

The Effect of the Combination of 2,4-D and BAP Concentrations on Callus Induction of Rice (*Oryza sativa* L. var. *siam epang*) from East Kotawaringin Regency

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Abstract

In 2023, rice production for population consumption in Central Kalimantan decreased by 7.80 thousand tons (3.82%), due to a 13.14 thousand-ton decrease in dry milled grain (GKG). This is due to environmental stress and resistance to diseases and pests, which affect rice quality and productivity. Rice (*O. sativa* L. var. *siam epang*) is a rice that has advantages such as resistance to acidic soil pH, high rainfall, resistance to pests, and diseases. The success and occurrence of callus regeneration in vitro are generally influenced by PGR with appropriate concentrations. This study aimed to determine the most appropriate concentrations of PGRs 2,4-D and BAP to induce callus in Siam Epang rice. This study is an experimental factorial Completely Randomized Design (CRD) with two treatment factors, namely 2,4-D and BAP, at concentrations of 1 ppm, 2 ppm, and 3 ppm, respectively. Data analysis was conducted on qualitative and quantitative data, including research parameters such as callus formation time, callus weight, callus color, callus texture, and the percentage of explants forming callus. The results showed that the combination of 2,4-D and BAP PGR concentrations affected the growth of Siam Epang rice seed scutellum explants. The addition of 1 ppm 2,4-D and 0 ppm BAP PGR resulted in the fastest callus formation time, namely 3 days. Meanwhile, the addition of 3 ppm 2,4-D and 1 ppm BAP PGR resulted in the highest percentage of explants forming callus (100%) across all treatments. 1 ppm 2,4-D and 0 ppm BAP produced a fresh weight of 0.15 g, a dry weight of 0.03 g, and a callus weight of 0.09 g, the highest among all treatments. The callus formed was ivory white, yellowish white (cream), light yellow, and blackish brown with a compact texture. Thus, the combination of PGR concentrations that produced the highest percentage of callus formation in Siam Epang rice callus was 3 ppm 2,4-D and 1 ppm BAP, with 100% of explants forming callus. Further research is needed to evaluate the effects of various combinations of 2,4-D and BAP concentrations on Siam Epang rice callogenesis.

Keywords: 2,4-dichlorophenoxy acid; 6-benzyl amino purine; callus; concentration; Siamese rice.

INTRODUCTION

The agricultural sector makes a strategic contribution to economic growth by providing a redundant supply of food to meet food needs (Ayun *et al.*, 2020; Rasman *et al.*, 2023). Rice plants (*Oryza sativa* L.) belong to the Poaceae family in botanical classification (Ho *et al.*, 2018) and are one of the most widely cultivated cereal crops in the world (Fanata & Qudsiyah, 2020). Rice is the main food commodity in Indonesia, produced by rice plants (*O. sativa*) (Elbaar *et al.*, 2024). In 2023, Central Kalimantan province experienced a decline in rice production for population consumption of 7.80 thousand tons, or 3.82%, due to a 13.14 thousand tons decline in dry milled grain (DMG), or 3.82% (BPS, 2023). This phenomenon was caused by many factors, such as environmental stress (Noor *et al.*, 2022), resistance to

diseases and pests which have an impact on decreasing the quality and productivity of rice (Daud *et al.*, 2023). Rice (*O. sativa* L. var. *siam epang*) is a superior local rice variety from East Kotawaringin (BSIP Kalimantan Tengah, 2023) which has several advantages such as resistance to acidic soil pH, high rainfall, and resistance to pests and diseases (HPT) (Adhavi & Permadi, 2023). HPT capabilities are widely possessed by local rice and wild rice (Hairmansis *et al.*, 2015; Santoso *et al.*, 2020). Brown planthopper (BPH), tungro, blast, cercospora leaf spot, brown spot by *Helminthosporium* sp. (Norjamilah *et al.*, 2021), attack *Sitophilus oryzae* (Kamsiati *et al.*, 2013) and bacterial leaf blight (BLB) (Asroh *et al.*, 2019) as the main HPT cause of rice puso (Rohaeni *et al.*, 2016). This Siam Epang rice has a unique taste and excellent quality, with a slightly dry texture (Bandrang,

2023). Regeneration in rice plants (*O. sativa*) can come from the root (Abiri *et al.*, 2017; Yu *et al.*, 2017), leaf (Karthikeyan *et al.*, 2011), immature seeds (Hiei & Komari, 2008), mature seeds (Islam *et al.*, 2014), anther (Tripathy *et al.*, 2019), and protoplasts by forming callus (Poddar *et al.*, 2020). But in rice callus induction (*O. sativa*) the use of seeds as a source of explants is better than other sources of explants (Rahman *et al.*, 2015). Callus functions as an object of gene modification and transformation to improve plant traits (Maulidia & Fanata, 2019). Callus formation occurs under certain environmental conditions. The success of the in vitro callus regeneration process is generally determined by the plant's genotype, explant type and part, culture media formulation, plant growth regulator (PGR) use, and culture conditions (Khattak *et al.*, 2017; Ho *et al.*, 2018; Carsono *et al.*, 2021). Plant growth regulators (PGRs) at appropriate concentrations can induce callus proliferation and differentiation (Hamzah & Azma, 2021). The combination of PGRs 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyl amino purine (BAP) in callus induction plays a role in inducing somatic embryogenesis traits by regulating callus morphogenesis and growth through proliferation (Azizah *et al.*, 2017). According to Rahman's research *et al.*, (2020) the development of MR219 rice callus increased in the combination of 1.0 mg/L 2,4-D + 1.0 mg/L BAP, which produced 80.83% globular callus. Meanwhile, according to Muktirianur's research *et al.*, (2022) the combination of 1 mg/L 2,4-D + 0.5 mg/L BAP produced the best callus yields on local rice varieties Buyung, Siam, Ketalun Tawar, and Serai Gunung with the highest percentage of non-compact callus structure with yellowish white (cream) and white colors. This study aims to determine and determine the most suitable combination of 2,4-D and BAP PGR concentrations as an effort to induce callus in rice (*O. sativa*) superior local variety Siam Epang from East Kotawaringin.

MATERIALS AND METHODS

Time and Place

This research was conducted from December 2024 to August 2025 at the Plant Tissue Culture Laboratory of the Balai Pengembangan Produksi Benih Tanaman Pangan dan Hortikultura (BPPB-TPH) in Palangka Raya City and Gedung Pusat Pengembangan IPTEK dan Inovasi Gambut (PPIIG) Palangka Raya University. Data analysis was conducted at the Biology Study Program Laboratory, Faculty of Mathematics and Natural Sciences, Palangka Raya University.

Tools and materials

The tools used in this study were laminar air flow (BioChamb BC 1300), culture rack (Biorack type 2 Bio Chamb BC-300 BR), autoclave (All American Model 1941X), stereo microscope (OPTIKA Italy MX/SFX

Series) pH meter, hot plate (B-ONE Analog Hotplate Stirrer (Ceramic Plate) Type AHS 19C), magnetic stirrer, analytical balance (BEL Engineering M5-M214Ai), oven (Thermo Scientific – Heratherm General Protocol Incubators), culture bottle, petri dish, bunsen, beaker glass, measuring cup, syringe, Erlenmeyer flask, spatula, plastic (plastic warp), tweezers, dropper pipette, measuring pipette, rubber/bulb, aluminum foil and mini studio.

