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Research Article

Effect of Different Concentrations and Combinations of Benzyl Aminopurine and Indole-3-Butyric Acid on Micropropagation of *Vanilla Planifolia*

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ABSTRACT
Micropropagation of explants *in vitro* has potency to address propagule demands for promoting large-scale vanilla production. Plant growth regulators (i.e., cytokinin, auxin) are necessary for the plant micropropagation success. Objective of this study is to determine the shoot multiplication and root development of *Vanilla planifolia* under the influence of different concentrations and combinations of BA and IBA for micropropagation. Sixty stem nodal segments of *Vanilla planifolia* were cultured on MS medium supplemented with IBA (0, 0.5, or 2.0 mg/L) and combined with BA (0, 0.5, 1.0, or 2.0 mg/L). Shoot multiplication and root induction were measured after 60 days of culture. The results show that the MS medium with 1 mg/L IBA hindered shoot growth, while the medium containing 1 mg/L BA yielded the highest number or weight of shoots per explant. For the root development, supplementing the medium with 0.5 mg/L or 1 mg/L IBA improved root length or number of roots per explant, respectively. This research establishes a valuable approach for vanilla micropropagation by utilising low concentrations of plant growth regulators and a rapid protocol. This paves the way for significant advancements in large-scale commercial production.

INTRODUCTION
Vanilla planifolia, commonly named 'flat-leaved vanilla', is a species of vanilla orchid, native to Mexico, Central America, Colombia, and Brazil (Sinha et al. 2008). According to Royal Botanical Gardens, Kew (RBG Kew 2023) vanilla is one of the most popular flavors in the world, and the second most expensive spice after saffron because of the intensive labor required to grow and produce its pods (fruits). Vanilla plants have been introduced worldwide to tropical areas including Cambodia (Ramachandra Rao & Ravishankar 2002; McGregor 2004; Cameron 2011). The pod or tiny seeds of vanilla are used as flavoring agents (vanillin) in food, including ice cream, chocolate, liquor, soft drinks and candies, and for other purposes, e.g., cosmetics and pharmaceuticals as well.

Vanilla is perennial climbing orchid with a thick, cylindrical, succulent green stem and an evergreen vine that can reach up to 15 m in length (Janarthanam & Seshadri 2008). Stem cutting is the common

propagation method for this plant (Anuradha et al. 2013; Baqueiro-Peña & Guerrero-Beltrán 2017; Arya et al. 2021), with labour intensive and time consuming (Kalimuthu et al. 2006). Rapid micropropagation is needed to meet demands of the vanilla world market that remaining consists about 70% of synthetic vanilla extract, which is produced from chemical components (DMNP 2023). Micropropagation has become the cornerstone of a massive commercial plant propagation industry, with hundreds of laboratories around the globe utilizing this technique.

Several studies on multiplication of *V. planifolia* have been done, using different parts of vanilla plant, e.g., callus culture, protocorms, shoot tips, stem nods, root tips, shoot tips (e.g. Philip & Nainar 1986; Giridhar & Ravishankar 2004; Kalimuthu et al. 2006; Janarthanam & Seshadri 2008), and different supporting materials, light intensity or media types (e.g., Kunwanlop et al. 2018; Erawati et al. 2021). Plant growth regulators (PGRs), known as plant hormones, are essential for plant micropropagation because they play critical role in directing the development and multiplication of plant cells and tissues in a controlled environment. PGRs influence the way plant tissues develop into specific structures like shoots or roots; for instance, cytokinin (e.g., Benzyl Aminopurine – BA, Kinetin) promote shoot multiplication, while auxin (e.g., Naphthaleneacetic Acid – NAA, Indole-3-Butyric Acid – IBA) can stimulate root initiation (Bhatla & Lal 2023). Previous studies on the effects of cytokinin and auxin on *V. planitolia* micropropagation investigated the use of various combinations, such as BA or kinetin with NAA supplemented MS medium (George & Ravishankar 1997; Ayele et al. 2017; Izzati et al. 2013), BA and kinetin with MS medium (Erawati et al. 2021), and BA alone with Gamborg's B5 medium (Kunwanlop et al. 2018). de Oliveira et al. (2013) report the influence of BA on shoot multiplication of *V. planifollai*, and IBA on rooting in double-phase culture system.

