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Full Length Research Paper

Efficient *in vitro* multiplication protocol for *Vanilla planifolia* using nodal explants in Ethiopia

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***Vanilla planifolia* Andr. is a tropical commercial spice crop known for its popular flavoring substance called vanillin. Vanillin is the second most expensive spice in the world market, next to saffron. Ethiopia has favorable environment for vanilla production and the crop has huge local and international market. There is a soaring investors' interest to produce vanilla in the country provided there is plenty and reliable planting material supply. In line with this, we report efficient and reproducible tissue culture based mass propagation protocol for elite vanilla clones introduced into the country. Significant difference ($p < 0.0001$) was observed among selected hormone combinations for rate of shoot multiplication. An average number of 3.12 to 4.17 shoots were obtained after 45 days of nodal culture on MS media supplemented with BA combined with KIN and NAA. The optimum level found was 1 mg/L BAP combined with 1.5 mg/L KIN. Both hormone free and MS media supplemented with different levels of NAA gave 100% rooting for shoots separated and transferred onto rooting media. More than 85% survival rate was achieved during acclimatization. The availability of this protocol is a key step towards large scale vanilla production in Ethiopia.**

Key words: Nodal culture, mass propagation, *Vanilla planifolia*.

INTRODUCTION

Vanilla planifolia Andr. is an important tropical spice crop, commercially cultivated for its pods (beans). The pods (beans) of vanilla are the source of the popular flavoring substance called vanillin. Vanillin is mainly used in flavoring cakes, ice creams, sweets, chocolate and beverages. It is also used in the cosmetics and perfume industry (Goodenough, 1982). This has made it the second most expensive spice in the world market, next to saffron (Ranadive, 1994). The world production of vanilla is estimated to be about 3500 tones per annum and nearly 300 tones are consumed in USA alone. Malagasy Republic grows 70 to 80% of the worlds' vanilla crop (Geetha and Shetry, 2000).

In an effort to change the countries murky image in the eyes of the world, Ethiopia is implementing agricultural development led industrialization policy. Like any other developing country, where capital is limiting while labour and land are ample, our competitive advantage in the world market is supplying standard agricultural products. In line with this, availability of favorable environment (Ranadive, 1994) and colossal local and international market potential has prompted large scale production of vanilla in Ethiopia for some time now. Hence, it has been long since investors started knocking at the door of JARC, the national coordinating centre for coffee and spice research, for vanilla planting material provision. The main set back, however, remained how to produce enough planting material out of the handful number of vines available in the country in order to meet this huge demand.

Vanilla is a vine orchid, generally propagated by stem cutting of mature vine. But, the method is slow, labour

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Abbreviations: BAP, benzyl amino purine; **KIN,** kinetin; **NAA**, naphthalene acetic acid; **MS,** Murashige and Skoog media; **RH,** relative humidity.

intensive and time consuming. It is also uneconomical as the harvest of stems for propagation arrest the growth and development of the mother plant (Geetha and Sheety, 2000; Giridhar et al., 2001). Moreover, the market demand can hardly be met with stem cutting from the handful set of vines available in the country. Hence, it is imperative to exploit the potential of tissue culture to effectively multiply and supply the required amount of planting materials for large scale plantation.

By employing tissue culture techniques, vanilla has been propagated both through direct organogenesis (Philip and Nainar, 1986) and indirect organogenesis (Davidonis and Knorr, 1991; Gu et al., 1987). Different explant sources have been utilized for *in vitro* propagation of vanilla through direct organogenesis. Geetha and Sheety (2000) and Kononowicz and Janick (1984) reported the use of shoot tip and nodal segments for efficient micro-propagation protocol, whereas George and Ravishanker (1997) used axillary buds for *in vitro* multiplication of vanilla. In most cases, shoot proliferation was achieved by axillary bud growth or protocrm formation. However, commercial level of mass propagation demands simple, economical, high multiplication rate and highly reproducible protocol without an intervening callus or protocorm phase so as to give true-to-type clones. Here, we report efficient *in vitro* mass multiplication protocol for vanilla clones found in Ethiopia using nodal segment as an explant source.

MATERIALS AND METHODS

Regeneration and multiplication pathway followed in the experiment is summarized in Figure 1. Nodal explants harvested from screen house grown vanilla vines (Figure 2a) were gently washed with liquid detergent and left under running tap water for 30 min. The size of the explants was reduced to two nodes each with hanging internode in both sides and soaked in 3 g/l “Kocide” solution for 30 min. After rinsing them 3 - 5 times in distilled water, the nodes were dipped in 70% alcohol for 5 min inside the laminar flow cabinet. The nodes were then rinsed 3 - 5 times in distilled water and transferred into sterile bottle containing 0.1% $HgCl_2$ solution for 5 min where the bottle was gently shaken. The explants were then rinsed again with sterile distilled water 3 - 5 times to completely get rid of $HgCl_2$.

