

## Research Article

# Micropropagation of Superior Variety of Vanilla (*Vanilla planifolia* Andrews) Through *In Vitro* Indirect Organogenesis

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### ABSTRACT

Two varieties of *Vanilla planifolia*, var. Sovania Agribun and Hivania Agribun, were nationally released in 2021. These varieties are categorised as superior due to their greater resistance to stem rot diseases compared to existing varieties. Micropropagation is a viable method for producing disease-free vanilla seedlings. This study aimed to identify the optimal combination of auxin and cytokinin for callus initiation and regeneration into plantlets of the superior vanilla variety, Hivania Agribun. The research used a completely randomised design with two factors: explant type and hormone combinations. A combination of 2,4-D, at a higher concentration than kinetin, was used to induce callusing, while a combination of 2,4-D with a higher concentration of kinetin was applied for callus proliferation; both steps were performed in the dark. The treatments with 2,4-D (0.5–1.5 mg L<sup>-1</sup>) and kinetin (0.1 mg L<sup>-1</sup>) were most effective in inducing callus formation on the first node and apical shoot explants. The combination of 2,4-D (0.1 mg L<sup>-1</sup>) and kinetin (0.5–1 mg L<sup>-1</sup>) was effective for callus proliferation and the formation of organogenic callus. Organogenic callus regeneration was achieved under illumination, with the medium containing BAP (0.5 mg L<sup>-1</sup>) producing the most abundant regenerating shoot buds and shoots.

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## INTRODUCTION

Propagation of vanilla plants is typically done vegetatively using tendril cuttings. However, propagating plants by that way requires a large amount of plant material, especially if extensive planting or rejuvenation is needed. Furthermore, according to Hu et al. (2019), long-term propagation by cuttings can lead to genetic erosion, which resulted in low genetic diversity and reduced the ability of vanilla plants to cope with environmental changes (Elestad et al. 2022). Another issue is disease susceptibility, particularly to pathogenic fungi. *Fusarium oxysporum* f.sp. *vanillae* has long been identified as the most dominant fungus responsible for causing stem rot diseases (SRD) (García et al. 2015).

In 2021, we under the Indonesian Agricultural Research and Development Agency released two varieties of *Vanilla planifolia*, named Sovania Agribun and Hivania Agribun. These varieties are classified as superior due to their higher resistance to SRD compared to existing varieties. The Official Letter of Decision of the Republic Indonesia Minister of Agriculture No. 115/Kpts / KB.020 / 12/ 2022 mentioned that *V. planifolia* var. Hivania Agribun was the result of hybridisation between the parents Vania 1 and Vania 2, both previously developed by the Research Institute for Spices and Medicinal Plants (Balittro) in Bogor. The vanillin content of Hivania Agribun is 2.87 %. Its unique characteristics include straight pods of equal size from base to tip, small flowers, and leaves positioned at a 45° angle to the stem/vine. This variety exhibits stable growth and is capable of adapting to a wide range of environmental conditions (Shukri 2023).

*In vitro* plant multiplication can serve as an alternative method to increase vanilla seed production. The balance between auxin and cytokinin is known to regulate callus formation and organogenesis (Zhao et al. 2021). The auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is used to induce callus initiation by promoting the dedifferentiation of explant cells through cell division (Özkul et al. 2016; Anhuji et al. 2023). In general, cytokinin 6-furfurylaminopurine (kinetin) stimulates cell division and controls cell morphogenesis, while BAP (6-benzylaminopurine) is commonly used to promote shoot development and to increase the percentage of shoot regeneration from callus (Hardjo et al. 2023). In *Vanilla planifolia* Vania 1, Ajijah et al. (2010) achieved callus induction rates of up to 60 % using 0.5 mg L<sup>-1</sup> kinetin combined with 2–3 mg L<sup>-1</sup> 2,4-D. Meanwhile, Erawati et al. (2021) reported that a combination of 0.5 mg L<sup>-1</sup> BAP and 2 mg L<sup>-1</sup> kinetin was effective in enhancing shoot proliferation from nodal cultures of vanilla.

