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In Vitro Selection of Four Soybean Genotypes using PEG for Drought Tolerance

Seleksi In Vitro Empat Genotipe Kedelai menggunakan PEG untuk Toleransi Kekeringan

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ABSTRACT

The use of somatic embryo for in vitro selection program is very useful since the selected traits will be inherited in the progeny. This study was aimed to obtain somaclonal variants for drought tolerances of soybean genotypes from in vitro selection using polyethylene glycol (PEG). The experiments were set up in two factors with completely randomize design and each treatment was replicated ten times. Four soybean genotypes (Tanggamus, Yellow Biloxi, CG-22-10, and SP-10-4) were used in this study as first factor experiment. In vitro selection was in embryogenic callus level using media containing PEG (0, 5, 10, 15, and 20%) as second factor experiment was done to all genotypes. The results showed that after 3 months in the selection medium, SP-10-4 and Tanggamus genotypes showed higher percentage of fresh callus (surviving callus) and number of embryogenic callus, compared to CG-22-10 and Yellow biloxi. In contrast, CG-22-10 had the lowest number of fresh callus and number of embryogenic callus compared to other genotypes. PEG in high concentration decreased the percentage of fresh callus and number of embryogenic callus in all genotypes. Tanggamus was the only genotype that survived until cotyledonary-stage embryos after transferring in MS0 regeneration medium. Seven Tanggamus somatic embryos from PEG selection succesfully germinated and regenerated into plantlet as drought-tolerant somaclone candidates.

Keywords: abiotic stress, embryogenic callus, Glycine max, somaclone

ABSTRAK

Penggunaan embrio somatik pada program seleksi in vitro sangat dibutuhkan untuk mendapatkan karakter yang diinginkan serta dapat diturunkan pada generasi berikutnya. Tujuan dari penelitian ini adalah untuk mendapatkan varian somaklon tanaman kedelai yang toleran kekeringan melalui seleksi in vitro dengan agen seleksi PEG (polyethylene glycol). Penelitian ini menggunakan rancangan percobaan acak kelompok dengan dua faktor perlakuan dan masing-masing perlakuan diulang 10 kali. Empat genotipe kedelai (Tanggamus, Yellow Biloxi, CG-22-10, and SP-10-4) digunakan dalam percobaan ini sebagai faktor perlakuan pertama. Seleksi ini vitro dilakukan pada tahap kalus embriogenik seluruh genotipe yang dicoba menggunakan media dengan penambahan PEG (0, 5, 10, 15, and 20%). Hasil percobaan menunjukkan bahwa setelah 3 bulan dalam media seleksi, genotipe SP-10-4 dan Tanggamus menunjukkan persentase kalus segar (kalus yang hidup) dan jumlah kalus embriogenik lebih tinggi dibanding genotipe CG-22-10 dan Yellow biloxi. Sebaliknya, genotipe CG-22-10 memiliki persentase kalus segar dan jumlah kalus embriogenik terendah dibanding genotipe lainnya. Penggunaan konsentrasi PEG yang makin tinggi menurunkan persentase kalus segar dan jumlah kalus embriogenik seluruh genotipe yang diuji. Tanggamus merupakan satu-satunya genotipe yang bertahan hidup (survive) hingga embrio tahap kotiledon setelah dikulturkan dalam media regenerasi MS0. Pada percobaan ini tujuh embrio somatik genotipe Tanggamus hasil seleksi PEG berhasil membentuk kecambah dan beregenerasi menjadi planlet sebagai kandidat somaklon yang toleran kekeringan.

Kata kunci: cekaman abiotik, Glycine max, kalus embriogenik, somaklon

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INTRODUCTION

Soybean (*Glycine max* (L) Merr.) is an important crop in Indonesia for food and as a component of fodder (Suharsono and Jusuf, 2009). As an important source of protein for many Indonesian, the demand of soybean for food is increasing every year. The increasing demand of soybean is not met by national production mainly due to decreasing planting area. Thus, extensification of soybean is seemly the alternative program to increase soybean production. The extensification may use less favorable areas i.e. drought-prone areas as a targeted program to minimize competition with other more profitable crops such as corn and rice. The development of high yielding soybean varieties may require crop improvement programs even in abiotically stress condition including drought.