The sterilized nodal segments were laid on prior autoclaved sterile petridishes to remove bleached part at both ends. Then explants were cultured in conditioning media and maintained for three days. After three days clean explants were transferred into initiation media to increase the number of nodes for multiplication. The initiation media contains MS media (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 8 g/L agar, 1 mg/L BAP and 0.5 mg/L GA3. After 45 days the elongated shoot with 6 - 8 nodes were obtained and cut into segments of two nodes each. Nodes were cultured on multiplication media while the contact with the media was at an inclined position to encourage sprouting of multiple shoots around the node. The multiplication media was MS media supplemented with 30 g/L sucrose and 8 g/L agar, different levels of BAP (0, 1, 2 and 3 mg/L) combined with NAA (0.0, 0.5, 0.75, 1.0 and 1.25 mg/L) or Kin (0.0, 0.5, 1.0 and 1.5 mg/L). The multiplied shoots were separated and transferred into fresh media every 45 days for 4 - 5 subcultures. The multiplied shoots were finally transferred into hormone free MS media for rooting and elongation for a month before they were taken to screen-house for acclimatization. The hardening was started in a tunnel covered with polyethylene plastic sheet and sprayed everyday to maintain the RH (relative humidity) as high as possible to simulate the RH of the culture bottles. The RH was lowered gradually from 100 to 60% with in two weeks. The reduction of the RH was continued for another two weeks towards the regular RH of the environment. Concurrently, the light intensity was increased gradually towards full light intensity from 30% cut off at the beginning of the hardening. In the process more than 85% survival was achieved and seedlings were then planted in regular nursery pot.

RESULTS AND DISCUSSION

Using the sterilization technique outlined above, 90% contamination free explants were obtained of which 60% survived the sterilization pressure. Conditioning media, prepared in test tube, was included in the procedure to transfer clean explants onto initiation media (Figure 2b). Four nodal segments were cultured per jar in initiation media. After 45 days the two nodes produced 6 - 8 nodes. Inclusion of GA3 facilitated the upright growth of the node with increased inter-node length.

Differential response was obtained by using different levels of BAP combined with Kin or NAA. Significant difference ($p < 0.0001$) was obtained among treatments for multiple shoot initiation from each explant. After the first subculture, 45 days, an average number of 3.42 to 4.17 shoots that can easily be separated and cultured on fresh media (Figure 2c) were produced for the above mentioned hormone combinations (Table 1). However, clearly separated and fully grown multiple shoots were mainly observed when 1mg/L BAP alone was used (Figure 2d). There was no significant difference for multiple shoot induction between hormone free media and media supplemented with 2 or 3 mg/L of BAP combined with different levels of NAA (0.5, 0.75, 1.0 and 1.5 mg/L). This attributed to the tendency of more number of tiny multiple shoot production at early stages which eventually develop callus through time, especially after the first subculture when high level of BAP and Kin was used. This result is concurrent with results obtained by Geeth and Sheety (2000), George and Ravinshaker (1997) and Kalimuthu et al (2006) of induced multiple shoots by combining BAP with Kin and NAA.

Number of multiple shoots recorded after the second subculture showed a significant difference ($p < 0.0006$) and average number shoots increased from a minimum of 1 to 1.33 and a maximum of 4.17 to 5 (Figure 2e). The maximum number of shoots per node recorded throughout the entire experiment was 12 while the minimum was 1. Separating the multiplied shoots after every subculture and culturing them on fresh multiplication media resulted in three fold increase in average shoot number as compared to sub-culturing multiplied shoots on fresh media without separating individual shoots (Figure 2f and g). Apart from media supplemented with 2 mg/L of BAP where callusing was observed, the top six hormone com-

