Callus Induction of Justicia Gendarussa Leafexplant (Justicia Gendarussa Burm.f.) with growth regulator 2,4-D, IBA and Kinetin

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Abstract: ***The Justicia Gendarussa (Justicia Gendarussa Burm.f.) is an Indonesian medicinal plant which contains a flavonoid compound that is act as antifertility. The study aimed to understand the effect of growth regulator 2,4-D, Indole Butyric Acid (IBA) and kinetin toward the callus induction from Justicia Gendarussa leaf explant. This study was used factorial design with 24 combinations and 3 replications. The basic medium used were Murshige and Skoog (MS) medium with growth regulator 2,4-D and IBA (0.5 mg/l; 1mhg/l; 1.5mg/l) with kinetin (0.5mg/l; 1mg/l; 1.5mg/l; 2mg/l). The data analysis was used two ways multivariate Anova and Duncan test. The result showed growth regulator 2,4-D with kinetin had longest time of the formation on 5th ,6th and 7th days with 100% callus formation. The best weight of callus with growth regulator 2,4-D with 1.5 mg/l and 1.5 mg/l of kinetin (1.8147 ±0.1147 g), while best dry weight of callus with 1 mg/l of growth regulator 2,4-D with 0.5mg/l (0.1240 ±0.0277 g) which callus had brown colour and crust texture. Meanwhile, the combination of IBA with kinetin had longest time of callus formation was at 6th and 7th day with 100% callus formation. The highest weight loss of callus in growth regulator with IBA 1mg/l with 1.5 mg/l of kinetin (0.4688±0.0958 g), while best callus dry weight in IBA 1 mg/l with 2 mg/l of kinetin (0.0895 ± 0.0089 g) with brown callus colour and compact texture. These results had proved growth regulator 2,4-D with kinetin and IBA with kinetin was able to induce callus on Justicia Gendarussa leaf explant.***

I. INTRODUCTION

Justicia Gendarussa is referring to the Acanthaceae family which use in traditional medicine for headache, fever, rheumatism, myalgia, back pain and respiratory disorders [1,2]. The chemical content in the leaves such as O-disubstituted aromatic amines, 2-(2'aminobenzyl) amino benzyl alcohol, 2-aminobenzyl alcohol, O-methyl ethers, friedelin, lupeol and β-sitosterol [3]. Justicia Gendarussa plant are used by Indonesians as pain medication, gout, headache, lumbago, abscess, bruises, sprains and rheumatic.

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In India, the traditional plant was done through seeds and cuttings. The weakness of this technique are low seed germination rate, limited plant sources and genetic variation [4]. Meanwhile, plant propagation technique which can use to provide modern crops is plant tissue culture. Plant tissue culture is plant propagation technique by taking plant parts such as tissues. organs or cells then cultured on the sterile, artificial medium which part of the plant are able to regenerate and differentiate into completed plant.

The tissue culture technique is more efficient for the plant propagation since required large crop quantities in short time space, small area, reduce plant damage risk by the pest or diseases and obtains secondary metabolites in a short time [5,6]. The in vitro culture technique developed is callus culture technique. The callus is continuing cell division and uncontrollably to form unorganized mass of the cells. The callus culture is generally intended to take secondary metabolites and enzymes from the plant as seeds for vegetative propagation of plants and to obtain plant resistant toward virus or fungi. Higher chances in getting large quantities seeds as more callus is formed.

The growth regulator has important role in the growth and plant division in in vitro cultures. The growth regulator substances widely used in in vitro cultures are auxin and cytokines. Auxin is phytohormones which play roles in elongation induction and cell division. Indole Butyric Acid (IBA) is a synthesis auxin which more stable chemical properties and low plant mobility. In 2,4-Dichlorophenoxy Acetic Acid (2,4-D) is more stable than auxin since it's not readily decomposed by releasing enzyme by the plant cells or by heating in the sterilization process. Meanwhile, adenine derivatives play roles in encouraging cell division and stimulating multiplication of the buds. Kinetin is preferred cytokinin type due to its resistance toward degradation and cheaper.

Several studies were related to the callus induction for Justicia Gendarussa Brum.f. had been done with several growth regulator. Fadilah[7] had mentioned callus was formed at 0.5 mg/l NAA with 1.5 mg/l BAP. Ayob[8] found maximum callus formation was obtained at concentration of 1 mg/l growth regulator and 3 mg/l kinetin. Meanwhile, Armid et al. [9] found maximum callus formation at concentration of 1 mg/l NAA and 0.5 mg/l BAP [9].

