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Various Infection Time of *Agrobacterium rhizogenes* Strain LB510 for Hairy Root Induction on *Justicia gendarussa* Burm.f.

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Abstract : This study aimed to determine the effect of various infection time *Agrobacterium rhizogenes* strain LB510 towards hairy root induction on *Justicia gendarussa* Burm.f. leaf explants. Various infection time are 10, 20, 30, 40, 50, and 60 minutes. Explants co-cultivated for 2 days in free-hormone solid medium, then transferred to solid MS0 medium supplemented with 300 ppm cefotaxime antibiotics. Culture incubated without light. Various data were collected, namely transformation efficiency, root formation duration, amount and length of hairy root. Observation conducted every week for 6 weeks. Data analyzed statistically using Kruskal-WallisTest and followed by Mann-Whitney Test. Result shows that on each treatment of *Agrobacterium rhizogenes* strain LB510 infection duration influence the formation of hairy root from gandarusa leaf explants. On this study, 10 minutes is the best infection time in order to induce hairy root with highest transformation efficiency (100%), 2 hairy roots formed, the lenghtof hairy root are 3.18 cm, and hairy root formation in 14 days.
Keywords : *Agrobacterium rhizogenes* strain LB510, hairy root, infection time,*Justicia gendarussa* Burm.f..

Introduction

Gandarusa is used as a male contraceptive by society Sentani, Irian Jaya[1]. Gandarusa compound that serves as antifertilitas ingredient is flavonoids[2].Utilization of gandarusa massively confronted with problems of availability of raw materials, because these plants have not been cultivated. The flavonoid compundis produced in small amounts, so we need a tissue culture techniques. One approach technology that can be used to increase the content of plant secondary metabolites[3].

Gandarusa secondary metabolites can be produced by the hairy root culture. The advantages of hairy root culture such as relatively fast growth, productive and stable in generating the desired compound[4]. The growth of hairy root can be optimized by inoculating *Agrobacterium rhizogenes*[5,6]. *Agrobacterium* is a type of soil bacteria that have the ability to transfer T-DNA of the Ri plasmid (root inducing plasmid) into plant cells through wounding[7].

The success of the infection and gene transfer by *Agrobacterium*, determined by the type and condition of explant, presence or absence of explant wounding, the density and infection time of bacteria, and

cocultivation length time[8,9,10]. The infection time also determines the success of a bacterial infection of the soybean crop explants[11].The 5 minutes was the best infection time to hairy roots induction on *Talinum paniculatum*[12]. The research was carried out by Lokhande showed that 10-20 minutes infection time could improve significantly the average efficiency transformation of *Agrobacterium rhizogenes* on *Withania somnifera* L.[13]. Previous research on the culture of hairy root on gandarusa has not been widely reported .

In previous studies have successfully performed the induction of hairy root on the leaves of *Justicia gendarussa* Burm.f. using various strains of *Agrobacterium rhizogenes*, which the best strain is strain LB510[14]. The next stage is to find the optimum conditions between the density and the infection time to obtain the transformation efficiency optimum.The infection time correlated with the density of *Agrobacterium*[15]. Therefore, this study was conducted to determine the effect of the infectiontime of *Agrobacterium rhizogenes* strain LB510 to the hairy root induction on gandarusa leaf explants.

Experimental

Materials used are leaf explants *Justicia gendarussa* Burm.f. which obtained from the Institute of Materia Medika Batu Malang. *Agrobacterium rhizogenes* strain LB510 was obtained from the Research Center of Biotechnology, Indonesian Institute of Sciences (LIPI), Bogor, Indonesia. The chemicals used are alcohol 70 %, 1N HCl, KOH 1N, Clorox 10 %, distilled sterile, spritus and chemicals of Murashie and Skoog (MS) medium[16], Luria Bertani(LB)medium (10g Tryptone, 5g yeast extract and 10g NaCl/l), and the antibiotic cefotaxime.

