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Induction and Growth of Endosperm Callus of Rimau Gerga Lebong (RGL) Citrus on Several Media Composition

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ABSTRACT

Rimau Gerga Lebong (RGL) variety is one of the main orange fruit commodities in Lebong Regency of Bengkulu Province, which has a competitive advantage and has good market potential. However, high number of seed characteristic makes this orange fruit becomes less popular. Triploid genotype formation through endosperm culture in vitro is an alternative solution to develop seedless orange fruit. The objective of this study was to determine the best composition medium for callus induction of endosperm of RGL orange seeds as the foremost step of in vitro triploid plant development. The research was conducted from August 2017 until December 2018 at the Plant Tissue Culture Laboratory, Department of Agronomy, Faculty of Agriculture, University of Bengkulu. The experiment was arranged in a completely randomized design, with eight treatment combinations i.e. G1 (MT + 5 ppm BAP + 2 ppm 2,4-D), G2 (MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH), G3 (MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm + 0.5 ppm Kinetin), G4 (MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0.5 ppm to 500 ppm Kinetin + ME) , G5 (MS + 5 ppm BAP + 2 ppm 2,4-D), G6 (MS + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH), G7 (MS + 5 ppm BAP + 2 ppm 2,4- D + 500 ppm CH + 0.5 ppm Kinetin), G8 (MS + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0.5 ppm to 500 ppm Kinetin + ME), with three replications. The experimental unit was five culture bottles containing three explants per bottle. Observations were conducted on days to callus formation, rate of callus formation, callus weight, callus diameter, callus color and callus texture. The results showed that media of MS + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH was the best media for callus induction, and MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH was the best media for callus development.

INTRODUCTION

Citrus is horticultural commodities that serve as a source of nutrition, a source of income, and a source of foreign exchange. The contribution of citrus agro-industry in increasing revenue will foster new citrus development centres. The availability of superior varieties, both quality and productivity by consumer needs, is an absolute that must be met in the global free market. Achieve a balance between demand and supply; national citrus production needs to be increased continuously (Karsinah et al., 2002).

Production of tangerines in Indonesia in the last five years fluctuated, in 2012 the production of tangerines amounted to 1,498,396 tons in 2013 of 1,548,401 tons in 2014 amounted to 1,785,264 tons in 2015 reached 1,744,339 tons and in 2016 reached

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2,014,214 tons, while in Bengkulu province the production of tangerines in 2012 amounted to 10,319 tons in 2013 amounted to 9,440 tons, in 2014 7,263 tons in 2015 increased again by 9,049 tons and in 2016 it decreased by 7,169 tons (BPS, 2017).

One type of local tangerine developed in Bengkulu Province is the Gerga Lebong orange which is now registered with the name of the orange variety Rimau Gerga Lebong or RGL (Kementan, 2012). The orange is a superior commodity of Lebong Regency because it has a competitive advantage that is fruiting throughout the year, namely the fruit is yellow-orange, large fruit size 200-350 grams, high juice content and has good market potential. RGL oranges bear fruit all the time, one tree exists 4-6 generations, in one tree, there are flowers, young fruit until the fruit is ready for harvest (Rambe et al., 2012).

The obstacle in the development of local tangerines is the character of the fruit with a large number of seeds so that they cannot compete with imported products. One way to get triploid plants is by inducing endosperm tissue. The endosperm is a plant tissue that is triploid because it comes from the fertilisation of two polar nuclei and one sperm (Berger, 2003).

Research to arrange triploid plants made from endospermic tissue has been carried out on the citrus of Siem Simadu (Husni et al., 2010), inter -cyclone Siem cross oranges with tangerines and large oranges (Sunyoto et al., 2010), Lemon Ali and Mirza (2006)), Katsuri orange (Mahadi et al., 2016) but no one has done it on RGL citrus varieties. Based on this, this research needs to be done to get the citrus triploid plants of RGL varieties through endosperm tissue. This study aims to obtain the best media composition for the induction and development of endosperm tissue callus varieties of Rimau Gerga Lebong (RGL).