This study aims to identify the optimal combination of auxin and cytokinin for callus formation and its regeneration in the propagation of the Hivania Agribun variety of vanilla.

## MATERIALS AND METHODS

### Plant Materials

The genetic material used was stem cuttings of *Vanilla planifolia* (Andrews) var. Hivania Agribun. It belongs to the Research Institute for Spices and Medicinal Plants (ISMCR). Shortcuttings of one single leaf node were planted on a sandy planting medium in polybags and maintained in a greenhouse. They were sprayed with fungicide Propineb 70 % (2 g L<sup>-1</sup>) every two days, three times starting from one week before new shoots were collected for tissue culture works. It was done as a standard procedure to minimise fungal contamination.

### Methods

#### Explant Sterilisation

Explant material was taken by cutting the 3rd segment of the new shoot. The leaves on the 1st and 2nd nodes were removed, leaving the base of the leaf.

External sterilisation was done by soaking the explants in a liquid detergent solution for 30 minutes. The next procedure followed Ajijah et al. (2010) with some improvements. The explants were then rinsed in running tap water and soaked again in a solution of 70 % Propineb fungicide (2 g L<sup>-1</sup>) and 20 % Streptomycin sulfate bactericide (2 g L<sup>-1</sup>) for 60 minutes, respectively. The explants were then rinsed with sterile distilled water before taking them to the sterilisation stage in the laminar airflow hood. The explants were then soaked successively in 70 % ethanol for 1 minute, 20 % NaOCl, 10 % NaOCl, and 10 % Iodopovidon solution for 5 minutes each. This sterilisation ended with rinsing the explants three times using sterile distilled water in every successive sterilisation process.

### Callus Induction

Sterilised explants were divided into three types: the apical shoot tip, the first node, and the second node. Each explant was slightly wounded in several areas and cultured horizontally on MS basal medium with nine different treatments—one control and eight combinations of plant growth regulators (PGRs). The treatments used in this study were adapted from the work of Ajijah et al. (2010) on the Vania 1 variety. The combinations consisted of 2,4-D (0.5; 1.0; 1.5; 2.0 mg L<sup>-1</sup>) and kinetin (0.1; 0.3 mg L<sup>-1</sup>). Each treatment consisted of 3 types of explants, each with seven replications. Each replication in a 75 mL glass vessel contained one explant. The cultures were placed in a dark room with a temperature of  $\pm 25$  °C. The parameters observed were the time of callus' first appearance, the percentage of explants producing callus, and the visual callus texture.

### Callus Proliferation

The callus originated from the best induction media, giving rise to viable (greenish-white) callus, was multiplied by slicing it into several portions, 0.4 to 0.6 cm in diameter, and each portion was cultured on a growing medium with a combination of 2.4 D (0.1 mg L<sup>-1</sup>) and increased kinetin concentrations (0.5; 1.0; 1.5 mg L<sup>-1</sup>). Each treatment combination was made in five replications; each replication consisted of two calli. The cultures were maintained in the dark. The parameters observed were the visual shape of the callus and the increase in callus volume by measuring every week the length, width, and height (p x l x t) compared to the initial size of the callus portion.

### Adventitious Shoot Regeneration

Callus from the best-performing treatments were transferred to the regeneration stage. The selection criteria for optimal callus were based on visual assessment, including viability, texture (compact to slightly friable), and a diameter of  $\geq 0.7$  cm. Each callus cluster was divided and subcultured in 3 regeneration media, which was MS supplemented with BAP (0.1; 0.3; 0.5 mg L<sup>-1</sup>), instead of kinetin. Four replicates for each treatment, and each replicate consisted of two callus clumps. The cultures were kept in a dark room for the first week, then moved under the light at 800-1100 lux for 16/24 hours. The parameters observed were the initial shoot emergence time and the average number of shoots and shoot buds per callus explant.

