Somatic Embryogenesis in Different Soybean Varieties

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ABSTRACT

National demand for soybean (*Glycine max* L. Merrill) is higher that its production, so that Indonesia needs to import the commodity. The government has planned to extensity the crop to acid soil areas, which has not been managed at the maximum capacity. However, many problems faced in acid soil, particularly high content of aluminum and low pH, which inhibit the growth of plant. Soybean variety tolerant to the condition is still limited. To improve the tolerance to aluminum, a study was conducted through *in vitro* selection. One of the problems needs to be accomplished is the method of cell regeneration, since no standard (repeatable) method has been established. Therefore, it is necessary to study the effect of genotypes (varieties), source of explants, time of subculturing/physiologycal condition of mother plant and other factors on the success of somatic embryogenesis. The study was conducted in two steps. In the first step, 7 sources of explants respectively collected from 10 soybean varieties were tested for their response to callus induction on 18 formulation media and to embryo maturation on 25 formulation media. Callus induction media were Murashige and Skoog (MS) or Phillips and Collins (PC-L2) in the combination with 2,4-D (0-40 mg/l), picloram (0-0.01 mg/l), BA (0-0.5 mg/l), kinetin (0-0.1 mg/l), NAA (0-10 mg/l), and several amino acids. Meanwhile for somatic embryo maturation basal media MS or PC-L2 had been used in the combination with 2,4-D (0-0.05 mg/l), BA (0-0.5 mg/l), NAA (0-0.3 mg/l), zeatin (0-1 mg/l), GA_3 (0-0.1 mg/l), manitol, and charcoal. The best explants from first experiment were then used in second experiment. Results showed that 5 varieties (Bromo, Tambora, Wilis, Black Manchu, and Argomulyo) were responsive to the treatments. From 18 media formulations, 5 formulae gave good results for embryonic callus, which developed into embryosomatic structures. Culture in MS media with high concentration of auxin, NAA (10 mg/l) or 2,4-D (40 mg/l) and amino acid, followed by subculture in the media with low concentration of 2,4-D produced embryonic callus which was able to develop to bipolar embryo. For maturation and germination, somatic embryo structures were subcultured on a media without auxin. The best methods of somatic embryogenesis produced in this study were repeatable and relatively produced high regeneration ability.

Key words: *Glycine max*, aluminum, *in vitro*

INTRODUCTION

National demand for soybean is higher than its production, so that the country needs to import the commodity in a large quantity every year. To reduce the import of the product, Indonesia has programmed to increase soybean production, particularly in acid soil which covers 101.519 million hectares through out the country, consisting of Podsolic, Lateritic, and Hidromorphic alluvial soils (Notohadiprawiro, 1983).

The problem in soybean production in acid soil, is that the soil has a low pH, high content of Al, low content of nutrient particularly of N, P, K, Ca, Mg, and Mo and low activities of soil microorganism. High content of aluminum will result in toxicity to plant

(Rao *et al.*, 1993). General symptom of toxicity is a retardation of root development (short and thick) as a result of inhibition in cell elongation. The existing soybean varieties generally need a higher pH (±6) and susceptible to high aluminum content. To develop soybean in acid soil with low pH, it is necessary to provide farmers with soybean variety resistant to low pH and high aluminum content. The variety could neutralize a toxic released by Al in the plant or reduce the excessive Al ion by the roots.

As the source of Al resistance of soybean is very limited, the improvement of the characters was approached through *in vitro* selection. The method has been adopted in improving the resistance characters in different crops either to biotic or abiotic factor (Stavarek and Rains, 1984; Ahloowalia, 1986). *In vitro* selection to produce cells resistant to aluminum had been applied on tomato and potato (Stavarek and Rains, 1984) and sorghum (Smith *et al.*, 1983).

Cell and callus are generally used for *in vitro* selection, and the problem that frequently arises is regeneration system of cell or callus. Ojima and Ohira (1982), found the cells of carrot resistant to aluminum and the callus was not able to regenerate.

Cell regeneration may be conducted in two directions, through organogenesis and somatic embryogenesis. In crop improvement through biotechnology, somatic embryogenesis is more preferred, as it can be developed from a single cell so that the progenies genetically are definitely homogenous. Soybean is a species that is very difficult to be regenerated *in vitro* (Graybosch *et al.*, 1987). Tissue culture of soybean has been started since 1960, but until 1970's no report stated that the cell of the crop could differentiate or able to be regenerated (Beversdorf and Bingham, 1977). Since 1970's regeneration systems in soybean has been studied through somatic embryogenesis, but the frequency of succes was low, sporadic, and unrepeatable. Based on these results the research, were initiated by testing different sources of explants from various varieties for their response to *in vitro* regeneration on different media formulations.

