Micropropagation of Vanilla Planifolia Andrews on Commercial-Scale

Tran Ngoc Tu Uyen, Bui Thanh Hoa, Pham Hong Diep, Tran Van Minh*

School of Biotechnology, International University, Vietnam National University HCM

***Corresponding Author**

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ABSTRACT

Vanilla planifolia, a high value crop due to vanillin extract in its fruits, is a tropical orchid that can adapt and be profitably cultivated in Vietnam. This study describes a micropropagation of *V. planifolia* on commercial scale by using shoot-tip culture techniques. Three different strengths of MS medium containing 100 ml/L coconut water (CW) were screened for their effects on shoot regeneration and growth. Results indicated that 1/2xMS medium was a promising basal medium for vanilla tissue culture. The addition of 1 mg/L BA to that medium significantly produced the highest multiplication rate ($p < 0.001$) with 3.67 ± 0.08 shoots per explant. Regenerated shoots were transferred to 1/2xMS medium supplemented with 0.1 mg/L BA and three types of organic additives for growth and development. Banana homogenate (100 g/L) significantly promoted shoot extension (6.22 ± 0.25 cm), leaf development (5.03 ± 0.03 leaves per shoot with a mean length of 3.10 ± 0.13 cm), root formation (3.83 ± 0.07 roots per shoot) and root elongation (5.42 ± 0.21 cm) compared to CW (100 ml/L) and peptone (1 g/L). The combination of NAA to medium containing CW (100 ml/L) and BA (0.1 mg/L) did not significantly improve root production ($p = 0.178$) and elongation ($p = 0.231$) after 3 weeks of culture. The findings in this study would help to establish an efficient and viable protocol for commercial-scale micropropagation to provide adequate planting materials of *V. planifolia* in Vietnam.

Keywords: Vanilla planifolia, micropropagation, shoot regeneration, organic additives

INTRODUCTION

The vanilla plant (*Vanilla planifolia* Andr.) is a species of vanilla genus belonging to the family Orchidaceae. Vanilla is climbing vine orchid with perennial succulent stem, oblong lanceolate leaves and aerial axial roots arising opposite each leaf [1, 2]. Although orchids are famous and widely cultivated for its beautiful blooms, the genus vanilla is commercially grown for its fruits. Vanilla fruit, commonly called as “beans” or “pod” in the vanilla market, after undergoing fermentation and curing process, is the main source of natural vanillin (4hydroxy-3-methoxybenzaldehyde), one of the most used flavoring agents. Vanillin is widely used in food and beverages, especially in the ice cream and chocolate industry, because its sugary sweet aroma can impart sweetness and reduce the use of sugar and other artificial sweeteners [2, 8]. In addition, vanillin is also employed in perfumes, cosmetics and pharmaceutical industries [3]. It has been reported that vanillin has numerous medicinal effects including but not limiting to anticlastogenic [4], antimutagenic [5], anticarcinogenic [6], antisickling [7], anti-inflammation [9, 10], antioxidant [11, 12, 13] and antimicrobial properties [10]. Such findings make vanillin a potential food preservative as well as pharmaceutical applications [14, 15].

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Synthetic vanillin has been accepted with great demand and mostly used by companies for years. The desire for natural vanillin has been increasing as people become more health-conscious in recent years. However, extracting vanillin from vanilla fruits is complicated and time-consuming. Vanilla flowering usually occurs over a period of 2 months, once a year, but it is observed that each flower stays in bloom for less than 24 hours and must be hand-pollinated at the right time (8 – 11 am) for fertilization and fruit development [16]. It requires 10-12 months for the pods to fully mature. After harvesting, these pods are subjected to a complex fermentation and curing process for vanillin production. As a result, natural vanillin is one of the most expensive spices in the world market.

