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Hairy Root Induction on *Justicia gendarussa* by Various Density of *Agrobacterium rhizogenes* strain LB 510

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Abstract

Gandarusa (*Justicia gendarussa* Burm.f.) is an Indonesian medicinal plant that has many benefits as drug and male contracetive. For industrial needs, Gandarusa must be available in large quantity. Hairy root culture is one of methode to produce phytochemistry compound. The objective of the study was to examine the effect of various density of *Agrobacterium rhizogenes* strain LB510 on hairy roots induction of gandarusa (*Justicia gendarussa* Burm.f.) leaf plant. Leaf explants were inoculated in MS liquid medium with various density of OD_{600} = 0.1; 0.2; 0.3; 0.4; and 0.5. Explants were co-cultivated for 2 days on MS solid medium without any hormone then sub-cultured on MS solid medium containing antibiotic cefotaxim 300 ppm, in dark condition. The data were analyzed descriptively and statistically. The results showed that various density of *Agrobacterium rhizogenes* strain LB510 was affected the lenght of hairy roots induction of *J. gendarussa* Burm.f., but these was not effected toward lenght formation time and number of hairy root. The treatment of OD_{600} 0.2 was the best treatment for hairy root induction on *Justicia gendarussa* Burm. f. This data could be used for optimized the quality of methode of hairy root induction.

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INTRODUCTION

Gandarusa (*Justicia gendarussa* Burm.f.) is an Indonesian medicinal plant. It has many benefits as analgesic, antipyretic, diaforetic, diuretics, and sedatives. It root is used as drug for malaria (Braidot et al., 2008). Leaves of gandarusa are used as a pain killer in medication, gout, headache, back pain, and facilitate menstruation. In West Papua, roots and leaves of gandarusa are used as a male contraceptive (Prajoga et al., 2009).

For industrial need, gandarusa must be available in large quantity. The problem encountered is that there is limitation of gandarusa production, specifically for the production of drugs. Tissue culture technique is deemed efficient for plant propagation. One of the tissue culture techniques has been developed for the production of secondary metabolites is hairy roots culture. The advantages of using hairy roots culture is the high genetic stability and the absence of any growth regulators (Pirian et al., 2012).

The other advantages of using hairy roots culture are the ability to produce chemical compounds in a short time and the ability to produce compounds that are difficult to obtain naturally (Syukur et al., 2009). According to Manuhara et al. (2015) and Park et al. (2011), the advantage of the hairy roots culture is often demonstrated high capacity in the production of secondary metabolites that is almost equal to or greater than the parent plants. Lee et al. (2010) succeeded in obtaining alizarin of 4.3 mg/g dry weight and purpurin of 4.9 mg/g dry weight of the hairy roots cultures of *Rubia akane* Nakai. Hairy roots production by *Agrobacterium rhizogenes* transformation produced trigonellin, three to five times of plant origin (Raheleh et al., 2011).

The hairy root induction are influenced by several factors, such as *Agrobacterium rhizogenes* strains, the addition of growth regulators on the media, parts of the plant are used as explants, and the density of bacteria (Ermayanti et al., 2009; Lee et al., 2010; Swain et al., 2011). The research was carried out by Chakravarty & Pruski (2010) showed that OD_{600} = 0.2 could improve significantly the average efficiency transformation of *Agrobacterium rhizogenes* on potato.

There is a few reported on hairy root induction of *Justicia gendarussa* Burm. f.. Wahyuni et al. (2015a) determine the effect of five strains of *Agrobacterium rhizogenes* (LB510, LB509, YMB072001, A4T, and ATCC 15834) to induce hairy root on leaf explants of *Justicia gendarussa* Burm. f., the strain LB510 is the best strain. A preliminary study is needed to determine the appropriate method for the hairy roots induction of gandarusa. Therefore, this study was aimed to obtain scientific information on the effect of the *Agrobacterium rhizogenes* strain LB510 density toward gandarusa hairyroots induction.

A protocol for hairy root induction of *Justicia gendarussa* Burm.f. has not been established and the present study of the influence of some factor, expecially *Agrobacterium rhizogenes* density, on hairy root induction in *Justicia gendarussa* Burm. f. was an attempt to develop a technique for hairy root production in *Justicia gendarussa* Burm.f.

