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CALLUS INDUCTION AND PROPAGATION OF COCOA (*Theobroma cacao* L.) CLONES SULAWESI 2 IN VARIOUS CONCENTRATION OF 2,4-D, BAP ADDING WITH COCONUT WATER

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ABSTRACT

The goals of this experimen were to obtain the best concentration of 2,4-D with 15% coconut water to induced cacao callus, and the best concentration of BAP with 15% coconut water in callus propagation. This was experimental research which arranged by Completely Randomized Design, consists of two steps, callus induction and callus propagation. Callus induction from cacao flowers used several concentrations of 2,4-D combined with 15% of coconut water. In callus propagation, the medium treated by various concentrations of BAP with 15% of coconut water. This research was undertaken during period of time February until April 2016 at Laboratory of Forestry Biotecnology Forestry Faculty of Tadulako University. Data was analysed with analysis of variance and difference between treatment were determined by using BNJ at 5%. The result showed that the best medium for callus induction was 2.0 mg/L of 2,4-D with 15% of coconut water (K3). This medium produced callus in 13.50 days after induction with 99,17% of explants producing callus. Treatment of 2 mg/L of 2,4-D + 1.0 mg/L of BAP + 15 % of coconut water (SK6) was giving best response in callus propagation. This medium produced 0.55 ml volume of callus, white-yellowish callus with crumb textured which were active in cell proliferation.

Key Words : BAP, Cacao clone Sulawesi 2, 2,4-D, callus induction, callus propagation, coconut water.

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is an important crop for Indonesia as a third foreign exchange earner after oil and rubber and the third place as the world cocoa producer after Ivory Coast and Ghana (Rosmana, 2005). Sulawesi contributed the largest production compared to other provinces, this suggests that the development of cocoa in Sulawesi plays an important role in the development of cocoa in Indonesia (Fahmid, 2013).

The development of the cocoa crop can not be planted in monoculture anymore. Cocoa plants require shading to growth by planting agroforestri patterns. Kakao shaded more favorable than that not shaded, where shade trees beneficial to increase cocoa biophysical conditions and contribute to biodiversity (Obiri *et al.*, 2007). Nair (1993) reported that cocoa can be combined with the concept of agro forestry plants in one hand the public can get a result and soil conservation on the other hand can be maintained. Agroforestry interpreted by planting forests in agricultural land. The development of the cocoa crop in Central Sulawesi involves many varieties or clones. Cocoa development in Sulawesi including Sulawesi 1 (S1), Sulawesi2 (S2), MCC 01, MCC 02, panther clones and medan clones from Sumatra, Irian clones from Irian (Muslim, 2015).

Sulawesi cocoa clones S2 is one of the clones that can be planted with agroforestry pattern, this clone is relatively resistant to disease Vascular Streak Dieback (VSD) and root disease. Klon S2 is more resistant than clone S1 (Muslim, 2015). It is therefore necessary for the propagation of clones cocoa S2 compliance with the

patternof cocoa seedlings of agroforestry in the future. One of method that can be performed for the multiplication of cocoa clones S2 is by doing the multiplication and development of cacao clones that have superior genetic potential through multiplication culture techniques that is somatic embryogenesis.

Somatic embryogenesis is the process by which somatic cells developed to form a new plant through specific stages of embryonic development without going through the fusion of gametes. Embryogenesis that occur indirectly initiated by callus formation. Calus is a collection of amorphous cells that occur from tissue cells defend themselves continuously. The purpose of the callus culture is to obtain embryonic callus embrionic. Calus can be generated via callus culture and cell suspension with the addition of 2,4-D, or in combination with plant growth regulator (PGR) another or other organic compounds.

RESEARCH METHODS

This study is based on a completely randomized design (CRD), which is carried out in two stages, namely the first stage of the experiment is callus induction consisting of five treatments and six replications so that there are 30 experimental units, media treatment are used as follows :

K_1= 1,0mg/l 2,4-D +coconut water 15%
K_2= 1,5mg/l 2,4-D+ coconut water 15%
K_3= 2,0mg/l 2,4-D +coconut water 15%
K_4= 2,5mg/l2,4-D +coconut water 15%
K_5= 3,0 mg/l 2,4-D + coconut water 15%

Variables observed in this research were the rise of callus, percentage of explants formed callus, callus color and texture of the callus.