The materials used in this study were whole embryo explants (scutellum) of rice seeds (*O. sativa* L. var. *siam epang*) originating from Lampuyang Village, Teluk Sampit District, East Kotawaringin Regency, Central Kalimantan Province, scutellum aged five months and 11 days, medium murashige and skoog (MS), agar powder, sucrose, myo-inositol, antibiotic plant preservative mixture (PPM™), PGR 2,4-dichlorophenoxyacetic acid (2,4-D) 0 ppm, 1 ppm, 2 ppm, 3 ppm, 6-benzyl amino purine (BAP) 0 ppm, 1 ppm, 2 ppm, 3 ppm, 70% alcohol, spirits, 100% sodium hypochloride or bayclin (5.25% active chlorine), black cloth, cardboard, NaOH, KOH 0.1 M, HCL 0.1 M, and distilled water.

Types of research

This research is an experimental study that uses a factorial Completely Randomized Design (CRD) with two treatment factors, namely the concentration of 2,4-D with four treatment levels (0 ppm; 1 ppm; 2 ppm; 3 ppm), and the concentration of BAP with four treatment levels (0 ppm; 1 ppm; 2 ppm; 3 ppm). Factorial RAL with two treatment factors is a research design that is applied when the treatment to be tested is a combination of several factors that are crossed, not nested, and applied in homogeneous environmental conditions (Susilawati, 2015; Hidayat *et al.*, 2018). Details of the combination of PGR treatments on callus induction (*O. sativa*) 'Siam Epang' is presented in Table 1.

Table 1 Combination of PGR treatment concentrations on rice callus induction (*O. sativa* L. var. *siam epang*).

2,4-D	BAP			
	B0	B1	B2	B3
D0	D0B0	D0B1	D0B2	D0B3
D1	D1B0	D1B1	D1B2	D1B3
D2	D2B0	D2B1	D2B2	D2B3
D3	D3B0	D3B1	D3B2	D3B3

Information: 0 ppm 2,4-D and 0 ppm BAP (D0B0), 1 ppm 2,4-D and 0 ppm BAP (D1B0), 2 ppm 2,4-D and 0 ppm BAP (D2B0), 3 ppm 2,4-D and 0 ppm BAP (D3B0), 0 ppm 2,4-D and 1 ppm BAP (D0B1), 1 ppm 2,4-D and 1 ppm BAP (D1B1), 2 ppm 2,4-D and 1 ppm BAP (D2B1), 3 ppm 2,4-D and 1 ppm BAP (D3B1), 0 ppm 2,4-D and 2 ppm BAP (D0B2), 1 ppm 2,4-D and 2 ppm BAP (D1B2), 2 ppm 2,4-D and 2 ppm BAP (D2B2), 3 ppm 2,4-D and 2 ppm BAP (D3B2), 0 ppm 2,4-D and 3 ppm BAP (D0B3), 1 ppm 2,4-D and 3 ppm BAP (D1B3), 2 ppm 2,4-D and 3 ppm BAP (D2B3), 3 ppm 2,4-D and 3 ppm BAP (D3B3).

Based on Table 1, there are 16 experimental treatment combinations used in this study. Each treatment

combination will be repeated three times, that the total number of experimental treatment units obtained is 48. Each experimental treatment unit consists of two explants. A total of 96 unit experimental will be needed in this study.

Procedure

Sterilization of Tools and Workspace

Clean research equipment including glassware such as petri dishes, beakers, erlenmeyer flasks, spatulas, tweezers, and culture bottles using washing soap, then rinse with clean water, and dry. Then, research equipment (except culture bottles) is wrapped in paper, then put in heat-resistant plastic and will be sterilized using an autoclave at a temperature of 250 °C for 30 minutes. Before use, clean the laminar air flow using 70% alcohol around the work table and dry it, then turn on the UV light for 60 minutes.

Making Plant Growth Regulator (PGR) Stock 2,4-D

Preparation of 2,4-D ZPT stock solution with a concentration of 100 ppm in 100 ml. In making the 2,4-D stock solution, first weigh 10 mg (0.01 g) of 2,4-D ZPT powder then put it into a 100 ml Erlenmeyer flask then drip KOH first to homogenize it, then add sterile distilled water and insert a magnetic stirrer into the Erlenmeyer flask then place it on a hotplate and turn on the speed (rpm) until the 2,4-D PGR stock solution is homogeneous (marked by the absence of molecules in the solution that clump).

Making Plant Growth Regulator (PGR) Stocks BAP

Preparation of BAP ZPT stock solution with a concentration of 100 ppm in 100 ml. In making the BAP stock solution, first weigh 10 mg (0.01 g) of BAP PGR powder then put it into a 100 ml Erlenmeyer flask then drip HCL first to homogenize it, then add sterile distilled water and insert a magnetic stirrer into the Erlenmeyer flask then place it on a hotplate and turn on the speed (rpm) until the BAP PGR stock solution is homogeneous (marked by the absence of molecules in the solution that clump).

Dilution of Plant Growth Regulator (PGR) Stock Solution

Dilution of the PGR stock solution is carried out by calculating the 2,4-D and BAP stock solutions using the solution dilution formula (Salikode *et al.*, 2024) below:

$$V1.M1 = V2.M2$$

Information:

V1 = Availability of original solution volume

M1 = Availability of original solution concentration

V2 = Volume of a desired solution

M2 = Concentration of a desired solution

Making Callus Induction Media

The callus induction media was made by first weighing the media ingredients which included 0.443 gr of Murashige and Skoog (MS), 3 gr of sucrose, and 0.1 myo-inositol using an analytical balance, then mixing the ingredients in a 1000 ml beaker glass that had been filled with a magnetic stirrer and 100 ml of distilled water, then adding 0.1 ml of antibiotic plant preservative mixture (PPM™), then homogenizing using a magnetic stirrer on a hotplate, then adding the PGR hormone 2,4-D and BAP according to the treatment code. Then, it was stirred using a magnetic stirrer, and the pH was adjusted to 5-6. Then put the agar powder into a beaker, stir with a magnetic stirrer, and heat on a hotplate until boiling. After boiling, pour the media solution into a glass jar or bottle, then cover the lid with plastic wrap. The glass jar bottle filled with media is placed in an autoclave and sterilized for 30 minutes at 250 °C. After completion, the media is stored at 20 °C and is ready for use after 3-7 days.

*Preparation of Rice Seed Explants (*O. sativa* L. var. *siam epang*)*

The part of the rice plant that will be used as an explant for callus induction is the intact embryo (scutellum) of the rice seed. The selected seeds are uniform in size, retain an intact embryo or scutellum, and are free of fungal contamination or black spots. To sterilize the explant, first the rice seed is de-husked, then the seed is cut in half and the scutellum is taken, then the scutellum is soaked in 70% alcohol for 1 minute, rinsed with sterile distilled water, then soaked again in a 100% sodium hypochloride solution or Bayclin for 2 minutes, rinsed with sterile distilled water three times with a rinsing time of 5 minutes each. Then the scutellum is air-dried on filter paper placed in a petri dish (Anggoro, 2021). The entire explant sterilization process is carried out in the LAF.