MATERIALS AND METHODS

This research was conducted from August 2017 to December 2017 in Laboratory of the Division of Biotechnology and Plant Tissue Culture, Department of Crop Production Laboratory of the , Faculty of Agriculture, University of Bengkulu. The material used was RGL orange originated in one of the Lebong Regency farmers' gardens which was 11-13 weeks after the anthesis and ± 35 mm in diameter. The media used are those commonly used for citrus culture, namely MT (Murashige Tucker) and MS (Murashige Skoog) which were modified.

This experiment uses Complete Randomized Design, with eight treatments (Table 1). Each experimental unit consisted of MS and MT media which treated with Plant Growth Regulator (PGR) according to the treatment combination, which was repeated three times with 5 sample bottles, each composed of 3 explants in one bottle.

Table 1. Composition of callus induction media from endosperm tissue of oranges RGL

Notation	Treatment
G1	MT + 5 ppm BAP + 2 ppm 2,4-D
G2	MT + 5 ppm BAP + 2 ppm 2,4-D +
G3	MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0,5 ppm Kinetin
G4	MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0,5 ppm Kinetin + 500 ppm ME
G5	MS + 5 ppm BAP + 2 ppm 2,4-D
G6	MS + 5 ppm BAP + 2 ppm 2,4-D +
G7	MS + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0,5 ppm Kinetin
G8	MS + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0,5 ppm Kinetin + 500 ppm ME

The observed variables were time callus formed (DAP), percentage of explants formed callus (%), callus color, callus texture, callus weight (g), callus diameter (mm). The Observation data were analysed using analysis of variance at the F level of 5%. If the data is not enough for statistical analysis, then the calculation is done descriptively with picture illustrations. Determination of the best media to induce and callus development based on rank (scoring) and callus growth variables, the percentage of explants forming a callus, callus weight, and callus diameter, callus color, and callus texture.

RESULTS AND DISCUSSIONS

When the 30 DAP induction treatment begins to show a response to the planted explant, it is first seen through a change in color from the

explant from transparent to milky white. Endosperm cells then divide and are characterized by an increase in explant volume, then explants form a callus. The treatment of G1 (MT + 5 ppm BAP + 2 ppm 2,4-D) and G5 (MS + 5 ppm BAP + 2 ppm 2,4 -D) did not show a response until 12 weeks after culture (Table 2). In line with the results of Graitter et al. (1990) which showed that sweet orange endosperm lacked response to MT or MS base media added by BAP, but strongly responded to the two primary media when combined with kinetin and Malt Extract. The results of the test analysis at the 5% level indicate that the requirements for review are not enough so that the difference in the average value ± standard error.

Table 2. Effect of media composition on induction and development of endosperm callus Orange Rimau Gerga Lebong (RGL)

Media	Variable Observed			
	TCF (DAP)	PEFC (%)	CW (g)	CD (mm)
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
G1	-	-	-	-
G2	37,2 ± 0,6	63,0 ± 7,4	0,59 ± 0,1	1,85 ± 0,1
G3	35,5 ± 0,6	48,1 ± 8,1	0,47 ± 0,0	1,50 ± 0,1
G4	36,8 ± 0,3	38,9 ± 5,6	0,26 ± 0,0	1,23 ± 0,1
G5	-	-	-	-
G6	36,2 ± 0,6	86,7 ± 9,9	0,58 ± 0,1	1,33 ± 0,1
G7	36,5 ± 0,5	33,3 ± 0,0	0,49 ± 0,0	1,05 ± 0,2
G8	37,0 ± 0,0	33,3 ± 0,0	0,52 ± 0,0	1,71 ± 0,0

Notes : TCF = Time Callus Formed; PECF = percentage of explants formed callus; CW= callus weight; CD = callus diameter, DAP= Day After Planting,; SE = Standard Error

Effect of Media Composition on Callus Induction

Based on observations it can be seen that the treatment combination of G3 media is the fastest yielding mean while growing callus with an average of 35.5 DAP followed G6 treatment with an average of 36.2 DAP, G7 is 36.5 DAP, G4 with an average of 36.8 DAP and G8 has an average of 37.0 DAP, while G2 has the longest average when growing callus which is 37.2 DAP (Table 2).

