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Agrobacterium rhizogenes Mediated Hairy Root Induction in *Justicia gendarussa* Burm.f

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ABSTRACT

Five strains of *Agrobacterium rhizogenes* (LB510, LB509, YMB072001, A4T, and ATCC 15834) were tested to determine their effects on the induction of hairy root on leaf explants of *Justicia gendarussa* Burm. f.. Leaf explants were sterilized, and then infected within liquid MS medium + sucrose containing *A. rhizogenes* OD600 = 0.1, for 20 minutes while being shaken gently by hand. Teh explant was co-cultivated on Murashige and Skoog (MS) solid medium at 25°C for 48 hours. After 48 hours of co- cultivation, explants were transferred to MS solid medium with the addition of 250 mg/L cefotaxim, and repeated 1-2 times. Observations were carried out during 6 weeks to observe the specified parameters. Data were analized by Kruskal-Wallis Test and followed by Mann-Whitney Test. All strains of *A. rhizogenes* were used in this study were able to produce hairy root. The treatment of LB510 and YMB072001 strain was the best strain in the hairy root induction of *J. gendarussa* Burm. f.. The infection frequency of LB510 strain was highest (100 %), longest hairy root (2.23 cm), and highest of hairy root wet weight (0.0243 g). The treatment of YMB072001 strain was the highest number of hairy roots (3.3) per explant and the highest number of hairy roots dry weight (0.0098 g).
KEYWORDS: *Agrobacterium rhizogenes*, hairy root, *Justicia gendarussa* Burm. f.

INTRODUCTION

Gandarusa (*Justicia gendarussa* Burm. f.) is an Indonesian medicinal plant, Acanthaceae family. Gandarussa is used for migraine headaches, fever, hemiplegi, paralysis of facial muscles, swelling, ear pain, inflammation, bronchitis, dyspepsia, eye diseases, bleeding, muscle pain, antirheumatic, antinociception, antihepatotoksic and anti malaria [1, 2]. Based on ethnomidicine study, gandarusa (local name) is used for male birth control drug in the Irian Jaya, Indonesia [3]. Prajogo et al. [4] proved the male antifertility gandarusa efficacious clinically. According Prajoga et al. [5] gandarusa is also useful as antiviral.

Utilizations of gandarusa in the industrialized world are faced with a problem its availability in nature. Gandarusa has not been cultivated and grow wildly. Gandarusa cultivation is a relatively long time traditionally. The level and quality of the desired active compounds are not as expected. Levels of 0.9% of the total flavonoid extraction, while gendarusin A 0.03% [6]. Modern biotechnology interventions are needed to address this problems, one of which is the hairy root culture.

Hairy root culture is the hairy root induction techniques by genetic transformation using *Agrobacterium rhizogenes*. Excess hairy root culture is capacity in the production of secondary metabolites similar to or greater than the parent plant [7]. Trigonelline production in *Trigonella foenum-graecum* hairy root cultures produces three to five times that of plant origin [8]. In industry, the hairy root culture is much more effective than cell culture because of its genetic stability. Culture of the hairy roots can also produce recombinant proteins for potential pharmaceutical industry [9].

A. rhizogenes is a genus of gram-negative soil bacterium, is responsible for the formation of hairy root at the site of infection. These bacteria can transfer the T-DNA, which is in Ri (root inducing) plasmid, the size of few hundred kb, from bacteria to plant cells [10]. Many factors affect the success of the hairy root culture. That are strains of bacteria [11,12,13,14,15], the type of plants [11,13,15,16], parts of plants [11,13,15,16,17], and culture medium [7,11,15,18,].

The study of the efficiency of various strains of *A. rhizogenes* on induction on hairy root of gandarusa plants still exist. This study aims to find the right kind of strain for the optimization of the hairy root induction of gandarusa leaf.

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MATERIALS AND METHODS

Rejuvenation of *A. rhizogenes*

A. rhizogenes strain LB510, LB509, YMB072001, A4T, and ATCC15834 was obtained from the Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI) Bogor. The bacteria culture medium were LB and YMB medium of solid and liquid. The bacteria was streaked on solid media. Isolates were stored in the refrigerator for 1 week. The bacteria has been rejuvenated to each 25 mL of YMB medium and liquid LB medium. Then left at room temperature for 24 hours.