MATERIALS AND METHODS

Research on somatic embryogenesis of soybean was conducted from April 1998 to March 1999. The study consisted of two activities. In the first study different kinds and formulations media were applied on different explants of various varieties of soybean. The best media formulation and most responsive explants of certain varieties in producing somatic embryo were used in the second study.

In the first study 18 media formulations for callus induction and 25 formulation for embryo maturation were respectively applied on different sources of explants (immature seed, cotyledone, the edge of young leaves, immature zygotic embryo, mature zygotic embryo, epicotyls, hypocotyls, and root tip) from 10 varieties of soybean (Malabar, Kerinci, Tambora, Agromulyo, Black Manchu, Tidar, Orba, Wilis, Bromo, and Krakatau).

Two basal media were used for callus induction, i.e. Murashige and Skoog (MS) and Philips and Collins (PC-L2) which was respectively combined with growth

regulators, i.e. 2,4-D (0-40 mg/l), picloram (0-0.001mg/l), BA (0-0.5 mg/l), kinetin (0-0.1 mg/l), NAA (0-10 mg/l), and several amino acids as the source of organic N. To prevent an excessive cell division resulted from the application of high dosage of 2,4-D (40 mg/l), the callus were subcultured on a media with gradually reduced auxin content. The media for somatic embryo maturation consisted of 25 formulations which were respectively made up from two basal media (PC-L2 and MS) combined with different growth regulators 2,4-D (0-0.05 mg/l), BA (0-0.5 mg/l), NAA (0-0.3 mg/l), zeatin (0-1 mg/l), GA_3 (0-0.1 mg/l), manitol, and charcoal.

Explant sources were collected from young pod, leaves from *in vitro* cultures, and mature seeds. The explants were sterilized with alcohol 70% and clorox 20-30%. Sterile explants were than incubated on callus induction media illuminated with 1000 lux for 16 hours. The growing embryonic callus, which produced somatic embryos, was subcultured on a media for embryo maturation.

In the second study, the best media (which produced somatic embryo) were applied on the responsive explants of the responsive varieties obtained in the first studies. The parameters used for evaluating the treatments were the formation of embryogenic callus, embryosomatic structure, and number of the embryo structures.

RESULTS AND DISCUSSION

First Experiment

From 10 varieties with respectively 8 sources of explant, showed that the callus of Wilis, Bromo, Black Manchu, and Argomulyo were more embryogenic than that of others varieties. The callus generally showed higher regeneration ability through somatic embryogenesis introducing embryoid, which had similar development patern with zygotic embryo (Cheng and Raghavan, 1985). By using the tested explants and media formulations Tidar and Malabar varieties were not able to form any embryonic callus. Both varieties produced a compact callus that tended to produce adventives roots. This evident was also reported by Cheng and Raghavan (1985), in solanaceae, where the compact callus produced roots while friable one produced somatic embryo. From various media and source of explants tested, it was shown that Black Manchu variety produced more embryonic callus than Orba but somatic embryo structures only develop from cotyledone and mature zygotic embryo.

From the 5 varieties that were responsive to *in vitro* treatments, the embryogenic cells successfully developed to somatic embryo generally came from MS media containing different nitrogen and phosphate content from PC-L2. In other varieties, Phillips and Collins found that PC-L2 was better than MS in somatic embryo production. This implies that various factors affect cell development such variety, nutrients, growth substances and the sources (kinds) of explants. Auxin of strong activity (2,4-D, NAA or its combination with cytokinin of low concentration) is generally used for embryonic callus

induction (Sellars *et al.*, 1990). Table 1 and 2 show that auxin application at different concentrations affected embryogenic cell development.

