In vitro Elicitation of Vanilla planifolia Using Methyl Jasmonate and Ferulic Acid Enhances Vanillin Production

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Abstract

Vanillin is a high-value phenolic compound widely utilised in the food, beverage, pharmaceutical, fragrance, and cosmetic industries. Yet its natural production from *Vanilla planifolia* pods remains limited. Hence, the present study aimed to establish an efficient in vitro elicitation to enhance vanillin accumulation in different vegetative tissues (root, stem and leaf) of *V. planifolia*. Initially, nodal explants were cultured on MS medium supplemented with BAP alone or in combination with NAA to induce plant regeneration. Subsequently, elicitation was performed by treating the tissues with MeJA, FA and AC applied singly or in combination. In vitro regeneration was successfully achieved, with BAP alone promoting shoot proliferation, stem thickening and callus formation, while BAP and NAA in combination supported shoot elongation. In vitro elicitation further encouraged root, leaf and node formation of the plantlets. Vanillin content was quantified using HPLC reverse-phase coupled with a DAD detector, using a wavelength of 280 nm, following in vitro elicitation. Leaves from the in vitro plantlets treated with FA alone exhibited a higher vanillin content. Meanwhile, the stems and roots yielded higher vanillin accumulation in the combination of MeJA and AC, and in FA and AC, respectively. The findings provide a significant in vitro elicitation strategy for sustainable vanillin production that may help meet the demand for natural vanillin across various industries.

Key message

This study established a novel in vitro elicitation using MeJA, FA singly or in combination with AC, treated on the vegetative tissue-specific of , enhancing the vanillin content.

Introduction

Vanilla planifolia is a climbing orchid native to Mexico and Central America that belongs to the family Orchidaceae. The species thrives under tropical conditions, requiring high humidity, moderate shade, and warm temperatures for optimal growth, whereas sufficient light intensity is crucial for flowering (Nugraha et al. 2024). Vanillin is one of the most economically important chemical compounds, known for its flavour and aroma (Arya et al. 2021). Natural vanillin is the second most expensive spice after saffron and is widely used in the food, beverage, pharmaceutical, perfumery, and cosmetic industries (Karatoprak 2022; Olatunde et al. 2022).

Despite its high demand, natural vanillin production remains limited due to low yield and its cultivation and curing process is labour-intensive (D'Arrigo et al. 2024). Vanillin is naturally present in vanilla pods, where the aroma is strongly developed after the curing process (Peña-Barrientos et al., 2023). Furthermore, vanilla content typically accounts for less than 2% of the dry weight of cured pods (Gallage & Møller 2015). This scarcity has resulted in a excessively dependence on synthetic vanillin, which dominates the global market. However, growing consumer preference for naturally produced compounds has raised interest in alternative production strategies (Walton et al. 2003; Feron et al. 2023; Liu et al. 2023).

Biotechnological approaches, particularly plant tissue culture incorporated with elicitation strategies, offer promising alternatives for enhancing secondary metabolites production (Khan et al. 2021). Plants naturally synthesize secondary metabolites as part of their defence and stress response mechanisms. Vanillin is a secondary metabolite developed from the biosynthetic pathway in *V. planifolia* (Kundu et al. 2017). Vanillin biosynthesis originates from the phenylpropanoid pathway, beginning with phenylalanine (PAL) and proceeding through intermediates such as cinnamic acid, p-coumaric acid, caffeic acid, and ferulic acid, which are subsequently converted into vanillin (Havkin Frenkel & Belanger 2007; Walton et al. 2003). Alternative routes in vanillin biosynthesis involving benzoate intermediates have also been investigated (Havkin Frenkel et al. 1999). Nonetheless, the vanillin biosynthetic pathway remains partially studied due to its biochemical complexity.

Elicitors are known as molecules that enhance the synthesis of secondary metabolites (Patel & Krishnamurthy 2013) by activating defence-related signalling pathways that significantly impact bioactivities, mitigation of environmental stress, plant survivability and productivity (Jan et al. 2021). Methyl jasmonate (MeJA) is a well-established elicitor stimulate the accumulation of secondary metabolites, increased activity of antioxidant enzymes and the expression of genes (Murthy et la. 2014; Ho et al. 2020). Ferulic acid (FA) is a key intermediate in the phenylpropanoid pathway and is identified as a direct precursor of vanillin in *V. planifolia*, whereby vanillin synthase (VpVAN) catalyses its conversion to vanillin (Gallage et al. 2014; Lee et al. 2020; Jiang et al. 2023). Activated charcoal (AC) serves key roles in plant tissue culture by adsorbing inhibitory compounds, toxic metabolites, and phenolic compounds. It has a porous structure that enables remarkable capacity to adsorb various chemical compounds (Isalar 2025).

Based on the biochemical framework, the exogenous application of FA in combination with a signaling elicitor such as MeJA may enhance metabolic flux toward vanillin biosynthesis. However, the effects of MeJA and FA in combination that are associated with vanillin production in *V. planifolia* have not been extensively studied, particularly with respect to tissue-specific responses. Understanding of these responses is crucial for preliminary insight into the vanillin biosynthetic pathway and accumulation of vanillin in vitro. Therefore, the present study aimed to evaluate the effects of MeJA and FA applied singly and in combination with AC on vanillin accumulation in different *in vitro* tissues (leaves, stems, and roots) of *V. planifolia*.

Material and Methods

Plant material

In vitro plantlets of *V. planifolia* aged 6 months obtained from the Plant Tissue Culture Laboratory, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS) were used in this study.

In vitro Regeneration

Nodal explants approximately 1.0 cm in size were excised from in vitro plantlets of *V. planifolia* and then cultured on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 6-Benzylaminopurine (BAP) singly (0.0, 1.0, 2.0, and 3.0 mg/L) or in combination with 0.5 mg/L α-Naphthaleneacetic acid (NAA) (Duchefa Biochemie). All treatments were supplied with 30.0 g/L sucrose (R & M Chemicals) and solidified with 3.0 g/L Gelrite (Duchefa Biochemie). The pH was adjusted to 5.8 before being autoclaved at 121°C, 15 psi for 20 minutes. In this study, 35 explants were individually cultured per treatment in a 300 mL culture jar containing 50 mL medium. The cultures were incubated in a controlled growth room at 25 ± 1°C under a 16-hour photoperiod provided by cool white, fluorescent light (Philips TL-D LIFEMAX Super 80 linear fluorescent tubes, 36 W, T8, G13 bi-pin) for two months.