METHODS

Plant preparation

Plants used as explants are leaves of gandarusa (*Justicia gendarussa* Burm.f.). This plant was obtained from the Institute of Materia Medical, Batu, Malang, Indonesia. Leaves were taken from the second-third nodal segment. The leaves were washed under running water for ten minute and then disinfected by anti-fungiside "topsin" with 1% (v/v), followed by several rinses in sterilized distilled water and sterilized by 10% Clorox "baycline" for ten minute, followed by three times rinses in sterilized distilled water.

Bacterial strain and culture media

LB510 strain of *Agrobacterium rhizogenes* was obtained from the Research Center of Biotechnology, Indonesian Institute of Sciences (LIPI), Bogor, Indonesia. The LB510 strain was grown at room temperature (±27°C) in Luria Bertani medium (LB) (per l) (10g trypton, 5g yeast extract and 10g NaCl) of solid and liquid. Media was sterilized by autoclave at 1.2 atm 121°C for 20 minutes.

The bacteria was stroke 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. *Agrobacterium rhizogenes* strains LB 510 was prepared with various dencity of OD_{600}: 0.1 (P1); 0.2 (P2); 0.3 (P3); 0.4 (P4); and 0.5 (P5).

Induction of hairy roots by *A. rhizogenes*

The sterile explant were infected by inserting into MS medium (Murashige and Skoog, 1962) liquid + sucrose containing *A. rhizogenes* OD600 = 0.1, 0.2, 0.3, 0.4, 0.5 for 10 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 transformation frequency, length of hairy root transformation time, number of hairy root and length of hairy root. The transformation efficiency data were shown by percentage. The number of hairy root data were analyzed by Anova Test at 5% level and followed by Duncan Test at 5% level. The length of hairy root and length of hairy root formation time data were analyzed by Brown-Forsythe Test at 5% level and followed by Games-Howell Test at 5%. Picture data were analyzed descriptively.

RESULTS AND DISCUSSION

The transformation efficiency

The number of explants induced is 4-10 explants per treatment. Table 1 shows that OD_{600} = 0.2 has the highest transformation efficiency (100%), followed by OD_{600} = 0.1 (90%), while treatment OD_{600} = 0.3 and OD_{600} = 0.5 has a value transformation efficiency equal value (50%). The lowest transformation efficiency obtain for OD_{600} = 0.4 (40%). The negative control, hairy root formation is not observed.

Based on the percentage of transformation efficiency data, the treatment OD_{600} = 0.2 has the highest transformation efficiency (100%). This is consistent with research conducted by Kereszt et al. (2007) that the K999 strain is able to induce hairy roots from cotyledon explants Zigongdongdou effectively on a low density. This is due to the high density of bacteria that can damage cells or plant tissue. Basu et al. (2015) study on *Plumbago zeilanica* reported that the OD_{600} > 0.1 is best density of *Agobacterium rhizogenes* strain LBA 9402.

The results of this study indicated that the density of the *Agrobacterium rhizogenes* strain LB510 effected on the hairy roots induction of gandarusa leaf explants (*Justicia gendarussa* Burm.f.). Hairy roots that appears was an adventitious roots that grow after 2 weeks of infected and cultured in MS solid medium containing cefotaxime.

Hairy roots are formed because of *Agrobacterium rhizogenes* T-DNA transfer from ri-plasmid (root inducing plasmid) into the genome of the host plant. The transformation will induce hairy roots on the infected part. Opin compounds will be produced and serves as a source of nutrients for the bacteria). T-DNA also contain oncogenes, ie. genes that contribute to encode growth hormone auxin and cytokinin. Oncogene expression in ri-plasmid characterized the formation of adventitious roots on a large scale in the infected areas and known as 'hairy root' (Manuhara, 2014).

Density *Agrobacterium rhizogenes* and the infection duration determine the success of transformation. This is due to the high density of *Agrobacterium rhizogenes* that causes the death of explants, although the medium added by antibiotic. Antibiotics are not able to inhibit the growth of *Agrobacterium rhizogenes* in excessive growth. This is supported by Hu et al. (2006) research, transformation by high density bacteria on *Lycium barbarum* causes excessive growth of bacteria. Manuhara et al. (2012) research showed that higher density was not able to increase the transformation efficiency of *Agrobacterium rhizogenes* on *Talinum paniculatum* leaf explants both in strain LB510 and YMB072001.