Drought is one of the most common environmental stresses affecting plant growth and productivity. Drought has been one of the major causes of low yields in soybean production as well as in other crop production in the dry areas in Indonesia. The development of crop cultivars with increased tolerance to drought, both by conventional breeding methods and by *in vitro*, is an important strategy for agricultural production in the dryland. The use of drought-tolerant cultivars is a more practical and a cost-efficient approach in comparison to improving the cultural techniques and facilities for dryland farming (Hemon and Sudarsono, 2010).

It has been widely accepted that applying drought stress during callus or plant cells proliferations is efficient for selecting drought tolerant cell lines and the regeneration of tolerant plants (Sakthivelu *et al*., 2008). Different responses for drought tolerance, such as a higher membrane stability and a better yield when grown under low water potential, have been identified from somaclonal variants (Matheka *et al*., 2008; Mahmood *et al*., 2012a).

In vitro regenerated plants often exhibit phenotypic differences. Some of these differences may have been inherited in the progeny generations, and are exploited for developing new germplasm with improved characters such as tolerance to abiotic stress (Raia *et al*., 2011). Somaclonal variation may be the results of a number of DNA changes (Mujib *et al*., 2007) since some of the observed variation is heritable.

Plant cell and tissue culture has been a useful tool to study stress tolerance mechanisms under *in vitro* conditions. *In vitro* culture techniques minimize environmental variations due to defined nutrient media, controlled conditions and homogeneity of stress application. In addition, the simplicity of such manipulations enables studying large plant population and stress treatments in a limited space and short period of time (Sakthivelu *et al*., 2008).

Somatic embryogenesis is one of tissue culture techniques that can be used for *in vitro* selection. The use of somatic embryo (SE) for *in vitro* selection program is very valuable since the selected traits will be inherited in the progenies. Using selective agents variability of regenerants from somatic embryogenesis (especially via callus phase) could enhance genetic variation. Polyethylene glycols (PEG) of high molecular weights have been used to simulate drought stress in plants as non-penetrable and non-toxic osmotic agents lowering the water potential in a way similar to soil drying (Muhammad *et al*., 2010; Khodarahmpour, 2011).

In-vitro selection by culturing plant cells and tissues on medium supplemented with polyethylene glycol (PEG) or mannitol have identified drought tolerant tissues of potato and rice (Gopal and Iwama, 2007; Wani *et al*., 2010; Raia, 2011). Successful *in vitro* selection for drought tolerance using PEG has been reported for maize (Matheka, 2008), peanut (Rahayu and Sudarsono, 2015), sorghum (Tsago, 2014), banana (Bidabadi *et al*., 2012) and *pelargonium* (Hassanein, 2010). *In vitro* selection using PEG was also reported for several genotypes of soybean such as Tidar, B3731, and MSC8606 (Widoretno *et al*., 2003) and JS 335, Hardee (Indian cultivars), Collina and Korada (introduced in Bulgaria) (Sakthivelu *et al*., 2008).

The previous study have developed efficient procedures for inducing somatic embryos from soybean immature cotyledons explants (Yang *et al.*, 2009; Khumaida and Handayani, 2010). Widoretno, *et al*., 2003 have demonstrated the tolerant soybean embryogenic callus (EC) could survive in such selective medium supplemented with PEG and might regenerate into drought tolerant plantlets. The main goal of this study was to develop an effective protocol for regenerating drought tolerant somaclonal variants of soybean. Specific objectives were to regenerate somaclonal variants among embryogenic callus, to select drought tolerant lines on a medium containing polyethylene glycol (PEG) using histological analysis, and to evaluate the regenerated lines against drought stress under *in vitro* conditions.

MATERIALS AND METHODS

The research was conducted in Laboratory of Tissue Culture and Laboratory of Microtechnic, Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University (IPB) in February 2012 to March 2014.

Explant Preparation

Four soybean genotypes (Tanggamus, Yellow Biloxi, CG-22-10, and SP-10-4) were used in this study. Tanggamus and Yellow Billoxi are national varieties, and CG-22-10 and SP-10-4 are the F7 of crossing obtained from Laboratory of Plant Breeding Research IPB. After plants were grown in pots under field conditions, immature pods containing immature cotyledons (3-5 mm) were harvested 2 to 3 weeks after flowering.