### Histology Incision

The incision was made by cutting the callus samples radially using a razor blade. The incision results were placed on a glass slide, given a drop of distilled water, then covered with a slip glass. Samples were observed using a compound light microscope (Olympus CX33) and photographed with an Indomikro HDMI camera at 40 - 100x magnification.

### Data analysis

The results of visual observations of the callus were analysed descriptively, while the quantitative data were statistically analysed using a factorial completely randomised design (CRD) with a Two-Way Analysis of Variance (ANOVA). If significant differences were found, the analysis was followed by the Duncan's Multiple Range Test (DMRT) at a 5 % level of confidence, using Statistical Product and Service Solutions (SPSS) software, version 26.0.

## RESULTS AND DISCUSSION

### Callus Induction

Callus formation began with the swelling of the explant on the induction medium. This swelling occurred as a result of the explant's absorption of nutrients and water from the medium. Wounding the explant triggered callus formation as a natural response of the tissue to heal the wound. Callus formation was first observed between the second and fifth week after planting. The calli were primarily compact and yellow. Statistically, both the explant type and the combination of 2,4-D with kinetin significantly influenced callus formation, although no interaction between these two factors was observed (Table 1). The best explant types for inducing callus were the first node and the shoot tip, with average percentages of 46.03 % and 42.86 %, respectively.

**Table 1.** Percentage of explants *Vanilla planifolia* var. Hivania Agribun producing callus in various combinations of 2,4-D and kinetin according to explant type\*

Induction treatment**	% explants producing callus			Average
	Shoot tip	1 <sup>st</sup> node	2 <sup>nd</sup> node	
DK0 (D0Kin0)	0	14.29	0	4.76 <sup>b</sup>
DK1 (D0.5Kin0.1)	71.43	57.14	0	42.86 <sup>a</sup>
DK2 (D0.5Kin0.3)	28.57	42.86	42.86	38.10 <sup>a</sup>
DK3 (D1Kin0.1)	57.14	71.43	0 0.00	42.86 <sup>a</sup>
DK4 (D1Kin0.3)	28.57	57.14	14.29	33.33 <sup>ab</sup>
DK5 (D1.5Kin0.1)	71.43	28.57	42.86	47.62 <sup>a</sup>
DK6 (D1.5Kin0.3)	57.14	71.43	14.29	47.62 <sup>a</sup>
DK7 (D2Kin0.1)	42.86	28.57	28.57	33.33 <sup>ab</sup>
DK8 (D2Kin0.3)	28.57	42.86	0	23.8 <sup>ab</sup>
Average	42.86 <sup>a</sup>	46.03 <sup>a</sup>	15.87 <sup>b</sup>	

\*31 weeks after planting (WAP). \*\*D=2,4-D at 0, 0.5, 1.0, 1.5, and 2.0 mg L<sup>-1</sup>; Kin=kinetin at 0, 0.1, 0.3 mg L<sup>-1</sup>. The numbers followed by the same letter in the same column and row indicate not significantly different according to the DMRT at  $\alpha = 5$  %.

