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The Effect of Squalene Addition In-Vitro to Increase Asiatiaticoside Hairy Root Culture of *Centella Asiatica* (L.) Urban

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Abstract. The work on the potential of *C. asiatica* as a source of asiaticoside has been done very little in Indonesia. However, it is very promising economically. By using the progress in biotechnology in this case the use of the culture of hairy roots is expected to become a prospective method for producing the secondary compound from C. *asiatica*. Culturing the hairy roots is a method of producing secondary compound with many benefits compared to cell culture or suspension. The objectives of this research were to obtain the best Squalene concentration is important to obtain high production of triterpenoid secondary metabolite. The research consist 4 treatments in Completely Randomized Design (CRD) with 6 replications. As a treatment was concentration 0 ppm Squalene (control), 150 ppm, 300 ppm, 450 ppm. The results showed that strains R.1000 of *Agrobacterium rhizogenes* and 300 ppm Squalene concentration increased *asiaticoside*.

1. Introduction

Centella asiatica (L.) urban is one of medicinal plants in Indonesia which belongs to the group umbelliferae/apiaceae. It produces secondary metabolites which have the potential to be developed as medicines to cure various kinds of diseases. The metabolites can stimulate collagen biosynthesis and they are used in the treatment of leprosy, scars after operation or burn, fibrosis and radiotherapy. There are various types of compounds contained in *C. asiatica* namely asiaticocide, asiatat and madekasat acid [1]. In addition to as treatment of diseases, they can also improve intelegency question (iq), mental ability, and can overcome mentally retarded children. One research proved that *C. asiatica* plants could upgrade learning ability and memory of someone. Because of that benefits the plant is also known as food of the brain [2].

The needs for raw material used as ingredients will keep increasing in line with the needs of medicine. On the other side, naturally production of the secondary compound is very limited. Commercially, the source of secondary compound is still taken from plant extracts in field, so there has been a problem in the availability of raw materials.

Until now, the work on potential of *C. asiatica* as a source of triterpenoid has been done very little in Indonesia. However, it is very promising economically. By using the progress in biotechnology in this case the use of the culture of hairy roots is expected to become a prospective method for producing the secondary compound from *C. asiatica*. Culturing the hairy roots is a method of producing secondary compound with many benefits compared to cell culture or suspension. This is because the culture of hair roots is more stable genetically so issue like genetic variation causing decline in production could be avoided. In some plants, production of secondary metabolites is higher from hairy roots compared to the roots of normal plants or from his native plants [3]. Manipulation to

increase productivity of hairy roots can be increased by precursor through addition of Squalene into medium . The objectives of this research were to determine a proper concentration of Squalene in order to increase triterpenoid production by *C. asiatica.*

2. Materials and Methods

The research was done in the laboratory of plant physiology and tissue culture of department of Biology, Faculty of Math and Natural Science, Andalas University, Padang. Design used was a randomly complete design (RAL). The treatment was Squalene concentrations: control (a0), 150 ppm (a1), 300 ppm (a2), 450 ppm (a3). Each treatment was given six replications with the total experiment units were 24.

2.1. Research Procedures

2.1.1. Induction of hairy roots by A. rhizogenes R.1000. Leaves of *C. asiatica* (L.) urban were used as explants. Then transformation and co-cultivated were done for 30 minutes in bacterial suspension. Before bacteria was used, it was cultured for 48 hours in the medium yeast mannitol broth (YMB). Ex-plant was moved several times in the same medium until it was free from bacteria. Then ex-plant was moved to the base of solid MS medium without ampicillin. Then culture was incubated d2 and the gus assay was done.

2.1.2. Test gus was done to confirm that T-DNA plasmid Ri had been integrated into the plant genome [6]

2.1.3. Propagation of hairy roots of C. asiatica (L.) Urban. The hairy roots of C.asiatica that had been successful to be induced by A. rhizogenes were propagated in MS ½ liquid macro nutrient medium by adding 30 mg/sucrose in a dark room at a temperature 250C.

2.1.4. Addition of Squalene. Hairy roots already propagated were cut more or less 2 centimeters and cultured in 250 ml erlemeyer containing 50 ml liquid medium. Precursor treatment was done by adding Squalene solution based on treatment through milipore membrane (0.22 m) into medium aseptically. Then hairy roots were incubated.

2.1.5. Variables observed. Variables observed were time of hairy roots initiation, the percentage of hairy roots emergence, wet weight of hairy roots, and test of triterpenoid compound content.

2.1.6. Data Analysis. Data were analyzed using ANOVA with 5 percent level of confidence to know the difference of wet weights of hairy roots and a mixture of triterpenoid content. Test of smallest significant difference with 5 percent level of confidence was used to compare average response of treatments used [4].

3. Results and Discussions

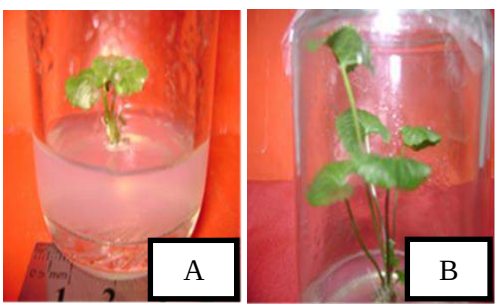

Figure 1. Cultures of *C.asiatica,* A. 1 month. B. 3 months

The results of this culture are used as explant source in transforming *A. rhizogenes* to form the hairy roots.

