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Enhanced Protocol Development for *in vitro* Multiplication and Rooting of Vanilla (*Vanilla planifolia* Andr.) Clone (Van.2/05)

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Authors' contributions

This work was carried out in collaboration between all authors. Author YBA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors WT and KB managed the analyses of the study. Author KB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Vanilla (*Vanilla planifolia Andr.*) is a multipurpose spice in the world. The crop had been introduced to Ethiopia from Mauritius and it is still under maintenance at the Tepi National Spices Research Center (TNSRC). It is commonly propagated through stem cuttings which could arrests subsequent plant growth and development and serves as an ideal means for the spread of varied diseases. To solve the mentioned problems, no efficient *in vitro* protocol had so far been developed to propagate this vanilla clone in Ethiopia. Therefore, this study was initiated to address this gap by developing efficient protocol for the enhanced multiplication of this accession (Van.2/05). In this study plant material was taken from TNSRC. The experiments were carried out in Jimma Agricultural Research Center (JARC) at Biotechnology Laboratory. Nodal explants and Murashige and Skoog (MS) (1962), basal medium were used exclusively throughout the experiments. The experiments were laid out in Completely Randomized Design (CRD) with factorial treatment combinations and

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replicated three times for plant growth regulator (PGR) assisted experiments. In the study, shoot multiplication, the combined use of 2 mg l^{-1} 6-Benzylamino Purine (BAP) and 0.5 mg l^{-1} Naphthalene Acetic Acid (NAA) *was* proved to be the best providing the highest shoot mean number (5.33) and length (4.9 cm) of shoots after five weeks of culture. The combined use of different MS basal medium strengths and Indol -3-Acetic Acid (IAA) concentrations were employed. Therefore, ½ MS strength combined with 0.5 mg l^{-1}IAA produced mean number of 4.00 roots per plantlet with mean length of 6.1cm. The average rate of *ex-vitro* survival was 83.4%. Therefore, the advent of this protocol could have considerable value to enhance the expansion of vanilla cultivation in Ethiopia.

Keywords: Micropropagation; PGR; nodal explants; TNSRC.

1. INTRODUCTION

Vanilla belongs to the family *Orchidaceae*, of which some 110 species are reported so far [1]. Currently, only three of these species are under commercial production, i.e. *Vanilla planifolia* Andr. (Mexican Vanilla), *Vanilla pompona* Schiede (West Indian Vanilla) and *Vanilla tahitensis* (Tahitian Vanilla) [2]. However, *V. planifolia* Andr. is the most popular of these three cultivated species with regard to its area of production, productivity and product quality, as well.

Vanilla planifolia Andr. is a diploid species with 2n = 32 chromosomes [3], which is originated from the dense tropical forests of Mexico and Central America, and had been domesticated and cultivated in the last 1000 years. However, Mexico is reported to have the largest genetic diversity of *V. planifolia* as compared to other vanilla-producing countries [4]. It is herbaceous perennial climbing terrestrial vine adapted to the warm humid tropics [5].

Vanilla is the only spice plant from the family *Orchidaceae* that is economically valued for its cured fragrant beans, which make one of the most expensive spices in the international trade, second to saffron [6]. Being one of the world's most popular flavoring material, vanilla finds extensive applications in confectionery and perfumery industries, thus it is among the top most marketed flavoring spices in the world [7]. Vanilla is commonly propagated through the vegetative means that commonly uses cuttings collected from a healthy and vigorously growing mother plant [8].

The accession (Van.2/05) was introduced from Mauritius in 2005 and among the accession available information from its origin had confirmed that this to be the best commercial cultivar, both in its productivity and quality. This particular clone had also adapted well under Tepi and its surrounding agro-ecologies [9].

Shortage of planting material is the critical bottleneck that hampered wider dissemination and cultivation of vanilla in Ethiopia. Therefore, this particular clone is among the top nationally prioritized crop species for rapid mass multiplication using the modern tissue culture technique [10]. Therefore, any work targeting to resolve the prevailing shortage of planting materials would in turn help a lot to respond to the national strategic plan that targets enhancing diversification of the national export commodities [11].

It has been long since investors started knocking the doors of the Tepi National Spice Research Center, which is the center of excellence and national coordinating center for spices research, thereby the mandated institution to supply planting material of elite varieties together with improved technology packages for the production of vanilla [11]. However, the major setback to respond to the prevailing demand is associated with the inability of the center to produce sufficient quantities of planting materials using the conventional vegetative propagation technique, out of the handful number of vines found at its backyard [12].

Therefore, up scaling of vanilla production to a commercial level would face difficulties in Ethiopia at this moment, due to the inefficiency of the conventional vegetative propagation technique currently employed. This is mainly attributed to the limited number of stock plants the center has at hand that makes collection of sufficient quantities of stem-cuttings from the existing few mother plants nearly impossible, as it would result in retarded plant growth and development. Therefore, in order to respond to the prevailing demand for propagules of elite materials, the rapid regeneration of such specific clones through

modern techniques, like tissue culture, becomes imperative. Consequently, adoption of the modern micropropagation technique with optimum subculture stages enables production of millions of plantlets from a single mother plant within a short period of time becomes so vital [13]. However, effective exploitation of the technique requires development of the most efficient procedures at all stages of the process that suit the specific species and clone at hand.