Embryo Somatic Induction and Proliferation

The solidified MS medium containing B5 vitamin and gelrite 2 g L^{-1} was used for somatic embryo (SE) induction. The medium was added with 3% sucrose and growth regulator of 2,4-D 5 mg L^{-1}combined with NAA 5 mg L^{-1} (MS-D5N5

medium). Pods were surface-sterilized by immersion for 1 min in 70% ethanol and 15 min in 1% solution of sodium hypochloride and then rinsed three times in sterile water. In a laminar flow hood, immature cotyledons were aseptically removed from the pods and the end containing the embryonic axis was cut off and discarded. After the seed coats were removed, the two cotyledons were separated and the abaxial side was placed on the media. The pH of the media was adjusted to 5.6-5.8 and autoclaved at 121 ^{o}C for 15 min. Four cotyledons of each genotypes were placed on each flask and incubated under white fluorescent light of 1,500 lux at 24 h photoperiod at 24 ± 3 ^{o}C. The 5 weeks-old somatic embryo clusters of globular stage or embryogenic callus were harvested and proliferated in the same media (MS-D5N5 medium). The harvested embryogenic callus (EC) with somatic embryos (SE) structure after proliferation will further used in *in vitro* selection using PEG for drought tolerance.

In Vitro Selection Using PEG

The experiment was set up in a two-factor experiment with completely randomize design. *In vitro* selection to drought stress was done to all genotypes (Tanggamus, Yellow Biloxi, CG-22-10, and SP-10-4) as first factors experiments. Drought was simulated by subsequently the added polyethylene glycol (molecular weight 6000) at concentrations of 0, 5, 10, 15, and 20% (w/v) to the media MS-D5N5 (MS media containing 2,4-D 5 mg L^{-1} and NAA 5 mg L^{-1}) as second factors experiments.

In vitro selection was done at embryogenic callus (EC) level, the callus clumps harvested in proliferating medium were excised and planted in the selection medium. Each experimental unit consisted of four clumps of soybean EC cultured in one culture vial (250 mL) containing 25 ml of MS-D5N5 medium. Each clump of the EC contained 2 to 4 SEs. Ten replicates were prepared for each of PEG concentration. This study used liquid PEG-supplemented MS-D5N5 medium to proliferate soybean EC and SEs as agar would not solidify in the presence of a high concentration of PEG 6000. Sterilization of the media used standard heat sterilization methods at 120 °C, 1.5 kg/cm^2 using autoclave. To prevent explants from drowning, a raft covered with a single layer of filter paper (Whatman, grade 6 filter paper) was floated on the liquid medium. Soybean EC and SE were laid on top of the filter and cultured on the medium for a total of three months period. The EC were transferred onto fresh medium every month. The cultures were incubated under white fluorescent light of 1,500 lux at 24 h photoperiod at 24 ± 3 ^{o}C until they initiate EC and SE. The percentage of explants performing EC survival and the number of proliferated SEs at 1; 2 and 3 month after cultures (MAC) on selective medium were recorded. An analysis of variance was performed to all quantitative data obtained in this study, and significant differences among treatment means were calculated by the Duncan's Multiple Range Test at a probability level of 0.05.

Regeneration of Drought Tolerant Somatic Embryo Variants

At the end of the selection period, surviving EC with globular structure of four genotypes tested were further cultured in SE recovery and maturation medium (MS0). After four weeks in MS0 medium, EC with the mature SEs were transferred to SE germination medium (MS0 + 1 g L^{-1} activated charcoal) to regenerate plantlets of somaclone variants with drought tolerance. In both culture steps, the EC were incubated in culture room with a constant temperature of 25 °C and a continuous lighting at approximately 1,000 lux provided by cool white fluorescent tube lamp (100 W each). The lamps were placed at 50 cm above the culture rack. The developing SE and number of initiated plantlets formed during incubation were recorded.

RESULTS AND DISCUSSION

Embryogenic Callus Induction and Proliferation

All genotypes (Tanggamus, Yellow Biloxi, CG-22-10, and SP-10-4), started to form embryogenic callus at five weeks after culture. The yellowish colour and compact structure were the characteric that founded in this embryogenic callus. Callus masses that have yellowish colour and compact structure indicate embryogenic capacity (Kumari *et al*., 2006). The formed somatic embryo stages was another characteristic that exhibited in embryogenic callus. They emerged among callus masses, particularly for stage of embryo globular (Figure 1). The number of embryogenic callus with globular embryo increased after transferring to proliferation media (Figure 2). This embryogenic callus were further cultured in PEG selection medium for in vitro selection.

