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Effects of hypoxia condition in embryogenic callus growth of soybean cell culture

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Abstract. The study was performed at Tissue Culture Laboratory, Agrotechnology Department, University of Sumatera Utara, to investigate the effect of plant growth regulator (PGR) and embryogenic callus performance soybean cultivars on hypoxia condition. This research had two stages, induction of embryogenic callus and analysis metabolism of callus after hypoxic condition with T-test. The analysis was used factorial Completely Randomized Design with two factors. The first factors were cultivars of soybean (Baluran, Gepak Kuning, and Grobogan) and the second factors were combinations of PGR (5 mg/l 2,4-D + 1 mg/l BAP, 10 mg/l 2,4-D + 1.5 mg/l BAP, and 15 mg/l 2,4-D + 2 mg/l BAP). The result showed the cultivars, combination of PGR, and interaction between cultivars and PGR gave significant effect to weight callus. The result of T-test showed that in hypoxic condition, POD enzyme exercise on Gepak Kuning's callus in 5 mg/l 2,4-D + 1 mg/l BAP was different before and after hypoxic condition.

1. Introduction

The soybean is one of the most important food commodity in Indonesia. In year 2015, soybean production increased 4.5% based on Central Statistics Agency (BPS) which reached 998.870 tons of soybean dry seeds. The increasing production is supported by the addition of harvest area of soybean plants [1]. Soybean plants susceptible to flooding. Puddles cause premature aging so that the leaf chlorosis, necrosis, fall and stunted plant growth, which in turn decreases the results. Loss of the vegetative phase is smaller than in the reproductive phase, ie 17-43% in the vegetative phase and 50-56% in the reproductive phase [2]. Plants that were flooded in a short time will have the condition of hypoxia (lack of O_2) [3].

Selecting cultivars for tolerant to flooding can be done with some experiment in the field or in the laboratory [2]. With *in vitro*,is expected to provide solutions resistant cultivars, tolerant or sensitive to flooding and is expected to maintain its integrity and grow into fully plants [4]. Somatic embryogenesis offers an in vitro method for mass propagation of important plant species. In addition, somatic embryogenesis enables to produce large numbers of plantlets [5] and also suitable for germplasm conservation, the selection of genetic variants for desirable characters, the generation of somaclonal variants and for performing cellular genetic manipulations [6].

In vitro culture techniques provide a means of rapid plant propagation and a tool for crop improvement. The success of tissue culture techniques is determined by the type and concentration of plant growth regulator used. Growth regulator auxin and cytokinin are needed to stimulate cell division and formation of callus [7]. Auxin has a role on the growth of cells, the apical dominance and

callus formation. Cytokinins are growth regulators a role in regulating cell division and differentiation shoots influence on callus tissue [8]. By mixing some combination of growth regulators 2,4-Dichlorophenoxyasetat (2,4-D) and Benzylaminopurine (BAP) to the culture medium is able to stimulate the formation of callus on the explants. Based on [9], in young cotyledons soybean explants have started callus at the age of 1-2 weeks after explant callus culture with percentage 75%, in the treatment of 10 mg/l 2,4-D + 10 mg/l NAA. Research conducted by [10] on the culture *in vitro* of fruit Napier stated that BAP 1.5 mg/l is the optimum concentration in macassar fruit seed growth *invitro* for the purpose of propagation.

One of the alternatives that can be done to produce hypoxic-tolerant soybean with techniques *in vitro* in embryogenesis using combination of plant growth regulator (PGR) 2,4-D (2,4 Dichlorophenoxyacetic acid) and BAP (6-Benzylaminopurine) to induce embryogenic callus of selected soybean cultivars.

2. Materials and Methods

This study was conducted at Tissue Culture Laboratory of the Faculty of Agriculture, University of Sumatera Utara, Medan, with two phases of activity that embryogenic callus formation using a completely randomized design factorial and test the metabolic activity of callus on hypoxic conditions using t-test analysis Minitab 16. The study was consists of two factors, which as first factor, soybean cultivars (Baluran/V_1, Gepak Kuning/V_2, and Grobogan/V_3) and second factor, plant growth regulator (PGR) treatment with a combination of 5 mg/l 2,4-D + 1 mg/l BAP (Z_1), 10 mg/l 2,4-D + 1.5 mg/l BAP (Z_2), and 15 mg/l 2,4-D + 2 mg/l BAP (Z_3).