Sterile explant was cut with a size of approximately $1cm^2$. Six pieces of sterile erlenmeyer prepared for the transformation of the media's treatment, containing liquid MS medium which has been added a bacterial suspension of *A. rhizogenes* with OD600 = 0.1. The explants were put in a sterile erlenmeyer containing media transformation and shaked with a wide range of time (10 / P1, 20 / P2, 30 / P3, 40 / P4, 50 / P5, and 60 minutes / P6).

Furthermore explants grown in a MS solid medium for 2 days, dark incubation, at room temperature of 25^oC. Each treatment were repeated 10 times. Co-cultivation was carried out for 2 days, then explants were rinsed with sterile liquid MS medium containing cefotaxime. The explants were planted in MS medium containing cefotaxime 300 ppm. Observations was carried out for 6 weeks. The parameters werehairy root length, number of explants that formed the hair roots, number of hairy roots, and the length of formation time of hairy roots.

Data of this research were a quantitative and qualitative data. Qualitative data was hairy root morphology (structure, texture, color and growth starting position of hairy root). that were analyzed descriptively. Quantitative data were the number and length of hairy root, that were analyzed using Kruskal-Wallis Test followed by Mann-Whitney Test level of 5 % .

Result and Discussion

The TransformationEfficiencyand TheHairy Roots Formation Time

The treatment of 10 minutes, 40 minutes, and 60minutes has highest tranformation efficiency (100%), follow by 20 minutes infection time (80%), while 30 minutes infection time (50%), and the lowest tranformation efficiency obtain for 50 minutes infection time (30%). The negative control, hairy root formation is not observed.

The growth of the hairy roots from the explants is an indication of the transfer of T - DNA derived from *A. rhizogenes*(Ri plasmid) into the genome of a plant cell gandarusa . *Agrobacterium* is a bacteria that has the ability to transfer T-DNA plasmid, known as Ri plasmid (root inducing plasmid) into plant cells through wounding[7]. T-DNA will be integrated into the plant chromosome and will express the genes to synthesize compounds opine. The resulting compound opine serves as a provider of nutrients for bacteria (*Agrobacterium*)[17] .

Table 1.The transformationefficiencyand the length of hairy roots formation time

Treatment	Repetition	The number of eksplan can form hairy root	Transformasi Efficiency (%)	Length of hairy roots formation time (days)
P0 (control)	10	0	0	-
P1 (10minutes)	10	10	100	21,48 ± 6,37[a]
P2 (20 minutes)	10	8	80	25,21 ± 7,26 [a]
P3 (30 minutes)	10	5	50	21,20 ± 5,89[b]
P4 (40 minutes)	10	10	100	21,55 ± 3,50[b]
P5 (50 minutes)	10	3	30	27,66 ± 6,35[c]
P6 (60 minutes)	10	10	100	26,20 ± 4,66[c]

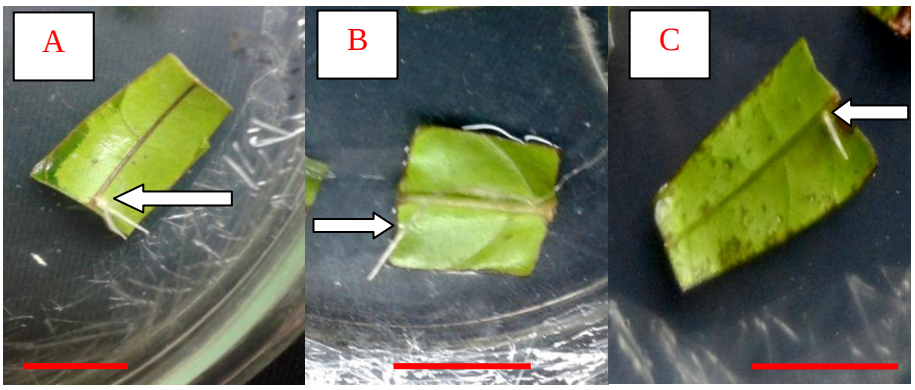

Figure 1. (A) Hairy roots appears at the edge of the midrib, Treatment of 10 minutes infection time in 13th days observation. (B) Treatment of 40 minutes in 22nd days observation; and (C) Treatment of 20 minutes infection time in 24thdaysobservation. Bar = 1 cm

In addition, T - DNA also contain oncogenes are genes that play a role to encode growth hormone auxin and cytokinin. Oncogene expression on plasmid Ri is characterized the formation of adventitious roots on a large scale in places infected and is known as hairy root[7].

