The Effect of Elisitor on Growth and Ginsenoside Level in Hairy Root Culture of Panax ginseng Cultivated in Shake Flasks

Johan Sukweenadhi*, Stefan Pratama Chandra1, Leonardo Satriono Putra1, Yoanes Maria Vianney1, Theresia Lilian2, Merlyn Wongso2, Melisa Widjaja2, Sari Pramadiyanti2, Pissa Christanti2, Kim–Jong Hak3, Deok–Chun Yang3, and Asad Jan4

1Faculty of Biotechnology, University of Surabaya, Raya Kalirungkut, Surabaya 60293, East Java, Indonesia
2PT.Bintang Toedjoe, Pulomas, Jakarta 13210, Indonesia
3Graduate School of Biotechnology, College of Life Science, Kyung Hee University, Yongin–si, Gyeonggi–do 17104, Republic of Korea
4Institute of Biotechnology & Genetic Engineering, University of Agriculture Peshawar, Peshawar, 25130, Khyber Pakhtunkhwa, Pakistan

Abstract. In recent years, plants have become an important part of traditional medicine. Although the medicinal potential of the plant looks very promising, there are great difficulties that inhibit products for the production of herbs on a large scale. Panax ginseng C.A. Mey. is a plant often used in traditional medicine in various countries because ginseng can cure various diseases in humans. The main bioactive component in ginseng is the triterpene saponin compound, namely ginsenosides. Ginseng needs to be cultivated at least 4 yr before the root can be harvested. In addition, a special environment is needed for ginseng to develop properly. One way to obtain ginsenosides is to use root hair culture. However, until now it has been reported that the accumulation of ginsenoside in root hair cultures is still low. Elicitation effective method to increase the production of secondary metabolites in vitro culture. The aim of this research was to observe the effect of elicitor in the form of yeast extract and coconut water to the media on the growth and levels of ginsenoside in flask scale Panax ginseng root hair culture. From the data obtained, it can be concluded that the addition of yeast extract from the start with a concentration of 20 mg L–1 cannot increase the levels of ginsenoside in the hairy root culture of P. ginseng. The addition of coconut water from the beginning with a volume of 10 mL increases the biomass but cannot increase the ginsenoside levels in the hair culture of P. ginseng.

Keywords: Herbal product, in vitro, micropropagation, plant tissue culture, traditional medicine,

1 Introduction

In recent years, plants have become an important part of traditional medicine. Therefore, demand for herbal products is increased. Although the medicinal potential of the plant appears to be very promising, there are great difficulties that inhibit products for the production of herbs on a large scale. Some of these are lack of reproducibility in plant extracts, difficult standardization, genetic variation, and environmental conditions. In addition, secondary metabolites which have a therapeutic effect, are produced in small amounts, influenced by the physiological and developmental stages of the plant [1].

Panax ginseng C.A. Mey. is a plant often used in traditional medicine in various countries, such as Korea, China, and Japan [2]. Panax means "can cure all diseases" because ginseng can cure various diseases in humans. The main bioactive compound in ginseng is triterpene saponin, namely ginsenosides. Of the many species of ginseng, research focuses on examining the ginsenoside found in Panax ginseng (Korean ginseng), Panax notoginseng (Chinese ginseng), Panax japonicum (Japan ginseng), and Panax quinquefolius (American ginseng) [3]. Ginsenosides have many benefits for human health. Ginsenoside consists of two groups, namely the major ginsenosides consisting of the Rb group (Rb1, Rb2, Rb3, Rb4), and the second group is the Rc group (Rc1, Rc2, Rc3, Rc4), and the Rd group (Rd1, Rd2, Rd3, Rd4).

* Corresponding author: sukwee@staff.ubaya.ac.id
Rb2, Rc and Rd) and the Rg group (Re and Rg1) and minor ginsenosides such as Rh1, Rh2, and compound C. There have been many studies in vivo and in vitro which indicates that ginsenosides can act as anti-stress, anti-cancer, neuroprotective agents, and have benefits in the cardiovascular system and nervous system [4].

The main ginsenosides producer is the root where ginseng root need to be cultivated at least 4 yr before it can be harvested. In addition, cultivation requires special conditions where it is not exposed to direct sunlight. Ginseng is also sensitive to various diseases and requires quite expensive prevention costs. Therefore, plant tissue culture such as callus culture, callus culture, and root hair culture were developed to produce ginsenosides. Plant tissue culture is an alternative technique that can be used to produce high value secondary metabolites. Plant tissue culture are usually used on plants that have a high value but are difficult to develop conventionally, take a long time to harvest, and have low yields [5]. Several plant tissue culture that can be used to produce ginsenoside are adventitious shoot culture, callus culture, cell suspension culture, protoplast culture, bioreactor scale propagation, in vitro mutagenesis, polyploidization induction, and root hair culture [6, 7]. Root hair culture has a number of advantages, that is a stable growth phase, genetically stable, capable of producing secondary metabolites in greater numbers than the actual plant, and producing new secondary metabolites that are not found in the original plant [6]. However, until recently it was reported that the accumulation of ginsenoside in root hair cultures was still low. Therefore, several treatments are needed that can increase ginsenoside production, one of which is elicitation [4].

