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EMBRYOGENIC CALLUS INDUCTION OF KATOKKON CHILI (*CAPSICUM CHINENSE*) HYPOCOTYL AT VARIOUS CONCENTRATIONS OF 2,4-D

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SUMMARY

Katokkon chili (*Capsicum chinense* Jacq.) is a unique chili plant belonging to the family of Solanaceae. This chili is one of the site-specific genetic resources found in Tana Toraja and North Toraja Regencies, South Sulawesi Province, Indonesia. The presented research sought to determine the use of different 2,4-D (Dichlorophenoxy acetic acid) concentrations for callus initiation and further in-vitro mutation breeding program, conducted at the Hasanuddin University, Makassar, Indonesia. The chili seeds' germination occurred in sterile environment, with the hypocotyl part of the sprouts cut and placed on Murashige-Skoog (MS) medium containing 2,4-D concentrations (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg L^{-1}). The experimental layout was a completely randomized design (CRD) with three replications. Results showed 2,4-D concentration 0.5 mg L^{-1} had a significant effect on callus weight (24.23 mg), while concentration 1.5 mg L^{-1} at the rate of callus appearance and callus formation proportion (6.73 days after culture and 86.7%, respectively). Overall, the callus colors were gray-yellow, yellow-white, orange-white, gray-white, and gray-brown, with friable texture. In addition, the callus was embryonic at concentrations 0.5, 1.0, and 1.5 mg L^{-1}. The next research plan is to explore the regeneration potential by analyzing somaclonal variation in the regenerated plants.

Keywords: Katokkon chili (*Capsicum chinense*), peppers, 2,4-D concentrations, callus, in-vitro mutation breeding

Key findings: In Katokkon chili (*Capsicum chinense*) plants, the tissue culture technology for using 2,4-D to stimulate callus formation originating from the chili hypocotyl is unknown. The presented study determined the most effective 2,4-D concentration to induce callus in chili katokkon hypocotyl derived from sterile sprouts for further in-vitro mutation breeding programs.

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INTRODUCTION

Chili is a horticultural commodity commercially cultivated in tropical countries, including Indonesia. Katokkon chili (*Capsicum chinense*) is a unique local chili of South Sulawesi, Indonesia, which grows in the highlands around 1000–15,000 m above sea level. This chili is one of the location-specific genetic resources (GR) found in Tana Toraja and North Toraja Districts, South Sulawesi Province, Indonesia (Julianto, 2016). Katokkon chili is the same as Paprika chili, but smaller and round, with a size around 3–4 cm. Katokkon chili fruits turn purple-green when young and red when ripe, taste very spicy, and have a distinct aroma (Iryani and Bali, 2021). The Katokkon chili has good prospects for further development as an agribusiness commodity because its price is relatively stable compared with other chili types (Julianto, 2016; PPVPP, 2017; PIHPSN, 2021).

Conventionally, using seeds in propagation is most common in chili plants. However, tissue culture techniques could support the development of chili quality and enhance the adaptability and quality of chili fruits (Soelaiman and Ernawati, 2013). This technique can benefit genetic transformation and regeneration of plant cells, one of which is by callusing parts of the leaves, stems, and roots. Therefore, the callus availability is essential and very effectively, its production can be through tissue culture. The callus culture essentially creates a large population of undifferentiated cells. It is advantageous for mutation breeding because mutations are random events (Wijerathna-Yapa *et al.*, 2022). Having a larger cell population increases the chance of obtaining the desired mutations in genes controlling specific traits.

The successful tissue culture of shoot multiplication, organogenesis, and somatic embryogenesis depends on several factors, including external and internal factors. One of the important external factors affecting the explant maturity process is growth regulators (Wahyuni *et al*., 2020). Widely used growth regulators in tissue culture are auxins and cytokinins. One of these regulators classified as auxin is 2,4-D (Dichlorophenoxy acetic acid) (Riyadi and Tirtoboma, 2017). In crop plants, the auxin application can stimulate cell division and enlargement located at the top of the plant and cause the growth of new shoots. The addition of larger and more stable doses of 2,4-D tends to cause callus growth from explants and inhibit plant shoot regeneration. Previous research by Chaudhary *et al.* (2006) has reported the use of 2,4-D at a concentration of 2 ppm gave the best results on chili plants.

