Development of Hairy Root Culture of Andrographis Paniculata for in Vitro Adrographollide Production

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***Abstract*—Development the hairy root culture of *A. paniculata* was conducted for growing the hairy roots and production of andrographollide. Different strains of *Agrobacterium rhizogenes* (R-1000, A4, ATCC 15834), different types of explants (cotyledons, hypocotyls, and leaves) and different infection time of *A. rhizogenes* (1, 2, 3 days) were tested to induce hairy roots of *A. paniculata*. The results indicated that the best strain, type of explants and infection time for hairy roots induction were found in strain ATCC 15834, the explants of cotyledon and the 2 days of infection, respectively. The best medium for growing the hairy roots was liquid half strength MS medium with the addition of 5.0 µM IBA. The highest amount of andrographollide was observed in the medium with the addition of 5.0 µM IBA on the week of two, as much as 0.54%. Integration of T-DNA of *A. rhizogenes* in hairy roots was confirmed by polymerase chain reaction (PCR) analysis with specific primer for *rol*A and *rol*C genes of the plasmid. Visualization of the PCR products on agarose gel electrophoresis showed two fragments with lengths of 248 bp and 490 bp which corresponds to *rol*A and *rol*C genes from Ri plasmids of ATCC 15834.**

***Index Terms*—*A. paniculata*; hairy roots; andrographollide; *A. rhizogenes*; *rol*A and *rol*C genes.**

I. Introduction

Andrographis paniculata (Burm.f.) Wallich ex Nees is one of highly valuable medicinal plant which is distributed in many tropical countries such as Indonesia. The primary medical constituen found in *A. Paniculata* is andrographolide, a diterpene secondary metabolite. The compound was mainly detected in leaves of the plant as much as 2.39% [1] and was also detected in the roots but in much lower (0.15%) than that of amount in the leaves. Andrographollide showed a broad range of pharmacological effects, such as anti-HIV [2], antifertility [3], anticancer [4], antimicrobials [5], analgesic, antipyretic, antiinflamation [6], and hepatoprotectiv[7].

To meet the market demand of andrographollide, leaves and roots of *A. paniculata* have been used as resources for crude extract or single compound of andrographollide. This could threaten the population of *A. paniculata* when harvesting the leaves and roots continuously. For this reason, alternative resources need to be developed in order to provide andrographolide without destroying population of the plant. For many years, plant tissue culture (plant *in vitro* culture) has been developed as alternative resources for secondary metabolites production [8]. Our previous study on production of andrographolide *in vitro* indicated that andrographollide production in callus and cell culture of *A. paniculata* as much as 0.49% and 0.23%, respectively. Meanwhile, [9] reported that andrographllide in adventitious roots culture could reach 7.3%. This finding indicates that differentiated tissue (root) culture of *A. paniculata* is promising system for adrographollide production.

It has been known for over twenty years that induction of root culture can be done by transforming plant explants with soil bacterium, *A. rhizogenes*. After infection, the inducted roots can grow faster on free hormone media. Therefore hairy roots culture has been widely used as an alternative method for the production of secondary metabolites [10]. The advantages of hairy root culture over the adventitious root culture are fast growth in hormone free media, low doubling time, genotypic and biochemical stability, and can be cultured in bioreactor for large scale secondary metabolite production [11].

The successful for induction of hairy roots by *A. rhizogenes* is affected by several factors, such as type of explants, strain of *A. rhizogenes* strain, time of infection of the bacteria into plant tissue, and regeneration media [12]. Therefore in order to develop the hairy roots of *A. paniculata* for *in vitro* adrographollide production, the presence study was conducted with the objectives to determine the most compatibly of strains of *A. rhizogenes* with the tissue of *A. paniculata*, the best time of infection , the best types of explants, and the best medium for culturing hairy roots.

II. Materials and Methods

A. Plant Material

Plant leaves and seeds of *Andographis paniculata* (Burm.f.) Wallich ex Nees were collected from BALITTRO (Research Institute of medicinal and aromatic plants). Explants of leaves were obtained from

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2-3 months old plant. The explants were surface-sterilized with 1.04% sodium hypochlorite (v/v) solution containing 0.15% tween 20 for 15 minutes, followed by rinsing 3 times with sterile water. They were cut into 1 × 1 cm^2 segments and precultured on Murashige and Skoog (MS) agar medium for five days.