Preparation of Elicitor Stock Solutions

A stock solution of MeJA (Sigma-Aldrich) was prepared by dissolving 2.31 mL of MeJA into 100.0 mL of absolute ethanol (HmBg), resulting in a final concentration of approximately 100 mM. Meanwhile, a stock solution of FA (Happy Formulating, Malaysia) was prepared by dissolving 1.94 g of FA powder in 100.0 mL of absolute ethanol, yielding a final concentration of approximately 100 mM. All stock solutions were stored at 4°C until further usage. In addition, a 1.0% (w/v) activated charcoal (AC) solution was prepared by dissolving 0.01 mg of AC in 1 L of distilled water.

In vitro Elicitation

After two months of in vitro regeneration, the healthy plantlets were selected and transferred to fresh MS basal medium and maintained in the culture room for two weeks to allow for the depletion of previous hormone residues. In this study, the plantlets were cultured individually in 50 mL culture medium in a 300 mL culture jar. After that, elicitors (MeJA, FA, and AC) were aseptically applied in each treatment either alone or in combination. Overall, 22 explants were treated per treatment, and the cultures were subsequently incubated in the same culture room as in the in vitro regeneration study for a month.

Preparation of Plant Extracts

After one month of in vitro elicitation, the plantlets' growth was monitored. Then, the plantlets were harvested and separated accordingly into different vegetative parts, such as leaves, stems, and roots. The extraction and analysis of vanillin from these samples followed the previous method (Ferreira et al., 2018) with minor modifications. Initially, fresh tissues were oven-dried at 55°C until the samples reached constant weight. Subsequently, the samples were separately ground into a fine powder using a clean mortar and pestle. One gram of the dried powder sample was dissolved in 150 mL of 50% (v/v) ethanol (HmbG→ Chemicals), which was reflux- extracted for 30 minutes at 75°C using a heating mantle (MTOPO MS – ES303). The reflux solution obtained was cooled to room temperature and filtered

through Advantec filter paper (pore size 0.09 µm, No.5A, 110mm) before being subjected to a rotary evaporator (EYELA) to remove the solvent and obtain a concentrated crude extract.

Quantitative Analysis of Vanillin using HPLC-UV/Vis

Quantitative analysis of vanillin was performed using a Shimadzu LC-20A HPLC reverse-phase on a C^{18} column (5 µm pore size; 250 mm × 4.6 mm internal diameter), Thermo Scientific, USA, and UV detection at 280 nm wavelength with minor modification (Diaz-Bautista et al., 2022). The mobile phase consisted of Solvent A (methanol), Solvent B (water), and Solvent C (acetonitrile). The percentage of Solvent A (40%) and C (60%) remained constant, while Solvent B varied (0–40% and 100%). Solvent B began with 100% at 1 min, decreased to 0% at 3 min, and held for 7 min, then returned to 100% at 10 min. Meanwhile, Solvent A and C started at 3 min and held for 7 min. The column oven was maintained at 55°C, and the HPLC was run following the gradient elution time at a flow rate of 1.0 mL/min. The standard vanillin solution was used to prepare the calibration curve containing multiple concentration points ranging from 0.00079 to 0.1 mg/mL. After that, all concentrated crude extracts were diluted with 10 mL of HPLC-grade methanol (Sigma-Aldrich→), vortexed for 2 minutes, and then filtered using 0.22 µm micropore H-PTFE (HmbG). Subsequently, both the vanillin standard and crude extracts (5 µL) were injected separately into the HPLC system in triplicate. Vanillin was identified by comparing the retention times of the standard and the crude extracts in the HPLC chromatograms. Vanillin concentration (mg/mL) was converted to vanillin content (mg/g DW) based on the dry mass of each sample.

Statistical Analysis

Data obtained from in vitro regeneration and elicitation experiments were analysed using one -way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at a significance level of $p < 0.05$, using IBM SPSS Statistics version 27. The data are presented as mean values ± standard error (SE) of parameters measured from 35 explants per treatment. Meanwhile, the vanillin content quantification data obtained from HPLC analysis are presented as mean values ± standard deviation (SD) of three technical injections from a single biological sample per treatment.

Results

Effects of BAP and NAA Applied Singly or In Combination

The effects of BAP and NAA on the *in vitro* regeneration of *V. planifolia* nodal explants after two months of culture are presented in Table 1. The application of plant growth regulators, either singly or in combination, significantly influenced both shoot and root proliferation, elongation, callus formation, node, leaf and stem development.

Table 1
Effects of Different Concentrations of BAP singly or BAP and NAA Combined on Mean Number of Shoot (NOS), Length of Shoot (LOS), Number of Root (NOR), Length of Root (LOR), Percentage of Explant Producing Callus (Callus), Number of Leaf (NOL), Number of Node (NON) and Width of Stem (WOS) After 2 Months of Culture.

