Morphological Changes of Cell in Relation to Increased Catharanthine Content of *Catharanthus roseus* Cell Aggregate Culture after Tryptophan Treatment

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***Abstract*— A study on changes in cell morphology and its association with increased catharanthine content in *Catharanthus roseus* cell cultures after tryptophan treatment has been carried out. This study was part of a strategy to improve the content of catharanthine. One strategy used was the addition of tryptophan as a precursor. Increased content of catharanthine is essential in *in vitro* culture in order to keep the catharanthine content at least equal to those grown in nature (*in vivo*). The research was conducted in the laboratory using MS medium with tryptophan 50-250 mg/L. The results showed that cell morphology was essentially the same for each treatment. The apparent discrepancy was the presence of long cells in higher number in the treatment of tryptophan. The highest ratio was obtained from the treatment of 150 mg/L tryptophan on day 14 of culture. There was relation between changes in cell morphology (more long cells) with the increased content of catharanthine after tryptophan treatment. The highest content occurred on day 14 after 150 mg/L treatment of tryptophan with 75% long cells (specialized cells) and 50.96 µg/g dry weight of catharanthine content.**

***Index Term*— *Catharanthus roseus*, cell culture, cell morphology, tryptophan, catharanthine.**

I. INTRODUCTION

VINCA (*Catharanthus roseus* (L.) G. Don) is an annual shrub widely cultivated as ornamental and medicinal plants. In medicine it is widely used to treat hypertension, diabetes, bleeding due to decreased platelet count, chorionic epithelioma, acute lymphocytic leukemia, acute monocytic leukemia, lymphosarcoma, and reticulum cell sarcoma [1]. Around 100 kinds of alkaloids have been identified in this plant [2, 3], among others are anti-cancer alkaloids such as vinblastine, vincristine, catharanthine, lauricidin and leurosine. Commercial vindoline, vincristine and vinblastine are mostly extracted from Vinca, especially the white flowers [4].

Production of alkaloids and catharanthine from Vinca has been done *in vitro*. Yet the content of alkaloids and catharanthine fluctuated according to type of produced bioactive. Therefore, a strategy to increase the content of alkaloids and catharanthine has been tested with various methods such as addition of precursors, elicitation and immobilization [5]. From the three strategies, the addition of precursor was a sustainable improvement, because the cells grew well but the content of the catharanthine increased from its control.

Our previous study [6] found that the addition of precursor approach has been performed. Tryptophan treatment on Vinca callus could increase the catharanthine content to 950.536 µg/g dry weights (d.w.). Generally, Vinca plant with low callus growth produces more catharanthine than that with high callus growth. From the study we also found the optimum growth was at treatment of 175 mg/L tryptophan precursor. Cells undergoing specialization and differentiation produce high secondary metabolites in *in vivo* cultures [7]. Cells undergoing differentiation show slower growth than the undifferentiated ones. In addition, cell morphology in callus cultures of red beet affects betalain pigments accumulation [8]. The morphology of cylindrical cells contains higher alkaloids than the spherical cells, the longer cells contain the higher the alkaloids content [9]. The addition of tryptophan precursor can increase the catharanthine content [6], and might also increase the number of cylindrical or long cells.

Our study aimed to investigate the response of cell growth of Vinca that was tryptophan treated and its cell morphological changes in relation to specialized cells under tryptophan treated. We also studied morphological changes of cell in relation to increased catharanthine content of Vinca cell aggregate culture after tryptophan treatment.

II. MATERIALS AND METHODS

A. Materials

The plant used as the source of explants in the experiments was white flower Vinca (*Catharanthus roseus* (L.) G. Don). The

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leaves that are still actively growing at 3-4 leaves from the apex shoots were used as the explants.

B. Research Design

Our study utilized a CRD (completely randomized design) with six treatments of tryptophan, i.e. control (T0), tryptophan 50 mg/L (T1), 100 mg/L (T2), 150 mg/L (T3), 200 mg/L (T4), and 250 (T5) mg/L [10] that were carried out in 100-mL Erlenmeyer flasks. Each experimental unit consisted of three replications that consisted of three bottles of culture each.

