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Sistyananda FN · Suminar E · Nuraini A · Kadapi M · Murgayanti · Mubarok S · Budiarto R · Renaldi E · Kusumiyati

Optimization of *Physalis angulata* L. callus induction and salinity-induced antioxidant production

Abstract. Groundcherry (*Physalis angulata* L.) is a plant with many medicinal potentials due to its rich secondary metabolites such as phenolic and flavonoid. However, conventional agriculture practices are still limited, especially in Indonesia. This study was divided into two stages. The first stage was conducted to determine optimal 6-BAP and 2,4-D combination for callus induction, while the second stage was conducted to determine callus phenolic, flavonoid, and antioxidant response to salinity stress. The first stage was arranged in factorial completely randomized design with two factors: 6-BAP (0, 2, and 4 mg/L) and 2,4-D (0, 0.5, 1, and 1.5 mg/L). The second stage was arranged in simple completely randomized design with different NaCl concentration (0, 25, 50, 75, 100 mM) as treatments. The results showed significant interaction ($p < 0.05$) between 6-BAP and 2,4-D on callus induction. Combination of 2 mg/L 6-BAP and 1 mg/L 2,4-D showed the highest callus formation percentage (46% increase), callus size (60.12% increase), and fresh weight (179.69% increase), and greener compact callus. Application of NaCl as salinity stress at second stage experiment served as an elicitor to enhance callus antioxidant capacity. Salinity level at 100 mM NaCl showed the most accumulation of phenolic content (17.8% increase), flavonoid content (25.17% increase), and antioxidant activities (6.84% IC_{50} decrease). This study demonstrates plant growth regulator optimization with salinity stress elicitation integration as an effective strategy to enhance antioxidant production in *P. angulata* callus, providing a practical approach for controlled secondary metabolite production.

Keywords: Flavonoid · Groundcherry · Phenolic content · Salinity stress · Secondary metabolites

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Sistyananda FN*[1] · Suminar E[2] · Nuraini A[3] · Kadapi M[3] · Murgayanti[2] · Mubarok S[2] · Budiarto R[2] · Renaldi E[4] · Kusumiyati[3]
[1]Agrotechnology Student, Faculty of Agriculture, Universitas Padjadjaran. Jl. Raya Bandung Sumedang Km 21, Jatinangor, Sumedang 45363, West Java, Indonesia
[2]Agrotechnology Study Program, Faculty of Agriculture, Universitas Padjadjaran. Jl. Raya Bandung Sumedang Km 21, Jatinangor, Sumedang 45363, West Java, Indonesia
[3]Agronomy Study Program, Faculty of Agriculture, Universitas Padjadjaran. Jl. Raya Bandung Sumedang Km 21, Jatinangor, Sumedang 45363, West Java, Indonesia
[4]Agribusiness Study Program, Faculty of Agriculture, Universitas Padjadjaran. Jl. Raya Bandung Sumedang Km 21, Jatinangor, Sumedang 45363, West Java, Indonesia
*Correspondence: firstian21001@unpad.ac.id

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Introduction

Groundcherry is one of the medicinal plants that grows in Indonesia. *P. angulata* can thrive in various places such as rice fields, gardens, forests, and even roadsides. However, this plant becomes harder to find because it is often considered as a weed and eradicated by many farmers (Nuranda et al., 2016). Any research and agricultural practices in Indonesia are relatively narrow and limited unlike Brazil and Mexico (da Silva Ramos et al., 2021; Vargas-Ponce et al., 2016). *P. angulata* has been widely used as a raw material in biopharmaceutical and non-biopharmaceutical products in various regions due to its many benefits. *P. angulata* has many potentials as antioxidant, anti-inflammation, anticancer, antibacterial, antidiabetic, and antidiarrheal agent due to its rich of contents (Fadhli et al., 2023). The imbalance between its high utilization potential and the limited cultivation development suggests structural challenges in the management of *P. angulata* in Indonesia.

The medicinal potentials of *P. angulata* are derived from the secondary metabolites contained within the plant, such as phenolic content and flavonoid content. Phenolic compound is a large group of secondary metabolites containing one or more aromatic rings with hydroxyl groups (Xu & Wang, 2025). Phenolic compound can be found in many parts of plant with various physiological function, such as antioxidant, protection from ultraviolet radiation, and protection mechanism from pests and diseases (Balasundram et al., 2006). One group derived from phenolic is flavonoid group. Flavonoid is a polyphenol compound contain three aromatic rings as their main structure (Shen et al., 2022). Flavonoid play role in stress reducing mechanism, as a pigment to attract pollinators, and protect plant from pests and diseases (Samanta et al., 2011). Both phenolic and flavonoid compound have pharmaceutical values as antioxidant, anti-inflamation, anti-aging, metabolism regulation, and prevent cardiovascular diseases (Oluwole et al., 2022).

Obtaining a raw material for pharmaceutical products from *P. angulata* is a big challenge in Indonesia due to the lack of conventional cultivation. Moreover, the level of secondary metabolites can be influenced by environmental factors, makes it more difficult to meet the demand (Chaidir et al., 2015). Due to these challenges, one solution to propagate *P. angulata* and obtain its secondary metabolites in a short time, large quantities, and under stable environmental conditions is through *in vitro* callus culture. *In vitro* culture allows us to modify environmental conditions, as well as the quantity and quality of the plant materials to meet industrial demands (Mastuti & Rosyidah, 2021). The purposes of propagating plants through *in vitro* callus are for mass propagation and production of secondary metabolites. This is because callus tissue can be multiplied rapidly and has potential to produces higher levels of secondary metabolites compared to whole plants (Sitorus et al., 2011). Plant tissue culture also allows exposure of controlled stress to enhance the production of secondary metabolites without viability lost.

