yield and relative grain yield per plant of Al grown tolerant plants was due to the higher of number of pods and dry weigh of seed (Table 3**).** Analysis of variance showed that main effects of treatment and genotype were significant ($p < 0.05$), means were therefore presented for those effects (Fig. 3). Number of pods and dry weigh of seed were significantly lower under aluminum compared to control treatments (Table 3).

The results revealed that 15 ppm Al produced the largest difference in plant growth between the five mutant lines and three varieties (Fig. 3).

Table 3. Effects addition of AlCl3 at 15 ppm on yields of soybean mutant lines and three varieties grown hydroponically.

Soybean lines	AlCl3 concentration (ppm)	
	0	**15**
	Number of filled pod per plant	
Mutant G1	13.5	**9.4**
Mutant G2	11.1	7.5
Mutant G3	10.6	8.2
Mutant G4	10.4	7.2
Mutant G5	12.0	6.8
RajaBasa	12.3	8.5
Taggamus	17.3	8.8
Lumut	12.3	4.3
	Dry weight of seeds per plant (gr)	
Mutant G1	2.06	**1.72**
Mutant G2	1.82	1.48
Mutant G3	1.65	1.41
Mutant G4	1.63	1.40
Mutant G5	1.68	1.37
RajaBasa	1.66	1.34
Taggamus	1.59	1.30
Lumut	1.49	1.20

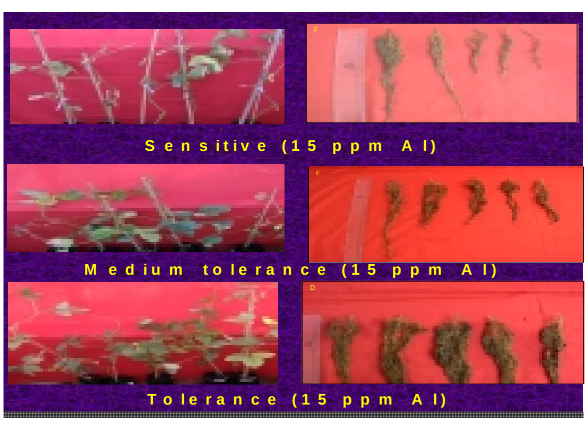

Fig. 3. Response of soybean plants grown Hydroponically medium with AlCl3.

G1 mutants line were also identified as more tolerance than that Tanggamus since the G1 mutant line produced higher number of pods and dry weigh of seed per plant (9.4 and 1.72 gr) Tanggamus produced number of pods and dry weigh of seed per plant 8.8 and 1.3 mg (Table 3). On the other hand, inhibitory effect on water uptake, growth and grain yield in soybean were observed [1]. Tolerant genotype responded to high Al condition by low decreasing dry seed weight per plant. Concentration of Al at 15 ppm was effective to differentiate responses of the tested soybean mutant lines and cultivars under *in vitro* callus growth and seed germination tests, and hydroponic condition test, respectively.

Potted plants in soil acid selection

The effect of aluminium in soil on number of filled pod per plant and dry weight seeds per plant (g**)** of the soybean mutant lines are described in this section. Aluminium affected growth number of filled pod per plant and dry weight of seeds of different mutant lines and varieties of soybean. Significant difference for tap root length in 75 days old mutant lines ware detected using the soil containing hight Al (Fig. 4). G4 mutant line showed longest taproot length than Tanggamus and Lumut showed shortest tap root length (data not shown). It can be seen from Fig. 4 that number of filled pod per plant and dry weight of seeds decreased in soybean plants under hight Al toxicity in soil (Table 4).