The difference of thewillow leaf explantsresponse to *Agrobacterium* infection is caused the difference ofexplant genotype[18]. This is supported by Potrykus that there are several factors that determine the competence of explants to form hairy root , such as explant genotype, type of organ, organ development levels, historical even of each explants were used[19]. Besides the explant response, the ability of *Agrobacterium* to induce hairy root in plants, such as density of bacteria, inoculum immersion time, and the *Agrobacterium* strain[20].

The length of hairy root formation time is various. Hairy roots begin to form in the range of 14-38 days . The treatment of 10 minutes infection time is fastest, on the 2nd week. This result differs from previous studies conducted by Anekawaty, that the effect of inoculum *A. rhizogenes* strain LB510 to hairy root induction with various density of OD_{600} 0.1; 0.2; and 0.3 in explant *Catharanthus roseus*,only forming callus at 16 weeks of culture[21].

This indicates that the same strain infected to different crops there will be a difference in the length of time the formation of hairy root. Hairyroot are formed in a short period of time, varying from one week to more than a month, it depends on the difference in plant species[17]. A similar study in leaf explants gandarusa treatment of 40 minutes infection time by strain YMB 072 001 is capable of forming hairy roots on 15thdays and strain A4T capable of forming hairy root on 27thdays with a time of infection 10 minutes[22] but differ in *Talinum paniculatum*[23]. *A. rhizogenes* strain LB510, treatment of OD_{600} = 0.1 for 20 minutes[24]. This is indicates that the *Justicia gendarussa* Burm.f. more suitable if transformed by *A. rhizogenes* strain LB510, for 10 minutes.

The infection time correlated with the density of *Agrobacterium*, therefore it is necessary to find the optimum conditions between the density and the infection in order to obtain maximum transformation efficiency. The density of bacteria low and the duration of infections are short will lead to low efficiency of transformation, but the density of bacteria is high and the duration of infections is long will result in the growth of bacteria that are not controlled (overgrowth) that will contaminate explants and than explant can not grow and die finnaly[15].

The number of hairy roots

The various infection time is affected the number of hairy roots. The treatment 10 minutes infection time has highest average number of hairy root (2 hairy roots), follow by 40 minutes (1.7), 20 minutes (1.2), 50 minutes (0.7) and the lowest is 30 minutes (0.5). The negative control, hairy root formation is not observed (Figure 2).

These results are contrast to these studies that the hairy root induction gandarusa by the strain YMB 072 001 can produces 5 hairy roots by the treatment of OD_{600} = 0.3 for 20 minutes infection time, whereas A4T formed one hairy root by the treatment of OD_{600} = 0.2 and 0.5 for 20 minutes[14]. The treatment *Agrobacterium* strains R1601 produces 5.3 hairy rootsin *Rubia akane* Nakai[9] and the study showed that the OD_{600} = 0.1 had the highest average number of hairy roots (2.13 hairy roots) for 10 minutes infection time[23].

