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Induction of Hairy roots of Pegagan (Centella Asiatica (l.) Urban using Several Explant Sources with Several Agrobacterium Rhizogenes Strains in Vitro

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***Abstract*— Induction of hairy roots of pegagan using A. rhizogenes strains is important to obtain high production of triterpenoid as secondary metabolite. The objectives of this research were to obtain the best A. rhizogenes strains and the best explant sources for C. asiatica hairy root induction. The research consisted of two stages. Stage I was A. rhizogenes arranged in Completely Randomized Design (CRD) with 3 treatments and 6 replications. As a treatment was inoculation with A. rhizogenes strains LBA 9457, A4, R1000 and control. Stage II was explant sources trial arranged in CRD with 3 treatments and 6 replications. The treatments were: internodus, apical bud and leaves. The results showed that all three strains of A. rhizogenes were able to induce C. asiatica plant to produce hairy roots, with R1000 as the best strain.The best explant source was explant leave with 15 day after inoculation and the percentage of C. asiatica hairyroot formation was 100 %. The β-glucoronidase (GUS) test result has proven that Ri plasmid T-DNA of A. rhizogenes was integrated into the genome of C. asiatica plant.**

***Keywords*— Agrobacterium rhizogenes, Centella asiatica (L.) Urban, hairy root.**

INTRODUCTION

Pegagan (Centella asiatica (L.) Urban belongs to the family of Umbelliferae. This plant produces secondary metabolites that have the potential to be developed into drugs of various diseases [1]. Various types of compounds contained in C. asiatica are asiaticoside and madecassic acid which are active ingredient capable of stimulating the production of collagen and protein biosynthesis helpful as a driver for the process of wound healing in humans [2].

This plant contains triterpenoid, secondary metabolites, mainly asiaticoside, asiatic acid and madecassic acid, used to treat bone loss for the olds, increase intelligence in children, youth drug, medicinal skin diseases, for cosmetics, as a potent anticancer drug for ovarian, improve nerve and circulatory disorders, as raw material for drugs useful as anti-dementia and anti-stress [3].

The need for raw materials used as ingredients is increasing along with the increasing need for medication. On the other hand, producing secondary metabolites naturally is still very limited Secondary metabolites production for commercial sources is effectively done by using plant extracts from field. The problem is often the availability of secondary metabolites is low. In addition, the content of secondary metabolites is also influenced by the natural conditions such as geographical factors, climate and pests and diseases [4].

One of the technique to induce higher secondary metabolites production is via in vitro culture [5]. In vitro multiplication is an alternative to get the plants in large quantities [6]. Plant tissue culture is a technique to develop the plant, either in the form of organ, tissues and cells under aseptic in vitro conditions. This technique is based on the totipotensi theory cells in aseptic conditions, the use of artificial culture media with a complete nutrient content and plant growth regulators [7].

Hairy root cultures as a form of tissue culture techniques are the result of transformation using Agrobacterium rhizogenes genetically. Hairy root cultures provide many benefits, among others are having biosynthesis ability as in normal plant roots with the growth rate is much faster than normal roots or equal to the cell suspension cultures [8], and is able to express the formation of metabolite products stable over the period of culture[9]. In some plants, the production of secondary metabolites was higher in hairy roots compared to normal roots of plants or the ones produced by origin plant [10]

Several factors are known to affect the success and efficiency of transformation via Agrobacterium including tissue culture conditions and co-cultivation conditions, the suitability of the bacterial strains and selection of plant tissue as starting materials (explants source) [11]. From a research conducted on two types of quinine (Cinchona ledgeriana and C. succirubra), out of 10 strains tested, only A. rhizogenes strain LBA 9457 were able to induce hairy roots of both types of quinine. Furthermore, from the two types of quinine it is known that the explants derived from apical buds and leaves in vitro is the best source of explants to form hairy roots [12]. Explant source selection is very influential for the success of the hairy root culture. Research conducted by [13] on plants of Patchouli (Pogostemon cablin Benth) showed the best explant to induce hairy root is from the leaves. The results of a study done by [14], on Ophiorrhiza pumila plant, indicated that the best explants to induce hairy roots derived from the buds of shoots.

