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Callus Induction of Gendarussa (*Justicia gendarussa*) by Various Concentration of 2,4-D, IBA, and BAP

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Abstract

Justicia gendarussa Burm.f., a medicinal plant, is Acanthaceae that has many functions. Furthermore, the compounds in gendarussa must be produced in high quantity and quality by applying callus culture method. Accordingly, it is important to study the effects of plant growth regulators (2,4-D, IBA, and BAP) on callus induction of gendarussa leaves. This research design utilized a factorial design with two factors (2,4-D and IBA: 0.5, 1, 1.5 mg/L and BAP: 0.5, 1, 1.5, 2 mg/L). The experiment consisted of 24 treatments, each of which was repeated 3 times. Observation was carried out in 6 weeks. Data on the time of callus formation, percentage of explants formed callus, and callus morphology were analyzed descriptively,while data on fresh and dry weight were analyzed by Two-Way ANOVA ($\alpha = 0.5$). Interestingly, the results showed that various concentration of plant growth regulators (2,4-D, IBA, and BAP) affected callus induction from leaf explants of gendarussa. We concluded that the most optimal treatment combination of concentration of plant growth regulators in inducing callus from leaf explants of gendarussa is 1.5 mg/L 2,4-D and 2 mg/L BAP with a relatively long period of callus formation at the earliest, i.e. on day 5, 2.247 g of fresh weight, 0.108 gof dry weight, white callus translucent, and friable. Moreover, the optimum treatment will be used to produce secondary metabolite and seed synthetic by cell suspension culture.

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INTRODUCTION

Gendarussa (*Justicia gendarussa* Burm.f.), a member of Acanthaceae, is a plant that has been used as a traditional medicine. Some of its benefits include remedies for headaches, fever, hemiplegia, facial paralysis, swelling, ear pain, inflammation, bronchitis, dyspepsia, eye diseases, bleeding, muscle pain, antirheumatic, antinociceptive, antihepatotoxicity, malaria, and male contraceptive (Prajogo *et al.*, 2009; Thomas & Yoichiro 2010).

Various studies have shown that gendarussa contains compounds of alkaloids, triterpenoids, tannins, justicine, steroids, and flavonoids (Prajogo *et al.*, 2009) serving as secondary metabolites that can be utilized in various medicinal applications. However, massive utilization of gendarussa in various drug applications poses some problems regarding the availability of the raw materials since the secondary metabolites has very limited amount in nature. If the content of gendarussa is obtained directly from the plant, its quality often does not meet the expectations and is inconsistent.

Gendarussa is a wild plant that has not been widely cultivated yet. Thus far, gendarussa has been conventionally reproduced by cuttings and planting seeds. However, this conventional propagation has some drawbacks including low multiplication rate and time-consuming, very low seed viability, and requiring extensive land for sustainable farming (Thomas & Yoichiro, 2010). Therefore, research development is required in search of effective and efficient method to meet the supply of useful secondary metabolites from gendarussa.

Plant tissue culture methods or in vitro culture can be used as alternative solutions to increase crop productivity, such as producing secondary metabolites in medicinal plants, propagation, and plant breeding (Isda & Sulianyah, 2009). Plant tissue culture can produce secondary metabolites with high economic value in a relatively short time, take place continuously, and result in more consistent and controlled quality as well as higher content levels than direct harvesting (Sitorus *et al.*, 2011; Ariati *et al.*, 2012). The success of callus culture depends on the use of the basic media (Kaviani 2010), a combination of plant growth regulators (Shirin *et al.*, 2007; Hoesen *et al.* 2008; Jahan *et al.*, 2009), appropriate environmental factors, and development of explants (Ibrahim *et al.*, 2010; Reddy *et al.*, 2011).