The seeds of *A. paniculata* were surface-sterilized with 0. 52% sodium sodium hypochlorite (v/v) solution containing 0.15% tween 20 for 10 minutes, followed by washing 3 times with sterile water. They were germinated on solid MS medium at 25℃ in the dark. After 21 days, cotyledons and hypocotyls excised from the germinated seedling and were precultured on Murashige and Skoog (MS) agar medium for five days.

B. Strain of Agrobacterium Rhizogenes

Three different strains of *A. rhizogenes*: R-1000, A4, ATCC 15834 was used to select the most compatible *A. rhizogenes* that can cause the formation of hairy root on the explants of *A. paniculata*. They were obtained from Biotechnology Research Centre, Indonesian Institute of Sciences Cibinong, West Java. A single clone from each strain of *A. rhizogenes* R-1000 , A4, ATCC 15834 was cultured on 50 ml Luria Bertanie (LB) liquid medium in the dark, at 28 ℃, on a rotary shaker at 100 rpm/min, until cell density reached 2 x 10^6 cell/ml or equivalent with OD_{600} = 0.5. The bacterial suspensions were then centrifuged at 4,000 rpm/min for 20 min, followed by re-suspension in half strength liquid MS medium (Murashige and Skoog) for inoculation.

C. Induction of Hairy Root

To induce hairy roots, three different explants (cotyledons, hypocotyls, and leaves) from precultured media were infected by the three different strains of *A. rhizogenes* R-1000, A4, ATCC 15834 suspension (OD_{600}= 0.5) containing 100 µM acetosyringone for 60 minutes. Following infection, the infected explants were then co-cultivated on Murashige & Skoog agar medium in three different time of infection (1, 2, and 3 days). After co-cultivation, each explant was washed with 250 mg/L cefotaxime for 30 minutes. The explants were then transferred into MS medium containing 200 ppm cefotaxime for two days to inhibit the growth of *Agrobaterium*. This step was repeated three times to eliminate *Agrobacterium*. After 3 passages of disinfections, the explants were placed on growth regulator- free MS solid medium containing 250 mg/L cefotaxime and kept in the dark for 1 week, followed by transfering the explants into free-hormone MS medium without cefotaxime to induce hairy roots. As a control, non-infected explants were cultured on free-hormone MS medium. Induced hairy roots were left on the medium to allow further growth of roots.

D. Cultured of Hairy Root

After numerous hairy roots were appeared from the best types of explant and the best time of infection, they were separated from explants and transferred into liquid full strength or half-strength MS medium with addition of two different concentration of IBA (0.5 and 5.0 µM IBA) and without IBA as a control. To measure growth of the cultures, each hairy root culture in Erlenmeyer flask was harvested at one week interval within six weeks period, dried and weighted to constant weight. Each treatment was repeated triple and data was presented as their average.

E. High Performance of Liquid Chromatography (HPLC) Analysis of Andrographolide

The amount of andrographollide in the hairy root culture was analyzed by using High Performance of Liquid Chromatography (HPLC) equipped with ODS-C18 column (particle size 5 µm, 15cm x 4.6mm) and UV detector (λ=254 nm). Analysis was conducted in mobile phase of methanol: water (6:4) at flow rate of 0.7 mL/minutes throughout the analysis. Quantitative analysis was conducted with referred to the calibration curve of authentic andrographollide.

F. Identification of Transformed A. paniculata Hairy Root by Polymerase Chain Reaction

1) Isolation of DNA

Isolation of DNA from the hairy roots and non transformed roots was conducted according to the procedures of [13] with a minor modification. In this procedures, NTES (0.1M NaCl; 0.01M Tris-HCl, pH 7.5; 1.0M EDTA and 1% sarkosyl) was used for extraction. One gram of harvested hairy roots was frozen in liquid nitrogen, grounded into fine powder and added with 1.5 mL of a mixture Phenol: Chloroform: Isoamyl alcohol (25:24:1) and 2.5 mL of NTES. The sample was transferred to a microtubes containing 200 µL of a mixture of Phenol: Chloroform: Isoamyl alcohol (25:24:1) and 300 µL of NTES. The samples were mixed gently and centrifugated at 5000 x g for 10 minutes. The supernatants (aqueous layer) were transferred to new microtubes and DNA was precipitated by adding 1 volume isopropanol and 1/10 volume of 3.0 M Sodium acetate, pH 6.0. DNA was collected by centrifugation at 5000 x g for 10 minutes. The supernatant was discarded and the pellet was wash with 200 µL of 70% ethanol followed by drying. The pellet was resuspended in 30 µL of Te RNAse and incubated at 37 ℃ for 1 hour.