Phenolic and flavonoid production can be induced under stress condition, such as salinity or salt stress. Salinity stress causes two phases of stresses to plant, osmotic stress and ionic stress (da Silva et al., 2021). Osmotic stress is an inability of plant water intake, causing temporary dehydration; while ionic stress occures when there is excessive ion accumulation of Na^+ and Cl^- inside plant cells causing metabolism abnormalities (Munns & Tester, 2008). Stressed plant will induces reactive oxygen species (ROS) signaling mechanism to activates antioxidant activity, osmoprotectant synthesis, and ion transport protein expression and enhances plant tolerance against salt stress (Ighodaro & Akinloye, 2018). Increased ROS level will activates phenilpropanoid biosynthesis pathway, producing antioxidant compounds such as phenolic and flavonoid to mitigate the oxidative damaging effects (Ahmed et al., 2015). This study is also expected to serve as a reference for industrial-scale herbal medicine manufacturers, enabling them to efficiently obtain high yields of phenolic and flavonoid compounds.

Materials and Methods

Time and location of research. The study was conducted in two stages. The first stage focused on callus induction, aimed at determining the optimal combination of plant growth regulators (PGRs) for callus induction, while the second stage involved the elicitation of callus using NaCl as a salinity stress treatment to identify the optimal concentration for maximizing phenolic and flavonoid accumulation. The first stage was

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carried out from February to March 2025 at the Plant Tissue Culture Laboratory, Faculty of Agriculture, Universitas Padjadjaran. The second stage conducted from August to December 2025 at the Plant Tissue Culture and Post-Harvest Analysis Laboratory, Faculty of Agriculture, Universitas Padjadjaran.

Tools and materials. The materials used in this experiment included: *P. angulata* seeds collected from Ciparanje Experimental Garden, Universitas Padjadjaran; 1.05% sodium hypochlorite; Murashige and Skoog (MS) medium; 6-benzylaminopurine (6-BAP); 2,4-dichlorophenoxyacetic acid (2,4-D); sodium chloride (NaCl) salt; 80% methanol; distilled water; 10% Folin-Ciocalteu reagent; 7.5% sodium carbonate; 10% aluminum chloride; gallic acid and quercetin standards; 1 M potassium acetate; 2,2-diphenyl-1-picryl-hydrazyl (DPPH); sodium hydroxide; and hydrochloric acid.

The equipment used in this experiment included: a hot plate magnetic stirrer, beakers, an analytical balance, a pH meter, culture bottles, an oven, an autoclave, spatulas, pipettes, micropipettes, forceps, scalpels, Petri dishes, culture scissors, hand sprayers, a spirit lamp, a laminar air flow cabinet (LAFC), culture racks equipped with LED (light-emitting diode) lamps, a thermo-hygrometer, a refrigerator, test tubes, graduated cylinders, volumetric flasks, a mortar and pestle, a shaker, a vortex mixer, a centrifuge, microtubes, cuvettes, and a UV-Vis spectrophotometer.

Experimental Design. First stage of experiment consisted of 12 treatments with 3 replications. Ten culture bottles used for each replication, which containing 3 explants per bottle. The experiment was arranged in a factorial completely randomized design (CRD) with two factors. The first factor was the concentration of the plant growth regulator 6-BAP with three levels of treatments (0, 2, and 4 mg/L), while the second factor was the concentration of the plant growth regulator 2,4-D with four level of treatments (0, 0.5, 1, and 1.5 mg/L).

The optimal medium identified in the first stage of the experiment was utilized as the basal combination for callus induction in the second stage. The second stage consisted of five treatments with four replications. Each replication consisted of 15 culture bottles with three calli per bottle. The experiment was arranged in a single-factor completely randomized design (CRD), with NaCl concentration as the treatment factor at levels of 0, 25, 50, 75, and 100 mM.

First Stage Experiment. *Physalis angulata* L. seeds were obtained from the Ciparanje Experimental Garden, Universitas Padjadjaran. The seeds were harvested from physiologically mature fruits, indicated by their yellow calyx. Seed dormancy was broken using 100 mg/L gibberellin (GA3) solution for 24 hours. The seeds were then sterilized with a 1.05% sodium hypochlorite solution for 15 minutes and rinsed with sterile distilled water three times for 5 minutes each time (Mastuti et al., 2020). The sterilized seeds were then initiated on MS basal medium without any supplementation under aseptic conditions in LAFC. After eight weeks incubation, leaves from the plantlets were used for callus induction. Leaves from the grown plantlets were collected as explants. The criteria of the leaves included located at fourth from both the base and the apex, green in color and not yellowish, and fully opened. The explants were then cut into 1 cm × 1 cm pieces and sub cultured into the treatment medium, with three explants per bottle, under aseptic conditions in the LAFC. The explants were incubated for four weeks in an incubation room at room temperature with 16 hours photoperiod per day.

Second Stage Experiment. Callus from the first experiment were cut into 1 cm × 1 cm x 1 cm pieces and sub cultured into a second stage medium. The basal composition of the medium consisted of MS medium with 1 mg/L BAP and 0.5 mg/L 2,4-D, supplemented with different concentrations of NaCl as experimental treatments. The calluses were then cut into pieces measuring 1 cm × 1 cm. Each bottle consisted of three calluses were incubated for 5 weeks in an incubation room at room temperature with 16 hours photoperiod per day.