The most influential factors of in vitro plant growth are the interaction and balance between the plant growth regulators endogenously and exogenously by cultured cells (Sen *et al.*, 2014). Callus culture of *Justicia gendarussa* Burm.f. (Indian varian) has been successfully performed using a combination of growth regulation NAA and BAP (Amid *et al.*, 2011). Research on callus induction from gendarussa (Indonesian variant) leaf explants was investigated by Wahyuni *et al.* (2014) who used a combination of growth regulators of NAA, IAA, and BAP. The results of the study showed that gendarussa leaf explants successfully induced callus, however, the callus growth tended to be slow, underwent browning and had compact texture. Meanwhile, a combination of growth regulators 2,4-D, IBA, and BAP has never been utilized on callus induction from gendarussa leaf explants. Combination of concentration from different plant growth regulators can induce different responses. Therefore, this study was conducted to optimize the induction of callus from leaf explants of gendarussa (*Justicia gendarussa* Burm.f.).

METHODS

The explants used were gendarussa leaves (*Justicia gendarussa* Burm.f.) obtained from the 2nd to the 3rd leaves from the shoots. Explant sterilization was done by using a fungicide (ditane) 1 g per 500 mL in a beaker glass and then shaken for 8 minutes after being washed under running water 3-5 times. Sterilization was continued in the Laminar Air Flow (LAF) using a 50% solution of clothing bleach solution for 8 minutes, and finally the explants were rinsed with sterile distilled water 3 times for 3 minutes. Gendarussa leaf was placed in a petri dish that has been lined with filter paper. Gendarussa leaf was cut to a size of approximately 1 cm^2 and then planted in a culture bottle on MS solid medium (Murashige & Skoog, 1962) according to treatment combined concentration of plant growth regulators (2,4-D, IBA, and BAP).

This study used factorial design with two factors. The first factor was the concentration of 2,4-D (D) and IBA (I), which was 0.5; 1; 1.5 mg/L combined with the second factor using the concentration of BAP (B), which was 0.5; 1; 1.5; 2 mg/L. The experiment consisted of 24 treatments and each treatment was repeated 3 times. Observations were made weekly for 6 weeks. The cultures were incubated at 25±2°C by irradiating fluorescent lamp (Philips energy saving LED 8-Watt) continuously.

Data were collected for callus formation time (days), the percentage of explants forming

callus (%), callus fresh weight (g), callus dry weight (callus dried in 50°C oven until the weight is stable/g), and callus morphology (changes in colour and texture). These data were then analyzed descriptively. Data on fresh and dry weight of callus were analyzed statistically using Two-Way ANOVA test with significant level (α) 0.05 and then tested using a Games-Howell to determine significant differences between the treatments.

RESULTS AND DISCUSSION

Callus Formation Time and the Percentage of Explants Forming Callus

Parameter of callus formation time was intended to determine whether the concentration of growth regulators was faster in inducing callus and callus proliferation with the use of plant growth regulators (2,4-D, IBA, and BAP). The addition of plant growth regulators on tissue culture medium influenced the growth rate of the explant cultured cells. Based on the observations, the formation of callus started from day 5 to day 7 after planting. This indicated the effect of plant growth regulators (2,4-D, IBA, and BAP) even though it was not too significant. The most rapid formation of callus occurred in the combination treatment of 2,4-D and BAP, followed by the combination of IBA and BAP, namely concentration $D_{1.5}B_{0.5}$; $D_{1.5}B_{1.5}$; and $D_{1.5}B_2$ on the 5th day after planting. Although there were similarities in the duration of callus formation, it was also observed that the treatment with $D_{1.5}B_2$resulted in relatively more callus mass gain than other treatments. The result was consistent with previous studies conducted by Wahyuni *et al.* (2014) on a gendarussa leaf explants of callus formation indicating the duration ranging from day 5 to day 7, with the treatment of 1 mg/L NAA and 1.5 mg/L BAP being the fastest. Arianto *et al.* (2013) states that 2,4-D is a plant growth regulator mostly used on callus culture due to its strong activities to stimulate cell dedifferentiation process, press organogenesis, and maintain callus growth. The 2,4-D demonstrated stronger and more optimal activity as compared to other auxins due to the separation of carboxyl groups by carbon or carbon and oxygen (Manuhara, 2014).