C. Callus Cultures and Subcultures to Acquire Callus Stock

Callus culture and subculture techniques were performed with aseptic technique as in our previous study [10]. Subculture of callus was transferred to a new medium with the same composition with callus production medium. The callus was transferred to MS solid medium that was added with PGR 2 mg/L NAA and 0.2 mg/L kinetin. The subculture was carried out continuously to multiply the callus as the source of explants on cell aggregate cultures in Erlenmeyer flask and bioreactor. The subculture of callus was conducted every 21 days and the callus used has been subcultured for 1 year 2 months.

D. Cell Aggregate Culture

Cell aggregate cultures following the method of previous study [10] with some modifications. Cell aggregate cultures were carried out in a 250-mL Erlenmeyer flask. Each flask contained 50 mL of liquid MS medium with the same combination of PGR to callus production medium. The callus used was about 1 year old subcultured. An amount of 5 g of callus was transferred to 50 mL of liquid MS medium and was added with 2 mg/L NAA and 0.2 mg/L kinetin (NK medium or T0) [11]. The cell aggregate cultures were incubated at room temperature and agitated at a speed of 120 rpm. Subcultures were made after 14 days by replacing the old liquid medium with new liquid medium of similar nutrient composition.

E. Tryptophan Treatment in Erlenmeyer

Cell aggregates obtained from the cultures were separated from the media. The remaining media of the cell aggregates were dried using suction papers in a sterile petri dish and then weighed. The culture container used was a-100 mL Erlenmeyer flask. Each flask contained 25 mL of liquid MS medium with the same combination of PGR to callus production medium but without agar. Subcultures in Erlenmeyer flasks with tryptophan treatments of 0, 50, 100, 150, 200, and 250 mg/L were made directly at the second subculture. Each treatment was made four replications. The inoculum weight used in the culture was 2 g of cell aggregates that was weighed aseptically in a laminar air flow while being subcultured.

The growth of cell aggregates was determined by wet weighing the cell aggregates on a digital scale to the nearest four decimal digits on each harvesting day or each sampling, i.e. on day 0 (approximately 6-8 hours after treatment), 4, 7, 10, 14, 17, and day 21 following the method of previous study [10].

F. Harvest and Sampling

Prioritized cell aggregates harvested were those from day 14 after tryptophan subculture and were wet weighted. Cell aggregates was dried in a freeze dryer for use in the analysis of secondary metabolites such as catharanthine content by HPLC.

G. Observations of Cells Speciation through Morphological Observations of the Cells

Observations on cell specialization through single cell morphology changes due to treatment of tryptophan precursor were conducted using the method of Kim *et al*. [9, 12]. Observations were made by taking a drop of just stirred fresh culture medium. Medium droplets were deposited on an object glass to be observed under a microscope (Nikon Halogen 100 W) with a magnification of 10 × 10. Each field of view was photographed with a digital camera (Nikon DXM 1200F). Cell shape was observed from the images whether they are cylindrical and spherical cells with the focus of attention was to the long (cylindrical) cells. The cell is said to be cylindrical or long cells if the ratio of length-to-diameter ≥ 1.5 [9]. The percentage of long cells was calculated by dividing the number of long cells with the total cells, as follows [12]:

$$N(\%) = \frac{\text{Number of cylindrical cells or ratio} \geq 1.5}{\text{Number of total cells per field of view}} \times 100\% \quad (1)$$

H. Data Analysis

Data were collected, tabulated and statistically analyzed to draw conclusions. Analysis of variance (ANOVA) was used to determine the content of catharanthine in cells and medium in a completely randomized design (CRD) at a-95% confidence level. If there are any significant differences, post hoc test DMRT (Duncan's multiple range test) at 95% confidence level was undertaken.

III. RESULTS AND DISCUSSION

Effect of tryptophan treatment on cell growth in a visual observation suggested that increasing concentrations of tryptophan in the tryptophan treatment did not necessarily improve cell growth (Fig. 1) similar to the results of previous research [10]. The observations pointed that the observation day also affected the growth of cell aggregates. Cell aggregates in T2, T3, and T4 appeared more in number and more resistant to contaminants. T2-T4 cultures were not contaminated in the four repetitions. It related only to the tryptophan treatments. At such concentrations the optimum cell growth occurred on days 14 to 21 (Fig. 1). The observations were conducted on day 0, 4, 7, 10, 14, 18, and 21.