Table 1. Embryogenic callus formation of different explants, formulation media in 10 varieties of soybean 10 weeks after culturing

Varieties and explant sources	Media formulations																	
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18
1. Wilis																		
Immature seed	-	-	-	-	-	-	-	-	-	-	v	v	-	v	v	v	-	-
Cotyledon	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
The edge of young leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	v	v	v	v	-	-	v	v	v	v	v	v	-	v	v	v	-	-
Mature zygotic embryo	v	v	v	v	-	-	v	v	v	v	v	v	-	v	v	v	-	-
Epicotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypocotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root tip	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2. Bromo																		
Immature seed	-	-	-	-	-	-	-	-	-	-	v	v	-	-	-	-	-	-
Cotyledon	-	-	-	-	-	-	-	-	-	-	v	v	v	v	v	v	-	-
The edge of young leaves	-	-	-	-	-	-	-	-	-	-	-	-	v	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	v	v	-	v	v	v	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	v	v	-	v	v	v	-	-
Epicotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypocotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root tip	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3. Tambora																		
Immature seed	-	-	-	-	-	-	-	-	-	-	v	v	-	v	v	v	-	-
Cotyledon	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
The edge of young leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	v	v	v	v	-	-	v	v	v	v	v	v	-	v	v	v	-	-
Mature zygotic embryo	v	v	v	v	-	-	v	v	v	v	v	v	-	v	v	v	-	-
Epicotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypocotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root tip	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4. Argomulyo																		
Immature seed	-	-	-	-	-	-	-	-	-	-	v	v	-	v	v	v	-	-
Cotyledon	-	-	-	-	-	-	-	-	-	-	v	v	-	v	v	v	-	-
The edge of young leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	v	v	-	v	v	v	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epicotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypocotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root tip	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 1. Continued

Varieties and explant sources	Media formulations																	
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18
5. Black Manchu																		
Immature seed	-	-	-	-	-	-	-	-	-	-	v	v	-	v	v	v	-	-
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
The edge of young leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	v	v	-	v	v	v	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epicotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypocotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root tip	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6. Krakatau																		
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cotyledone	-	v	-	-	-	v	-	v	-	-	v	v	-	v	v	v	-	-
The edge of young leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	v	v	-	v	v	v	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epicotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypocotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root tip	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7. Orba																		
Immature seed	v	-	-	-	v	-	-	-	-	-	v	v	-	v	v	v	-	-
Cotyledone	v	-	-	-	v	-	-	-	-	-	v	v	-	v	v	v	-	-
The edge of young leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	v	-	-	-	-	-	v	v	-	v	v	v	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epicotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypocotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root tip	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8. Tidar																		
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
The edge of young leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epicotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypocotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root tip	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9. Kerinci																		
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cotyledone	-	-	-	v	-	-	-	-	-	v	-	-	-	-	-	-	-	-
The edge of young leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	v	-	-	-	-	-	v	-	-	-	-	-	-	-	-
Mature zygotic embryo	-	-	-	v	-	-	-	-	-	v	-	-	-	-	-	-	-	-
Epicotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypocotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root tip	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 1. Continued

Varieties and explant sources	Media formulations																	
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18
10. Malabar																		
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
The edge of young leaves	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epicotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypocotyls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root tip	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Notes: MI = PC-L2 + 2,4-D 0.5 mg/l + kinetin 0.1 mg/l + ABA 0.05 mg/l, M2 = PC-L2 + Na_2HP0_4 80 mg/l + adenine sulfate 6 mg/l + casein hydrolisate 1 g/l + 2,4-D 2 mg/l + BA 0.5 mg/l + ABA 0.05 mg/l, M3 = PC-L2 + 2,4-D 0.5 mg/l + kinetin 0.1 mg/l + ABA 0.075 mg/l + picloram 0.01 mg/l + BA 0.1 mg/l, M4 = PC-L2 + Na_2HP0_4 80 mg/l +adenine sulfate 6 mg/l + casein hydrolisate 1 g/l + 2,4-D 0.5 mg/l + BA 0.1 mg/l + ABA 0.075 mg/l + picloram 0.01 mg/l + kinetin 0.1 mg/l, M5 = PC-L2 + 2,4-D 0.5 mg/l + kinetin 0.1 mg/l + ABA 0.05 mg/l + sucrose 2.5%, M6 = PC-L2 + Na_2HP0_4 80 mg/l + adenine sulfate 6 mg/l + casein hydrolisate 1 g/l + 2,4-D 2 mg/l + BA 0.5 mg/l + thiamin 1 mg/l + sucrose 2.5%, M7 = MS + 2,4-D 0.5 mg/l + kinetin 0.1 mg/l + ABA 0.075 mg/l, M8 = MS + Na_2HP0_4 80 mg/l + adenine sulfate 6 mg/l + casein hydrolisate 1 g/l + 2,4-D 2 mg/l + BA 0.5 mg/l + ABA 0.05 mg/l, M9 = MS + 2,4-D 0.5 mg/l + kinetin 0.1 mg/l + ABA 0.075 mg/l + picloram 0.01 mg/l + BA 0.1 mg/l, MI0 = MS + Na_2HP0_4 80 mg/l + adenine sulfate 6 mg/l + casein hydrolisate 1 g/l + 2,4-D 0.5 mg/l + BA 0.1 mg/l + ABA 0.075 mg/l + picloram 0.01 mg/l + kinetin 0.1 mg/l, M11 = MS + 2,4-D 40 mg/l + sucrose 6%, M12 = MS + NAA 10 mg/l + casein hydrolisate I g/l + amino acid, M13 = MS + NAA 10 mg/l, M14 = MS + 2,4-D 40 mg/l, M15 = MS + 2,4-D 40 mg/l + amino acid, M16 = MS + 2,4-D 40 mg/l + Sucrose 6% (pH = 4), M17 = MS + BA 0.3 mg/l + thidiazuron 1 mg/l + kinetin 0.1 mg/l, M18 = MS + thidiazuron 0.2 mg/l + kinetin 0.2 mg/l + 2,4-D 2 mg/l

