GENOTYPE SPESIFIC ON SOMATIC EMBRYO AND GERMINATION OF SOYBEAN

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ABSTRACT

This research aims to study somatic embryo development of four soybean genotypes and their germination and conversion into intact plant. The explants used were clumps of embryogenic callus containing globular stage embryo(s). This experiment was designed using entirely randomized factorial model. Four soybean genotypes were combined with nine origin induction medium and four combinations of histodifferentiation regulators medium. Result showed that genotype and origin induction medium interacted in promoting cotyledon-stage embryos. Interaction of Ceneng and IK-12 medium (NAA 53.71 µM + 2.4-D 67.87 µM) resulted the highest efficiency in inducing cotyledon-stage embryo. The highest average number of cotyledon-stage embryo was reached by combination of 2.69 μM NAA + 13.32 µM BA. Genotype Ceneng indicated the highest percent of germination and index of vigour. CG76-10 has the fastest germination rate, however the conversion efficiency was poor in all treatments.

Key words: Embryogenic callused, cotyedone-stage embryo,vigour index.

INTRODUCTION

Successfully grown in vitro somatic embryo will means nothing if it can't pass the germination stage. It could not be regenerated into a plantlet. A significant process, which has occurred since embryo's development, is the differentiation of shoot and apical meristem.

The controlling mechanism differentiate plant's somatic embryo vaguely, and it makes a signal that dissolved molecules become active or working. It is proven that embryogenic culture media could trigger embryogenesis process (Von Arnold *et al*., 2002). Globular somatic embryo grown from embryogenic callus must be sub cultured into medium without auxin in order to differentiate (Umahera and Kamada, 2005). Nevertheless, some cases of somatic embryo development and maturity occurred on the medium with same concentration of plant growth regulator as the callus induction medium (Martin, 2003).

According to Stasolla and Yeung (2003) germination refer to root growth/elongation which is not always be followed by shoot growth, however conversion is the development of root system and functional shoot to successfully grown a plantlet. In the other statement, Ranch (1992) stated that conversion is analogue germination of seed, which is characterized by functional root and apical meristem. The germination processes of both on somatic and zygotic embryo were preceded by maturity process. Somatic embryo has faced some morphology and biochemical process conversion during maturity phase. Incomplete maturity of somatic embryo is the most limiting factor of conversion embryo to a plantlet (Hussein *et al*., 2006).

Maturation is the final process of embryogenesis and characterized by the presences of completely morphology mature embryo traits. Somatic embryo doesn't undergo a better maturity

process, while zygotic embryo does. Some evidence showed that somatic embryo has fast yet gradual growing process to germination (Walker and Parrot, 2001; von Arnold *et al*., 2002). The difficult maturity process that has been the main obstacle in successful somatic embryogenesis did not only happened on soybean, but also other plants (Kőrbes and Droste, 2005).

Number accumulation materials stored in embryo is the factor that determines embryo capability to regenerate into plantlet (Bozhkov and von Arnold, 1998). Kőrbes and Droste (2005) concluded that soybean genotype determines the morphology of mature embryo and affected the capacity of an embryo to convert into plantlet. Generally, conversion rate of plant from somatic embryo is about 0-50%. This is obviously lower than germination process through zygotic embryo that could reach more than 90% (Jayasankar *et al*., 2001; Lee *et al*., 2002; Gray, 2005).

Plant growth regulator (PGR) substance is the key compound to induce somatic embryogenesis. In this experiment, NAA and four types of Cytokinins including kinetin, Thidiozuron, BA, and Zeatin were used as the combination of histodifferentiation PGR. The low concentration of NAA is supposed to be able to keep embryogenic cell proliferation, while cytokinin is supposed to stimulate globular embryo to change from conversion phase.

This research aims to evaluate the development of somatic embryo from four genotypes of soybean on histodifferentiation medium and to analyze its germination and conversion process from somatic embryo into plantlet.