Furthermore callus obtained from the best treatmentof callus induction stage (stage I) then sub culture/propagated at the callus stage (stage 2), which consists of six treatments and six replications so that there are 36 experimental units, with the treatment were used as follows :

SK_1 = 2mg/l 2,4-D + coconut water 15%
SK_2 = 2 mg/l 2,4-D + 0,2 mg/l BAP + coconut water 15%
SK_3 = 2mg/l 2,4-D + 0,4 mg/l BAP + coconut water 15%
SK_4 = 2mg/l 2,4-D + 0,6 mg/l BAP + coconut water %
SK_5 = 2mg/l 2,4 D + 0,8 mg/l BAP + coconut water 15%
SK_6= 2 mg/l 2,4-D +1,0 mg/l BAP + coconut water 15%.

The variables measured were callus color, texture callus, callus cell morphology and the volume of callus.

This research conducted in the laboratories of the Faculty of Forestry Science University Tadulako Palu, begins in April 2016.

Activities on callus induction stage included sterilization of tools, preparationand sterilisassi of growing media, sterilization of explants (planting material), cultivation of explants and observations. While on the callus propagation stage is similar to callus induction stage, the difference is the type and concentration of growth regulators are used and the type of explants used. In the induction stage using cocoa flower explants (staminodes) clones Sulawesi 2 while the propagation stage using callus best results of the induction phase of callus then subcultured/propagation in each treatment were tested at the stage of callus.

RESULTS AND DISCUSSION

Induction Callus Satge.

Time of Formed Callus. The fastest formed callus obtained at thet K3 treatment (2.0 mg / l 2.4-D coconut water + 15%) with an average of 13.50 HSK. While the slowest formed callus contained in K2 treatment (1.5 mg / l 2.4-D + coconut water 15%) with an average of 19.83 HSK. The average time formed callus is presented in Table 1.

Callus formation starts with the swelling size of this eksplant followed by the formation of callus on the tip explants (former incision). According Pierik (1987) and Suryowinoto (1999), the occurrence of callus injuries caused by the stimulation, the

stimulation causes the equilibrium on the cell wall changing directions, some protoplasm flowing out so that begins to form callus. Ulfa (2011) also states that the callus will appear at the incision, with the incision is easier for a 2.4-Diclorophenoxyacetic Acid (2,4-D) diffuses into the plant tissue, so that 2,4-D is added to the media culture willhelping auxin contained in tissue explants stimulates cell division, especially the cells located around the injured area.

The formation of callus also due to cells in contact with the media as well as the addition of PGR (2,4-D) to the culture medium, thus compelled become meristematic and subsequent cleavage of such active tissue wound closure. According Hagio (2002) and Sujatha and Prabakaran (2001) PGR of groups such as 2,4-D auxin is important for callus induction. In addition auxin can also lead to differentiated cells capable of undergoing de-differentiation.
According to the results of observation showed that the concentration of PGR (2.4-D) different produce different responses to the current emerging staminodes explants callus on cocoa at culture. Media Murashige and Skoog (MS) added 2.4-D with a concentration of 2.0 mg/l produce the most rapid response in staminodes cocoa stimulate callus formation, followed by a concentration of 2.5 mg / l. The average time to form callus on this treatment are 13.50 and 16.33 DAC. This indicated that at these concentrations are in balance the interaction between PGR (2.4-D) and coconut water are added to the culture medium with endogenous hormones containedin the cultured plants. According to Gunawan (1987), different explants respond so differently to the same treatment. Yelnititis research results (2008) states that the callus induction from explants pieces Shoreapinanga young embryo occurs on average 10 days after culturing. However, callus induction from cotyledon pieces of Pinusradiata occur 4-5 weeks after culturing (Schestibratov *et al.*, 2003). This indicates that the callus from different explants formed at different times as well.

Table 1. The Average Time to form Callus Days After Culture (DAC)

Perlakuan	Rata-Rata	BNJ 5%
K1	18.17^{bc}	
K2	19.83^{c}	
K3	13.50^{a}	2.70
K4	16.33^{b}	
K5	16.83^{b}	

Description : The Figure Followed by The Same Letter are not Significantly Different in Test HSD 5%.

Tabel 2. Average Percentage Eksplan form Callus

Treatments	Average	BNJ 5%
K1	93.33^{ab}	
K2	93.33^{ab}	8.46
K3	99.17^{b}	
K4	98.33^{ab}	
K5	90.00^{a}	

Description : The Figure Followed by the Same Letter are not Significantly Different in Test HSD 5%.

