Effect of *Agrobacterium rhizogenes* and elicitation on the asiaticoside production in cell cultures of *Centella asiatica*

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ABSTRACT

Background: *Centella asiatica* (L.) Urb. (Apiaceae) is an important medicinal plant, and it has been using to prepare herbal medicines. The compounds responsible for the biological activity of *C. asiatica* are triterpenoids such as asiaticoside. Asiaticoside is also important as a marker for standardization of *C. asiatica*. Due to the low content, there is a need to enhance the production of asiaticoside of *C. asiatica*. The biotechnological approach is one of the methods that can be used to enhance its production. **Objectives:** This study was designed to enhance the production of asiaticoside from *C. asiatica* using *A. rhizogenes* and elicitation experiments. **Materials and Methods**: Callus cultures were initiated using Murashige and Skoog (MS) medium supplemented with 1.0 mg/L indole-3-acetic acid (IAA) and 1.0 mg/L 6-benzylaminopurin (BAP). All media were supplemented with 4% (w/w) sucrose and solidified with 0.9% agar. Elicitations were done using pectin, methyl jasmonate, and Cu^{2+} ions. Transformed hairy root cultures were performed using *A. rhizogenes*. **Results:** Callus culture of *C. asiatica* was successfully initiated. Enhancement of the production of asiaticoside in the callus culture by elicitors pectin was up to 31%; methyl jasmonate (50 μM) in cell suspension cultures at day 14 was up to 171% compared to explant and 494% compared to control callus; copper ion (25 μM) at day 21 was up to 144% compared to explant, and 676% compared to control cell suspension cultures. While enhancement by genetic transformation using A. rhizogenes was 166-172% compare to untransformed roots **Conclusion:** Elicitation and genetically transformed hairy root cultures of *C. asiatica* produced asiaticoside up to 172% higher than untreated callus.

Key words: *Agrobacterium rhizogenes*, asiaticoside, *Centela asiatica*, elicitation, medicinal plant

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INTRODUCTION

Centella asiatica (L) Urb. (Apiaceae) is commonly known as pegagan and used in Indonesian traditional medicine to treat cough, dysentery, as an antipyretic, diuretic, to treat skin inflammations, bronchitis, abdominal pain and as an anthelmintic.[1,2] Phytochemical and biological investigations of *C. asiatica* have been published. Asiatic acid, madecassic acid, asiaticoside, and madecassoside are the principle triterpenoids found in *C. sciatica*.[2] A topical application of ethanol extract (100 mg/kg body weight) showed wound healing properties in rabbit with burns.[3] Wound healing activity has also been reported from clinical studies with patients with burns, cellulitis, leprotic infections, and skin ulcers.[4] *C. asiatica* also displayed pharmacological activities different from those mentioned in the traditional use. It was shown that the total triterpenoid fraction from aerial parts of *C. asiatica* was useful in patients with diabetic microangiopathy. It improved microcirculation, decreased capillary permeability, and protected against the deterioration of the microcirculation.[5] A cardioprotective effect of an aqueous extract of *C. asiatica* on the antioxidant tissue defence system during doxorubicin-induced cardiac damage has been reported in rats and ascribed to the triterpenoid fraction.[6] The total triterpenoid fraction of *C. asiatica* improved the signs and symptoms in patients with venous hypertension, correlated well with the improvement of the microcirculation and capillary permeability.[7] Moreover, asiaticoside has been reported to promote angiogenesis and to stimulate blood vessel formation and mucosal cell regeneration.[8]

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Asiaticoside has important pharmacological activities. In contrast, the production of secondary metabolites from plants such as asiaticoside is usually low. This is a bottleneck in attempting to develop the medicinal plants. Due to this problem, there is a need to establish the method that can be used to solve the problem. The biotechnological approaches have been used to enhance the production of such active compounds. Cell cultures have been used to enhance the production of secondary metabolites from plants.[9] A method for enhancing secondary metabolite production is by transformation using natural vector system *Agrobacterium rhizogenes*, the causative agent of hairy root disease in plant. Genetically transformed hairy root by infection of plants with *A. rhizogenes*, a gram-negative soil bacterium, has been used for secondary metabolite production. The fast growing hairy root can be used as a continuous source for the production of valuable secondary metabolite. A better understanding of the molecular mechanism of hairy root development, which is based on the transfer of *A. rhizogenes* T-DNA into the plant genome, has facilitated its increasing use in metabolic engineering. Hairy root has been widely studied for the *in vitro* production of the secondary metabolite.[10,11] These methods were applied to enhance the production of active compounds from an Indonesian medicinal plant.