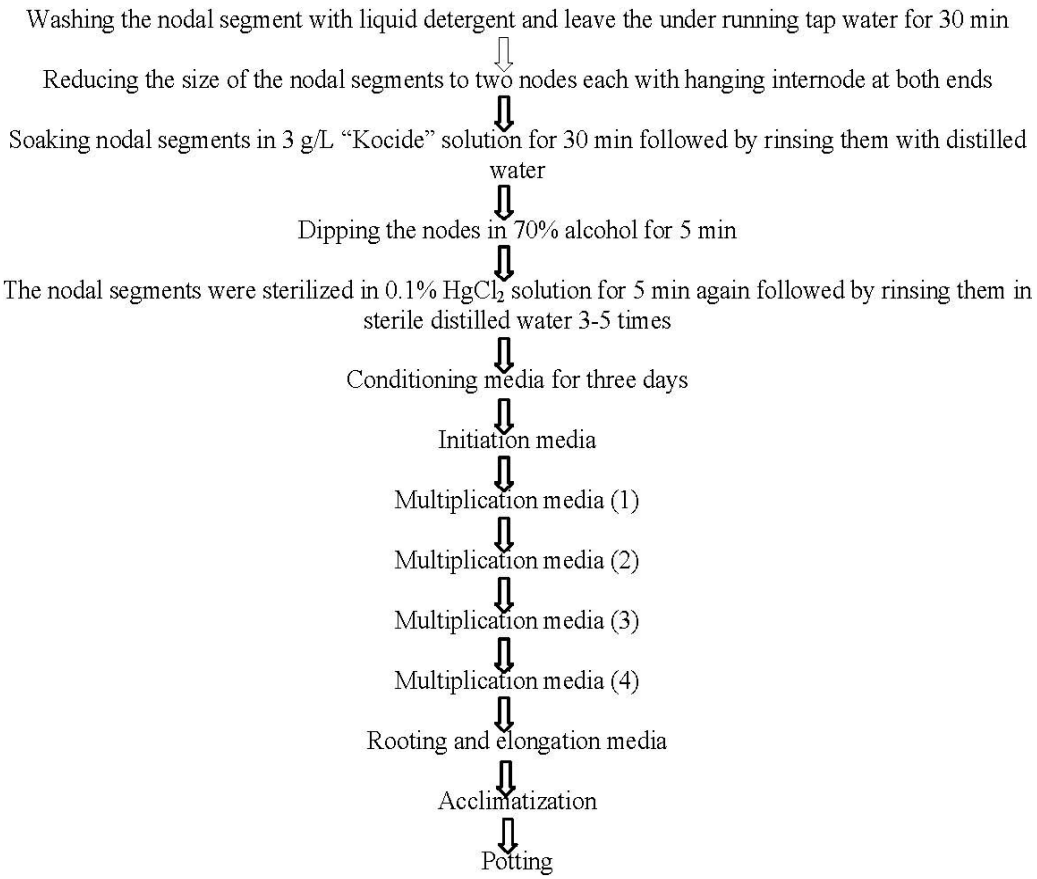

Figure 1. Schematic representation of the vanilla multiplication process.

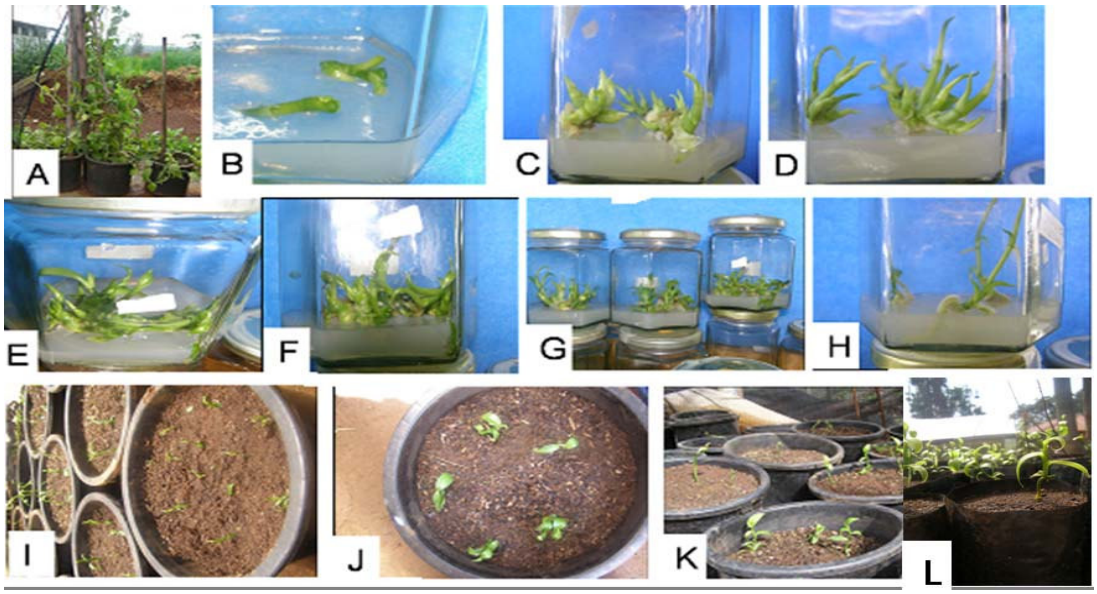

Figure 2. (A) Vanilla vine in screen house used for nodal source. (B) Node cultured on multiplication media. (C) Shoot multiplied after 45 days. (D) Shoot multiplied after 60 days. (E) Shoot multiplied after two subcultures. (F and G) Shoot multiplication continued. (H) Rooting. (I) Plantlets transferred to soil mix for acclimatization. (J) Plantlets acclimatized for two weeks. (K) Seedlings ready for potting. (L) Potted seedlings.