3.1. Time of initiation of hairy roots of Centella asiatica (l .) Urban

The results of inoculation of *A. rhizogenes* R.1000 in C.asiatica plants could induce the formation of a hairy root with an average time of initiation 15 days after inoculation (dai)(Table 1). On the control, hairy root was not formed. This was caused by no bacterial t-DNA transfer into the genomes of plants. Transfer of plasmid DNA into the plant cells is through initiation of transfer process that occurs when the wounded plant releases phenols with low Molecule weight namely asetosiringon (AS). AS will induces genes vir which encode protein. Proteins produced have important role as an intermediary in transformation of Agrobacterium. Protein vir A serves as a genetic transduction sensor signal and protein vir G is a component regulator. Protein vir A is a membrane functioning as chemo receptors to catch the AS compound. Detection of AS by receptors vir A means AS will be transducted from sensor vir A to the regulator vir G, so that vir G becomes active. Then vir G will activate other vir genes followed by changes of T-DNA. Protein of vir D1 and D2 participated in the process of T-DNA transfer. Vir D2 is a specific strand and has a specific sequence as endonuclease. Cutting of Ri plasmids by vir D2 on T-DNA border produces a form of single strand of T-DNA (DNA-ssT) known as the T-Strand. Gene vir E produces a protein vir E2 which play a role in processing T-DNA strand into a single strand and binds with single-strand T-DNA to form single strand DNA binding and protein (ssDNA binding protein). Besides protein vir E2, other proteins that bind with single strand DNA namely vir D2 that bind at the end of 5, would serve to protect T-DNA strand from nuclease degradation. Then T-DNA integrate into a plant genome. T- strand is intermediate molecule transferred from A. rhizogenes to plant cells. Vir D2 is covalently bound at the end of 5 t-strand and interacts with a nick in plant DNA. Single stranded T-DNA attached to DNA strand of plant and the twisting occurs in plant DNA producing second nick (point of intersection). Each strand of T-DNA ligation with plant DNA yielding homologous strand. The realignment and replication of point of intersection into successive plant DNA yielding duplicate and changes in the structure of target DNA [3]

.

Table 1. Initiation time and percentage of hair roots emergence in induced *C. asiatica*

No	Treatment	Initiation time (DAI)	Hairy roots emergence (%)
1	Control	0	0
2	Strain R.1000	15	100

3.2. Percentage of hairy roots formation of Centella asiatica

Strain R1000 could induce 100% formation of hairy roots. The success of DNA transfer depends on compatibility between plant and A . rhizogenes isolate used. Compatibility is usually indicated by the ability of *A. rhizogenes* to receive cue from wounded plant and followed by induction of virulent factors necessary in the process of inoculation [8] . No formation of hairy root due to no integration between t-DNA plasmid plant genome. The compatibility between plant genome with bacterial isolates and the ability of *A.rhizogenes* to infect plants also depends on endogenous hormonal balance of the plants or sps being induced. When oncogen is exspressed, there will be rapid growth of cells (Table 2). Strain R1000 could initiate 100 % exsplant of *C. asiatica* in accordance with statements of Ref. [9] that the expression of an oncogen gene in Ri plasmid characterizes the great formation of adventive roots in the wounded sites, known as hairy roots.

3.3. Wet weight of hairy roots culture of C. asiatica after adding Squalene

Wet weight of hairy roots seemed to increase in average until the end of observation. The addition of 300 ppm Squalene gave the average highest wet weight 447.10 mg and significantly different from the one 150 ppm , 450 ppm and control (Table 2).

Table 2. Wet weight of hairy roots culture of C. asiatica after adding of Squalene

No	Treatment	Wet weight (mg))	Asiaticoside (mg)
1	No treatment	130 a	1.30 a
2	Squalene, 150 ppm	310 b	3.10 b
3	Squalene, 300 ppm	447.10 c	4. 07 c
4	Squalene, 450 ppm	308 b	3.08 b

Note: numbers followed by the same letters at the same column no significant different at 5% confident level.

The addition of high Squalene could affect the osmoticum media because Squalene is macromolecule so it affects the absorption of nutrient, primary metabolite is used more as a source of energy to absorb nutrient than for growth of hairy roots. The absorption of nutrient in high osmotic pressure takes much energy obtained from primary metabolite decomposing. The success of adding precursor to biotransformation into components expected must meet the requirements: (1) culture should have the enzyme needed to transform the precursor to products, (2) product is formed more quickly than the following metabolite products, and (3) precursor and product produced are not poisonous in culture [10].

3.4. Content of triterpenoid compound in the culture of hairy roots of C. asiatica

Data on content of secondary metabolite statistically show the significant differences among treatments. Addition 300 ppm Squalene could increase triterpenoid content higher than the treatment of 150 ppm, 450 ppm and control. Squalene is a mediator compound that is formed near triterpenoid so it can increase triterpenoid content. Squalene is known as precursor of oksido Squalene is also called epoxy-squalene, which is an intermediate compound to form triterpenoid. According to [6], the addition of Squalene can increase activity an enzyme like HMGR.

Figure 2. Culture of hairy roots of *C . asiatica* after addition of Squalene

4. Conclusions

The addition of Squalene in vitro could increase the content of secondary metabolite in the culture of hairy roots of *Centella asiatica* (L.) Urban. The addition of 300 ppm Squalene could increase the highest triterpenoid content 4.07 mg.

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