2) PCR analysis

To confirm integration of Ri T-DNA of *A. rhizogenes* into genome of *A.paniculata*, PCR analysis was performed using DNAs from the hairy roots as template and non transformed roots as control, respectively, according to the methods of [14] with a minor modification. Moreover, two specific primer were used for amplification of *rol*A and *rol*C sequences: *rol*A (forward 5' –CGTTGTCGGAATGGCCCAGACC-3'; reverse 5'-CGTAGGTCTGAATATTCCGGTCC-3') and *rol*C (forward 5'-TGTGACAAGCAGCGATGAGC-3'; reverse 5'- GATTGCAAACTTGCACTCGC-3'). The amplification was performed as follow, an initial denaturation step of 3 minutes at 95 ℃, followed by 30 cycles of denaturation at 95 ℃ for 30 seconds, annealing at 50 ℃ for 40 seconds, and extension at 72 ℃ for 2 minutes, and final extension at 72 ℃ for 5 minutes. The PCR products were fractioned by electrophoresis on a 1.5%

agarose- ethidium bromide gel using Tris-acetate-EDTA buffer and photographed under UV lamp at λ 380 nm.

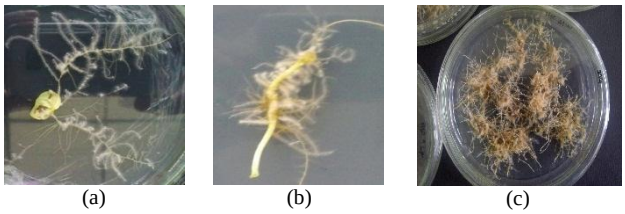
III. Results and Discussions

A. Establishment of Hairy Roots

Among the tested explants (cotyledons, hypocotyls, and leaves segments), cotyledons tissue more susceptible to infection by strain R-1000, ATCC 15834 and A4 of *A. rhizogenes* rather than hypocotyls and leaves segments . This was indicated by the formation of hairy root when the infected explants were placed on hormone-free medium (Fig. 1). The highest infection frequency on cotyledons was observed with 2 days time of infection by *A. rhizogenes* R-1000 (60%), followed by the infection with ATCC 15834 (20%) and the lowest infection frequency was observed by infection with A4 (13%) as presented on Table I. Hairy roots were also induced on hypocotyls tissue when infected by R1000 with the infection frequency 30% on day-1, 50% on day 2 and day-3. However, no hairy roots were obtained when the strains of *A. rhizogenes* inoculated to leaves segments. These means that only cotyledon and hypocotyls explants could be transformed with strain R-1000, ATCC 15834 and A4 of *A. rhizogenes* to regenerate hairy roots on hormone-free medium. The effect of explants types, strains of *A. rhizogenes* (R-1000, ATCC 15834 and A4) , and time of infection on frequency of hairy roots induction is presented on Table I.

TABLE I. The frequency of Infection by R-1000, ATCC 15834 and A4 of *A. rhizogenes* to Different Types of Explant in Different Time of Infection

Percent (%) of infection frequency on		Strain of *A. rhizogenes*		
Explants	Day	R-1000	A4	ATCC 15834
Cotyledon	1	50	<5	<5
	2	60	13	20
	3	35	<5	<5
Hypocotyls	1	30	<5	<5
	2	50	<5	<5
	3	50	<5	<5
Leaf segments	1	0	0	0
	2	0	0	0
	3	0	0	0



(a) (b) (c)

Figure 1. Hairy roots induced from the cotyledon (a), hypocotyls (b) explants after 21 days infection by *A. rhizogenes* R-1000 and cotyledon explants (c) after 21days infection by *A. rhizogenes* ATCC 15834 on hormone-free MS medium

Even though, the highest infection frequency (60%) on cotyledon was obtained by infection with strain R-10000 , however the highest number of roots induced was obtained on the cotyledonery explants which was infected by *A. rhizogenes* strain ATCC 15834 (Fig. 1). Therefore further investigation was performed with the hairy roots which induced by *A. rhizogenes* strain ATCC 15834.

In this experiments, the differences in efficiency of hairy roots which are induced on cotyledons, hypocotyls, and leaves might be caused by the differences of endogenous hormones concentration in different tissues and/or different stages of growth. In these experiments, cotyledons tissue seems more susceptible to infection by strain R-1000, ATCC 15834 and A4 of *A. rhizogenes* rather than hypocotyls and leaves segments. Possibly, cotyledonery explants has appropriate amount of auxin for root inductions when infected by *A. rhizogenes*. This finding consistent with the report of [15] which mentioned that appropriate amount of auxin in plant tissues was required for the formation and growth of hairy roots.