However, there are no reported studies specifically investigating the combined effects of BA and IBA concentrations on the micropropagation of *V. planifolia*. The objective of the present study was to determine the effects of different concentrations and combinations of BA and IBA on shoot multiplication and root induction of vanilla plants (*Vanilla planifolia)*, using stem nodal segments for *in-vitro* culture.

MATERIALS AND METHODS

Experimental design

The experiment was conducted at the AgroBio4Cam (ABC) Laboratory (latitude: 13.0897004 N, longitude: 103.233008 E) in Battambang, Cambodia, from February 20th to April 22nd, 2023. ABC, a private company established in Battambang, Cambodia in 2021 with Registration No: KH/88412/22 under the Ministry of Commerce, provided the laboratory facilities. *Vanilla planifolia* explants were obtained from a commercial farm in Siem Reap and brought to the ABC laboratory in 2022.

A complete randomised design was used for this experiment, with 12 treatments and 5 replicates, one cutting in each culture vessel or replicate. Combinations of different concentrations of IBA and BA were prepared for each treatment to test their effects on shoot and root initiation of *in vitro* propagation of vanilla plants (*V. planifolia*). Combinations of different concentrations of IBA and BA were prepared for each treatment to test their effects on shoot and root initiation of *in vitro* propagation of vanilla plants (Table 1).

Table 1. Detailed treatments of the effect of BA (Benzyl Aminopurine) and IBA (Indole-3-Butyric Acid) on shoot and root multiplication of vanilla plants (*Vanilla planifolia*) micropropagation.

Treatments	Plant growth regulators (mg/L)	
	BA	IBA
T0	0	0
T1	0.5	0
T2	1	0
T3	2	0
T4	0	0.5
T5	0.5	0.5
T6	1	0.5
T7	2	0.5
T8	0	1
T9	0.5	1
T10	1	1
T11	2	1

Culture conditions

This study employed stem nodal segments (2 – 3 cm in length with a single axillary bud) from healthy young shoots of *V. planifolia* for micropropagation. Following sterilisation protocols established by Abebe et al. (2009), explants were first washed with a 3 g/L detergent solution (three times, 10 minutes each) followed by a 30-minute fungicide soak (3 g/L copper hydroxide). After rinsing with sterile distilled water (three times), explants were transferred to a laminar flow cabinet and sequentially treated with 70% alcohol (5 minutes) and 0.1% mercuric chloride (5 minutes), with thorough rinsing with sterile water after each step.

Following sterilization, individual explants were cultured aseptically on basal MS medium (Murashige & Skoog 1962) supplemented with 3% sucrose, 0.4% agar (gelling agent), and various combinations of IBA (0, 0.5, or 2.0 mg/L) and BA (0, 0.5, 1.0, or 2.0 mg/L). Prior to agar addition, the medium pH was adjusted to 5.7 ± 0.1 using 1 N KOH or HCl. The medium was sterilized by autoclaving at 121°C for 20 minutes. Each explant was inoculated into a 250 mL vessel containing 50 mL of the designated medium. Cultures were incubated in a plant growth room at 25 ± 1°C under a 16-hour photoperiod provided by cool white, fluorescent light (1,000 – 2,000 lux) for 60 days (Figure 1). Daily observations were made, and growth and development measurements were recorded after 60 days of culture.

Statistical analysis

To evaluate plant growth and development across all treatments and the control, the following parameters were measured after 60 days in culture, including shoot formation time (days to first appearance), number of shoots per explant, shoot fresh weight, shoot length, number of leaves per shoot, number of roots per explant, root length, and root fresh weight.