Treatment (mg/L)	NOS	LOS (cm)	NOR	LOR (cm)	Callus (%)	NOL	NON	WOS (cm)
Control	0.60_{b} ± 0.08	0.41_{b} ± 0.06	3.34_{a} ± 0.15	4.48_{a} ± 0.33	00.00_{c} ± 0.00	2.60_{ab} ± 0.28	3.94_{a} ± 0.13	0.23_{c} ± 0.01
1.0 BAP	2.49_{a} ± 0.37	0.49_{b} ± 0.07	0.00_{c} ± 0.00	0.00_{d} ± 0.00	23.00_{b} ± 0.07	0.51_{c} ± 0.16	1.77_{c} ± 0.23	0.26_{bc} ± 0.04
2.0 BAP	2.57_{a} ± 0.35	0.37_{b} ± 0.06	0.00_{c} ± 0.00	0.00_{d} ± 0.00	40.00_{a} ± 0.08	0.17_{c} ± 0.09	1.71_{c} ± 0.22	0.34_{ab} ± 0.04
3.0 BAP	1.80_{a} ± 0.33	0.42_{b} ± 0.10	0.09_{c} ± 0.06	0.15_{cd} ± 0.13	34.00_{ab} ± 0.08	0.23_{c} ± 0.14	1.83_{c} ± 0.23	0.43_{a} ± 0.05
0.5 NAA + 1.0 BAP	0.74_{b} ± 0.18	0.44_{b} ± 0.10	2.77_{b} ± 0.34	1.61_{b} ± 0.27	3.00_{c} ± 0.03	2.86_{ab} ± 0.37	3.80_{a} ± 0.37	0.25_{bc} ± 0.02
0.5 NAA + 2.0 BAP	1.91_{a} ± 0.41	0.65_{ab} ± 0.13	0.49_{c} ± 0.16	0.78_{c} ± 0.30	3.00_{c} ± 0.03	2.14_{b} ± 0.41	2.80_{b} ± 0.47	0.23_{c} ± 0.04
0.5 NAA + 3.0 BAP	1.71_{a} ± 0.27	0.86_{a} ± 0.11	0.40_{c} ± 0.18	0.64_{cd} ± 0.26	3.00_{c} ± 0.03	3.14_{a} ± 0.38	3.83_{a} ± 0.38	0.25_{bc} ± 0.03

Data represent the mean value ± standard error (SE). Means with different letters in the same column are significantly different at $p < 0.05$, using Duncan's Multiple Range Test.

Shoot proliferation and elongation

In general, approximately 1 to 3 shoots were produced per explant, with BAP alone promoting a greater number of shoots (Fig. 1a). The shoot production was lower in nearly all BAP and NAA combinations and in the control. Explants cultured on MS medium supplemented with 2.0 mg/L BAP gave the highest mean number of shoots ($2.57 \pm 0.35_{a}$) compared with the other treatments. Nonetheless, the difference in shoot proliferation was not significant in most treatments except for 0.5 mg/L NAA + 1.0 mg/L BAP and the control. Shoot elongation was more pronounced when BAP was combined with NAA, in comparison with BAP alone and the control. Most explants had shoot length less than 0.5 cm, while the longest shoot ($0.86 \pm 0.11_{a}$ cm) was obtained from those cultured in MS medium containing 0.5 mg/L NAA and 3.0 mg/L BAP combined (Fig. 1b), which is comparable to those explants treated with 0.5 mg/L NAA and 2.0 mg/L BAP combined. The presence of either BAP applied singly or in combination with NAA

markedly increased the shoot quantity and size, respectively. Hence, clarifying the significant effects of BAP alone and BAP in combination with NAA on the shoot proliferation and elongation.

Root proliferation and elongation

In contrast, root production followed a different pattern, with the control exhibiting superior effects on both root number and root size (Fig. 1c). The presence of either BAP singly or BAP in combination with NAA noticeably reduced the rooting efficiency of the explants. Explants cultured in the control treatment produced the highest mean number of roots ($3.34 \pm 0.91_a$) and the longest root ($4.48 \pm 0.33_a$ cm). Subsequently, followed by those explants treated with combined treatment, 0.5 mg/L NAA and 1.0 mg/L BAP. The combination treatment ranked second after the control in terms of the root proliferation ($2.77_b \pm 0.34$) and root elongation ($1.61_b \pm 0.27$). Therefore, indicating treatments with BAP and NAA in combination showed better explant rooting compared to those in BAP alone.

Callus and node formation

Callus formation was primarily observed in BAP-treated explants, with the highest percentage ($40.00_a \pm 0.08$) recorded at 2.0 mg/mL BAP. However, the callus production showed no significant difference from that produced in 3.0 mg/L BAP (Fig. 1d). This explained the essential role of BAP alone in callus formation. In contrast, explants treated with BAP and NAA in combination supported node production. Although the number of nodes produced was the highest ($3.94_a \pm 0.13$) in the control, the results showed no statistical difference from almost all the treatments in the combination.

Leaf and stem development

Leaf production followed a slightly similar trend to the shoot elongation, with the highest number of leaves ($3.14_a \pm 0.38$) recorded in explants treated with 0.5 mg/L NAA + 3.0 mg/L BAP. Indeed, the combination of BAP and NAA produced a higher number of leaves in comparison to BAP alone. Conversely, BAP alone exhibited better stem development, with the greatest stem width ($0.43_a \pm 0.05$ cm) observed at 3.0 mg/L BAP (Fig. 1e). Besides, increasing BAP concentration was also associated with increased stem thickness, which indicated the significance of BAP alone in the stem development.

Effects of MeJA, FA and AC Applied Singly or In Combination

The effects of MeJA, FA, and AC on vegetative growth of *V. planifolia* after one month of in vitro elicitation are presented in Table 2. The elicitor application influenced several growth parameters, including both shoot and root proliferation, elongation, node, leaf and stem development.

Table 2
Effects of MeJA, FA and AC applied singly and in combination on Mean Number of Shoot (NOS), Length of Shoot (LOS), Number of Root (NOR), Length of Root (LOR), Percentage of Explant Producing Callus (Callus), Number of Leaf (NOL), Number of Node (NON) and Width of Stem (WOS) After 1 Month of *In vitro* Elicitation.

