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Growth and production of secondary metabolites in the callus of Bima Brebes shallot varieties

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Abstract. *Habibah NA, Yuniastuti A, Susanti R, Lisdiana, Mustikaningtyas D, Lutfiah A, Aulia SN, Rabbani T. 2024. Growth and production of secondary metabolites in the callus of Bima Brebes shallot varieties. Biodiversitas 25: 2811-2820.* Shallots contain bioactive compounds that can be produced through callus culture. This study investigates the effects of growth regulators 2,4-D, picloram, and kinetin on the growth and production of secondary metabolites in shallot bulb callus. A Complete Randomized Design (CRD) with two factors was used in the experiment. The first factor was the concentrations of 2,4-D (0, 1, 2, and 3 ppm) and picloram (0, 1, 2, and 3 ppm). The second factor was kinetin concentrations (0, 0.25, and 0.5 ppm). The parameters evaluated were callus fresh weight, callus dry weight, percentage of callus formation, callus induction time, callus morphology, phytochemical components, and antioxidant activity. The optimal growth medium is crucial for developing growth curves. Bioactive compounds were identified using Liquid Chromatography-Mass Spectrometry (LC-MS). Results indicated that explants grown on a medium combining picloram and kinetin exhibited better growth than those grown on a 2,4-D and kinetin combination. The callus colors varied from white to yellowish-white, yellow, and brownish-yellow, with a crumbly texture. Callus from all treatments contained bioactive compounds indicated by antioxidant activity, flavonoid content, and total phenolics. LC-MS analysis identified 87 types of secondary metabolite compounds. Callus treated with picloram and kinetin consistently produced flavonoids and phenolics and exhibited antioxidant activity.

Keywords: Bioactive, callus, kinetin, picloram, shallots

INTRODUCTION

Plants are a rich source of phytochemicals, and their secondary metabolites have been used in medicines, supplements, and cosmetics. However, the yield of these secondary metabolites from plants is generally low, and their industrial-scale synthesis is difficult due to their complex chemical structures. Plant cell culture is a promising alternative for producing desired secondary metabolites by applying specific Plant Growth Regulators (PGRs) (Twaij and Hasan 2022). Callus culture has proven an effective strategy for manipulating and producing valuable secondary metabolites. The callus is a mass of cells that have undergone dedifferentiation and regained totipotency, enabling it to regenerate the entire plant body. Dedifferentiation involves transforming cells from a specialized state to a more primitive, stem cell-like state (Jiang et al. 2015). The addition of PGRs can trigger this reversal. Callus formation occurs at appropriate concentrations when explants are cultured on media containing PGRs, specifically from the cytokinin and auxin groups (Dar et al. 2021).

The choice of media and the appropriate combination of PGRs are crucial for the successful induction of bioactive compounds in explants. A combination of auxin and cytokinin is typically used for plant callus induction. In in vitro tissue culture, cytokinins function as shoot inducers (Šmeringai et al. 2023) by stimulating auxin biosynthesis (Di et al. 2016; Yan et al. 2017). Auxin, a phytohormone that moves directionally in the plant, is crucial in inducing polarity and regulating plant morphogenesis in vitro, including the formation of shoots and roots (Pasternak and Steinmacher 2024). By providing a balanced concentration of auxin and cytokinin, explants are stimulated to form basal stem cells, increasing the callus' fresh and dry weight (Hunaish and Almasoody 2020). Auxins commonly used for callus induction include 2,4-D and picloram (Saikia et al. 2013; Vats and Kamal 2014; Daud et al. 2022). The specific growth regulators required for callus induction can vary depending on the type of explant and plant species (Singh et al. 2014; Veraplakorn 2016; Habibah et al. 2016, 2019, 2020). For example, the growth of *Allium hirtifolium* callus is also affected by the type of growth regulators (Farhadi et al. 2017). The growth and production of secondary metabolites in callus cultures are influenced by the selection of growth regulators and the conditions of the culture environment. *Stelechocarpus burahol* callus cultured on Murashige and Skoog (MS) medium with various PGRs yielded different concentrations of flavonoids

(Habibah et al. 2016). Similarly, the impact of growth regulators on flavonoid and phenolic content has been documented in *Elaeocarpus grandiflorus* callus (Habibah et al. 2019; 2023).

Shallots (*Allium cepa* L.) are valuable horticultural plants with numerous applications and significant economic importance. They contain medicinally beneficial nutrients and bioactive compounds, including quercetin, kaempferol (Shahrajabian et al. 2020), alkaloids, cysteine, cycloalliin, saponins, tannins, phenols, steroids, and triterpenoids (Boukeria et al. 2016). Qualitative analysis of the 70% ethanol extract from Brebes shallot varieties revealed the presence of volatile oil compounds, saponins, tannins, flavonoids, and terpenoids (Nurcahyo et al. 2022). The primary volatile compounds in shallots, particularly those grown in Indonesia (including Bima Brebes, Biru Lancor, Saptosari, and Filipin varieties), are disulfides containing sulfur, thiophene, and various functional groups. The most prevalent volatile compounds in Bima Brebes shallots include compounds containing sulfur (40.61%), various functional groups (28.43%), and disulfides (17.51%) (Indrasari et al. 2021). Shallots are reported to possess bioactive compounds with various beneficial activities, including antidiabetic, antimicrobial, antiplatelet, anticancer, antidepressant, antioxidant, antihypertensive, antiparasitic, immunomodulatory effects, anti-inflammatory, and SARS-CoV-2 inhibitory (Farag et al. 2017; Ko et al. 2018; Zhao et al. 2021).

No studies have been reported on the optimal medium for the growth and production of secondary metabolites in the callus of Bima Brebes shallots. This study investigates the growth and production of secondary metabolites in Bima Brebes shallot callus using MS medium supplemented with combinations of 2,4-D and kinetin, as well as picloram and kinetin.