Effect of PEG on Developing EC and ES

The analysis of Kruskal Wallis Test (α=0.05) showed the interaction between PEG concentration and genotypes on percentage of fresh callus (surviving callus) at 4, 8 and 12 weeks after culture (WAC) in the selection medium (Table 1-3). The highest percentage of fresh callus was obtained from interaction of 0-20% PEG concentration (all concentration levels of PEG) and SP-10-4 genotypes. The interaction of Tanggamus genotypes and PEG concentration of 0-20% (4 WAC), 5% and 20% (8 WAC) and 5% (12 WAC) also gave similar results. Based on those results, SP-10-4 and Tanggamus were the genotypes that were capable of producing fresh callus at the whole range of PEG concentration. Furthermore, Tanggamus produced the largest callus diameter that was related to percentage of callus survival (Figure 3).

Polyethylene glycol (PEG) may have no injurious or toxic effects on the plant, but inhibit growth by lowering the water potential of the medium so that cultured explants are unable to take up water. The reduction of callus growth

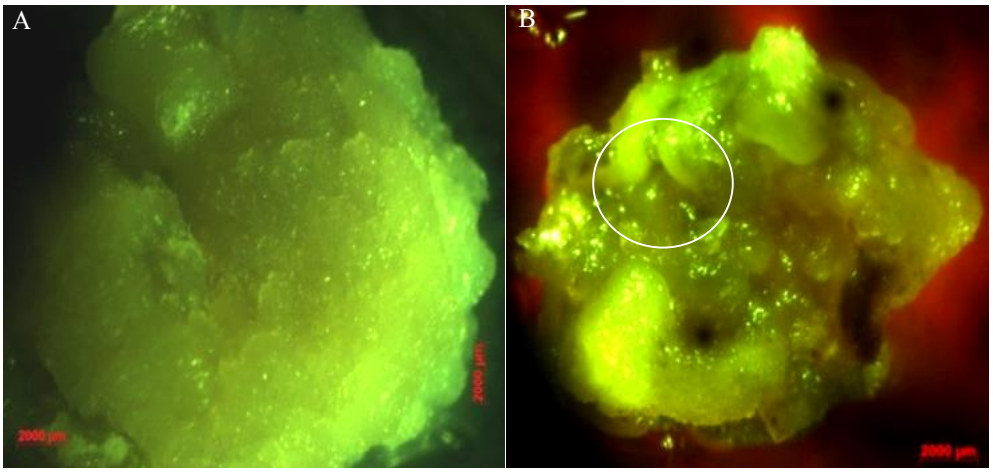

Figure 1. Non embryogenic callus of SP-10-4 genotype cultured on induction media containing 3% sucrose + 2,4-D 10 mg L^{-1} + NAA 10 mg L^{-1} after 6 weeks of culture (A) and embryogenic callus of SP-10-4 genotype cultured on induction media containing 3% sucrose + 2,4-D 5 mg L^{-1} + NAA 5 mg L^{-1} after 6 weeks of culture (B)

was probably due to reduction of cytoplasmic and vacuolar volume resulting from removal of water from cytoplasm by a lowered cellular water potential (Bartels and Sunkar, 2005). A lowered external osmotic potential is detrimental as cells are unable to take up water and nutrients from the external environment, leading to decline in growth. As showed in Table 1-3, SP-10-4 and Tanggamus survived better (performing the highest fresh callus until 3 MAC for each) in the selection medium of all PEG concentration used. Widoretno et al. (2003) reported that 15% PEG resulted in