Callus formation is marked by the emergence of cell clumps of yellowish green or light green on the wound. Furthermore, these clots will form a mass of cells called callus. Correspondingly, George and Sherington (1992) state that cell division that leads to the formation of callus on their response to cuts and supply of endogenous or exogenous hormones into explants. Closure of callus tissue is derived from parenchymal cells. The fastest growth of callus formation occurs in the peripheral areas of the region due to the availability of nutrients and better oxygen.

The percentage of callus formation indicates the response level of the explants on the use of tested plant growth regulators (Rasud, 2012). Observations on the administration of plant growth regulator (2,4-D, IBA, and BAP) on gendarussa leaf explants showed that it had no effect on the percentage of explants induced callus as in all treatments100% of explants formed callus. The results of this study are similar to a previous study conducted by Isda and Sulianyah (2009), in which callus grows in all combinations of concentrations of plant growth regulators IBA and BAP whereas none of the explants form callus on media that has not been added with growth regulators on *Centella asiatica*leaf explants. Research conducted by Sen *et al.* (2014) on leaf explants of *Achyranthes aspera* L. added with plant growth regulators 2,4-D and BAP at various concentrations has successfully induced callus on all treatments. The composition of the combination of concentrated growth regulators used in this study has proven to be able to induce callus and not to hamper growth. This is consistent with the previous study conducted by Rashmi and Trivedi (2014), using 2,4-D and BAP at various concentrations ranging from 0.5 to 10 mg/L on *Nerium odorum*leaf explants. The research results indicate the presence of callus growth at low concentrations, whereas at high concentrations of 3 to 10 mg/L BAP and 2,4-D no callus growth was shown.

Fresh and Dry Weight of Callus

An increase in the callus fresh weight is due to an increasing number of cells (cell division) and the increase in cell size (cell enlargement). According to Indah and Ermavitalini (2013), fresh weight physiologically consists of two contents, namely water and carbohydrates. The fresh weight of callus is caused by the high content of water. The amount of fresh weight produced highly depends on how fast these cells divide, multiply, and then grow callus (Andaryani, 2010).

Results of Two-Way ANOVA statistical tests (α = 0.05) showed that plant growth regulators (2,4-D, IBA, and BAP) concentration significantly affected the fresh and dry weight of callus from gendarussa leaf explants. In general, the combination of 2,4-D and BAP had higher fresh and dry weight of callus as compared to the combination of IBA and BAP. Combination treatment of the concentration of 2,4-D and BAP

had the highest fresh weight in $D_{1,5}B_2$ treatment, which amounted to 2.247 ± 0.044 g.

This study demonstrated better results than other studies using a combination of plant growth regulator (NAA, IAA, and BAP) on *Justicia gendarussa* Burm.f. leaf explants with the highest callus fresh weight of only 1.372 g in 1 mg/L NAA and 0.5 mg/L BAP treatment. Another study conducted by Bhagya *et al.* (2013) reported that a combination of optimal concentration, i.e. NAA 1 mg/L + BAP 0.1 mg/L induced callus from the leaf of *Justicia gendarussa* Burm.f. (Pakistani variant). The difference between the combination of optimal concentration on the research showed that plant also affected the use of plant growth regulators such as the type, concentration and interaction between hormones.