Besides, Janarthanam et al. [10] found optimum callus induction from Justicia gendarussa Burm.f. with combination of 0.5 mg/l BAP and 10% coconut water.

There is an increment in total concentrations of phenolics, flavonoids, alkaloids and phytosterols in callus-rich cultures compared with native plants [11]. Therefore, callus culture can be considered as better method for secondary metabolite production. In general, there is limitation in callus formation in Indonesia and it's important for production development of beneficial materials especially secondary metabolites. The study aims was to understand the effect of growth regulator 2,4-D, Indole Butyric Acid (IBA) and kinetin toward the callus induction from Justicia Gendarussa leaf explant.

II. METHODOLOGY

The study had been conducted at Plant Physiology Laboratory, Faculty of Science and Technology, Airlangga University within four months from January until April 2013. In this study, Justicia Gendarussa leaves were used (Justicia Gendarussa Burm.f.) from 2nd to 4th leaf from the shoots. This plant was obtained from Health Office of East Java Province, House of Materia Medica, Batu City, Malang.

Meanwhile, the chemical had been used were Murashige and Skoog (MS) medium included 2,4-DIchlorophenoxy Acetic Acid (2,3-D), Indole Butyric Acid (IBA) and kinetin, Indole Butyric Acid (IBA) and kinetin,spirtus,steriale aqueous, clorox for sterilization of explant as well as alcohol for space sterilization.

The tools included autoclave, Laminar Air Flow (LAF), analytical scales, electric stove, culture bottle, petri dish, beaker glass, Erlenmeyer flask, pipette, scapel, tweezers, measuring cup, Bunsen, umbrella paper and aluminium foil.The micronutrient stock solutions preparation was carried out by prepared 100ml of supply with weighing and dissolving one by one of micronutrient material in MS medium into 200ml of Erlenmeyer flask contains approximately 80 ml of aqueous, while homogenizing using magnetic stirrer until the solution became colourless and add aqueous until 100ml. The Erlenmeyer was covered with aluminium foil and labelled MIKRO MS100X, 1 ml/l, then deposited micronutrient stock solution in the refrigerator.

The growth regulator 2,4-Dichlorophenoxy Acetic Acid (2,4-D) and 100ml IBA by weighed on 10mg in each growth regulator and dissolved in a few drops of KOH1N. The solution became colourless and aqueous was added into 100ml. The stock was kept in refrigerator. The kinetin was carried out by weighed 10 mg of kinetin and dissolved in few drops of HCL1N. The solution became colourless and the aqueous was added until 100ml with continuously stirred by using magnetic stirrer. The stock solution was kept in the refrigerator.

The Justicia Gendarussa explant planting for callus induction was done by cutting the leaves with an area approximately 1 cm2 and disposed the edge leaves. The leaves were placed into culture bottle contained callus bottle which covered with aluminium foil. The sample was maintained in incubation room at temperature, 25C±2C with 20 watt TL filter discharge.

The independent variables were growth regulator 2,4-D, IBA and kinetin with various concentration. The growth regulator 2,4-D and IBA concentrations were 0.5 mg/L, 1mg/l and 1.5 mg/l, while kinetin concentrations were 0.5 mg/l, 1mg/l, 1.5 mg/l ad 2 mg/l. The dependent variables consisted callus duration (days), callus formation percentage, wet callus weight and dry callus weight (gram) and callus morphology (colour and texture).The control variables included the explant, pH, light intensity, medium and temperature.

The qualitative data was analyzed descriptively with obtain data description. Meanwhile, quantitative data was in form of wet callus weight and dry callus weight were measured using the statistical test. The statistical test used were normality and homogeneity test (Kolmogorof-Smirnov). Furthermore, the abnormal distributed data would with non-parametric test (Kruskal Wallis and Mann Whitney test) and if the data was normally distributed would continued by parametric test (Anova).

III. RESULT AND DISCUSSION

The combination of growth regulator 2,4-D with kinetin on Justicia Gendarussa leaves explant with various concentration had influenced on wet callus weight and dry callus weight.Table 1 showed each treatment had mean wet and dry callus weights. The highest wet callus weight was obtained at concentration factor of 1.5 mg/l growth regulator with 1.5 mg/l kinetin which was 1.8147±0.1147 g. The lowest wet callus weight was gained at concentration factor of 0.5 mg/l growth regulator with 2 mg/l kinetin for 1.1000± 0.1276 g. Meanwhile, highest dry callus weight was gained at concentration factor of 1 mg/l growth regulator with 0.5 mg/l kinetin (0.1240±0.0227 g) and lowest of dry callus weight was gained at concentration factor of 1.5 mg/l growth regulator with 2 mg/l kinetin (0.0550±0.0149 g).