MATERIALS AND METHODS

Research design

This research employed a Complete Randomized Design (CRD) with two factors. The first factor is the 2,4-D concentration (0, 1, 2, and 3 ppm) and picloram concentration (0, 1, 2, and 3 ppm). The second factor is kinetin concentration (0, 0.25, and 0.5 ppm). The total treatments were 24, i.e., D0K0: 2,4 D 0 ppm + kinetin 0 ppm, D0K25: 2,4 D 0 ppm + kinetin 0.25 ppm, D0K50: 2,4 D 0 ppm + kinetin 0.5 ppm, D1K0: 2,4 D 1 ppm + kinetin 0 ppm, D1K25: 2,4 D 1 ppm + kinetin 0.25 ppm, D1K50: 2,4 D 1 ppm + kinetin 0.5 ppm, D2K0: 2,4 D 2 ppm + kinetin 0 ppm, D2K25: 2,4 D 2 ppm + kinetin 0.25 ppm, D2K50: 2,4 D 2 ppm + kinetin 0.5 ppm, D3K0: 2,4 D 3 ppm + kinetin 0 ppm, D3K25: 2,4 D 3 ppm + kinetin 0.25 ppm, D3K50: 2,4 D 3 ppm + kinetin 0.5 ppm. P0K0: picloram 0 ppm + kinetin 0 ppm, P0K0: picloram 0 ppm + kinetin 0.25 ppm, P0K25: picloram 0 ppm + kinetin 0.25 ppm, P0K50: picloram 0 ppm + kinetin 0.5 ppm P1K0: picloram 1 ppm + kinetin 0 ppm, P1K25: picloram 1 ppm + kinetin 0.25 ppm, P1K50: picloram 1 ppm + kinetin 0.5 ppm, P2K0: picloram 2 ppm + kinetin 0 ppm, P2K25: picloram 2 ppm + kinetin 0.25 ppm, P2K50: picloram 2 ppm + kinetin 0.5 ppm, P3K0: picloram 3 ppm + kinetin 0 ppm, P3K25: picloram 3 ppm + kinetin 0.25 ppm, and P3K50: picloram 3 ppm + kinetin 0.5 ppm.

Explant sterilization

The shallot bulb explants of the Bima Brebes variety were first cleaned under running water for 15 min, then treated with a detergent solution (1 g/L) for 1 h, followed by a bactericide and fungicide solution (1 g/L) for 24 h. It was followed by immersion in a 10% bleaching agent for 10 min, then a 5% bleaching agent for another 10 min. Each stage was followed by rinsing the explants thrice with sterile distilled water for 3-5 min. Before planting in the medium, the outer layer of the tuber was burned off, and the tuber was peeled in 2-3 layers.

Callus induction

The medium used for culturing shallot bulb explants was MS, supplemented with different combinations of plant growth regulators: 2,4-D (0, 1, 2, and 3 ppm) + kinetin (0, 0.25, and 0.5 ppm) and picloram (0, 1, 2, and 3 ppm) + kinetin (0, 0.25, and 0.5 ppm). The explants were incubated for 40 days. Growth parameters assessed included callus fresh weight, callus dry weight, percentage of callus formation, callus induction time, and callus morphology.

Development of a growth curve

The growth curve was established by harvesting the callus every 5 days until reaching the stationary phase. At each harvest, the callus was weighed and then dried in an oven at 60°C for 48 h. After drying, the callus was weighed again. Growth was assessed based on both the fresh and dry weights of the callus.

Assessment of bioactive compounds and antioxidant activity

The dry callus (0.5 g) of shallots was ground into a powder using a mortar and pestle. The powder was then macerated with either water or methanol for 2 days. The resulting extract was dried and resuspended in the respective solvent. Subsequently, a spectrophotometer analyzed the extract for total flavonoid, phenolic, and antioxidant activity. Total flavonoid content was determined using the method of Zou et al. (2004). A 0.5-mL aliquot of sample solution was mixed with 2 mL of distilled water and 0.15 mL of a 5% NaNO2 solution. After 6 minutes, 0.15 mL of a 10% $AlCl_3$ solution was added. Then, the mixture was added with 2 mL of 4% NaOH solution, and water was added to the final volume of 5 mL and allowed to stand for another 15 min. The absorbance of the mixture was determined at 510 nm using a spectrophotometer UV-Vis (Thermoscientific). Water was used as a blank sample. Quercetin was used as a positive control. The concentration of total flavonoid content in the test samples was calculated as mg quercetin equivalent (QE)/g of dried callus based on a standard curve of quercetin ($y = 0,0532x + 0,2448$, $R = 0,9947$). The total phenolic content was measured using the Folin-Ciocalteu method. The sample (0.2 mL) was mixed with water (0.6 mL) and Folin-Ciocalteu's phenol reagent

(0.2 mL). After 5 minutes, saturated sodium carbonate solution (8% w/v in water, 1 mL) was added to the mixture, and distilled water was added to bring the volume to 3 mL. The reaction was carried out in the dark for 30 min, and the absorbance from different samples was measured at 765 nm. The phenolic content was calculated as Gallic Acid Equivalents (GAE)/g of dry callus based on a standard curve of gallic acid (y = 0,0796x + 0,132, R = 0,999). All determinations were carried out in triplicate. Antioxidant activity was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method following the method of Phuyal et al. (2020).

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

The shallot callus extract was dissolved in methanol to achieve a concentration of less than 100 ppm, using a pipet to ensure a homogeneous solution. The sample was centrifuged at 8,000 rpm for 10 min to separate the pellet from the supernatant. The supernatant was collected to precipitate protein. Two milliliters of the supernatant were transferred to a centrifuge tube, mixed with 3 mL of acetonitrile acidified with 0.2% formic acid, and centrifuged at 8,000 rpm for 30 s. The resulting supernatant was used for purification via solid-phase extraction. The solution was filtered through a 0.45 µm cellulose acetate filter membrane and degassed before being injected into the Liquid Chromatography-Mass Spectrometry (LC-MS) (Shimadzu LCMS-8,040, with a Shimadzu Shim Pack FC-ODS column (2 mm × 150 mm, 3 µm). The injection volume was 1 µL, with a capillary voltage of 3.0 kV and a column temperature of 35°C. The analysis was performed in isocratic mobile phase mode, with a flow rate of 0.5 mL/min and a mobile phase of 90% methanol and water. The mass spectrometer operated in ion mode [M]+, with a collision energy of 5.0 V, desolvation gas flow of 60 ml/h at 350°C, and a fragmentation method using low energy CID, ESI ionization, scanning of 0.6 s/scan (Mz: 10-1,000), source temperature of 100°C, and run time of 80 min.