Treatment (mg/L)	NOS	LOS (cm)	NOR	LOR (cm)	Callus (%)	NOL	NON	WOS (cm)
Control	$0.55_{b} \pm 0.19$	$0.27_{ab} \pm 0.09$	$8.23_{a} \pm 0.79$	$6.32_{ab} \pm 0.52$	0.00 ± 0.00	$6.27_{a} \pm 0.55$	$7.05_{a} \pm 0.54$	$0.30_{c} \pm 0.00$
MeJA	$1.18_{ab} \pm 0.28$	$0.41_{a} \pm 0.08$	$6.59_{a} \pm 0.87$	$4.22_{c} \pm 0.58$	0.00 ± 0.00	$4.86_{ab} \pm 0.60$	$6.45_{a} \pm 0.50$	$0.33_{a} \pm 0.01$
FA	$0.64_{b} \pm 0.23$	$0.22_{ab} \pm 0.08$	$8.64_{a} \pm 0.70$	$6.15_{ab} \pm 0.36$	0.00 ± 0.00	$4.68_{ab} \pm 0.60$	$7.32_{a} \pm 0.52$	$0.33_{ab} \pm 0.01$
MeJA + FA	$1.55_{a} \pm 0.35$	$0.40_{a} \pm 0.06$	$7.36_{a} \pm 0.83$	$5.05_{bc} \pm 0.76$	0.00 ± 0.00	$3.64_{b} \pm 0.51$	$7.23_{a} \pm 0.52$	$0.30_{bc} \pm 0.01$
AC + MeJA	$0.59_{b} \pm 0.20$	$0.15_{b} \pm 0.05$	$7.00_{a} \pm 0.51$	$4.95_{bc} \pm 0.47$	0.00 ± 0.00	$3.23_{b} \pm 0.38$	$6.14_{a} \pm 0.35$	$0.29_{c} \pm 0.01$
AC + FA	$0.73_{b} \pm 0.20$	$0.22_{ab} \pm 0.05$	$8.68_{a} \pm 0.69$	$6.97_{a} \pm 0.53$	0.00 ± 0.00	$4.77_{ab} \pm 0.57$	$7.36_{a} \pm 0.57$	$0.31_{abc} \pm 0.00$
AC + MeJA + FA	$1.23_{ab} \pm 0.30$	$0.33_{ab} \pm 0.05$	$6.50_{a} \pm 0.68$	$4.71_{bc} \pm 0.57$	0.00 ± 0.00	$4.27_{b} \pm 0.59$	$7.23_{a} \pm 0.48$	$0.33_{a} \pm 0.02$

Data represent the mean value ± standard error (SE). Means with different letters in the same column are significantly different at $p < 0.05$, using Duncan's Multiple Range Test.

Shoot proliferation and elongation

A lesser number of shoots was produced after a month of in vitro elicitation, whereby only 1–2 shoots were obtained from the in vitro plantlets. The highest mean number of shoots ($1.55_{a} \pm 0.35$) was yielded in the combination of MeJA and FA (Fig. 2a). The result was statistically different from almost all treatments, including control, except for those treated with MeJA alone or AC, MeJA and FA in combination. Although the highest shoot elongation was reported for both MeJA alone ($0.41_{a} \pm 0.08$ cm) (Fig. 2b) or MeJA and FA in combination ($0.40_{a} \pm 0.06$ cm), the results were statistically comparable with the control and nearly all treatments, excluding plantlets treated with AC and MeJA combined. Thus, suggesting MeJA alone and MeJA in combination with other elicitors had remarkable effects on the shoot proliferation and elongation.

Root proliferation and elongation

A similar pattern of root development was discovered in both in vitro regeneration and elicitation. The root proliferation and elongation were correlated, whereby a higher root number ($8.68_{a} \pm 0.69$) and the

highest root length ($6.97_a \pm 0.53$ cm) were recorded in the same treatment, FA in combination with AC (Fig. 2c). Nonetheless, the roots were more actively developed after a month of the in vitro elicitation, as the plantlets produced within the range of 7–9 roots with a length of 4–7 cm in size. Even though all plantlets had slightly different shoot numbers in all treatments, the difference was not statistically significant. The difference was significant in the root elongation between some of the treatments tested. Thus, the application of FA and AC combined assisted in root development.

Callus and node formation

In vitro elicitation exhibited an adverse effect on callus formation. All plantlets treated with various elicitors singly or in combination did not produce any callus. Indeed, the plantlets were actively involved in the node formation. All the plantlets produced within the range of 6–7 nodes, with no statistical difference between treatments. A higher number of nodes ($7.36_a \pm 0.57$) was obtained when the plantlets were applied with FA and AC in combination (Fig. 2c). Hence, demonstrating the combination of elicitors had superior effects on the number of nodes produced, besides root development.

Leaf and stem development

In general, the plantlets produced within the range of 4–6 leaves, whereby the highest leaf number ($6.27_a \pm 0.55$) was obtained from the control (Fig. 2d). Although no statistical difference in leaf production after a month of elicitation in vitro, the elicitor applied singly productively resulted in a higher number of leaves. Meanwhile, those plantlets applied in combinations yielded a lower number of leaves. In contrast, the elicitors applied singly and in combinations had comparable effects on stem width, whereby the same stem thickness was obtained when the plantlets were treated either with MeJA alone ($0.33_a \pm 0.01$ cm) or AC, MeJA and FA in combination ($0.33_a \pm 0.02$ cm).

Quantification of Vanillin Accumulation

The effects of MeJA, FA, and AC on vanillin accumulation in various vegetative parts of *V. planifolia* in vitro were quantified and are presented in Table 3. In vitro elicitation stimulated the vanillin content in root, stem and leaf samples. A standard curve was generated, where a linear regression equation with a specific correlation coefficient $(y = 1E + 8x + 196796,\ R^2 = 0.9981)$ was obtained for the identified vanillin. Vanillin was detected at 8.36 minutes retention time with a total analysis time of 17.5 minutes (Supplementary data).

Table 3
Vanillin content in different tissues of *V. planifolia* after *in vitro* elicitation with MeJA, FA, and AC.

Treatments	Roots (mg/g)	Stem (mg/g)	Leaves (mg/g)
Control	$0.44_{b} \pm 0.23$	$0.24_{cd} \pm 0.07$	$0.34_{b} \pm 0.11$
MeJA	$0.59_{b} \pm 0.10$	$0.46_{bcd} \pm 0.16$	$0.52_{ab} \pm 0.17$
FA	$0.60_{b} \pm 0.10$	$0.73_{b} \pm 0.01$	$1.03_{a} \pm 0.42$
MeJA + FA	$0.68_{b} \pm 0.09$	$0.33_{cd} \pm 0.10$	$0.49_{ab} \pm 0.21$
AC + MeJA	$0.56_{b} \pm 0.04$	$1.05_{a} \pm 0.05$	$0.65_{ab} \pm 0.06$
AC + FA	$1.05_{a} \pm 0.05$	$0.17_{d} \pm 0.13$	$0.44_{b} \pm 0.14$
AC + MeJA + FA	$0.67_{ab} \pm 0.12$	$0.51_{bc} \pm 0.07$	$0.57_{b} \pm 0.31$

Data represent the mean value ± standard deviation (SD) of three technical injections from a single biological sample per treatment.