Duration of co-cultivation is also an important factor for genetic transformation by *A. rhizogenes*, since the integration of T-DNA into the plant genome occurs within a certain period of time, and depending on the genotype of explants and bacterial strains [16]. The result indicated that the highest efficiency of hairy roots induction on cotyledons was attained in 2 days co-cultivation, while the highest efficiency of hairy roots induction on hypocotyls was attained in 2 or 3 days co-cultivation. [17] also showed that co-cultivation time with *Agrobacterium* needed was 2 to 3 days. Increasing the duration of co-cultivation from 72 hours negatively affected the explants. Affected areas in explants became black, and overall, the explants became soft and lost their regeneration capacity [18]. Moreover, the longer duration of co-cultivation frequently resulted in *Agrobacterium* overgrowth.

B. Hairy Root Culture A. paniculata

Culture of the hairy root which derived from cotiledonary explants that infected for two days by *A. rhizogenes* strain ATCC 15834 showed better growth in liquid half-strength MS medium than in full- strength MS medium. In these experiments, hairy roots culture could grow on hormone free medium but it was not optimum. The optimum culture conditions can be obtained by adding of plant growth regulator, such as indole butyric acid (IBA), the most commonly plant growth regulator used for roots growth. IBA was the most efficient plant growth regulator in stimulating hairy roots growth and branching of Coffee [19]. Thus the medium for rapid growth of hairy roots of *A. paniculata* has been optimized by adding IBA.

The highest growth of the roots was obtained in the addition of 5.0 µM IBA (0.0735 ± 0.0153 g dry weight) on the weeks-5, followed by the addition of 0.5 µM IBA which generate 0.0429 ± 0.0012 g dry weight after 3 weeks of culture, and the lowest growth was observed in the medium with no addition of IBA i.e. 0.0463 ± 0.0010 g dry weight after 5 weeks of culture (Fig. 2). Overall results showed that the highest hairy root growth was obtained in liquid half-strength MS medium with addition of 5.0 µM IBA. Most of the hairy roots that culture in the liquid half-strength MS medium showed

fast growth and high lateral branching (Fig. 3). This result was consistent with previous study that the addition of IBA could stimulate the formation and growth of lateral roots and root primordial because auxin (IBA) could stimulate pericycle cells to divide [20]. In this experiments the hairy roots culture which was established in liquid half strength MS medium grew better with addition of 5.0 µM IBA (Fig. 3C) compared to the addition with 0.5 µM IBA (Fig. 3B) and with no addition of IBA (Fig. 3A). Similar phenomenon also reported in hairy roots culture of *Pueraria phaseoloides* [21].

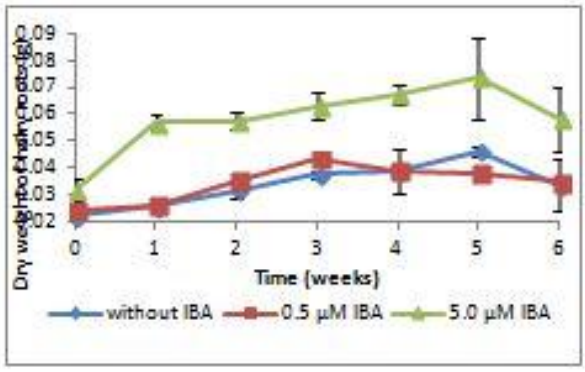


Figure 2. Growth curve of hairy roots of *A. paniculata* in ½ MS medium with addition of 0.5 µM; 5.0 µM IBA and without addition of IBA

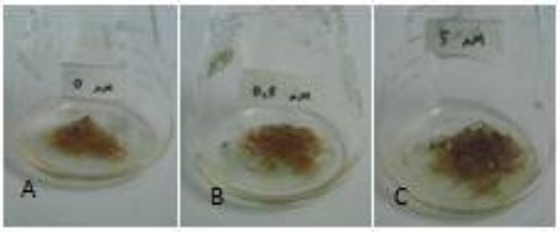


Figure 3. Hairy roots culture of *A. paniculata* in liquid half –strength MS medium without IBA (A), with 0.5 µM IBA (B), and with 5.0 µM IBA (C)