Data analysis

Data on callus fresh weight, dry weight, percentage of callus formation, callus induction time, flavonoid content, phenolic content, and antioxidant activity were statistically analyzed using a factorial Analysis of Variance (ANOVA), followed by Duncan's Multiple Range Test. Callus morphology and phytochemical components were analyzed descriptively.

RESULTS AND DISCUSSION

Callus induction of Bima Brebes shallots on MS medium treated with the growth regulator combination of 2,4-D and kinetin or picloram and kinetin resulted in various callus growth (Tables 1 and 2). The treatment combination of 2,4-D and kinetin did not significantly affect the percentage of callus formation, callus induction time, fresh weight, or dry weight of the Bima Brebes shallot callus (Table 1). The treatment combination of picloram and kinetin significantly affected the percentage of Bima Brebes shallot callus formation. The highest percentage of callus growth of Bima Brebes shallots was obtained in the treatments of P1K0, P1K25, P1K50, P2K0, P2K25, and P3K0 (Table 2).

Tables 1 and 2 show that callus growth varied in each combination of growth regulators. Explants cultured on media with a treatment combination of 2,4-D and kinetin had the highest callus percentage of 66.67%, while those on media with a combination of picloram and kinetin reached 100%. Explants with the fastest callus induction occurred in the combination of 3 ppm of 2,4-D without kinetin (0 ppm), i.e.,12 Days After Planting (DAP). In contrast, the fastest callus induction on picloram and kinetin was obtained in 1 ppm picloram + 0 ppm kinetin and a combination of 3 ppm picloram + 0 ppm kinetin, i.e., 5.67 DAP.

Table 1. Percentage of callus formation, callus induction time, fresh weight, and dry weight of Bima Brebes shallot callus with the treatment combination of 2,4-D and kinetin treatment

Treatment combination of		Percentage of callus (%)	Callus induction time (DAP)	Fresh weight (g)	Dry weight (g)
2,4-D (ppm)	Kinetin (ppm)				
0	0	0	0	0	0
	0.25	0	0	0	0
	0.50	0	0	0	0
1.00	0	33.33	30	0.34	0.03
	0.25	66.67	21	0.21	0.02
	0.50	33.33	30	0.20	0.02
2-00	0	0	0	0	0
	0.25	0	0	0	0
	0.50	0	0	0	0
3.00	0	66.67	12	0.25	0.03
	0.25	0	0	0.13	0.01
	0.50	0	0	0	0

Table 2. Percentage of callus formation, callus induction time, fresh weight, and dry weight of Bima Brebes shallot callus with the treatment combination of picloram and kinetin treatment

Treatment combination of		Percentage of callus (%)*	Callus induction time (DAP)	Dry weight (g)	Fresh weight (g)
Picloram (ppm)	Kinetin (ppm)				
0	0	0^{c}	0	0	0
	0.25	0^{c}	0	0	0
	0.50	0^{c}	0	0	0
1.00	0	100^{a}	5.67	0.11	1.52
	0.25	100^{a}	6.00	0.13	1.46
	0.50	100^{a}	13.00	0.15	1.18
2.00	0	100^{a}	7.00	0.15	1.35
	0.25	100^{a}	9.00	0.10	1.36
	0.50	33.33^{b}	10.00	0.04	0.98
3.00	0	100^{a}	5.67	0.10	0.60
	0.25	66.67^{b}	8.00	0.14	1.52
	0.50	100^{a}	6.67	0.14	2.02

Note: *Different superscript letters in the same column indicate significant differences

The highest average fresh weight of callus was in the 1 ppm2,4-D + 0 ppm kinetin treatment, with an average of 0.34 g. In contrast, the highest fresh weight for the picloram and kinetin combinations was obtained in the 3 ppm picloram + 0.5 ppm kinetin, with an average of 2.02 g. The highest average callus dry weight was observed in the 1 ppm 2,4-D + 0 ppm kinetin and 3 ppm 2,4-D + 0 ppm kinetin treatments, yielding 0.03 g. For the picloram and kinetin combinations, the highest dry weight was obtained with 1 ppm picloram + 0.5 ppm kinetin and 2 ppm picloram + 0 ppm kinetin, yielding 0.15 g.

The induction of shallot callus is marked by the formation of a yellowish-white cell mass at the base of the shallot bulb. Callus begins to develop from the wounded area of the explant and continues to grow. This process is closely associated with cell division, enlargement, and elongation, which are influenced by adding PGRs to the culture medium. PGRs stimulate cell division and enlargement in the explants, promoting callus induction and growth. The roles of auxin and cytokinin in callus induction are related to the activation of transcription factors and cofactors (Figure 1). Callus induction by auxin was achieved by inhibiting responsive factors (arfs) by the protein indole-3-acetic acid (aux/IAA). Cytokinins activate callus induction through type b Arabidopsis response regulators (arr). Auxin and cytokinin regulate cell division by influencing transcription factors, including proporz1 (prz1), arf-lbd factors, type b arrs, and obf binding protein 1 (obp1). Subsequently, the transcription factor e2fa, cyclin-dependent protein kinases d (cycd), and the inhibitory protein (kip)-related proteins (krp) regulate the cell cycle processes involved in callus induction (Perianez-Rodriguez et al. 2014).

The callus produced under the PGR combination exhibits different colors, but all have the same crumbly texture (Table 3). The morphology of shallot callus in the combination medium of 2,4-D and kinetin is illustrated in Figure 2. The morphology in the combination medium of picloram and kinetin is shown in Figure 3.

Callus texture indicates callus quality, whether the cells are actively dividing or stagnant. In this study, the shallot callus exhibited a crumbly texture, characterized by a loose arrangement of cells in the intercellular spaces. The crumbly texture of the callus is influenced by several factors, such as the type of PGRs used, the type of explant, and the culture medium (Guo and Jeong 2021).