Observed Parameters. In the first experiment, leaves explants dedifferentiated into callus. The observed parameters of this stage are the response of callus growth and development to medium with varying concentrations of 6-BAP and 2,4-D. The observed callus growth parameters including the percentage of explants forming callus, callus size, callus fresh weight, callus consistency, and callus color. Callus size was assessed using score diagram on a scale of 1–23 (Figure 1). Callus consistency was evaluated using a consistency scale of 0–5 (Table 1) based on the study by Hidayat et al. (2021). All calli in the

first stage experiment were evaluated after four weeks of treatments.

In the second stage of experiment, the observation parameters are including callus secondary metabolites accumulation and antioxidant activity to varying concentrations of NaCl as a salt stress treatment. Callus secondary metabolites accumulation included total phenolic content in gallic acid standard and total flavonoid content in quercetin standard (Yari et al., 2025); while callus antioxidant activity was assessed using IC_{50} DPPH method (Hussen & Endalew, 2023; Shimamura et al., 2014). After five weeks of salinity stress treatment, the callus was dried in an oven at 36°C for 72 hours and subsequently ground into powder. The sample powder was then macerated in 80% methanol for 24 hours while being agitated on a rotational shaker at 100 rpm. Finally, the extract was centrifuged at 4,800 × G for 5 minutes.

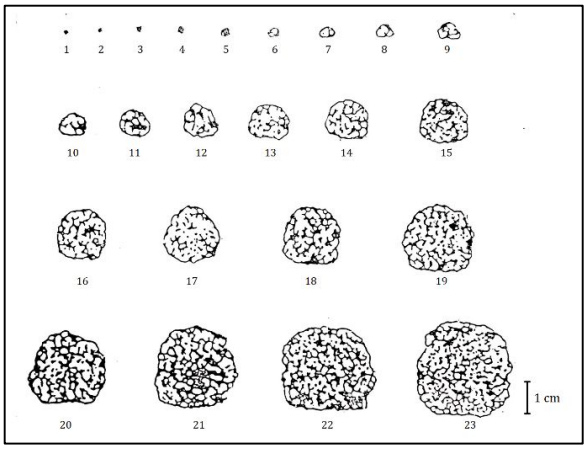

Figure 1. Callus size score

Total Phenolic Content (TPC) was determined by mixing 80 µl of extract or standard solution with 200 µl of 10% Folin-Ciocalteu reagent and homogenized using a vortex for four minutes. Subsequently, 2 mL of 7.5% sodium carbonate was added followed by the addition of distilled water to reach its final volume of 5 mL. The solution was then incubated in the dark room at room temperature for 30 minutes. Absorbance was measured using spectrophotometer at 760 nm wavelength with triplicate analysis. Gallic acid at 25, 50, 100, 200, and 400 mg/L concentration was used as a standard to generate a calibration curve. The TPC values were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW).

Table 1. Callus consistency score

Score	Consistency	Note
0	Dead and did not form callus	Undifferentiated
1	Friable 1 (cotton-like structure)	Callus with a cotton-like structure
2	Compact callus	Callus surface is glossy and entirely smooth
3	Compact with nodules	Appears on the wounded area of the explant
4	Friable 2 (easily crumbled structure)	Callus with an easily crumbled (friable) structure
5	Friable 2, followed by nodule formation	Callus followed by nodule formation

Total Flavonoid Content (TFC) was determined by mixing 120 µl of extract or standard solution with 1.5 mL of 80% methanol, 100 µl of 10% aluminum chloride, 100 µl of 1 M potassium acetate, and distilled water to reach its final volume of 5 mL. The solution was then incubated in the dark room at room temperature for 40 minutes. The absorbance was measured using spectrophotometer at a wavelength of 415 nm. Quercetin was used as the standard at concentrations of 25, 50, 100, 200, and 400 mg/L to establish a calibration curve. The TFC values were expressed as milligrams of quercetin equivalents per gram of dry weight (mg QE g^{-1} DW).

Antioxidant activity was determined using DPPH method. Callus extracts were diluted into 50-2000 mg/L concentrations, and each 2 mL concentration or 2 mL of control was added by 2 mL 0,1 mM DPPH solution. The solutions were then incubated in dark room at room temperature for 30 minutes. The absorbance was measured using spectrophotometer at a wavelength of 517 nm. Inhibitory concentration (IC_{50}) was calculated using direct interpolation method, where antioxidant activities close above and below 50% were selected to determine its regression line and equation according to Li et al. (2015).

$$IC_{50} = x_1 + \frac{50\% - y_1}{y_2 - y_1} \times (x_2 - x_1)$$

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Where x_1 and x_2 are the concentrations between 50% inhibitory, while y_1 and y_2 are the inhibitory percentage.

Statistical Analysis. The experimental design used a factorial completely randomized design for the first stage and single completely randomized design for the second stage. Quantitative data were analyzed using analysis of variance (ANOVA) based on the F-test at a 5% significance level. If a significant effect was observed (F calculated > F table), the analysis was followed by Duncan's Multiple Range Test (DMRT)at the 5% level. The analysis was conducted using SPSS software.

