

The Effect of NAA and BA on In Vitro Callus Induction of Biduri (*Calotropis gigantea*)

TA Wulandari¹, AT Sakya¹, S Hartati^{1,2}, Samanhudi^{1,2}, M Rahayu¹, A Setyawati¹

¹Agrotechnology Department, Faculty of Agriculture, Universitas Sebelas Maret, Indonesia

²Research and Development Center for Biotechnology and Biodiversity, Universitas Sebelas Maret, Indonesia

Email: tyas_alivia01@student.uns.ac.id

Corresponding Author: amaliatetrani@staff.uns.ac.id

Abstract: Biduri (*Calotropis gigantea*) is a medicinal plant group with various properties. Biduri contains chemical compounds like alkaloids, glycosides, phenolics, tannins, flavonoids, terpenes, and sterols. Biduri callus induction through tissue culture is one of the efforts to increase the production of secondary metabolites. The study aimed to obtain the right concentration of growth regulators for the induction of callus biduri. The study used a Completely Randomized Design (CRD) which was arranged in a factorial manner consisting of 2 treatment factors with 25 treatment combinations and was carried out 3 times. The first factor was the growth regulator NAA and the second was the growth regulator BA. The data obtained were analyzed using ANOVA analysis of variance 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 application of 1 ppm NAA and 3 ppm BA produced a compact, green callus texture. At the same time, the treatment without an NAA application could not have a callus on biduri. Further research can be done by subculture callus biduri when the callus is still fresh and green to produce secondary metabolites.

Keywords: Auxin, Cytokinin, Plant tissue culture, Secondary metabolites

1. Introduction

Biduri contains chemical compounds like alkaloids, glycosides, phenolics, tannins, flavonoids, terpenes, and sterols[1]. However, currently, the use of biduri plants is still not maximized. Even in some areas, it is still considered a weed, so biduri cultivation efforts through tissue culture are needed. The height of the biduri plant can reach 3 meters, producing a white, watery, bitter-tasting, intense, and toxic sap that comes out of the plant's stem. The sap has properties in the health sector such as asthma, dizziness, bronchitis, tumours, leprosy, dyspepsia, and digestive diseases. The public can use biduri leaves to treat scabies, wounds, canker sores, itching in chickenpox, measles, fever, and coughs, while biduri flowers have tonic and appetite-enhancing properties [2].

Cultivation of biduri through tissue culture is one of the efforts that can be taken to induce callus of biduri in the production of secondary metabolites. Tissue culture can produce many plants in a shorter time, is free from pests and diseases, requires less seed and is not seasonally dependent [3]. However, there are also obstacles encountered in tissue culture cultivation, namely the occurrence of fungal and bacterial contamination in both the media and the planted explants[4].

Biduri callus production through tissue culture was supported by adding growth regulators in the form of auxins and cytokinins, namely Napthalene Acetic Acid (NAA) and Benzyl Adenine (BA), to MS media (Murashige and Skoog). NAA has more stable properties than other types of auxin, such as Indole Acetic Acid (IAA). NAA is also not easily decomposed by enzymes released by cells or heating in the sterilization process [5]. BA is more intense than kinetin, so it is often used to stimulate shoot multiplication. Generally, plants have a better response to BA than to kinetin, so BA is more effective for shoot production [6]. The proper concentration of auxin and cytokinin can help form callus biduri.

2. Materials and Methods

The research was carried out for approximately four months, from August 2021 to 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, petri dishes, and matches. Fire, label paper, plastic wrap. The materials used in this study were explants of the biduri plant in the form of the second segment from the top, MS media components, agar - agar, sugar, auxin (NAA), cytokinins (BA), sterile distilled water, 70% alcohol, and chlorox.

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 3 times so that there were 75 experimental units. The first factor was the growth regulator NAA (0; 0.5; 1; 1.5; and 2 ppm), and the second was the growth regulator BA (0, 1, 2, 3, and 4 ppm). The variables measured in the study were callus appearance time, percentage of callus appearance, callus texture, and callus colour. The data obtained were analyzed using ANOVA analysis of variance 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 initialization stage begins with taking explants in a greenhouse, then taking them to the laboratory and washing them thoroughly using sufficient detergent. The explants were then rinsed using running water and distilled water until clean. After that, the explants were soaked in a solution of bactericide and fungicide with a concentration of 1 g of bactericide and 1 g of fungicide. Then, added with distilled water up to 1000 ml for 1 hour 45 minutes. After that, the explants were rinsed again using distilled water before being brought to LAF. The first step during sterilization in LAF was washing the explants in sterile distilled water 2 times while shaking for 1 minute. Next, the explants were immersed in chlorox (100% bayclin) for 1 minute while shaking. Then the explants were immersed in 70% alcohol for 1 minute while shaking. After that, rinse the explants using sterile distilled water 2 times for about 1 minute while shaking. After the sterilization process, the explants are 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. The culture bottles were then covered with plastic wrap to keep them tightly closed and to avoid contamination.

3. Results and Discussion

3.1. Callus emergence time

The beginning of callus formation is characterized by swelling at the bottom of the explant, which then continues to grow. The callus is initially white or green but will turn brown over time, so it must be immediately subcultured. The results of the analysis showed that the treatment of giving ZPT single NAA had a significant effect on the time of callus appearing in biduri. According to [7], auxin plays an essential role in the callus induction process. The 0 ppm NAA treatment was significantly different from the 0.5 NAA treatment; 1; 1.5; and 2 ppm. The time for callus to appear in the 0.5 to 2 ppm NAA treatment was when the explants were 15-16 DAP.