Table. 1 Mean of wet and dry callus weight based on combination of growth regulator 2,4-D with kinetin

Callus weight (g)		Callus weight (g)	
Growth regular 2,4-D	**Kinetin**	**Wet**	**Dry**
0.5	0.5	1.6311 ± 0.1571^{de}	0.1014 ± 0.0117^{de}
0.5	1.0	1.1304 ± 0.1025^{a}	$0.0677 \pm 0,.0138^{abc}$
0.5	1.5	1.483 ± 0.1500^{bcd}	0.0782 ± 0.0118^{abcd}
0.5	2.0	1.1000 ± 0.1276^{a}	0.0846 ± 0.018^{bcd}
1.0	0.5	1.4762 ± 0.0791^{bcd}	0.1240 ± 0.0277^{e}
1.0	1.0	1.6084 ± 0.3110^{cde}	0.0860 ± 0.0115^{bcd}

1.0	1.5	1.1888 ± 0.0881[a]	0.0754 ± 0.008[abcd]
1.0	2.0	1.3042 ± 0.0739[ab]	0.0903 ± 0.0128[cd]
1.5	0.5	1.1070± 0.0818[a]	0.0596 ± 0.0045[ab]
1.5	1.0	1.4591 ± 0.1648[bc]	0.0600 ± 0.0091[ab]
1.5	1.5	1.8147 ± 0.1147[e]	0.0682 ± 0.0173[abc]
1.5	2.0	1.7348 ± 0.1126[de]	0.0550 ± 0.0149[a]

Meanwhile based on Table 2, Kolmogorov-Smirnov test showed normal distributed since p wet weight = 0.923 > 0.05 and p dry weight = 0.798 > 0.05. The data also tested homogeneity with Levene test resulted in homogenous data p wet weight =0.190 and p dry weight = 0.182 >0.05. Meanwhile, two ways Anova multivariate test indicated combination of growth regulator 2,4-D with kinetin affected on wet and dry callus weights, (p=0.00< 0.005).

Table. 2 Statistical analysis of wet and dry callus weight based on combination of growth regulator 2,4-D and kinetin Normality One-Sample Kolmogorov Smirnov Test One-Sample Kolmogorov Smirnov Test

		Residual for wet weight of growth regulator 2,4-D	**Residual for dry weight of dry weight of growth regulator 2,4-D**
N		36	36
	Mean	0.0000	0.0000
	Standard deviation	0.1195	0.1182
	Absolute	0.092	0.108
Most Extreme Differences[a,b]	**Positive Negative**	0.092 -0.080	0.108 -0.108
Kolmogorov Smirnov Z		0.550	0.646
Asymp. Sig. (2-tailed)		0.923	0.798

a. Test distribution is normal
b. Calculated from data

Table 3 showed combination of IBA and kinetin with various concentration factors had different mean of wet and dry callus weight. The highest mean wet callus weight was obtained at concentration factor of 1mg/l IBA with 1.5 mg/l kinetin (0.4688±0.0958) and lowest wet callus weight was gained at concentration factor of 1.5 mg/l IBA with 1 mg/l kinetin (0.1509±0.0275 g). Meanwhile, highest dry callus weight was 0.0895 ± 0.0089 g which the combination concentration was 1 mg/l IBA with 2 mg/l kinetin and lowest dry callus weight was 0.0617±0.0042 g at combination concentration 1 mg/l IBA and 1 mg/l kinetin.