In general, vanilla is commercially planted in Madagascar, Indonesia, Mexico, China and many other tropical or subtropical countries. Requiring optimum temperature ranges from 21- 32 °C with rainfall between 2000 – 2500 mm annually, evenly distributed in ten months, and having a two-month dry period, vanilla can adapt to Vietnam conditions. The fact that it has been successfully cultivated in Bình Thuận, Đắk Lắk, Thừa Thiên - Huế, Bình Dương [17, 18]. With ample land and labor, Vietnam has a potentially competitive advantage in the world vanilla market if we expand its cultivation. Therefore, providing enough planting material is the paramount. Conventional breeding by stem cutting is not efficient, time-consuming, and may arrest the mother plants [19]. Thus, plant tissue culture is an effective way to propagate and supply the demand number of plantlets for large scale plantations.

In vitro multiplication of *V. planifolia* has been reported through both direct and indirect organogenesis [20] by using shoot tip and nodal segment [21, 22, 23, 24, 25], leaf portion [26, 18], as well as aerial root [27] as the explant. Apart from normal solid and semi-solid culture, the effects of other systems such as double-phase system and immersion system [28] have also been investigated. However, there are very limited reports on *in vitro* propagation protocols of *V. planifolia* in Vietnam. Thus, the aim of this research is to establish an economically efficient protocol for micropropagation of vanilla native clones found in Vietnam using shoot-tip culture techniques on commercial-scale.

MATERIALS AND METHODS

Materials

Plant materials

Explants used in this experiment were 2 month acclimatized vanilla (*V. planifolia*) provided by TPECO Biotechnology Corporation (Ho Chi Minh City, Vietnam).

The basic nutrient medium is MS (Murashige and Skoog, 1962) [29], MW (Morel & Wetmore, 1951) [42], supplemented with 30 g/L sucrose, 10% coconut water, and 8 g/L agar, pH was adjusted to 5.8; and growth regulators BAP (6-benzylaminopurine), NAA (α-naphthalene acetic acid)

Conditions: The medium was distributed in aliquots of 60 ml into 330 ml glass jars and capped with closure prior to autoclaving at 121ºC, 1 atm. for 20 minutes. Cultures were maintained at a temperature of 26 ± 2 ºC with a 16-hour photoperiod provided by white LED (light-emitting diode) lights.

Methods

Surface sterilization and preparation of initial culture

The plants were cut into shoot-tip and nodal segments about 1.5 – 2 cm. After being soaked in liquid detergent for 20 min and washed under running tap water, the nodes were first brought into the laminar air

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flow chamber and transferred to a sterile bottle containing 70% ethanol for 5 min where the bottle was gently shaken. Next, they were dipped into 50% of commercial bleach for 20 min followed by 0.1% $HgCl_2$ solution for 10 min. Finally, the explants were rinsed 5 times with sterile distilled water to completely get rid of $HgCl_2$. The surface sterilized nodes were laid on sterile petri dishes to remove damaged parts of both ends and then were cultured in MS [29] medium. Non-contaminated samples after 7 days were used for the experiment.

Effects of strength-MS media on shoot-tip and axillary bud regeneration of vanilla

Contamination-free explants after 7 days in initial cultures were transferred to full-strength, half strength (1/2) or one-third strength (1/3) MS medium supplemented with 100 ml/L coconut water (CW). The parameters evaluated at 5 weeks of culture were: shoot length (cm), number of nodes and number of leaves.

Effects of BA on in vitro shoot multiplication of vanilla

Regenerated shoots from strength of MS media experiment were aseptically collected and divided into nodal segments of roughly 1 cm before being cultured into MS medium supplemented with 100ml/L CW and different concentrations of BA (0-0.1-0.3-0.5-1.0 mg/L). After 4 weeks of inoculation, the following variables were evaluated: number of shoots, number and nodes, and shoot height (cm).

Effects of organic additives on in vitro shoot growth of vanilla

Vanilla shoots derived from 0 and 0.1 mg/L BA treatments were used. Shoots that had two nodes and were about 2 – 2.5 (from the lower node to the shoot tip) were cut out from the original nodes and transferred to 1/2xMS medium containing 0.1 mg/L BA and different organic supplemented with coconut water (100 ml/L), banana (100 g/L) and peptone (1 g/L). The length (cm) and number of nodes, shoots, roots and leaves were recorded after 4 weeks of culture.