Table 2. Growth of embryogenic structures at different media formulations in 10 varieties of soybean 10 weeks after culturing

Varieties and explant sources	Media formulations																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. Wilis																									
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
2. Bromo																									
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
3. Tambora																									
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
4. Argomulyo																									
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v

Table 2. Continued

Varieties and explant sources	Media formulations																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
5. Black Manchu																									
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
6. Krakatau																									
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
7. Orba																									
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v
8. Tidar																									
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9. Kerinci																									
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10. Malabar																									
Cotyledone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mature zygotic embryo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Immature seed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Notes: 1 = PC-L2 + picloram 0.001 mg/l + BA 0.15 mg/l, 2 = PC-L2 + picloram 0.001 mg/l + BA 0.15 mg/l + 2,4-D 0,1 mg/l, 3 = PC-L2 + picloram 0.001 mg/l + BA 0.15 mg/l + 2,4-D 0.3 mg/l, 4 = PC-L2 + picloram 0.001 mg/l + BA 0.15 mg/l + 2,4-D 0.5 mg/l, 5 = PC-L2 + NAA 0.3 mg/l + BA 0.05 mg/l + 2,4-D 0.05 mg/l, 6 = PC-L2 + BA 0.01 mg/l, 7 = PC-L2 + manitol 15 g/l + charcoal 2.5 g/l + GA_3 0.1 mg/l + zeatin 0.5 mg/l, 8 = PC-L2 + manitol 15 g/l + charcoal 2.5 g/l + GA_3 0.1 mg/l + zeatin 1 mg/l, 9 = MS + manitol 15 g/l + charcoal 2.5 g/l + GA_3 0.1 mg/l + zeatin 0.5 mg/l, 10 = MS + manitol 15 g/l + GA_3 0.1 mg/l + zeatin I mg/l, 11 = MS + maltose 6% + charcoal 0.5 g/l, 12 = MS + maltose 6% + charcoal 0.5 g/l + PEG 2.5%, 13 = MS + maltose 6%+ charcoal 0.5 g/l + GA_3 0.1 mg/l + zeatin 1 mg/l, 14 = MS + maltose 6% + charcoal 0.5 g/l + GA_3 0.1 mg/l + 2-iP 1 mg/l, 15 = MS + maltose 6% + charcoal 0.5 g/l + GA_3 0.1 mg/l + 2-iP 0.5 mg/l, 16 = MS + Y-GG 2.5%, 17 = MS + GA_3 0.1 mg/l + zeatin 1 mg/l, 18 = MS + charcoal 0.5 g/l + GA_3 0.1 mg/l + 2-iP 1 mg/l, 19 = MS + charcoal 0.5 g/l + GA_3 0.1 mg/l + 2-iP 0.5 mg/l, 20 = MS + sucrose 3% + manitol 1.5 g/l + GA_3 0.1 mg/l + zeatin 1 mg/l, 21 = MS + picloram 0.001 mg/l + ABA 0.05 mg/l + GA_3 0.1 mg/l, 22 = MS + BA 0.5 mg/l + zeatin 0.1 mg/l + betain 100 mg/l, 23 = MS + BA 0.5 mg/l + picloram 0.01 mg/l + betain 100 mg/l, 24 = MS + BA 0.5 mg/l + kinetin 0.1 mg/l + betain 100 mg/l, 25 = MS0

Auxin application at high concentration (10-40 mg/l) showed better effect on embryogenic callus development. Wetherell and Dougall (1976), Kigosoe *et al.* (1993) used high concentrations in carrot and other species. So did Komatsuda and Ohiyama (1988), Komatsuda *et al.* (1992), and Li *et al.* (1996) on somatic embryogenesis in soybean.