MATERIAL AND METHODS

Somatic Embryo Histodifferentiation

Embryogenic callus of four soybean genotype that contains globular somatic embryo, which is a result of proliferation process are taken apart from its clump and subcultured into histodifferentiation medium. Histodifferentiation medium itself consist of macro and micro element of MS medium (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al*., 1968), 3% sucrose, pH 7.0, 0.2% gelrite, and plant growth regulator of appropriate treatment. Each genotype that shows best callus induction rate was cultured into treatment medium. Culture was incubated in a culture room with 28^{o}C, light intensity 1.500 lux in 24 hours photoperiod.

Experimental design was complete randomized design and factorial model. The first factor was genotype which consist of Ceneng (G1), Godek (G2), CG 30-10 (G3), and CG 76-10 (G4). The second factor was source media they were cultured (best callus induction medium) consist of IK-5 to IK-13. The third factor was four combination of regulators which including: MH1: 2.69 µM NAA + 13.32 µM BA, MH2: 2.69 µM NAA + 13.94 µM Kinetin, MH3: 2.69 µM NAA + 6.81 µM thidiazuron, and MH4: 2.69 µM NAA + 13.70 µM Zeatin.

Observation on conversion was done for 5 weeks after cultured (WAC) as follow: the number and percentage of cotyledonary embryo; color of embryo; normal embryo amount and its percentage; abnormal embryo percentage. The data was analyzed with ANOVA, and if the result differs significantly, the data will be subjected to Duncan Multiple Range Test.

Somatic Embryo of Germination

Cotiledonary embryo was separated one by one and then cultured into maturation medium. The maturation medium composition was similar with the previous histodifferention medium. The Cotiledonary embryo was cultured for one month and then sub cultured to germination medium, MS0 (Murashige and Skoog, 1962).

The experimental design was a complete randomized design (CRD). Single factor experimental was soybean genotype with four levels: Ceneng (G1), Godek (G2), CG 30-10 (G3) and CG 76-10 (G4). Germination evaluation was conducted in every 5 days interval up to 25 days after culture, which was marked by the appearance of hair structure on the top of apical or root. Evaluations that were conducted are: sprout endeavour, abnormal shoot percentage, germination rate (GR), and maximum potential growth (PTM).

Histology Analysis

Observation of somatic embryo ontogeni's character on various development stage should be done trough histology study by making preparation with paraffin method developed by Khasim (2002).

RESULT AND DISCUSSION

Cotiledonary Embryo

Somatic embryo development has been induced by transferring embryo to medium with auxin or combination of auxin and low concentration of cytokinin. Some histodifferentiation steps occurred because of conversion type and plant growth regulator (PGR) concentration. Zimmerman (1993) stated that synthetic auxin such as 2.4-D was effective to get and proliferate embryogenic callused, even though it was difficult to dissolve in cell metabolism. Elimination or reduction of 2.4-D concentration in histodifferentiation medium will also eliminate the factor that was exhibiting gene expression for transition, which was required in globular transition step into heart phase.

The other plant growth regulator, cytokinins, was needed for embryo induction on some dicotyledons plants. In some cases cytokinins was only one used to stimulate development of embryogenic culture. Another commonly used cytokinin substance are BA, thidiazuron (TDZ), kinetin, and natural cytokinin (Raemakers *et al*., 1995; Gray, 2005). In this research, the highest number of cotyledonary embryos was reached by interaction between genotype Ceneng and IK-12 medium (NAA 53.71 µM + 2.4-D 67.87 µM), which could produce 1.00 cotyledonary embryo (Table 1).