In line with research Sunyoto et al. (2010) callus formation in oranges as a result of cross-Siam crossing with tangerines and large oranges, the composition of the best growing media to stimulate callus formation was MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0.5 ppm Kinetin + 500 ppm ME. Casein Hydrolysate and Malt extract are substances that can stimulate callus formation. Kinetin is a substance that acts to encourage cell morphogenesis. The addition of kinetin causes the transcription and translational phase of RNA to take place rapidly (Wijayani 2002).

The provision of complex organic compounds and the addition of cytokinins in the callus induction have a significant effect on callus formation. The media content of cytokines and auxins do not have an impact on explants, but according to Rahmi et al. (2010) in Kanchi citrus addition of growth regulators is one way that can be done to be able to induce and increase callus growth in culture media. Low concentration of 2,4-D can stimulate cell division and callus formation, especially in dicotyledonous. Cytokinins (benzyl amino purine /BAP) encouraged cell division in small amounts (Mahadi et al., 2014).

The embryogenic callus that results from combining auxin and cytokinin have a good influence on callus formation and growth and will affect secondary metabolite production (Bienaime et al., 2015). The highest percentage of explants forming callus was obtained from the G6 treatment (MS + 5 ppm BAP + 2 ppm 2,4-D by adding 500 ppm CH) followed by G2 (MT + 5 ppm BAP + 2 ppm 2,4-D) with the addition of 500 ppm CH (Table 2). Similar results have been reported by Kosmiatin et al. (2014) with MS, and MW vitamin modification media can induce somatic embryogenesis from the endosperm tissue of Siam orange (Citrus nobilis Lour.) Cv Simadu with the highest percentage of embryogenesis callus formation obtained from the addition of 500 ppm CH, that is 84.0%.

MS media with modification of 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH has the highest average of 86.7% followed by primary MT media with modification of 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH which is 63 0% (Table 2). The very different percentages of explants indicate that the diversity in explants

is high, due endosperms are tissues that have different stages of cell development and cell function so that each cell responds differently to media formulations (Kosmiatin, 2013).

The treatment of G1 (MT + 5 ppm BAP + 2 ppm 2,4-D) and G5 (MS + 5 ppm BAP + 2 ppm 2,4-D) without CH, Kinetin and ME did not influence the percentage of explants forming callus while CH singly gives the best results compared to combined with Kinetin and ME. This is caused by the addition of excess auxin, kinetin and amino acid Malt Extract resulting in G3, G4, G7, G8 no better than G6 and G2 treatment.

Casein hydrolysate (CH) and malt extract (ME) are complex organic compounds which are a collection of many amino acids that cannot be clearly defined in their composition (Nair, 2008). High availability of organic material in the media can increase the accumulation of amino acids as a constituent of proteins so that the accumulation of proteins increases, the formation of proteins in cells, especially protein storage is needed to form somatic embryos (Deo et al., 2010).

The best media to induce callus is G6 medium with MS + 5 ppm BAP composition + 2 ppm 2,4-D + 500 ppm CH on the callus growing variable with a score of 5 and on the variable explant percentage forming a callus with a score of 6 so as to obtain the highest score of 11 (Table 3).

Table 3. Determination of the best media to induce endospermic callus tissue

Media	Time Callus Formed		Percentage of explants formed a callus		Total Scoring
	Rank*	Score	Rank*	Score	
G1	-	-	-	-	-
G2	6	1	2	5	6
G3	1	6	3	4	10
G4	4	3	4	3	6
G5	-	-	-	-	-
G6	2	5	1	6	11
G7	3	4	5	2	6
G8	5	2	6	1	3

Notes: * = *Rank based on the best means value*

Effect of Media Composition on Callus Development

Callus weight per explant shows the variation in weights between treatment media due to different media compositions (Table 1). The biggest callus weight was produced by G2 that is 0.59 g followed by G6 that is 0.58 g while the lowest callus weight was produced in the G4 weighing 0.26 g. Media contain 2,4-D, and BAP did not produce callus. According to Rahayu et al. (2003), a large callus weight is due to the high water content in the callus. The resulting wet weight is very dependent on the speed of the cells to divide, multiply and proceed with the enlargement of the callus.