Effects of NAA on root formation of vanilla

Shoots from previous experiments were cut to remove all existing roots and obtain shoots with three nodes before being cultured on 1/2xMS medium containing 100 ml/L CW, 0.1 mg/L BA and varying concentrations of NAA (0, 0.1, 0.3, and 0.5 mg/L). After 3 weeks of inoculation, the percentage of shoots forming root, number of roots and root length were recorded to evaluate the effect of NAA on in vitro rooting of vanilla.

Data analysis

The experimental designs followed completely randomized design (CRD) with three replicates. One replicate was performed 5 bottle. One bottle was cultured with 3 explants. Data analyses were performed using IBM SPSS Statistical 26 Software (SPSS v.20 Inc., Chicago, USA). The means and standard errors (indicated as ± values) were calculated for the treatment responses and then analyzed with one-way analysis of variance (ANOVA) and differences among treatments were further analyzed using Duncan's test at 5% significance level.

RESULTS

Effects of strength-MS media on shoot-tip and axillary bud regeneration of vanilla

This experiment was conducted to investigate the effects of strengths of MS medium on axillary bud regeneration of vanilla shoot-tip and nodal explants, of which results are shown in Table 1 and Figure 1.

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Shoot initiation was observed from all explants after 10 days of culture and each explant produced only one shoot after 35 days of culture. It was noted that shoot length was increased by increasing salt strength in MS medium. While there was significantly lower ($p < 0.01$) in shoot elongation ability of *V. planifolia* cultured on MS medium containing 1/3x salt strength (3.91 ± 0.08 cm) compared to half and full-strength, there was no significant differences in shoot length between two latter groups. On the other hand, vanilla shoots tended to generate more nodes in weaker on strength MS medium. Shoots initiated on full strength MS cultures had average 2.83 nodes per shoot, which was significantly ($p = 0.011$) less than shoots on 1/2 and 1/3xMS medium (3.06 nodes per shoot). The highest number of fully opened leaves was observed in 1/2xMS cultures with a mean of 3.75 leaves per shoot.

Table 1. Effect of different strengths of MS media on axillary bud regeneration of vanilla

Full MS + 10% (v/v) CW	Shoot length (cm)	No. of nodes per shoot	No. of leaves per shoot
One-third strength	3.91 ± 0.08b	3.06 ± 0.03a	3.31 ± 0.10b
Half strength	4.30 ± 0.10a	3.06 ± 0.03a	3.75 ± 0.05a
Full strength	4.34 ± 0.06a	2.83 ± 0.05b	3.42 ± 0.08ab

The results represent the mean ± standard error of the mean (SEM) of three replicated experiments after 5 weeks of culture. Means indicated with the same letter were not significantly different based on analysis of variance (ANOVA) followed by Tukey's HSD test at $P < 0.05$.