The purpose of the research was to elicit the callus culture of *C. asiatica* in order to enhance the production of asiaticoside from *C. asiatica* using cell cultures and genetically transformed hairy root cultures.

MATERIALS AND METHODS

Plant material, solvents and chemicals

Centella asiatica (L.) Urb. (Apiaceae) was collected in December 2007 from Bandung, West Java, Indonesia. The plant samples were authenticated at the School of Life Sciences and Technology, Institut Teknologi Bandung (Indonesia). The leaves of plant were used as explants to initiate cell and callus cultures of *C. asiatica*. All solvents and chemicals were purchased from Sigma-Aldrich (Singapore).

Innitiation of callus and cell suspension cultures

The sterile leaves were cut into slices and callus induction was obtained using media with different compositions. These media were modifications of the Murashige and Skoog (MS).[12] For *C. asiatica*, MS medium was supplemented with 1.0 mg/L indole-3-acetic acid (IAA) and 1.0 mg/L 6-benzylaminopurin (BAP). All media were supplemented with 4% (w/w) sucrose and solidified with 0.9% agar. The callus cultures were grown under an L/D regime (16/8 h: 3,000 lux) at 26 °C. After 4–6 weeks, callus were used as an explant for hairy root culture for the analysis of secondary metabolite contents. Cell suspension cultures were initiated using the same medium with callus withouth agarose.

Elicitation

To stimulate enhancement of asiaticoside production, some elicitors have been fed to the cultures of *C. asiatica*. The elicitors were pectin with various concentrations, i.e. 0.05, 0.1, and 0.2% w/v for callus cultures, while for cell suspension cultures were methyl jasmonate (50, 100, and 200 μM) and Cu^{2+} ions (10 and 25 μM). Pectin, methyl jasmonate, and Cu^{2+} ions were added to reach different final concentrations above. These compounds were dissolved using 0.2 mL of 96% ethanol and transferred to a sterile 500 mL Erlenmeyer flask containing the cell suspension cultures of *C. asiatica* after inoculation into the fresh medium (at the start of the growth cycle). Callus and suspension culture were harvested in certain days after elicitation.

Induction the hairy root culture using *Agrobacterium rhizogenes*

A. rhizogenes ATCC 15834 strain was cultured using a YMA medium for 2 days at 25 °C. A part of plant leaves or callus culture called explants was sterilized using water-sterilization liquid then incubated in the *A. rhizogenes* culture which was called the disc method. Explants were transferred to the *A. rhizogenes* suspension culture for 40 min. Then, infected plants were rinsed with sterile water and moved to the original medium. The cultures were grown in the solid MS medium containing 1.0 mg/L IAA and 1.0 mg/L BAP and sucrose 25 mg/L. The infected explants were transferred to the MS medium containing cefatoxime 0.2 g/L.

Control for genetic transformation

The integration of *rol A* and *rol C* genes from *A. rhizogenes* into the plant genome, which is the genetic evidence for hairy roots transformation, was checked by PCR. Therefore, the following specific primers were designed: for *rol* A gene, nucleotide positions 21–42 (5'-CGTTGTCGGAAT-GGCCCAGACC-3') and 268–246 (5'-CGTAGGTCTGAATAT-TCCGGTCC-3'), totally 248 bp; for *rol* C gene, positions 51–70 (5'-TGTGA-CAAGCAGCGATGAGC-3') and 550–531 (5'-GATTGCAAACTTGCACTCGC-3'), a fragment of 490 bp totally. Vir D_2 gene is not involved in the plant genome during the transformation. The specific primers for the detection of vir D_2 are: primer A (5'-ATGCCCGATCGAGCTCAAGT-3) and primer E (5'-CCTGACCCAAACATCTCG-GCTGCCCA-3'), ending in a fragment of 338 bp. The DNA of *A. rhizogenes* was isolated using plasmid purification kits (Bio Basic Inc. Canada) while DNA of uninfected and infected roots was

isolated according to a protocol for rapid isolation from dry and fresh samples.[13] The DNA of *A. rhizogenes* and uninfected plants was used as positive and negative control, respectively. All PCR reactions were performed in an AB Applied Biosystem 2720 thermal cycler with recombinant taq DNA polymerase (Fermentas GMBH, St. Leon-Rot, Germany). The PCR program was 5 min at 95°C, 35 cycles of 30 s at 95 °C, 40 s at 50 °C, 2 min at 72 °C, and a final step of 5 min at 72 °C.