Even though histodifferentiation medium shows significantly different effect, it has no interaction with other factors. It means the cotyledonary embryo was freely formed, affected only by histodifferentiation medium. The highest average number of cotyledonary embryo stage was 0,245 reached by combination of 2.69 µM NAA + 13.32 µM BA (MH1). It was followed by MH2 medium (2.96 µM NAA + 13.94 µM kinetin) with 0.15 embryo, MH3 (2.69 µM NAA + 6.81 µM thidiazuron) with 0.078 embryo and MH4 (42.69 µM NAA + 13.70 µM zeatin) with 0.038 cotyledonary embryo phase, respectively (Figure 1).

Barwale *et al*. (1986) experiment's showed that organogenic callus culture was grown from young embryo which was cultured on 13.3 µM BAP and 0.2 µM NAA medium. Radhakrishnan and Ranjithakumari (2007) reported that additional 4.4-22.2 µM BAP which was combined by some auxin concentration gave better result on soybean's embryogenesis than kinetin.

Normal embryos variable was significantly influenced by interaction between genotype and induction medium. Thus, these two factors influence each other in normal growth induction and development of somatic embryo. Normal embryo criteria based on morphology such as proved by Hiraga *et al*. (2007) including: monocotyledon, dicotyledons, polycotyledon and branches embryo.

Cotyledonary embryo that develops normally was shown on Figure 2. The highest value for average normal embryo cotyledonary was gained from combination between Ceneng genotype and IK-12 medium (NAA 53.71 µM + 2.4-D 67.87 µM) with average 0.9 embryo (Table 2).

For normal embryo variable, all factors could not show significant effect. Abnormal cotyledon was determined by observing morphology appearance as stated by Hiraga *et al*. (2007) which are: fused cotyledon, long hypocotyle with vestigial cotyledon, and micro cotyledon (data not shown). Abnormality embryo with morphology criteria as above was a factor that could decrease germination rate (Bucheim *et al*., 1989). Parrot *et al*. (1988) and Fernando *et al*. (2002) explained that abnormality of morphology and soybean somatic embryo's development related to 2.4-D level.

Table 1. The effect of medium and genotype interaction on average number of cotyledonary embryo's.

Code	Primary Medium		Genotype			
	PGR (μM)		Ceneng	Godek	CG30-10	CG76-10
	NAA	2,4-D				
IK-5	26.85	0.00	0.353b	0.000c	0.100bc	0.182bc
IK-6	26.85	22.62	0.188bc	0.188bc	0.154bc	0.000c
IK-7	26.85	42.25	0.125bc	0.000c	0.000c	0.111bc
IK-8	26.85	67.87	0.214bc	0.000c	0.000c	0.000c
IK-9	53.71	0.00	0.222bc	0.133bc	0.000c	0.100bc
IK-10	53.71	22.62	0.000c	0.000c	0.167bc	0.200bc
IK-11	53.71	42.25	0.000c	0.000c	0.000c	0.000c
IK-12	53.71	67.87	1.000a	0.000c	0.000c	0.000c
IK-13	80.56	0.00	0.000c	0.083c	0.000c	0.000c

Note: value which is followed by same alphabet not real different according to DMRT at $\alpha = 0.05$. Data was transformed with $\sqrt{x + 0.5}$.

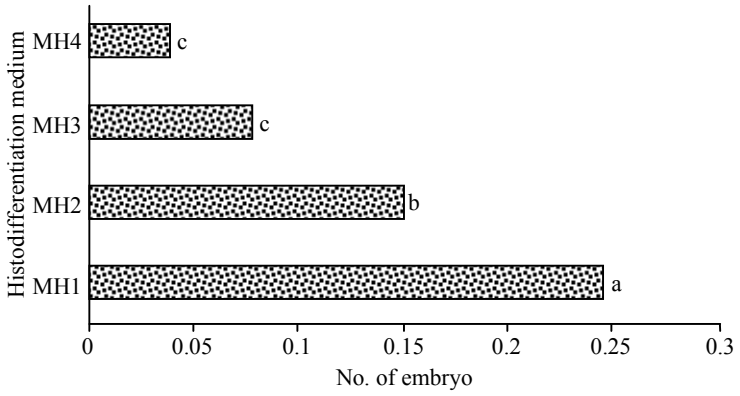

Figure 1. Histodifferentiation medium influence on average number of cotyledonary embryo's.