The color of the callus indicates its growth phase. Shallot callus color ranged from yellowish-white to brownish-yellow. A white-to-yellow color indicated active cell division, while a reddish-to-brown color suggested maturity (Salvaña 2019). Additionally, callus color can vary depending on the type of explant. In the 1 ppm 2,4-D + 0.25 ppm kinetin treatment, some parts of the callus exhibited a brownish-yellow color, indicating the accumulation and oxidation of phenolic compounds (Liu et al. 2021).

Table 3. Morphology of the callus of shallot

PGR	Callus morphology		PGR	Callus morphology	
	Color	Texture		Color	Texture
D0K0	-	-	P0K0	-	-
D0K25	-	-	P0K25	-	-
D0K50	-	-	P0K50	-	-
D1K0	Yellow	Crumb	P1K0	Brownish-yellow	Crumb
D1K25	Brownish-yellow	Crumb	P1K25	Dark yellow	Crumb
D1K50	Yellow	Crumb	P1K50	Yellow	Crumb
D2K0	-	-	P2K0	White	Crumb
D2K25	-	-	P2K25	White	Crumb
D2K50	-	-	P2K50	Yellowish-white	Crumb
D3K0	Yellow	Crumb	P3K0	White	Crumb
D3K25	-	-	P3K25	White	Crumb
D3K50	-	-	P3K50	Light yellow	Crumb

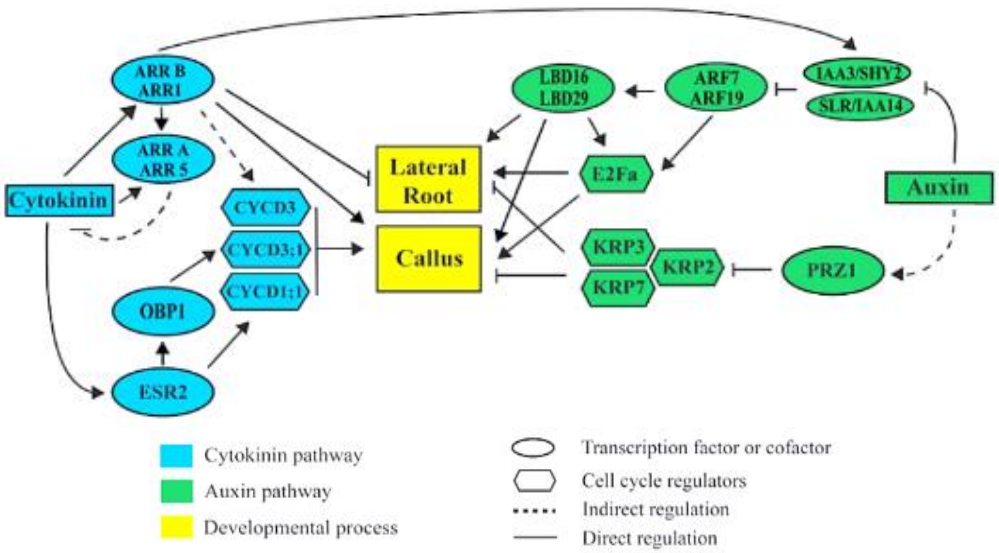


Figure 1. Mechanism of auxin and cytokinin in callus induction (Perianez-Rodriguez et al. 2014)

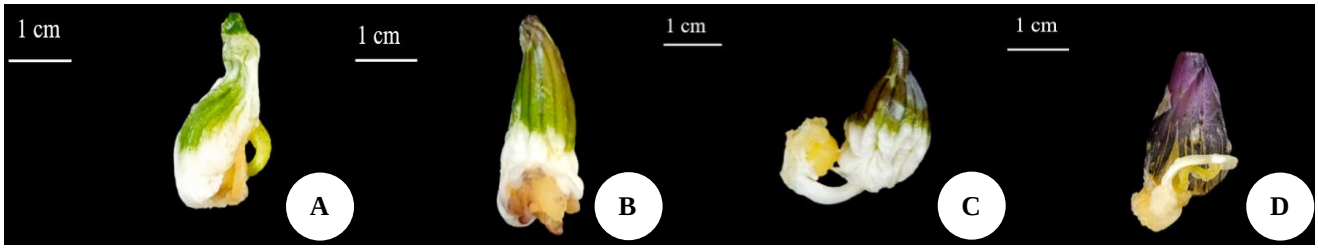


Figure 2. Shallot callus cultured on MS medium supplemented with 2,4-D and kinetin: A. D1K0; B. D1K25; C. D1K50; and D. D3K0