Sterilization Leaf Eexplants

J. gendarussa Burn f. obtained from the Institute of Materia Medica, Batu, Malang. The explants was second leaf of shoots. The explants were washed, then rinsed with running water. Explants were rinsed with water three times. The explants were shoaked in 1% fungicide solution for 10 minutes. Explants were rinsed with water three times. The explant was sterilized with 50% Clorox for 5 minutes, then rinsed with sterile distilled water, 3 times.

Induction of hairy roots by *A. rhizogenes*

The sterile explant were infected by inserting into MS medium [19] liquid + sucrose containing *A. rhizogenes* OD600 = 0.1, for 20 minutes. After 48 hours of co-cultivation, the explants were transferred to MS medium with the addition of 250 ppm of cefotaxime, and repeated 1-2 times. Observations were carried out for 6 weeks to observe the parameters determined. Each treatment was repeated 10 times.

Data Analysis

The data were the frequency of infection, length of time the inception of roots, root length, number of roots and root morphology. Quantitative data obtained from this study were analyzed by the Kruskal-Wallis Test at 5% level, followed by The Mann-Whitney Test at 5%. Qualitative data were analyzed descriptively.

RESULTS AND DISCUSSION

The frequency of infection (percentage of explants growing roots compared to the overall number of explants in one treatment) can be determined as shown in table 1. The infection frequency of strain LB510 was highest (100%), 80% of YMB072001 strain, 70% of ATCC15834, 30% of LB509 and 20% of A4T (Table 1). These results was in line with research Manuhara et al. [20] that strain LB510 had the optimal frequency of infection for *Talinum paniculatum*, but is not optimal for *Artemisia annua* and *Artemisia cina* [13]. Chandran and Potty [15] showed that different strains affected to different infection frequency of *Ipomoea batatas*, *Solenostemon rotundifolius*, *Vigna vexillata*, and *Canavalia* sp., which were the strain ATCC 15834 had the best results in all species. Strain ATCC 15834 and YMB072001 best for *Artemisia annua* and *Artemisia cina* [13]. Strain 15834 was best for *Solenostemon scutellaroides* [14]. The results showed the frequency of infection of A4T strains was lowest, in contrast to studies of Swain et al. [7] showed that the strain A4T was best for *Clitoria ternatea*. According Akramian et al. [21] that the different strain of *A. rhizogenes* had the variation of tranformation ability. Differences of the virulence of *A. rhizogenes* can be caused by the bacterial plasmids.

Table 1. The frequency of infection of various strains of *A. rhizogenes* on leaf explants *J. gendarussa* Burm. f.

Treatment	Number of infected explant	Replicated	Frequency of infection (%)
Negative Control	0	10	0
LB510	10	10	100
LB509	3	10	30
YMB072001	8	10	80
4T	2	10	20
ATCC15834	7	10	70

Hairy root are formed because of *A. rhizogenes* T-DNA transfered Ri (root inducing) plasmid into the genome of the host plant. The transformation resulted hairy root on the infected part. Opin compounds will be generated and served as a source of nutrients for bacteria [22]. Based on the type of opin, *A. rhizogenes* strains can be separated into five groups, namely octopin, agropin, nopalin, mannopin, and cucumopin [23]. Agropin group was the most commonly used strain [12]. In this study, there were two strains agropin group, including the ATCC15834 and A4T

strains. The LB510, LB509, and YMB072001 strain have not been studied, so that references of all three strains was very little.

Other parameters in this study is the length of hairy root formation time (Figure 1). The hairy root was starting to grow in wound site of the leaf explants *J. gendarussa* Burm. f., on day 14-26 after infected. Based on the average length of the hairy root formation time, the A4T strain was able to induce the growth of hairy root on 14 days, in line to studies on *Amaranthus spinosus,* hairy root emerged on 15-18 days of *A. rhizogenes* strain A4T treatment [24]. This suggested that if the same strain are infected to different species, it will show the difference in the length of hairy root formation time. According to Hu and Du [12] the hairy root will be induced in a short period of time, ranging from one week to over a month, depending on plant species diversity.

Treatment of strains *A. rhizogenes* also affected the number of hairy root formed. The mean number of hairy root was different on each treatment (Figure 2). The average number of hairy root number of strain YMB072001 was 3.3 hairy root (highest), ATCC15834 and LB510 strain was 1.4 hairy root, LB509 strain was 0.5 hairy root and strain A4T was 0.2 hairy root. The results obtained in line with Wahyuni et al. [25] that the number of hairy root of *J. gendarussa* Burm. f. by YMB072001 strain treatment was more than the A4T strain treatment.