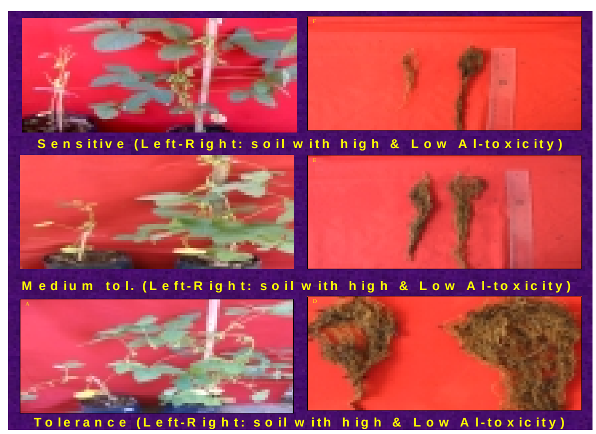

Fig. 4. Response of soybean plants grown soil acid containing high Al toxicity and without Al (Lumut control of sensitive variety; Tanggamus control of tolerant variety and mutant line G4 detect more tolerant than Tanggamus) respectively.

According to the data (Table 4) the promising mutant lines G4 showed better yields than Tanggamus on acid soil containing high Al-toxicity. G4 mutant line also produced more dry weight seed under stress Al condition 1.26 g per plant compared than that of Tanggamus 0.86 g per plant. There were significant difference under hight Al condition between mutant G4 and Tanggamus. These results suggest that mutant lines

G4 is more tolerant to aluminum than Tanggamus (control of Al tolerant variety). The lowest dry weight of seed was in Lumut variety. Aluminium has influenced soybean production base on Number of pods and dry weight of seed.

Table 4. Effects soil with low and high AlCl3 toxicity on yields of soybean mutant lines and three varieties.

Soybean lines	Soils type	
	Low Al toxicity	Hight Al toxicity
Number of filled pod per plant		
Mutant G1	5.19ad	2.67a
Mutant G2	4.69c	2.11b
Mutant G3	4.93cde	2.21b
Mutant G4	**4.67c**	**2.61a**
Mutant G5	5.19cd	2.07 b
RajaBasa	5.44c	2.29ab
Taggamus	5.86b	2.47ab
Lumut	6.7a	2.16 b
Dry weight of seeds per plant (gr)		
Mutant G1	3.01ab	1.05ab
Mutant G2	2.99ab	1.01ab
Mutant G3	2.84b	0.97ab
Mutant G4	**3.61ab**	**1.26a**
Mutant G5	2.29c	0.95b
RajaBasa	2.40b	0.89b
Taggamus	3.01ab	0.86b
Lumut	2.31c	0.75b

Excess Al in soil enters roots, resulting in reduced plant vigor and yield [6]. Moreover, **G4** mutant line also produced more Number of filled pod and dry seed weight per plant than Tanggamus when they were grown on high aluminum stress in acid soil (Table 4). Higher concentrations of Al' affected plant growth, depressed photosynthesis, enhanced transpiration, and induced lipid per oxidation [15].

Plant Tolerant to Al

Plant tolerance to Aluminum was using Sensitive Index (S) and the results are presented in Table 5-7. Based on calculation on S value on callus dry weight and root dry weight, five mutant lines the in vitro selection were classified as tolerant with S value - 7.57 and 0.37 respectively. Raja Basa and Lumut variety were sensitive had S value 5.82 and 7.85 (Table 5 and 6).

In addition, this selection method has reduced the number of sensitive lines with S >1. These results demonstrated that tolerance level to Al can be improved using this technique. Similar result was reported in rice and in soybean [16,17]. The used PEG media was effective to select drought tolerant callus cells, which subsequently regenerated to developed lines with higher tolerance to drought stress compare the parental line [16]. Selection in 15% PEG media was effective to produce mutant cell. This cell can regenerate to develop drought tolerant mutant [17].

Table 5. Al Sensitivity Index of agronomical characters of mutant lines and three varieties of soybean.