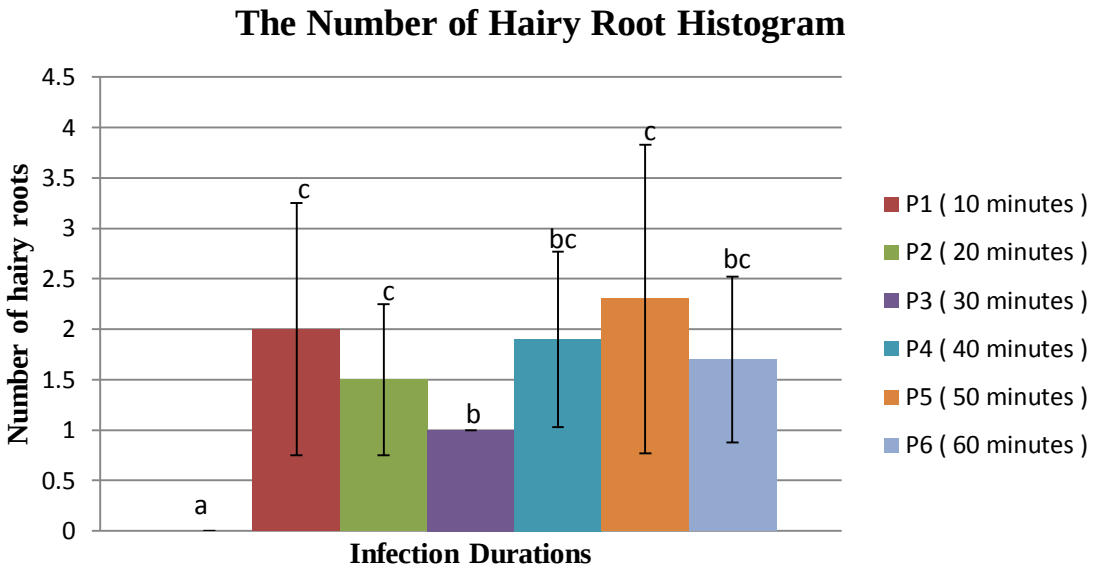

Figure 2.The average number of hairy roots formed on explants *Justicia gendarussa* Burm.f. (The difference letters above diagram shows a significant difference from the results by Mann-Whitney Test at significant level 5%).

There is not hairy root in negative control, caused due to the time of immersion of explants in the bacterial suspension for too long causing bacterial infection too much so the plant tissue to be stressful and can not grow[25]. Another factor that led to explant can not form the hair roots as explants can not remove phenolic compounds in an amount sufficient to stimulate bacterial chemotaxis to be easily attached to the plant cell[14].

The length of the hairy roots

Treatment of infections time affected the hairy root length. Lenght of hairy roots is varying for each treatment (Figure 3). The average length of the hairy roots is highest for 10 minutes infection time (3.18 cm), followed by 60minutes infection time (0.8 cm), 30 minutes (2.99cm), 40 minutes (2.83 cm), 20 minutes (2 ,4 cm), and 50 minutes (1.08 cm).

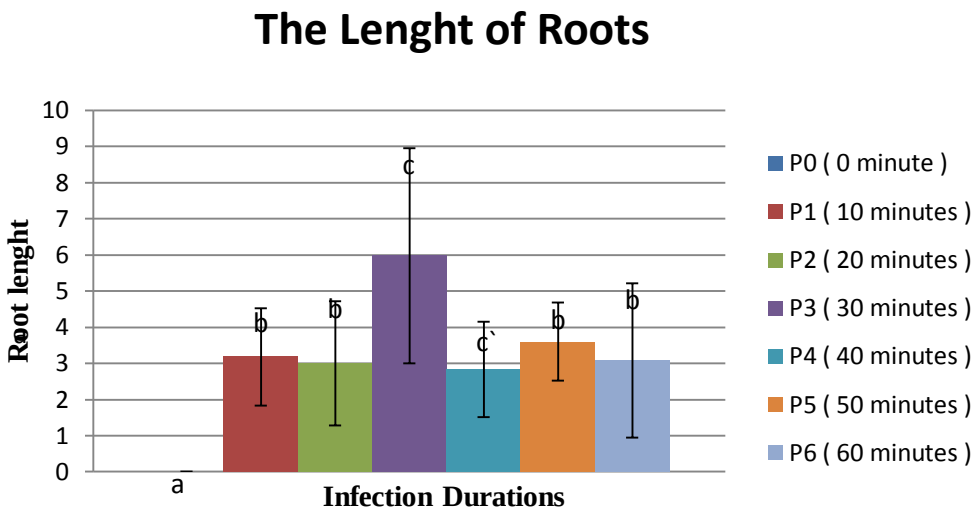

Figure 3. The average length of hairy roots formed on explants *Justicia gendarussa* Burm.f. (The difference letters above diagram shows a significant difference by Mann-Whitney Test at significant level 5%)