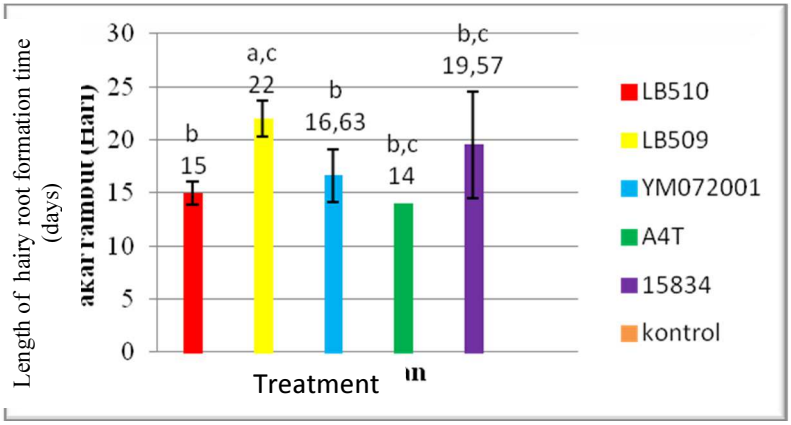

Figure 1. Graph of the average length of hairy root formation time on leaf explants *J. gendarussa* Burm. f. with different strains of *A. rhizogenes* treatments (different letters indicate significance at the Mann-Whitney Test with a level of 5%).

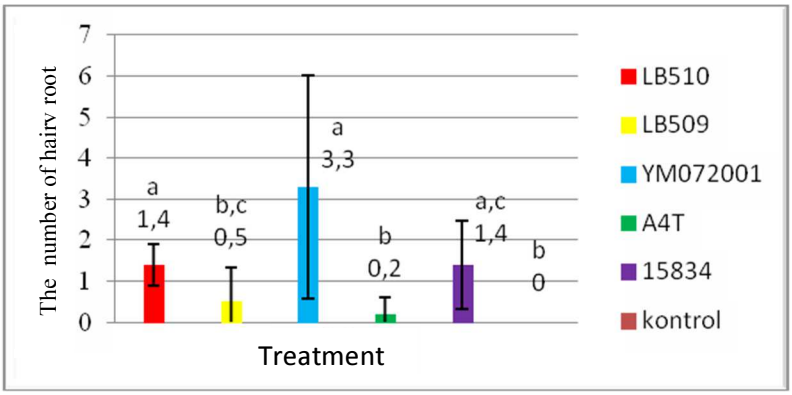

Figure 2 Graph of the average number of hairy root on leaf explants of *J. gendarussa* Burm. f. with different strains of *A. rhizogenes* treatment at 6th week (different letters indicate significance at the Mann-Whitney test with a level of 5%).

The observation of hairy root length for six weeks was between 0 to 6.6 cm (Figure 3). The results of these studies was varied in each treatment. LB510 strains induced longest hairy root. In contrast to Puspitaningsih [26] study that strain YMB072001 treatment induced hairy root of *Talinum paniculatum* Gaertn. longer than LB510 strain treatment.

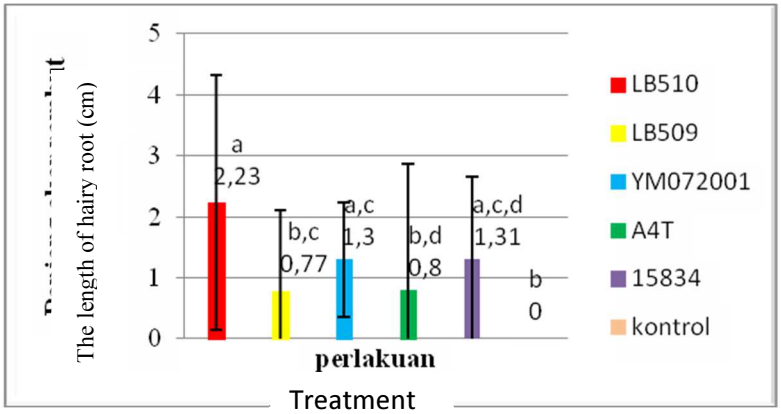

Figure 3. Graph of the average length of hairy root on leaf explants of *J. gendarussa* Burm. f. with different *A. rhizogenes* treatment at 6th weeks (different letters indicate significance at the Mann-Whitney test with a level of 5%).