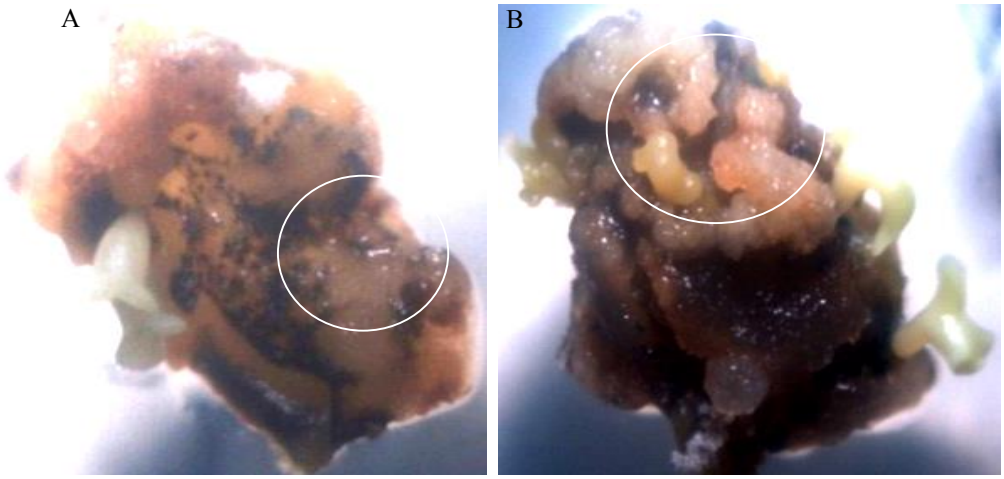

Figure 2. Proliferation of SE on Tanggamus genotypes : Globular and torpedo SE before proliferation (A), proliferation of globular SE (circle line) with some cotyledonary SE following proliferation (B)

Table 1. Effect of PEG concentration and genotypes on percentage (score) of EC survival after 1 month of culture

PEG conc. (%)	Genotypes			
	CG-22-10	SP-10-4	Tanggamus	Yellow biloxi
0	5.0a	4.9ab	5.0a	5.0a
5	4.2b-e	5.0a	5.0a	3.6de
10	3.0e	5.0a	4.2abc	3.7cde
15	3.1e	4.9ab	4.2abc	3.7cde
20	3.4e	4.6ab	4.3a-d	3.3e

Note: Values followed by the same letter at the rows and columns were not significantly different according to Kruskal Wallis Test (α = 0.05). Scoring: 5 = EC survival 80-100%, 4 = EC survival 60- < 80%, 3 = EC survival 40- < 60%, 2 = EC survival 20- < 40%, 1 = EC survival 0- < 20%

Table 2. Effect of PEG concentration and genotypes on percentage (score) of EC survival after 2 months of culture

PEG conc. (%)	Genotypes			
	CG-22-10	SP-10-4	Tanggamus	Yellow biloxi
0	5.0a	4.9ab	5.0a	5.0a
5	3.8c-f	5.0a	5.0a	3.6def
10	3.3f	4.9ab	4.1b-e	3.5ef
15	3.2f	4.8ab	4.2b-e	3.8c-f
20	3.2f	4.5abc	4.3a-d	3.1f

Note: Values followed by the same letter at the rows and columns were not significantly different according to Kruskal Wallis Test (α = 0.05). Scoring: 5 = EC survival 80-100%, 4 = EC survival 60- < 80%, 3 = EC survival 40- < 60%, 2 = EC survival 20- < 40%, 1 = EC survival 0- < 20%

Table 3. Effect of PEG concentration and genotypes on percentage (score) of EC survival after 3 months of culture

PEG conc. (%)	Genotypes			
	CG-22-10	SP-10-4	Tanggamus	Yellow biloxi
0	4.9ab	5.0a	5.0a	5.0a
5	3.7d-i	5.0a	5.0a	3.4ghi
10	3.3hi	4.8abc	4.2b-e	3.5e-i
15	3.2i	4.7abc	4.0c-h	3.7e-i
20	3.2i	4.5a-d	4.1c-g	3.3hi

Note: Values followed by the same letter at the rows and columns were not significantly different according to Kruskal Wallis Test (α = 0.05). Scoring: 5 = EC survival 80-100%, 4 = EC survival 60- < 80%, 3 = EC survival 40- < 60%, 2 = EC survival 20- < 40%, 1 = EC survival 0- < 20 %

90% explant survival of B3731, 54% of Tidar (moderately tolerant) and 30% of MSC 8606 (sensitive to drought). Related to that, the resulted freshly embryogenic callus of all genotypes in our study might be the somaclone variants that have putative tolerance to drought.

In this experiment, the unfreshed callus was also characterized by browning, the calli turned brown then black and eventually died, particularly CG-22-10 which had much greater callus mortality. Tissue browning and death maybe the consequence of water loss due to exposure to severe osmotic stress. Variations in survival to water stress could not be attributed to the genotypes. However, other studies have demonstrated a genotype-dependent response to water stress induced by PEG or ABA (Lutts *et al*., 2004). PEG stress tolerant callus tended to show less browning and higher survival rates under high level of PEG 6000 (Mahmood *et al*., 2012b). Our results were in line with results of those studies on PEG stress tolerance in plants. By adding PEG 6000 in the selective medium, callus proliferation reduced as well as *in-vitro* regeneration capacity of potato (Gopal and Iwama, 2007), soybean (Sakthivelu *et al*., 2008), tomato (Aazami *et al*., 2010), and wheat (Sayar *et al*., 2010).