Table. 3 Mean wet and dry callus weight based on combination of IBA and kinetin

Concentration (mg/l)		**Callus weight (g)**	
IBA	**Kinetin**	**Wet**	**Dry**
0.5	0.5	0.2871 ± 0.0228[b]	0.0633 ± 0.0028[ab]
0.5	1.0	0.2999 ± 0.0060[b]	0.0603 ± 0.0101[ab]
0.5	1.5	0.3785 ± 0.0416[bcd]	0.0678 ± 0.0153[ab]
0.5	2.0	0.2689 ± 0.0242[b]	0.0560 ± 0.0044[a]
1.0	0.5	0.3472 ± 0.0655[bc]	0.0681 ± 0.0119[ab]
1.0	1.0	0.2989 ± 0.0571[b]	0.0617 ± 0.0042[ab]
1.0	1.5	0.4688 ± 0.0958[d]	0.0848 ± 0.0093[c]
1.0	2.0	0.4222 ± 0.0618[cd]	0.0895 ± 0.0089[c]
1.5	0.5	0.4469 ± 0.0269[d]	0.0741 ± 0.0079[bc]
1.5	1.0	0.1509± 0.0275[a]	0.0658 ± 0.0090[ab]
1.5	1.5	0.3488 ± 0.0135[bc]	0.0646 ± 0.0042[ab]
1.5	2.0	0.4500 ± 0.0576[d]	0.0744 ± 0.0029[bc]

Based on Table 4, the Kolmogorov Smirnov showed normal distributed since p wet weight = 0.953 > 0.05 and p dry weight = 0.996> 0.05. The Levene test showed p wet weight =0.128 and p dry weight =0.081>0.05. Meanwhile, two-way Anova Multivariate test indicated combination of IBA with kinetin had influenced on wet and dry callus weight because p wet weight =0.00<0.05 and p dry weight = 0.002< 0.05.

Table. 4 Statistical analysis of wet and dry weight callus weight based on combination IBA with kinetin Normality One-sample Kolmogorov Smirnov test One-sample Kolmogorov Smirnov test

		Residual for wet weight of IBA	Residual for dry weight of dry weight of IBA
N		36	36
	Mean	0.0000	0.0000
	Standard deviation	0.0437	0.0070
	Absolute	0.086	0.068
Most Extreme Differences[a,b]	**Positive**	0.086	0.059
	Negative	-0.068	-0.068
Kolmogorov Smirnov Z		0.516	0.408
Asymp. Sig. (2-tailed)		0.953	0.996

a. Test distribution is normal.
b. Calculated from data.

IV. DISCUSSION

The callus induction formation is an important step in plant tissue culture which through this process until secondary metabolite production had been done. The growth regulator helps stimulate the callus formation. The callus formation was caused by contact cells with the medium and convert to meristematic and actively had division such as tissue cover the wound.

The result showed the callus was formed in formed in first week in both, combination of growth regulator 2,4-D with kinetin and combination of IBA with kinetin. The combination of growth regulator 2,4-D with kinetin had rapid induced callus at concentration of 0.5mg/l growth regulator 2,4-D with 0.5mg/l, 0.5 mg/l growth regulator 2,4-D with 1 mg/l kinetin, 0.5mg/l growth regulator 2,4-D with 1.5mg/l kinetin, 1 mg/l growth regulator with 0.5 mg/l which showed the callus growth on 5th day. Meanwhile, combination of IBA and kinetin showed the rapid inducing callus on the concentration of 0.5 mg/l IBA with 1.5 mg/l kinetin, 0.5mg/l IBA with 2 mg/l kinetin, 1 mg/l IBA with 1 mg/l kinetin, 1 mg/l IBA with 1.5 mg/l kinetin, 1 mg/l IBA with 2 mg/l kinetin and 1.5 mg/l IBA with 0.5 mg/l kinetin which observed callus growth on 6th day. Besides, most callus were formed on 7th day which involved all factors of combination concentration.

These results were different from Ayob[8] that showed rapid callus formation induced from Justicia Gendarussa explants with combination of growth regulator 2,4-D with kinetin at 2nd weeks. Meanwhile, Fadilah [7] had proved that callus induction from Canna explants was able to induce callus on 5th day with combination of 1 mg/l 1-Naphthaleneacetic acid (NAA) with 1.5 mg/l 6-Benzylaminopurine (BAP) [7]. The study by Rout and Sahoo [12] showed callus induction from Elephantopusscaber L. (Elephant's foot) was induced callus on 12th day with combination of growth regulator 2,4-D with kinetin.

All combination of growth regulatory concentrations had been able induced the callus in short term time which indicated all of these treatments had same ability to induce callus formation. The different treatments had observed on differences of response toward the callus formation which indicated by the leaves strand that begun to be curve, widen, swollen and followed by the callus growth on the leaf edge.

The callus had observed the discoloration and growth in each week.