The growth of the hairy root caused weight gain. Wet weight hairy root obtained is very small, as much as 0.0243 grams LB510, YMB072001 of 0.0241 grams and 0.0112 grams of ATCC15834, whereas LB509 and A4T is zero grams (the wet weight was very small, could not be detected by the balance analytic used in this study). In contrast to the data of wet weight of roots, the highest dry weight was YMB072001 of 0.0098 grams, whereas LB510 lower at 0.0063 grams and 0.0033 grams of ATCC15834.

Table 2. The wet weight and dry weight of hairy root on leaf explants of *J. gendarussa* Burm. f. with different strains of *A. rhizogenes*.

Treatment	Wet weight (gram)	Dry weight (gram)
Negative control	0	0
LB510	0,0243	0,0063
LB509	0	0
YMB072001	0,0241	0,0098
A4T	0	0
ATCC15834	0,0112	0,0033

Hairy root on leaf explants of *J. gendarussa* Burm. f. by LB510 strain treatment was long and branched (Figure 4C). In one explant, there was one or two hairy root. In the treatment of LB509 strain, the roots was thicker at the base (Figure 4B). In the treatment of YMB072001 strain the hairy root was short size, 1-8 hairy root (Figure 4A). In the treatment of A4T strain, the hairy root was different on each explants. The one was length and branched, but the other one was short and unbranched (Figure 4E). In the treatment of ATCC15834 strain, the hairy root was varied on each explants. The one was long and branched but the other one short and unbranched (Figure 4D).

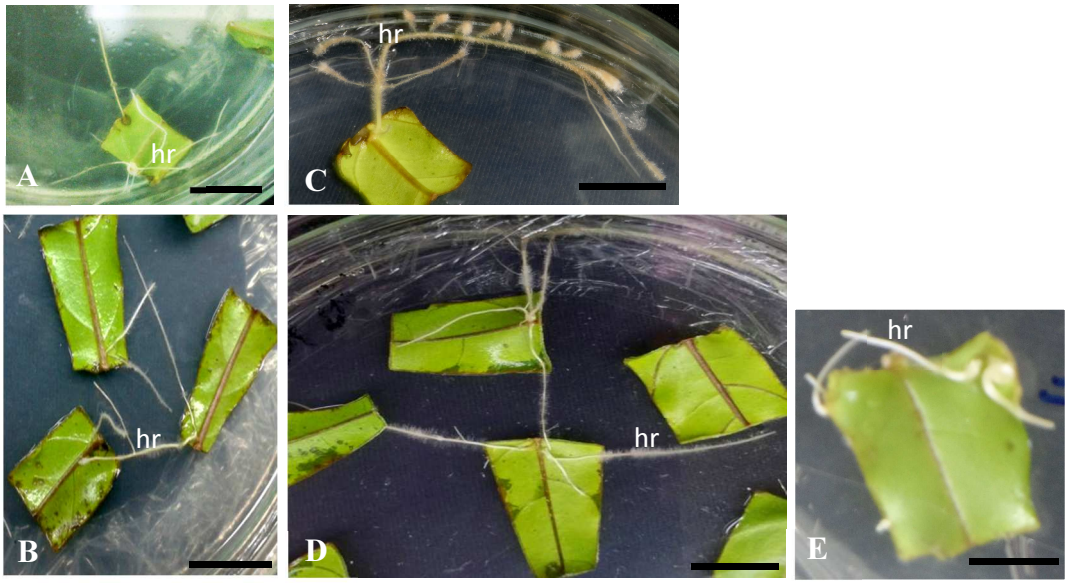

Figure 4 . Hairy root on *J. gendarussa* Burm. f. by *A. rhizogenes* strain treatment. A. YMB072001, B. LB509, C. LB510, D. ATCC15834, E. A4T. hr. hairy root. Bar = 1 cm.

Figure 5 showed that the hairy roots appear on midrib area (Figure 5.1A and 5.2A). It supported Ove et al . [29] study, that hairy root induction genes (*rol* genes) had similar tissue spesific expresion pattern and are mainly confined to root meristems and the phloem. Danial et al. [30] study showed that the hairy roots of *Eurycoma longifolia* are formed on hypocotyl explant only. In detail analysis showed that whereas the *rolB* promoter seems to be generally expressed in phloem, phloem parenchyma [31]. The *rolC* promoter is spesifically expressed in phloem companion cells [32]. The root stem of explants formed roots slightly (Figure 5.2B) was bigger than root stem of the explants formed many roots (Figure 5.1B). The root hair at the ends of branched hairy root (Figure 5.1C) more than unbranched hairy root (Figure 5.2C).