To this end, no standardized protocol had been availed so far for efficient micropropagation of this vanilla clone (Van.2/05), which had been introduced recently from Mauritius. Thus, it became crucial to develop protocol for the rapid mass propagation and dissemination of this potential vanilla clone to exploit effectively the existing agroecologic potentials for vanilla cultivation in Ethiopia, thereby benefiting the nation from both import substitution and export of this invaluable spice. Therefore, this research was conducted with the following objectives:

- To determine the effects of combinations and concentrations of BAP and NAA on multiplication (growth and development) of vanilla shoots *in vitro.*
- To test the different concentrations of IAA for best *in vitro* root initiation of vanilla shoots using different strengths of MS basal media.

2. MATERIALS AND METHODS

2.1 Experimental Site

The experiment was conducted under laboratory condition at JARC at Biotechnology Laboratory, which is located 363 km South West of Addis Ababa at 7°40'N latitude and 36°47'E longitudes. The Center has maximum and minimum temperature of 26.2ºC and 11.3ºC, respectively and with 1529.5 mm average rain fall.

2.2 Plant Material

A recently introduced commercial vanilla (*Vanilla planifolia* Andr.) clone (Van. 2/05) was used in this study. Fifty Plantlets of the stated clone, propagated through the conventional vegetative cutting system, were obtained from TNSRC and maintained under the greenhouse condition of JARC, and used as stock plants. The stock plants were treated mixed Fertilizer containing Nitrogen, Phosphorus and Potassium at a mix of 17:17:17 and the planting materials were also treated with fungicide to reduce fungal contaminants.

2.3 Explant Culture

Actively growing explants were collected from the stock vanilla plant and washed thoroughly using tap water using liquid soap to reduce contaminant load and dipped in a 70% ethanol solution for one minute and rinsed four times with sterilized distilled water and finally the explants were exposed to 5% active chlorine local bleach (*Berekina*®) for 20 minutes and rinsed 4 times with sterilized distilled water to avoid the burning effect of the sterilant on explants.The surface sterilized explants were laid on sterile petri dishes in the laminar flow hood and further excised with the help of sterile forceps and blade to remove the chlorine affected tissues at both ends. In due time, their sizes were reduced to a single node with internodes at both sides. Then after, the trimmed explants were cultured in 370 cm^3 volumes Manson jars containing 40 ml of the conditioning medium, i.e. a PGRs free MS basal medium, vanilla nodal explants of approximately 2 cm length were inoculated on to the conditioning medium. After culturing, the mouth of each jar was properly closed with its cap and sealed with a strip of parafilm.

The jars were then labelled, indicating the media code, culturing date, and name of the clone before they were randomly placed on the Dixon shelves within the growth room. After three days culture on the conditioning medium, non-contaminated and surviving cultures were transferred to the shoot initiation mediumso as to produce sufficient number of explants for the subsequent multiplication experiment. This particular medium consists of MS basal medium supplemented with 30 g l^{-1} sucrose, and added with 2 mg l^{-1} BAP and 0.5 mg l^{-1} GA_3. Shoots initiated at this stage were sub-cultured onto a PGR free medium for two weeks prior to their use in subsequent experiments, so as to remove any carry-over effect of the PGRs that were used at the previous culture stages.

Unless stated otherwise, in all cases the culture jars containing the explants were placed randomly in the growth room having an average of 50 - 60% relative humidity, temperature of 24-26ºC and 2500 lux of light intensity with 16 hrs duration of light [14].

2.4 Experimental Layout

2.4.1 Experiment I: The effects of combinations and concentrations of BAP and NAA on growth and development of vanilla shoots *in vitro*

After five weeks of culture on the initiation medium, elongated shoots were collected and cut into segments of two nodes. These two node segments of vanilla explants were cultured for five weeks on the multiplication medium at an inclined (45º) position to encourage sprouting of multiple shoots around the nodes [15]. In this experiment, MS basal medium supplemented with 30 g l^{-1} sucrose with four levels of BAP (0, 1, 2 and 3 mg l^{-1}) in combination with four levels of NAA (0.0, 0.5, 1.0 and 2.0 mg l^{-1}). The P^H of the medium was adjusted to 5.75 before it autoclaved and gelled with 1% agar. Therefore, the experiment was laid in a 4 x 4 factorial combination in CRD, whereby the two factors being BAP and NAA each at 4 treatment levels with three replications and three explants per treatment. 25% L-cystine and 100g per L myo-inosetol were added as additives. Shoots multiplied at this stage were sub-cultured onto PGRs free medium for at least two weeks prior to their use in subsequent experiments, so as to remove any carry-over effect of the PGRs that were used at the previous culture stages.