Mature seeds of the three selected soybean cultivars were dehusked and surface sterilized with 70% (v/v) ethanol for 2 min followed by 40% (v/v) sodium hypochlorite for another 20 min shaking. After rinsing five times with sterile distilled water, the sterilized seeds were used for callus induction. Soybean callus induction was following the method of [11]. The mature seeds were placed on MS medium [12] supplemented with 2,4 D + BAP, sucrose (30 g l^{-1}), casein hydrolysate (0.4 g l^{-1}) and gelrite (2.75 g l^{-1}). The pH of the media was set to 5.8 and added the PGR prior to autoclaving at 121^0C for 25 min. The cultures were incubated at 25^0C ± 2^0C under dark condition. After 2 weeks of culture, the primary callus was removed from the endosperm parts, and subcultured every 2 weeks in the same media for up to 8 weeks.

Planting was carried out in the laminar air flow (LAF), the matured seed that had been sprayed with 70% alcohol are planted soybean as cotyledon parts. The seeds were placed on petridish, then separated the embryo and endosperm part by using tweezers and a scalpel. Then the endosperms were planted into the media culture flask which has been prepared according to treatment. Each bottle consists of 1-2 explant culture and after planting is completed culture bottle rack placed in the culture of light such as light fixtures.

The bottles have been planted explant cultures were sprayed with 70% alcohol every day to be free from microorganisms (bacteria and fungi) that cause contamination. The temperature of the culture that is used is 18-22 $^{\circ}$C and light intensity of 2000 lux.

3. Results and Discussion

3.1.Growth of Callus

The percentage of callus growth has shown at Fig. 1. It showed that callus growth of Baluran cultivar was (0%), Gepak Kuning (100%) and Grobogan (100%). The percentage of callus growth was observed at 4 weeks after planting. Baluran cultivar stagnated (no growth) because of contaminated and therefore can't be forwarded to the advanced stage.

The results showed that the cultivar of Baluran not induced callus. Explants of cultivar baluran stagnated which started from planting until a certain time is not dead but is not growing. There are several factors that influence the success of tissue culture one of which is the explants. Callus growth is the result of a very complex interaction between explant, medium composition and environmental conditions during the incubation period.

Grobogan cultivar capable of forming callus at 1 week and Gepak kuning capable of forming callus on 2 weeks. Explants callus marked by the growth of cells in which tissue explant growth and raised lumps cell mass was yellowish white. The callus that formed were in the injured area on the explants. Callus formed generally yellowish white color and textured friable. Media fastest induce callus in this study is 15 mg/l 2,4-D + 2 mg/l BAP.

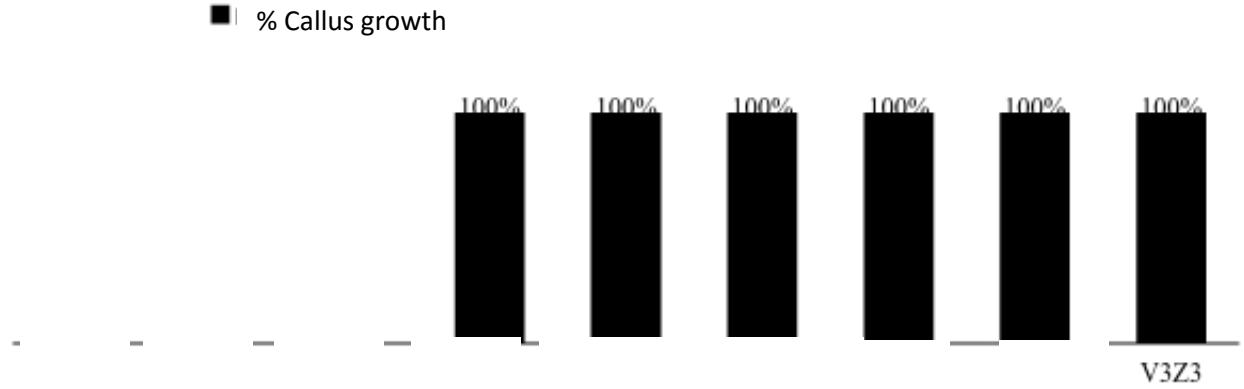


Figure 1. Histogram percentage growth of callus with different combinations of plant growth regulator at the age of 4 weeks