Some hairy roots can continue to grow, but there are not others gaining long hairy root meaning. The hairy roots do not grow, gradually becomes brown and eventually die. The average length of the hair roots is highest in the 10minutes treatment (3.18 cm). This is in contrast with previous studies that the hairyroots are formed on the explants *Justicia gendarussa* Burm.f. induced by strain LB510 is longer than the hairy roots induced by other strains (LB509, YMB 072 001, A4T, and ATCC 15834) for 20 minutes[22].

Morphology of Hairy Root

In the 10minutes treatment, the explant growth curve in the first week, in the second week hairy root begin formed and at the end of the observation portion leaf explants brown, brown leaf explants edge and grow roots. Roots were formed on each explant ranged 1-5, branched and branches have the texture of hair root (Figure 4). In the treatment of 20, 40, 60 minutes have the variousthe number of hairyroot. Hairyroot are formed in white, appeared most of the leaves and bones are also in the area around wounding. When the roots grow first, towards growing up or growing in media surface and then grow toward the bottom or base to grow through the media (Figure 4). In the treatment of 30 and 50 minutes every 1-2 explants only capable of forming roots of the hair, white. The first hairy roots grow very long and branched, but both short hairy roots (Figure 4). Overall the hairyroot are formed have fine hairy root on the surface, the base of the hairy root has a thicker texture and root ends of the taper. On the negative control treatment, whole explants not form the hair roots from the first week to the sixth week, but there is growing callus on midrib and around the edges of the leaves are bruised. Callus that formed the white and has a callus types friabel and begin to form in the 2nd week of the culture period (Figure 4).