Table 1. Effect of NAA on callus emergence time (DAP) in biduri explants

NAA treatment (ppm)	Callus Emergence Time (DAP)
0	0 a
0.5	15.47 b
1	15.00 b
1.5	16.73 b
2	16.73 b

Note: Numbers followed by the same letter in the same column are not significantly different at the 5% level DMRT

Table 1 showed that the application of NAA with low and high concentrations induced the appearance of callus in biduri explants. However, treatment without NAA could not induce callus in biduri explants. This was presumably because the culture media only contained BA, which did not play a role in callus formation. According to [8], callus initiation can be influenced by the type of explant and the kind of Plant Growth Regulator (PGR) added to MS media.

3.2. Callus colour

The colour that appears in the callus indicates the chlorophyll content in the tissue, the greener the colour of the callus, the more chlorophyll content in the callus. The colour of the callus produced in each treatment was different and underwent a colour change from the beginning of the callus appearance to the last observation. According to [9] the resulting colour variation may be due to the various types of PGR used, differences in PGR concentrations and the type of explant.

Table 2. Effect of NAA and BA on callus colour in biduri aged 10 WAP

BA (ppm)	NAA (ppm)				
	0	0.5	1	1.5	2
0	-	Yellowish white	Yellowish white	Yellowish white	Green
1	-	Brownish-yellow	Brownish-yellow	Brownish-yellow	Yellowish white
2	-	Brownish-yellow	Yellowish white	Yellowish white	Yellowish white
3	-	Brownish-yellow	Green	Brownish-yellow	Brownish-yellow
4	-	Yellowish white	Brownish-yellow	Yellowish white	Brownish-yellow

The combination of auxin and cytokine can produce a greener callus colour caused by cytokines, which tend to promote the formation of chlorophyll. According to [10] various conditions of callus colour can be caused by pigmentation, the influence of light, and the part of the plant used as a source of explants. The resulting callus colours were green, yellowish white, and brownish yellow. According to [11], green, white, and yellow indicate that the cells are still actively dividing, while brown and black show signs of ageing in cells. Changes in the colour of the callus mean a change in the growth phase, from actively dividing cells to mature cells.

The results showed several callus colours: green, yellowish white, and brownish yellow. The colours produced at the end of the observation were yellowish white and brownish yellow, while the least was green. This is presumably because the callus has entered the ageing phase, so if it is going to be subcultured or propagated through callus culture, it is better when the colour of the callus is still green and fresh. According to [12], color changes in the callus indicate a change in the growth phase and can be caused by synthesising phenolic substances in the callus.

3.3. Callus texture

The texture formed in callus is generally divided into 3 types: compact, crumb, and a combination of compact and crumb. A compact callus is a callus that has a dense cell arrangement, while a crumb callus is a callus that has a loose and fragile cell structure. According to [13], the Callus's brittle texture indicates that the callus will develop into a somatic embryo.

Table 3. Effect of NAA and BA on callus texture in biduri aged 10 WAP

BA (ppm)	NAA (ppm)				
	0	0.5	1	1.5	2
0	-	Crumb	Crumb	Crumb	Crumb
1	-	Crumb	Crumb	Compact	Crumb
2	-	Crumb	Crumb	Compact	Crumb
3	-	Crumb	Compact	Compact	Crumb
4	-	Crumb	Crumb	Compact	Compact

Callus texture was observed at the end of the observation at the age of 10 WAP. Based on the table 3, the texture of the callus formed was compact and crumbly. Most of the callus texture produced was a crumb, and only a few callus had a compact texture. According to [14], compact callus texture is good because it can produce more secondary metabolites.

According to [15], This varied callus texture depends on the variant of the explant, the media used, and growth regulators.

Table 3 shows that the most compact callus was produced in the ZPT BA treatment combined with 1.5 ppm NAA. According to [16], the compact texture formed on the callus can be caused by the formation of lignification so that the callus becomes hard and is also the effect of cytokinins that work in the nutrient transport process. Based on visual observations, the crumb callus texture formed on the biduri can be separated easily using tweezers but is easily broken, and the cells are easily attached to the tweezers. Compact callus texture is dense, rigid when cut, and not easy to separate.

3.4. The percentage of callus emergence time

The percentage of callus emergence showed the effect of the addition of NAA and BA on callus emergence. The results showed that only explants planted on media without NAA could not produce callus, while in other treatments, callus was formed with a percentage of 100%. According to [17], the application of NAA in MS media can affect callus formation faster. The callus formed can be grown into more numerous shoots in a specific time.

Table 4. The effect of NAA and BA for percentage of callus emergence time

BA (ppm)	NAA (ppm)				
	0	0.5	1	1.5	2
0	0%	100%	100%	100%	100%
1	0%	100%	100%	100%	100%
2	0%	100%	100%	100%	100%
3	0%	100%	100%	100%	100%
4	0%	100%	100%	100%	100%

Media without NAA is thought unable to induce callus because no auxin plays a role in stimulating callus formation. In addition, although the explants had endogenous auxin, it was insufficient to form callus in the biduri. Therefore, adding NAA is needed in the callus formation process to be more optimal.

4. Conclusions and Recommendations

The results showed that the application of 1 ppm NAA and 3 ppm BA produced a compact and green callus texture. At the same time, the treatment without an NAA application could not produce callus on biduri. Further research can be done by subculture callus biduri when the callus is still fresh and green to produce secondary metabolites.

5. Acknowledgments

The authors are grateful to Universitas Sebelas Maret, which has funded this research through the scheme of Penelitian Unggulan Terapan (PUT) of Non-APBN UNS for the 2021 budget year.

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