Low density is considered more efficient for hairy roots induction although extend the duration of the transformation because it makes the delivery T-DNA is more efficient. Chakravarty & Pruski (2010) study on potato, OD_{600} = 0.2 increases the average transformation efficiency significantly.

Xu et al. (2006) explained that the transformation ability of *Agrobacterium* to plant is

Table 1. Efficiency transformation of gandarusa leaf explants by *Agrobacterium rhizogenes* strains of various density LB510 for 6th week (n = 10)

Treatment	Number of explant formed hairy roots	Repetition	Eficiency Transformation (%)
P1 (Negatif Control)	0	10	0
P2 (OD_{600}=0,1)	9	10	90
P3 (OD_{600}=0,1)	10	10	100
P4 (OD_{600}=0,1)	5	10	50
P5 (OD_{600}=0,1)	4	10	40
P6 (OD_{600}=0,1)	5	10	50

different. Park et al. (2011) revealed the success of transformation and T-DNA delivery depends on the cultivars compatibility and *Agrobacterium*. Compatibility is demonstrated by the ability of *Agrobacterium* to receive signals from wounding plant and followed by inducer factors. Based on that, there is not any formation of hairy roots, due to incompatibility of the possibility of gene T-DNA of *Agrobacterium* to the plant chromosome is brook. T-DNA is not integrated with the plant cell genome. There is a fairly large DNA sequences that encodes proteins inactive, if the position of the T-DNA inserted is not random in the plant genome and only integrated in the DNA, that will not active then it will not be expressed (Pal et al., 2013).

The length of hairy roots formation time

Formation of hairy roots on gandarusa leaf explants was characterized by the appearance of small white bumps around the wounding area infected by *Agrobacterium rhizogenes* strain LB510. The length of hairy roots formation time was 15-30 days. Average length of hairy roots formation time was 20, 17, 23, 20 and 21 days for OD_{600}=0.1; 0.2; 0.3; 0.4 and 0.5 respectively. On the negative control, hairy root formation was not observed (Figure 1). Gomes Howell Test indicated that there was a significant difference between the number of hairy roots of negative control and treatment but among the treatment (OD_{600} = 0.1; .2; 0.3; 0.4; 0.5) there was not significant difference.

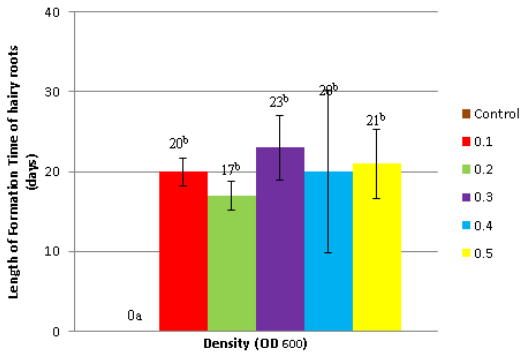

Figure 1. The average length of hairy roots formation time on gandarusa leaf explants by various densities of *Agrobacterium rhizogenes* strain LB510.

Based on the length of hairy roots formation time, *Agrobacterium rhizogenes* strain LB510 is able to induce the hairy roots on the leaf explants gandarusa (*Justicia gendarussa* Burm.f.) in the range of 15-30 days. Meanwhile, the explants in the negative control treatment are not able to form the hairy roots until 6th weeks of culture period. Explants on treatment OD_{600} = 0.2 are able to form the hairy roots in the fastest time of 17 days, OD_{600} = 0.4 and OD_{600} = 0.1 on 20th days (Figure 1).

