

Application of BAP and IBA for In Vitro Callus Formation of Biduri (*Calotropis gigantea*)

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Abstract. Biduri (*Calotropis gigantea*) is a herbaceous plant that has benefits as a medicinal plant. Secondary metabolites are contained in several organs: leaves, roots, and flowers. A callus is a collection of amorphous cells that accumulate secondary metabolites. Callus production can be stimulated through plant growth regulators induction. The research objective was to obtain the optimal concentration of BAP and IBA for callus formation in biduri. The explants used were the first stem segments of biduri which were 1,5 months old and 2 cm long. A Fully Randomized Design was arranged in a factorial design used with 2 factors and each was repeated 3 times. The first factor was BAP concentrations (0, 1, 2, 3, 4 ppm) and the second factor was IBA concentrations (0, 1, 2, 3, 4 ppm). The variables observed included the callus induction time, callus morphology (color and texture), and percentage of callus appearance. The results showed that the concentration of 4 ppm BAP with 1 ppm IBA was able to produce green and compact callus. Increasing the concentration of BAP up to 4 ppm increased the percentage of callus appearance and decreased the callus induction time.

Keywords: Auxin, callus, cytokinin, plant growth regulator, tissue culture

1. Introduction

Calotropis gigantea is known as Biduri or Widuri in Indonesia. Biduri is a herbaceous plant originating from Southeast Asia and can be found in Indonesia, the Philippines, Cambodia, Malaysia, Thailand, India, China, and Sri Lanka [1]. This plant is widely grown in Indonesia as a wild plant and is used as a traditional medicine for various diseases by utilizing the root bark, flowers, leaves, and sap. This plant can grow wild in tropical climates with abundant populations and grows in areas around the coast or on dry land. However, biduri in Indonesia is still not optimal in its utilization.

Biduri plants have several active compounds in several organs, namely leaves, roots, and flowers. The leaves of the biduri plant are known to contain flavonoid compounds, polyphenols, tannins, saponins, and calcium oxalate. The roots of the biduri plant contain alkaloids, phenols, saponins, and steroids [2]. While the flower of biduri contains phenolic compounds, flavonoids, sugars, steroids, saponins, and quinones. Active compounds in biduri can be used as medicines. There are several benefits of the biduri, namely as an antimicrobial, antioxidant, antipyretic, cytotoxic, and procoagulant [3]. In addition, the sap that comes out of the stem of the biduri plant has health benefits, namely for herbal medicine for asthma, bronchitis, dyspepsia, leprosy, and tumors [4].

Methods were being made to be able to produce biduri plants quickly, namely by in vitro culture. Tissue culture technique has aseptic culture conditions, controlled culture room, and the use of culture media with the addition of nutritional content and growth regulators. Tissue culture techniques are also needed in optimizing plant secondary metabolites. Secondary metabolites can also be produced from callus. Therefore, callus induction through tissue culture is necessary. Callus induction is the initial stage of in vitro culture technique which aims to produce and multiply callus cells in bulk. Callus is an important source of planting material in regenerating plants because each plant cell has the ability to form new individuals [5].

Factors that affect the growth of explants are the need for nutrients, both macro and micro, growth regulators, and the environment around the culture. Media is an important factor in the supply of nutrients for explants. Media commonly used in tissue culture is Murashige and Skoog (MS) media which contains macro

and micro nutrients needed by plants for growth and development. Tissue culture media not only provide nutrients but also provide sugar, vitamins, and plant growth regulators [6].

Plant growth regulators have a role in controlling biological processes in plant tissues, such as regulating the speed of tissue growth. Benzyl Amino Purine (BAP) is a growth regulator of the cytokinin group which plays a role in stimulating cell division and shoot formation. While Indole 3-Butyric Acid (IBA) is a growth regulator of the auxin group that plays a role in stimulating root and callus growth. Chemically, IBA has an effective root stimulation because it is more stable and quite difficult to be influenced by sunlight [7]. The research objective was to obtain the optimal concentration of BAP and IBA for callus formation in biduri.

2. Materials and Methods

The research was carried out for approximately four months from August 2021 – December 2021. The research location was at the Laboratory of Plant Physiology and Biotechnology, Faculty of Agriculture, Sebelas Maret University, Surakarta. The tools used in the study were culture bottles, autoclaves, Laminar Air Flow (LAF), measuring cups, beakers, erlenmeyer, scalpel, bunsen, analytical scales, pH meter, tweezers, hot plates and magnetic stirrer, petridishes, label paper, plastic wrap. The materials used in this study were explants of biduri plants in the form of stems from the first segment above, components of MS media, agar, sugar, auxin (IBA), cytokinins (BAP), sterile distilled water, 70% alcohol, and clorox.

The study used a completely randomized design (CRD) which was arranged in a factorial design consisting of 2 treatment factors with 25 treatment combinations and was carried out three times so that there were 75 experimental units. The first factor was the growth regulator BAP (0, 1, 2, 3, and 4 ppm) and the second was the growth regulator IBA (0, 1, 2, 3, and 4 ppm). The variables observed included the callus induction time, callus morphology (color and texture), and percentage of callus appearance. The data obtained were analyzed using Analysis of Variance (ANOVA) with a 5% level test. If there was a significant difference, it was continued with the Duncan Multiple Range Test (DMRT) at a 5% level.