The root-to-shoot length ratio was calculated by dividing root length by shoot length. To assess mean differences across multiple groups, one-way analysis of variance (ANOVA) was used for normally distributed data. For data with non-normal distributions, the Kruskal-Wallis test (Kruskal & Wallis 1952) was employed. Post-hoc analysis for

both tests involved Tukey's Honestly Significant Difference (HSD) test (Tukey 1949) to compare all pairwise means at $\alpha = 0.05$. All statistical analyses were performed using R statistical software version 3.6.3 (R Core Team 2020), and data visualization was accomplished with the 'ggplot2' R package (Wickham 2011).

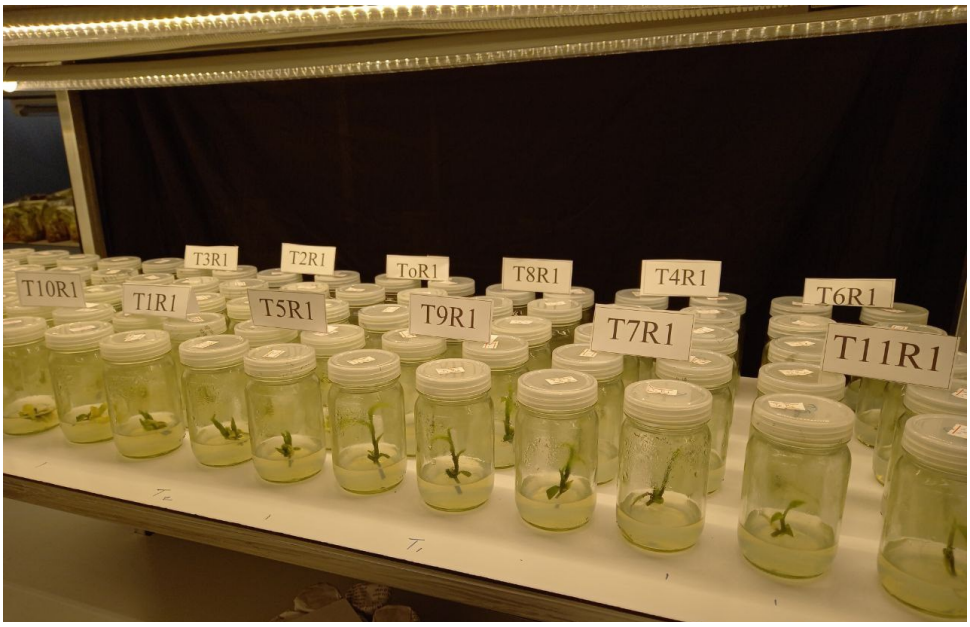

Figure 1. The vanilla plants (*Vanilla planifolia*) *in vitro* culture in the culture room of the AgroBio4Cam laboratory were observed at 40 days after inoculation.

RESULTS AND DISCUSSION

All *in vitro* cultures of *Vanilla planifolia* were free of contamination (Figure 1 & 2). Application of different BA and IBA concentrations and combinations significantly affected shoot and root growth and development of *V. planifolia* micro-propagation (Figure 3 & 4; $P < 0.001$).

Shoot Initiation and Multiplication

Supplementation with BA alone led to the earliest shoot development observed within 7 – 8 days (Figure 3a). Conversely, media lacking plant growth regulators or containing only IBA delayed shoot development. Interestingly, the greatest shoot length (5.37 cm) was achieved on medium containing 1 mg/L IBA (Figure 3b), while the medium with 1 mg/L BA resulted in the highest number (4.93 shoots per explant) and weight (828 mg) of shoots per explant (Figure 3c, d). The control group (medium without plant growth regulators) exhibited poorly developed shoots.

This study suggests that cytokinin alone (BA) can promote single shoot regeneration from *V. planifolia* nodal explants cultured on MS media containing 1 mg/L IBA with 0 or 0.5 mg/L BA. In contrast, shoot multiplication required supplementation with both BA (1 mg/L) and IBA (0.5 mg/L) in the MS medium. These findings align with Zuraida et al. (2013), who reported the greatest shoot number and length for *V. planifolia* cultured on MS medium with 1 mg/L BA for stem nodal segments, or 2 – 3 mg/L BA for shoot apex explants (cultured for 45 days). Erawati et al. (2020) found that adding 3 mg/L BA to the MS medium yielded the best results for *V. planifolia* multiplication, with 3 – 4 shoots emerging per explant, each measuring 2 – 2.5 cm in length, at 28 days after inoculation.