The addition of coconut water into the media also support the formation of callus. According Seswita (2010), the addition of coconut water in callus initiation media is very influential on the rise of the number of callus and callus generated presentation. The existence of the components contained in coconut water can interact with endogenous hormones owned byeach explant so as to stimulate cell division (Surachman, 2011).

Percentage of Eksplan Forming. The percentage of explants formed callus was the highest obtained at K3 treatment (2.0 mg / l 2.4-D coconut water + 15%) with an average that is 99.17%, while the percentage of explants formed callus obtained at the lowest K5 treatment (3.0 mg / l 2.4-D + 15% coconut water) is 90.00%. The average percentage of explants formed callus is presented in Table 2.

Based on the average percentage of explants formed callus, treatment K3 is an optimal concentration for callus in greater numbers. According to George and Sherrington (1984), that the optimal concentration of

auxin that induces callus faster and in greater numbers. The big difference in the percentage of explants formed callus on each treatment due to the concentration of 2,4-D different, and the addition of coconut water. This is in line with Ariati research results (2012), who reported that the addition of 2,4-D and coconut water in the media gives a different response and very fast in generating embryonic callus growth of explants cocoa seeds.

Their slow explants formed callus is due to the differences in each explant in the speed of absorption of nutrients and plant growth regulator (PGR). This is consistent with research Ibrahim *et al.* (2010), using explants ginger are also often fail to form a callus. Moreover, the existence of other factors that could cause failures in vitro culturingie multiple explants undergo secondary metabolism.

Color of Callus. The results of visual observation of the color of callus at the end of the observation are presented in Table 3.

Table 3 shows the color of the current callus formed callus formation until the end of the observation callus showed a color change that is of white color and then changed to a yellowish white which then becomes yellow or brownish yellow. Callus color change from white to yellowish white about four to five weeks after the formation of callus, then the next two weeks callus staminodes cocoa clones S2 turns into yellow or brownish yellow. George and Sherrington (1984) states that the callus color change was caused by the synthesis of phenolic substances on cells (callus).

Table 3. Warna Callus Explants Staminodes Flowers Cocoa at Various Treatment

Perlakuan	Ulangan	Warna Kalus	Persentase (%)			
			P	PK	K	KC
K_1	I	KC	0%	50%	16,67 %	33,33 %
	II	K				
	III	PK				
	IV	PK				
	V	PK				
	VI	KC				
K_2	I	PK	0%	66,67%	0 0%	33,33 %
	II	KC				
	III	PK				
	IV	PK				
	V	PK				
	VI	KC				
K_3	I	PK	0%	100%	0 0%	0 %
	II	PK				
	III	PK				
	IV	PK				
	V	PK				
	VI	PK				
K_4	I	PK	0%	100%	0%	0 %
	II	PK				
	III	PK				
	IV	PK				
	V	PK				
	VI	PK				
K_5	I	PK	0%	50%	16116,67 %	33,33 %
	II	KC				
	III	K				
	IV	PK				
	V	PK				
	VI	KC				

Note : P = White, K = Yellow, PK = White to Yellow, KC = Yellow to Bown.

White White Yellowish Browing

Figure 1. Color Callus in Callus Induction Stage.

Table 4. Tekstur Callus on the Different Treatment of Age 8 Weeks After Culturing (WAK)

P	Replications						Percentage (%)		
	I	II	III	IV	V	VI	K	I	R
K_1	K	I	I	I	I	K	33,3%	66,6%	0 %
K_2	I	I	I	I	I	K	16,6%	83,3%	0 %
K_3	R	R	I	I	I	R	0 %	50 %	50%
K_4	I	I	I	R	R	R	0 %	50 %	50%
K_5	I	K	I	I	I	K	33,3%	66,6%	0 %

Note : K = Non Friable, I = Intermediates, R = Friable.

The callus color change indicates a decline in the growth of the cells of the callus. According Widayanto (2004), callus color change from white to yellowish white that the cells were still active easily defended, yellow or brownish yellow or brown indicates symptoms of aging cells. Furthermore Rasud (2012), suggests callus color change indicates a change in the growth stage cells and regeneration of cells. The white color indicates which cells are actively dividing young, yellow color shows that cells grown towards the stage of active division and brown or amber showing symptoms of aging cells. Callus color describes the visual appearance of callus that can be known activity level a cell. The change callus color can be seen in Figure 1.

Texture Callus.The results of visual observation of the texture of the callus is presented in Table 4.