The material used in the form of explants of biduri plants was obtained by breeding biduri using planting media in polybags. The explant used was the first segment of the plant stem, which was 1.5 months old. The tools used in the laboratory for initiation are sterilized first using an autoclave with a pressure of 1.5 psi. Murashige and Skoog (MS) culture media was prepared by taking each stock solution such as macronutrients, micronutrients, vitamins, Fe-EDTA, and growth regulators according to the treatment and size that had been determined.

The explants used were sterilized outside the LAF by washing thoroughly using detergent and rinsing using running water, then followed by washing with distilled water. Sterilization outside the LAF was done by immersing the explants into 1000 ml of a fungicide and bactericide solution with a concentration of 1 g each for 1 hour 45 minutes and then rinsing with distilled water before being brought to LAF. Explant sterilization in LAF began with washing the explants in sterile distilled water two times while shaking for 1 minute. Then, the explants were immersed in clorox (100% bayclin) for 1 minute while shaking. Then the explants were immersed in 70% alcohol for 1 minute while shaking. After that, washed the explants in sterile distilled water two times while shaking for 1 minute. After the sterilization process, the explants were cut along approximately 2 cm. Then, the explants were dried on sterile tissue. The explants were passed before the fire and then planted in culture bottles. Then, closed the culture bottles and covered with plastic wrap to keep them tightly closed and avoid contamination.

3. Result and Discussion

3.1. Callus induction time

The callus induction time on the explants indicated the rate of cell division speed. The callus that appears began with swelling of the lower part of the biduri explant that has been injured. Next, a greenish-white clump of cells appeared which continued to grow as the plant cells. DMRT at 5% significance level in Table 1 shows that interaction between BAP and IBA had no significant effect while BAP had a significant effect on the callus induction time.

Table 1. Effect of BAP on callus induction time in biduri explants (DAP)

BAP treatment (ppm)	Callus induction time (DAP)
0	2.67 a
1	8.87 b
2	6.40 b
3	6.87 b
4	8.87 b

Note: Numbers followed by the same letter in the same column are not significantly different at 5% level of DMRT; DAP: Day After Plantation

The results of the analysis showed that the fastest callus induction time was found in the 0 ppm BAP treatment, which was 2.67 DAP. The treatment of BAP 0 ppm was significantly different from all other treatments, namely BAP 1; 2; 3; and 4 ppm. Meanwhile, application BAP 1 and 4 ppm showed the longest

callus induction time, which was 8.87 DAP and was not significantly different from BAP treatment 2 and 3 ppm. In this study, media without BAP (BAP 0 ppm) showed the fastest callus appearance. This research is in line with Suhartanto [8], states that callus induction requires a low concentration of cytokinins. The addition of cytokinins in the form of BAP reduces the rate of callus formation.

3.2. Callus texture

Callus quality can be seen visually, namely from the resulting callus texture. Callus texture shows the cell regeneration ability in these tissues because the composition of cells, both in terms of density and size, is different. Callus texture became the parameter used to evaluate callus growth [9]. Callus texture is divided into compact and friable callus. Based on visual observations made at 65 DAP, the callus texture data were compact and friable (Table 2).

Table 2. The effect of combination treatment of BAP and IBA concentrations on the texture of biduri callus (65 DAP)

IBA (ppm)	BAP (ppm)				
	0	1	2	3	4
0	-	-	-	-	Friable
1	-	-	Compact	Compact	Compact
2	Compact	Compact	Friable	Compact	Compact
3	-	Compact	Friable	Compact	Compact
4	Compact	Compact	Compact	Compact	Compact

Information: ppm: *part per million* (mg/l); DAP: Day After Plantation

The final observation resulted in compact callus texture which was more dominant than friable callus. Based on these data, it can be seen that the concentration of BAP given to MS media did not show any effect on callus texture. Callus with friable texture was found in the combination treatment of BAP 2 ppm + IBA 2 and 3 ppm, as well as in the treatment of BAP 4 ppm + IBA 0 ppm. Table 2 shows that IBA 2 and 3 ppm with BAP 2 ppm could produce friable textured callus. The addition of IBA 2 and 3 ppm affected the ability of the tissue to absorb nutrients in the media so that the auxin content in the explants increased. This causes the arrangement of cells in friable callus to be stretched. IBA is an auxin type growth regulator, which has a function to increase cell elasticity. Loose cell walls cause water to enter the cells and cell wall lignification is inhibited.

The texture of friable callus is indicated by a texture that has spaces between cells, is brittle, and easy to separate, while compact (non-friable) callus is characterized by the form of callus in the form of dense lumps and is difficult to separate [10]. The cell regeneration ability of friable callus tends to be better than that of compact callus. The composition of the culture media and application of plant growth regulators caused differences in callus texture. Differences in callus texture led to differences in the ability to produce secondary metabolites. Compact textured callus has better accumulation of secondary metabolites than friable callus [11]. Therefore, compact callus has a higher secondary metabolite ability than friable callus.