Dichlorophenoxy acetic acid (2,4-D) is one of the pesticides used to cultivate somatic tissues, such as, young stems and leaves. The composition of 2,4-D presumably will direct the callus formation process. Among the auxin group, in tissue culture media, the commonly used auxins are 2,4-D and IAA (Indole-3-acetic acid). Compared with IAA, 2,4-D has more stable properties because it does not decompose easily by enzymes released by plant cells and by heating in the sterilization process (Ermavitalini and Indah, 2013). In callus formation, variations can happen, such as, callus weight produced due to the influence of added endogenous auxin concentrations in plants. The required exogenous auxin in culture media depends on the type of explants used, as well as, the growth regulator type and concentration added in the media (Faramayuda and Ramelan, 2016; Pakum *et al*., 2021).

In the initial stage of the culture technique, the callus induction aims to produce and multiply the cells in massive quantities. Callus is a crucial source of plant material in plant regeneration because every plant cell can develop a new organism (Rasud and Bustaman, 2020). Using in-vitro culture, the callus formation can grow using explants from various sources, such as, young shoots, leaves, root tips, fruits, and floral parts. However, the best explant for callus induction is the tissue of seedling parts germinated in vitro. The greatest potential use of callus culture is where the separation and induction of callus cells can occur to differentiate into embryo somatic (Luqman, 2012). Based on the above discussion, the presented research aims to study the use of different concentrations of

2,4-D to form callus as an initiation of in-vitro mutation breeding in Katokkon chili (*Capsicum chinense*).

MATERIALS AND METHODS

Katokkon chili (*Capsicum chinense*) samples as explant's sources came from Lembang Madandan, Rante Tayo District, Tana Toraja Regency, Indonesia (119̊55″14.640″T – 3̊2″12.042″S). In vitro culture progressed from October 2021 to May 2022 at the Laboratory of Bioscience and Plant Reproduction Biotechnology, Faculty of Agriculture, Hasanuddin University, Makassar, Indonesia.

The material used comprised Katokkon chilies as a source of explants, Murashige and Skoog (MS) media with inorganic and organic components, growth regulators (2,4-D), 96% alcohol, Clorox, distilled water, and adhesive tape. The study used equipment as follows: digital analytical scales (Mettler Toledo), Benchtop pH meter (WTW Inolab PH 7310), autoclave (TRADE Raypa), and Laminar Air Flow Cabinet (LAFC). Other tools included microscope, hot plate, magnetic stirrer, culture bottle, cup petri, Erlenmeyer, measuring cup, pipette, tweezers, spatula, scalpel, millimeter block, and stationery.

The presented study employed the one-factor completely randomized design (CRD) with three replications with 2,4-D different concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg L^{-1}). Each treatment in each replication comprised five treatment bottles, and experimental units totaled 105.

Research procedure

Tools preparation

Tools, such as, Erlenmeyer, Petri dishes, tweezers, bottles, scalpels, measuring cups, and spatulas first reached well washing with soap, then, rinsed, and dried. The tools (except culture bottles) bore paper wrapping and sterilization using an autoclave (with a temperature of 121 °C and pressure of 17.5 psi, for 30 min).

Media preparation

Using the Murashige and Skoog (MS) media consisted of one-half (½) MS for germination and the callus initiation stage. The MS media preparation contained MS stock solutions (A, B, C, D, E, F, and vitamins) (Table 1). The solution received sugar (30 g L^{-1}) and Gellan Gum (2 g L^{-1}). Next, measuring the pH of the solution helped obtain the number 6.0 (adding NaOH to increase the pH and HCL to lower the pH). Then, distilled water addition continued until the solution volume was sufficient for one liter. Specifically for callus initiation media, incorporating MS media with the 2,4-D compound was according to the specified treatment. The MS media's heating to boiling proceeded its pouring into culture bottles, as much as, 25 ml per bottle. Then, sterilizing the bottles used an autoclave (with temperature of 121 °C and pressure of 17.5 psi, for 15 min).

In vitro seed germination

Selected Katokkon chili fruits (healthy and with no pests) achieved soap washing and thorough rinsing, with the last rinse using distilled water. The cleaned chili fruits underwent heat sterilization (flaming) on the fruit's surface three times, cutting them to get the seeds. The seeds proceeded soaking in chlorine for two minutes, then rinsed with distilled water three times, and drained for 30 min. After the seeds have dried, the seeds' separation from the afterbirth continued sowing in culture bottles containing ½ MS media. Each culture bottle contains the seeds from each chili fruit.