Results and Discussion

Percentage of Explants Forming Callus. The first stage of experiment demonstrated interactive effects of the cytokinin 6-BAP and the auxin 2,4-D treatments. Analysis of variance for the percentage of callus formation indicated there is a significant interaction ($p < 0.05$) between both plant growth regulators. The interaction graph of 6-BAP and 2,4-D showed the optimal concentration for each combination after four weeks application (Figure 2). Control treatment induced callus formation percentage only 54%. The application of 6-BAP and 2,4-D to the explants increased the percentage of callus formation compared to the control. The interaction graph of 6-BAP and 2,4-D showed that all concentrations of 6-BAP induced highest callus formation percentages when combined with 2,4-D at 1 mg/L and 1.5 mg/L.

One of the indicators affecting callus induction is the dedifferentiation of explants followed by callus formation. Callus is a mass of unorganized and non-specialized plant cells that has been widely applied in various biotechnology practices such as plant propagation, genetic modification, and secondary metabolite mass production (Thorpe, 2012). Callus *in vitro* culture success rates are influenced by the type of explant, composition of the medium, and plant growth regulators (PGRs). Dedifferentiation and callus formation occurs when explants are treated with appropriate ratios of PGR such as auxin and cytokinin (Su et al., 2011). Cytokinin 6-BAP and auxin 2,4-D are known for their stable structure and have good efficacy for *P. angulata in vitro* culture.

Cytokinin 6-BAP and auxin 2,4-D indicate an interaction in callus formation. These combinations can trigger dedifferentiation and callus formation in plant cells (Su et al., 2011). Explants without 6-BAP and 2,4-D treatment had a potential to fail to form callus or small callus formation. This case happened due to the existence of endogenous hormones from the explant, allowing some cells to divide (Sari et al., 2014). In addition, explant wounding while subculture can be another factor to induce and stimulate cell division and callus formation (Aziz et al., 2014). Wounding in leaf tissue can trigger expression of the cdc2 gene, increasing the competency of cells to undergo cell division (Hemerly et al., 1993).

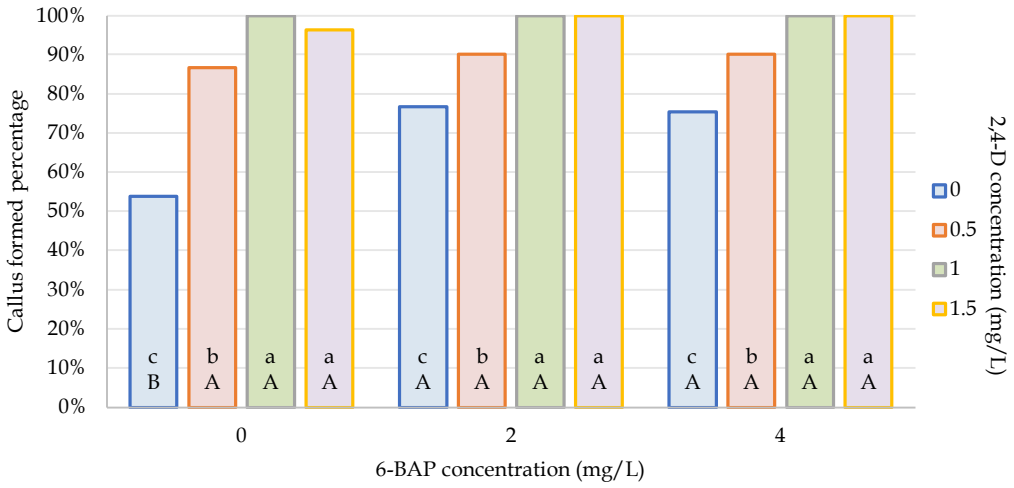

Figure 2. Interaction effect of 6-BAP and 2,4-D on the percentage of callus formation
Note: Means followed by lowercase letters indicate significant differences among 2,4-D concentrations within the same 6-BAP level, while uppercase letters indicate significant differences among 6-BAP concentrations within the same 2,4-D level.

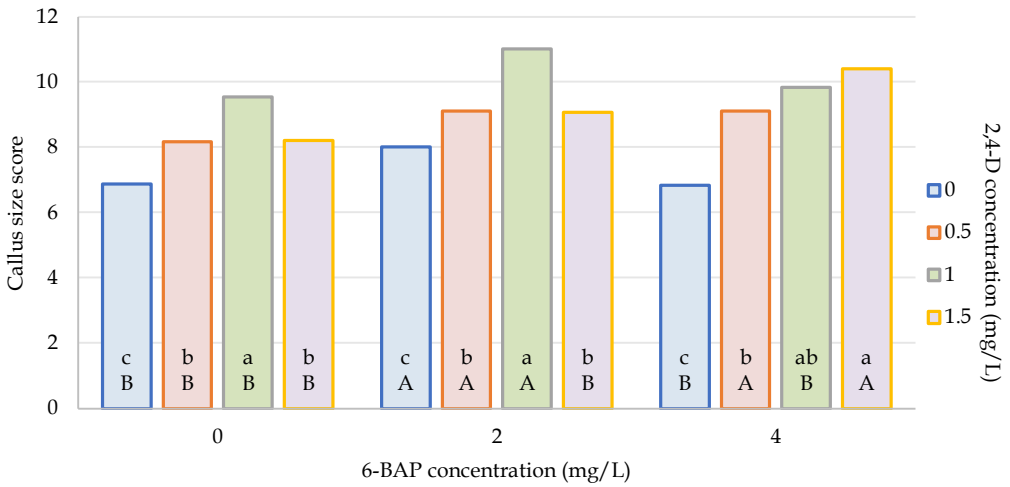

Figure 3. Interaction effect of 6-BAP and 2,4-D on callus size scale

Note: Means followed by lowercase letters indicate significant differences among 2,4-D concentrations within the same 6-BAP level, while uppercase letters indicate significant differences among 6-BAP concentrations within the same 2,4-D level.