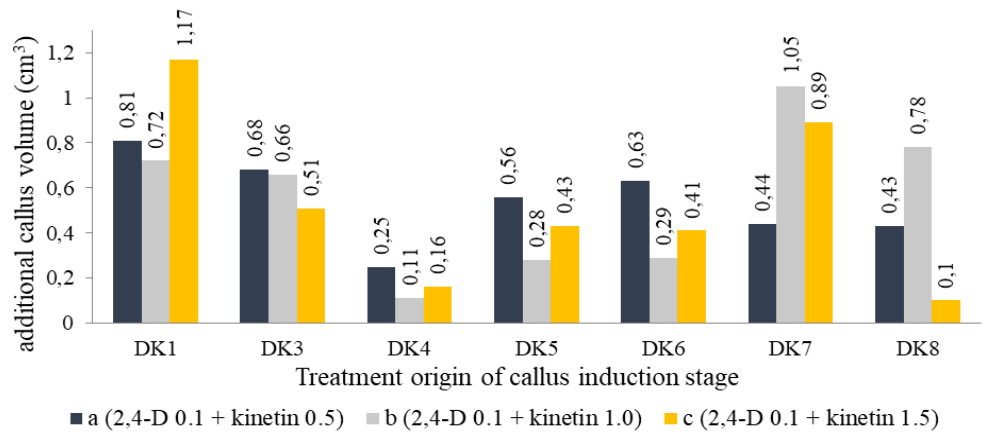
The percentage of callus formation in Hivania Agribun was observed in response to treatments with 2,4-D at concentrations of 0.5–1.5 mg L<sup>-1</sup>, in combination with kinetin at 0.1 and 0.3 mg L<sup>-1</sup>. The combination of 2 mg L<sup>-1</sup> 2,4-D with the same kinetin concentrations resulted in a lower percentage of callus formation. The addition of 2,4-D to the induction medium enhances cell wall permeability, inducing dedifferentiation, stimulating swelling, and altering callus color (Yaowachai et al. 2023). The presence of kinetin contributes to increased callus cell proliferation and differentiation through cell division (Sankepally & Singh 2016). Several callus turned brown after a few weeks in the induction medium. Tissue browning can be attributed to the oxidation of phenolic compounds by polyphenol oxidase (PPO) activity (Pompili 2023). The accumulation of oxidised phenolic compounds damages the cells, leading to cell death (I'anutushshoimah et al. 2020).

### Callus Proliferation

The calli developed from the seven induction treatments were transferred to the callus proliferation stage. Rapid callus growth occurred between the third

and eighth weeks after subculture. The yellow, compact-textured callus continued to proliferate, and its yellow color became paler. Statistical analysis of variance showed that the origin of the callus (induction media) and the combinations of 2,4-D with kinetin at high concentrations (proliferation stage) had no significant effect on callus enlargement, nor did the interaction between treatments. However, the data presented in Figure 1 show that the most significant increase in volume occurred in the DK1 callus, which received treatment c (0.1 mg L<sup>-1</sup> 2,4-D + 1.5 mg L<sup>-1</sup> kinetin), followed by the DK7 callus, which received treatment b (0.1 mg L<sup>-1</sup> 2,4-D + 1.0 mg L<sup>-1</sup> kinetin).

Within ten weeks of observation, it was found that the combination of 2,4-D (0.1 mg L<sup>-1</sup>) and elevated kinetin concentrations (0.5, 1.0, 1.5 mg L<sup>-1</sup>) produced proliferative callus, as evidenced by an increase in callus size. A lower auxin level combined with high cytokinin levels can synergistically stimulate optimal callus cell division and shoot regeneration (Ramabulana et al. 2021). Ajijah et al. (2010) reported much higher concentration of 2,4-D (2-3 mg L<sup>-1</sup>) in combination with 0.5 mg L<sup>-1</sup> kinetin that provided the most prolific calli in Vania 1 variety. Vania 1 is one of the parent plants from which Hivania Agribun was derived. At this stage, we observed that some calli became crumbly, but most maintained a white-yellow, compact texture with a smooth white surface. This white surface later transformed into a more watery callus.