*Explant Planting of Rice Seeds (*O. sativa* L. var. *siam epang*)*

The sterile explants were dried on filter paper to prevent them from getting wet, then planted in the prepared induction medium. First, the explants were taken using sterilized tweezers, dipped in 70% alcohol, and burned with a Bunsen burner each time. Then, two explants were placed in a glass jar containing the callus-induction medium, with the scutella positioned on the surface. The glass jar containing the explants was then closed and covered with plastic wrap. Then, it was placed in a growth chamber at 22 °C in the dark (Azizah *et al.*, 2017; Mostafiz & Wagiran, 2018; Noor *et al.*, 2022).

*Observations of Rice Seed Explants (*O. sativa* L. var. *siam epang*)*

Observations were conducted daily to assess callus development and contamination. Observations were conducted on explants that had formed callus (Sumadji,

2015) by referring to research parameters, callus formation time, callus weight, callus color, callus texture, and the percentage of explants forming callus. Then, observations on the planted explants were conducted for 21 days (3 weeks) (Mostafiz & Wagiran, 2018; Khan, 2019; Kok *et al.*, 2020; Saad *et al.*, 2023).

Research Parameters

Callus Formation Time

The observation process involved recording the time of initial callus formation, which was calculated as days after growth (HST). Indicators of callus formation include greenish-white tissue and surface swelling on the explant (Deng *et al.*, 2020; Isda & Salsabilla, 2022).

Callus Weight

Callus weight was determined by measuring fresh and dry weight. Fresh weight (FW) of callus was determined in an empty petri dish by weighing the callus without the culture bottle and growth medium. Dry weight (DW) was determined in an empty petri dish by weighing the callus after it was placed in an oven at 60°C for 3 days (72 hours) (Mostafiz *et al.*, 2018).

Then there is the growth index, calculated from FW and DW, which aims to provide data on overall growth (Godoy-Hernández & Vázquez-Flota, 2012). The growth index can be calculated using the following equation (Dayal *et al.*, 2025):

$$\text{Formula} = \frac{[FW + DW]}{2}$$

The growth efficiency index (%) is another way to provide an idea of how efficiently plants convert available resources into dry matter by providing the percentage of total FW converted into dry matter and if the growth efficiency value is higher it will indicate that a greater proportion of FW is due to biomass, not water content (Godoy-Hernández & Vázquez-Flota, 2012; Daud *et al.*, 2023; Dayal *et al.*, 2025). Determination of the growth efficiency index can use the equation below (Dayal *et al.*, 2025):

$$\text{Formula} \frac{FW}{DW} \times 100$$

Callus Color

Observation of callus color was carried out by categorizing the color results of callus growth based on visual descriptions, as shown in Figure 1.

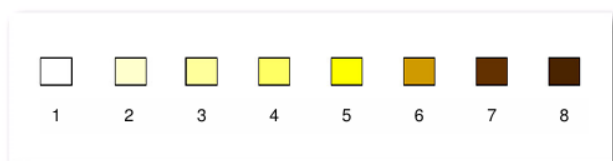


Figure 1. Callus color categories, 1 = white, 2 = ivory white, 3 = yellowish white (cream), 4 = light yellow, 5 = yellow, 6 = light brown, 7 = brown, and 8 = blackish brown (Wanichananan *et al.*, 2010; Hamzah & Azma, 2021).

Callus Texture

Observation of callus texture was carried out by observing the morphology of the callus formed, namely, callus with a crumbly texture (non-embryonic) and a compact texture (embryonic), as in Figure 2.

Compact callus is characterized by a dense structure, smooth surface, pale yellow color, and relatively uniform size (Wu *et al.*, 2022), whereas friable callus is characterized by a texture that is easily disintegrated because it is composed of loose cells and generally forms a root-like structure without going through the globular embryogenic phase (Ho & Vasil, 1983; Fitriani, 2008; Yinxia & Te-chato, 2012; Sidek *et al.*, 2022).

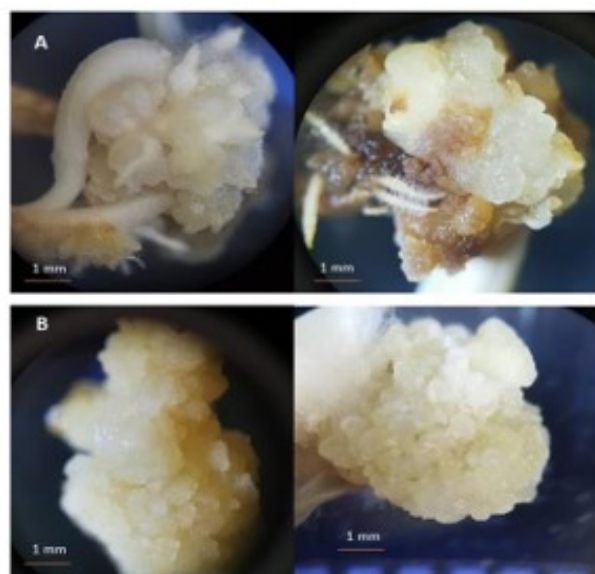


Figure 2. Callus texture categories (A) non-embryogenic callus, (B) embryogenic callus (Sidek *et al.*, 2022).

Percentage of Explants Forming Callus

The percentage of explants forming callus is calculated based on the number of explants in the growth medium that successfully grew and formed callus compared to the total number of explants that have been cultured. The percentage value (%) is obtained by dividing the total number of explants that grew and produced callus by the total number of explants that have been planted in the medium, then multiplying by 100%. The percentage of explants forming callus can be calculated using the equation below (Shahsavari *et al.*, 2010; Mostafiz & Wagiran, 2018; Noor *et al.*, 2022):

$$\text{Callus Induction Frequency} = \frac{\text{Total Callus Growth}}{\text{Total Explants}} \times 100$$

Data analysis

This study obtained two types of data: qualitative and quantitative. Qualitative data were obtained through visual observations of callus color and texture and analyzed descriptively. Quantitative data were obtained through observations of callus formation time, callus weight, and the percentage of explants that formed

callus. Quantitative data are then presented as mean $\pm$ standard deviation (SD) and minimum and maximum values (range).

RESULTS AND DISCUSSION

The study was conducted to determine the effect of the combination of concentrations of the PGR 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyl amino purine (BAP) on the time of callus formation, the percentage of explants forming callus, fresh callus weight (BS), dry callus weight (BK), growth index, growth efficiency index (%), callus color, and callus texture from rice scutellum explant callus (*O. sativa* L. var. *siam epang*). The results of the study with various

treatments combining 2,4-D and BAP PGR concentrations showed a variety of responses in rice callus after 3 weeks (21 days) of explant culture.