Analysis of the produced active compounds

Asiaticoside, an active compound of *C. asiatica* from original plant, callus, elicited callus and genetically transformed hairy root was analysed using a TLC scanner (CAMAG, Germany), 100 mg of powdered sample was extracted using 1 ml methanol, and filtered. Methanol extracts were dried, and re-dissolved by 0.1 ml methanol. An amount of 20 µl were spotted into silica gel GF_{254} plate, then eluted using toluene–acetone (9:1). The bands were visualized using anisaldehyde in H_2SO_4. The TLC plate was then measured using a TLC scanner. The concentrations of asiaticoside production were calculated using standard asiaticoside as a reference. A series concentration of asiaticoside standard was used to make a calibration curve.

RESULTS

Callus culture of *C. asiatica* was successfully initiated. Callus started to be formed after 2–3 weeks incubation then continuously growing. The best medium was the MS medium supplemented with 1 mg/L IAA and 1 mg/L BAP. Callus was subculture every 6 weeks. Cell suspension was also successfully initiated from callus culture and subculture every 4 weeks. Subculturing was done until amount of callus and cell suspensions was enough for all elicitation experiments. Elicitation of pectin on callus culture resulted in an enhancement of asiaticoside production up to 31%. However, this enhancement was much lower than in transformed hairy root. Elicitation of methyl jasmonate and copper ions on cell suspension culture resulted enhancement of asiaticoside production. Enhancement of asiaticoside production from elicited cell suspension cultures with methyl jasmonate at 100 µM on day 14 compared to *pegagan* leaves, control suspension and 8 weeks callus was 171%, 125%, and 494%, respectively. Elicitation with copper 25 µM on day 21 enhanced asiaticoside compared to *pegagan* leaves, control suspension and 8 weeks callus 144%, 676%, and 416%, respectively [Table 1].

Induction of callus using *A. rhizogenes* successfully produced hairy root culture [Figure 1]. *Agrobacterium*-transformed hairy root enhanced the production of asiaticoside up to 172% compared to untransformed callus [Figure 2]. To check whether DNA of *A. rhizogenes* was transformed into plant cells, DNA of transformed hairy root, untransformed

Table 1: Asiaticoside production after elicitation with methyl jasmonate and copper ion compared to controls (leaves, cell suspension culture and 8 weeks callus of *Centella asiatica*). All control considered to be 100%.

Elicitation sample	Day 14 (%)			Day 21 (%)		
	a	*b*	*c*	*a*	*b*	*c*
Methyl jasmonate 50 µM	121	88	349	21	99	61
Methyl jasmonate 100 µM	171	125	494	35	165	102
Methyl jasmonate 200 µM	166	121	479	52	243	149
Copper ion 10 µM	86	63	248	48	224	137
Copper ion 25 µM	129	94	372	144	676	416

a = compared to *Centella asiatica* leaves, *b* = compared to control cell suspension cultures, and *c* = compared to 8 weeks callus

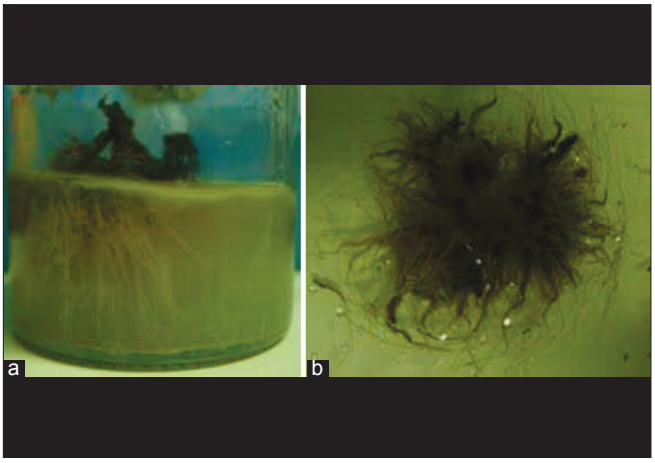

Figure 1: Genetically transformed hairy roots cultures of *C. asiatica* (a), without induction by *Agrobacterium rhizogenes* (b)

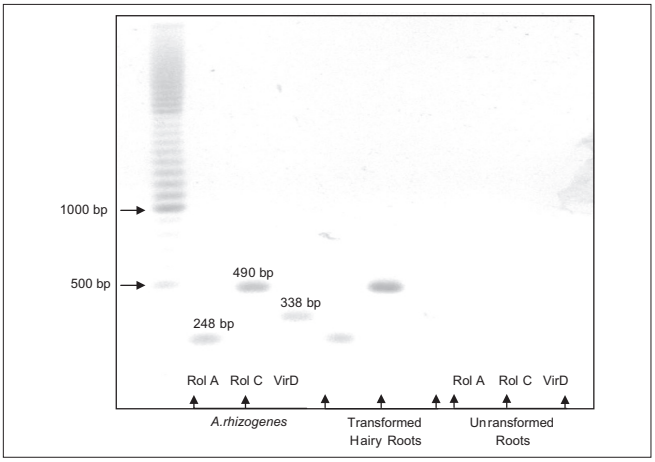

Figure 2: Gel electrophoresis of the PCR product from *Agrobacterium rhizogenes*, transformed hairy root and untransformed root of *Centella asiatica*