Based on the results of visual observation of the texture of the callus that forms on eight DAK, the cocoa clones S2 obtained callus ie crumb texture, compact and intermediet. Callus the dominant type of crumb is produced in media which added2.0 mg / l 2,4-D + coconut water 15% (K3) and 2.5 mg / l 2,4-D + coconut water 15% (K4), while the dominant type of compact callus and intermediates produced 1.0 mg / l 2,4-D (K1), 1.5 mg / l 2, 4-D (K2) and1.5 mg / l 2,4-D (K3) with the additionof coconut water 15% in each of those treatments.

Callus with crumb structure (friable) seemed tenuous bonds between cells, callus be easily separated by using tweezers and easily broken, partly sticky callus on tweezers. While the callus with a compact structure (non-friable) is callus cell is compact, difficult to separate and very solid (Lizawati, 2012). Turhan (2004), callus which intermediates are partly compact and partly friable. Based on type of callus produced, callus-type friable is callus which has good quality because it is easily separated into single cells (Arianto, *et al.*, 2013). According Widyawati (2010), the formation offriable callus spurred by the presence of endogenous auxin hormone produced internally by explants cultured.

Tabel 5. Callus Color at Callus Propagation Stages

Treatments	Replication	Callus Color	Percentage (%)	
			PK	K
SK_1	I	PK	33,33%	66,67%
	II	K		
	III	K		
	IV	K		
	V	PK		
	VI	K		
SK_2	I	K	33,33%	66,67%
	II	PK		
	III	K		
	IV	K		
	V	K		
	VI	PK		
SK_3	I	K	66,67%	33,3%
	II	PK		
	III	PK		
	IV	PK		
	V	K		
	VI	PK		
SK_4	I	PK	66,67%	33,33%
	II	PK		
	III	K		
	IV	PK		
	V	PK		
	VI	K		
SK_5	I	PK	100%	0%
	II	PK		
	III	PK		
	IV	PK		
	V	PK		
	VI	PK		
SK_6	I	PK	100%	0%
	II	PK		
	III	PK		
	IV	PK		
	V	PK		
	VI	PK		

Note : PK = White to Yellow K = Yellow.

Propagation Callus Stages. Callus obtained from the best treatment then propagated by subculturing of that callus on media propagation stage. Visual observations showed that all treatments were tested to produce callus size much larger and also grows much faster than at this stage compare to induction stage. This indicates that in order to obtain friable callus with a callus size large and continuously need to do subculture. Subcultures callus can be performed on the same treatment or in combination with other plant growth regulator as well as the different treatment.

According to Gunawan (1998), to save the life and continuous propagation, the resulting callus need to be sub culture. Furthermore, the callus mass in the right media will lead to run out of nutrients and water. In addition, in the callus cells also release compounds that inhibit the metabolism of callus growth itself, so that with the subculture treatment can be obtained amount of callus aboundly.

Color Callus. The results of visual observation of the color of callus on propagation stage are presented in Table 5.

Indicators of the growth explants *in vitro* propagation is callus color which is a visual representation that can be known that callus formed callus cells are still actively growth or diei. Kalus formed from an explant raises different color. The color of callus produced on this stage is yellowish white. Yellowish white callus indicates that the cells are still easy and actively defend. Callus with yellowish white color is also one of the characteristics of embryogenic callus. This is in agree with the results of research Lizawati (2012) on embryogenic callus induction explant apical buds of Jatropha (*Jatrophacurcas* L.) with the use of 2,4-D and Thidiazuron (TDZ), embryogenic callus which one of them characterized by colored callus white or yellowish. Yellow on callus occurs in most treatments of the level of concentration of 2.5 ppm, 5 ppm, 7.5 ppm, 10 ppm 2,4-D supplemented with 0.5 ppm, 1 ppm, 1.5 ppm and 2 ppm TDZ.

Texture Callus. The results of visual observation of the texture of the callus on the callus stage ispresented in Table 6.