Elicitation is effective strategy to increase production of secondary metabolites in in vitro culture. This process involves adding an elicitor to the growth medium. Elicitor is a molecule that can stimulate the plant defense system so that it can increase the production of secondary metabolites. Elicitors can be classified into biotic and abiotic elicitors. Biotic elicitor is an elicitor derived from living things, either in the form of exogenous elicitor derived from pathogens or endogenous elicitor derived from plants. Biotic exogenous elicitor is an elicitor in the form of a compound released by a microorganism or a component of the microorganism such as a cell wall, whereas a biotic endogenous elicitor is an elicitor in the form of a degradation product from the plant cell wall, protein, or molecule synthesized by plants in response to stress from the attack of microorganisms and pathogens. Abiotic elicitors are elicitors that do not come from living things such as UV radiation, osmotic stress, drought, floods, heavy metals, fungicides, and herbicides [4].

One of the commonly elicitors that can be used to increase the production of secondary metabolites is yeast extract. A certain concentration of yeast extract in culture media will affect the biosynthesis of various metabolites and the optimum concentration of yeast extract is different for each plant species. It was reported that yeast extract has been able to increase the production of secondary metabolite compounds in several plants such as Psoralea corylifolia L., Hyoscyamus niger L., Pueraria candolleti Wall. ex Benth., and Portulaca oleracea L. Coconut water (Cocos nucifera L.) is a beverage that is famous internationally and always consumed. In addition, cultivation requests every year. Coconut water has the main composition, namely sugar and minerals so that it can increase the ability of cell division. Coconut water can be used as an elicitor by accelerating the culture to reach its stationary phase to produce ginsenoside production [8].

From previous research [9], it was obtained that the formulation B with a medium volume of 13 L was able to produce a fresh weight of about 2.7 kg and after drying, the dry weight average 109 g. However, the accumulation of ginsenoside in the root hair culture was still very small, around 2%. Although the dry weight has not reached the target, optimization is also carried out to increase ginsenoside levels using elicitation. The aim of this research was to observe the effect of elicitor in the form of yeast extract and coconut water to the media from the start on the growth and levels of ginsenoside in flask scale root hair culture of P. ginseng. It is expected that the use of elicitors can increase ginsenoside levels by more than 5%. The concentration of yeast extract used was 20 mg L⁻¹ while the volume of added coconut water was 10 mL.

2 Material and methods

2.1 Media preparation

For this research, the composition of the media that was used was formulation B and the elicitor was added since the beginning of the media preparation. The control medium was prepared by mixing all the ingredients into the container then adding Reverse Osmosis (RO) water using Pure RO II. After the RO water was added, the media was stirred using a homogenizer at 400 rpm (1 rpm = 1/60 Hz) for 40 min. After being evenly mixed, the pH of the medium was adjusted 5.8 to 6.0. After adjusting the pH, the media was poured 100 mL into a 250 mL flask and then covered with aluminum foil. Media was sterilized using Autoclave Hankuk HK–AC200P at 121 °C, pressure of 1.5 atm for 15 min. Then let the media cool it self for at least 3 d before it can be used. The experimental medium had the same steps as the control medium, only the addition of 20 mg L⁻¹ of yeast extract (MERCK) or 10 mL of coconut water into the medium. The total medium that is prepared are 50 flasks, consisting of 10 flask of control medium, 20 flask of yeast extract medium, and 20 flask of coconut water medium.
2.2 Incubation and incubation of hairy root culture in flask

Inoculation of hairy root was carried out in sterile Laminar Air Flow (LAF) and carried out aseptically. Hairy root was cut into small pieces (± 1 cm) before being put into the medium. After being cut, put ± 10 strands of root hair into the medium. The inoculation process was carried out two times where the first inoculation only used 30 flasks, that is 10 flask of control medium, 10 flask of yeast extract medium, and 10 flask of coconut water medium then next week with addition of 10 flask of yeast extract medium and 10 flask of coconut water medium. After the inoculation process, the flask is placed on the shaker and then stirred at 110 rpm for 7 wk.