Having long exposure of 2.4-D level during induction process will influenced embryo development on histddifferent stages.

When forming of cotyledonary embryo's phase was observed as a part of genotype, Figure 3 shows that there are significant differences between Ceneng genotype and other genotypes whether in total embryo variable or normal embryo. Total percentage of cotyledonary embryo and normal embryo are 26.76% and 23.24% respectively. This marks the significant differences of normal embryo percentage between Ceneng and three other genotypes, which is about 4.90% to 7.14% and 1.96% to 4.76%. In addition, there is no significantly different between abnormal embryo and genotype's variables.

The graphic clearly shows that Ceneng genotype as shade tolerant genotype could not succeeded its embryogenic character to its progeni, which are CG30-10 and CG76-10. It clearly shows that tolerant character was unrelated with embryogenic capacity. Embryogenic capacity on genotype CG30-10 and CG76-10 was quantitatively same with shade sensitive genotype Godek. Thus it could be assumed that somatic embriogenetic regeneration capability on four genotypes above are controlled by homozygote recessive genes.

Embryo color is related to somatic embryo's development. On hevea tree, white embryo will regenerate into planlets, while green embryo will become embriogenetic callus through secondary embryogenesis process (Vesseire *et al*., 1994). Somatic embryo color has also been used in estimation of carrot synthetic seed (Sakamoto *et al*., 1992) and somatic embryo conversion potency of sweet potato (Padmanabhan *et al*., 1998).

During this research, two colors occurred on cotyledonary embryo's phase, pale green and yellowish. It relatively doesn't change until the end of research. The analysis of variance shows that

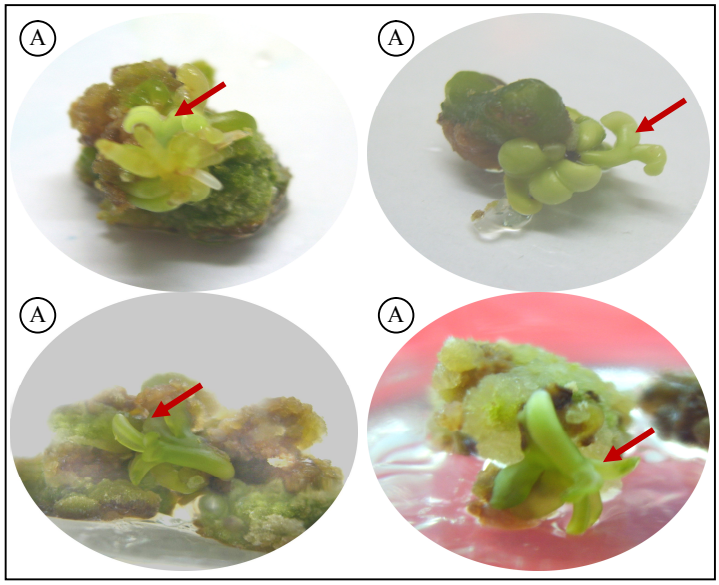

Figure 2. Cotyledonary embryo phase that grow normal (as shown by archer). A = Ceneng genotype from primary medium IK-12 on medium MH1 (2.69 µM NAA + 13.32 µM BA), B = Godek genotype from primary medium IK-6 on MH2 medium (2.69 µM NAA + 13.94 µM Kinetin, C = Genotype CG30-10 from primary medium IK-10 on MH1 (2.69 µM NAA + 13.32 µM BA), D = Genotype CG76-10 from primary medium IK-10 on MH1 (2.69 µM NAA + 13.32 µM BA).

there was no variable, which significantly influence pale green color occurrences. While yellowish one significantly influenced by callus induction medium and interaction between callus induction medium and histodifferentiation medium.