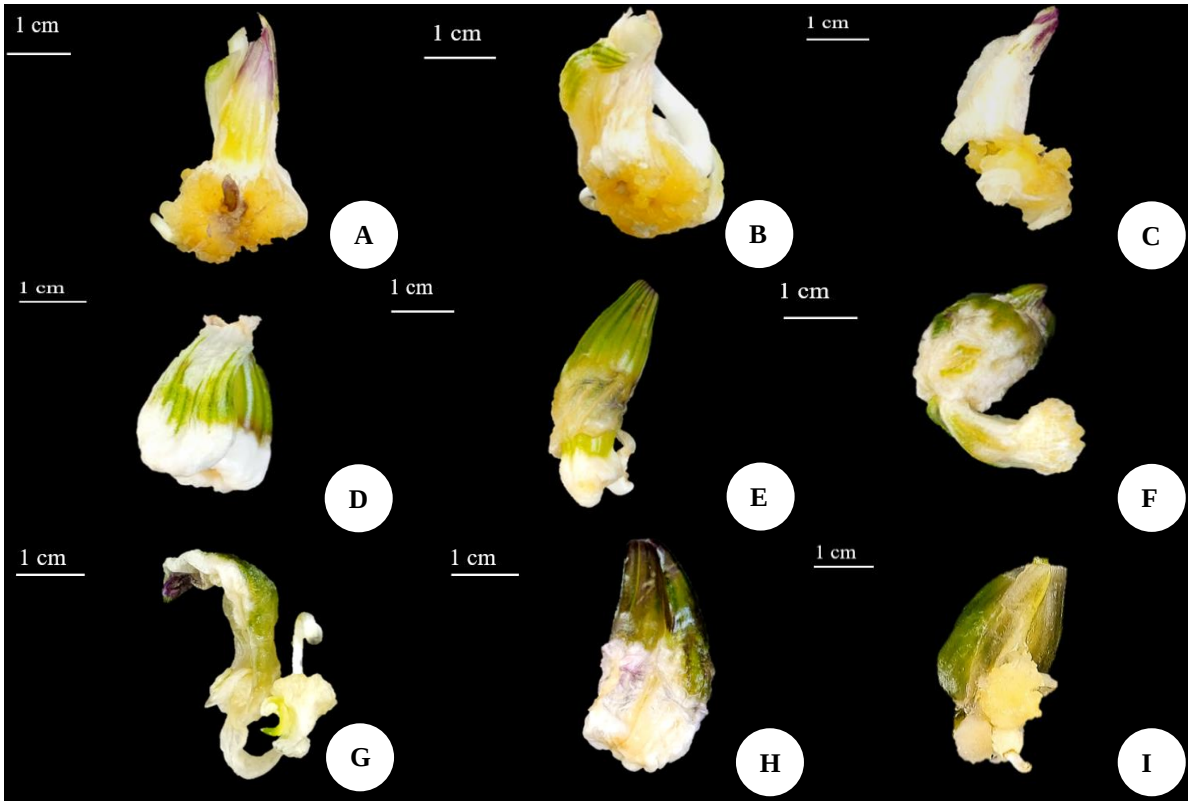


Figure 3. Shallot callus was cultured on an MS medium supplemented with picloram and kinetin: A. P1K0; B. P1K25; C. P1K50; D. P2K0; E. P2K25; F. P2K50; G. P3K0; H. P3K25; and I. P3K50

This research also determined the total phenolic content, total flavonoids, and antioxidant activity of the callus of Bima Brebes shallots due to picloram and kinetin application. Table 4 shows that all shallot callus samples contained flavonoids and phenolics at various concentrations and exhibited antioxidant activity. Although the biosynthesis of flavonoids in plants follows a common pathway, the concentration of flavonoids produced can differ due to variations in the expression patterns of genes related to key enzymes and transcription factors. These variations are influenced by several factors, such as organ development stages, hormonal treatments, and responses to wound stimulation (Zhao et al. 2013). Exogenous plant growth regulators influence the accumulation of secondary metabolites by modulating the expression of genes involved in their synthesis. This regulation occurs at the transcriptional level, where gene transcription factors play a crucial role in plant development and the synthesis of secondary metabolites (Zhao et al. 2011; Rosa et al. 2013).

The highest flavonoid levels were achieved in callus maintained on a medium supplemented with 1 ppm picloram and 0.25 ppm kinetin. The highest phenolic content was observed in the medium with 1 ppm picloram. The highest antioxidant activity was found in the callus maintained on medium with 3 ppm picloram and 0.5 ppm kinetin. These results suggest that Bima Brebes shallot callus could be a source of flavonoids, phenolics, and antioxidants. Callus from *Allium cepa* L., *Allium fistulosum* L., *Allium tuberosum* Rottler ex Spreng., *Stelechocarpus burahol* (Blume) Hook.f. & Thomson, *Dioscorea esculenta* (Lour.) Burkill, and *Elaeocarpus grandiflorus* Sm. had been reported to produce flavonoids, phenolics, and exhibit antioxidant activity (Habibah et al. 2016, 2021; Yoshimoto et al. 2022). However, the composition of secondary metabolites varies among these species. The secondary metabolite composition of the bulb and callus of Bima Brebes shallot was obtained using LC-MS (Table 5).

High content of phenols and flavonoids are beneficial

for health due to their potent antioxidants that aid in disease prevention (Nadeem et al. 2018). The antioxidant activity of plant-derived polyphenols is attributed to their ability to neutralize free radicals (Krzysztoforska et al. 2019; Liu et al. 2019; Santos-Buelga et al. 2019; D'Angelo et al. 2020; Jamshidi-kia et al. 2020).

A previous study by Habibah et al. (2024) showed that Bima Brebes shallot bulbs produced 99 secondary metabolites, while the callus from these bulbs yielded 87 secondary metabolites. The predominant group of secondary metabolites in the bulbs and calluses was flavonoids, including kaempferol and quercetin. However, several compounds found in the Bima Brebes shallot bulbs were not detected in the callus, such as propanethial S-oxide, 3,4-dihydroxybenzoic acid, p-coumaric acid, ascorbic acid, L-methionine sulfone, L-selenomethionine, cyanidin, glutathione, cyanidin 4′-glucoside, peonidin 3-arabinoside, 5-carboxypyranocyanidin 3-O-β-glucopyranoside, and cyanidin 3-(6″-malonylglucoside), 5-carboxypyranocyanidin 3-O-(6″-O-malonyl-β-glucopyranoside), cyanidin 3-laminaribioside, peonidin 3-(6′-malonylglucoside)-5-glucoside, malvidin 3,5-diglucoside, and cyanidin 3-(3-glucosyl-6-malonylglucoside)-4′-glucoside were detected in the bulbs but not in the callus. Conversely, some compounds such as 2,4-dimethylthiophene, 2-ethyl-5-methyl-3-thiazoline, zwiebelane A, 4-vinyl guaiacol, and lunularin appeared in the callus but were absent from the tubers. This discrepancy may be due to low concentrations of these compounds in the bulbs, making them undetectable, or the lack of necessary substrates or components for their synthesis in the growth medium.

The unique secondary metabolites in *Allium* plants include S-alk(en)ylcysteine sulfoxides (CSOs), sulfur-containing compounds known for their distinctive taste and health benefits. Previously identified CSOs include S-methylcysteine sulfoxide (methiin), S-allylcysteine sulfoxide (alliin), S-trans-1-propenylcysteine sulfoxide (isoalliin), and S-n-propylcysteine sulfoxide (propiin) (Yoshimoto and Saito 2019). In this study, the CSO metabolites detected in Bima Brebes shallot callus were alliin and cycloalliin. This study identified precursor compounds such as glutathione and intermediate compounds in the CSO biosynthesis pathway. These include γ-glutamyl-S-methylcysteine (a precursor to methiin) and S-(2-carboxypropyl) glutathione (precursors to alliin and isoalliin). The CSO biosynthesis pathway is illustrated in Figure 4. However, other CSO compounds, such as methiin, isoalliin, and propiin, were undetected. This could be due to the low expression levels of genes encoding the enzymes involved in CSO biosynthesis. Cycloalliin, a more stable cyclic form of isoalliin, is formed through cyclization, typically during heating (Lee et al. 2016). Cycloalliin in the shallot callus may have resulted from heating during extraction or LC-MS analysis preparation.