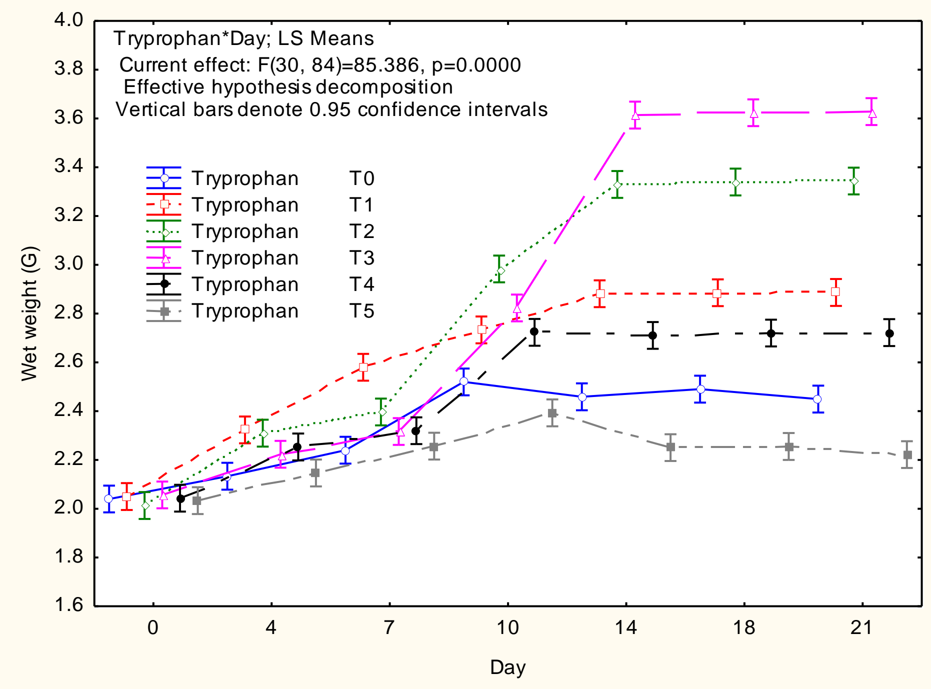

Fig. 1. The growth of cell aggregates of *C. roseus* measured in wet weight (g) in Erlenmeyer with tryptophan treatment on different culture days with tryptophan treatment (in mg/L) 0 (T0), 50 (T1), 100 (T2), 150 (T3), 200 (T4), and 250 (T5).