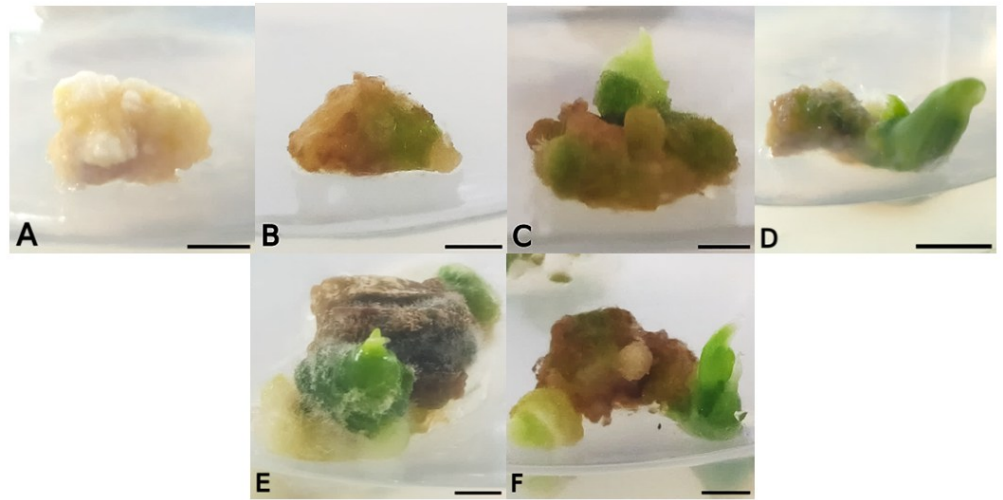
**Figure 1.** Increase in callus volume of *Vanilla planifolia* var. Hivania Agribun in the proliferation media (a, b, c) based on the origin of the induction media at 10 weeks after planting (WAP).

The callus produced at this stage emitted a fragrant aroma, which may indicate the presence of specific secondary metabolites of vanilla. According to Efferth (2019), the formation of secondary metabolites can be influenced by external factors, such as the pH of the culture medium, temperature, light, and culture aeration. Maintaining cultures in agar media containing certain nutrients and vitamins can also act as a trigger. High concentrations of ammonium ions or low phosphate levels stimulate the formation of secondary metabolites in *in vitro* cultures (Efferth 2019). The presence of oxidative stress, pathogen attacks, and physical and chemical stimuli serves as an elicitor to modulate gene expression and induce enzyme synthesis, which promotes the production of secondary metabolites, such as flavonoids, terpenoids, alkaloids, phenylpropanoids, and polypeptides (Al-Khayri et al. 2023), as demonstrated by Hasibuan et al. (2021), who reported that a *Diaporthe* fungal filtrate-derived elicitor enhanced quinidine production in Quina cell cultures.

### Adventitious Shoot Regeneration

During the regeneration stage, the callus color changed to green, indicating the formation of chlorophyll in the presence of light, a process also promoted by cytokinin (BAP). Light is an abiotic factor that significantly affects plant

morphogenesis and growth. In addition to its role in photosynthesis, light can influence the expression of genes that control plant metabolism, which can impact growth and development, a process known as photomorphogenesis (Weremczuk et al. 2021). Figure 2 illustrates the callus regeneration into shoots.



**Figure 2.** Callus regeneration of *Vanilla planifolia* var. Hivania Agribun to adventitious shoots on the media with BAP. (A) Callus on the day of subculture; (B) Callus turning green; (C - F) Adventitious shoots from callus, aged 4 WAP in regeneration stage. Scale = 5 mm.

According to Cortleven and Schmuelling (2015) and Cortleven et al. (2016), cytokinins directly regulate chlorophyll biosynthesis genes through the process of light harvesting during the transition from etioplasts to chloroplasts. Signals from cytokinin receptors activate the expression of the HEMA1, and the other genes. These genes encode the enzyme GluTR (Glutamyl-tRNA Reductase), which catalyzes the synthesis of 5-aminolevulinic acid (ALA). ALA is then converted into protochlorophyllide. The presence of light will activate NADPH-dependent protochlorophyllide oxidoreductase, facilitating the regeneration of chlorophylls. The formation of chlorophyll, beginning with ALA synthesis, marks the initiation of the phytychromobilin pathway, which originates from phytyochrome apoproteins (Sanchez-Barcelo et al. 2016). Phytychromes are synthesised in the cytosol; the inactive form, Pr, acts as a photoreceptor that mediates photomorphogenic responses to red light. Upon exposure to light, Pr is converted to the active Pfr form, which then moves to the nucleus. The photoactivated phytyochrome suppresses the activity of constitutive photomorphogenic 1 (COP1), thereby promoting photomorphogenesis (Sanchez-Barcelo et al. 2016).

