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Content of Ajmalicine on Cultured Callus *Catharanthus roseus* (L.) G. Don with Tryptophan Treatment

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Abstract: ***Catharanthus roseus (L.) G. Don is cultivated as an ornamental plant and also has a high economic value because it contains terpenoid indole alkaloids such as ajmalicine that have pharmacological benefits. This research was conducted to investigate the content of ajmalicine in callus culture that was given tryptophan treatment with concentrations of 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L and 250 mg/L. Identification and analysis of ajmalicine using HPLC Shimpak VP-ODS C18 150 x 4.6 mm at a wavelength of 254 nm. The results showed that the ajmalicine content was lower after the tryptophan treatment, where the minimum ajmalicine content of the tryptophan 200 mg/L, ie 0.346 µg/g dw. Ajmalicine content of the treatment of 250 mg / L tryptophan, is the highest content in all treatments, ie 2.686 µg/g bk. So it can be concluded that the treatment of tryptophan in callus culture Catharanthus roseus can decrease the content of ajmalicine.***

Keywords: Ajmalicine, Tryptophan, Callus culture, *Catharanthus roseus* (L.) G. Don

1. Introduction

Catharanthus roseus (L.) G. Don. is a plant that is widely used as a medicinal plant. *C. roseus* contains many terpenoid indole alkaloids (TIA) [1]-[2]. Ajmalicine is one of the TIAs in *C. roseus* that is useful for the treatment of blood circulation-related diseases especially to treat circulatory disorders to the brain [3]-[4]. Ajmalicine also has been used for the treatment of senility syndrome or dementia [5]. *In-vitro* culture has gained much attention as an effective technology for producing useful secondary metabolites [6], [13] and being an alternative technology of conventional cultivation in the soil [7]. The use of this method has the advantage of being able to produce a culture free from contamination because it is carried out under controlled environmental conditions and is independent of the climate and soil conditions. In addition, *in-vitro* cultures provide an opportunity to modify and regulate genetic information in order to produce economically valuable metabolites with better properties [8].

Alkaloids production from *C. roseus* has been done *in-vitro* but the resulting alkaloid content is still fluctuating. It is possible to increase the production of alkaloid compounds in cell culture using a strain selection method that has high production or by optimizing the composition of the medium, elicitation, and the addition of precursors from secondary metabolite biosynthesis [9]. Use of the elicitor from *Pythium aphanidermatum* (Edson) Fitzp on the root culture of *C. roseus* significantly affects the ajmalicine content [10].
Tryptophan as a precursor can significantly increase the production of TIA in cell suspension cultures. Addition of precursors such as a tryptophan treatment of 150 mg/L can increase the catharanthine content of 50.96 µg/g dw [11].
On the other hand, tryptophan treatment does not always increase cell growth in some concentrations. The concentration of tryptophan 150 mg/L showed optimum cell growth in *C. roseus* [12], [13]. TIA content is also different in each type of *in-vitro* culture. Furthermore, [14] explained that the production of alkaloids and callus growth are interrelated. High cell growth produces low alkaloid, and vice versa. In compact callus, synthesis of ajmalicine is higher than in cell cuspension [16].

This research aims to analyze the content of ajmalisin on cultured callus *C. roseus* treated tryptophan.

2. Research Methods

Source of explant is *C. roseus* leaves that are still active to grow, leaves of the 3rd or 4th of the shoots. Callus is induced in MS medium with PGR 2 mg/L 2,4-D and 0.2 mg/L kinetin [12]. After 28 days (4 weeks), the callus is separated to be sub-cultured on a new medium to multiply the callus. The media used are the same as the previous medium which added 2 mg/L NAA and 0.2 mg/L kinetin [12]. At the time the callus was 12 weeks old, the callus weighing 1 gram was transferred to the treatment medium which is the same previous medium as subculture medium with the addition of tryptophan according to the treatment of 0 mg/L tryptophan as control, 50 mg/L, 100 mg/L, 150 mg/L, 200 mg L and 250 mg/L [13]. After 14 days, callus was cultured on the treatment medium. It was dried by using freeze dryer.

Callus extraction for the ajmalicine analysis using Pandiangan [13] method was modified. Callus samples were dried, then crushed with a mortar and dissolved in 10 mL of methanol analytical. Samples were agitated for 4 hours at a speed of 120 rpm. Mixture of methanol and the precipitate is

separated and then the methanol extract was evaporated to dryness at a temperature of 25°C for 24 hours. Evaporation of the residue was acidified with 0.3 N HCl to reach a pH of 1.5 and then extracted with 10 mL of dichloromethane. Acid phase was then basified by adding 4 M NaOH up to pH 11 and then extracted with 10 mL dichloromethane 2 times. Dichloromethane fraction was evaporated in a temperature of 25°C during the day. Evaporation of the residue was dissolved in 2 mL of methanol and prepared for HPLC analysis [11].

Identification of ajmalicine content was performed using the High Performance Liquid Chromatography (HPLC). The qualitative analysis was conducted by comparing the retention time of ajmalicine standard under the same conditions. If a compound has the same retention time as ajmalicine standard, the compound is ajmalicine. Quantitative analysis was done by converting the the sample area with the area of standards of known concetrations [15].