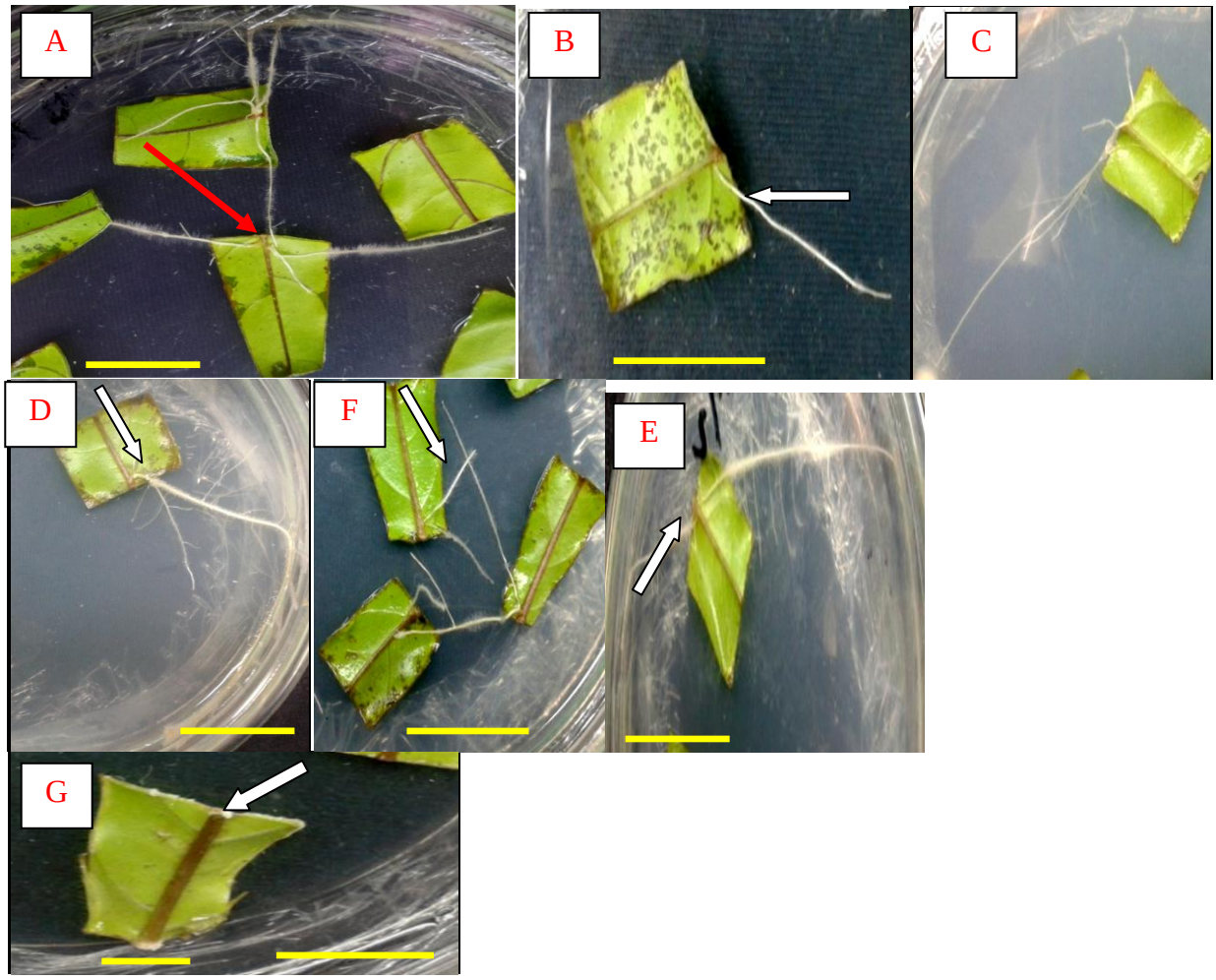

Figure 4. Hairy root of *Justicia gendarussa* Burm.f. (A)10 minutes infection time at 29th days observation; (B) 20 minutes infection time at 34 days observation; (C) 30 minutes infection time at 42nd days observation;(D) 40 minutes infection time at 42nd days observation; (E) 50 minutes infection time at 42nd observation; (F) 60 minutes infection time at 36th days observation; (G) negative control at 38th observation.Bar = 1 cm.

Hairyroot are formed on gandarusa leaf explants is the result of genetic transformation of bacteria *A. rhizogenes* strain LB510 with genomic DNA of plant cells gandarusa. The cells were successfully transformed plants will increase the sensitivity to auxin and has the ability to produce auxin, causing the formation of hairy roots[26]. This can be justified because during the period of cultivation on MS medium capable of forming hairyroot branches .

Based on the results of this study indicate that various infection time of *A. rhizogenes* strain LB510 effected on the hairy root induction on leaf explants *Jucticia gendarussa* Burm.f . The best infection time is 10 minutes. This is indicated by the value of the transformation of 100 %, the number of hairy roots formed the most explant (on average 2 hairy roots per explant), the longest hairy root (3.18 cm), and fastest time in inducing hairy roots (14 days).

The time variation of infection determines the success of genetic transformation *A. rhizogenes* to that plants are able to produce hairyroot. Time of infection is determined by the type of plant and the method of transformation. In *Talinum paniculatum* by way shaken at 30 rpm for 5 minutes[12], the *Withania somnifera* L. manner shaken slowly for 10-120 minutes[13], on *Fagaricum tataricum* by immersion for 10 minutes[27], on *Theobroma cacao* by soaking for 30 minutes[28], on *Potulaca oleracea* by soaking for 20 minutes[29]. For further

research is recommended to test the effect of the transformation method *A. rhizogenes* transformation efficiency in plants *Justicia gendarussa* Burm f ..