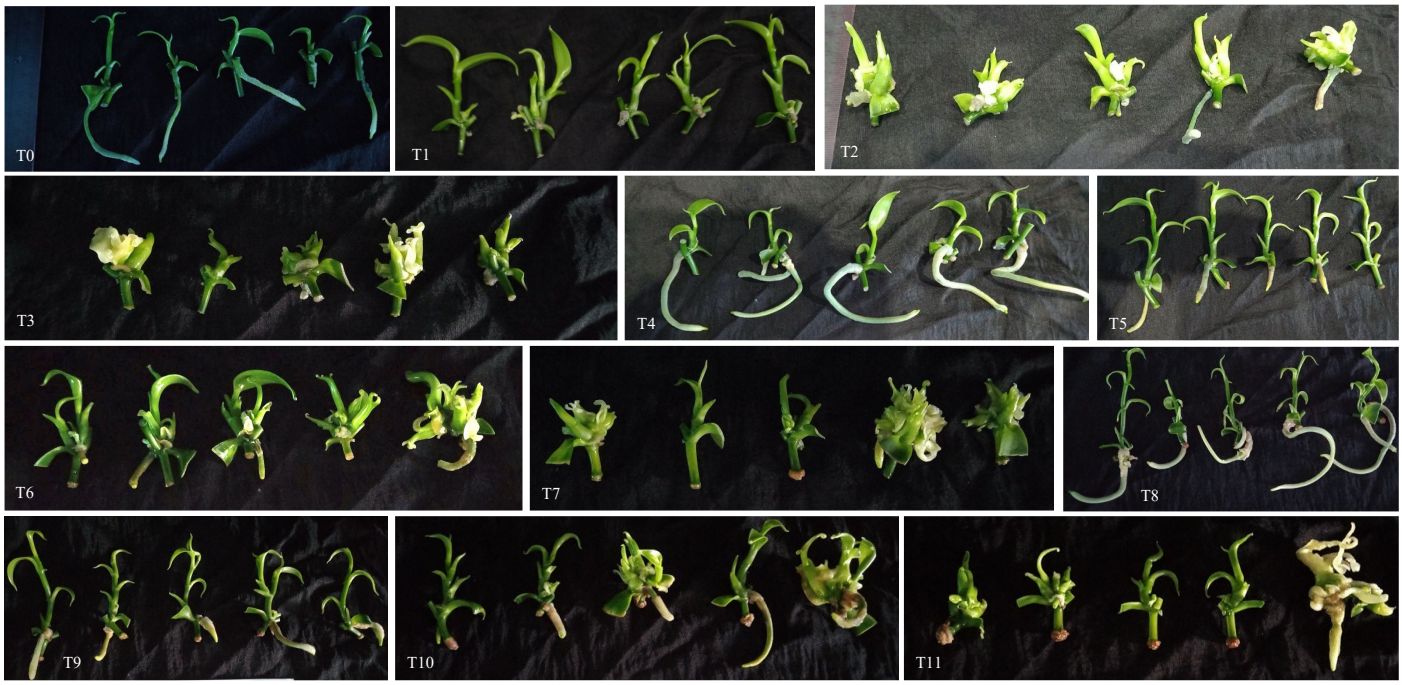

Figure 2. Effect of different BA and IBA concentrations and combinations on the propagation of Vanilla (*Vanilla planifolia*) after 60 days of *in vitro* culture.
Note: T0: 0 mg/L BA + 0 mg/L IBA; T1: 0.5 mg/L BA + 0 mg/L IBA; T2: 1 mg/L BA + 0 mg/L IBA; T3: 2 mg/L BA + 0 mg/L IBA; T4: 0 mg/L BA + 0.5 mg/L IBA; T5: 0.5 mg/L BA +0.5 mg/L IBA; T6: 1 mg/L BA + 0.5 mg/L IBA; T7: 2 mg/L BA + 0.5 mg/L IBA; T8: 0 mg/L BA + 1 mg/L IBA; T9: 0.5 mg/L BA + 1 mg/L IBA; T10: 1 mg/L BA + 1 mg/L IBA; and T11: 2 mg/L BA + 1 mg/L IBA.