Callus induction

The hypocotyls of Katokkon chili sprouts (after two weeks of sowing) sustained cutting into three parts, each measuring 5 mm. The hypocotyl sliced pieces will facilitate the absorption of the media to enable the quick callus formation. Then, placing the hypocotyl pieces continued in the culture bottles for each treatment. Each bottle consists of three hypocotyl pieces obtained from one plantlet. Embryogenic callus reached selection from callus induction with 2,4-D.

Table 1. MS Stock Solutions.

Stocks	Chemical material	Concentration of compounds in the media (mg/L)	Pipette volume
A	NH_4NO_3	1.650,000	20
B	KNO_3	1.900,000	10
C	KH_2PO	170,000	
	H_3BO_2	6,200	
	KL	0,830	10
	$Na_2M_0O_42H_2O$	0,250	
	$C_oCL_26H_2O$	0,025	
D	$CaCl_22H_2O$	440,000	10
E	$MgSO_47H_2O$	370,000	
	$MnSO_47H_2O$	22,300	
	$ZnSO_47H_2O$	8,600	10
	$CuSO_45H_2O$	0,025	
F	Na_2 EDTA	37,300	
	$FeSO_47H_2O$	27,800	10
Vitamin	Glycine	2,000	
	Nicotonic Acid	0,500	
	Myo-inositol	100,000	20
	Pyridoxine-HCL	0,500	
	Thiamine-HCL	0,100	
Sucrose		30 g	
Agar		8 g	

Embryogenic callus induction

The callus obtained continued transplanting on MS media to reduce the effect of 2,4-D and stimulate the formation of embryogenic callus.

Data collection and analysis

At the end of the study, measuring callus formation comprised the percentage of hypocotyls forming callus out of the total number of hypocotyls cultured in the same treatment. The callus emergence rate was inclusive on the length of time between culture initiations until the callus was first visible on the surface of each explant. At the end of the observation period, weighing ensued on the callus sample in each trial. The recorded data assessment used the analysis of variance (ANOVA) method. The obtained means incur further testing with Honest Significant Differences (HSD) for significance at the probability level of 0.01%. The correlation analysis also proceeded to determine the relationship among the observed parameters.

RESULTS

The percentage of callus formation with 2,4-D concentration treatments showed significant differences. The average percentage of callus formation appears in Table 2. The 2,4-D concentration (1.5 mg L^{-1}) produced the highest average percentage of callus formation (86.7%), significantly different from the control treatment (Table 4). However, it did not differ significantly from other 2,4-D concentrations (0.5, 1.0, 2.0, 2.5, and 3.0 mg L^{-1}).

The 2,4-D concentrations (1.0 and 3.0 mg L^{-1}) produced the fastest time of callus formation (5.13 days after planting) and emerged remarkably dissimilar from the control treatment (Table 3). However, they were nonsignificantly different from the four other 2,4-D treatments, viz., 0.5, 1.5, 2.0, and 2.5 mg L^{-1}. The 2,4-D concentration 0.5 mg L^{-1} produced the maximum average of callus weight (24.23 mg) (Table 4). The said promising treatment was also notably different from the control treatment and 2,4-D concentration at 1.0 mg L^{-1}. However, the said

Table 2. Percentage of callus formation.

Treatments	Percentage of callus formation (%)	CV HSD 5%
Control (t0)	0.0[b]	
0.5 mg L^{-1} 2.4-D	75.6[a]	
1.0 mg L^{-1} 2.4-D	68.9[a]	
1.5 mg L^{-1} 2.4-D	86.7[a]	33.0
2.0 mg L^{-1} 2.4-D	80.0[a]	
2.5 mg L^{-1} 2.4-D	80.0[a]	
3.0 mg L^{-1} 2.4-D	68.9[a]	

Notes: The average value followed by the same letter is not significantly different at the Tukey HSD = 0.05.

Table 3. Average of callus formation (DAP).