Length of Callus Formation Time (HST) and Percentage of Explants Forming Callus (%) of *Scutellum* Rice Seeds (*O. sativa* L. var. *siam epang*) with a combination of 2,4-D and BAP plant growth regulator concentrations

This research was conducted to obtain callus from scutellum explants of Siam Epang rice seeds. The explants were grown on MS medium with 16 combinations of 2,4-D and BAP concentrations, yielding varying responses in terms of callus formation time and the percentage of explants forming callus, as shown in Table 2.

Table 2. The average length of time for callus formation (HST), and the percentage of explants forming callus (%) from scutellum explants of Siam Epang rice seeds on MS media with various combinations of 2,4-D and BAP PGR concentrations during three weeks of culture.

Treatment	Callus Formation Time (HST)		Percentage of Explants Forming Callus (%)
	Mean $\pm$ SD	Range	
D0B0 (control)	0 $\pm$ 0	0 – 0	0
D1B0	2.50 $\pm$ 1.22	0 – 3	83.33
D2B0	5.33 $\pm$ 4.41	0 – 11	66.67
D3B0	7.33 $\pm$ 5.68	0 – 11	66.67
D0B1	2.33 $\pm$ 5.72	0 – 14	16.67
D1B1	2.83 $\pm$ 6.94	0 – 17	16.67
D2B1	4.67 $\pm$ 3.61	0 – 7	66.67
D3B1	7 $\pm$ 0	0 – 7	100
D0B2	0 $\pm$ 0	0 – 0	0
D1B2	0 $\pm$ 0	0 – 0	0
D2B2	5.83 $\pm$ 2.86	0 – 7	83.33
D3B2	4.67 $\pm$ 3.61	0 – 7	66.67
D0B3	0 $\pm$ 0	0 – 0	0
D1B3	0 $\pm$ 0	0 – 0	0
D2B3	4.67 $\pm$ 3.61	0 – 7	66.67
D3B3	3.50 $\pm$ 3.83	0 – 7	50.00
Total			42.71%

Table 2 shows that the fastest callus formation time (HST) occurred in the combination treatment of PGR concentration of D1B0 with a value of 2.50 $\pm$ 1.22 and a range of 0 – 3, then the treatment of D2B1 with a value of 4.67 $\pm$ 3.61, D3B1 with a value of 7 $\pm$ 0, D2B2 with a value of 5.83 $\pm$ 2.86, D3B2 with a value of 4.67 $\pm$ 3.61, D2B3 with a value of 4.67 $\pm$ 3.61, and the treatment of D3B3 with a value of 3.50 $\pm$ 3.83 and a range of 0 – 7, then treatment of D2B0 with a value of 5.33 $\pm$ 4.41 and D3B0 with a value of 7.33 $\pm$ 5.68 and a range of 0 – 11, then treatment of D0B1 with a value 2.33 $\pm$ 5.72 and range 0 – 14, and the longest callus formation time occurred in the treatment of D1B1 with a value of 2.83 $\pm$ 6.94 and range 0 – 17.

Table 2 shows that the results of the treatment of D3B1 showed that all explants formed callus with the percentage of explants forming callus being 100%, then the treatment of D1B0, D2B2 with the percentage of explants forming callus being 83.33%, then the treatment

of D2B0, D3B0, D2B1, D3B2, D2B3 with the percentage of explants forming callus being 66.67%, then the treatment of D3B3 with the percentage of explants forming callus being 50.00 %, and the treatment of D0B1, D1B1 with the percentage of explants forming callus was 16.67%.

Fresh Weight (FW), Dry Weight (DW), and Callus Weight of *Scutellum* Rice Seeds (*O. sativa* L. var. *siam epang*) with a combination of 2,4-D and BAP plant growth regulator concentrations

In the callus induction stage, callus weight is a parameter that indicates the level of callus growth. The results of weighing the callus FW, callus DW, and callus weight that had been treated with various combinations of 2,4-D and BAP PGR concentrations showed differences in weight and average values as shown in Table 3.

Table 3. The average fresh weight, dry weight, and callus weight of Siam Epang rice seed scutellum explants on MS media with various combinations of 2,4-D and BAP plant growth regulator concentrations during three weeks of culture.

Treatment	Fresh Weight of Callus (FW) (g)		Callus Dry Weight (DW) (g)		Callus Weight (g)
	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD
D0B0 (control)	0 $\pm$ 0	0 - 0	0 $\pm$ 0	0 - 0	0 $\pm$ 0
D1B0	0.15 $\pm$ 0.09	0 - 0.24	0.03 $\pm$ 0.02	0 - 0.06	0.09 $\pm$ 0.09
Treatment	Fresh Weight of Callus (FW) (g)		Callus Dry Weight (DW) (g)		Callus Weight (g)
	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD
D2B0	0.05 $\pm$ 0.04	0 - 0.11	0.02 $\pm$ 0.01	0 - 0.03	0.03 $\pm$ 0.03
D3B0	0.03 $\pm$ 0.03	0 - 0.07	0.01 $\pm$ 0.01	0 - 0.02	0.02 $\pm$ 0.02
D0B1	0.02 $\pm$ 0.04	0 - 0.10	0.00 $\pm$ 0.01	0 - 0.02	0.01 $\pm$ 0.03
D1B1	0.01 $\pm$ 0.03	0 - 0.06	0.00 $\pm$ 0.01	0 - 0.02	0.01 $\pm$ 0.02
D2B1	0.04 $\pm$ 0.03	0 - 0.08	0.01 $\pm$ 0.01	0 - 0.02	0.02 $\pm$ 0.03
D3B1	0.08 $\pm$ 0.05	0 - 0.15	0.02 $\pm$ 0.00	0 - 0.02	0.05 $\pm$ 0.04
D0B2	0 $\pm$ 0	0 - 0	0 $\pm$ 0	0 - 0	0 $\pm$ 0
D1B2	0 $\pm$ 0	0 - 0	0 $\pm$ 0	0 - 0	0 $\pm$ 0
D2B2	0.05 $\pm$ 0.03	0 - 0.10	0.01 $\pm$ 0.01	0 - 0.02	0.03 $\pm$ 0.03
D3B2	0.03 $\pm$ 0.02	0 - 0.05	0.01 $\pm$ 0.01	0 - 0.01	0.02 $\pm$ 0.02
D0B3	0 $\pm$ 0	0 - 0	0 $\pm$ 0	0 - 0	0 $\pm$ 0
D1B3	0 $\pm$ 0	0 - 0	0 $\pm$ 0	0 - 0	0 $\pm$ 0
D2B3	0.12 $\pm$ 0.18	0 - 0.48	0.01 $\pm$ 0.01	0 - 0.02	0.07 $\pm$ 0.13
D3B3	0.10 $\pm$ 0.21	0 - 0.54	0.01 $\pm$ 0.01	0 - 0.02	0.06 $\pm$ 0.15

Table 3 shows the results of the treatment of D1B0, which showed the highest average value of callus FW, with a value of 0.15 g, and the highest callus DW, with a value of 0.03 g. In the treatment of D1B1, the average value of callus FW was 0.01 g, and in the treatment of D0B1, D1B1 had the lowest average value of callus DW, with an average value of 0.00 g. The average callus value for each MS medium and 16 treatments combining 2,4-D and BAP PGR concentrations varied.