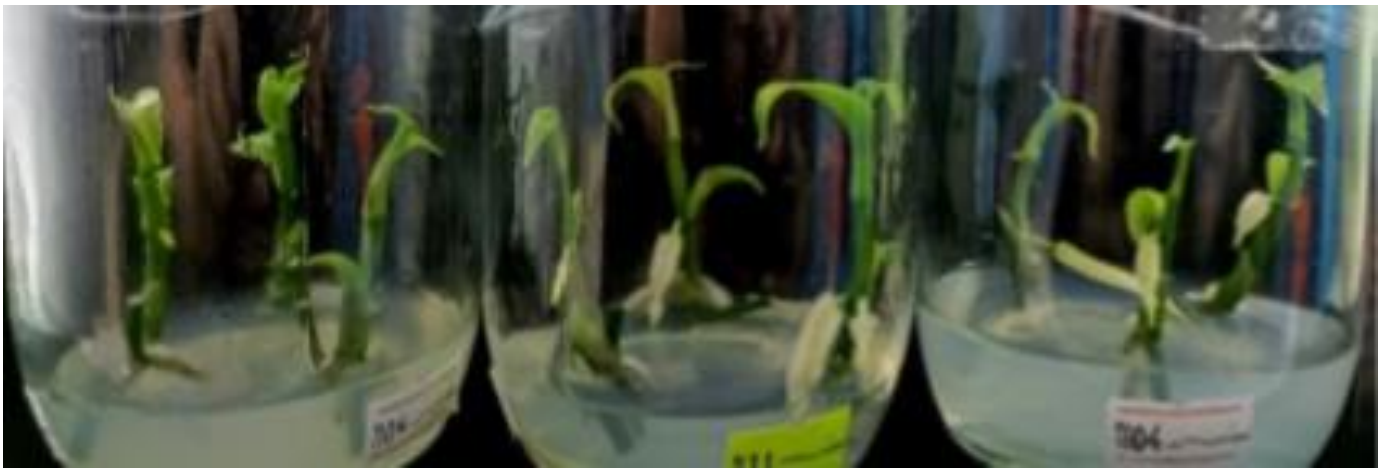

Figure 1. Regenerated shoots in 1x, 1/2x and 1/3xMS medium (from left to right)

Effects of BA on in vitro shoot multiplication of vanilla

The results observed for the number of shoots, shoot length and number of nodes per shoot in reference to treatment with BA are shown in Table 2. The increase of BA significantly improved shoot multiplication. Among all treatment tested, the highest number of shoots per explant (3.67 ± 0.08 shoots) was observed from the medium supplemented with 1.0 mg/L BA while the plant growth regulator (PGR) free medium showed the lowest number of shoots and well as nodes, which could be used as explant for shoot proliferation.

The average shoot length was found to be significantly decreased in cultures containing 1.0 mg/L BA. It was observed that axillary buds initiated on the medium from third to fourth week and began to elongate from fifth week onwards [30]. As treatment with 1.0 mg/L BA initiated significantly more shoots, many shoots (Figure 2). Shoot initiated on 1/2xMS medium supplemented with CW (100 ml/L) and BA at 4 weeks were small bulbous shoots, resulting in lower mean shoot length. It was also observed that the correlation

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between the number of shoots and shoot length were highly significant (p = 0.008) and negative, with a coefficient of – 0.66.

Table 2. Effect of BA concentration on shoot proliferation of vanilla

BA concentration (mg/L)	No. of shoots per explant	No. of nodes per shoot	Shoot length (cm)
0.0	1.00 ± 0.00c	2.58 ± 0.06c	3.87 ± 0.35a
0.1	1.00 ± 0.00c	2.89 ± 0.06ab	3.42 ± 0.22ab
0.3	1.02 ± 0.02c	3.04 ± 0.06a	3.56 ± 0.14ab
0.5	1.58 ± 0.12b	2.78 ± 0.02bc	3.16 ± 0.19ab
1.0	3.67 ± 0.08a	2.91 ± 0.05ab	2.82 ± 0.08b