Callus Size. There is a significant interaction ($p < 0.05$) between cytokinin 6-BAP and auxin 2,4-D for callus size. This study showed that 6-BAP at 0 and 2 mg/L should be combined with 2,4-D at of 1 mg/L to produce the largest callus size. Cytokinin 6-BAP at 4 mg/L produced the largest callus size when combined with 2,4-D at 1.5 mg/L, although it was not significantly different from the combination with 1 mg/L 2,4-D. High concentrations of 6-BAP combined with 2,4-D at 0 and 1 mg/L tended to reduce callus size (Figure 3). The combination of 6-BAP and 2,4-D increased callus size compared to the control after four weeks (Figure 4).

Culture medium and PGRs composition can greatly affects *P. angulata* callus growth. Callus size is one indicator to assess its suitability to the composition of medium and PGRs. The faster the callus size increased, the faster mass propagation can be done to achieve industrial demands. Tissue swelling is a stage in callus formation that indicates active cell division and elongation (Anggraeni et al., 2022). Proper hormone ratios and tissue woundings in explant trigger continuous cell division and enlarging callus size (Aziz et al., 2014). This condition may involve cdc2 gene expression to prepare cell division and increase tissue growth (Hemerly et al., 1993).

Applying right concentration of PGRs to explants enhances callus size. Tissue swelling and size increase indicate that PGRs combination promotes cell division in callus tissue (Anggraeni et al., 2022). Wounded explant tissue allows easier diffusion of nutrients and PGRs from external medium, which accelerate cell division and tissue swelling (Fitroh et al., 2018). While right concentration of PGRs can increase callus growth, excessive concentrations of PGRs can be toxic, disrupting hormonal balance in the plant tissue, causing stress and inhibiting cell division (Wartina et al., 2011).

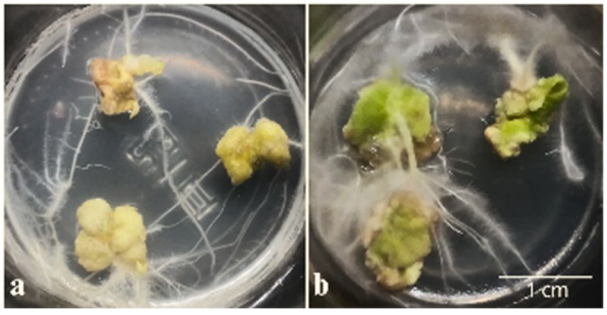

Figure 4. Comparison of callus size on (a) untreated medium and (b) medium with a combination of 2 mg/L of 6-BAP and 1 mg/L of 2,4-D

Callus Fresh Weight. Callus fresh weight was significantly affected ($p < 0.05$) by 6-BAP and 2,4-D interaction. Control treatment produced callus only 83.7 mg of fresh weight at four weeks after subculture. The application of 6-BAP increased callus fresh weight with different

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combinations of 2,4-D, although there was no significant difference between 2 mg/L and 4 mg/L of 6-BAP concentrations (Figure 5). The application of 2,4-D increased callus fresh weight until reaching its maximum at 1 mg/L concentration, although there was no significant difference between 0 mg/L of 6-BAP combined with either 1 mg/L or 1.5 mg/L of 2,4-D. At 2 mg/L and 4 mg/L of 6-BAP, the combination with 1.5 mg/L of 2,4-D reduced callus fresh weight and made it not significantly different from 0.5 mg/L of 2,4-D.

Callus growth can be measured through the increase in callus fresh weight. An increase in callus fresh weight indicates optimal cell division (Anggraeni et al., 2022). Application of cytokinins such as 6-BAP and auxins like 2,4-D at appropriate concentration and ratio can be capable to increase callus fresh weights (Fitroh et al., 2018). A synergistic interaction between auxins and cytokinins can stimulate explant's cell division and dedifferentiation, increasing their fresh weight (S. M. Li et al., 2021). High callus fresh weight comes from rapid and active cell division, callus morphology, and high water content (Hidayat et al., 2021). However, excessive concentration of auxin such as 2,4-D can accelerate cell aging, while excessive concentration of cytokinin such as 6-BAP is potentially trigger programmed cell death, hindering cell division and growth (Carimi et al., 2003; Peterson et al., 2016). Callus browning can also inhibit cell division, slowing down the increase in fresh weight (Rybin et al., 2024). On the other hand, too low concentration or inapropriate ratio of PGRs may fail to induce and stimulate cell division (Yücesan et al., 2015)

Callus Consistency and Color. Callus visual observation by its consistency and color can indicate the response of callus development. In general, callus consistencies are categorized into two types: compact and friable. However, Hidayat et al. (2021) utilize a scoring system that classifies callus consistency into six classes for more specific details. This study showed that most calluses had a consistency score 2, which indicate compact callus texture (Table 2). Compact callus usually formed when explants are newly dedifferentiated and the cell structures remain relatively dense and compact. Callus consistency can change over time after being sub cultured onto new medium.