In the root sample, a higher vanillin accumulation was recorded mostly in the combined elicitor compared to those applied singly. AC and FA in combination demonstrated remarkable vanillin content ($1.05_{a} \pm 0.05$), which verified that better root development correlated with the increasing of vanillin accumulation. The vanillin content was nearly 2-fold higher than that recorded in the control and the result was significantly different from other treatments except for AC, MeJA and FA in combination. An inconsistent trend was observed in the stem sample, though the highest vanillin accumulation was obtained from the stem sample treated with a combination of elicitors. AC and MeJA in combination promoted the highest vanillin content ($1.05_{a} \pm 0.05$). Interestingly, the amount was equivalent to the vanillin content in the root sample. However, the vanillin content was slightly 4-fold higher compared to that recorded in the control. Unlike root and stem samples, the accumulation of vanillin was higher in the leaf sample treated with the elicitor applied singly. FA applied singly demonstrated a higher vanillin content ($1.03_{a} \pm 0.42$), which was not significantly different from some of the treatments. The amount of vanillin was 3-fold higher than in the control. Collectively, each sample can produce approximately 1.0 mg vanillin / g extract (DW). Furthermore, better production of vanillin was observed in the samples treated with elicitors in combination.

Discussions

Effects of PGRs on In Vitro Growth

Micropropagation of *Vanilla spp.* has been done using various types of explants such as axillary bud, shoot tip, nodal segment, leaf, protocorm, bulbous shoot bud, and immature seed in vitro fragmented explant (Gantait & Kundu, 2017). Nodal explant is prevalent in the micropropagation of *Vanilla spp.*

(Mushomiyimana et al., 2011; Tan et al., 2013; Sharma & Bora, 2015; Gantait & Kundu, 2017) and well-known as an effective explant source for micropropagation of plants due to proficiency in huge and rapid plant reproduction (Şaşkın et al., 2022). In the present study, nodal explants of *V. planifolia* responded positively to MS medium supplemented with BAP, either singly or in combination with NAA. Higher concentrations of BAP promoted shoot proliferation and stem thickening, while the inclusion of NAA enhanced shoot elongation and leaf development.

Previous studies on other plant species reported that MS supplemented with 3.0 mg/L BAP resulted in a higher mean number of shoots (Kanchanapoom et al., 2012; Klaocheed et al., 2020; Septianingsih et al., 2024). There are related studies on micropropagation of *V. planifolia* that reported BAP promoted optimal shoot induction as well as shoot formation from nodal explants (Ab Rahman et al., 2013; Asid et al., 2025). The application of cytokinin, especially BAP, in the present study had positively increased the stem width. Meanwhile, previous findings clarified the increased of bud length and diameter (Mahardhini et al., 2023). The findings supported the application of BAP for stem thickness. The IPT7 gene synthesizes bioactive cytokinin, thereby increasing cambium cell division (Kim et al., 2022). In the molecular mechanism, the binding of IPT7 and the PtHK3a stimulates the phosphorelay cascade, which activates the Type-B response regulators (RRs). Thus, turned on the WOX4/Cyclins, leading to an increase in stem thickness. Nonetheless, rising concentration of BAP caused deformity and shrinking in the phloem and xylem region that led to shortening in the stem radius (Kuplemez & Yildirm, 2020).

Cytokinin incorporated with auxin plays a crucial role in the vascular cambium development (Kim et al., 2022). The present study resulted in a higher length of shoots and leaf numbers when the explant was cultured in a combination of NAA and BAP. This combination stimulates shoot length due to the balance of cytokinin-driven proliferation with auxin's role in cell elongation and vascular development (Sosnowski et al., 2023). In addition, higher concentrations of BAP in combination with NAA prevent excessive callus and rooting while enhancing the shoot length and number of leaves. A similar finding was recorded previously, whereby the longest shoot and the highest number of leaves were obtained in the same treatment with the higher concentration of BAP in combination with NAA (Gebeyehu, 2015). Besides, this combination also provides a better number of leaf production (Wijaya et al., 2022). These findings are consistent with previous reports highlighting the synergistic role of cytokinins and auxins in regulating shoot organogenesis in *Vanilla spp.* (Divakaran & Babu, 2009).

Control promoted a higher number of roots, root length, and number of nodes in the present study. Similar findings were recorded in an earlier study of *V. planifolia*, which mentioned that control exhibited higher root number and length (Asid et al., 2025). The occurrence may be due to reliance on endogenous auxin levels, which promote natural and balanced rhizogenesis. Sufficient endogenous auxin biosynthesis in the root primordia facilitates root initiation, resulting in greater root number and length (Yu et al, 2017). Lateral roots arise from single-layered pericycle cells, maintained the ability to undergo cell division upon activation (Roychoudhry & Kepinski, 2021). In the molecular mechanism, the binding of Aux/IAA proteins and auxin response factors (ARFs) turns on the genes that precede root formation (Leyser, 2018). Inhibition of primary root elongation and reduction of root number are probably due to

overexpression of the Gretchen Hagen 3 (GH3) gene, accompanied by a substantial reduction in free auxin (Ai et al., 2023). Manipulation of the GH3 gene expression level resulted in variations in root length (Di Mambro et al., 2017). In terms of node production, the KNOX gene confines node differentiation and allows the stem to elongate by repressing the YABBY gene, which promotes differentiation of the node (Tsuda et al., 2024).