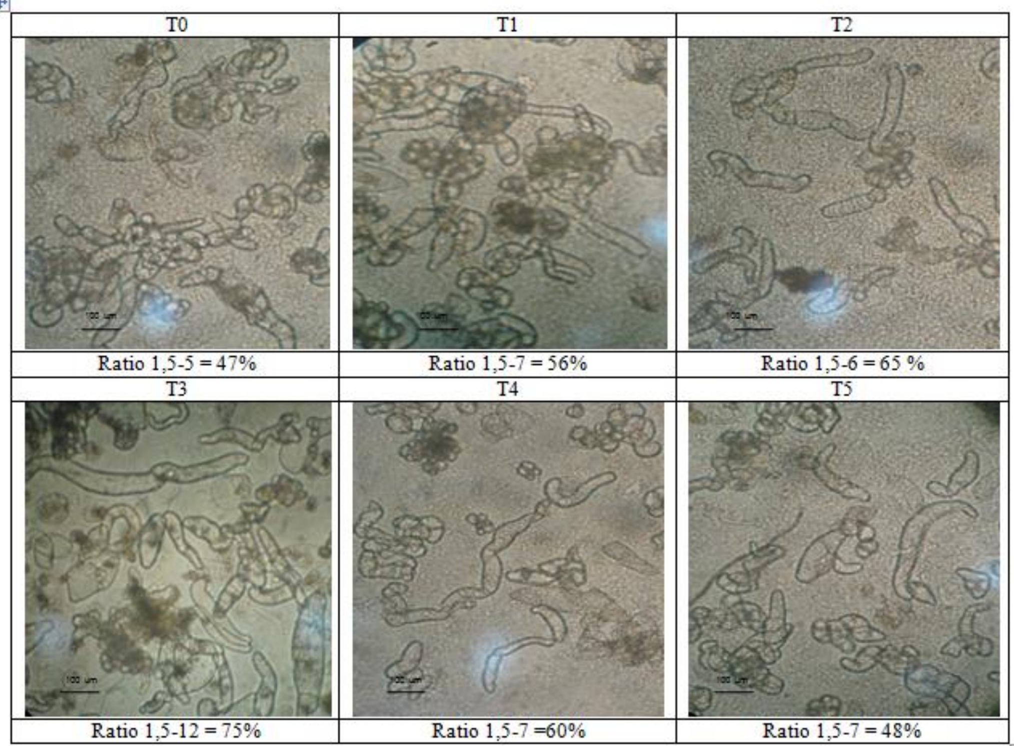

Fig. 2. Specialization of cells through morphological observation on *C. roseus* L (G) Don cells on day 14 after culture in tryptophan precursor treated media (in mg/L): 0 (T0), 50 (T1), 100 (T2), 150 (T3), 200 (T4), and 250 (T5). The cell ratio of each treatment is shown at the bottom of the picture.

TABLE I
EXAMPLES OF QUALITATIVE OBSERVATION OF CELL AGGREGATES OF VINCA (*C. ROSEUS*) CULTURED TREATED WITH TRYPTOPHAN (MG/L): CONTROL (T0), 50 (T1), 100 (T2), 150 (T3), 200 (T4) AND 250 (T5) AFTER 14 DAYS CULTURE IN TREATMENT MEDIUM.

Variable Observations	T0	T1	T2	T3	T4	T5
Sightings culture	somewhat murky flaxen	somewhat murky flaxen	somewhat murky flaxen	somewhat murky flaxen	somewhat translucent yellowish	yellowwish,, little fawn
The percentage of cell length	47 %	56%	65%	75%	60%	48%
Cell Morphology	Cells small round elongated cell	Cells round & elongated cell	round cell & elongated cell	round cell & elongated cell	round cell & elongated cell	round cell, & elongated cell
Change Morphology						
Ratio (length/width)	1,5-5 = 47%	1,5-7 = 56%	1,5-6 = 65%	1,5-12 = 75%	1,5-7 = 60%	1,5 -7= 50%
Wet weight (g)	2,545	2,775	3,275	3,555	2,665	2,015
Increase the percentage of wet weight	27,25%	38,75%	63,75%	77,75%	33,25%	0,75%
The color cell	White and Blackish	White	brownish	gray-white	Fawn-colored	Fawn-colored

The qualitative observations of aggregates and cells from culture results in the treatment of a wide variety of tryptophan can be seen in Table I. The tables only show observation on day 14 in tryptophan precursor treatment. The cell growth could also be seen from the color change of cell aggregates. Well grown cells tend to have flaxen color to yellowish while the color of those not well grown will be yellow to brown.

The morphological microscopic observation of the cells produced in cell aggregate cultures also showed a significant effect of tryptophan treatment. The effect was in the cell specialization. The results can be seen in Table I and Fig. 2. The morphology of the cells in the control group was dominated by spherical cells (the length:width ratio) is small, while the number of long (specialized) cells in the tryptophan treatment groups increased with the increased of tryptophan concentration , such as from T0 to T4. The length:width ratio could reach 8-12 (Table I and Fig. 2). The morphology of long cells in T3 and T4 seems to relate with the activity of Tryptophan Decarboylase (TDC) [10], protein content, and catharanthine content.

Tryptophan precursor treatment significantly affected the specialization of cells. The percentage of control cells with length:width ratio between 1.5 to 5 was 47%; while the tryptophan treatment groups had a very large ratio to reach 12, although the ratio did not always increase with increasing tryptophan concentrations. The highest ratio was obtained in treatments between 150 and 200 mg/L tryptophan. Darsini [13] have observed the development of the cells laticifer in callus of Vinca and showed that the alkaloid-producing laticifer cells were long in shape. Cell elongation in tryptophan treatment was a specialized response on alkaloid secondary metabolites formation as well.

The day of culture also determines the changes in cell ratio and size. The maximum cell ratio of about 10-12 was obtained on days 14 to 21. On these days the cells appeared to enlarge and was about to divide. The ratio on day 28 was smaller (data not shown) that was thought to have underwent cell division.

Another thing that was related to the resistance of the tryptophan treated cell aggregates might be caused by the expression of proteins that play a role in the immune response. The immune response in *Arabidopsis thaliana* plants is illustrated in Fig. 2 [14].