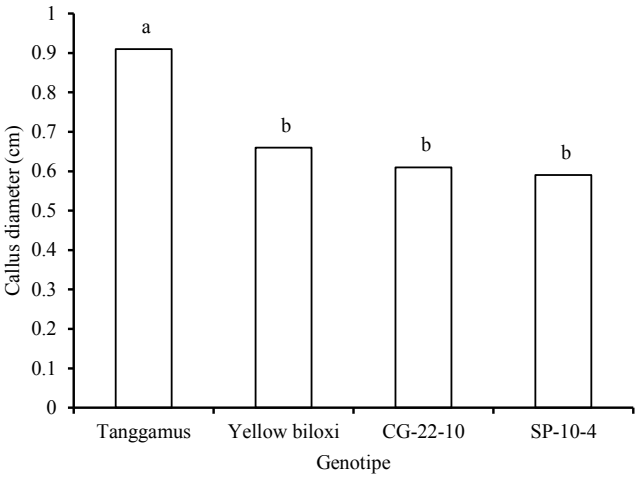

Figure 3. Effect of genotypes on calli diameter cultured in PEG selection medium. Means with the same letters are not significantly different according to DMRT Test (α = 0.05)

PEG concentration affected number of embryonic callus. The results after 3 months in the selection medium showed that higher concentration of PEG lead to the lower number of embryogenic callus in all genotypes. In this case, the embriogenic capacity of the callus was reduced by increasing concentration of PEG applied (Table 4). This was similar to the results of Widoretno *et al*. (2003) that selective media with PEG inhibited the growth and development of the soybean explants and decreased the number of the somatic embryo. The increasing amount of PEG added to the selective media deteriorated somatic/callus embryo. Water stress by PEG in the media reduced the number of embryogenic callus of all genotypes in this study (Table 4). The decreased water potential by PEG in media not

Table 4. Effect of PEG concentration on number of SE per explant

PEG concentration (%)	Number of SE per explant		
	1 MAC	2 MAC	3 MAC
0	11.08a	11.45a	11.60a
5	10.10a	10.00b	10.13b
10	9.85b	9.53b	9.70b
15	9.98b	9.70b	9.73b
20	9.05b	8.53c	8.25c

Note: Values followed by the same letter at the same columns were not significantly different according to DMRT (α = 0.05). MAC = months after cultures

only affected callus growth but also to the ability of cells in callus masses to form embriogenic cells with somatic embryo differentiation (Tereso *et al*., 2007). This inhibitory effects of PEG might be due to the decreased endogenous polyamines in explant tissues under PEG stress (Hemon and Sudarsono, 2010).

Identification of PEG Stress Tolerant EC and ES

After 3 months of culture, number of SEs on 5% PEG, 10% PEG and 15% PEG were not significantly different (P>0.05) indicated that EC and SE of all genotypes at 15% PEG survived, looked fresh and different from 20% PEG which gave the lowest number of SE. Tanggamus was the only genotype that survived and grew normally until cotyledonary-stage embryos after transferring in MS0 regeneration medium. PEG could be applied for stimulating drought because it could inhibit water in such a way that no water is provided for somatic cell, except for the callus/ somatic cell which has particular mechanism for absorbing water. Only the tolerant callus which bears PEG media selection could increase its tolerance against drought stress (Lestari, 2006). The presence of survival ECs and SEs in this experiment were also demonstrated by histological examination as shown in Figure 4. Similar to control, there were not damage cells on that survived somatic embryos. According to these results, the normally growing SEs on selective MS-D5N5 medium containing 15% PEG were PEG stress tolerant; therefore, these activities identified the presence of PEG stress tolerant EC and SEs. Since addition of PEG in selective media causes dehydration stress, PEG stress tolerant EC and SEs could also be drought stress tolerant SE variants. Hence, soybean plants regenerated from PEG stress tolerance EC and SEs could also be drought stress tolerance.

Supplementing PEG 6000 into MS-D5N5 medium resulted in inhibition of EC and SE proliferation of four soybean genotypes. The higher PEG concentration added in the medium, the lower the development and proliferation of SE. Matric forces of ethylene oxide sub-units of the PEG polymer results in low osmotic pressure in the medium. The ethylene oxide sub-units retain water through the formation of hydrogen bonds (Abdel-Raheem *et al*., 2007; Sayar *et al*., 2010). Therefore, in the selective medium supplemented with PEG 6000, water molecule in the medium become less available and they cannot be absorbed directly by the growing explants. Lower osmotic pressure might inhibit EC and SEs proliferation and development on the selective medium containing 20% PEG.