Table 1. Average of fresh and dry weight of callus on gendarussa leaf explants with various combinations of growth regulators 2,4-D and BAP (n=3)

Treatments (mg/L)	Wet Weight of Callus(g)	DryWeight of Callus(g)
$D_{0,5}B_{0,5}$	0.718 ±0.111^{b}	0.061 ±0.006^{ab}
$D_{0,5}B_{1}$	0.263 ±0.020^{a}	0.040 ±0.003^{a}
$D_{0,5}B_{1,5}$	1.500 ±0.444^{ef}	0.091 ±0.005^{cd}
$D_{0,5}B_{2}$	1.230 ±0.117^{cd}	0.078 ±0.008^{abc}
$D_{1}B_{0,5}$	1.373 ± 0.099^{de}	0.078 ± 0.003^{bc}
$D_{1}B_{1}$	0.722 ±0.148^{b}	0.063 ±0.011^{abc}
$D_{1}B_{1,5}$	1.233±0.066^{cd}	0.078 ±0.004^{bc}
$D_{1}B_{2}$	1.156 ±0.067^{c}	0.076 ±0.008^{abc}
$D_{1,5}B_{0,5}$	1.669±0.115^{f}	0.084 ±0.005^{bc}
$D_{1,5}B_{1}$	1.489 ±0.154^{ef}	0.083 ±0.005^{bc}
$D_{1,5}B_{1,5}$	1.321 ±0.200^{cde}	0.107 ±0.022^{cd}
$D_{1,5}B_{2}$	2.247 ±0.044^{g}	0.108 ± 0.005^{d}

Description: Score averages followed by the same letter show no real differences according to Duncan test (α = 0.05). D: 2,4-D, B: BAP.

Callus Morphology (Colour and Texture of the Callus)

Morphology of callus on thecombination of growth regulators 2,4-D and BAP concentration was different as compared to the morphology of the callus on a combination of IBA and BAP concentration. This indicated that the administration of growth regulators (2,4-D, IBA, and BAP) had some effects on callus morphology (colour and texture of the callus). Morphological observation on callus with a combination of 2,4-D and BAP concentration showed that in general the colour change began with a callus turning from yellowish green to yellowish white, then at the end it varied from translucent white, brown, and blackish yellow, however, there was also a white callus that remained unchanged until the end of the observation. The texture of the callus on a combination of 2,4-D and BAP concentration showed callus with friable texture. Morphological observation on callus with a combination of IBA and BAP concentration showed that in general the colour changes that occurred as the callus turned from light green to brownish yellow, then turned into brown or green and brown on the edges of callus contacted with the media. Texture of the callus on a combination of IBA and BAP concentrations showed callus with compact texture.

Table 2. Average of freshand dry weight (g) of callus from gendarussa leaf explants with various combinations of growth regulators IBA and BAP (n=3)

Treatments (mg/L)	Wet Weight of Callus(g)	DryWeight of Callus(g)
$I_{0,5}B_{0,5}$	0.365±0.045^{bc}	0.089±0.011^{b}
$I_{0,5}B_{1}$	0.133±0.033^{a}	0.036±0.008^{a}
$I_{0,5}B_{1,5}$	0.220±0.031^{ab}	0.053±0.004^{a}
$I_{0,5}B_{2}$	0.372±0.020^{c}	0.078±0.015^{ab}
$I_{1}B_{0,5}$	0.254±0.007^{ab}	0.055±0.003^{a}
$I_{1}B_{1}$	0.163±0.039^{a}	0.039±0.004^{a}
$I_{1}B_{1,5}$	0.467±0.103^{c}	0.082±0.011^{b}
$I_{1}B_{2}$	0.224±0.013^{ab}	0.056±0.003^{a}
$I_{1,5}B_{0,5}$	0.216±0.060^{ab}	0.055±0.012^{a}
$I_{1,5}B_{1}$	0.367±0.006^{c}	0.074±0.010^{ab}
$I_{1,5}B_{1,5}$	0.440±0.035^{c}	0.081±0.000^{b}
$I_{1,5}B_{2}$	0.576±0.095^{c}	0.091±0.004^{b}

Description: Score averages followed by the same letter show no real difference by Games-Howell Test (α = 0.05). D: 2,4-D; B: BAP.