Effect of tryptophan treatment on cell growth (wet weight) of cell aggregates is depicted in Fig. 1. The Table shows that the treatment of tryptophan does not always enhance cell growth. The statistical analysis also showed that the days of observation also determine the growth of cell aggregates.

The concentration of tryptophan of 100 and 150 mg/L or T2 and T3 cell aggregates yielded optimum cell growth on days 14 to 21 (Fig. 1 and Table I). Our previous study on culture in Erlenmeyer found that tryptophan effect occurred on days 10-14 [5]. However, this study demonstrated that cell aggregate cultures that used callus that has been subcultured for more than one year showed optimum growth on days 14, 18, and 21 (Fig. 1). Statistically, the three days of observation were not significantly different.

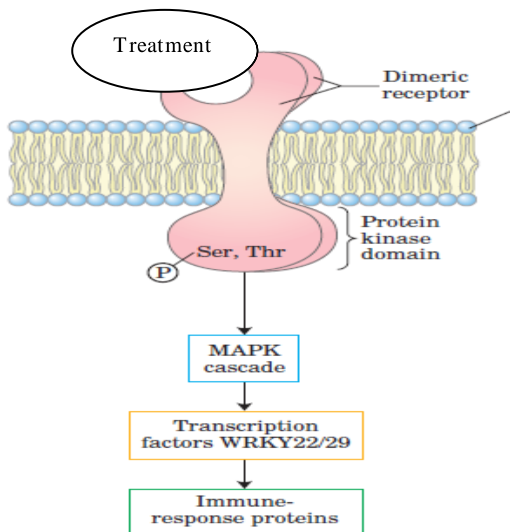

Fig. 3. Immune response proteins in plants (adopted from [14]).

Therefore, we recommend harvest and subculture were conducted on day 14 for efficiency and economical considerations. In addition, treatment of tryptophan significantly influenced the changes in the wet weight of the Vinca cell aggregates on T3 treatment. The highest increase in wet weight was also achieved in T3 that was equal to 77% and occurred on day 14 (Table 1). The highest wet weight 3.63 ± 0.05 g was achieved on day 14, and had no significant different from days 18 and 21 (Fig. 1).

Effect of tryptophan on catharanthine content after 14 days of culture was presented in Figs. 3 and 4. These results were also supported by the presence of long cell morphology with ratio ≥ 1.5. Pattern of both parameters depicted in Fig. 5. In general, a high percentage of long cells also produces high catharanthine. Kim *et al*. [9] reported that the production of indole alkaloids, ajmalicine and catharanthine was significantly greater in long cells than in the spherical ones

Based on the results of ANOVA analysis, tryptophan treatment of 50, 100, 150, 200, and 250 mg/L had a significant influence on the catharanthine content in cell aggregates. The maximum catharanthine content was obtained on T3 treatment about 50.96 μg/g d.w. (Fig. 4). Duncan test (DMRT) was performed on the most influential treatment. The Duncan test showed that the treatment of tryptophan had a significant influence on the catharanthine content of cell aggregates on day 14. It was revealed that the optimum treatment was the T3 treatment (150 mg/L tryptophan). Based on the analysis, it can be concluded that the addition of 50-250 mg/L tryptophan to the culture of cell aggregates could increase the catharanthine content of the *C. roseus* cell aggregates with optimum concentrations of T3 (150 mg/L). This result indicates that the hypothesis of treatment of tryptophan precursor may affect the catharanthine content is accepted. The effect there of is a positive effect, e.g. increase in the content of catharanthine.

The pattern of increase in wet weight or growth of cell aggregates treated with tryptophan in Vinca was similar (Fig. 1). However, it is different from the control (T0) where the maximum biomass occurred on day 10 only and decreased as was shown in our previous study [10] that the pattern of cell aggregates growth of tryptophan treated was longer to reach its stationary phase. The control achieved it on day 10, while treatment groups had it on days 14 to 21.