3. Results and Discussion

3.1 Callus induction

Callus induction from *C. roseus* inoculated on induction medium, ie MS (Murashige and Skoog) medium with addition of PGR 2 mg/L 2,4-D and 0.2 mg/L kinetin began to show the initial response of callus induction on 5th day to 6th day. It is characterized by the appearance of explant start curved. Medium MS with 2,4-D and kinetin is good for callus induction. The use of 2,4-D will only produce a whole callus with a lot of quantity fast [16].

On 8th day, there is proliferation of cells on the edge of explants former incision, then form the callus. This is thought to occur because of the cell response to the incision [18]. Based on visual observation, the formed callus grows more enlarged, yellowish white and has a weakening structure as seen in Figure 1.

Until the 28th day after culturing, callus continues to enlarge and no differentiation takes root. This is in accordance with that proposed by [13] that on callus subculture using 2 mg/L 2,-D and 0,2 mg/L kinetin there is no root formation but only callus [14].

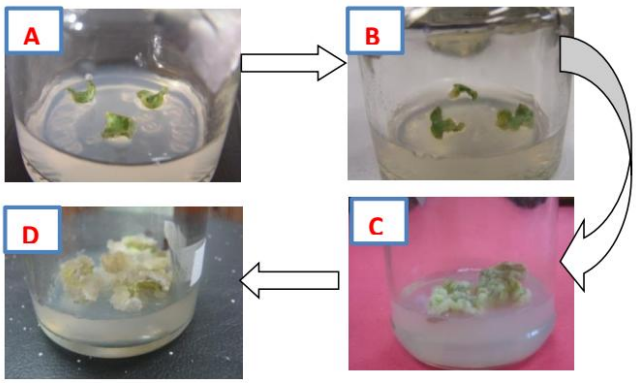

Figure 1: Stages of callus induction on C. roseus on MS medium with addition of 2 mg/L 2,4-D and 0,2 mg/L kinetin. (A) 6th day, (B) 8th day, (C) 14th day, (D) 28th day.

3.2 Callus Subcultured

Callus which was 8 weeks old, then sub-cultured for callus propagation using MS medium with addition of 2 mg/L NAA and 0.2 mg/L kinetin. On the 4th day after subculture, callus changes color from yellowish white to brownish yellow. Suspected browning occurs due to callus adaptation to the composition of PGR on the medium, which was originally 2.4-D replaced with NAA. On the 14th day after the subculture, the callus appears to have a good growth characterized by the presence of new cells on the surface of the callus. On the 21st day, the culture looks more enlarged with the growth of young yellow callus. Callus growth is indicated by increasing callus size due to cell division or cell size increase. Auxin can stimulate cell extension while cytokines can stimulate cell division [20].

The callus produced at this stage is compact and solid as shown in Figure 2. This is consistent with previous studies that the addition of 2 mg/L NAA and 0._2 mg/L kinetin produced compact callus [13]. Compact callus is also produced at a certain level of differentiation of cells and tissues from *C. roseus* stem and leaves explants on MS medium added 5.37 µM NAA and 4.65 µM kinetin [17]. At the age of 12 (twelve) weeks, callus was produced with a slightly turbid white color.

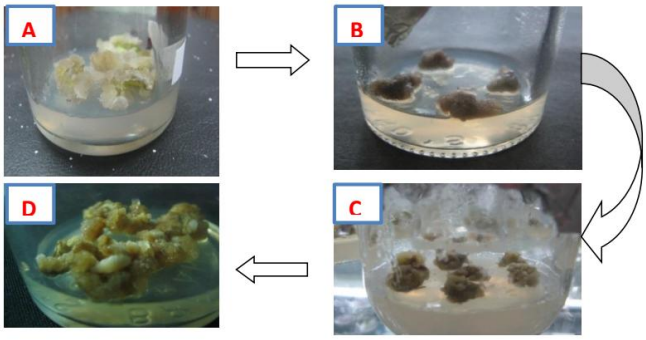

Figure 2: Callus subcultured on MS medium with addition of 2 mg/L NAA and 0.2 mg/L kinetin. (A) callus 8 weeks old from production medium, (B) callus 4th day on subculture medium, (C) callus 14th day on subculture medium, (D) callus 21st day on subculture medium.

3.3 Subculture Callus on Treatment Medium

Callus grown on tryptophan-treated medium, visually showed increased size until the 14th day. Callus on treatment 250 mg/L tryptophan seen the size of callus with dry weight of 0.14 grams. The highest dry weight of callus was at the treatment of 50 mg/L tryptophan, ie 0.26 gram as in Figure 3.