Table 3 shows that the D1B0 treatments had the highest average callus weight, at 0.09 g. In the D0B1

treatments, D1B1 had the lowest average callus weight at 0.01 g.

Growth Index (Growth Index) (GI) and Growth Efficiency Index (Growth Efficiency Index) (%) (GEI) of Scutellum Rice Seeds (*O. sativa* L. var. *siam epang*) with a combination of 2,4-D and BAP plant growth regulator concentrations

The combination of 2,4-D and BAP PGR concentrations on GI and GEI showed different average values and are shown in Table 4.

Table 4. Average growth index (GI) and growth efficiency index (GEI) of scutellum explants of 'Siam Epang' rice seeds on MS media with various combinations of 2,4-D and BAP plant growth regulator concentrations during three weeks of culture period.

Treatment	Growth Index (growth index) (GI)		Growth Efficiency Index (growth efficiency index) (%) (GEI)
	Mean $\pm$ SD	Range	
D0B0 (control)	0 $\pm$ 0	0	0
D1B0	0.09 $\pm$ 0.05	0 - 0.15	17.51
D2B0	0.03 $\pm$ 0.03	0 - 0.07	22.74
D3B0	0.02 $\pm$ 0.02	0 - 0.04	17.95
D0B1	0.01 $\pm$ 0.02	0 - 0.06	2.18
D1B1	0.01 $\pm$ 0.02	0 - 0.04	4.97
D2B1	0.02 $\pm$ 0.02	0 - 0.05	19.87
D3B1	0.05 $\pm$ 0.02	0 - 0.09	27.10
D0B2	0 $\pm$ 0	0	0
D1B2	0 $\pm$ 0	0	0
D2B2	0.03 $\pm$ 0.02	0 - 0.06	18.85
D3B2	0.02 $\pm$ 0.01	0 - 0.03	18.08
D0B3	0 $\pm$ 0	0	0
D1B3	0 $\pm$ 0	0	0
D2B3	0.07 $\pm$ 0.09	0 - 0.25	9.87
D3B3	0.06 $\pm$ 0.11	0 - 0.28	11.27

The average GI results for each MS medium, with 16 treatments of the combination of 2,4-D and BAP plant

growth regulator concentrations, showed varying responses. In the callus induction stage, GI is an index

value that reflects overall growth. Table 4 shows that the treatment of D1B0 had the highest GI value (0.09 ± 0.05), followed by D0B1, and D1B1 had the lowest value (0.01 ± 0.02).

The average GEI results for each MS medium, with 16 treatments of the combination of 2,4-D and BAP plant growth regulator concentrations, showed varying responses. GEI is an index that provides an overview of how efficiently plants convert available resources into dry matter; a higher GEI indicates a greater proportion of FW due to biomass rather than water content.

Table 4 shows that the D3B1 treatment had the highest GEI among all PGR 2,4-D and BAP combination treatments, with a value of 27.10%. Meanwhile, the treatments D0B1 and D1B1 showed the lowest GEI

values among all treatments, at 2.18% and 4.97%, respectively.

Morphology of Color and Texture of Callus from *Scutellum* Rice Seeds (*O. sativa* L. var. *siam epang*) with Various Combinations of PGR 2,4-D and BAP Concentrations

This research phase involved weekly observations to determine the morphology of the callus from the moment it began to grow until the third week of the callus culture period. These observations were descriptive, and the results varied across treatments. The results of the color and texture morphology observations are shown in Table 5 and Figure 3.

Table 5. Morphology of color and texture of callus from scutellum explants of Siam Epang rice seeds on MS media with various combinations of 2,4-D and BAP plant growth regulator concentrations during three weeks of culture.

Treatment	Color Score	Callus Color	Callus Texture
D0B0 (control)	-	-	-
D1B0	2	Ivory white	Compact
D2B0	3	Yellowish white (cream)	Compact
D3B0	3 and 8	Yellowish white (cream) and blackish brown	Compact
D0B1	4	Light yellow	Compact
D1B1	3	Yellowish white (cream)	Compact
D2B1	8 and 3	Dark brown and yellowish white (cream)	Compact
D3B1	8 and 3	Dark brown and yellowish white (cream)	Compact
D0B2	-	-	-
D1B2	-	-	-
D2B2	3	Yellowish white (cream)	Compact
D3B2	3 and 8	Yellowish white (cream) and blackish brown	Compact
D0B3	-	-	-
D1B3	-	-	-
D2B3	8 and 3	Dark brown and yellowish white (cream)	Compact
D3B3	8	Dark chocolate	Compact

The results of morphological observations on each MS medium, with 16 treatments of the 2,4-D and BAP PGR combination, showed varying callus colors. The results of this study showed that four distinct callus colors were obtained, with all callus textures being compact. The colors recorded and observed were ivory white (2), yellowish white (cream) (3), light yellow (4), and blackish brown (8). The determination of callus color

is based on the research by Wanichananan et al. (2010) and Hamzah & Azma (2021).

In the treatment of D1B0, the color was dominated by ivory white callus color in all explants, then the treatment of D2B0 produced a callus color dominated by yellowish white (cream) callus color in all explants, and the treatment of D3B3 produced a brownish (brownish) callus color with a blackish brown callus color in all explants.

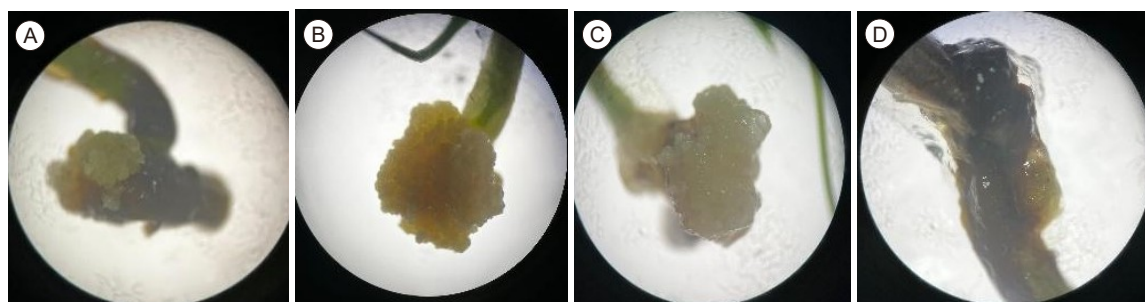


Figure 3. Morphology of callus of scutellum explant of rice seed (*O. sativa* L. var. *siam epang*) A). Callus color is light yellow; B). Callus color is ivory white; C). Callus color is yellowish white (cream); D). Callus color is blackish brown.