Table 1. Average number of multiple shoots produced for each treatment.

MS media supplemented with (mg/L)	Shoot number/ 45 days	MS media supplemented with (mg/L)	Shoot number/ 45 days	MS media supplemented with (mg/L)	Shoot number/ 45 days	MS media supplemented with (mg/L)	Shoot number/ 45 days
BA (0)+Kin (0)	1.25ih	BA (2)+Kin (1)	2.67fdecg	BA (0)+NAA (0.5)	1.00i	BA (1)+NAA (1)	3.17bdec
BA (1)+Kin (0)	3.17bdec	BA (3)+Kin (1)	3.58bac	BA (0)+NAA (0.75)	1.18ih	BA (2)+NAA (1)	3.08bdec
BA (2)+Kin (0)	3.42bdac	BA (0)+Kin (1.5)	2.67fdecg	BA (0)+NAA (1)	1.08i	BA (3)+NAA (1)	2.41feg
BA (3)+Kin (0)	3.08bdec	BA (1)+Kin (1.5)	4.17a	BA (0)+NAA (1.25)	1.25ih	BA (1)+NAA (1.25)	2.75fbdecg
BA (0)+Kin (0.5)	2.58fdeg	BA (2)+Kin (1.5)	2.25feg	BA (1)+NAA (0.5)	2.33feg	BA (2)+NAA (1.25)	1.83ihg
BA (1)+Kin (0.5)	2.33feg	BA (3)+Kin (1.5)	3.17bdec	BA (2)+NAA (0.5)	2.92fbdec	BA (3)+NAA (1.25)	2.08fhg
BA (2)+Kin (0.5)	2.58fdeg	BA (0)+NAA (0)	1.08i	BA (3)+NAA (0.5)	2.42feg		
BA (3)+Kin (0.5)	3fbdec	BA (1)+NAA (0)	3.42bdac	BA (1)+NAA (0.75)	3.50bdac		
BA (0)+Kin (1)	3.67ba	BA (2)+NAA (0)	2.08fhg	BA (2)+NAA (0.75)	1.92ihg		
BA (1)+Kin (1)	2.67fdecg	BA (3)+NAA (0)	2.75fbdecg	BA (3)+NAA (0.75)	3.17bdec		

There was high significant difference observed among treatments ($p < 0.0001$). Media supplemented with BA (1) + Kin (1.5), BA (0) + Kin (1), BA (3) + Kin (1), BA (1) + NAA (0.75), BA (2) + Kin (0) and BA (1) + NAA (0) were the top six media combinations. Media supplemented with BA (0) + NAA (1), BA (0) + NAA (0) and BA (0) + NAA (0.5) were the bottom three media combinations.

Table 2. Average node production and rooting.

Variable	Hormone combinations					
	0 mg/l NAA	0.5 mg/l NAA	0.75 mg/l NAA	1. mg/l NAA	1.25 mg/l NAA	0.5 mg/l NAA +1 mg/l BAP
Node	3.94a	3.50ab	3.89a	2.83b	4.11a	3.75ab
Root	100a	100a	100a	94.44ab	100a	66.67b

binations remained unchanged.

Non significant difference was observed between hormone free media and media supplemented with different levels of NAA (0.5, 0.75, 1.0 and 1.5 mg/L) for rooting ($p < 0.431$) and elongation ($p < 0.0843$). The maximum average number of nodes recorded after 1 month was 4.11 for MS media supplemented with 1.25 mg/l of NAA while the minimum was 2.83 for MS media supplemented with 1 mg/L of NAA (Table 2). Non significant difference was observed between hormone free MS media and the top media for elongation. Among the four media combinations, 100% rooting was recorded for hormone free MS media (Figure 2h). This is in agreement with the findings of George and Ravishanker (1997) and Philip and Nainar (1986). More than 85% survival was recorded during acclimatization (Figure 2i, j and k).

In conclusion, the efficiency of this first report on vanilla multiplication was found to be comparable with reports available so far (Geeth and Sheety, 2000) and can be used for large scale planting material supply. The protocol can also be utilized to conserve the few vines of *v. planifolia* introduced into the country.

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