Application of amino acid to the media containing high concentration of auxin could improve somatic embryogenesis (Wetherell and Dougall, 1976: Johansson 1986). The application of amino acid (glutamine, casein hydrolisate, arginin) in this study showed the same results. A specific reduction nitrogen like NH_4^+, glutamine or adenine could increase somatic embryo formation, since in the chloroplast the amino acids could act as a precursor in the production of protein, nucleic acid, and others cellular processes.

From different explant tested it was found that zygotic embryos from cotyledone were more responsive in producing embryogenic callus. Embryo and meristemmatic cells of the reproductive tissue more responsive than that of mature cells. Teixeira *et al.* (1993) reported that types of explant frequently give more effect on somatic embryo genesis than media composition or oilier environmental factors.

For maturation, embryogenic cells were subcultured on a media without growth regulator (Table 2). The use of simple media (MS = without growth regulator) showed better result than other growth media. Media without growth regulator for maturation of somatic embryo was also used by Komatsuda and Ohiyama (1988) and Li *et al.* (1996). Embryogenic callus from Tidar and Kerinci varieties did not produce any mature somatic embryo structure. In other varieties embryogenic cells produced bipolar embryo particularly in Wilis, Tambora, Bromo, Black Manchu, and Argomulyo. In Bromo variety, embryogenic callus from all the sources of explants developed to form the mature bipolar embryos.

Explant from cotyledone, zygotic embryo, and immature seed were generally more embryogenic and formed bipolar embryo faster than mature zygotic embryo. In the complex media they were not able to produce torpedo structure except in MS media containing manitol 15 g/l + GA_3 0.1 mg/l + zeatin 1 mg/l, and MS containing GA_3 1 mg/l + zeatin I mg/l.

To find out the best method (repeatable), the responsive varieties and explants, and media formulations producing the best regeneration will be repeated in the second experiment.

Second Experiment

From the second experiment, it was seen that embryonic callus development ranged from 17.86-100% (Table 3). From 5 varieties tested, Wilis variety showed the highest percentage of callus formation (90.09%), followed by Black Manchu (82.14%), Tambora (79.02%), Bromo (77.77%), and Argomulyo (70.00%). From 5 media formulations, MS + NAA 10 mg/l + casein hydrolisate I g/l + amino acid (M12), MS + 2,4-D 40 mg/l + 2 amino acids (M14), MS + 2,4-D 40 mg/l + 3 amino acids (M15),

produced the highest percentage of embryogenic callus formation for all the varieties tested. NAA was also used by Barwale *et al.* (1986) for regenerating zygotic embryo, and by Komatsuda *et al.* (1992) for regenerating cotyledone.

In maturation phase, the highest percentage (50%) of somatic embryo structures was produced from Wilis cultured on MS media 2,4-D 40 mg/l + 2 amino acid. Bipolar embryos were varied in shape, two of them were trumpet and cotyledon us. Somatic embryo structures did not develop in Bromo and Argomulyo varieties when they were cultured on MS + 2,4-D 40 mg/l + 2 amino acids or on MS + 2,4-D 40 mg/l + 3 amino acids (Table 4). The same formulation will give different effect if the pH of the formulation were different. Somatic embryo structures will be inhibited at a pH of about 4 (i.e. Black Manchu and Argomulyo cultured on MS + 2,4-D 40 mg/l + sucrose 6%, pH = 4). In low pH, some organic or inorganic compound (e.g. $P0_4^{3-}$, NH_4^+ or vitamin B1) are not perfectly dissolved in the media, resulting in the nutrients are not available to the embryogenic cells.