Discussion

Length of Callus Formation Time (HST) and Percentage of Explants Forming Callus (%) of *Scutellum* Rice Seeds (*O. sativa* L. var. *siam epang*) with a combination of 2,4-D and BAP plant growth regulator concentrations

In some treatments, a callus formed in the first week of explant culture, whereas in others it formed in the second week. Callus in rice began to appear, characterized by greenish-white tissue with surface swellings on the explant (Deng *et al.*, 2020; Isda & Salsabilla, 2022). According to Garcia *et al.* (2019), callus formation can occur when the plant tissue is exposed to the hormone's auxin and cytokinin, with the ratio of these hormones determining the level of cell differentiation and dedifferentiation. In research, Naik *et al.* (2017) showed that callogenesis largely depends on the availability of exogenous auxin, its type, and concentration.

In addition, the growth conditions during induction (Meneses *et al.*, 2005; Sosnowski *et al.*, 2023), and components in the growth media will also have an effect on callogenesis (Pérez-Bernal *et al.*, 2009; Barman *et al.*, 2017). Table 2 shows that the 1 ppm 2,4-D and 0 ppm BAP treatments showed the fastest callus formation time, is 3 days, while the 1 ppm 2,4-D and 1 ppm BAP treatments showed the longest callus formation time, 17 days. The study obtained varying callus formation times: 3, 7, 11, 14, and 17 days. According to Libin *et al.*, (2012) several previous studies related to callus induction in rice, have reported that PGR 2,4-D is an auxin hormone that is very suitable for callus induction, but it is necessary to optimize the optimal concentration, because the concentration required will vary depending on the source of the explant and the rice genotype to be used (Verma *et al.*, 2011; Dalila *et al.*, 2013; Roly *et al.*, 2014).

Manimaran *et al.*, (2013) noted that young callus has a higher regenerative potential than older callus. This is due to the presence of large numbers of actively dividing cells, making young callus more responsive to callus initiation, both in callus proliferation and differentiation towards shoot formation. Differences in 2,4-D concentration requirements for callus growth are likely influenced by genetic variations that determine the plant's response to hormone concentration intervals, which affect callogenesis and callus proliferation (Verma *et al.*, 2011; Poraha *et al.*, 2016). In addition to genetic factors, non-genetic factors also influence callogenesis, callus proliferation, and shoot regeneration, explant age (Hoque & Mansfield, 2004) and culture conditions (Husaini *et al.*, 2011; Lardon & Geelen, 2020).

Several studies have shown that the synthetic auxin 2,4-D plays a key role in the success of callus induction in rice (Joyia & Khan, 2013). However, other studies have reported that its effectiveness can be increased by combining it with the synthetic cytokinin BAP (Sahoo *et al.*, 2011). A high concentration of 2,4-D (above 4.02 mg

L-1 for 48 hours) is known to reduce the mitotic index, thereby inhibiting cell division (Özkul *et al.*, 2016). Several previous studies recommended the use of 2,4-D PGR alone at 1.5-2.0 mg/L in callus induction of Malaysian upland rice cultivars Lamsan and Siam (Shahsavari *et al.*, 2010), Biris cultivar rice from Sarawak (Libin *et al.*, 2012), varietas Sita, Rupali, Swarna Masuri MTU-7029 (Upadhyaya *et al.*, 2015), and rice variety MR219 (Abiri *et al.*, 2017).

In research by Radhakrishnan *et al.* (2001) and Pérez Bernal *et al.* (2014), it is known that cells utilize the PGR 2,4-D according to their physiological needs; however, excess concentration of this PGR can trigger herbicide effects, which will actually inhibit the callus induction process (Carsono *et al.*, 2021). This inhibition is related to the possible toxic effects of high auxin concentrations in the culture medium (George *et al.*, 2008; Teoh *et al.*, 2023). At low concentrations, 2,4-D induces a hormetic response, in which the compound stimulates biological activity at low concentrations but causes toxic effects at higher concentrations (Bondy, 2023). Increasing the concentration of auxin over cytokinin in the culture medium will generally direct differentiation towards root formation, while higher concentrations of cytokinin over auxin will generally stimulate shoot formation. However, there is no definite ratio between auxin and cytokinin that applies generally to all plant species or plant organ parts to be cultured. Therefore, the optimal composition of auxin and cytokinin hormones, along with the PGRs derived from them, will depend on the species, tissue type, and on research results and empirical experience (Reddy & Obaid, 2017).

In research, Ferdous *et al.* (2008) and Mahmud *et al.* (2024) reported that the optimal concentration of 2,4-D for callus induction in rice was 2.5 mg L-1. Then Shweta *et al.*, (2020) showed that 2.5 and 3 mg L-1 were the optimal concentrations of 2,4-D for callus induction in CO 51 rice and Malaysian lowland rice (Mostafiz & Wagiran, 2018). Callogenesis generally occurs simultaneously with tissue reunification or cork tissue formation and can sometimes progress to organogenesis (Sugiyama, 2015). In vitro organogenesis is thought to involve three interrelated stages. In the initial stage, cells respond to organogenesis induction signals, for example through plant growth regulators (PGR); then in the second stage, competent cells are directed and determined through the balance between PGRs appropriate for specific organogenesis (organogenesis induction) and the final stage is characterized by organ development through morphological differentiation that occurs independently of external PGR (Sugiyama, 2015).

In the current trend of callus induction culture research, the most effective auxin for inducing callogenesis is the PGR 2,4-D, although some researchers prefer the natural auxin IAA or the synthetic auxin NAA (Ngomuo *et al.*, 2013). According to Rahman *et al.* (2007) and Abiri *et al.* (2017), 2,4-D can

disrupt actin structures and slow cytoplasmic flow. The actin cytoskeleton plays an important role in cell division, expansion, differentiation, and organ initiation by altering cell morphology and cytoarchitecture (Abiri *et al.*, 2017). Disruption of actin structure by the PGR 2,4-D can cause various physiological effects, such as leaf epinasty, changes in mitochondrial and peroxisome mobility, and affect cell metabolism, due to their close relationship with chloroplasts. In addition, peroxisome activity, which suppresses the production of reactive oxygen species (ROS), will decrease when actin polymerization and interactions are affected by auxin herbicides. This condition will cause cells to experience severe oxidative stress due to the disruption of peroxisome and mitochondrial function (Rodríguez-Serrano *et al.*, 2014). Deposition of structural cell wall components, such as pectin, hemicellulose, and cellulose, plays a very important role in induction, differentiation, and cell proliferation. Callus formation can occur due to mutations that cause loss-of-function in the cell wall (Iwai *et al.*, 2002; Abiri *et al.*, 2017).

In the combination treatment of 3 ppm 2,4-D and 1 ppm BAP, it was found that all explants formed callus, with the percentage of explants forming callus being 100%. Then, in the treatment of 1 ppm 2,4-D and 0 ppm BAP, 2 ppm 2,4-D and 2 ppm BAP, the percentage of explants forming callus was 83.33%. According to Ali *et al.* (2004), the combination of 2,4-D with BAP or NAA resulted in a stronger callus-induction response in lowland rice. In research, Din *et al.* (2016) confirmed that the addition of 2,4-D either singly or in combination can increase the success of callus induction. According to Ikeuchi *et al.* (2016), among the various factors that influence callus induction and regeneration, plant genotype and culture media composition are the two main factors that determine the direction of in vitro culture development.