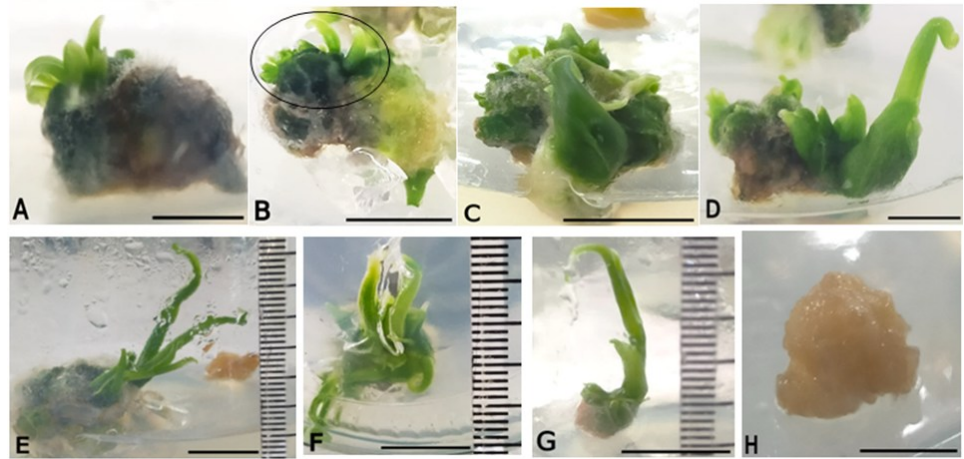
BAP in combination with NAA or 2,4-D promotes callus induction and multiplication, stimulates shoot emergence, and increases the number of proliferated shoots and shoot length in vanilla (Chai et al. 2024) and in Phalaenopsis orchid (Sarmah et al. 2024). In another herbaceous plant, *Curculigo orchioides*, Umar et al. (2025) reported the highest callus formation and regeneration when using a combination of BAP and IBA. Other auxins, such as 2,4-D and picloram, resulted in lower culture development. According to the statistical analysis of variance, there was an interaction between the previous treatments (induction + proliferation media) and various concentrations of BAP (Table 2). The callus treatments DK3(a)B 0.5 and DK1(c)B 0.5 resulted in the highest number of shoots (36.25 and 26.25, respectively), with DK1(c)B 0.5 being the most effective in producing potential buds that developed into shoots (28.75). The development of adventitious shoots on the callus is shown in Figure 3.

**Table 2.** Regeneration of adventitious shoots of *Vanilla planifolia* var. Hivania Agribun as influenced by the previous treatments on callus and BAP, observed eight weeks after subculture.