The present study showed notable callus formation with BAP applied singly in the micropropagation of *V. planifolia*, which is also comparable to the discovery in a prior study (Asid et al., 2025). Nevertheless, the finding was in contradiction to the earlier finding, whereby the combination of BAP and NAA resulted in a higher callus formation (Chai et al., 2024). Another study indicated BAP in combination with auxin achieved 100% callogenesis and organogenesis (Puspaningtyas et al., 2025). In the molecular mechanism, the callogenesis and organogenesis are stimulated by Type B response regulators (RRs) such as ARR1 and ARR12. The binding of ARR1 to CLAVATA3 (CLV3) contributed to the ARR12-dependent inhibitory effect on callus formation and shoot regeneration (Liu et al., 2020). Plantlets established in the present study on in vitro regeneration of *V. planifolia* are healthy and morphologically stable, suitable for subsequent elicitation experiments.

Effects of Elicitors on In Vitro Growth

Efficient in vitro regeneration is a critical prerequisite for further successful elicitation studies. Elicitation is commonly used in in vitro cultures to obtain secondary metabolites using various elicitors (Jadczak et al., 2019). Elicitors are associated with a variety of microbial, physical and chemical factors (Miladinova-Georgieva et la., 2022) that enhance metabolite biosynthesis by triggering plant defense signaling pathway (Razak et al., 2025).

In the present study, the application of an elicitor such as MeJA negatively affected the growth and development of *V. planifolia*. The finding was supported by a previous study, which mentioned that the fresh weight of calli, shoot and root decreased in MeJA treatment (Demirci, 2022). Nonetheless, other studies indicated that the application of MeJA had a positive impact on plant growth, whereby it can increase plant size (Wang et al., 2023) and leaf area (Susilowati et al., 2023). Application of MeJA significantly increased shoot length and shoot dry weight, number of leaves (Rasouli et al., 2021; Rahmawati et al., 2023; Danish et al., 2024). A trade-off between plant growth and secondary metabolite signaling pathway was observed in MeJA treatments, associated with a complex interaction between metabolites and endogenous hormones (Kumari et al., 2017).

Likewise, FA treatment adversely impacts the growth and development of *V. planifolia*, with no effect on root, shoot and calli recorded in the present study. The finding was supported by an earlier study, which demonstrated that the application of FA inhibits root growth and development (Singh et al., 2014). Meanwhile, other studies showed that the addition of FA enhanced the plant's ability to maintain cellular homeostasis and regulate stress-responsive gene expression (Khan et al., 2024). FA inhibited the enzyme to control the cell wall during plant growth (Leontakianakou et al., 2025) and reduced the stress gene expression by neutralising reactive oxygen species (ROS) (Khan et al., 2024). Hence, promotes

plant growth, especially in root formation (Li et al., 2025) and biomass production (Gorni et al., 2021). The application of FA markedly increases the number of adventitious roots *in vitro* and acts as an antioxidant that protects indole-3-acetic acid (IAA) from decarboxylation (De Klerk et al., 2011).

Although utilization of multi-elicitor treatment is constrained as the induction primarily relied on two combinations of elicitors, those combinations have been found to exhibit synergistic amplification effects and accumulation of secondary metabolite products (Wen et al., 2023). In the present study, a combination of MeJA and FA resulted in a higher number of shoot production. Yet no specific research has previously mentioned such a combination. However, a study on the association between MeJA and other elicitors, such as salicylic acid (SA), has shown an impact on shoot multiplication (Sliwinska et al., 2021). The effect of MeJA and FA applied together might cause plant cells to become shoot competent. Furthermore, a positive effect on shoot and stem development was recorded in the FA treatment with the addition of other elicitors (Litvinovskaya et al., 2025).

The present study promoted a better root length in treatment FA combined with AC, which supported the root elongation of *V. planifolia*. FA is a key intermediate in the phenylpropanoid pathway (Gao et al., 2023). Meanwhile, AC is known to adsorb inhibitory phenolic compounds and residual plant growth regulators, thereby moderating elicitor-induced stress and improving rooting success *in vitro* (Thomas, 2008; Martins et al., 2024). Moreover, AC in combination with other elicitors enhanced various growth parameters and phytochemical characteristics (Najafi et al., 2025).

Another combination of elicitors evaluated in the present study includes AC fortified with MeJA or MeJA and FA. Both combinations evidently portrayed adverse effects on plant growth and development of *V. planifolia*. Further evaluations are necessary to address the limitations of the current research, mainly in shoot, leaf, root and callus formation.

Quantification of Vanillin Accumulation

In general, vanillin is a derivative of phenylalanine (PAL) via the phenylpropanoid pathway, which is usually detected during vanilla pod development and maturation (Fock-Bastide et al., 2014). Our findings indicated that vanillin accumulation varied among tissues and elicitor treatments. However, there is a limited study on the induction of vanillin accumulation by elicitors in roots, stems and leaves of *V. planifolia.* Earlier study identified various metabolites, including vanillin and its glucosides from roots, stems and leaves (Hernández-Peña., 2025). Accumulation of vanillin is due to the expression of the vanillin synthase (VpVAN) gene with the action of precursors (Gallage et al., 2014). Vanillin content was higher in the root compared to the aerial part due to more antifungal properties available in the part (Huang et al., 2024).

Single application of elicitors in the present study demonstrated that similar vanillin content was extracted from roots, which is not significantly different from the control. The earlier study revealed that the VpVAN gene enhanced the vanillin production in the transgenic hairy root, and subsequent elicitation of MeJA further improved the production of the compound (Husain et al., 2024). In *V. planifolia* pod,

VpVAN converted FA and its glucosides to vanillin and its glucoside in the vanillin biosynthetic pathway (Zorzi et al., 2021). A study previously mentioned that MeJA enhanced the bioactivity and accumulation of vanillin in the root (Nandy et al., 2021). Besides, MeJA also applied to the leaves to enhance the phenolic compounds (Villamarin and Salome, 2017; Chutimanukul et al., 2025).