Based on the results of visual observation of the texture of the callus formed at the stage of callus, the cocoa clones S2 obtained callus friable texture in all treatment. Callus with friable texture with a yellowish white color is characteristic callus embriogenik. The formation embryogenic callus in treatment SK5 and SK6 marked with callus textured friable with a yellowish

white color, because influenced by the composition of the medium used, which use a combination of auxin (2,4-D) with cytokinin (BAP or kinetin) at appropriate concentrations can enhance the callus induction process. Parott *et al.,* (1988) reported that many auxin used for somatic embryos is 2,4-D. Research Hofmann *et al.*, (2004), showed that embryogenesis was significantly affected by genotype, sucrose, and the type of auxin used. Furthermore, the results of research (Khumaida & Hand, 2010) also reported a callus induction medium containing 2% sucrose, 100 mg / l glutamine, with a combination of auxin and cytokinin (1.0 mg / l 2,4-D and 3.0 mg / l BA) successfully induce embryogenic callus ginger, both in the treatment of explant origin rhizomes are harvested young and old. Lizawati research results, (2012) reported the percentage of embryogenic callus formed from shoot tip explants of axillary Jatropha, shows that the highest percentage of embryogenic callus occurred in some treatment with 2,4-D concentration level of 2.5 ppm.

Callus/cell embryogenic will face a series of morphological changes and biochemistry and finally formed somatic embryos (Zimmerman, 1993; Jimenez, 2001). All somatic cells in plant consist of informationneeded toproduce whole plants and functional, somatic is the basic form of totipotensi (total genetic potential) cells, a unique nature of high level plant (Quiroz-Figueroa *et al.*, 2006).

Table 6. Tekstur Callus in Propagation Stage

Treatmen	Replivation						Percentage (%)		
	I	II	III	IV	V	VI	K	I	R
SK_1	R	R	R	R	R	R	0 %	0 %	100%
SK_2	R	R	R	R	R	R	0 %	0 %	100%
SK_3	R	R	R	R	R	R	0 %	0 %	100%
SK_4	R	R	R	R	R	R	0 %	0 %	100%
SK_5	R	R	R	R	R	R	0 %	0 %	100%
SK_6	R	R	R	R	R	R	0 %	0 %	100 %

Note:K = Non Friable, I =Intermediet, R = Friable.

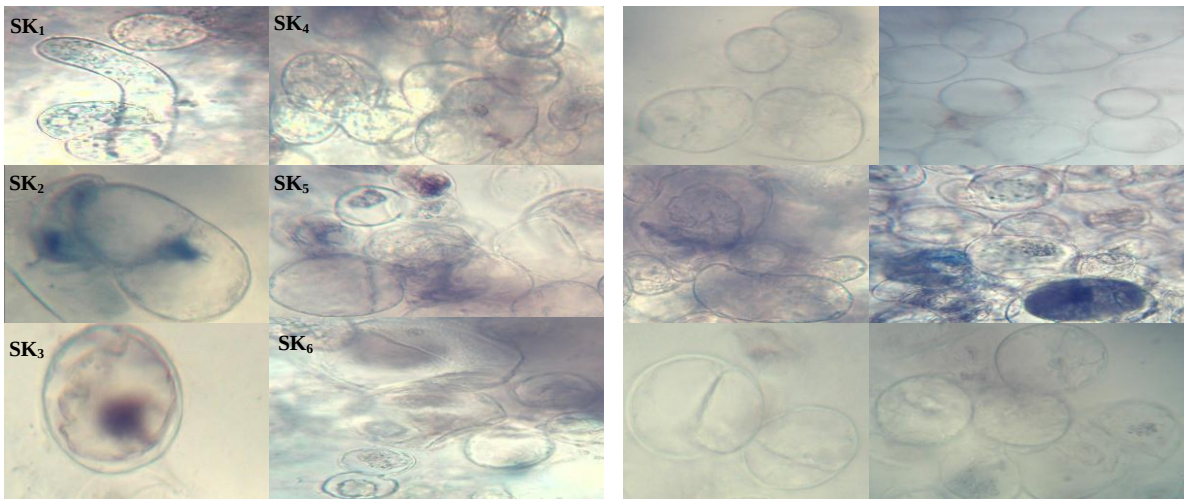

Figure 3. Form of Callus Cells Staminodes Clones of Cocoa (*Theobroma cacao* L.) from Each Treatment Showed a Collection of Cells. Black Line Below The Image Shows The Scale of 20 µm.

Perlakuan	Rata-rata	BNJ 5%
SK_1	0.23^{a}	
SK_2	0.35^{ab}	
SK_3	0.37^{bc}	0.09
SK_4	0.45^{cd}	
SK_5	0.48^{de}	
SK_6	0.55^{e}	

Description : The Figure Followed by The Same Letter are not Significantly Different in Test HSD 5%.