2.3 Harvest and drying hairy root culture

After the incubation period is over, the hairy root were harvested. The media was removed and then the hairy root is collected in a container. The hairy root was rinsed using tap water 1 time. After rinsing using tap water, rinse the root hair using RO water 1 time. The root hair was drained first using a perforated container for 1 h to minimize the water attached to hairy root before being put into a container. The fresh weight was measured using Mettler Toledo MS16001L. The hairy root were dried using a Memmert UF750 oven which had been adjusted to the temperature at 50 °C, 60 % fan, 60 % flap, for 7.5 h. After drying, the root hair dry weight was weighed using the Mettler Toledo MS16001L. After the dry weight is obtained, the yield percentage was calculated using the Equation (1):

\[
\% \text{ Yeald} = \frac{\text{Dry weight}}{\text{Fresh Weight}}
\]  

2.4 Extraction and sample preparation

The dried hairy roots were extracted using the Soxhlet method. 5 g of dry hairy roots were extracted in 300 mL methanol 80 % for 2 h. Then the extract was dried using 1 set of rotary evaporator (BUCHI). The dry extract was dissolved in 20 mL of RO water. The extract was extracted again using water-saturated butanol in a ratio of 1:1. The mixture was centrifuged at 8 000 rpm for 15 min. The organic phase was taken and entered into a 250 mL erlemeyer. The extract was extracted using water-saturated butanol until the organic phase became clear. The butanol extract was then dried again using a rotary evaporator (BUCHI). The butanol extract was evaporated to obtain a dry extract which did not contain the butanol scent.

2.5 Ginsenosides analysis

Samples were analyzed using HPLC Agilent 1260 Infinity II. The dried extract was dissolved in 50 mL of methanol-HPLC grade. Then the sample was filtered using a 0.2 µm PTFE filter. The stationary phase used was Kinetex C18 with a size of 50 mm × 4.6 mm and the mobile phase was an eluent with a gradient of acetonitrile (A) and water. The gradient eluent program is set as follows: 7 min 19 % A (isocratic); 7 to 11 min with 19 to 29 % A (isocratic), 11 to 14 min with 29 % A (isocratic); 30 to 31.5 min with 70 to 90 % A (isocratic); 31.5 to 34 min with 90 % A (isocratic); 34 to 34.5 min with 90 to 19 % A (isocratic); 34.5 min to 45 min with 19 % A (isocratic). The sample was injected as much as 5 µL with a sample flow rate of 0.6 mL min⁻¹. The sample was detected using a Diode Array Detector (DAD) at a wavelength of 203 nm. The quantitative calculation is done by comparing the peak area of the sample with the peak area of the reference standard.

2.6 Statistical analysis

All data were analyzed statistically using IBM SPSS statistics 25 software. The fresh weight, dry weight, % yield, and % ginsenoside will be tested using the One Way ANOVA test if the data is normal and homogeneous (parametric test) or tested using the Kruskal-Wallis test if the data is not normal or homogeneous (non-parametric test). Data were considered significantly different if the P value was < 0.05 [10].

3 Result and discussion

Elicitation is a effective strategy to increase the production of secondary metabolites in in vitro culture. This process involves adding an elicitor to the growth medium. Elicitors can be defined as compounds that can cause stress to plants when added in small amounts. Elicitors can also increase the biosynthesis of certain compounds because they are used by plants as precursor compounds. To maximize the use of elicitors, there are several things need to be considered, that is elicitor concentration, duration of exposure, age of culture, and media composition [1]. The amount of yeast extract added to the medium is 20 g L⁻¹, whereas because coconut water is a liquid, 10 mL are added. Yeast extract is not a strong elicitor, so it is added from the media preparation so the exposure occurs since the hairy root are inoculated into
the flask. Coconut water can be used as a component in growth media because it contains various kinds of carbohydrates, minerals, and amino acids which can support cell division so it is expected to increase the fresh weight of hairy root [8]. In addition, it is hoped that the addition of coconut water can also promote hairy root growth so that the hairy root enters the stationary phase faster. The stationary phase is the stage where cell division decreases but there is an accumulation of secondary metabolites in plant cells. Therefore, coconut water is also added to the media so that exposure occurs since the root hairs are cultured into the flask media. After incubation for 7 wk and based on the results of HPLC analysis, data obtained can be seen in Figure 1.