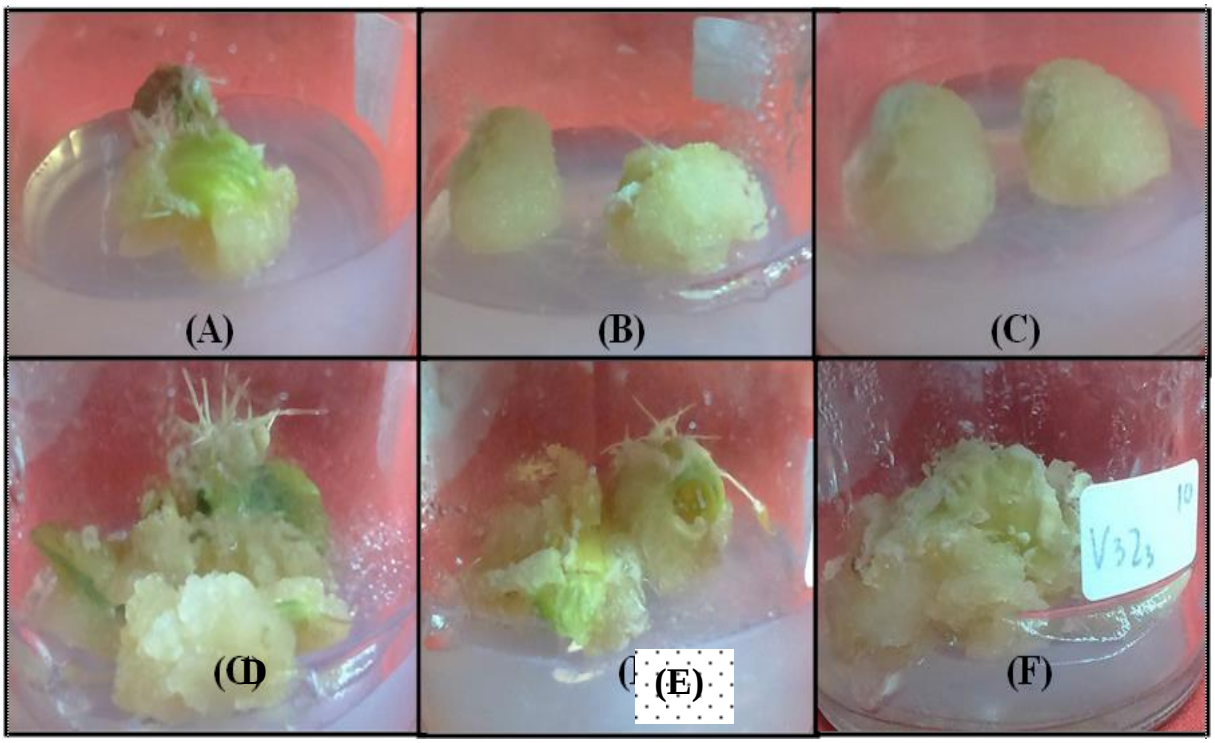
Plant growth regulators auxin and cytokinin stimulates cell division and the formation of callus. This is consistent with Khumaida and Handayani [9] reported that young cotyledons explants callus soybean crop began at the age of 1-2 weeks after explant percentage callus culture with 75% in the treatment of 10 mg/l 2,4-D + 10 mg/l NAA and the media 40 mg/l 2,4-D. Another research conducted by Manurung [10] on the culture *in vitro* of Makassar fruit (local fruit in South Sulawesi, Indonesia), giving the BAP cytokinin and auxin 2,4-D with different concentration level has a different response to the growth of fruit seeds makasar explants. The higher the concentration of BAP and 2,4-D, the higher the percentage of callus formation. BAP 1.5 mg/l is the optimum concentration in Makasar fruit seed growth *invitro* for the purpose of propagation.

3.2.Shape and Color of Callus

Figure 2 showed that the cultivar of Gepak Kuning produce callus denser texture than Grobogan cultivar and callus brighter colors. Callus was produced had a type of globular callus growth stage. Generally embryogenic callus can be induced using a plant growth regulator auxin or in combination with cytokines. Increased use of 2,4-D and BAP provide a good influence on the resulting structure. The higher the concentration of 2,4-D and BAP band is used then the friable callus produced.

Callus color tends to be yellowish white and bright yellow callus. Callus color formed indicates the activity of cell division that occurs in the callus. Callus is an collection of cells amorphous that occur from tissue cells that divide continually. This is consistent based on Lizawati [13] stated that the progress indicator on the cultivation of explants *in vitro* in the form callus visual representations that can be known that the callus which formed the cells are still actively dividing or die. Callus formed from an explant usually raises a different color.

Callus culture on every media appearance of callus induction showed almost the same. The callus that formed yellowish white, friable callus texture, there are nodules, and transparent. In relation to the embryogenic callus formed callus is embryogenic callus because of the color of a yellowish white callus and nodules. On visual observation is the embryonic stage known globular embryo stage. The size of callus induced from explants of Grobogan larger than Gepak Kuning cultivars. Yelnititis and Komar [14] stated that the embryogenic callus has friable structure, nodular and creamy. According to [15] embryogenic callus initiation occurs in response to the stress caused by the effect of auxin concentration is relatively high. Callus is a disorganized mass of cells.