The size of the callus diameter G2 is greater and significantly different from other treatments.The average callus G2 was 1.85 mm while for G3 is 1.50 mm, G4 is 1,23 mm, G6 is 1.33 mm, G7 measuring 1.05 mm and G8 measuring 1.71 mm. This shows that callus cells in the G2 treatment were differentiated faster than the other seven treatments (Table 2).

Optimal cell division in the treatment media G2 (MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH) will cause optimal callus growth, thus the faster the cells utilise growth hormone, the faster the number of cells increases, this will increase the diameter of the callus. Callus size produced in each treatment media is different, this is because by the ability of tissues to absorb water and nutrients, namely the ability to hold the process of diffusion, osmosis and turgor cell pressure (Sriyanti, 2000).

The observation of color callus using the Munsell Color Chart and it is doing in the last week. Callus produced from each treatment found the color and on average produced the same color, ranging from yellowish-white to dark green. In the treatment of G1 and G5 no color changes occurred in explants, the treatment media G2 produced a yellowish-white color on Munsell listed 8/4 2.5Y. In the treatment of G3 produced a Yellow callus is 7/6 2.5Y, on G4 which is 6/6 2.5Y brownish yellow, G6 produced 8/6 colors 2.5Y yellowish white. Yellowish green color is produced from G7 which is 8/4 2.5GY and G8 produced a green color, on Munsell it is listed 7/10 2.5GY (Figure 1).

The color of callus is green. The quality of the callus indicates the presence of chlorophyll in the tissue so that the green the color of the callus, the more chlorophyll content (Fatmawati,

2008). The G4 media (MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0.5 ppm Kinetin + 500 ppm ME) produced callus (with a brownish color. The metabolism of phenol compounds was toxic, which often aroused due to the error process in explant sterilization could inhibit growth or even causes tissue death (Yusnita, 2003).

Callus texture is one of the markers used to assess the quality of a callus. There were three types of the callus texture, namely compact (non-friable), intermediates, and crumbs (friable). The compact callus is a callus which is composed of nodular-shaped cells, with a dense structure and contains quite a lot of water (Manuhara, 2001). Compact callus is due to differences in the ability of plant tissue to absorb nutrients and growth regulators in the initiation media (Ibrahim, 2010). Based on observations, callus textures formed in each treatment are generally compact in texture and with varying colors such as seen in Figure 1.

Callus with crumb texture resulted from G2 and G8 while for G3, G4, G6, G7 is compact, for treatment of G1 and G5 are not formed (Table 4). Crumb callus texture occurs faster cell division than compact callus texture. The compact callus has a texture that is difficult to separate and looks solid Fitriani (2008). It was caused by the formation of lignification so that the callus becomes hard, which is a cytokinin effect that plays a role in nutrient transport (Mahadi et al., 2016) whereas the partially compact and crumb callus is called intermediate (Widiarso, 2010). Embryogenic callus cells have crumbly and easily decomposed callus texture, and callus color is white to yellow, it is not easy for the oxidation of phenolic substances so that the cell is easy to multiply (Mahadi, 2012). In line with research by Mahadi et al., (2016) for crumb callus which has faster callus growth rate and cell division, it is very suitable to be used as cell suspense culture to produce secondary metabolite material (Mahadi et al., 2016).

Table 4. Callus texture of RGL endosperm tissue

Media	Observation variable Callus texture
G1	-
G2	Crumbs, Yellowish White
G3	Compact, yellow
G4	Compact, Brownish Yellow
G5	-
G6	Compact, Yellowish White
G7	Compact, Yellowish Green
G8	Crumbs, Green