PEG-induced lower water potential might result in either cytoplasmic or vacuolar volume decline because of water removal from the cells (Chartzoulakis *et al*., 2002). Those conditions might also have happened in soybean EC and SE cultured on MS-D5N5 medium containing 20% PEG, leading to lower survival and reduced SE proliferation. Browning and dying of EC and SEs grown on PEG supplemented medium might also be due to water loss induced by osmotic stress or a high phenol production (Sakthivelu *et al*., 2008).

Plantlet Regeneration from Drought Tolerant EC dan ES

Embryogenic callus with somatic embryos survived from PEG selection recovered and matured in MS based

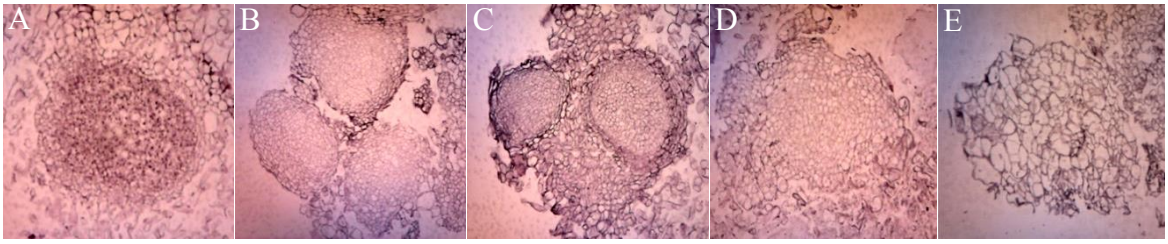

Figure 4. Histological observation of surviving somatic embryos of Tanggamus genotypes after selection in PEG media. Globular stage on 0% PEG (A), globular and heart stages on 5% PEG (B), globular and heart stages on 10% PEG (C), heart stages on 15% PEG (D), and globular stage on 20% PEG (E)

medium without growth regulator (MS0), and germinated in media containing MS0 and activated charcoal for plant conversion. The addition of activated charcoal was necessary to absorp the growth regulator residue and in turn to enhance germination process and regeneration into plantlets. In the present study, only somatic embryos from Tanggamus genotypes were germinated and regenerated into plantlets. From that, there were 7 survived somatic embryos germinated and regenerated into plantlet initiation (Figure 6), that were : 2 SE (from 5% PEG media), 3 SE (from 10% PEG media), and (2 SE from 15% PEG media). This seven regenerated plantlets were characterized by the leafy shoot with or without root emerged (Figure 5 and Figure 6), and therefore, 7 candidates of drought tolerant somaclone were obtained from this experimets, those were 2 candidates (from 5% PEG media), 3 candidates (from 10% PEG media), and 2 candidates (from 15% PEG media).

The addition of PEG to the MS medium decreased the water potential of the media inducing water stress that adversely affected the callus growth and *in vitro* regeneration capacity of the soybean cultivars (Sakthivelu *et al*., 2008). Matheka *et al*. (2008) reported delayed plantlet regeneration after PEG treatment, may be attributed to the detrimental effects of selection on regeneration. The number of shoots per regenerating PEG-selected callus was reduced compared to unselected callus (Matheka *et al*., 2008; Lutts *et al*., 2004). A low plantlet conversion is also due to abnormalities of somatic embryos produced. PEG causes the reduction in yield of somatic embryos as well as causing incomplete development and anatomical abnormalities of the somatic embryos (Tereso *et al*., 2007). Apparently, in our results, PEG treatment as well as genotypes influenced regeneration capacity after selection. Other reports also reported the strong influence of genotypes on regenerability under osmotic stress conditions (Lutts *et al*., 2004).

Continuously proliferated EC and SE for three months yielded these PEG 6000 stress tolerant EC and SEs from PEG sensitive standard soybean cv. 'Tanggamus'. Therefore, those EC and SE variants might acquire PEG stress tolerant mechanisms during EC or SEs proliferation. Surviving EC and SEs under such selective medium might have indicated they developed from mutant cells and tissues acquiring dehydration stress tolerance mechanisms. Regenerated plants from such EC and SEs might maintain the same mechanisms at plant levels and they might be the same mechanisms causing drought tolerance in identified variant lines. Although PEG induced dehydration stress may be different than drought stress in the field, the tolerance to both conditions might employ similar mechanisms such as maintaining high tissue water potential under the stress. Therefore, selecting for EC and SEs under PEG induced stress may end up with drought tolerant variants.