The distinctive flavor of *Allium* species is attributed to their sulfur-containing compounds, including thiosulfinates, thiosulfonates, and mono-, di-, and tri-sulfides (Liguori et al. 2017). Organosulfur compounds have also been known to help prevent chronic inflammation by reducing inflammatory mediators such as Nitric Oxide (NO), Prostaglandin (PG) E2, Interleukin (IL)-1β, IL-6, Tumor Necrosis Factor (TNF)-α, and IL-17 (Ruhee et al. 2020). The organosulfur compounds identified in the callus of Bima Brebes shallots are summarized in Table 6. In addition to CSO group compounds, other *Allium*-specific compounds were found in the callus of Bima Brebes shallots. Typical *Allium* compounds not included in the CSO group are presented in Figure 5.

Table 4. Antioxidant activity, total phenolic content, and total flavonoid levels in Bima Brebes shallot callus maintained on medium supplemented with picloram and kinetin

Sample	Antioxidant activity (IC_{50}; µg/mL)*	Total phenolic (mg GAE/g)*	Flavonoids (mg QE/g)*
P1K0	5.832^{h}	13.944^{b}	12.538^{a}
P1K25	6.741^{e}	21.237^{a}	7.556^{c}
P1K50	8.544^{a}	7.682^{e}	3.233^{def}
P2K0	7.212^{d}	7.556^{f}	4.455^{de}
P2K25	6.539^{g}	12.582^{c}	9.812^{b}
P2K50	6.741^{f}	8.958^{d}	3.045^{def}
P3K0	7.233^{c}	4.429^{g}	2.105^{f}
P3K25	7.968^{b}	2.833^{i}	4.944^{d}
P3K50	8.558^{a}	3.348^{h}	2.575^{ef}

Note: *Different superscript letters in the same column indicate significant differences

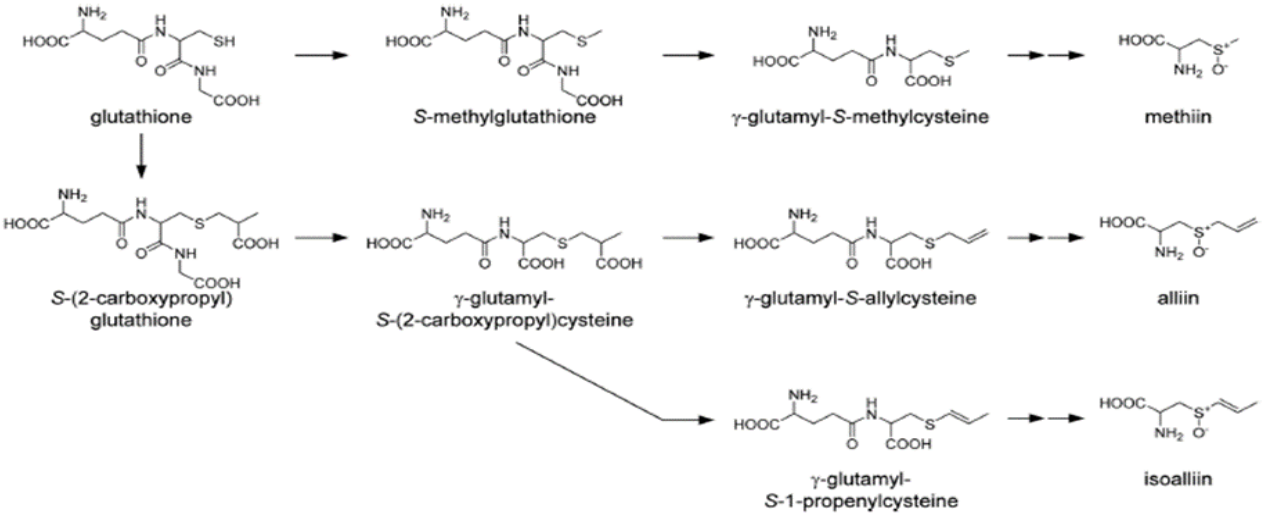
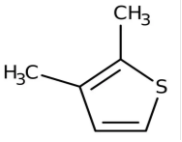
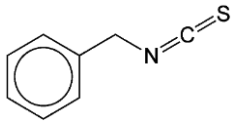
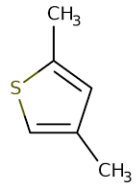
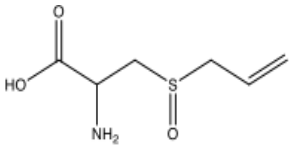
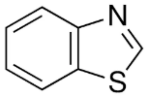
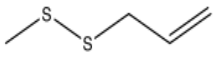
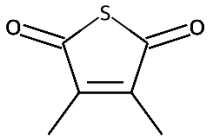
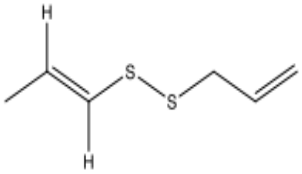
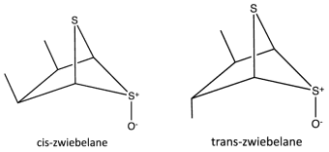
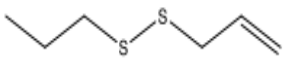
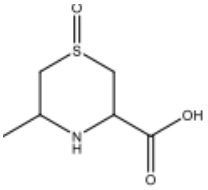
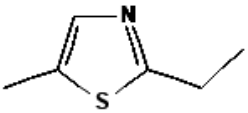
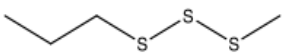
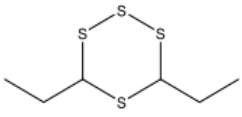