Fresh Weight (BS), Dry Weight (BK), and Callus Weight of *Scutellum* Rice Seeds (*O. sativa* L. var. *siam epang*) with a combination of 2,4-D and BAP plant growth regulator concentrations

According to Tariq *et al.* (2008) and Dayal *et al.* (2025), the highest average callus weight obtained with the use of 2,4-D reached 0.27 g. The increase in soft callus mass is thought to be related to the balance between the hormones auxin and cytokinin, along with internal cell regulatory mechanisms that induce cell division (Reddy & Obaid, 2017). Several previous studies have reported significant differences in FW or DW between various rice cultivars (Htwe *et al.*, 2011). In the research (Kadhimi *et al.*, 2016) it was found that the DW of Malaysian indica rice callus MR269 ranged between 0.16 g – 0.32 g. In addition, Azizi *et al.*, (2017) noted that maximum callus FW and DW were obtained in several Malaysian indica rice cultivars such as MR50, MR74, MR219, and MR276 with a range of 0.40 g - 0.45 g for FW and 0.01 g - 0.02 g for DW, respectively.

Research by Mostafiz & Wagiran (2018) found that combining multiple auxin types yields more effective results than using a single type alone. The use of 2,4-D alone is not sufficient to support proliferation or increase callus biomass (Abiri *et al.*, 2017). It has an inhibitory effect on callus proliferation and causes a brown discoloration of the callus. The increase in callus BS is thought to be influenced by the use of optimal concentrations of 2,4-D and BAP. The combination of BAP and auxin supports continuous cell division and development (Puspaningtyas *et al.*, 2025). When auxin and cytokinin concentrations are relatively balanced, cell mass accumulation tends to increase (Jadid *et al.*, 2024). Therefore, optimal cell division will support the growth of high-quality callus, thereby increasing callus FW (Puspaningtyas *et al.*, 2025).

The study obtained results from calculations of FW, DW, and callus weight that varied across all treatment combinations. Variations in callus weight reflect differences in plant cells' ability to absorb oxygen (Puspaningtyas *et al.*, 2025). BS is also influenced by the texture of the callus; namely, callus with a fragile texture generally undergoes faster cell division, thereby increasing its mass and weight (Puspaningtyas *et al.*, 2025). Differences in FW between calli can be caused by varying growth conditions. The increase in callus weight is mainly due to increased cell division, which increases the number of cells (Puspaningtyas *et al.*, 2025). In addition, the growth rate is influenced by the tissue's nutrient-absorption capacity, which is significantly affected by aeration and callus texture.

Dense and compact callus has a lower nutrient absorption capacity compared to looser (crumbly) textured callus (Rybin *et al.*, 2024). The addition of the PGR BAP to the proliferation medium is known to slightly reduce the size of the callus compared to the culture medium containing only 2,4-D, possibly due to an imbalance in the auxin-to-cytokinin ratio (Kakani *et al.*, 2009; Chitphet *et al.*, 2025). Adjusting the BAP concentration can increase callus proliferation, consistent with the findings of Kumari *et al.* (2021) and Dhamotharan *et al.* (2021), who reported that combining the PGR 2,4-D with the cytokinins kinetin or BAP at lower concentrations increased proliferation in various rice cultivars.

Growth Index (*Growth Index*) (GI) and Growth Efficiency Index (*Growth Efficiency Index*) (%) (GEI) of *Scutellum* Rice Seeds (*O. sativa* L. var. *siam epang*) with a combination of 2,4-D and BAP plant growth regulator concentrations

In analyzing callus growth dynamics, this approach is effective for determining the average subculture time. Callus cells exhibit high proliferation capacity for a certain period of time, then enter a stationary phase, where the rate of cell division decreases, their ability to divide decreases, and the cells undergo differentiation (Ibrahim *et al.*, 2019). Therefore, this GI method allows

the determination of conditions for cell maintenance and active division (Ibrahim *et al.*, 2019). The auxin hormone plays a role in stimulating the expression of the CDC2 gene, which encodes a protein kinase in cell cycle regulation that functions as a regulator of essential growth in the somatic embryogenesis process in plants (Wójcik *et al.*, 2020) and the PGR 2,4-D can trigger DNA hypermethylation that maintains cells in a mitotic state. In addition, the availability of carbon sources provides the energy plants need for various developmental processes, including shoot proliferation and root induction. Meanwhile, cytokinin hormones accelerate cell division by activating CDKs (*cdc2*, *cdk4*, *cdk6*) through interactions with specific cyclins, as well as by increasing transcription of the *cdc2* and *CyD3* genes, which promote cell proliferation (Ariani *et al.*, 2016). Then, auxin also plays a role in inducing cell elongation and increases BS by optimizing absorption.

The specific growth rate is determined when the cell population reaches half its maximum growth capacity, meaning that the increase in cell number follows a linear relationship (Pommerening & Muszta, 2016). Measuring callus DW cannot be done simply by comparing the highest value, because in each callus induction culture, cells undergo a growth cycle with distinct phases that occur at different rates. Therefore, this will have implications for determining the timing of callus harvest: primary metabolites are best harvested during the growth phase, while secondary metabolites are optimally produced under stress. Therefore, aspects of growth efficiency and the relationship between biomass accumulation and the time required to achieve it need to be considered (Setiaji *et al.*, 2020).

Morphology of Color and Texture of Callus from *Scutellum* Rice Seeds (*O. sativa* L. var. *siam epang*) with Various Combinations of PGR 2,4-D and BAP Concentrations

According to Jia *et al.* (2014) and Abiri *et al.* (2017), color in callus can be used as an effective selection indicator in classifying embryogenic callus. Several studies have reported that the inhibitory effect of 2,4-D on chlorophyll biosynthesis can lead to a whitish to yellowish callus color (Shukla *et al.*, 2014). The embryogenic callus that is formed will generally have a white and yellowish white callus color (Sharma *et al.*, 2017; Putri *et al.*, 2024). White callus generally indicates that cells are still young and actively dividing, while yellow or yellowish-white callus indicates that the cells have entered the mature stage or the final phase of division (Rasud & Bustaman, 2020). Furthermore, the yellow color of callus is often associated with good callus growth (Putri *et al.*, 2024). According to Indriani *et al.* (2020) and Das & Bandyopadhyay (2021), color variations are also influenced by cytokinins during plastid development, namely chloroplasts, which determine chlorophyll content; the greener the callus, the

higher its chlorophyll content. The change in callus color from green to yellow generally occurs due to chlorophyll degradation (Wahyuni *et al.*, 2020).

According to Gurav *et al.* (2020), a combination of 1.5 ppm 2,4-D and 1.5 ppm BAP treatment will produce green callus color. If there is an imbalance between the ratio of auxin and cytokinin, it will cause the callus color to be yellow to brown. In addition to being influenced by the auxin-to-cytokinin imbalance, the change in callus color is also influenced by reduced nutrient levels in the growth medium as the culture ages, which will accelerate culture degradation (Sari *et al.*, 2018). According to Mahadi *et al.* (2016), callus texture is divided into three types: crumbly, compact, and intermediate. The texture of the callus is an indicator of callus quality and can reveal cell physiological activity, namely whether cells are still actively dividing or have entered a resting phase (Haring *et al.*, 2024; Puspaningtyas *et al.*, 2025). Compact callus has a solid texture, is not brittle, and has the potential to develop into other plant organs such as roots or shoots (Wahyuni *et al.*, 2014). Compact callus texture is also considered suitable for the accumulation of secondary metabolites (Indah & Ermavitalini, 2013). Meanwhile, crumbly callus has a high-water content, is easily separated into cells, so it is suitable for use as an increase or addition of tissue and the development of somatic embryos (Sitorus *et al.*, 2011).