Treatments	Average	CV HSD 5%
Control (t0)	0.00[b]	
0.5 mg L^{-1} 2.4-D	6.40[a]	
1.0 mg L^{-1} 2.4-D	5.13[a]	
1.5 mg L^{-1} 2.4-D	6.73[a]	2.29
2.0 mg L^{-1} 2.4-D	6.07[a]	
2.5 mg L^{-1} 2.4-D	6.07[a]	
3.0 mg L^{-1} 2.4 D	5.13[a]	

Notes: The average value followed by the same letter is not significantly different at the Tukey HSD = 0.05.

Table 4. Average of callus weight.

Treatments	Average	NP HSD 5%
Control (t0)	0.00[c]	
0.5 mg L^{-1} 2.4-D	24.23[a]	
1.0 mg L^{-1} 2.4-D	17.97[b]	
1.5 mg L^{-1} 2.4-D	22.31[ab]	5.73
2.0 mg L^{-1} 2.4-D	22.83[ab]	
2.5 mg L^{-1} 2.4-D	19.55[ab]	
3.0 mg L^{-1} 2.4 D	19.45[ab]	

Notes: The average value followed by the same letter is not significantly different at the Tukey HSD = 0.05.

promising treatment did not differ significantly from four other 2,4-D concentration treatments, i.e., 1.5, 2.0, 2.5, and 3.0 mg L^{-1}.

The callus texture with 2,4-D treatments also produced a friable texture. The callus formation had a friable texture, except in the control treatment (Figure 1). The color of callus at the 2,4-D concentrations (0.5, 1.0, and 1.5 mg L^{-1}) showed the criteria of visual description of cells that were still actively dividing, i.e., moderate yellow-A (A), pale greenish yellow-C (B), pale yellow-A (C), and pale orange yellow-C (D) (Figure 2).

The appearance of embryogenic callus was indicative of the tendency of callus color, from moderate yellow, pale yellow to pale orange yellow (Figure 3). A bright yellow color indicates a healthy callus, a reddish color and brownish color implies the callus is mature, and a white color reveals an embryonic callus (Rahayu and Suharyanto, 2020). In the induction of somatic embryos, 2,4-D hormone