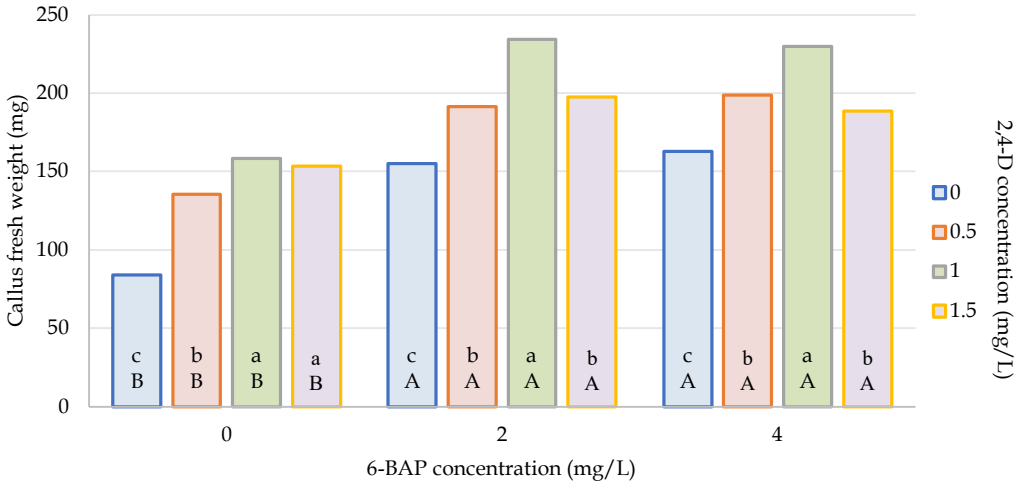

Figure 5. Interaction effect of 6-BAP and 2,4-D on callus fresh weight
Note: Means followed by lowercase letters indicate significant differences among 2,4-D concentrations within the same 6-BAP level, while uppercase letters indicate significant differences among 6-BAP concentrations within the same 2,4-D level

Table 2. Effect of 6-BAP and 2,4-D combinations on callus consistency

2,4-D Concentration	6-BAP Concentration		
	0 mg/L	2 mg/L	4 mg/L
0 mg/L	2	2	2
0.5 mg/L	2	2	2
1 mg/L	2	2	2
1.5 mg/L	2	2	2

Another callus development indicator that can be visually observed is callus color. This study showed that most treatments produced callus with color codes N144A, 151A, and N144C of Royal Horticultural Society Color Chart (The Royal Horticultural Society, London, 2007), which were categorized as yellow-green group (Table 3). Callus from control treatment, the combination of 0 mg/L 6-BAP + 1.5 mg/L 2,4-D, and 2 mg/L 6-BAP + 0.5 mg/L 2,4-D had the color code 151A, which more yellowish compared to other treatments. Treatments that maintained a greener callus color included the combinations of 2 mg/L 6-BAP + 1 mg/L 2,4-D and 2 mg/L 6-BAP + 1.5 mg/L 2,4-D, with color code N144C. This color can indicate that they were able to delay callus browning.

Callus consistency and color can describe its physiological response. Friable callus has loose arranged cells and can be easily broken apart, while compact callus has dense and tightly arranged cells with thick polysaccharide walls which making it harder to separate (Hidayat et al., 2021). Friable callus is suitable for propagation because it can be easily divided and scaled up via suspension cultures (Rasud & Bustaman, 2020), while compact callus is harder to propagate but is more efficient for secondary metabolites accumulation (Indah & Ermavitalini, 2013) due to its structured cell arrangement that potentially facilitates metabolite transport.

Callus color is an indicator that can reflects cell activity level (Rasud & Bustaman, 2020). White to yellowish callus indicates embryogenic and active dividing cells; green callus contains chlorophyll, while brown callus indicates phenolic oxidation or tissue aging (Anggraeni et al., 2022). Callus color may vary depending on the explant used (Hidayat et al., 2021). Low levels of 6-BAP and 2,4-D may trigger phenolic biosynthesis without inducing cell division, leading to phenolic accumulation without increasing its growth. In contrast, high concentrations of 6-BAP may induce programmed cell death, while 2,4-D can increase ethylene levels, accelerating cell senescence (Rybin et al., 2024).

Total Phenolic Content (TPC) Under Salinity Stress

Total phenolic content (TPC) was evaluated using gallic acid standard during second stage of this experiment. *P. angulata* callus showed an increasing trend of TPC although there is a slight decrease compared to control observed at the 25 mM treatment and gradual increase reaching the highest content at the 100 mM NaCl treatment (Table 4). This pattern indicates there are secondary biosynthesis activation of secondary metabolites as a response during salinity stress. Phenolic compounds are identified with its role as a non-enzymatic antioxidant to neutralize ROS accumulation inside plant cells during osmotic and ionic stress caused by salinity. It also can be implied that NaCl treatments at 0-100 mM was still within the callus tolerance threshold to perceive salinity stress.

Phenolic content biosynthesis under salinity stress is related to phenylpropanoid pathway. Salinity stress was divided into two phases, osmotic stress and ionic stress (da Silva et al., 2021). Excessive Na^+ accumulation inside cell during ionic stress will also increase ROS accumulation, which can damage cell by its oxidative reaction (Pungin et al., 2023). Intracellular Ca^{2+} level increased during stress and acting as second messenger to activate calcium sensors such as calcium-dependent protein kinase (CDPK), leading to activation of ROS signaling and transcription factors of defense-related genes and enzymes (Qari & Tarbiyyah, 2021). One of the key regulatory enzymes in phenolic production is phenylalanine ammonia-lyase (PAL) which catalyze phenilalanine, precursor of phenolic compounds (Kumari et al., 2023). Salinity stress signaling pathways allows for redirection of carbon and energy resources from primary metabolism to secondary metabolism for plants defense strategies (Ortiz & Sansinenea, 2023). The increased TPC in plant under salinity stress indicates that phenolic compounds play their role as ROS scavengers to maintain redox homeostasis (Gozdur et al., 2024).