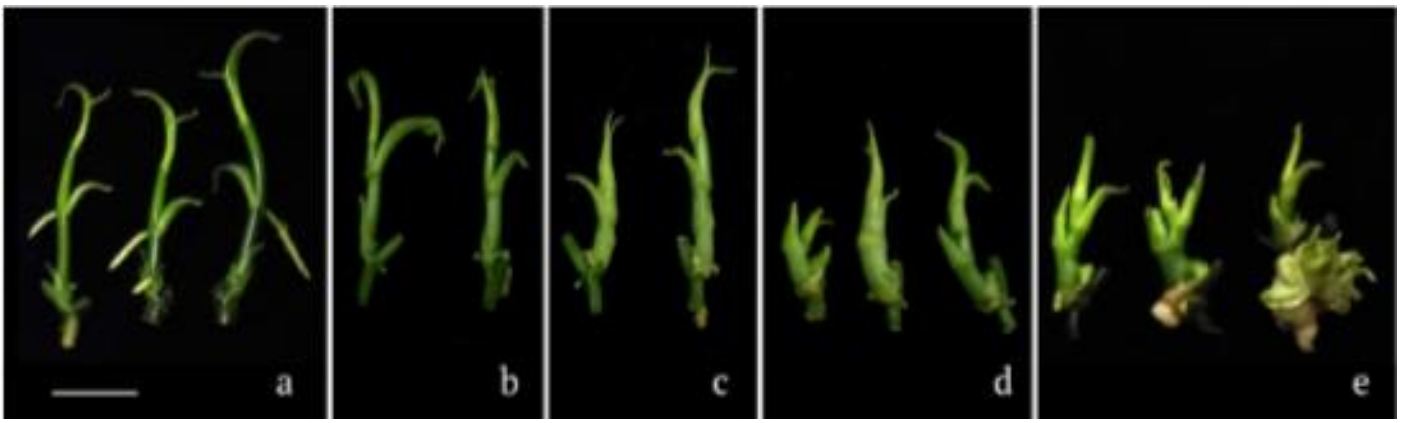

Figure 2. Shoot initiated on 1/2xMS medium supplemented with CW (100 ml/L) and BA. (a) 0 mg/L, (b) 0.1 mg/L, (c) 0.3 mg/L, (d) 0.5 mg/L, (e) 1.0 mg/L. Bar: 2.0 cm

Effects of organic additives on in vitro shoot growth of vanilla

Coconut water, banana, and many organic additives were found to effectively promote growth of in vitro orchids [31]. The response in shoot elongation, leaf development and root formation to the addition of coconut water, banana, and peptone in culture medium are shown in Table 4 and Figure 3. All tested treatments had rooting 100% and gave rise from 2 to an average of 5.42 ± 0.04 nodes per shoot after 4 weeks of culture, with no statistical differences among treatments. However, different organic additives caused significant differences in shoot and root length as well as number of roots. Banana (100 g/L) showed significantly higher mean shoot length (6.22 ± 0.07 cm per shoot) compared to peptone (1 g/L) (5.29 ± 0.10 cm per shoot). For root induction, the highest number of roots per explant (3.83 ± 0.07 roots) with the mean length of 5.42 ± 0.08 cm was observed from banana treatment, significantly differed from the second peptone treatment ($p < 0.001$) while coconut water (100 ml/L) supplement showed poorest rooting ability (1.63 ± 0.03 roots per shoot) as well as root length (1.19 ± 0.03 cm). Coconut water also showed significantly lower response for leaf development (4.53 ± 0.07 leaves per shoot with a mean length of 2.82 ± 0.05 cm) compared to banana and peptone treatments, which gave statistically similar results. Generally, in this experiment, the addition of 100 g/L banana to 1/2xMS medium containing 0.1 mg/L BA was best stimulated in vitro growth of vanilla shoots.

Table 3. Effect of different organic additives on in vitro shoot growth of vanilla

Organic additives	Shoot	Root	
	Shoot length (cm)	No. of leaves per shoot	Leaf length (cm)
CW 100 ml/l	5.73 ± 0.18ab	4.53 ± 0.07b	2.82 ± 0.09a

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Banana 100 g/l	6.22 ± 0.25a	5.07 ± 0.03a	3.10 ± 0.13a
Peptone 1g/l	5.29 ± 0.10b	5.03 ± 0.09a	3.06 ± 0.12a
	Node	Root	
	No. of nodes per shoot	No. of roots per shoot	Root length (cm)
CW 100 ml/l	5.43 ± 0.07a	1.63 ± 0.03c	1.03 ± 0.18c
Banana 100 g/l	5.50 ± 0.06a	3.83 ± 0.07a	5.42 ± 0.21a
Peptone 1g/l	5.33 ± 0.09a	2.67 ± 0.03b	2.92 ± 0.22b