Mostly, yellowish embryo occurred because of interaction between primary and histodifferentiation medium. As shown on Table 3, the highest percentage of yellowish embryo occurred on interaction between IK-5 with MH1 and MH4, IK-6 with MH3, IK-9 with MH1 and MH2, IK-12 with MH2 and MH4, and IK-13 with MH1, respectively. Every medium combination stated above was able to induce up to 100% yellowish embryo per explants.

Histology Analysis

Based on its initiation cell's amount, there are two ways a somatic embryo ontogenyc formed, namely unicellular and multicellular (Quiros-Figueroa *et al*., 2006). Histology analysis result mentioned two ways such as above. On Figure 4a, two shapes of globular phase embryo from Ceneng genotype was observed, which are the first and last globular phase. In the first phase, embryo was formed unicellularly. It showed the same structure as suspensor on basal embryo. It could be assumed that it was secondary embryo, which is formed from primary embryo. It is very easy to observe its network because of the purplish color of young cell or tissue composed of safranin. On the last globular embryo phase, it can be clearly observed starting from polarization of cell division that will develop into cotyledon and hypocotyle. Embryo was fully surrounded by non-embryogenic callus. Quiroz-Figueroa *et al*. (2006) concluded that the existence of non-embryogenic callus around embryo acted with nurse like function for the embryo. It is similar with endosperm function on zygotic embryo.

Figure 4b showed imperfect longitudinal piece of two types of globular embryo. On globular embryo advance phase, a part from explants (origin tissue) could be observed. On the first globular embryo, it seems that it is formed multicellularly where some embryo appeared closely to epidermal tissue. Figure 4c showed that there are two types of the first phase globular embryo of CG30-10 genotype. They were unicellular and multicellular. On the unicellular embryo, the structure clearly looks like suspensor. While on multicelluler embryo, it seems fusion happened to some embryos from subepidermal layer and part of embryo's basal were formed from united tissue with perfunctory net. It is possibly formed a direct embryogenesis. Every embryo has made pre-procambium with each other. According to dos Santos *et al.* (2006) structurally united first phase globular embryo will create the branch of cotyledon embryo phase.

On CG76-10 genotype (Figure 4d), longitudinal piece of last globular embryo's phase was observed. Biopolarisation of tissue was obvious, where part of apical, which will become cotyledon, has been formed in curve as the heart phase. Embryo seems obviously separated from explants. Friable callus structure that will initially form this embryo will cause it to be easily separated from callus phase.

Somatic Embryo Germination

Somatic embryogenesis is a complex process, where development, survival power, and final result quality depended on the first process. Von Arnold *et al*. (2002) stated that only mature embryo with perfect and complete required morphology and accumulated photosintate (carbohydrate, lipid,

and proteins) and also tolerant to desiccation will become the normal planlet/plants. Only plants that are gained by zygotic embryo will have same seedling.

A lot of approachment in somatic embryo germination of soybean has been done, but it wasn't efficient. In this research pre treatment, maturity process has done before germination process. Maturity process has done after histodifferentiation process to regenerate culture by doing subculture once in the same media. Color change in some embryo was observed after a month maturity process. Pale green embryo changes into yellowish, then into a cream color. It shows that there is a conversion to embryo maturity physiology.

Based on Balley *et al*. (1993) experiment, it is known that shoots structure without root and young shoot after 25 days cultured on MSO media rarely grow into plantlets. Only sprout structure with shoot can develop vigour root system after subcultured to new medium or planted on the field. Therefore, in this research germination is valued based on bud's appearance and root.