Previous research reports that the hairy root induction of the leaf explants *Justicia gendarussa* Burm.f. is formed on the hairy roots to 14-16 by *Agrobacterium rhizogenes* strain treatment A4T (Wahyuni et al., 2015b). Similarly, in a study conducted by Fu et al. (2005), a strain of *Agrobacterium rhizogenes* LBA9402, R1000, and R1601, are able to induce hairy roots within 2-5 weeks on leaf explants *Saussurea involucrate*. This result differs from previous studies conducted by Anekawaty (2011), that the effect of inoculum *A. rhizogenes* strain LB510 to hairy root induction by various density of OD_{600} 0.1; 0.2; and 0.3 in explant *Catharanthus roseus,*only forming callus at 16 weeks of culture. According to Hu and Du (2006) hairy roots induction were carried out in a short period of time, varying from one week to more than one month, it depends on the plant species diversity.

Agrobacterium rhizogenes has various ability to induce hairy roots on explants because explants can not produce a phenolic compound in an amount to sufficient stimulate bacterial chemotaxis. This is consistent with the explanation by Xu et al. (2006), the production of phenolic compounds as a result of wounding plants can make it easier to stick to bacterial cell walls of plants.

Explants from young tissues have not been able to produce enough phenolic compounds to stimulate bacteria, so it required additional inducer compounds to improve transformation process. Inducer compound is acetosyiringone. Acetosyiringone is an amino acid derivative compound that serves as a source of nutrients for the bacteria (Pirian et al., 2012). Acetosyiringone is reported to induce the formation of hairy roots in *Talinum paniculatum* Gaertn. The results showed that the level of transformation frequency is high enough (66% for the stem explants and 73% for leaf explants), both are transformed with *Agrobacterium rhizogenes* strain LBLB510 (Manuhara et al., 2012). This study do not use acetosyiringone that allegedly led to the transformation process between bacterial cells and plant cells. Hairy root is not observed in control treatment because there is not any transformation event.

The number of hairy roots

The number of hairy roots formed on gandarusa leaf explants is shown in Figure 2. The

number of hairy roots is 3.2; 2.8; 2.4; 2.2; and 2.2 for OD_{600} = 0.2; 0.1; 0.5; 0.3; and 0.4 respectively.

Based on the results of One Way Analysis of Variance (ANOVA) Test, there was an effect of *Agrobacterium rhizogenes* strain LB510 density to the number of hairy roots formed on gandarusa leaf explants. Duncan Test indicated that there was a significant difference between the number of hairy roots of negative control and treatment but among the treatment (OD_{600} = 0.1; .2; 0.3; 0.4; 0.5) is not significant difference (Figure 2).

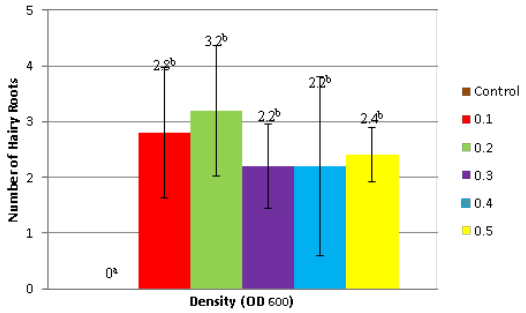

Figure 2. The average number of hairy roots formed on explants *Justicia gendarussa* Burm.f. (The difference letters above diagram shows a significant difference from the results by Duncan Test at significant level 5%).

The various density is affected the number of hairy roots. Treatment OD_{600}= 0.2 has highest average number of hairy roots (3.2 hairy roots) (Figure 2). Sukmawati's (2011) study showed that the OD_{600} = 0.1 had the highest average number of hairy roots (2.13 hairy roots). Ermayanti et al. (2009) explain the explants response was different because of the genetic potential of explant is different.

The length of the hairy roots

White bulge that appears on the midrib will grow and extends into the hairy roots (Figure 3). The hairy roots length is 5.1cm, 3.9 cm, 2.1cm, 2.0cm, and 1.4cm for OD_{600} = 0.2; 0.1; 0.3; 0.5 and 0.4 respectively.

Brown-Forsythe Test showed that there was an effect of *Agrobacterium rhizogenes* strain LB510 density toward the hairy roots length of gandarusa leaf explants. Based on Gomes-Howell Test showed that there was significant difference (Figure 4).