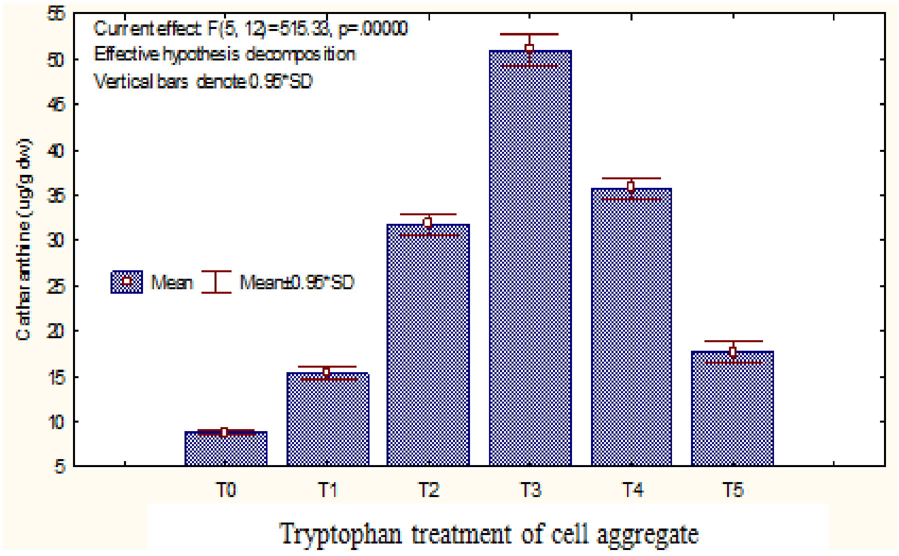

Fig. 4. Graph of catharanthine content (μg/g d.w.) in *C. roseus* cell aggregates treated with tryptophan (in mg/L): control (T0), 50 (T1), 100 (T2), 150 (T3), 200 (T4), and 250 (T5) on day 14 after culture in treatment medium.

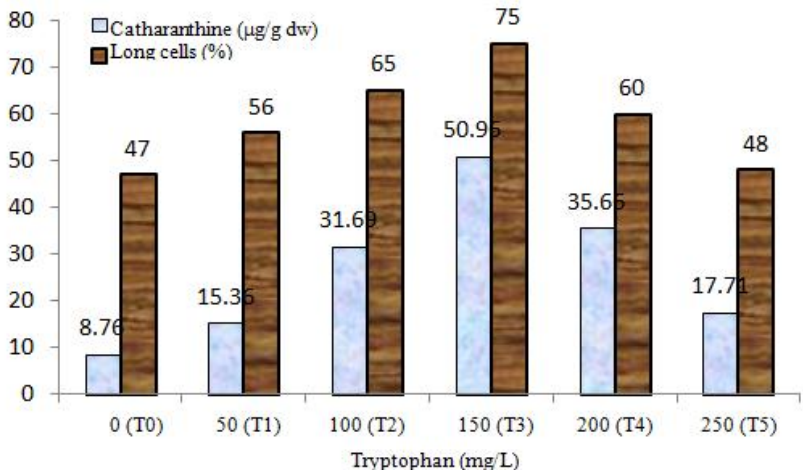

Fig. 5. Pattern of catharanthine content (µg/g d.w.) and specialized long cells (%) of the Vinca (*C. roseus*) cell aggregate that was added with tryptophan precursor (in mg/L): 50 (T1), 100 (T2), 150 (T3), 200 (T4), 250 (T5), and control (T0) in Erlenmeyer flask on day 14 after culture.

The emergence of morphological changes in cells that increasingly elongated in these Vinca cells associated with increased specialization to the concentration of 150 mg/L tryptophan is also something to do with the content of indole acetic acid (IAA) [12]. IAA as a plant growth regulator plays a role in cell elongation. This relationship has been reported by Pandiangan *et al*. [12]. with a positive correlation 0.80 or 80% support or complementary. The pattern of the relationship between IAA and specialized cells also expressed similar. It has also been reported by Pandiangan *et al.* [12].

IV. CONCLUSION

The cell morphology was essentially the same for each treatment. The significant difference was the presence of long cells more in the tryptophan treatment groups. The longest ratio was in treatment of 150 mg/L tryptophan on day 14 of culture. Cell elongation (specialization) was related to the increased in catharanthine content after tryptophan treatment. The highest content appeared on day 14 after treated with 150 mg/L tryptophan with 75% of long cells and catharanthine content of 50.96 µg/g dry weight.

This study has shortcomings in the use of tools to observe the cell growth that employed only a still camera on a microscope. In the next study we will utilize camera recorder to record the process of cell specialization dynamically after tryptophan treatment. Besides, it should also be investigated how the catharanthine content increase due to tryptophan treatment relates to other secondary metabolites on the same culture or cell aggregate culture of *Catharanthus roseus*.

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