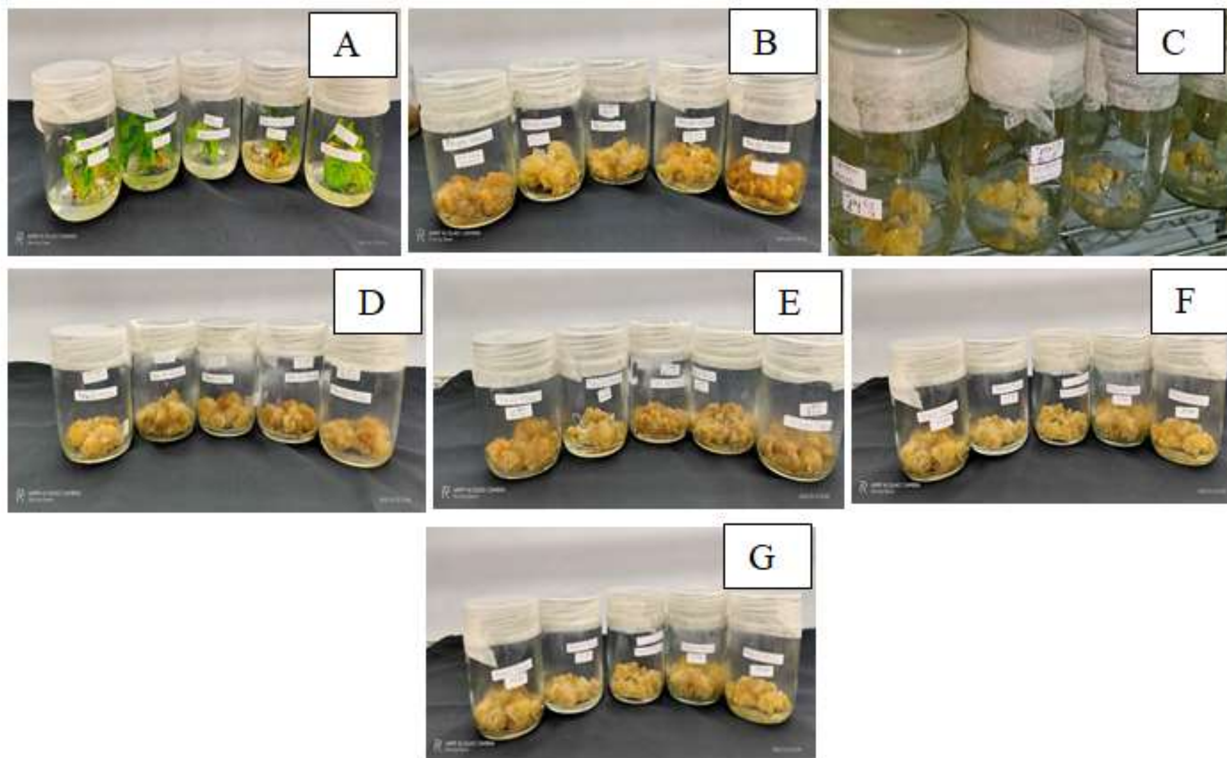

Figure 1. Callus texture of t0= 2,4-D 0.0 mgL-1control (A), t1= 2,4-D 0.5 mg L^{-1} (B), t2= 2,4-D 1.0 mg L^{-1} (C), t3= 2,4-D 1.5 mg L^{-1} (D), t4= 2,4-D 2.0 mg L^{-1} (E), t5= 2,4-D 2.5 mg L^{-1} (F), and t6= 2,4-D 3.0 mg L^{-1} (G).

Figure 2. Callus color of t1= 2,4-D 0.5 mg L^{-1} (A), t2= 2,4-D 1.0 mg L^{-1} (B), t3= 2,4-D 1.5 mg L^{-1} (C), t4= 2,4-D 2.0 mg L^{-1} (D), t5= 2,4-D 2.5 mg L^{-1} (E), and t6= 2,4-D 3.0 mg L^{-1} (F).

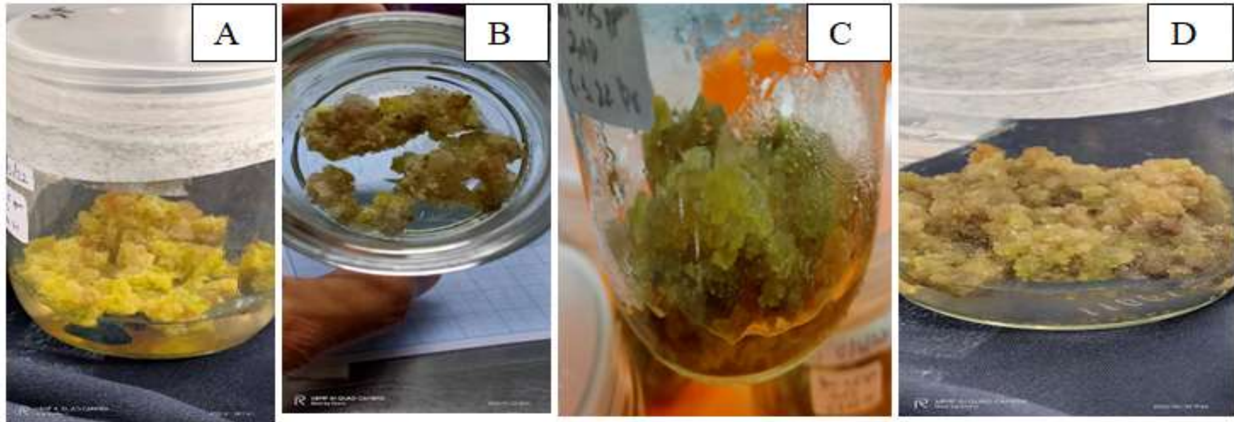

Figure 3. Embryogenic Callus of t1= 2,4-D 0.5 mg L-1 (A), t2= 2,4-D 1.0 mg L-1 (B), t3= 2,4-D 1.5 mg L-1 (C), and t4= 2,4-D 2.0 mg L-1 (D).