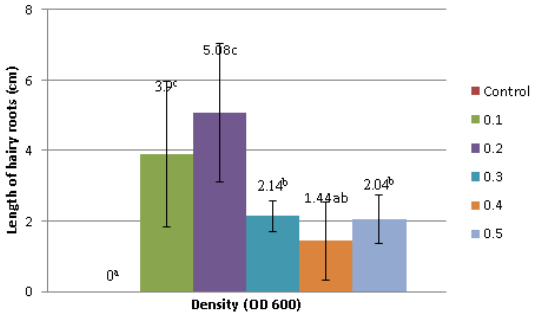

Figure 4. The average length of hairy roots formed on explants *Justicia gendarussa* Burm.f. (The difference letters above diagram shows a significant difference by Gomes Howell Test at significant level 5%)

The observation of hairy roots length showed the average of hairy roots length was 1.1 to 7.5 cm. The results of these studies varied in each treatment, as shown in Figure 4. The treatment of OD_{600}= 0.2 was able to induce the longest hairy roots. This is because the meristem tissue of explants was still able to perform activities of cell division.

Xu et al. (2006) explained that the transformation ability of *Agrobacterium* to plant is different. Park et al. (2011) revealed the success of transformation and T-DNA delivery depends on the cultivars compatibility and *Agrobacterium*. Compatibility is demonstrated by the ability of *Agrobacterium* to receive signals from wounding plant and followed by inducer factors. Based on that, there is not any formation of hairy roots, due to incompatibility of the possibility of gene T-DNA of *Agrobacterium* to the plant chromo-

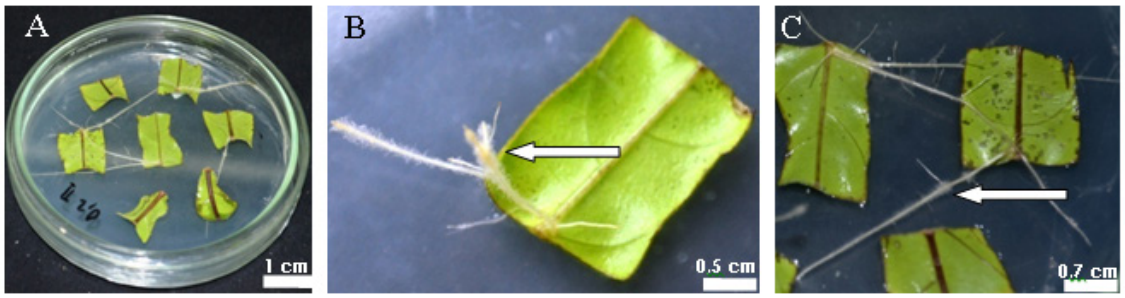

Figure 3. Hairy root development of Justicia gendarussa Burm.f. leaf explant. A. Explant growth on media, B-C. Hairy root growth from explant, arrow=hairy root.

some is brook. T-DNA is not integrated with the plant cell genome. There is a fairly large DNA sequences that encodes proteins inactive, if the position of the T-DNA inserted is not random in the plant genome and only integrated in the DNA, that will not active then it will not be expressed (Pal et al., 2013).

Hairy root formation from the leaf *Justicia gendarussa* Burm. f. demonstrate the development meristem in the leaf., and indicate that the hairy root induction has been sucsesfully, but the quality of hairy root (lenght, number, and morphology) must be improved. Futher improvement of the technique by manipulation of growth regulator, carbohydrate and temperature and by using a number of different genotype may enable the development of an effective methode for producing hairy root in *Justicia gendarussa* Burm.f. These limited results may be useful in the planning of any further attempts.

CONCLUSION

According to the result and discussion we conclude that *Agrobacterium rhizogenes* LB510 density (OD_{600}= 0.1; OD_{600}= 0.2; OD_{600}= 0.3; OD_{600}= 0.4; and OD_{600}= 0.5) effect toward the lenght of hairy roots, but theese do not effect toward lenght formation time and number of hairy root on *Justicia gendarussa* Burm. f. The OD_{600}= 0.2 of *Agrobacterium rhizogenes* LB510 is the best density to induce hairy roots on gandarusa leaf explants. The length time of the hiry roots formation is fastest time (17 days), the transformation efficiency is highest (100%), the number of hairy roots is highest (3.2 roots) per explant, and the hairy roots length is longest (5.1 cm).

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