The explant was growing on the MS medium with combination of growth regulator 2,4-D with kinetin which observed bended at sliced mark location on 3rd and 4th day and followed by callus formation on 5th day until 7th day. Meanwhile, combination of IBA and kinetin had observed explant was bended at the sliced mark on 4th and 5th days and followed the callus formation on 6^{th} and 7^{th} days. This observation was due to explant had absorbed the water from the medium in initial stage of growth process that resulted in enlarged cells. This stage the callus was not observed since explant was in lag phase which phase adaption with the medium. The callus formation was observed on 5th until 7th days which marked by white bulges on the sliced area.

The bended leaf strands are caused by the auxin influence and turgor pressure. The auxin present leads cell wall to be stretched. This cell wall loosening occurred due to secretion by activating an enzyme at certain pH. This enzyme will break the bonds between cellulose molecules in the cell wall. Turgor pressure occurs when the cell absorbs water molecules in response to dissolve growth regulator concentration in the vacuole [13].

The interaction and balance between growth regulating substances which provided to the medium and produced by the plant cells endogenously determined the direction of the culture development. The callus induction on Justicia Gendarussa Burm.f. with growth regulator 2,4-D and kinetin, IBA with kinetin showed explant were able to produce 100% of callus.

The highest wet callus weight was 1.8157 ±0.1147 with concentration factors of 1.5mg/l growth regulator 2,4-D with 1.5 mg/l kinetin and lowest callus weight was 0.1000±0.1276 g with combination of 0.5 mg/l with 2 mg/l kinetin. Meanwhile, highest wet callus weight was 0.4688±0.0958 g with combination of 1.5mg/l IBA with 1 mg/l kinetin and lowest wet callus weight was 0.1509±0.0275 g with combination 1.5 mg/l with 1 mg/l kinetin. These results suggested the combination of growth regulator 2,4-D with kinetin had produce higher wet callus weight than combination of IBA with kinetin for Justicia Gendarussa leaf explant.

Th callus ability to absorb and store water also affected by the callus texture. The cells located on the outer layer was easier to absorb the water than cells located in inner layer. Besides, uneven callus texture also caused certain cells are unable to contact with the medium especially

inner callus cells. the callus cells which had larger vacuoles would store more water than cells with small vacuoles. Meanwhile, lowest wet callus weight was produced from the combination of growth regulator due to explant only experienced callus formation in some explant surface [14]. The callus formation on some explant surfaces was also associated with relatively long initiation time of callus due to imbalance of combination of growth regulator 2,4-D with kinetin and IBA with kinetin which beginning treatment is suspected had less interaction with each other, hence explant metabolism process is disrupted.

The dry callus weight is measured by measured by drying to remove water content and stop metabolic activity in the material until constant callus weight is obtained. In this study, highest dry callus weight was 0.1240±0.0277 g with combination of 1 mg/l growth regulator 2,4-D with 0.5 mg/l kinetin and lowest dry callus weight was 0.0550 ± 0.0149 g at concentration factor of 1.5 mg/l growth regulator and 2 mg/l kinetin. Meanwhile, highest dry weight was 0.895±0.0089 g with combination of 1 mg/l IBA and 2 mg/l kinetin and lowest dry callus weight was 0.0617±0.0042 g at concentration factor of 1 mg/l IBA and 1 mg/l kinetin. These results showed dry callus weight based on combination of growth regulator 2,4-D with kinetin was greater than combination of IBA with kinetin. Besides, difference of wet and dry callus weights was influenced by the medium which combination treatment of auxin and cytokinin with various concentration factor so that different result would obtain from the culture explant.

V. CONCLUSION

In conclusions,treatments combination included growth regulator 2,4-D with kinetin and IBA with kinetin had affected the callus induction duration (days) and percentage of callus formation on Justicia Gendarussa leaves explant. Besides, both combinations also had affected on the morphology (colour and texture), wet and dry callus weight. The brown and crumb callus texture was observed in combination of growth regulator 2,4-D with kinetin. Meanwhile, the green and compact texture were observed in combination of IBA with kinetin. The highest wet callus weight was 1.4762±0.0791 g and dry callus weight was 0.1240±0.0277 g at concentration factor of 1 mg/l growth regulator 2,4-D with 0.5 mg/l kinetin at 5th days and 100% of callus formation. In combination of IBA with kinetin, wet callus weight was 0.4222±0.0618 g and dry callus weight was 0.0895±0.0089 g with 1 mg/l IBA and 2 mg/l kinetin at 6th days and 100% of callus formation.

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