Previous callus treatment	BAP concentration (mg L <sup>-1</sup> )	Average Number of shoots	Average Number of shoot buds	Shoot emergence time (weeks)
DK1(a)	B <sub>0.1</sub>	1.25 <sup>eph</sup>	0.00 <sup>c</sup>	7
	B <sub>0.3</sub>	2.50 <sup>def</sup>	0.00 <sup>c</sup>	5
	B <sub>0.5</sub>	3.75 <sup>def</sup>	11.25 <sup>bc</sup>	5 – 8
DK1(b)	B <sub>0.1</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
	B <sub>0.3</sub>	5.00 <sup>def</sup>	0.00 <sup>c</sup>	4 – 6
	B <sub>0.5</sub>	17.50 <sup>bcd</sup>	17.50 <sup>b</sup>	4 – 5
DK1(c)	B <sub>0.1</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
	B <sub>0.3</sub>	3.75 <sup>def</sup>	0.00 <sup>c</sup>	4 – 5
	B <sub>0.5</sub>	26.25 <sup>ab</sup>	28.75 <sup>a</sup>	4 – 6
DK3(a)	B <sub>0.1</sub>	16.25 <sup>bcde</sup>	7.50 <sup>bc</sup>	3 – 8
	B <sub>0.3</sub>	3.75 <sup>def</sup>	1.25 <sup>c</sup>	4
	B <sub>0.5</sub>	36.25 <sup>a</sup>	7.50 <sup>bc</sup>	2 – 7
DK3(b)	B <sub>0.1</sub>	1.25 <sup>ef</sup>	0.00 <sup>c</sup>	7
	B <sub>0.3</sub>	20.00 <sup>bc</sup>	7.50 <sup>bc</sup>	2 – 7
	B <sub>0.5</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
DK3(c)	B <sub>0.1</sub>	1.25 <sup>eph</sup>	0.00 <sup>c</sup>	7
	B <sub>0.3</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
	B <sub>0.5</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
DK5(a)	B <sub>0.1</sub>	3.75 <sup>def</sup>	0.00 <sup>c</sup>	6 – 7
	B <sub>0.3</sub>	20.00 <sup>bc</sup>	12.50 <sup>bc</sup>	4 – 5
	B <sub>0.5</sub>	11.25 <sup>cdef</sup>	2.50 <sup>c</sup>	4 – 8
DK5(b)	B <sub>0.1</sub>	1.25 <sup>eph</sup>	1.25 <sup>c</sup>	6
	B <sub>0.3</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
	B <sub>0.5</sub>	2.50 <sup>def</sup>	0.00 <sup>c</sup>	4
DK5(c)	B <sub>0.1</sub>	0.00 <sup>f</sup>	1.25 <sup>c</sup>	8
	B <sub>0.3</sub>	2.50 <sup>def</sup>	2.50 <sup>c</sup>	4
	B <sub>0.5</sub>	11.25 <sup>cdef</sup>	1.25 <sup>c</sup>	4 – 8
DK7(a)	B <sub>0.1</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
	B <sub>0.3</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
	B <sub>0.5</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
DK7(b)	B <sub>0.1</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
	B <sub>0.3</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
	B <sub>0.5</sub>	5.00 <sup>def</sup>	0.00 <sup>c</sup>	6 – 8
DK7(c)	B <sub>0.1</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
	B <sub>0.3</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–
	B <sub>0.5</sub>	0.00 <sup>f</sup>	0.00 <sup>c</sup>	–

The numbers followed by the same letter in the same column indicates that it is not significantly different according to the DMRT at  $\alpha = 5\%$ .

The ANOVA revealed significant effects of BAP alone on the number of shoots. The highest number of shoots and shoot buds was obtained with BAP at 0.5 mg L<sup>-1</sup> (Table 3). Callus regeneration also depends on the condition of the callus at the time it was first cultured. Two regeneration pathways are recognized in *in vitro* culture: organogenesis and somatic embryogenesis. The latter is more common in woody plants, such as *Falcataria moluccana* (Sunandar et al. 2017) and *Coffea arabica* (Avila-Victor et al. 2023). In this study, regeneration followed the organogenetic pathway. The condition of the callus, depending on which part of the source it was cut from, influences its capacity to respond during the regeneration stage. Most DK7 (D2Kin0.1) calli (Table 2) turned brown and showed no proliferation or differentiation. The failure to regenerate shoots from DK7 callus may be due to the high levels of 2,4-D (2 mg L<sup>-1</sup>) and cytokinin (kinetin and BAP), which resulted in the accumulation of phenolic compounds when the callus was exposed to light.



**Figure 3.** Growth and development of adventitious shoots of *Vanilla planifolia* var. Hivania Agribun on regeneration media, eight weeks after subculture. Developing shoots in the treatments DK1(c)B0.5 (A), DK1(a)B0.5 (B), DK1(b)B0.5 (C), DK3(a)B0.5 (D), elongating shoots (E-G), non-organogenic callus (H). Scale = 10 mm.