References

1. Prajogo BEW, Guliet D, Queiroz EF, Wolfender JL, Cholies NZ, Aucky H, Hostettmann. Isolation of male antifertility compound in N-Butanol fraction of *Justicia gendarussa* Burm f. leaves. Folia Medica Indonesia, 2009, 4(1): 28-31.
2. Braidot E, Zancani M, Petrussa E, Peresson C, Bertolini A, Patui S, Macri F, Vianello A. Review : Transport and accumulation of flavonoids in gravapine (*Vitis vinifera* L.). Plant Signaling & Behavior,2008, 3(9);626- 632.
3. Fowler MW, Stafford AM, Plant cell culture: process system and product synthesis. in FowlerMW, Moo-YoungM. Plant Biotechnology. Oxford, New York:*Pergamon Press*, 1991; 79-95.
4. Ernawati A. Produksi senyawa-senyawa metabolit sekunder dengan kultur jaringan tanaman. 1992; 169-208. Dalam: WattimenaG.A. MattjikN.A. SyamsudinE. WiendiN.M.A. and ErnawatiA.,Bioteknologi. Departemen Pendidikan dan Kebudayaan. Direktorat Jenderal Pendidikan Tinggi:*Pusat Antar Universitas*, Institut Pertanian Bogor.
5. Ercan AG,Taskin KM.*Agrobacterium rhizogenes*-mediated hairy root formation in some *Rubia tinctorum* L. population growth in Turkey. Tr. J. Bot, 1997,23; 373-377.
6. Hooykaas PJJ,*Agrobacterium*, anatural metabolic engineer of plants.in: Verpoorte R, AlfermannAW. Metabolic engineering of plant secondary metabolism. Netherlands:Kluwer Academic Publishers,2000; 51-67.
7. Nillson O, Olsson O. Getting to the Root: the role of The *Agrobacterium rhizogenes* rol genes in the formation of hairy roots. Physiol Plant, 1997, 100; 463-473.
8. Ermayanti TM, Al Hafizh E, Rahmawati S, Aryanti. Transformasi *Artemisia cina* dan *Artemisia annua* dengan *Agrobacterium rhizogenes*. Journal of Applied and Industrial Biotechnology in Tropical Region**,** 2009,2(2); 1-5.
9. Lee SY, Kim SG, Song WS, Kim YK, Park N I, Park SU. Influence of different strains of *Agrobacterium rhizogenes* on hairy root induction and production of alizarin and purpurin in *Rubia akane* Nakai. Romanian Biotechnological Letter, 2010, 15; 5405-5409.
10. Swain SS, Sahu L, Pal A, Barik DP, Pradhan C, Chand PK. Hairy root culture of butterfly pea (*Clitoria ternatea* L.): *Agrobacterium rhizogenes* x plant factors influencing transformation. World J Microbiol Biotechnol, 2011, 28; 729-739.
11. Pardal SJ, Wattimena GA, Aswidinnoor H, Herman M, Listanto E, Slamet. Transfer gen *Proteinase Inhibitor II*padakedelaimelaluivektor*Agrobacterium tumefaciens*untukketahanan terhadaphama penggerekpolong (*Etiellazinckenella* Tr.).JurnalBioteknologiPertanian, 2004, 9(1); 20-28.
12. Manuhara YSM, Kristanti AN, Utami ESW, Yachya A. Effect of sucrose and potassium nitrate on biomass and saponin content of *Talinum paniculatum* Gaertn. hairy root in ballon type bubble bioreactor. Asian Pac J Trop Biomed, 2015, 5(2); 1027-1032.
13. Lokhande VH, Kudale S, Nikalje G, Desai N. Hairy root induction and phytoremediation of textile dye, reactive green 19A-HE4BD, in a halophyte, *Sesuvium portulacastrum* (L.) L. Biotechnology Reports, 2015, 8;56-63.
14. Wahyuni DK, Vidianti F, Purnobasuki1 H, Ermayanti TM, Prajoga B, Utami ESW.*Agrobacterium rhizogenes* mediated hairy root induction in *Justicia gendarussa* Burm.f. J. Appl. Environ. Biol. Sci.,2015a, 5(4); 87-93.
15. Manuhara YSW. Kultur jaringan tumbuhan.Airlangga University Press.Surabaya, 2014.
16. Murashige T, Skoog F.A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologi Plantarum, 1962, 15: 437-497.
17. Hu Z,Du M. Hairy roots and itsapplication in plantgeneticengineering.Journal of Intergrative Plant Biology,2006, 48(2); 121-127.
18. Mathius NT, Reflini, Haris N, Santoso J, Roswiem AP. KulturAkarRambut*Cinchona ledgeriana*dan*C. succirubra*dalamKulturIn Vitro. Menara Perkebunan, 2004, 72(2); 72-87.
19. Potrykus I. Gene transfer to plants:Assessment of published approachesand result. Ann. Rev. Plant. Phisiol.Plant. Mol. Biol., 1990, 42: 205- 225.