The adaptive response is relevant in this experiment, where external stress factors influence cellular metabolism to reduce oxidative damages caused by stress and consistent with findings reported in another species. A study on *Rumex thyrsiflorus* exposed to salinity for four weeks showed an increase of accumulation of phenolic compound such as gallic acid at 43 mM NaCl concentration by 98.83% on male callus and 129 mM NaCl concentration by 185% on female callus (Gozdur et al., 2024). Another study also revealed that NaCl treatments at 84 and 250 mM for four weeks were able to increase total phenolic content of various *Nigella sative* genotypes (Golkar et al., 2020). These abilities of

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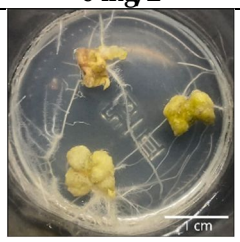
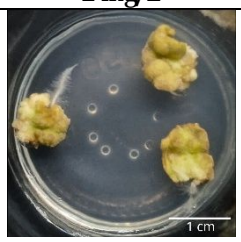
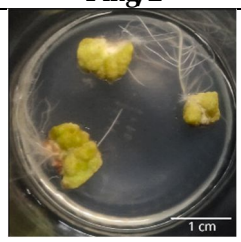
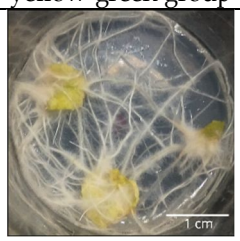
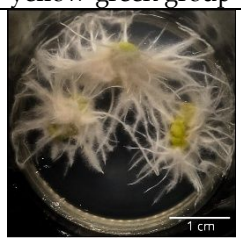
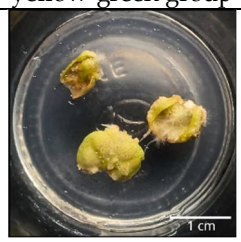
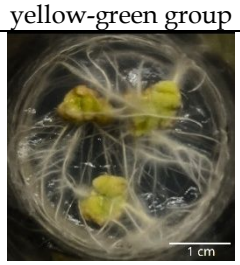
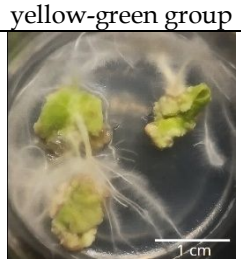
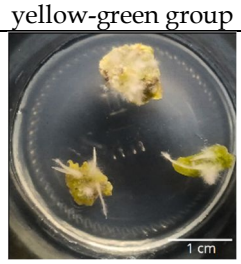
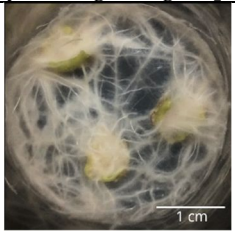
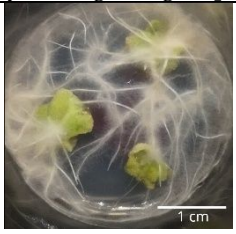
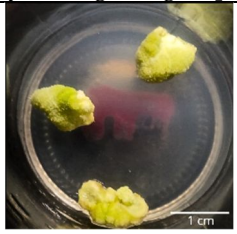
callus to enhance phenolic level under salinity stress indicates a strategic approach to stimulate phenolic accumulation for both physiological and biotechnological application. However, excessive NaCl concentration should be avoided to prevent over-toxicity and physiological damage, which directing into cell death (Bozaba & Kuru, 2024).

Total Flavonoid Content (TFC) Under Salinity Stress

Flavonoid content in *P. angulata* callus was evaluated using quercetin standard during second stage of this experiment. Callus TFC showed increase from control and reaching its highest accumulation at 100 mM NaCl treatment (Table 4). This pattern of value indicates that flavonoid biosynthesis in *P. angulata* callus is responsive to salinity stress treatment. Flavonoid compounds are one part of phenolic compounds that also play role at antioxidant activity to reduce oxidative damage caused by stress. Same with phenolic content, NaCl treatment at 0-100 mM also suggest that it was still within the callus tolerance threshold and acted as an elicitor to stimulate flavonoid biosynthesis. Flavonoid compounds play many roles in plant such as a defense mechanism against various biotics and abiotic stresses or as a non-enzymatic antioxidant (Panche et al., 2016).

Table 3. Callus colors after four weeks under different 6-BAP and 2,4-D combinations

2,4-D Concentration	6-BAP Concentration		
	0 mg/L	**2 mg/L**	**4 mg/L**
0 mg/L	 151A yellow-green group	 N144A yellow-green group	 N144A yellow-green group
0.5 mg/L	 N144A yellow-green group	 151A yellow-green group	 N144A yellow-green group
1 mg/L	 N144A yellow-green group	 N144C yellow-green group	 N144A yellow-green group
1.5 mg/L	 151A yellow-green group	 N144C yellow-green group	 N144A yellow-green group

Flavonoid compounds are a part of polyphenols and their biosynthesis also associated with phenylpropanoid pathway. Salinity-induced treatment is frequently used as a mediation to enhance secondary metabolites such as flavonoid by triggering plants adaptive response through its capability as free radical scavengers (Isah, 2019). During exposed to salinity stress, physiology abnormalities will be recognized and inducing many signaling to increase salt tolerance and activate antioxidant enzyme expression including for flavonoid biosynthesis (Chele et al., 2021). Not only PAL, but there are also chalcone synthase (CHS) and chalcone isomerase (CHI) acting as the key of regulatory enzyme controlling flavonoid compounds formation pathways from phenylpropanoid pathway (Mao et al., 2025). From this stage onward, a various kind of flavonoids are synthesized, tailored to their specific physiological roles and function (Kumari et al., 2023). Therefore, the flavonoid content observed in this study implies an adaptive metabolic response of callus tissue to salinity stress treatments.