Average number of somatic embryos, either globular or torpedo ranged from 0 to 5.4 (Table 5). The highest number of bipolar embryos produced by Wilis variety cultured on MS + 2,4-D 40 mg/l + 3 amino acids followed Bromo varieties cultured on MS + 2,4-

Table 3. Embryogenic callus formation in 5 varieties of soybean, 10 weeks after culturing

Treatments	Varieties					Average
	Wilis	Tambora	Bromo	Black Manchu	Argomulyo	
M11	78.57	62.50	25.00	92.86	75.00	66.79
M12	96.88	95.83	100.00	100.00	87.50	96.04
M14	100.00	96.88	85.71	100.00	75.00	91.52
M15	100.00	85.71	100.00	100.00	87.50	94.64
M16	75.00	54.17	78.13	17.86	25.00	50.03
Average	90.09	79.02	77.77	82.14	70.00	79.80

Notes: M11 = MS + 2,4-D 40 mg/l + sucrose 6%, M12 = MS + NAA 10 mg/l + casein hydrolisate 1 g/l + amino acid, M14 = MS + 2,4-D 40 mg/l + 2 amino acids, M15 = MS + 2,4-D 40 mg/l + 3 amino acids, M16 = MS + 2,4-D 40 mg/l + sucrose 6% (pH = 4)

Table 4. Somatic embryo structures formation in 5 varieties of soybean, 10 weeks after culturing

Treatments	Varieties					Average
	Wilis	Tambora	Bromo	Black Manchu	Argomulyo	
M11	17.86	12.50	8.33	25.00	29.17	18.57
M12	6.25	7.14	37.50	31.25	14.29	19.29
M14	50.00	28.13	16.67	17.86	16.67	25.87
M15	20.83	32.14	32.14	16.67	10.71	22.50
M16	12.50	16.67	17.86	0	0	9.41
Average	21.48	19.31	22.50	18.16	14.17	19.13

Notes: M11 = MS + 2,4-D 40 mg/l + sucrose 6%, M12 = MS + NAA 10 mg/l + casein hydrolisate 1 g/l + amino acid, M14 = MS + 2,4-D 40 mg/l + 2 amino acids, M15 = MS + 2,4-D 40 mg/l + 3 amino acids, MI 6 = MS + 2,4-D 40 mg/l + sucrose 6% (pH = 4)

Table 5. Average number of somatic embryo structures (globular, torpedo) in 5 varieties of soybean, 10 weeks after culturing

Treatments	Varieties					Average
	Wilis	Tambora	Bromo	Black Manchu	Argomulyo	
M11	2.40	3.00	4.50	2.29	1.43	1.19
M12	2.50	1.50	1.42	1.50	1.25	1.63
M14	3.00	4.00	1.25	1.20	2.75	2.44
M15	5.40	4.33	2.00	1.75	2.33	3.16
M16	1.25	2.50	2.20	0	0	2.72
Average	2.90	3.07	2.27	1.35	1.55	2.23

Notes: M11 = MS + 2,4-D 40 mg/l + sucrose 6%, M12 = MS + NAA 10 mg/l + casein hydrolisate 1 g/l + amino acid, M14 = MS + 2,4-D 40 mg/l + 2 amino acids, M15 = MS + 2,4-D 40 mg/l + 3 amino acids, M16 = MS + 2,4-D 40 mg/l + sucrose 6% (pH = 4)

D 40 mg/l + 3 amino acids + sucrose 6% (4.5). Teen weeks after culturing in the same media, the somatic embryos proliferate rapidly.

Subculturing embryogenic cells, on the media for embryo maturing will accelerate the production of larger bipolar embryo. Larger bipolar embryo is easy to be separated individually in somatic seed production. Somatic seeds produced showed variable performance, and the roots grew so intensive resulted in the growth of shoots were inhibited. To produce better performance of somatic seeds, in further experiment, different sources of cytokinin will be tested in low concentration.

From the two series of experiments, it was revealed that the best methods in the first experiment were repeatable and produced high percentage of regeneration therefore (50%), the method can be applied in the next study, i.e. the improvement of soybean resistance to Al and drought.

CONCLUSION

The best methods of somatic embryogenesis produced in this study were repeatable and relatively produced high regeneration ability (50%).

From 10 varieties tested, 5 varieties of soybean were responsive to somatic embryogenesis. The varieties were Bromo, Wilis, Tambora, Black Manchu, and Argomulyo.

The best explants to produce somatic embryo structures were the immature and mature zygotic embryos and cotyledone.

MS media enriched with 2,4-D 40 mg/l and several amino acid could induced embryogenic callus which was able to develop to bipolar embryo.

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