Genotype	Al (ppm)	Dry weight calluse		Dry weight root of seedling	
		Sensitfve Index	Penotype	Sensitive Index	
G1	15	-0.31	Tolerant	-6.16	Toleran
G2	15	-0.81	Tolerant	-6.68	Tolerant
G3	15	0.38	Tolerant	-1.27	Tolerant
G4	15	0.16	Tolerant	-7.57	Tolerant
G5	15	0.17	Tolerant	-3.97	Tolerant
Lumut	15	1.91	Sensitive	7.85	Sensitive
Rajabasa	15	1.68	Sensitive	5.82	Sensitive
Tanggamus	15	0.35	Tolerant	0.42	Tolerant

Based on ssensitive index on in vivo condition hydroponic we classified the genotypes into three group's viz., tolerant, medium tolerant and sensitive. G4 and G3 mutant lines had S value on seed dry weight 0.6 and 0.87 (Table 6). The S value on number of filled pod and seed dry weight G3 and G4 mutant's lines in hydroponic selection were classified as tolerant and midly tolerant. The other mutant lines (G1, G2 and G5) have S value <1. There were classified as middle tolerant to Al. Tanggamus, Raja Basa and Lumut variety based on S value on number of filled pod and seed dry weight were classified sensitive to Al. The S value on number of filled pod and seed dry weight G3 and G4 mutant's lines in hydroponic selection were classified as tolerant and middle tolerant (Table 6).

Table 6. Al Sensitivity Index of agronomical characters of mutant lines and three varieties of soybean.

Genotype	Al (ppm)	Dry weight calluse		Dry weight root of seedling	
		Sensitfve Index	Penotype	Sensitive Index	
G1	15	-0.31	Toleran	-6.16	Toleran
G2	15	-0.81	Tolerant	-6.68	Tolerant
G3	15	0.38	Tolerant	-1.27	Tolerant
G4	15	0.16	Tolerant	-7.57	Tolerant
G5	15	0.17	Tolerant	-3.97	Tolerant
Lumut	15	1.91	Sensitive	7.85	Sensitive
Rajabasa	15	1.68	Sensitive	5.82	Sensitive
Tanggamus	15	0.35	Tolerant	0.42	Tolerant

Results of the selection procedure for plastic pots with high Al soil, plant levels are summarized in (Table 7). Based on S value on number of filled pod all mutant lines tion were classified as middle tolerant except G5 was sensitive to Al. Three varieties (Tanggamus, Raja Basa and Lumut) based on S value on number of filled pod and seed dry weight were classified sensitive to Al. Calculation on S value on seed dry weight were classified G4 mutant line middle tolerant to Al. In Addition, this selection method has reduced the number of sensitive line with S value of >1 (Table 7). The results showed S value filled pod and seed dry weight G1, G2, G3, G5 and three varieties (Tanggamus, Raja Basa and Lumut) respectively were sensitive. Moreover, higher aluminum concentration significantly increased lipid per oxidation, decreased cell membrane stability, and changed the activities of super oxide dismutase (SOD) in the leaves of both plants [9].

Table 7. Al Sensitivity Index of agronomical characters of mutant lines and three varieties of soybean.

Genotype	Soils type	Number of filled pod per plant		Dry weight of seeds per plant	
		Sensitive Index	penotype	Sensitive Index	
G1	High Al	0.92	Midly tolerant	1.01	Sensitive
G2	High Al	0.99	Midly tolerant	1.01	Sensitive
G3	High Al	0.99	Midly tolerant	1.02	Sensitive
G4	High Al	0.86	Midly olerant	0.90	Midly tolerant
G5	High Al	1.05	Sensitive	1.02	Sensitive
Lumut	High Al	1.12	Sensitive	1.02	Sensitive
Rajabasa	High Al	1.02	Sensitive	1.03	Sensitive
Tanggamus	High Al	1.03	Sensitive	0.99	Midly tolerant

CONCLUSION

Effective concentration of AlCl3 needed in *vitro* medium, in hydroponic solution, and in soils in order to inhibit growth and development of tested soybean genotypes was 15 ppm. All mutant lines were more tolerance against AlCl3 toxicity than that of Tanggamus. Characteristics of the tested mutant lines were: G4 mutant line-tolerance against Al-toxicity in soil; G1 lines-against $AlCl_3$ toxicity in hydroponic condition; G3 mutant lines-tolerance against AlCl3 toxicity *in vitro* condition.

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