20. Lizawaty, Roedhy, Sobir, Tri. Pertumbuhanbibittanamanmanggis (*Gaciniamangostana* L.) setelahinokulasidenganberbagaigalur*Agrobacteriumrhizogenes*.BuletinAgronomi.2007, 35(2); 127-134 .
21. AnekawatyR. Pengaruh konsentrasi inokulum*Agrobacterium rhizogenes*terhadaptingkatkeberhasilan induksi akar eksplan daun *Catharanthusroseus*(L) G. Don. Skripsi. Surabaya: Departemen Biologi, Fakultas Sains dan Teknologi, Universitas Airlangga.2011.
22. Wahyuni DK, Nisa Q, Purnobasuki H, Ermayanti TM, Prajoga B, Utami ESW.Induksi akar rambut gandarusa (*Justicia gendarussa* Burm. f.) dengan perlakuan perbedaan lama waktu infeksi *Agrobacterium rhizogenes* strain YM072001 dan A4T. Jurnal Bioslogos, 2015b, 5(2): 38-45
23. Sukmawati.Pengaruhkonsentrasidanlamawaktuinokulasi *Agrobacterium rhizogenes* strain YMB 072001 terhadapefisisensiinduksiakareksplandaunginsengjawa (*Talinumpaniculatum*). Skripsi.Fakultas Sainsdan Teknologi, UniversitasAirlangga.Surabaya, 2011.
24. Purnamaningsih R. Introduksigen *DefH9-iaaM*dan*DefH9-RI-iaaM*kedalamgenomtanaman tomatmenggunakanvektor *Agrobacterium tumefaciens*.Jurnal AgroBiogen, 2010, 6(1); 18-25.
25. Wahyuningtyas E. Pengaruh konsentrasi dan lama waktu infeksi *Agrobacterium rhizogenes* strain 510 terhadap induksi akar eksplan daun ginseng jawa (*Talinum paniculatum* Gaertn).Skripsi. Departemen Biologi, Fakultas Sains dan Teknologi, Universitas Airlangga.Surabaya,2011,22 & 44.
26. Xu H, Zhou X, Lu, J, Wang J, Wang X. Hairy rootsinduced by *Agrobacterium rhizogenes* and production of regenerativeplantsand hairy rootculture in maize.Science in China Series C*:* Life Sciences, 2006, 49; 305-310.
27. Park NI, Li X, Uddin MDR, Park SU. Phenolic compound production by different morphological phenotypes in Hairy root cultures of *Fagophyrum tataricum* Gertn. Arch. Biol. Sci., Belgrade, 2011, 63(1); 193-198.
28. Syukur S, Aneloi ZN, Putri F. Transformasi *Agrobacterium rhizogenes* dan induksi akar rambut pada tanaman kakao (*Theobroma cacao*) untuk produksi senyawa antioksidan secara In Vitro. J. Ris. Kim., 2009, 2(1); 156-168.
29. Pirian K, Piri K, Ghiyasvand T. Hairy roots induction from *Portulaca oleracea* using *Agrobacterium rhizogenes* to noradrenaline's production. International Research Journal of Apllied and Basic Science, 2012, 3(3); 642-649.

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