![Fig 1. Bar chart showing average fresh weight (A), average dry weight (B), average % yield (C) average % ginsenoside (D) for each hairy root produced by each medium growth. Significant differences were indicated by the presence of signs (*) and (**) P value < 0.05](image)

The average fresh weight produced by the control medium, the yeast extract medium, and coconut water medium were 10.42 g ± 1.38 g, 3.80 g ± 1.54 g, and 16.50 g ± 2.86 g, respectively. Based on the results of statistical analysis, the fresh weight produced by each medium is significantly different from one another where the largest fresh weight produced by coconut water medium that is 16.50 g ± 2.86 g, while the smallest fresh weight is produced by yeast extract medium, that is 3.80 g ± 1.54 g. The fresh weight produced by the yeast extract medium has a smaller fresh weight produced by the control medium and coconut water medium. This can be caused by the yeast extract causes the hairy root become stress, thus inhibiting the cell division process. It is possible that carbon, minerals, and nitrogen in the medium are diverted to form secondary metabolites so that the biomass decrease. The biggest fresh weight was produced by coconut water medium. This is in accordance with the theory where the largest fresh weight will be produced by the media added with coconut water. This is because coconut water contains sugars in the form of sucrose, sorbitol, glucose, fructose, galactose, xylose, and mannose which can increase cell division. In addition, coconut water also contains various minerals such as potassium, calcium, sodium, magnesium, and many more where these minerals act as cofactors in an enzymatic reaction. Coconut water also contains amino acids such as Alanine, arginine, cysteine, and serine in higher amounts than cow's milk. This amino acid can be used as a nitrogen source to produce faster biomass growth [8]. In addition to increase the fresh weight, it is expected that hairy root that is inoculated in coconut water medium also have high ginsenoside because they enter the stationary phase faster.

After finished drying, the dry weight produced by each flask is very small. For analysis purpose, the dry hairy root produced by the control medium, yeast extract medium, and coconut water medium are each added together. Ten dry hairy root flasks were mixed together to obtain one dry weight data for control medium, two dry weight data for yeast extract medium, and two dry weight data for coconut water medium. The average dry weight produced by the control medium, the yeast extract medium, and the coconut water medium were 4.60 g, 4.70 g, and 11.50 g, respectively. Dry weight data cannot be analyzed statistically because the number of replications is very small. However, although it cannot be analyzed by statistical analysis, it can be seen that the dry weight produced by the coconut water medium is the largest and the dry weight produced by the control medium is the smallest. The dry weight produced by the coconut
water medium is the largest because it has the largest wet weight but the interesting part is the smallest dry weight is the dry weight produced by the control medium where the smallest fresh weight is owned by the yeast extract medium. This can be caused by difference in water content. Hairy root produced by yeast extract medium contains small amount of water so that even though the fresh weight is very small, it has a fairly large dry weight.

This can be seen in the average yield percentage produced by the control medium, yeast extract medium, and coconut water medium are 4.41 %, 6.18 %, and 3.48 %, respectively. From these data, it can be seen that the hairy root produced by yeast extract medium contains small amount of water, via versa, the hairy root produced by control medium and coconut water medium contains a lot of water so that it has a lower yield compared to the yield of the yeast extract medium.

The average levels of ginsenoside produced by the control medium, yeast extract medium, and coconut water medium were 1.39 % ± 0.02 %, 1.04 % ± 0.02 %, and 1.00 % ± 0.04 %, respectively. Based on the results of statistical analysis, the levels of ginsenoside produced by the control medium were significantly different from the levels of ginsenoside produced by the yeast extract medium and coconut water medium, while the levels of ginsenoside between the yeast extract medium and the coconut water medium were not significantly different. The levels of ginsenoside in hairy root extract produced by yeast extract medium and coconut water medium are not higher than control. In the case of yeast extract, this is inconsistent with the theory that the addition of an elicitor can increase the production of secondary metabolites. This can be caused by the use of the concentration of yeast extract that is not optimal. In a report reported by Kochan [4], the optimal concentration of yeast extract is 50 g L⁻¹ can increase the ginsenoside level in American ginseng. In addition, the duration of exposure also affects the accumulation of ginsenoside in hairy root. In a report by Kochan [4], it was also reported that ginsenoside levels would decrease with the length of exposure duration, where the peak of ginsenoside accumulation is on the 3rd d since yeast extract is given. In the case of coconut water, the levels of ginsenoside produced are the lowest. From these data it can be concluded that coconut water can increase biomass but cannot increase ginsenoside levels. This is because the hairy root are not in the stationary phase for a long period so that the accumulation of ginsenoside is not as much as the others.

The addition of yeast extract from the start with a concentration of 20 g L⁻¹ cannot increase the levels of ginsenoside in hairy root culture of P. ginseng. The addition of coconut water from the beginning with a volume of 10 mL increase the biomass but cannot increase the ginsenoside levels in the hair culture of P. ginseng. For the next experiment, yeast extract can still be used but the addition is not in the media preparation but after 27 d of incubation and incubated again for 3 d to obtain the biggest ginsenoside level.

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