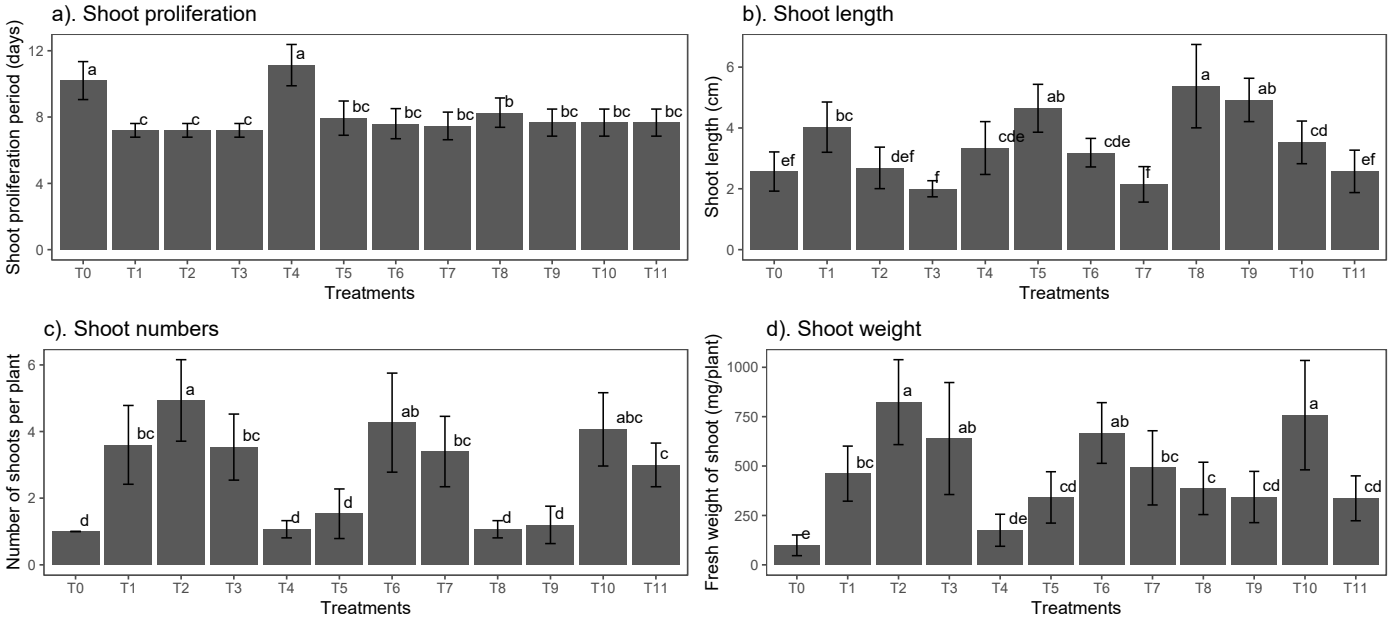

Figure 3. Effects of BA and IBA on shoot initiation and multiplication in vanilla plants (*Vanilla planifolia*) micropropagation: a). period of shoot proliferation (days); b). shoot length (cm); c). shoot number per explant; and d). shoot weight per explant (mg). Bars represent standard deviation. Different letters (a, b, c, d, e, f) within each panel indicate significant differences ($P < 0.05$) according to Tukey's HSD test.