Colour changes in callus that occurred during the 6-week observation indicated a change in the growth phase and the regeneration power of the cells. The colour of yellowish green, light green, translucent white, white, yellow, amber or green and brown showed that cells were still actively dividing (cleavage stage), while the colour of brown, yellow, black or brown showed symptoms of aging cells. Rasud (2012) states the colour of callus describes its visual appearance that indicates the activity level of a cell division. George and Sherrington (1984) also state that the callus colour change is caused by the synthesis of phenolic

substances on cells (callus). Phenol compounds appear to be toxic to cells when in excessive concentrations, which will inhibit growth (Hayati *et al.*, 2010). Widayanto (2004) further asserts that a change of colours on callus from yellowish white to brown indicates a decrease in callus cell growth. Such cells have very low cleavage activity so that their regeneration power has lessened.

Based on the six-weekobservation, the callus by combination of 2,4-D 1.5 mg/L and BAP 1.5 mg/L treatment and 2,4-D 1.5 mg/L and BAP 2 mg/L treatment showed colour changes more slowly than other treatments, and translucent white callus maintained its colour until the end of the observation. Meanwhile, callus in a combination IBA 1.5 mg/L and BAP 1.5 mg/L and IBA 1.5 mg/L and BAP 2 mg/L showed colour changes more slowly than other treatments. The colour of green callus, although turning into brown at the edge contacted with the media, were retained until the end of the observation. The result of this study is better than other studies using leaf explants of *Justicia gendarussa* Burm.f. administering growth regulator (NAA, IAA, and BAP), in which callus formed tended to undergo browning and had compact texture on all treatments (Wahyuni *et al.*, 2014). Manuhara (2014) suggested that the white or transparent callus is generally composed of meristematic callus tissue, while the green callus generally indicates a network which has undergone differentiation in embryonic tissue formation.

The texture of the callus is a marker used to determine the quality of callus produced by explant (Rasud, 2012). Friable callus grows separately into smaller parts, cuts off easily, and contains a lot of water (Sitorus *et al.*, 2011). On the contra, compact-type callus has a texture that is not easily cut off and looks solid (Amid, 2011). The good quality of callus texture depends on its purpose. Callus texture may vary from compact to crumble depending on the type of plants used, the composition of the nutrient media, the growth regulators, and the environmental conditions of the culture. In general, callus with friable texture is more embryonic than callus with solid texture (Manuhara, 2014).

Based on the observation of overall callus induction from gendarussa leaf explants treated with plant growth regulators of 2,4-D, IBA and BAP, the majority of parameter analysis showed that the optimal treatment is 2,4-D 1.5 mg/L and