In this research, result showed that Ceneng genotype has the most normal sprout (6 sprouts). While genotype CG30-10 and CG76-10 each has 3 normal sprouts respectively. On Godek genotype, none of cotyledon embryo's phase sprouted (Table 4). Based on number of normal sprout, germination rate performance could be derived, which are 40% on Ceneng genotype and 20% for CG 30-10 and CG76-10 respectively. Abnormal sprouts only occurred on Ceneng (3 sprouts). The vigour index value of Ceneng was 0.42 and CG30-10 and CG76-10 is 0.04 and 0.05 respectively. The fastest germination rate was reached by CG76-10 with average 20 days. Followed by Ceneng genotype with average 23.75 days and CG30-10 within average 25 days. Maximum growth potency on Ceneng genotype as much as 60% while on CG30-10 and CG76-10 genotype was 20% respectively.

On this research, the number of post maturity embryo that germinates normally was relatively low. Genotype Ceneng with the highest value of germination performance, which is 40%, still has lower viability compared to zygotic embryo. The same condition applies to Godek genotype where none of embryo showed germination. In certain condition, germination medium could cause embryo dormancy. If embryo has not germinated, it is possible that it was grown in environment where limiting factor is enforcing dormancy.

Germination performance is a potential viability measurement simulated from embryo's capability to grow and produce in optimum condition. The low embryo viability can be improved optimally by subjecting pre germination treatment. One of the alternatives that can be used is charcoal, to absorb remnant of 2.4-D, which was exhibiting germination (Li and Grabau, 1996).

Ghazi *et al.* (1986) obtained plantlets from somatic embryo, which was cultured in medium contain GA3 0.104 mg/l or zeatin 0.110 mg/l. Hammat and Davey (1987) gave desiccation of mature somatic embryo in sterile pipette until it shrink 40 to 50%. After desiccation process, germination performance of embryo increase to 30%. Ranch *et al*. (1986) in parrot *et al.* (1988) reported the use of maturity medium which contained 0.5% active charcoal and 10% sucrose. This high osmoticum medium is supposed to be able to maintain desiccation process. Then the embryo was transferred into hormone free MS medium until it germinates. Walker and Parrot (2001) reported that additional 5% polyethylene glycol or 1.5% sorbitol will increase germination of embryo and somatic embryo conversion.

The higher performance of CG76-10 genotype shows that genotype CG76-10 is stronger in unpredictable environment than the others. While the higher index vigor gained by Ceneng genotype shows that it has better capability of embryo to germinate normally on first germination stage.

Li and Grabau (1996) stated that conversion of embryo into root and shoot does not affected by genotype, even though somatic embryo induction efficiency and the result of primer somatic are affected by genotype. However, Tomlin *et al*. (2002) stated that maturity and germination capacity are influenced by genotype too. Maximum growth potency was reached by Ceneng (60%). Maximum growth potency is a rare variable in embryo viability test. Among other variable, only this variable shows the ability of embryo to grow. It was phenomenal growth medicated metabolism symptom.

In this research, only one sprout grows normally and regenerates into plantlet. This sprout extend epicotyle 1.6 mm per day and formed two leaves and two stems trifoliate leaf. The low rate of sprout conversion into plantlet may be caused by auxin effect during embryogenic callus induction, which was inhibiting sprout organogenesis capability. Furtheremore, conversion competency could also inhibited by abnormal maturation and no desiccation treatment prior germination.

CONCLUSION

Genotype and medium that were used on embryogenic callus induction could influence somatic embryo development. Both of them show significant interaction in cotyledonary embryo phase. Ceneng genotype interacted with callus induction medium that contain auxin with high concentration and showed higher efficiency rate whether in total embryo induction or normal cotyledonary embryo phase.

Histodifferentiation medium was freely affecting soybean's somatic embryo development. The higher average cotyledonary embryo was gained from NAA and BA. Two genotype histology analysis result (Ceneng and CG76-10) pointed the existence of globular embryo stage on some development step is higher in proliferation.

Each genotype show different viability of embryo. Higher germination performance and vigor index of Ceneng genotype, showed that it has more viability and vigor compared to the other genotypes. With faster germination rate, CG76-10 shows it has good vigor potency. Generally, sprout conversion rate into plantlet was low.

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