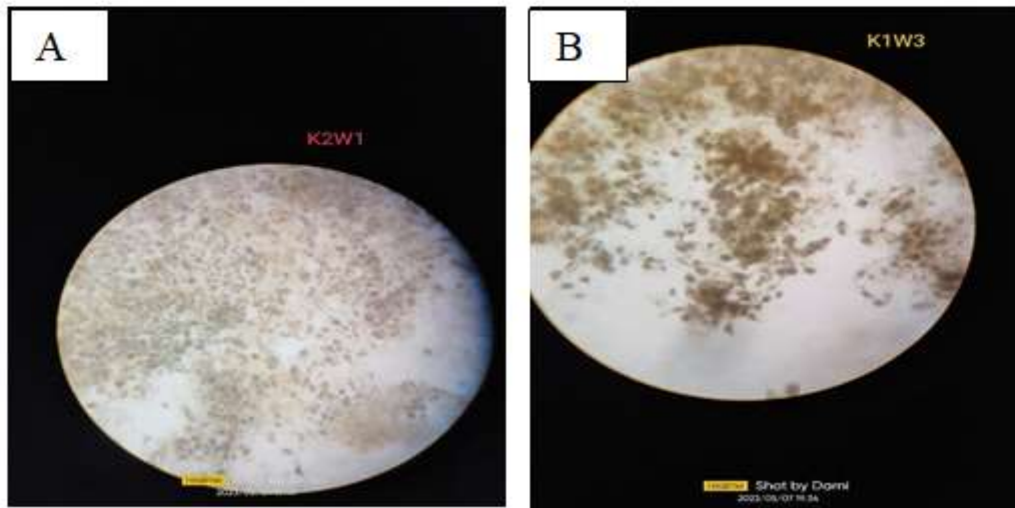

Figure 4. Globular cells of embryogenic callus for 4× magnification A) and 10× magnification (B).

tends to induce somatic embryos indirectly through the callus phase, thus, the number of embryos produced was numerous. However, various embryos produced were abnormal and difficult to germinate into plantlets (Khumaida and Handayani, 2010). Callus with a globular structure, green color, and a smooth texture has great potential to regenerate into plant plantlets (Khumaida and Handayani, 2010). Therefore, the callus colored moderate yellow-A to pale orange yellow-C has the potential to regenerate into plantlets, while in yellowish gray-B and light olive brown-B, the embryos produced are abnormal and difficult to produce plantlets.

According to microscopic observations, the embryogenic callus cells depicted cell shape in the form of granules containing cell nuclei (Figure 4). The embryogenic callus formed continued transferring to MS media without ZPT and sub-cultured every two weeks into the same media until the formation of somatic embryos in the globular, liver, torpedo, and mature cotyledon phases (Ajijah and Hartati, 2016).

DISCUSSION

The presented study used Katokkon chili (*C. chinense*) explants that experienced swelling around the hypocotyl's injury at 6–12 days after culture. The highest percentage of callus formation (%) was visible at the 2,4-D concentration (1.5 mg L^{-1}), with an average callus formation presentation of 86.7%. The appearing percentage of the number of callus and the number of embryogenic callus from Katokkon chili explants were the sign for the successful induction of explants used from hypocotyls by media added with 2,4-D. Hypocotyl is a young tissue in sprouts with the active division of the cells in this tissue. Similarly, it is very effective for the process of callus formation in other young tissues.

On average, above 50% of callus formation was evident, except in the control treatment. This can occur because of the use of hypocotyl tissue, actively dividing cells. The wounded plant surface allows endogenous auxin to stimulate cell division, especially around the wound. The callus induction was also faster because 2,4-D diffused more easily into the plant tissue through the wound, and thus, the added 2,4- D helped the endogenous auxin to stimulate the cell division, especially cells around the wound area (Waryastuti *et al*., 2017).

The growth progress of the callus emergence was apparent at the 2,4-D concentrations (1.0 and 3.0 mg L^{-1}) on day five. The variations in the speed of callus emergence bore influences from several factors, including the content of auxins and cytokinins found in the chili explants. The speed of callus emergence was not sequential according to the concentration and seemed influenced by the hormone content already existing in each explant. Therefore, the two 2,4-D concentrations have a greater impact on its growth. The appearance of callus on explants was an indicator of growth in in-vitro culture. In the presented study, the callus formation first appeared at the wound of the explant in contact with the media, and it started swelling around the opening of the explant curved.

Callus produced by in vitro culture emerged due to tissue wounds and a response to the hormone 2,4-D. In vitro growth and morphogenesis incurred effects from the interaction between the growth regulators added to the media and growth hormones produced by plant cells endogenously by cultured cells (Geetha *et al*., 2016). The formation of callus on the wounded part could be due to the stimulation of the tissue in the explant to cover the wound. Cell division that leads to callus formation occurs due to the response to the wound and the supply of natural and artificial hormones from outside into the explants (Geetha *et al*., 2016). In tissue culture, the callus growth on explants is one indicator of the growth. Callus is cells that appear in clusters on one or all slices of explants (Faramayuda and Ramelan, 2016).