A research was conducted entitled, "Induction of Hairy Root of Centella asiatica (L.) Urban Using Several Explant Source With Agrobacterium rhizogenes In Vitro. The objectives of the research were to obtain the best strains of A. rhizogenes and the best explant source to induce hairy roots of C. asiatica.

MATERIALS AND METHODS

A. Research Methods

This research was conducted at the Laboratory of Plant Physiology and Tissue Culture, Department of Biology, Faculty of Mathematics and Science, Andalas University, from November 2012 to June 2013. This study consisted of two satges. Stage 1: Trail of several A. rhizogenes strains arranged in a completely randomized design (CRD) with 4 treatments and 6 replications. The treatments were inoculation with A. rhizogenes strain LBA 9457, A4, R 1000 and control (without inoculation). Stage 2: Trial of explant sources arranged in CRD with 3 treatments and 6 replications. The treatments were sources of explants: stolon, apical buds and leaves. The parameters measured were C. asiatica hairy root initiation time, the percentage of living explants, the percentage of emergence of hairy roots on leaf explants, number of hairy roots. Data was analyzed statistically.

B. Working Procedure

A. rhizogenes strain R1000, LBA 9457 and A4, were maintained on agar slant YMB (Yeast Mannitol Broth) before inoculation. Inoculation was carried out following themethods as listed in Noli [12]. Explant of C. asiatica used to induce hairy roots derived from one week old cultured explants in vitro.

For bacterial decontamination, subcultures were performed on the same medium by adding Cefotaxin 100 mg/L. Subcultures were repeated 3 times until they were free of bacteria. Furthermore, explants were planted again on solid medium without cefotaxin.

A trial of β-glucoronidase (GUS) was performed to confirm the integration of T-DNA of Ri plasmid into the plant genome of C. asiatica. Working procedures followed methods of Jefferson [15] .

RESULTS AND DISCUSSION

A. Test of Some Strains of A. rhizogenes on production of hairy roots of C. asiatica

The results of testing several strains of A. rhizogenes in producing hairy roots of C. asiatica plants was performed in Table 1.

TABLE .I
EFFECT OF SEVERAL AGROBACTERIUM RHIZOGENES STRAINS TO HAIRYROOT. C. ASIATICA 30 DAYS AFTER INOCULATION (DAI)

No	Treatment	Initiation time (DAI)	Living explants (%)	Hairy roots emergence (%)
1	Control	-	-	-
2	Strain LBA 9457	24 a	33,33 c	33,33 c
3	Strain A_4	17 b	50 b	50 b
4	Strain R1000	15 b	100 a	100 a

Description: DAI = days after inoculation. The numbers in a column followed by the same lowercase letters are not different according to DNMRT 5%.

Results of A. rhizogenes inoculation on leaf explants of C. asiatica plants after cultures were 30 days old showed that strain R1000, A4 and LBA 9457 were able to induce the formation of hairy roots. Hairy root initiation time, the percentage of living explants and percentage of emergence of hairy root in plants of C. asiatica, transformed with R1000 strain, strain LBA 9457 and A4 strain of A. rhizogenes bacteria were presented in Table 1. It showed that LBA 9457, A4 and R1000 strains could induce the formation of hairy roots, with different initiation time. The fastest initiation time was shown in strain R1000. Explants inoculated with A. rhizogenes R1000 strain of bacteria, hairy roots began to form 15 DAI, while those inoculated with A4 (17 DAI) and LBA 9457 strains, hairy roots began to form 24 DAI. Differences in initiation time of C. asiatica hairy roots on each of the A. rhizogenes strain due to differences in genes on the Ri plasmid entering into the plant chromosome of C. asiatica.

Result of explant inoculation showed that only strain R1000 was able to induce 100% formation of hairy roots of the total replications. The successful formation of hairy roots from inoculated explants was an indication of the transferred of T-DNA from A. rhizogenes Ri plasmid into the genome of cells of C. asiatica. C. asiatica plant is susceptible to infection of A. rhizogenes. The three strains used showed the different responses to the infection of A. rhizogenes. The highest percentage of hairy roots emergence was obtained on treatment inoculated with A. rhizogenes, R1000 strain (100%), in contrast to the control treatment in which hairy roots did not emerge all. Inoculation with A4 strain treatment caused 50% hairy roots emergence and with LBA 9457 was 33.33%. The growth of hairy roots formed from explants of C. asiatica was very different both in number and percentage of emergence of hairy roots on each strain tested.