3.3. Callus color

Based on visual observations made at 65 DAT, scoring data was obtained with 4 categories, namely greenish white, dark green, brownish white, and brown (Table 3). The final observation resulted in the dominant callus color was brownish white while the least callus color was greenish white, namely in the treatment of BAP 3 ppm + IBA 3 ppm and BAP 4 ppm + IBA 0 ppm. Callus that is brownish in color indicates dead cells, this is due to the formation of phenolic compounds so that the callus does not grow [8]. In addition, the callus is brown because it has entered the aging phase, it is better to be sub-cultured when the callus color is still green and fresh.

Table 3. The effect of combination treatment of BAP and IBA concentrations on the color of callus (65 DAP)

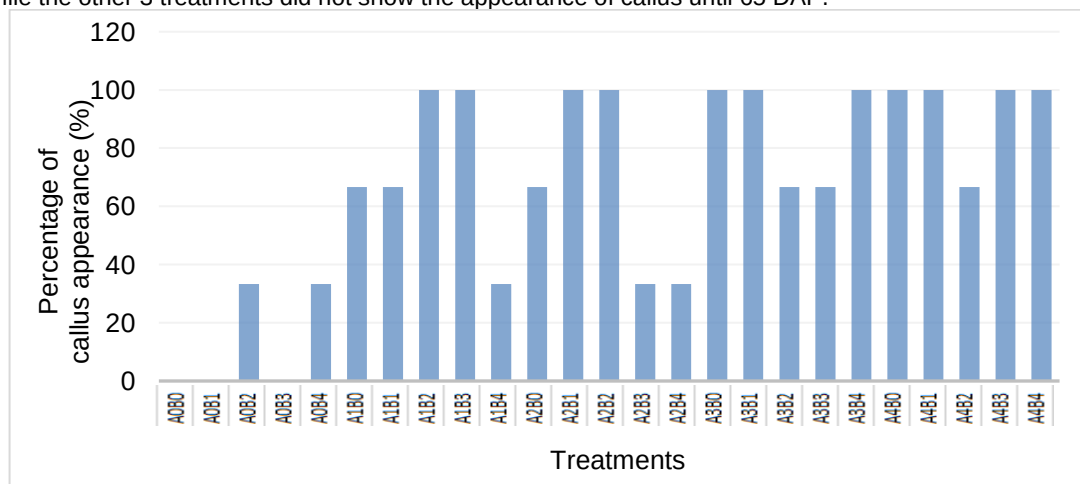
IBA (ppm)	BAP (ppm)				
	0	1	2	3	4
0	-	-	-	-	Greenish white
1	-	-	White brownish	Brown	Dark green
2	Dark green	Brown	White brownish	White brownish	Brown
3	-	White brownish	Brown	Greenish white	White brownish
4	White brownish	White brownish	Brown	Brown	Brown

Information: ppm: *part per million* (mg/l); DAP: Day After Plantation

The difference in callus color was caused by differences in the development of each callus. White and yellow pigments in callus indicated good callus growth [12]. This is clarified that greenish white callus occurs because it contains starch grains which gradually grow into a clearer membrane system and then form chlorophyll granules and can be said to have good callus quality [13].

3.4. Percentage of callus appearance

The results showed that the percentage of explants that appeared callus in each treatment combination was different (Figure 1). There were 11 treatments that showed a callus growth percentage of 100%, which means that callus appeared in explants in all replicates in that treatment. There were 6 treatments that showed a callus growth percentage of 66.67%, which means that callus appeared in 2 replications out of a total of 3 replications in that treatment. There were 5 treatments that showed a callus growth percentage of 33.34%, which means that callus appeared in 1 replication from a total of 3 replications in that treatment. While the other 3 treatments did not show the appearance of callus until 65 DAP.



Information:

A0 : BAP 0 ppm	B0 : IBA 0 ppm
A1 : BAP 1 ppm	B1 : IBA 1 ppm
A2 : BAP 2 ppm	B2 : IBA 2 ppm
A3 : BAP 3 ppm	B3 : IBA 3 ppm
A4 : BAP 4 ppm	B4 : IBA 4 ppm

Figure 1. Histogram of the effect of BAP and IBA on the percentage of callus appearance in biduri

The highest percentage tended to be dominated by 4 ppm BAP treatment with an average callus appearance of 93.3% for explants. While the treatment of 0 ppm BAP tends not to produce callus. In addition, the increase in the concentration of IBA was linear with the increase in the percentage of callus occurrence. The application of plant growth regulators especially auxin, is needed in the formation of callus and roots [14].

Conclusions and Recommendations

The results showed that the application of BAP 4 ppm with IBA 1 ppm had percentage of callus appearance 100% and produced green callus with compact texture. The treatment without BAP application increased the callus induction time. Further research can be done by analyzing the phytochemical compounds on biduri callus to know the amount of secondary metabolites.

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