P. angulata callus response of flavonoid accumulation to salinity treatment observed is aligned with findings reported in various species. A study on *Stevia rebaudiana* callus exposed to salinity for six weeks showed an increase of 40.95% accumulation of flavonoid compound at 100 mM NaCl concentration compared to control (Javed & Gürel, 2019). In *Rumex thyrsiflorus* callus, an increase of TFC, such as quercetin, was observed after four weeks of salinity exposure with 52% increase occurred in male callus at 43 mM NaCl and 221% in female callus at 129 mM NaCl (Gozdur et al., 2024). Another study also revealed that *Rhinacanthus nasutus* callus exposed to salinity stress at 100 mM NaCl concentration for seven days with 14 days of tissue recovery after exposure increased accumulation of total flavonoid content by 28.90% compared to control (Yaowachai et al., 2026). These studies support the observation that salinity stress can act as an effective elicitor to enhance flavonoid accumulation in callus cultures depending on species, NaCl concentration, and exposure duration, including *P. angluata* callus culture.

Antioxidant Activity Under Salinity Stress. Abiotic stress treatment in *P. angulata* callus is intended not only to observe its secondary metabolites content, but also to evaluate its antioxidant capacity. In this second stage of experiment, antioxidant activity was assessed by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay method. Lower IC_{50} value of DPPH reflects a higher ability of the extract to scavenge and neutralize DPPH free radical molecules. The result of this experiment showing gradual decrease of IC_{50} concentration indicates that salinity stress treatment can stimulate the production of antioxidant compounds and reach its higher antioxidant activity at 100 mM NaCl concentration (Table 4). The improvement of antioxidant activity under salinity stress can be associated with secondary metabolites accumulation with radical scavenging abilities.

Salinity stress induces oxidative stress through reactive oxygen species and disrupts cellular redox homeostasis. During salinity stress, secondary metabolites acting as a second line defense of antioxidant after enzymatic antioxidant by donating electrons and neutralize free radical molecules into their harmless form (Ighodaro & Akinloye, 2018). Secondary metabolites such as phenolics and flavonoids antioxidant capacity potential are influenced by its hydroxyl group structures (Gulcin, 2025). Therefore, phenolic and flavonoid content in this research also play role as radical scavenger. Antioxidant activity evaluation method in this experiment used DPPH free radical molecule to induce electron donation from non-enzymatic

Table 4. Comparison of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities after five weeks of callus exposure with different concentrations of NaCl

NaCl Concentration	TPC (mg GAE/g DW)	TFC (mg QE/g DW)	Antioxidant Activity IC_{50} (mg/mL)
NaCl 0 mM	3.316	0.433	0.935
NaCl 25 mM	3.309	0.450	0.912
NaCl 50 mM	3.350	0.451	0.905
NaCl 75 mM	3.364	0.469	0.885
NaCl 100 mM	3.494	0.542	0.871

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antioxidants, leading to reduction of DPPH molecules into their non-reactive state (Wołosiak et al., 2022). Salinity treatments were able to enhance *P. angulata* callus phenolic and flavonoid accumulation, which also potentially allows antioxidant capacity enhancement.

Several studies have reported antioxidant activity in callus *in vitro* cultures assessed by the DPPH assay, supporting the observation of enhanced radical scavenging capacity under salinity stress. In callus culture of *Thymus daenensis*, 14 days of 100 mM NaCl treatment increased antioxidant activity by 2%, within 0-250 µg/mL observation range of extracts concentration (Golkar et al., 2025). Another research in *Rhinacanthus nasutus* callus showed a decrease of IC_{50} value by 10.16% at 200 mM NaCl concentration exposure for 7 days (Yaowachai et al., 2026). In *Stevia rebaudiana* callus, antioxidant activity was increased by 13.66% at 100 mM NaCl treatment for 45 days (Javed & Gürel, 2019). Based on these studies, salinity stress can be considered as a promising abiotic elicitor to enhance antioxidant activity in *in vitro* callus cultures, including *P. angulata*, through regulation of secondary metabolite production.

Conclusion

Callus induction on *P. angulata in vitro* leaf explants can be triggered by combination of 6-BAP and 2,4-D. The combinations of 6-BAP and 2,4-D are capable to induce dedifferentiation of *P. angulata* leaf explants to form callus. Combination of 6-BAP at 2 mg/L and 2,4-D at 1 mg/L showed the largest callus size, highest fresh weight, and greener callus color compared to other treatments. All treatments produced callus with compact consistencies which are capable for secondary metabolites production. Callus of 6-BAP at 2 mg/L and 2,4-D at 1 mg/L is utilized as secondary metabolites and antioxidant producer by salinity treatments in second stage. Salinity stress treatments can induce *P. angulata* antioxidant levels especially phenolic content, total flavonoid content, and inhibitory concentration at 50% (IC_{50}). NaCl concentration at 100 mM showed the most phenolic and flavonoid accumulation with lowest IC_{50} value, indicates highest antioxidant activity, compared to control. These elevations indicated that salinity stress levels applied in this study did not exceed *P. angulata* callus physiological tolerance.

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