FA is a metabolic precursor in the vanillin production, and the production persists with further supplementation of FA to the plant tissues or biocatalyst systems (Gallage et al., 2014). The current study discovered that FA treatment in leaf tissue resulted in higher vanillin content compared to other treatments. This finding exhibited the role of FA as a direct precursor in vanillin biosynthetic pathways, which proceeds via phenylpropanoid intermediates in *V. planifolia* (Gallage et al., 2014; Lee et al., 2020). Further accumulation suggests that higher enzymatic activity is associated with FA conversion to vanillin content in leaf tissues. Previous study clarified that ^{14}C-FA fed to disks of green vanilla pods incorporated into glucovanillin or converted the vanillin within 24 hours (Negishi et al., 2009). The FA precursor feeding on cell suspension cultures yielded maximum accumulation of vanillin (Matam et al., 2017).

In the present study, the combination of AC and MeJA enhanced vanillin accumulation in the stem. To date, no studies have reported the application of AC and MeJA in combination as an elicitor to increase vanillin content in the stem. AC reduced inhibition on the phenylpropanoid pathway by absorbing the phenolic compound to increase the vanillin production. Incorporation of MeJA is attributed to upregulation of PAL that leads to more vanillin production.

This study clarified that root tissues exhibit higher vanillin accumulation in the combined treatment of AC and FA. The roots are often responsive to FA, and the adsorption properties of AC facilitate a more favourable metabolic balance for vanillin biosynthesis in plant culture, thus encouraging the increasing of the vanillin content (Meng-de et al., 2002). The presence of AC acts as a product ‘sink’ and relieves the product inhibition, thereby facilitating further metabolism in vanillin conversion (Barraclough et al., 1994). Besides, the addition of combined FA and AC increases the conversion rate of exogenous FA to vanillin several times faster than pods, which leads to an increase in vanillin content (Westcott et al., 1993). In root tissue supplied with FA, vanillin levels can be doubled within a few days, confirming that FA is an efficient precursor and roots are an active site of conversion (Barraclough et al., 1994; Gallage et al., 2014; Zorzi et al., 2021).

Other combinations in the present study evaluated the effects of MeJA and FA, and MeJA, FA, and AC treated in the root, stem and leaves. The finding disclosed that both combinations had no statistically significant differences in vanillin content compared to the control. Similarly, no specific study has previously investigated such research.

Conclusion

Based on our understanding, there is no published in vitro elicitation of MeJA, FA and AC singly or in combination treated in roots, stems and leaves of *V. planifolia*. This study established the first in vitro elicitation on different vegetative tissues that enhanced the vanillin content. Although elicitation only promoted a significant impact on shoot production, the finding would suggest that the elicitation enhanced the vanillin content association with VpVAN expression in all treated tissues. Root and stem treated with AC + FA, and AC + MeJA resulted in 1.05 mg vanillin, respectively. Meanwhile, leaves yielded slightly lower with 1.03 mg vanillin in FA applied singly. The present findings provide significant preliminary insights into tissue-specific elicitation strategies under controlled *in vitro* conditions. Continuation of the current study is necessary to address the research gap. Future studies should incorporate the quantification of total phenolic content and gene expression involved in vanillin biosynthesis in the root, stem and leaves of *V. planifolia.* The potential outcome may contribute to the production of natural vanillin utilised in various industries.

Declarations

Authors Contribution Statement

Nur Syahdina: The author involved in designing the experiment, performed data acquisition, data interpretation, graphic design, and manuscript writing.

Hashimah: The author contributed substantially to supervising and directing the overall research project, data interpretation, and manuscript writing. Besides, critically reviewed and approved the final version of the manuscript.

Nor Hisam: The author contributed substantially to supervising the extraction method and provided critical revisions to improve the scientific quality of the manuscript.

Muhammad Jeffry: The author assisted in experimenting with HPLC and data interpretation.

Nur Eynshierrah: The author assisted in the production of the in vitro plantlets and data interpretation.

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Funding Declaration

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Ethics Declaration

Not applicable.

The authors declare that this manuscript is original work, has not been published previously, and is not under consideration for publication elsewhere. Besides, there are no known competing financial interests or personal relationships that influence the research work.