According to Mahadi *et al.* (2016), compact callus formation was influenced by tissue lignification, which was reinforced by cytokinins' role in nutrient transfer. This was reinforced by the observation that high BAP concentrations (5–15 ppm) tended to produce densely textured callus (Mahadi *et al.*, 2016). Meanwhile, other research reported that the addition of PGR 2,4-D at high concentrations (2–3 ppm) had greater potential to produce callus with a crumbly texture (Maulana *et al.*, 2019). According to Astuti *et al.* (2020), a combination of 1.5 ppm 2,4-D and 1.5 ppm BAP produced callus with a compact texture and a greenish color. This compact texture is considered optimal for the accumulation of secondary metabolites, as the cell walls are dense and support the production of additional metabolites (Junairiah *et al.*, 2019). In general, the callus formed will have a yellowish-white morphology, with a compact texture, which is reinforced by the fact that embryogenic callus is characterized by meristematic cells with a high rate of cell division and isodiametric, dense cytoplasm (Vega *et al.*, 2009).

Optimal callus culture induction results are generally characterized by rapid growth and bright callus color, whereas callus with a fragile, easily destroyed texture (crumbs) is more suitable for subculture or suspension culture (Hamzah & Azma, 2021). It is known that 2,4-D is the most frequently used PGR for in vitro somatic embryogenesis induction due to its strong auxin-like activity, which influences the activity of other endogenous phytohormones such as IAA (Sidek *et al.*,

2022). Through morphological observations, callus can be classified as embryonic, bud, or root callus if there is plant organ regeneration, while callus that does not show plant organ regeneration will be compact or crumbly (Ikeuchi *et al.*, 2013). In indica rice, embryogenic callus is generally pale white to pale yellow in color with a fragile and loose texture, while non-embryogenic callus is characterized by a white color, rough texture, and a pointed shape (Abiri *et al.*, 2017).

In addition, the browning of the callus color observed with various combinations of 2,4-D and BAP PGR treatments is generally caused by the oxidation of phenolic compounds produced by plant tissue (Dridi *et al.*, 2022). The production of phenolic compounds can be triggered by physiological stress in plants, such as tissue injury or pressure from the culture medium, which can potentially inhibit and be toxic to explant growth (Dridi *et al.*, 2022). Callus blackening, or oxidative browning, is a common problem in tissue culture, generally caused by the accumulation and oxidation of phenolic compounds. The degree of callus blackening is greatly influenced by the activity of the enzymes polyphenol oxidase (PPO) and peroxidase (POD), which play a role in phenolic metabolism (Amini *et al.*, 2024).

Several preventive measures can be taken by adjusting the media composition, using appropriate types and concentrations of plant growth regulators (PGR), and selecting explants of the correct type and age. Several previous studies have reported that the addition of auxin, specifically the PGR 2,4-D, at high concentration can increase the intensity of browning in explants. In addition, variations in cytokinin type and concentration affect the level of callus browning. Other measures can also be taken by administering ascorbic acid 50-200 mg/L before planting the explants or conducting periodic subcultures at varying time intervals (Permadi *et al.*, 2023; Hamza *et al.*, 2024). If the concentration of the 2,4-D growth regulator is too high, it is known to inhibit callus induction while simultaneously triggering browning of the explants (Mostafiz & Wagiran, 2018; Fanata & Qudsiyah, 2020).

Several previous studies have shown a decrease in callus growth rate, often associated with the appearance of blackened or brown areas on explants. In certain conditions, it can even influence the formation of albino plants, which is influenced by low concentrations of 2,4-D and high temperatures. High concentrations of 2,4-D are known to inhibit normal embryo development and produce callus that tends to be wet and soft, whereas at low concentrations, it supports and increases the formation of compact, embryogenic callus (Fanata & Qudsiyah, 2020). The ability of callus to form embryogenic structures is an important aspect of in vitro transformation, because it determines the potential of the callus to produce somatic embryos and regenerate intact plantlets (Pérez Bernal *et al.*, 2014). Therefore, low concentrations of 2,4-D are generally used to trigger cell division, while higher concentrations are more

commonly used during early induction and subculture stages; however, residual 2,4-D in the culture medium can affect subsequent callus development.

This indicates that excessive administration of 2,4-D will reduce the totipotency of callus, due to the regulation of endogenous hormones by genes with a certain level of auxin sensitivity (Carsono *et al.*, 2021). In addition to hormones and plant growth regulators, browning is also influenced by the accumulation of secondary metabolites, prolonged callus incubation without subculture, and the age of the callus. According to Garcia *et al.* (2019), the stages of typical somatic embryo development in monocotyledonous plants include the formation of a proembryo, a globular embryo, a scutellar embryo, and a coleoptilar embryo. Somatic embryogenesis is a complex process involving physiological, morphological, and molecular changes across six main phases: induction, expression, multiplication, development, maturation, and germination (Ali *et al.*, 2007). According to Saad *et al.* (2023), optimal somatic embryogenesis will occur when achieved through a combination of 2,4-D and cytokinin. This process requires a specific PGR with the ability to induce cell dedifferentiation, chromatin restoration, gene expression rearrangement, and induction under stress conditions. In other studies, it has been reported that embryogenic callus derived from the scutellum of mature rice seeds is a highly promising explant source for callus induction and regeneration in vitro, due to its year-round availability and high embryogenic capacity (Sahoo *et al.*, 2011).

CONCLUSION

Based on the results of the research that has been conducted, it can be concluded that:

1. The combination of PGR D1B0 concentrations shows the fastest callus formation time with a value of 2.50 ± 1.22 and a range of 0 – 3 (3 days), and shows the highest average fresh weight of 0.15 g, the highest average dry weight of 0.03 g, and the average callus weight of 0.09 g. The combination of PGR D3B1 concentrations showed the highest percentage of explants forming callus across all treatments, namely 100%. This is due to several factors, including the optimal auxin-to-cytokinin ratio, media components, explant sources, and plant genotypes.
2. The morphology of the callus formed varied in color with a compact texture across all treatments. The callus formed was generally yellowish white (cream), blackish brown, or ivory white, with a compact texture, observed in the combination treatments D2B0, D2B2, D3B0, D1B1, D2B1, D3B1, D3B2, D2B3D3B3, and D1B0.
3. The optimal combination of 2,4-D and BAP PGR concentrations to produce the highest percentage of explants forming callus in rice callus (*O. sativa* L.

var. *siam epang*) is D3B1, with the highest percentage value of explants forming callus in all treatments, namely 100%.

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