BAP is the most commonly used cytokinin in *in vitro* plant multiplication. Erawati et al. (2021) achieved the highest number of shoots through direct organogenesis from vanilla nodal cultures using a combination of 0.5 mg L<sup>-1</sup> BAP and 2 mg L<sup>-1</sup> kinetin. Similarly, Rahmah et al. (2023) reported the highest percentage of nodal explants developing shoots, as well as the greatest shoot number in the Vania 2 variety, when cultured with 1.5 mg L<sup>-1</sup> BAP and 0.1 mg L<sup>-1</sup> IBA.

**Table 3.** Average number of shoots and shoot buds regenerated from callus of *Vanilla planifolia* var. Hivania Agribun based on BAP concentration.

BAP Treatment	Number of shoots	Number of shoot buds	Shoot emergence time (weeks)
B <sub>0.1</sub>	2.08 <sup>b</sup>	0.83 <sup>b</sup>	3 – 8
B <sub>0.3</sub>	4.79 <sup>b</sup>	1.98 <sup>b</sup>	2 – 7
B <sub>0.5</sub>	9.48 <sup>a</sup>	5.73 <sup>a</sup>	2 – 8

The numbers followed by the same letter in the same column indicates that it is insignificantly different according to the DMRT test at  $\alpha = 5\%$ .

### Histological Observation

Observations of some incised callus at 40x magnification showed that they are green, indicating the presence of chlorophyll in the regenerating callus cells (Figure 4A). The shoot meristem contained some leaf primordia, and vascular tissue had differentiated. In contrast, chlorophyll was absent in the non-organogenic area of the callus, which had a compact, slightly crumbly texture and was pale white (Figure 4B). However, this callus exhibited some areas with developing shoots in the meristematic zone.



**Figure 4.** Histological sections of shoot and callus. Structure of adventitious buds from regenerating callus (A), non-regenerated callus (B). Lp = Leaf primordia, Am = Apical meristem, Vt = Vascular tissue, Sc = Small cells (meristematic), and Lc = Large cells (non-organogenic). Scale = 100  $\mu\text{m}$  (A), 50  $\mu\text{m}$  (B).

According to Hernandez et al. (2020), the organogenic structure of vanilla callus begins with callus proliferation, forming a heterogeneous structure with two main areas: broad, vacuolated cells (non-organogenic) and small, well-demarcated cells with very dense nuclei, characteristic of meristematic cells. These latter cells then develop into shoot apical meristems, forming a meristematic dome covered by young leaf primordia. During the regeneration stage, several peripheral areas of the organogenic callus will also form mitotic zones, differentiating into additional shoot apical meristems.

## CONCLUSION

The shoot tip and first node are the most responsive explants. The combination of the auxin 2,4-D at higher concentrations (0.5-1.5 mg L<sup>-1</sup>) with the cytokinin kinetin (0.1 mg L<sup>-1</sup>) can induce callus formation in vanilla plants. The combination of a lower concentration of 2,4-D (0.1 mg L<sup>-1</sup>) and elevated kinetin (0.5-1 mg L<sup>-1</sup>) stimulates callus proliferation and accelerates organogenic callus formation. Single BAP at 0.5 mg L<sup>-1</sup> was the most effective for regenerating shoots on callus that had been previously treated with 0.5-1 mg L<sup>-1</sup> 2,4-D and 0.5-1.5 mg L<sup>-1</sup> kinetin during callus induction and proliferation. These results will support the distribution of this superior vanilla variety to farmers in order to increase the quality and quantity of vanilla bean production in Indonesia.

## AUTHOR CONTRIBUTION

E.H. initiated, designed, and supervised the research, and prepared the manuscript; M. carried out all the lab works and wrote the final report; D.R. designed, supervised and revised the manuscript; A.S. analysed the data and R.Y. wrote and arranged the manuscript.

## CONFLICT OF INTEREST

All the authors declare that there is no competing interest or financial relationship that could be construed as a potential conflict of interest.

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