Figure 4. Biosynthetic pathway for CSO secondary metabolite compounds (Yoshimoto et al. 2022)

Table 5. Phenolic compounds identified through LC-MS analysis of the bulbs and callus of Bima Brebes shallot

Compounds	Chemical structure	Percentage (%)	
		Bulbs	Callus P3K25
Polyphenolic			
Quercetin 3-sphoroside-7-glucoside	$C_{33}H_{40}O_{22}$	2.57	2.42
Quercetin	$C_{15}H_{10}O_7$	2.36	1.98
Quercetin 7,4′-diglucoside	$C_{27}H_{30}O_{17}$	2.28	1.14
Quercetin 3,7,4′-triglucoside	$C_{33}H_{40}O_{22}$	1.97	2.72
Quercetin-3-glucoside-7-rhamnoside	$C_{27}H_{30}O_{16}$	2.39	2.12
Quercetin-3-O- malonylglucoside	$C_{22}H_{24}O_{15}$	2.01	2.38
Quercetin 3-OL-rhamnoside	$C_{21}H_{20}O_{11}$	2.01	2.86
Quercetin 3,4′-di-O-β-glucopyranoside	$C_{27}H_{30}O_{17}$	2.67	2.81
Kaempferol-3-OD-glucoside	$C_{21}H_{20}O_{11}$	0.92	2.03
Kaempferol	$C_{15}H_{10}O_6$	1.72	2.61
Kaempferol 4′-glucoside	$C_{21}H_{20}O_{11}$	1.48	1.56
Kaempferol-3-sophoroside-7-rhamnoside	$C_{33}H_{40}O_{20}$	2.44	1.05
Kaempferol 3-sophoroside-7-glucuronide	$C_{33}H_{38}O_{22}$	2.09	2.76
Routine	$C_{27}H_{30}O_{16}$	1.55	1.86
Hirsutrin	$C_{22}H_{22}O_{11}$	1.86	3.20
Isoquercitrin	$C_{21}H_{20}O_{12}$	2.23	2.86
Isorhamnetin	$C_{16}H_{12}O_7$	0.61	1.68
Isorhamnetin 3-O-β-D-glucopyranoside	$C_{22}H_{22}O_{12}$	1.38	1.11
Tamarixetin	$C_{16}H_{12}O_7$	0.55	0.21
3-O-caffeoylquinic acid	$C_{16}H_{18}O_9$	1.15	2.05
Cyanidin	$C_{15}H_{11}O_6$	0.42	0
Cyanidin 4′-glucoside	$C_{21}H_{21}O_{11}$	1.04	0
Cyanidin 3-(6″-malonylglucoside)	$C_{24}H_{23}O_{12}$	1.13	0
Cyanidin 3-(3-glucosyl-6-malonylglucoside)-4′- glucoside	$C_{26}H_{43}O_{24}$	1.13	0
Cyanidin 3-laminaribioside	$C_{27}H_{31}O_{16}$	1.14	0
5-carboxypyranocyanidin 3-O-(6″-O-malonyl-β-glucopyranoside)	$C_{27}H_{23}O_{16}^{+}$	0.90	0
5-carboxypyranocyanidin 3-O-β-glucopyranoside	$C_{24}H_{21}O_{13}^{+}$	0.85	0
Peonidin 3-arabinoside	$C_{21}H_{20}O_{10}$	0.92	0
Peonidin 3-(6′-malonylglucoside)-5-glucoside	$C_{31}H_{35}O_{19}$	0.66	0
Malvidin 3,5-diglucoside	$C_{29}H_{35}O_{17}$	1.04	0
Taxifolin 7-O-β-D-glucopyranoside	$C_{21}H_{22}O_{11}$	1.91	0
Spiraeoside	$C_{21}H_{20}O_{12}$	1.71	2.54
Glutathione	$C_{10}H_{17}N_3O_6S$	0.23	0.17
Monophenolic			
Gallic acid	$C_7H_6O_5$	1.38	0.83
Sinapic acid	$C_{11}H_{12}O_5$	0.84	0.38
Methyl 4-hydroxycinnamate	$C_{10}H_{10}O_3$	1.11	1.90
Caffeic acid	$C_9H_8O_4$	0.66	1.40
Ferulic acid	$C_{10}H_{10}O_4$	0.90	0.52
Vanillic acid	$C_8H_8O_4$	1.04	0.64
Catechol	$C_6H_6O_2$	0.59	0.63
3,4-dihydroxybenzoic acid	$C_7H_6O_4$	0.66	3.51
4-hydroxybenzoic acid	$C_7H_6O_3$	0.83	0.38
p-coumaric acid	$C_9H_8O_3$	1.01	0
Acetovanillone	$C_9H_{10}O_3$	0.45	0.48