Plant responses to the induction of T-DNA from A.rhizogenes were different. Some formed callus first, some forms the hairy root and others remained alive without forming hairy roots, and some even had no response at all (dead). According to [16], in several species of plants, hairy

roots emerged directly at the site of inoculation, but in certain species initially appear on the inoculant callus which eventually form hairy root and hairy roots in normal circumstances would appear in the range of 1 to 4 weeks after inoculation.

Leaf explants of C. asiatica plants inoculated with some strains of A. rhizogenes also showed the formation of hairy root response (Figure 1) and the initial response of the explants was hairy root formation and explants that did not form the hairy roots (Figure 1). The growth of the hairy roots of C. asiatica transformed with each strain of A. rhizogenes was very different, especially in the number of hairy roots emerged (Figure 1).

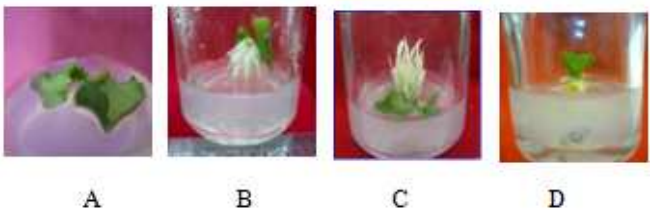

Figure 1. Response of C. asiatica leaf explants inoculated with some strains of A. rhizogenes
A. Control; B. strain A4; C. strain R1000; D. strain LBA 9457

B. Test of different sources of explants of C. asiatica plants transformed with A. rhizogenes bacteria, R1000 strain

The results of tests on several sources of explants producing the hairy roots of C. asiatica transformed with A. rhizogenes were presented in Table 2.

TABLE. II
EFECT SEVERAL EKSPLANS SOURCES WITH A. RHIZOGENES R. 1000 TO HAIRY ROOT

Explant source	Average time of initiation (DAI)	Average growth response		
		Living explants (%)	Hairy roots (%)	Number of Roots
1. Leaves	15 b	100	100 a	27 a
2. Stolon	24 a	100	77,7 b	16,7 b
3. Apical shoots	19,6 a	100	66,7 b	14 b

Remarks: The figures are marked with the same lowercase letters are not significantly different according to DNMRT 5%. DAI = days after

The difference in initiation time due to differences in the response of the plant taken as a source of explants. Explants derived from the leaves need a shorter time for initiation compared to explants derived from the stolon and apical buds. According to [17], the success factors of hairy root culture was on selection of plant tissue used as sources of explant.

The percentage of explants living in all parts of the plantlets used either leaves, apical buds and internodus showed insignificant results. Source of explants showed significant difference in the percentage of living explants. All explant sources can be used to form hairy roots, because after explants with A. rhizogenes transformation was initiated, all explants sources showed growth responses, good survival and the ability to form hairy roots. Percentage of living explants for all explants sources was 100%.

Growth response of C. asiatica transformed with several strains of A. rhizogenes was shown by the emergence of hairy root. The highest response (100%) was on leaf explants which was significantly different from the apical bud and stolon explants. The difference in the emergence of the hairy root might be possible because not all parts of the plantlets were suitable with bacterial isolates, so that not all bacterial T-DNA were integrated into the genome of the plants to form hairy roots. [18] stated that in higher plants there is a fairly large DNA sequences that do not encode active proteins. If the position of the T-DNA inserted in the genome was not random and only integrated plant in the DNA that are not active, then it can not be expressed by the plant.

The difference on the percentage of emergence of the hairy roots was also possible because of differences in age and physiological condition of explants used. Selection of the tissues as the initial material used was one of the most important factors affecting the success and efficiency of transformation. It depends on the age and physiological condition of the tissue. Differences in the ability of explant sources to form hairy roots after transformation with A. rhizogenes also affected the number of hairy roots formed. The average number of hairy roots was 16.7 from stolon explants which was insignificantly different from apical bud explants, 14 but significantly different from the leaf explants, 27. Results of some studies showed differences in the number of hairy roots produced on explants transformed with A.rhizogenes were influenced by the type of bacterial strains inoculating explants .