Descriptions :
(A). Treatment V_2Z_1 produces yellowish white callus with texture semi friable and types of development globular root
(B). TreatmentV_2Z_2 produces yellowish white callus with texture semi globular friable and types of development.
(C). Treatment V_2Z_3 produce callus cream-colored with texture semi friable and types of development globular + root
(D). treatment V_3Z_1 produce callus yellowish bright with texture friable and types of development globular + root
(E). treatment V_3Z_2 produce callus yellowish bright with texture friable and types of development globular root+
(F). treatmentV_3Z_3 produce callus yellowish with texture friable and types of development globular

Figure 2. Visualization Callus

3.3.weight of callus
The fresh weight obtained by weighing the callus using an analytical balance. The results obtained are presented in Table 1.

Table 1. Fresh weight of callus from Gepak Kuning and Baluran cultivars with different combinations of plant growth regulator (PGR) at age 6 Weeks

Cultivars	Growing Media			Mean
	Z_1	Z_2	Z_3	
	……..	g		
V_1(Baluran)	0.00 b	0.00 b	0, 00 b	0,00 a
V_2(Gepak Kuning)	2,60 a	4,13 a	3,10 a	3,28 b
V_3(Grobogan)	0.00 b	0.00 b	3.33 a	1,11 c
Mean	0,87 a	1,38 ab	2,14 a	1 , 46

Description: the numbers followed by the same letters in a row and the same column shows the real effect on DMRT at the level of 5%

Based on Table 1, the fresh weight of callus and results of variance showed treatment cultivars, and some combination of PGR and their interaction are both significant different at 6 weeks. The highest callus fresh weight on interactions obtained in combination Gepak Kuning and media 10 mg/l 2,4-D +

1.5 mg/l BAP.

Observations of variable weights callus observations indicate that genotype and media influence on the magnitude of the weight of the callus. Media interaction and the best cultivar found in the Gepak Kuning variety with a combination of growth regulators media 10 mg/l 2,4-D + 1.5 mg/l BAP. Growth regulator auxin and cytokinin plays a role in cell division and elongation so that the activity of both cause callus cell growth continuously. The better the callus growth then the resulting higher weight, greater callus clumps characterized by a growing callus mass. Indrianto [15] which states that the granting of cytokinin and auxin in the culture medium can stimulate cell division and morphogenesis. Based on research Khumaida and Handayani [9], the young cotyledons explants callus were best for soybean explant in the treatment of 10 mg/l 2,4-D + 10 mg/l NAA and also supported by Manurung [8] on the in vitro culture of Makassar fruit stating that BAP 1.5 mg/l is the optimum concentration in makassar fruit seed growth in tissue culture.

3.4.Protein concentration

Based on the results from Table 2, showed that the treatment inundation effect which was not significantly different to the increase or decrease in the protein concentration. On the average results of analysis of the protein concentration is known that the Gepak Kuning and Grobogan cultivars on the media 15 mg/l 2,4-D + 2 mg/l BAP to increase the protein concentration while two other treatment decreased protein concentration. Gepak Kuning and Grobogan cultivars showed metabolic response proteins in hypoxic conditions. The response to the waterlogged conditions caused changes to the process leading to the formation of protein. The use of cytokines to increase the rate of protein synthesis so that the faster the cell division process. In tissue cultures of nitrogen are building blocks of nucleic acids, proteins, and other compounds containing N. Dennis *et al.* [3] suggested that in conditions of hypoxia and anoxia tissues synthesize more soluble rice protein. In the second phase (4-24 hours) stages of the process plant responses to an oxygen-deficient condition is metabolic adaptation process. At this stage lasts induction of glycolysis and fermentation genes that are important for sustaining energy excretion. Metabolic response at this stage is more complex than expected because it involves changes in nitrogen metabolism.

Based on the analysis made known that the induced callus Grobogan cultivar of media 15 mg/l 2,4-D + 2 mg/l BAP have an increased metabolic activity after the hypoxic condition is the observation variable protein concentration and enzyme activity peroksidase (POD) and superoxide dismutase enzyme (SOD).

Table 2. Mean t-test against the protein concentration of soybean callus Gepak Kuning and Grobogan cultivars in different media combinations PGR

Treatment	Mean		t-Value
	Before hypoxic	After hypoxic	
V_2Z_1	1.265	1.133	0.070
V_2Z_2	1.217	1.205	0.759
V_2Z_3	0.853	1.228	0.390
V_3Z_3	0.084	0.476	0.408

4. Conclusion

Gepak Kuning and Grobogan cultivars are more responsive to the growth of embryogenic callus by percentage growth of 100%. Generally, the resulting callus creamy texture Interaction friable cultivar and the best growing media for embryogenic callus induction based on this research that Gepak Kuning cultivar on the media 10 mg/l 2,4-D + 1.5 mg/l BAP the fresh weight callus higher (4.13 g). In hypoxic conditions, the protein concentration callus Gepak Kuning and Grobogan cultivars induced by the media 15 mg/l 2,4-D + 2 mg/l BAP increased after hypoxic condition.

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