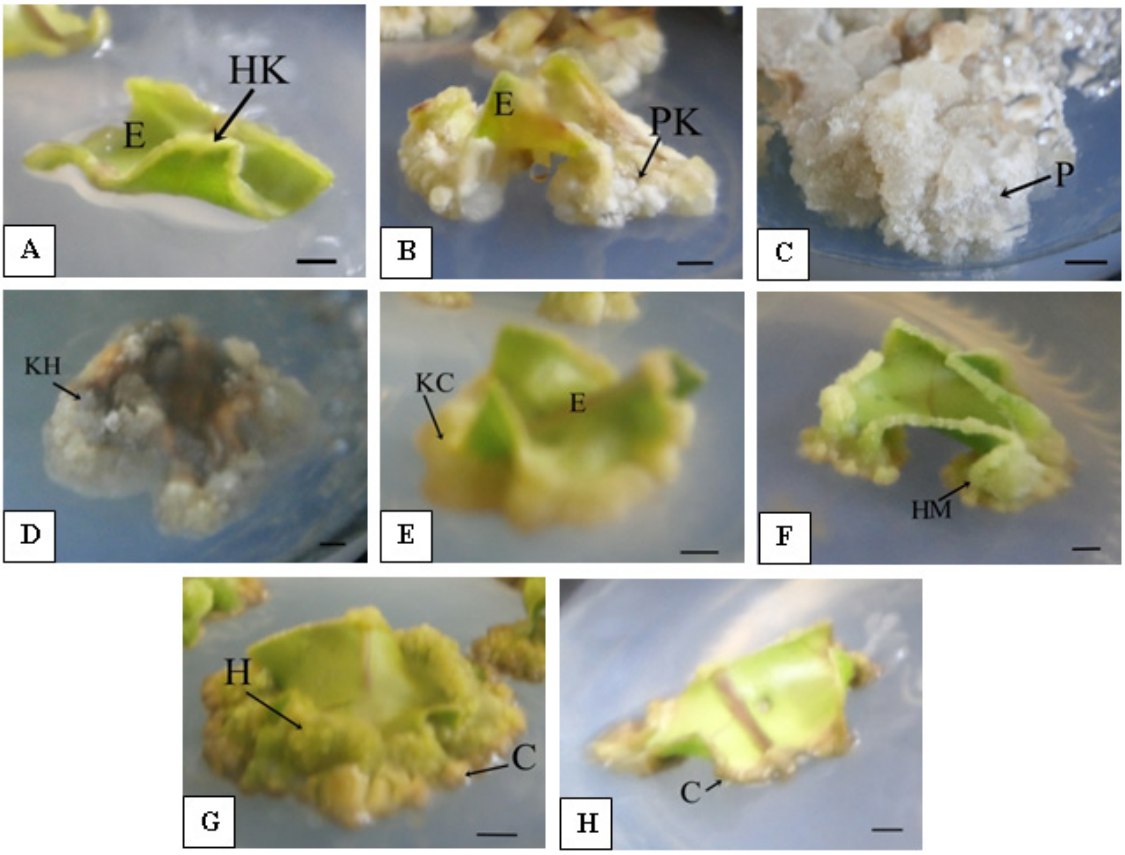

Figure 1. Callus colour (A) HK:yellowish green; (B) PK: yellowish withe; (C) P:transparent white; (D) KH:blackish yellow; (E) HM:light green; (F)KC:brownish yellow; (G) C: green and brown in the outside of callus; (H) C:brown, E: explant. Bar: 2.5 mm.

BAP 2 mg/L. The success of callus culture depends on the use of basic media, the combination of plant growth regulators, and appropriate environmental factors (George and Sherrington, 1992). In addition, Ajijah *et al.* (2010) suggests that the ability of each plant and plant tissue to form callus is not the same. Plant growth regulator widely used for callus induction is the combination of auxin and cytokinin (Zulkarnain, 2009), 2,4-D 3 mg/L + TDZ 1 mg/L optimum for callus induction of *Carica pubescens* Lenne &K.Koch. (Sari *et al.*, 2014), also 2.5 mg/L and Kinetin 0.5 mg/L optimum for callus induction of *Datura metel* (Nurchayati *et al.*, 2016). The findings are consistent with studies conducted by Gao *et al.* (2011) showing that the results of callus induction by the use of a combination of auxin and cytokinin for growth regulators is more effective than a single substance.

The finding of optimum hormone concentration in this study is useful for the production of the next callus. The obtained callus will be optimized for secondary metabolite production using callus culture or cell suspension culture. The obtained callus can also be used for the production of synthetic seeds through indirect embryogenesis. Furthermore, research on the production of secondary metabolites and synthetic seeds of *Justicia gendarussa* Burm.f. has never been done, although the research is very important to provide the needs of compounds as well as the needs of seeds, because the plant hasn't been cultivated.

CONCLUSION

This study concluded that various concentration of plant growth regulators (2,4-D, IBA, and BAP) affected callus induction of gendarussa leaf explants. Interestingly, we found that the most optimal treatment combination of concentration of plant growth regulators in inducing callus from leaf explants of gendarussa is $D_{1.5}B_2$ (1.5 mg/L 2,4-D and 2 mg/L BAP) with a relatively long period of callus formation at the earliest on day 5, 2.247 g of fresh weight and 0.108 gof dry weight, white callus translucent and textured friable. Moreover,optimum hormone concentration will be used to produce callus for secondary metabolite production and synthetic seed production by optimization according to culture purpose.

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