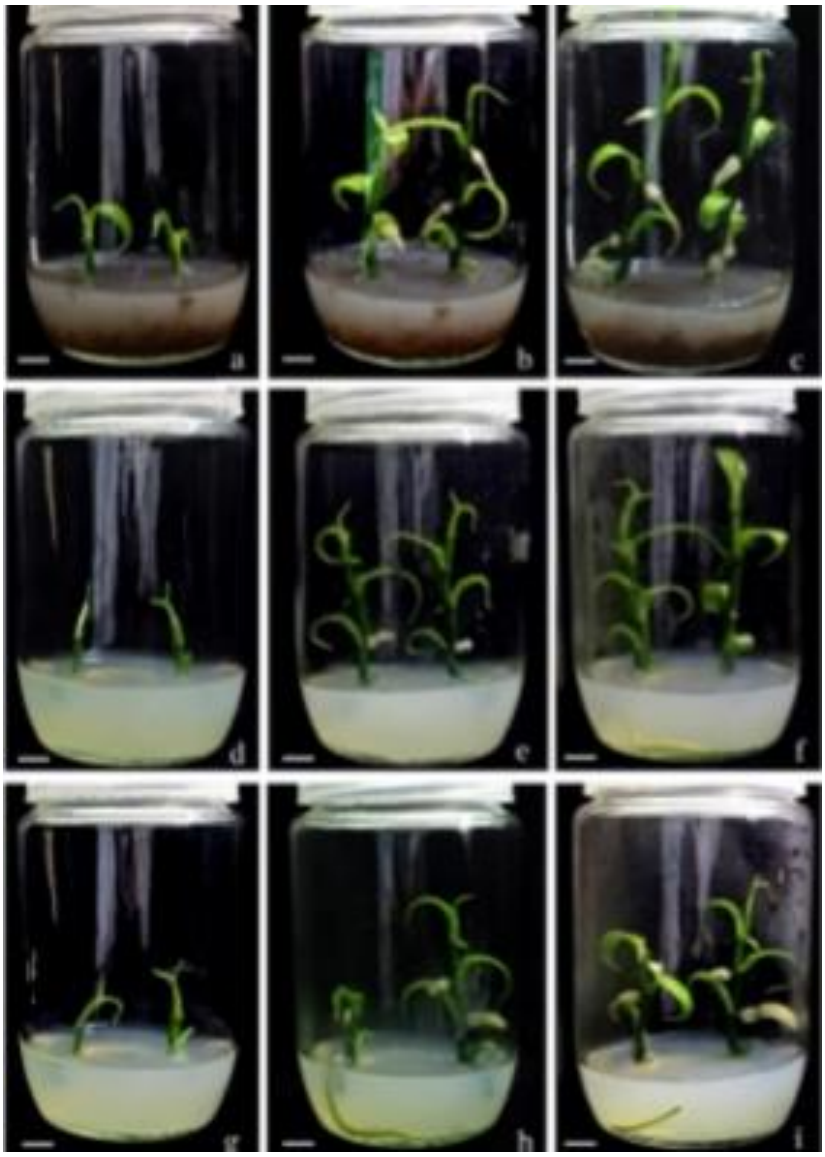

Figure 3. Vanilla shoots at 0, 2, 4 weeks (from left to right) in half strength MS medium containing 0.1 mg/L BA and different organic additives. (a, b, c) 100 g/L banana. (d, e, f) 100 ml/L coconut water. (g, h, i) 1 g/L peptone. Bar: 1.0 cm.

Effects of NAA on root formation of vanilla

In previous experiment of this study, cultures with 100 ml/L coconut water showed lowest root rate and elongation. Therefore, in this test, it was used and supplemented to the basal medium, and different NAA concentrations were added to the medium to investigate whether NAA can improve rooting ability of *V. planifolia* or not. Rooting percentage, length and number of roots are recorded after 3 weeks of

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inoculation were surveied. The observations showed that the addition of NAA did not enhance rooting rate of vanilla shoots. The number of roots generated per shoot and the shoot length were slightly increased, but the changes were not significant. Gopi et al. [30] reported that 1.0 mg/L IAA significantly improved root initiation as well as root elongation (3.50 roots per explant with an average length of 6.00 cm). However, it is not superior to the result obtained in cultures with 0.1 mg/L BA and 100 g/L banana in this study (3.83 roots per shoot with a mean length of 5.42 cm).