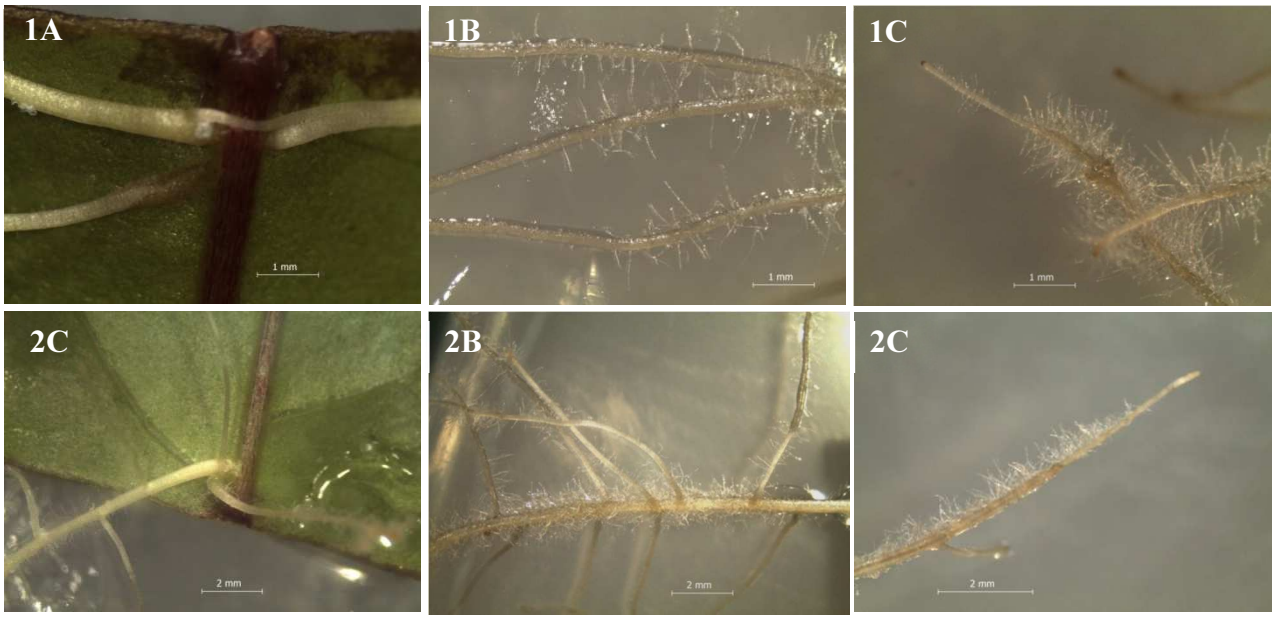

Figure 5. Structure of the hairy root *J. gendarussa* Burm.f. 1. the explants formed many roots. 1A. base of the root, 1B. stem root, 1C. root tip; 2. the explants formed roots slightly. 2A.base of root, 2B. stem root, 2C. root tip, rh. root hair, rb. root branch.

The results of this study shown that *A. rhizogenes* strain of LB510, LB509, YMB072001, A4T, and ATCC154 effected on *J. gendarussa* Burm. f. hairy root on. This is realized from the results of the Kruskal-Wallis statistical analysis that a strain of *A. rhizogenes* influenced of a long time to grow, the number and length of hairy root formed. These results were in line to the study of Lee et al. [10] that the strain of *A. rhizo genes* influenced the induction of hairy root and production of alizarin and purpurin on *Rubia akane* Nakai, hairy root growth and artemisinin production in *Artemisia annua* [27], and the growth of the hairy roots of *Vitis vinifera* [28].

Based on the resutls, it could be seen that the best strain of *A. rhizogenes* to induce hairy roots on the leaf explants of *J. gendarussa* Burm. f. was LB510 and YMB072001 strain. The infection frequency (100%)and root length (3.3 cm) of LB510 strain was highest. The number of hairy root (3.3) and dry weight of hairy root (0.0098 g) of YMB072001 strain was highest. The length time value of root hair formation, hairy root number and length of hairy root of two of these strains was not differ significantly based on the results of the Mann-Whitney Test. In the other words the ability of both strains to induce hairy root in *J. gendarussa* Burm. f. not significantly different.

CONCLUSION

Based on the results and discussion can be concluded that the strain of *A. rhizogenes* strain influenced the induction of hairy root on *J. gendarussa* Burm. f. and the best strains of *A. rhizogenes* in inducing the formation of hairy root in *J. gendarussa* Burm. f. was LB510 and YMB072001 strain.

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