C. Production of Andrographolide in the Hairy Root

The hairy root cultured in liquid half strenght MS medium with and without addition of IBA were harvested weekly within 6 weeks period and extracted for determination of andrographolide contents with HPLC. The results indicated that andrographolide was detected in the hairy root which was cultured in liquid half-strength MS medium with or without IBA. The maximum andrographolide content in the hairy root culture without addition of IBA (control) was attained after 3 weeks of culture (2.35 mg/g dry weight), or equivalent to 0.23% dry weight. The maximum andrographolide content in hairy root culture with addition of 0.5 µM IBA was also attained after 3 weeks of culture (2.14 mg/g dry weight), or equivalent to 0.21% dry weight, while in medium with addition of 5.0 µM IBA was attained after 2 weeks of culture (5.45 mg/g dry weight), or equivalent to 0.54% dry weight (Fig. 4). The addition of 5.0 µM IBA on the medium culture showed the highest amount andrographolide compared to the to the medium culture with the addition of 0.5 µM IBA or with no addition of IBA. Increasing amount of andrographollide following the addition of IBA might be related to auxin ability that can increase HMG-CoA reductase (HMGR) activity. HMGR is one of the key enzymes in andrographolide accumulation [22].

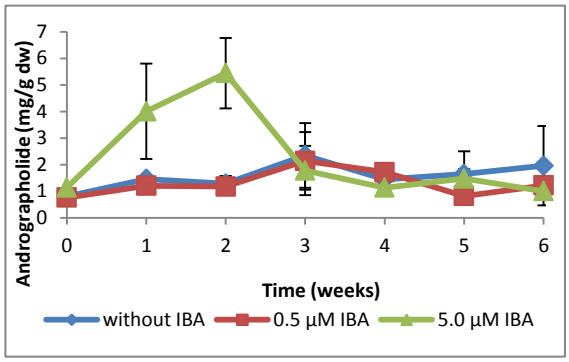


Figure 4. Andrographolide content in hairy roots culture in ½ MS medium with addition of 0.5 µM; 5.0 µM IBA and without addition of IBA

D. PCR Analysis of Transformed Hairy Root

Analysis of PCR product, which was amplified with *rol*A and *rol*C primers, on agarose gel showed the presence of the fragments with lengths of 248 bp and 490 bp in which corresponds to *rol*A and *rol*C genes from Ri plasmids of ATCC 15834. These fragments were only found in DNA samples isolated from the hairy roots but not found in untransformed roots (Fig. 5). These results indicated that the *rol*A and *rol*C genes from Ri plasmids of *A. rhizogenes* strain ATCC 15834 were integrated into the genome of *A. paniculata* hairy roots. Similarly, [23] reported that fragments corresponds to particular genes from Ri plasmids of *A. rhizogenes* only presence in transformed hairy root.

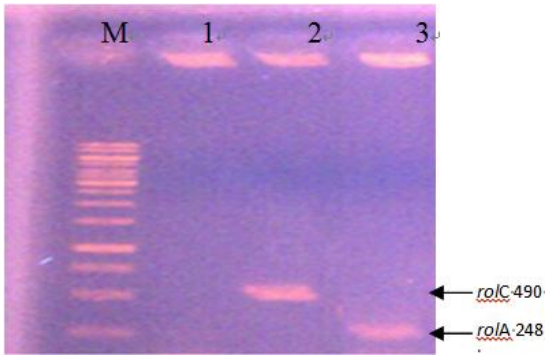


Figure 5. PCR analysis of the hairy roots of *A. paniculata* shows the fragments with lengths of 248 bp and 490 bp in which corresponds to *rol*A and *rol*C genes from Ri plasmids of ATCC 15834 (lane M:marker, lane 1: untransformed root, lane 2: *rol*C, lane 3: *rol*A)

IV. Conclusion

Efficiency transformation *A. paniculata* for hairy roots induction was affected by type of explants and duration of co-cultivation. The best explant for inducing hairy roots of *A. paniculata* was cotyledons, and most efficiently in 2 days of co-cultivation. The addition of

IBA into hairy roots medium culture could enhance the root growth and content of andrographolide. Concentration of IBA that promote the fastest growth and highest content of andrographolide was 5.0 µM. Polymerase chain reaction demonstrated the presence of two fragments with lengths of 248 bp and 490 bp which corresponds to *rol*A and *rol*C genes from Ri plasmids of ATCC 15834. These findings proved that T-DNA of *A. rhizogenes* was integrated into the genome of *A.paniculata* hairy roots.

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