Table 4. Effect of NAA concentration on in vitro rooting of vanilla

NAA concentration (mg/L)	shoots forming roots (%)	No. root per shoot	Root length (cm)
0.0	96.7	1.50 ± 0.30a	1.10 ± 0.18a
0.1	83.3	1.47 ± 0.07a	1.19 ± 0.16a
0.3	96.7	1.87 ± 0.03a	1.56 ± 0.12a
0.5	93.3	1.90 ± 0.06a	1.42 ± 0.16a

DISCUSSION

Effects of strength-MS media on shoot-tip and axillary bud regeneration of vanilla

Almost all of preceding studies about vanilla used full strength MS medium as the basal medium for multiplication stage [32, 22, 24] while 1/2xMS were used for rooting experiments [30, 33, 34]. In general, 1/2xMS medium gave the best response in regeneration of vanilla axillary buds. It was also reported that 1/2xMS medium containing 1 mg/L BA evoked response in vanilla shoot multiplication and root formation as well as full salt strength [35]. Presented no significant differences from full salt and better than 1/3xMS in shoot elongation as well as leaf development, 1/2xMS medium would be an appropriate basal medium for large scale tissue culture of *V. planifolia*, which achieved the best balance of costs and adequate quality.

Effects of BA on in vitro shoot multiplication of vanilla

This result is in agreement with earlier reports on vanilla shoot propagation [36, 22, 23, 37, 35], where BA (1.0 mg/L) showed highest multiplication rate. However, vanilla nodes cultured on 1/2xMS medium supplemented 1.0 mg/L BA produced relatively more shoots per explant as well as higher mean shoot length than that in 1xMS with similar additives at same period of culture reported by Abebe et al. [38] (2.30 shoots per explant with an average length of 1.8 cm) and Gopi et al. [30] (1.90 shoots per explant with an average length of 1.2 cm). Extra experiments should be performed to confirm the significant effects of 1/2x and 1xMS medium to multiplication stage of *V. planifolia*

Effects of organic additives on in vitro shoot growth of vanilla

Banana homogenates have been proved the benefits of healthy shoot development and root formation in other species of Orchidaceae family, especially Dendrobium orchids [39, 40, 31]. However, further optimization experiments with various concentrations of these three supplements should be conducted to define the most cost-effective treatment for industrial micropropagation

Effects of NAA on root formation of vanilla

This result is concurrent with the findings of previous studies about the effects of NAA to vanilla rooting [38, 30, 24]. It was reported that *V. planifolia* best rooted in MS free medium without auxins [38, 36, 41, 35]. The characteristics of vanilla plant, whereby a node always produces an aerial root, suggests that there is the presence of internal auxins that induces the root formation of vanilla. The effects of internal hormones

may not be affected by the addition of external NAA. In addition, the effects of other auxins to root formation of in vitro vanilla have been tested. Tan et al. [37] found that 2.0 mg/L IBA increased the number of roots per explant

CONCLUSION

This study demonstrates an efficient and simple protocol for vanilla multiplication using shoot-tip and nodal explants. Results indicated that 1/2xMS medium was a promising basal medium for vanilla tissue culture. The addition of 1 mg/L BA to that medium significantly produced the highest multiplication rate with 3.67 shoots per explant. Regenerated shoots were transferred to 1/2xMS medium supplemented with 0.1 mg/L BA and three types of organic additives for growth and development. Banana homogenate (100 g/L) significantly promoted shoot extension (6.22 cm), leaf development (5.03 leaves per shoot with a mean length of 3.10 cm), root formation (3.83 roots per shoot) and root elongation (5.42 cm) compared to CW (100 ml/L) and peptone (1 g/L). The combination of NAA to medium containing CW (100 ml/L) and BA (0.1 mg/L) did not significantly improve root production and elongation after 3 weeks of culture. The findings in this study would help to establish an efficient and viable protocol for commercial-scale micropropagation to provide adequate planting materials of *V. planifolia* in Vietnam.

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