Table 6. CSO group compounds found in the callus of Bima Brebes variety shallots

Cyclic monosulfide	Aliphatic monosulfides
	
2,3-dimethylthiophene	Benzyl isothiocyanate
	
2,4-dimethylthiophene	Alliin
	
Benzothiazole	Allyl methyl disulfide
	
3,4-dimethyl-2,5- thiophenedione	(E)-1-propenyl allyl disulfide
	
Zwiebelane A	Allyl propyl disulfide
	
Cycloalliin	2-ethyl-5-methyl-3-thiazoline
	
	Methyl propyl trisulfide
	
	4,6-diethyl-1,2,3,5-tetrathiolane

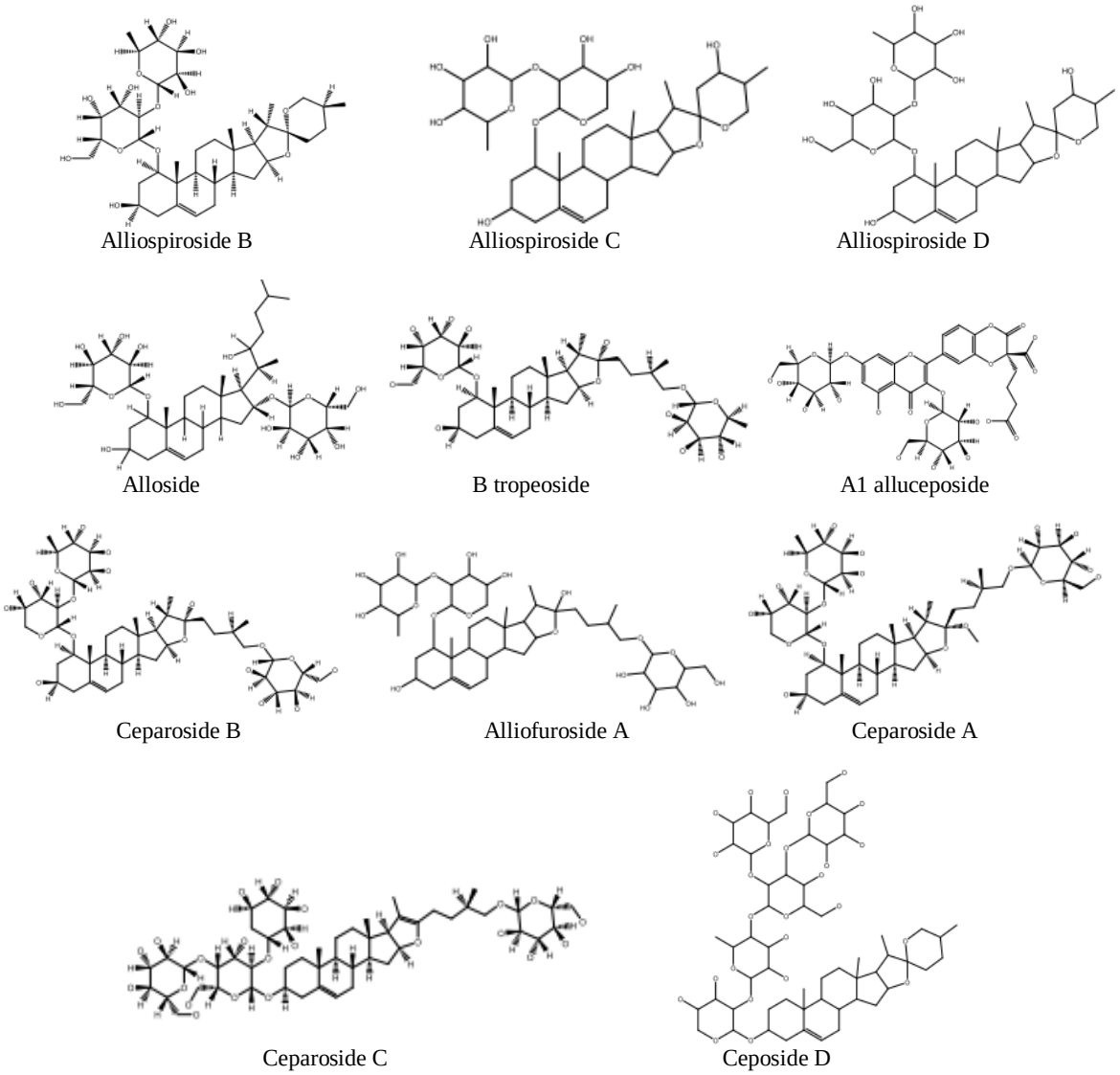


Figure 5. Typical *Allium* compounds that are not part of the CSO group

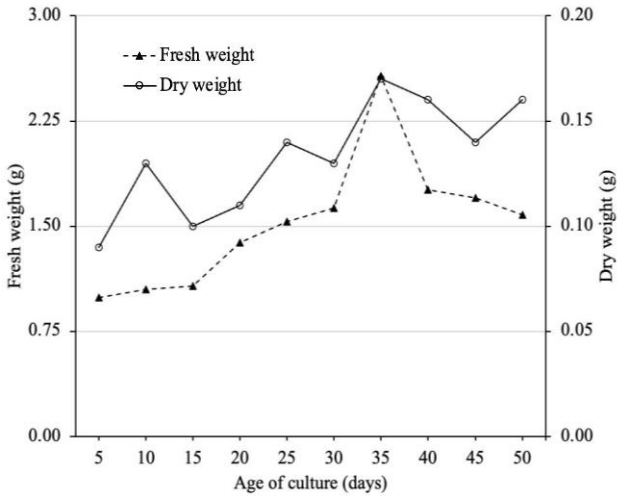


Figure 6. Growth curve of Bima Brebes shallot callus in 3 ppm picloram + 0.5 ppm kinetin (P3K50) media

The callus with the best growth performance was selected to develop a callus growth curve. Approximately 1 g of callus was subcultured into media containing 3 ppm picloram and 0.5 ppm kinetin and incubated for 50 days with harvest intervals of 5 days. The growth curve was designed to establish the optimal harvest period and the most effective elicitor time. Wet and dry weight growth curves are presented in Figure 6. Based on this figure, the highest average wet weight of the callus (2.57 g) was obtained at 35 days. This increase is likely attributed to the callus entering the log or exponential phase, where cell division is active. After 35 days, the wet weight of the callus declined until the 50th day because the callus entered the deceleration phase, characterized by reduced cell expansion and division (Bhatia et al. 2015).

Callus from Bima Brebes shallots grown on a combination of picloram and kinetin exhibited superior growth to those grown on 2,4-D and kinetin. The highest wet weight of callus was achieved at 3 ppm picloram + 0.5 ppm kinetin treatment. The highest dry weight was

obtained from treatments of 1 ppm picloram + 0.5 ppm kinetin and 2 ppm picloram + 0 ppm kinetin. Additionally, the percentage of explants producing callus was four times higher on the picloram + kinetin combination treatment than the 2,4-D + kinetin combination. The fastest callus induction was obtained on the picloram + kinetin combination, which was 2.12 times more quickly than the 2,4-D + kinetin treatment, i.e., 5 days after planting. The callus color ranged from yellowish-white to brownish-yellow, and its texture was crumbly. All callus samples treated with picloram and kinetin produced flavonoids and phenolics and possessed antioxidant activity. In total, 87 secondary metabolite compounds were identified in the callus. The highest fresh and dry weights were achieved on day 35 of callus growth, at 2.57 g and 0.17 g, respectively.

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