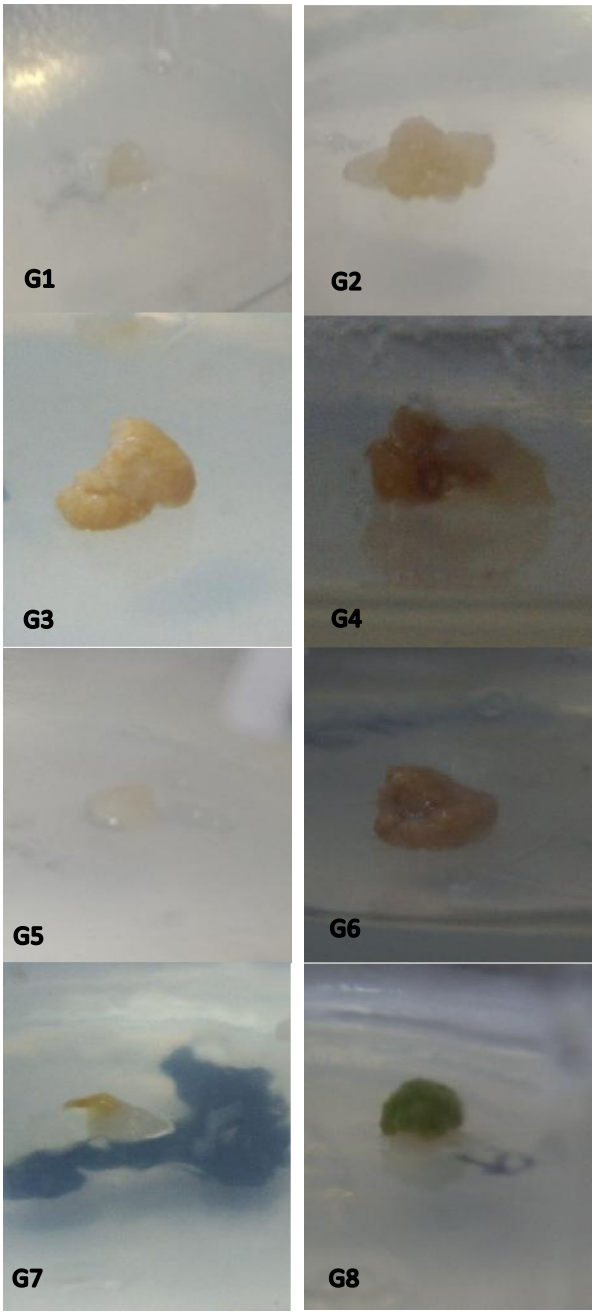

Figure 1. The color of the callus that arises from RGL orange endosprem tissue (G1 = MT + 5 ppm BAP + 2 ppm 2,4- D, G2 = MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH, G3 = MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0,5 ppm Kinetin, G4 = MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0,5 ppm Kinetin + 500 ppm ME, G5 = MS + 5 ppm BAP + 2 ppm 2,4-D, G6 = MS + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH, G7 = MS + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0,5 ppm Kinetin, G8 = MS + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH + 0,5 ppm Kinetin + 500 ppm ME)

The callus weight variable has a score 6, the callus diameter variable scores 5 and the callus color variable has a yellowish-white color with a score of 6 and the crumb callus texture with a score of 6, for the development of callus obtains the highest score of 23 (Table 5) so that the best media for callus development is G2 medium MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH.

CONCLUSIONS

The best medium for callus induction from RGL orange endosperm tissue is G6 namely MS + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH and the best medium for callus development is G2 MT + 5 ppm BAP + 2 ppm 2,4-D + 500 ppm CH. Further research needs to be carried out at the plant regeneration stage.

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Table 5. Determination of the best media for callus endosperm tissue development

	Callus weight		Callus Diameter		Callus colour		Callus texture		Total
	Rank*	Score	Rank*	Score	Rank**	Score	Rank***	Score	Score
G1	-	-	-	-	-	-	-	-	-
G2	1	6	1	5	1	6	1	6	23
G3	5	2	3	5	4	3	4	3	13
G4	6	1	5	3	5	2	6	1	7
G5	-	-	-	-	-	-	-	-	-
G6	2	5	4	6	2	5	3	4	20
G7	4	3	6	2	3	4	5	2	11
G8	*3*	*4*	*2*	*1*	*6*	*1*	*2*	*5*	*11*

** = Rank based on the best value; ** = Rank based on the best colour yellowish-white colour until brown; *** = Rank based on crumb texture to compact*

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