Conversely, Erawati et al. (2021) reported the highest number of shoots (6 shoots per explant) for *V. planifolia* micropropagation using MS medium supplemented with 0.5 mg/L BA and 2 mg/L Kinetin at 56 days after inoculation. Prabaninggar et al. (2021) found that BA at concentrations of 1, 2, or 3 mg/L had no effect on shoot proliferation in *in vitro* microcuttings of *V. planifolia*. Conversely, Ayele et al. (2017) reported that the combination of 2 mg/L BA and 0.5 mg/L NAA yielded the best re-

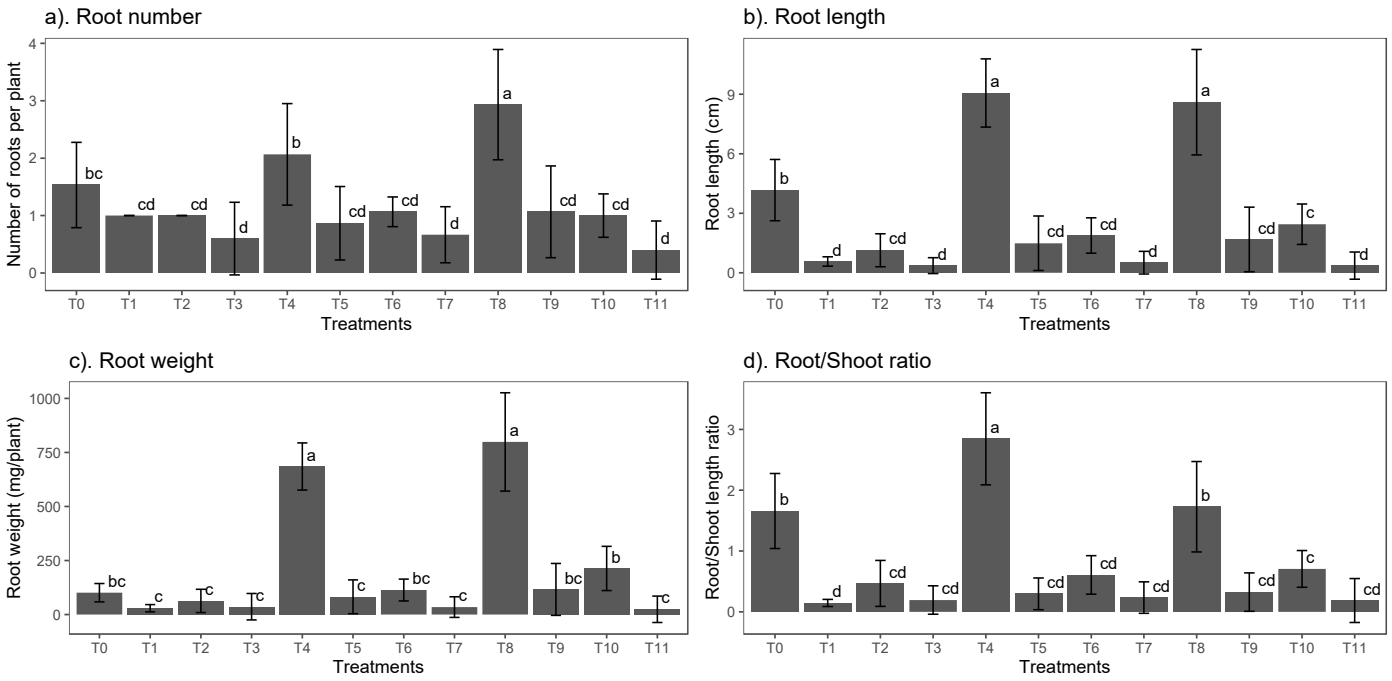

Figure 4. Effects of BA and IBA on the root growth and development: a). root number per explant; b). root length (cm); c). root weight per explant (mg); and d). root-to-shoot length ration. Bars represent standard deviation. Different letters (a, b, c, d) within each panel indicate significant differences ($P < 0.05$) according to Tukey's HSD test.