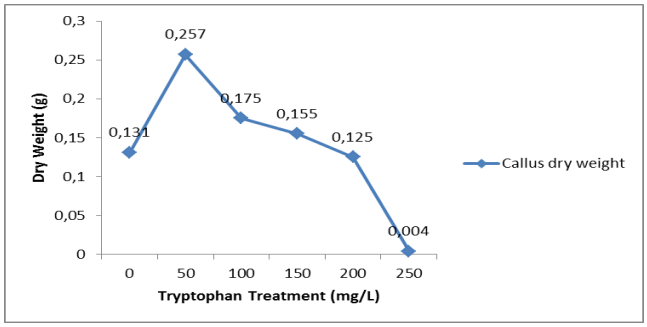

Figure 3: Callus dry weight that cultured on tryptophan-treated medium.

3.4 The content of ajmalicine with the addition of tryptophan

The results of variance analysis showed that the addition of tryptophan treatment compared to give a very different effect on the content of ajmalicine on cultured callus *C. roseus*. The analysis was continued by the LSD test to determine the differences between treatments. From these results, it was demonstrated that the ajmalicine content of callus-treated *C. roseus* callus decreased the ajmalicine content compared with no tryptophan treatment (control). The highest ajmalicine content in the controls was detected at 9,048 µg/g dw while the lowest ajmalicine content of the tryptophan treatment was 200 mg/L, ie 0.346 µg/g dw (Tabel 1).

The results of this research are in line with Merillon [21] research, in which the addition of tryptophan to cultured cell *C. roseus* on several different media ie subculture medium containing low sucrose and two media with high sucrose has no effect on the increase of ajmalicine and serpentine. Ajmalicine and serpentine are produced higher when added to the sekologanin than with the addition of tryptophan.

Canel [22] suggest the accumulation of alkaloids through unstable genetic engineering and is strongly influenced by culture conditions, such as the composition of hormones from the media and the availability of precursors. Zhao [23] also reported that growth and light regulators significantly influenced the production of alkaloids in *C. roseus*. 2,4-D can suppress the biosynthesis of all indole alkaloids including ajmalicine. In addition, alkaloid synthesis is also more on the callus which is yellow dark or yellow, and textured compact compared with a pale yellow callus and textured crumbs.

Table 1: Ajmalicine content of *C. roseus* callus cultured with tryptophan treatment

Tryptophan Treatment	Ajmalicine Content (µg/g dw) *)	Percentage of Ajmalicine Decrease (%)
0 mg/L (Kontrol)	9,048 ± 2,96 [a]	-
50 mg/L	1,908 ± 0,36 [b]	- 78,91
100 mg/L	2,266 ± 0,48 [b]	- 74,96
150 mg/L	0,395 ± 0,31 [b]	- 95,63
200 mg/L	0,346 ± 0,32 [b]	- 96,19
250 mg/L	2,686 ± 0,63 [b]	- 70,31

Note: *) The number followed by the same letter means no significant difference.

The ajmalicine curve pattern in callus cultures by tryptophan has a model that resembles a tryptophan at vindoline curve [15] There was a decrease in vindolin content in *C. roseus* on cell culture with a tryptophan treatment of 100 to 200 mg/L after 14 days of culture in the treatment medium. Vindolin began to increase in the treatment of tryptophan 250 mg / L.

The content of catharanthine on callus without tryptophan treatment is the lowest one. While the treatment 200 mg/L tryptophan yields the highest catharanthine content. The comparison of catharanthine content curve pattern [15] with the pattern of the ajmalicine content curve can be seen in Figure 4.

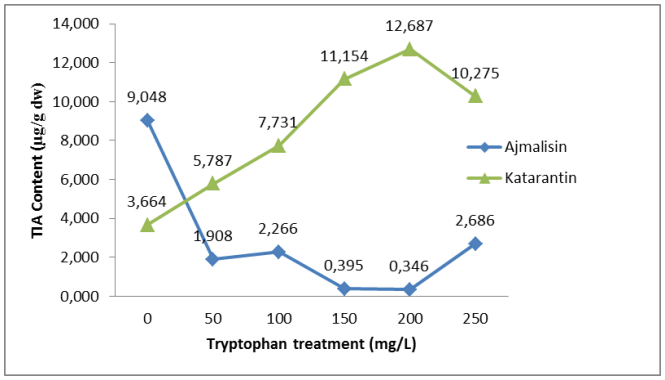

Figure 4: Pattern of curve catharanthine content [15] (modified) and ajmalicine content curve pattern by treatment of tryptophan.

The pattern is in contrast to the production of catharanthine, where tryptophan treatment significantly increases the catharanthine content [12]-[15]. The role of the TDC (tryptophan decarboxylase) enzyme may also be related to the decrease in the ajmalicine content. Pandiangan [14], reported that an increase in catharanthine or secondary metabolite content was also associated with an increase in TDC activity. There may also be a feed-back mechanism of a secondary metabolism [20].

4. Conclusions

The tryptophan treatment of *Cataharanthus roseus* (L.) G. Don callus culture affects the ajmalicine content. The results showed that the ajmalicine content was lower after the tryptophan treatment, where the minimum ajmalicine content of the treatment was 200 mg/L tryptophan, ie 0.346 µg/g dw. The treatment of 250 mg/L tryptophan, ajmalicine content is 2.686 µg/g dw which is the highest content in all treatments. So it can be concluded that the treatment of tryptophan in callus culture *C. roseus* can decrease the content of ajmalicine.

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