The promising results also showed the 2,4-D concentration (0.5 mg L^{-1}) produced the highest average weight of callus (24.23 mg). The maximum callus weight was due to auxin in the explants being able to induce callus and the 2,4-D concentration, which can enhance the volume of the obtained callus. A variation in callus weight occurred, which probably refer to the endogenous auxins in chili explants ably inducing callus. The need for additional exogenous auxin in the culture medium depends on the type of explants used, and the same is relevant to the type and concentration of growth regulators added to the media (Faramayuda and Ramelan, 2016).

Callus texture is one of the markers and signs used to evaluate the quality of the callus. The good callus will tend to have a friable texture (Andaryani, 2010). Friable and crumbly callus textures are considerably good because they allow for easy separation into single cells in suspension culture. Additionally, they also enhance oxygen aeration in the tissues. Thus, with these textures, the efforts to propagate for the number of calluses through suspension culture is easier (Andaryani, 2010; Nofanda *et al*., 2016).

Calluses formed from the explants usually have different colors. Some past studies reported good-quality callus is green (Ruspita *et al*., 2019). The difference in the

callus color indicates the different levels of the callus development. Generally, callus is green at the beginning of formation, then, as the callus develop, it begins to change color to yellow, brownish yellow to brown (Wahyuni *et al*., 2020; Syahid and Kristina, 2020). In callus color, the 2,4-D concentrations (2.5 and 3.0 mg L^{-1}) were notable with browning color due to the effects of the high 2.4-D concentrations. The interaction between growth hormones added to the media and the produced cells will endogenously determine the growth direction. The callus color in each 2,4-D treatment also varied. However, most of them were yellow to brownish, which is a sign of callus cell death. Explants initially experienced swelling, after which, an accumulation of phenol arises, causing a yellow color, and the accumulation of phenol in large quantities can change the color of the callus to blackish brown (Rahayu and Suharyanto, 2020).

In this study, the non-embryogenic callus tends to occur at several 2,4-D treatments with high concentrations. An indication of this is the decreasing growth of callus, which does not grow with time. In the first subculture with the same treatment media, the callus was difficult to separate and was soft, watery, and brownish-yellow. Browning color in callus was due to the metabolism of phenol compounds that were toxic and could inhibit growth and even cause tissue mortality. This is a sign of physiological deterioration of explants and indicates the synthesis of phenol compounds in the cultured samples (Lizawati, 2012; Gurung *et al*., 2020).

The obtained callus proceeded transferring to neutral media (MS), stimulating the formation of embryogenic callus. Decreasing the concentration of auxin (2,4-D) in the media aimed at increasing the number of globular embryos and increasing the number of differentiated embryos for further in-vitro mutation breeding. Embryogenic callus is obtainable by repeated subculture, and the callus with crumblier structure can result. Forming embryogenic calluses can be directly through repeated subcultures, either in the same treatment or in different treatments (Lizawati, 2012; Tarinta *et al*., 2020).

Using media repeatedly with the addition of auxin 2,4-D can increase callus formation in explants; however, the resulting cells may vary. By transferring to MS control media, one can expect the callus induction was able to enhance the cell sentience of the explant tissue. Therefore, the cell cycle and the initiation and embryo formation at the 2,4-D concentration, viz., 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg L^{-1}, were able to activate the specific genes for subsequent embryogenic callus induction (Khumaida and Handayani, 2010; Lizawati, 2012; Poudyal *et al*., 2023). The formation of embryogenic callus in this phase seemed to have a more stable cell condition, and it can be beneficial in further in-vitro mutation breeding programs.

CONCLUSIONS

The application of the 2,4-D with different concentrations can stimulate callus formation in Katokkon chili (*C. chinense*). In addition, based on the texture and color of the callus produced, embryogenic callus production succeeded at the 2,4-D concentrations (0.5–2.0 mg L^{-1}). In the 2,4-D treatment with higher concentrations (2.5 and 3.0 mg L^{-1}), the produced callus has a more brownish color, indicating a faster browning occurs in the callus.

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