The highest number of hairy roots formed was on R1000strain of A. rhizogenes treatment with leaf explants was 27 hairy roots and followed by stolon and shoot apical (Figure 2). Based on the average number of hairy roots, hairy root emergence, percentage of living explants and the average of initiation time, it could be stated that inoculation using R1000 strains showed the best results and the best source was leaf explants.

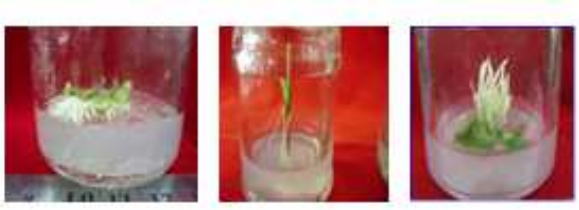

Figure 2. Response of some explants of C. asiatica inoculated with R1000 strain (A. Stolon, B. apical buds, leaf explants of C. asiatica)

Explant competence is determined by several factors, such as species or genotype explant origin, type of organ used as explants, the level of organ development, even history of each explant used.

C. *Integration of Plasmid Ri T-DNA into the genome of plants of C. asiatica (L.) Urban*

GUS test was performed to determine the integration of Ri T-DNA plasmid R1000 strain of A. rhizogenes bacteria into the genome of C. asiatica plants. In the hairy roots of C. asiatica which had been soaked in a solution of X-gluc for 24 h showed the formation of blue spots (Figure 3) in the root cells. This proved that the T-DNA of plasmid was integrated into the plant cell. [13] suggested that the gene w Gus gene was gene reporter that encoded the synthesis of β-glucoronidase, an enzyme that could hydrolyze a colorless substrate 5-bromo-3 indolyl 4 chloro glucuronide (X-gluc) became blue. [19] stated that the expression of β-glucoronidase gene activity (GUS) in transformed plant cells of the T-DNA could be used as an indicator of T-DNA integration into the plant genome. Expression of the cell strains of Agrobacterium infection is shown as blue spots by giving 5-bromo 4-3-indolyl cloro glucuronic acid (X-gluc). [20] explained that the expression of transgenic cells would be clear when analyzed using GUS parameters and could also describe the expression and function of the tissues of the transgene.

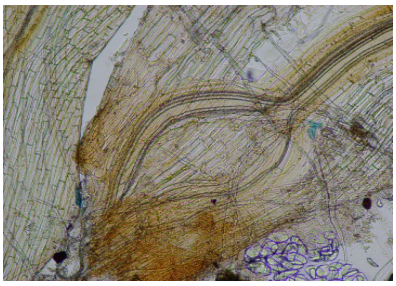

Fig. 3 Cross-section of hairy roots transformed with *A. rhizogenes* with blue color (designated by arrows).

Description: The blue color is the site where the DNA is integrated (as indicated by arrow). From the test results of GUS in the hairy roots it was showed that there was a blue color that covered the whole root or root portion only, or even not visible at all. This was possible because the transformation occured as a whole (cellular) or partially (chimera). [13] research also found the presence of hairy roots after being tested with GUS but blue spots was not formed. The GUS test was called positive if blue spots were formed on the tissues. The thicker and bluer, the higher the GUS gene expression. Other cause of invisible blue spots was possibly because the hairy roots was taken randomly, so it was possible that the hairy roots cut in GUS test was not part of the tissues of integrated with T-DNA.

CONCLUSIONS

The best strains of A. rhizogenes to induce hairy roots of C. asiatica was R1000 strain. Leaf explants was the best source of explants in inducing hairy roots.

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REFERENCES

[1] Maksum, R.. Peranan Bioteknologi dan Mikroba Endofit dalam Pengembangan obat Herbal.Majalah Ilmu Kefarmasian. Departemen Farmasi, Fakultas Matematika dan Ilmu Pengetahuan Alam (FMIPA) Universitas Indonesia. Depok. LI(3) : 437-441. 2005.

[2] Duke, J. A., M. J. B. Godwin, J. Du Cellier and P. A. K. Duke. 2002. Handbook of Medicinal Herbs. Second edition. CRC Press London-New York. Pp. 344-346.