In the present work, the methods of in vitro selection using PEG selection agent from several soybean genotypes have already developed for generating a putative somaclone drought tolerant. Seven regenerated plantlets of drought tolerant somaclone of Tanggamus genotype were obtained from this experiments; the four of that can be seen in Figure 6. This methods might also be applied to other soybean genotypes to obtain drought tolerant regenerant.

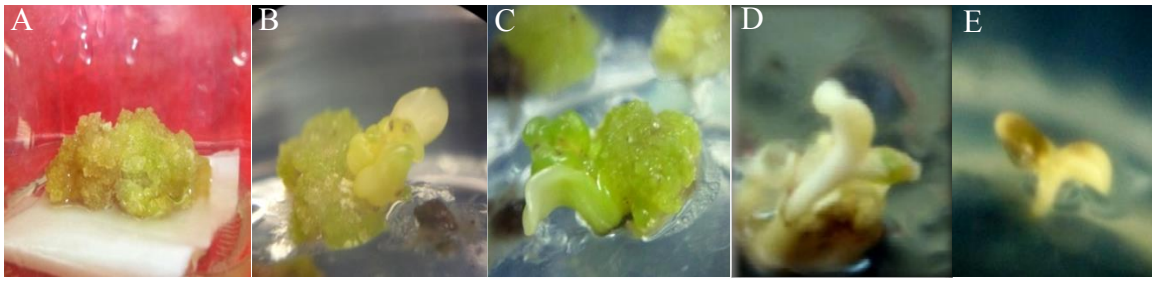

Figure 5. Development of embryogenic callus of Tanggamus genotypes on PEG media after 12 weeks of culture (A) and its regeneration on media containing MS0 + 1 g L^{-1} activated charcoal (B, C, D and E) after 8 weeks of culture

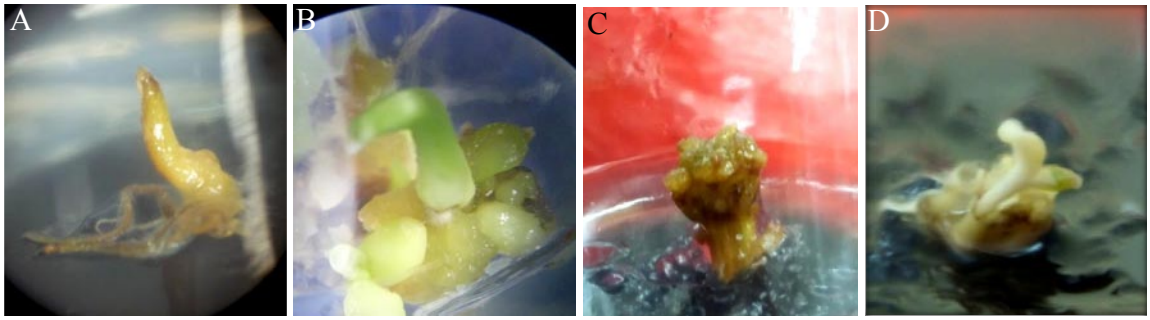

Figure 6. Plantlet initiation of Tanggamus genotypes on MS0 + 1 g L^{-1} activated charcoal regeneration media developed from surviving somatic embryo following selection in 5 % (B), 10 % (B and C), 15 % (D) PEG media

CONCLUSION

Somatic embryogenesis of four soybean genotypes was successfully induced using immature cotyledone explants, B5 vitamine and growth regulators of 2,4 D 5 mg L^{-1} combined with NAA 5 mg L^{-1} incorporated to MS media. In the selection medium of 0-20% PEG, the survived embryogenic callus with somatic embryos formed on all genotypes (Tanggamus, Yellow biloxi, SP-10-4 and CG-22-10). Higher concentration of PEG lowered the number of fresh callus and number of embryogenic callus in all genotypes. Tanggamus was the only genotype that survived until cotyledonary-stage embryos after transferring to MS0 media and have successfully regenerated into shoots or plantlets in the regeneration media. Therefore, this seven regenerated plants of Tanggamus genotypes were the candidates of drought tolerant somaclones, those were 2 candidates (from 5% PEG media), 3 candidates (from 10% PEG media), and 2 candidates (from 15% PEG media).

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