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Figures

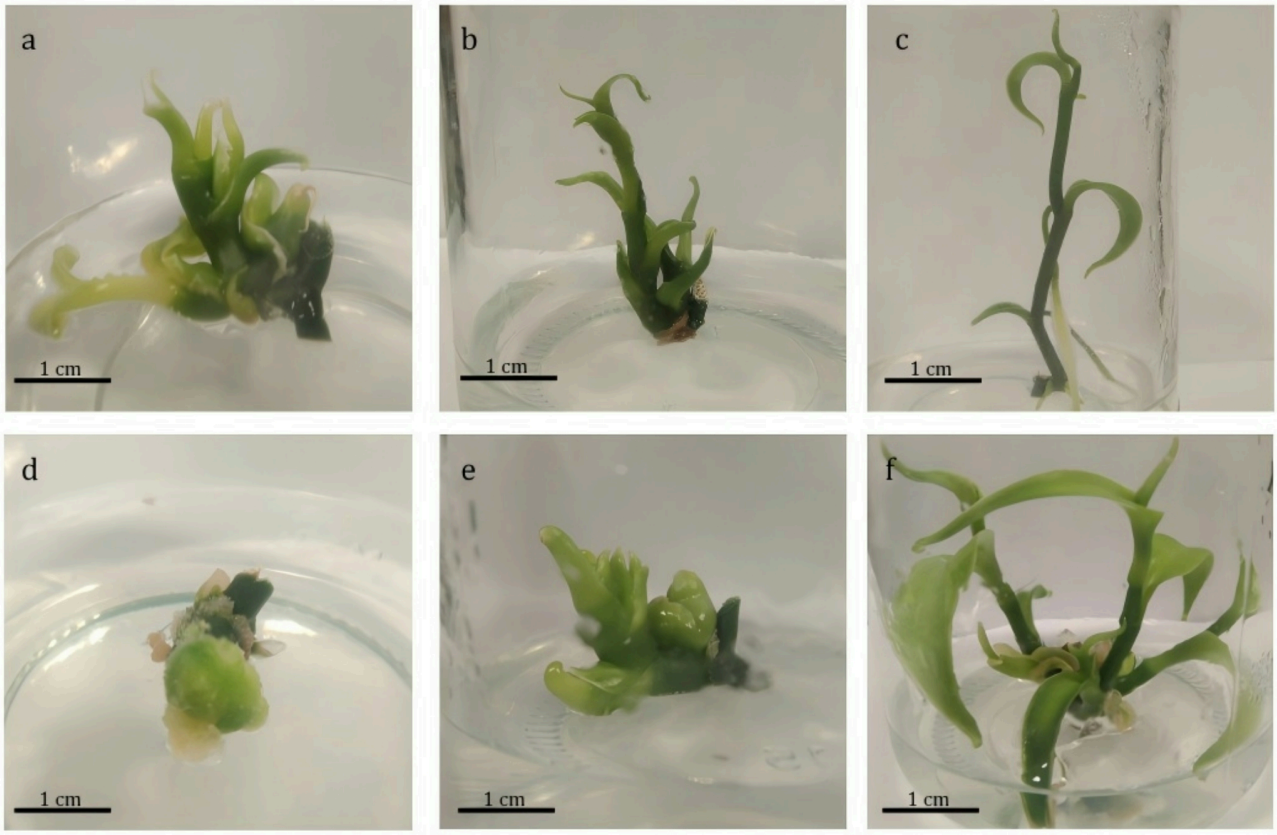

Figure 1

In vitro Regeneration of *V. planifolia* from nodal segment. **a)** Shoot multiplication on MS medium supplemented with 2.0 mg/L of BAP singly, **b)** shoot multiplication on MS medium fortified with 0.5 mg/L of NAA with 3.0 mg/L BAP in combination, **c)** root development of control explant, **d)** callus induction on MS medium supplemented with 2.0 mg/L of BAP singly, **e)** stem width development on MS medium containing 3.0 mg/L BAP singly and **f)**leaf proliferation on MS medium added with 3.0 mg/L BAP in combination obtained after 2 months of culture.

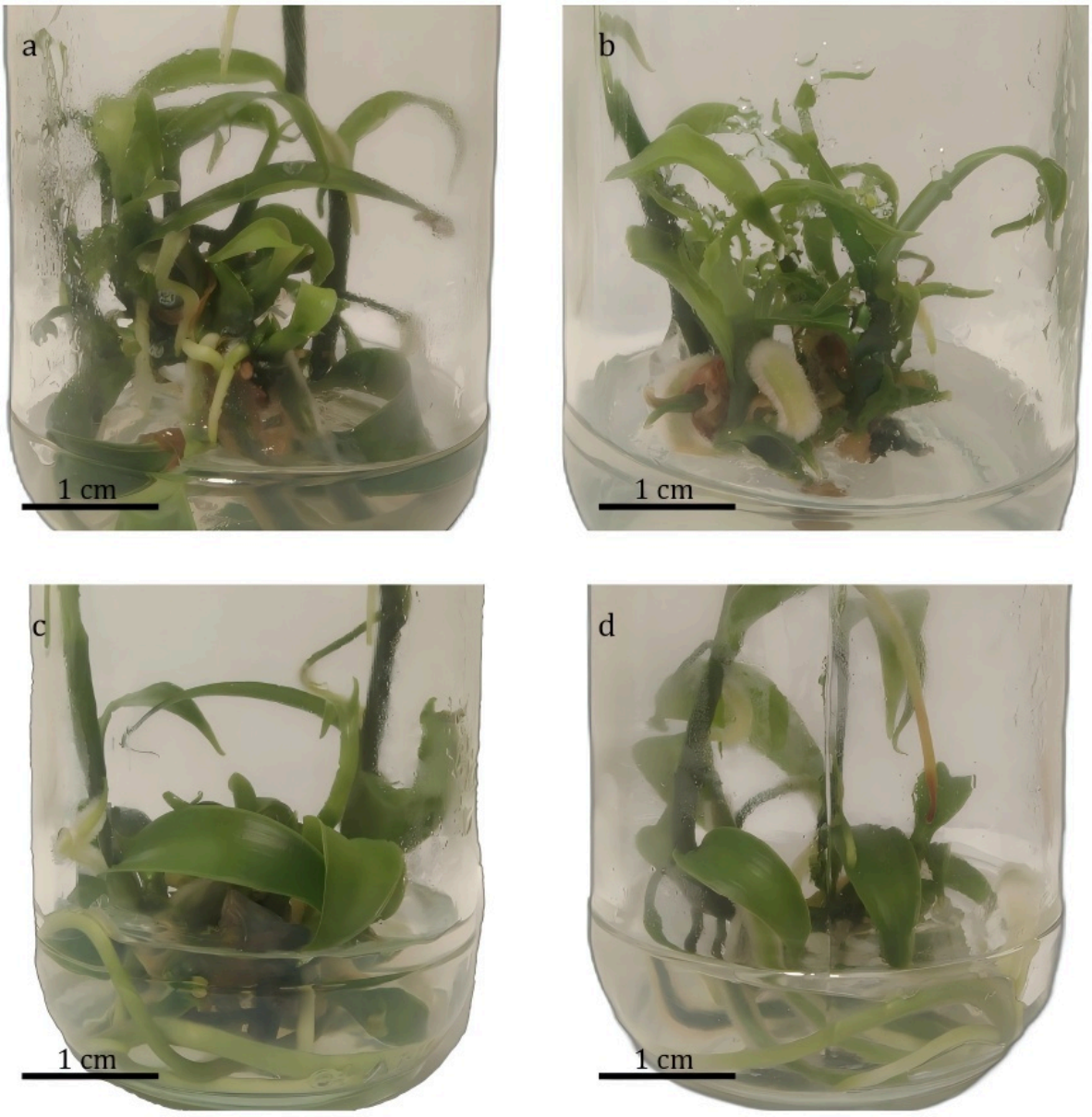

Figure 2

In vitro Elicitation of *V. planifolia* plantlets. **a)** Shoot formation on MS medium supplemented with MeJA and FA in combination, **b)**shoot elongation on MS medium fortified with MeJA applied singly, **c)**root and node development on MS medium supplemented with AC and FA in combination, and **d)** leaf proliferation on control for 1 month of culture.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryData.docx
- floatimage1.jpeg