[3] Nur K. K, A. Hawariah L. P. and Azizol A. K., Preliminary sraaning of antiproliferatve activity of selected extracts of Centella asiatica. Proceedings of the Seminar on Medicinal and Aromatic Plants. FRIM, 12-13 September 2000. Kuala Lumpur, Malaysia

[4] Lorence, A. F. Medina-Bolivar dan C. L. Nessler. . Camptothecin and 10-Hidroxycamptothecin from Camptothecin and 10-Hidroxycamptothecin from Camptotheca acuminate. Plant Cell Report. 22 (6): 437-441.2003

[5] Ling, P. K. A. . Triterpene Production In Centella asiatica (L.) Urban (Pegagan) Callus and Cell Suspension Cultures. PhD. Thesis, University Putra Malaysia.2004

[6] Nova, K. N. dan S. . Multiplikasi Tunas dan Aklimatisasi Pegagan (Centella asiatica L. Urban). Periode Kultur Lima Tahun. Jurnal Littri Vol. 14 No. 1, Maret 2008: 30-35.2008

[7] Yusnita. . Kultur Jaringan Cara Memperbanyak Tanaman Secara Efisien. Agromedia Pustaka: Jakarta. 2003

[8] Flores-Sanchez I. J., Ortega-Lopez J., Montes-Horcasitas M. C., Ramos-Valdivia A. C. . Biosynthesis of sterol and triterpenes in cell suspension culture of Uncaria tomentosa. Plant Cell Physiol. 43(12): 1502-1509.2002

[9] Rhodes M. J. C., Robins R. J., Hamiel A.J., Hilton M. G., Walton M.J. 1990. Poperties of transformed root cultures. London: Clarendon Pr: 201-205

[10] Ermayanti, T., M. L. Sari, E. M. R. Siregar dan D. Sudrajat. . Transformasi Mimba (Azadirachta indica A. Juss.) dengan Agrobacterium rhizogenes Galur ATCC-15834. Prosiding Seminar Nasional Bioteknologi Pertanian. Yogyakarta, 7-9 November 2000.

[11] Giri, A. and M. L. Narasu. . Transgenic Hairy Roots: Recent Trends and Applications. Biotechnology Advances. 18: 1-22.

[12] Noli, Z. A. Pertumbuhan dan Produksi Alkaloid Kinolina dari Kultur Akar Rambut Kina (Cinchona ledgerina Moens dan C. succirubra Pavon ex Klotzsch) Hasil Transformasi ZAgrobacterium rhizogenes Galur LBA 9457. Disertasi. Universitas Padjadjaran. Bandung. 2004

[13] Herlina, N. D. . Kepekaan Nilam (Pagostemon cablin Benth) terhadap infeksi Agrobacterium rhizogenes. Tesis. Jurusan Biologi. Institut Pertanian (IPB). Bogor. H. 2002.

[14] Watase. Regeneration of Transformed Ophiorrhiza pumila Plants Producing Camptothecin. Plant Biotechnology 21 (5): 337-342.2004

[15] Jefferson, R.A. . Assaying chimeric genes in plants: The GUS gene fusion system. Plant Molecular Biology Reports, 5, 387-405.1987

[16] Gunawan, L. W., N. A. Mattjik, E. Sjamsudin., A. Wiendi dan A. Emawati.. Bioteknologi Tanaman. Tim Laboratorium Kultur Jaringan Tanaman. Pusat Antar Universitas Bioteknologi (PAUB). Institut Pertanian Bogor (IPB). Bogor. 1991

[17] Gelvin, S. B. Agrobacterium Mediated Plant Transformation. The Biology behind the Gene- Jockeying Tool. Microbial. Mol. Rev. 67 (1): 37 .2000

[18] Toruan–Mathius, N. Pemanfaatan Teknologi in vitro Untuk Memproduksi Metabolit Sekunder. Seminar Sehari Peranan Fisiologi Dalam Menunjang Produksi dan Integritas Ekosistem. FMIPA Biologi IPB, Bogor. Tanggal 17 April 2003.

[19] Maysore, K. S., Burgund B., Xiao-bing D., Nune S. D. Andrel M., Walt R., and Stanton B. G. . Role of the Agrobacterium tumefacient Vir D2 Protein in T-DNA Transfer and Integration.MPMI 11(7): 668-683..1998

[20] Touminent, H., P. Laurence, R. Sharon, F. Sieghfried, O. Olof dan S. Bjorn. Cambial- Region Specific Expression of the Agrobacterium IAA Genes in Transgenic Aspen Visualized by a Linked vid A Reporter Gene. Plant Physiology. 12.