root, and *A. rhizogenes* were amplified using a PCR machine. Three pairs of primer were used; they were rol A and rol C which are responsible for hairy root induction and integrated to a plant cell, and Vir D2 which is not integrated to the plant cell. As showed in Figure 2 that the rol A and rol C could be found in *A. rhizogenes* and transformed hairy root, but they can be found in the untransformed root (callus). While Vir D2 could only be found in the *A. rhizogenes* but not in transformed and untransformed hairy roots. From these results, we concluded that the hairy root of *C. asiatica* was produced by *A. rhizogenes*-induced genetic transformation.

DISCUSSION AND CONCLUSION

Cell cultures were initiated with the general MS medium supplemented by growth hormone IAA and BAP with a ratio of 1:1. This ratio was suitable to initiate the callus culture. If the ratio was changed the formation of callus was interfered by the growing of shoot or root. This composition has been used for all callus and suspension culture experiment. For an elicitation experiment, all elicitor influenced the production of asiaticoside. Pectin as elicitor had effect on asiaticoside production. The mechanism of elicitation of pectin probably was because of its effect in the enzyme activity. It increases the activity of squalene synthase which catalyze squalene formation from farnesyl pyrophosphate (FPP).[14] Squalene is one of the substrates in biosynthesis of triterpenoid such as asiaticoside. However in this experiment, enhancement of asiaticoside after elicitation with pectin was not significant. Copper ion as elicitor has enhanced the production of asiatiocside. It was showed that the highest asiaticoside production was found at 21 days after elicitation with 25 μM copper ion compared to control cell suspension cultures. The offer production of asiaticoside did not effect on the growth or viability of the cell cultures. Interestingly, the cultures appear to have a long cycle. The copper ion appeared as a best elicitor used in relation to the production of asiaticoside which was up to 676%. This probably was because of the effect of copper ion functions as a cofactor for enzyme which are responsible for asiaticoside biosynthesis.[15] Methyl jasmonate has also enhanced the production of asiaticoside in the cell suspension culture of *C. asiatica* up to 494%. Jasmonate acid and its methyl ester have been reported to have an important role in the signal transduction process that regulates genes for plant protection, this mechanism is believed that methyl jasmonate can elicit the secondary metabolite production from plants.[16]

Surprisingly, the enhancement of asiaticoside production was also found in the transformed hairy root cultures 172% compared to the wild type. This enhancement probably caused through two mechanisms, elicitation and genetic transformation. Elicitation most probably happened due to the protection response of the plant culture against biological stimulus of bacteria, then plant cells were stimulate to produce secondary metabolites. While genetic transformation happened when bacterial DNA was integrated into plant DNA, thus may not only induce the hairy root formation but also influence the production of secondary metabolites. This result is in contrast with the study reported by Aziz *et al.*[17] In that study, asiaticoside and madecoside could not be detected from the transformed hairy root cultures. This may be caused by different strain of *A. rhizogenes* used for the study. In summary, genetically transformed hairy root culture of *C. asiatica* produced higher amounts of asiaticoside compared to untransformed hairy root. This genetic transformation was confirmed by the PCR reaction.

We performed DNA analysis in order to confirm the hairy roots transformation. The T_L region in plasmid T-DNA of agropine-type strain *A. rhizogenes* 15834 contains 18 open reading frames including several loci called *rol* (root loci). The products encoded by *rol A* and *rol C* genes were found to have a synergistic effect on root induction and induce increased sensitivity to auxin. PCR analysis showed that hairy roots from *C. asiatica* contain *rol C* and *rol A* genes (Figure 2, lanes 5 and 6) corresponding to the positive controls obtained by DNA from *A. rhizogenes* ATCC 15834 (lanes 2 and 3). Untransformed callus served as a negative control (lane 8, 9, and 10). Vir D_2 was not detected in the hairy roots (lane 7), thus showing that T-DNA is incorporated in the plant genome and it is not a residual bacterial contamination. From overall experiment, it was concluded that enhancement of asiaticoside was shown by elicitor treatments using pectin, methyl jasmonate, and Cu^{2+} ions as well as by genetic transformation using *A. rhizogenes*.

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