Callus Cells. Observations callus cells in various treatment showed activity of continuous cell division, it is characterized by the formation of cells in large amounts, elongated and clustered due to cell division continues. Good growth of embryos on mediaindicated by the division and accretion callussize. According to Wiendi *et al.,* (1991), callus embrionigenik is callus which has cells that small, dense cytoplasm, large nucleus, small vacuolization and contain starch grains. When viewed from the nature of the cell that continues to divide it can be said explant respond Callogenesis. According to Fatmawati *et al.,* (2010) Callogenesisisa callus that forms do not differentiate into organs, just change the size to be larger and effectively splitting. Callus growth in tissue culture involves a complex relationship between the planting material (explant) is used, the composition of the media, culture PGR and environmental conditions during incubation.

Volume Callus. The average volume of callus presented in Table 7. Based on the results showed that most callus volume obtained in the treatment SK6 (2 mg / l 2,4-D+ 1.0 mg / l BAP + coconut water 15%) with an average ie 0:55 ml. While most small callus volume obtained in perlakuan SK1 (2 mg / l 2,4-D + coconut water 15%) with an average that is 0:23 ml.

The results of the research in callus propagation known that the addition of various concentrations of Benzyl Amino Purine (BAP) in medium containing 2 mg / l 2,4-D and 15% coconut water provides different effects on callus culture. The higher the concentration of BAP is used, the resulting callus size bigger / much. This indicates that the addition of BAP to the culture medium containing 2 mg / l 2,4-D and 15% coconut water work synergistically and lead content of PGR in the network increased callus. Such improvements lead to increased callus tissue so cell division occurs continuously and eventually lead to callus size becomes larger (Figure 4).Morelarge size of the callus showed that the higher the activity, the number of tissue cells start dividing and the higher the determination owned (Yulia*et al.,* 2012).

Figure 4.Kalus Generated at Various treatments were attempted on Propagation Phase Callus. Black line below the picture showa scale of 1 cm.

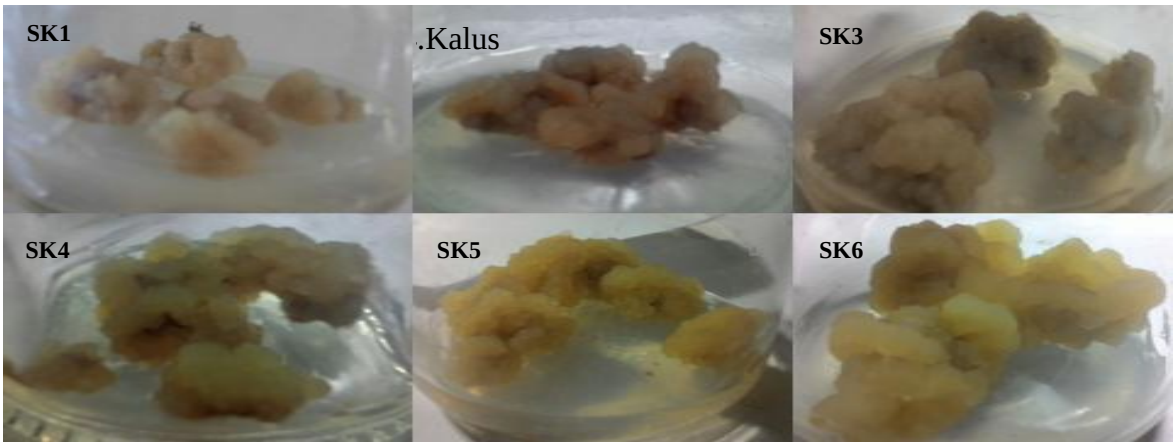

Figure 4. Callus Formed on Various Treatments at Callus Propagation Stage. Black Line Underdi Picture Shown The Scale 1 cm.

CONCLUSIONS AND RECOMMENDATIONS

Conclusion

The concentration of 2.0 mg / l 2.4-D + 15% coconut water (K3) is the best concentration for callus induction cocoa clones S2. In the medium composition formed callus fastest with an average of 13:50 Day After culture, with a percentage of explants formed callus is 99.17%.

The concentration of 2 mg / l 2,4-D + 1.0 mg / l BAP + 15% coconut water (SK6) is the best concentration for callus clones cocoa S2. On the composition of the media obtained callus greatest volume with an average is 0:55 ml and all callus formed yellowish white color which is one of the characteristics that embryogenic callus, textured friable and the resulting cells showed continuous cell division.

Suggestion

Culture media should add concentration of 2.0 mg/l 2.4-D + 15% coconut water and to callus can add 1.0 mg/l BAP. It is also recommended to conducting further research on cocoa flower callus induction to the germination stage.

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