sults for *V. planifolia* shoot multiplication. Notably, this treatment produced the highest number of shoots (5.33) and the longest shoots (4.9 cm) after five weeks of culture. Other studies by Sharma & Bora (2017) reported that MS medium supplemented with 3 mg/L BA and 1 mg/L NAA was most effective for maximum shoot bud differentiation of *V. planifolia* cultured from nodal segments. This medium resulted in the highest number of multiple shoots (11.6 per explant). Abebe et al. (2009) reported that for *V. planifolia* cultured on MS medium for 45 days, the combination of 1 mg/L BA and 1.5 mg/L Kinetin produced the maximum shoot multiplication (4.17 shoots per explant). Higher BA concentrations in the medium resulted in a decrease in shoot numbers for *V. planifolia*. However, supplementing liquid MS medium containing 1 mg/L BA with 15% coconut water improved shoot multiplication. Furthermore, research by de Oliveira et al. (2013) suggests that lower BA concentrations can enhance proliferation rates of vanilla plants in a double-phase *in vitro* culture system. Tan et al. (2011) observed the highest shoot multiplication with 9.6 shoots per explant and a shoot length of 4.7 cm, achieved using this approach.

Root Growth and Development

The medium supplemented with 1 mg/L IBA yielded the greatest number of roots per explant (2.93) (Figure 4a). For root length and weight, the medium containing either 0.5 mg/L or 1 mg/L IBA resulted in the highest values (9.07 cm and 799 mg per explant, respectively) (Figure 4b, c). Notably, the control group exhibited the least root growth. These findings suggest that auxin plant growth regulators can improve root growth and biomass. Explants were also able to develop roots on MS medium even without the addition of any plant growth regulators. The medium containing 0.5 mg/L IBA yielded the greatest root-to-shoot length ratio (Figure 4d).

This study found that auxin was necessary for improved root growth and biomass, although roots could grow on MS medium without auxin supplementation. Kunwanlop et al. (2018) reported similar findings

in their studies on two vanilla species. They observed the highest root induction in Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 1 mg/L BA, or 2 mg/L BA for *V. planifolia* micropropagation. Previous studies (Abebe et al. 2009; Zuraida et al. 2013) reported that root induction for *V. planifolia* micropropagation on MS medium did not necessarily require plant growth regulators. However, Ayele et al. (2017) found that half-strength MS medium supplemented with 0.5 mg/L IAA produced the highest number (4.0 roots per plantlet) and longest roots (6.1 cm) among the treatments they tested. In their study on *V. planifolia* micropropagation, Tan et al. (2011) found that 1 mg/L NAA in MS medium yielded the maximum root number, with 2.9 roots per explant. Besides plant growth regulators, other factors can influence shoot and root induction in vanilla plant micropropagation. These factors include medium type (solid or liquid), light intensity, plant hormone type, and more. Several studies have explored these influences (e.g. Srean et al. 2011; Tan et al. 2011; Bello-Bello et al. 2016; Sidek et al. 2018).

CONCLUSION

Our research established a streamlined method for large-scale production of vanilla plants. The investigation of different cytokinin (BA) and auxin (IBA) concentrations and combinations has revealed several options for plant hormone application in the *in vitro* culture of stem nodal segments for micropropagation of vanilla plants (*V. planifolia*). For shoot multiplication, using only 1 mg/L BA in the solid MS medium yielded the best results. Conversely, the highest root induction was achieved with 0.5 mg/L IBA in the same medium. This study demonstrates that single plant growth regulators can be effective for micro-propagating vanilla stem nodal segments. Supplementation with either 0.5 mg/L or 1 mg/L IBA promotes greater root length or more roots per explant, respectively. This research establishes a straightforward protocol for large-scale vanilla plant production. The key lies in using low concentrations of plant growth regulators, along with the rapid protocol developed. This approach can be effectively applied to various stages of vanilla micropropagation, paving the way for significant advancements in commercial production.

AUTHORS CONTRIBUTION

S.K. & C.H. designed the research and executed the experiment, S.K. analysed the data and wrote original draft preparation, S.R., S.R. & P.S. reviewed and edited the manuscript.

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CONFLICT OF INTEREST

The authors state that there was no conflict of interest in this research.

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