2.4.2 Experiment II: Effect of different concentrations of IAA for best *in vitro* root initiation of vanilla shoots on different strengths of MS basal medium

In vitro grown shoots of vanilla having an average length of 4 cm were used as explants in this rooting experiment and cultured on rooting media for about four weeks. Here, three different strengths of MS basal media (full MS, ½-MS and ¼-MS) supplemented with four levels of Indole-3-Acetic Acid (IAA) (0, 0.5, 1 and 1.5 mg l^{-1}). The medium was added with 20 g l^{-1} sucrose as a carbon source and 2 mg l^{-1} of activated charcoal to darken the medium and to enhance rooting as well. 25% L-cystine and 100 g per L myo-inosetol were added as additives. The P^H of the solution was adjusted to 5.75 before it autoclaved and gelled with 1% agar. The experiment was laid in a 3 x 4 factorial treatment combination in CRD with 3 replications.

2.5 Acclimatization

In this study, acclimatization of the vanilla plantlets was carried out following the recommendations of Zerihun Abebe et al. [12]. Accordingly, after initiating roots *in vitro*, the vanilla plantlets were taken from the culture jars, washed thoroughly with lukewarm water to remove the nutrient medium and solidifying agar from the root surface. Subsequently, the plantlets were planted in a polytube pots filled with sterilized potting mix of well decomposed coffee husk, forest soil and sand at a 1:1:1 ratio, respectively. The plantlets were then kept for two weeks under a plastic tunnel covered with 70% shade net to ensure provision of high Relative Humidity (RH) (80–90%) and temperature (24-26ºC), which was essential for their primary acclimatization. After two weeks the high RH level was gradually reduced to the ambient (60-70%) by removing plastic tunnel cover. Later, the plantlets were transferred to a 70% shade net without plastic tunnel for another two weeks for secondary acclimatization and hardening-off. Finally, they were transferred to a 50% shade house prior to their field transplanting, as vanilla would grow comfortably under partial shade in its natural agro ecology.

2.6 Statistical Analysis

The collected data were analyzed using the SAS statistical software (Version 9.2) and mean separation were made following the procedure of REGWQ (*Ryan-Einot-Gabriel-Welsch Multiple Range Test*). Linear correlation was applied for shoot parameters.

3. RESULTS AND DISCUSSION

3.1 The Effects of Combinations and Concentrations of BAP and NAA on Growth and Development of Vanilla Shoots *In vitro*

In the present study, the combined use of BAP and NAA had substantially promoted several growth parameters associated with the *in vitro* shoot multiplication of vanilla. Therefore, the two-way interaction of these two PGRs had revealed highly significant differences (P< 0.001) with regard to the average number of shoots, leaves and nodes, as well as mean length of the vanilla shoots produced *in vitro* after five weeks of culture (Table 1). The synergy of these two hormones avoids apical dominance of the cultured plantlets at early stage of development transferred from initiation medium (Fig. 1A) and later on enhanced multiple shoot development (Fig. 1B). Accordingly, the maximum mean number of shoots proliferated per explant (5.33)

and the longest shoots (4.9 cm) were recorded from those explants of vanilla cultured on MS basal medium added with 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA, while the minimum average shoot number (1.00) and the shortest shoots (1.9 cm) were obtained from the culture medium supplemented with 2 mg l^{-1} NAA only (Table 2). However, the average number of shoots recorded from the latter treatment was not statistically different from those supplemented with either 0.5 mg l^{-1} NAA or 1 mg l^{-1} NAA alone.

In vitro growing shoots of vanilla had revealed a tendency of callusing when cultured on a medium fortified with sole BAP, at a concentration of 3 mg l^{-1} (Fig. 1D). On the other hand, the use of NAA alone in the culture medium had negatively affected the average number of shoots and other shoot parameters in the course of vanilla *in vitro* culture. Besides, vanilla explants cultured on a PGR free MS medium revealed a tendency of root initiation, although they were cultured for shoot multiplication. In addition to this, the shoots produced on this particular medium were not good looking, hence indicating the necessity of using proper types and levels of PGRs to attain proliferation of good quality shoots.

In the present experiment, the highest mean number of leaves (2.41) and nodes (2.31) per explant were recorded from MS medium added with a combination of 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA (Table 2). The shoots obtained were thus stout, well elongated and healthy having fully unfolded leaves (Fig. 1C). However, the numbers of leaves recorded at this level were not statistically different when the culture media were fortified with the same level of BAP (2 mg l^{-1} BAP), but different rates of NAA, i.e. either 1 or 2 mg l^{-1} NAA. On the other hand, the lowest mean number of leaves and nodes (1.0 each) per explant were recorded from the treatment added with 2 mg l^{-1} NAA alone (Table 2). However, the average number of nodes recorded here was not statistically different from those treatments involving sole NAA at a rate of 1 mg l^{-1}.

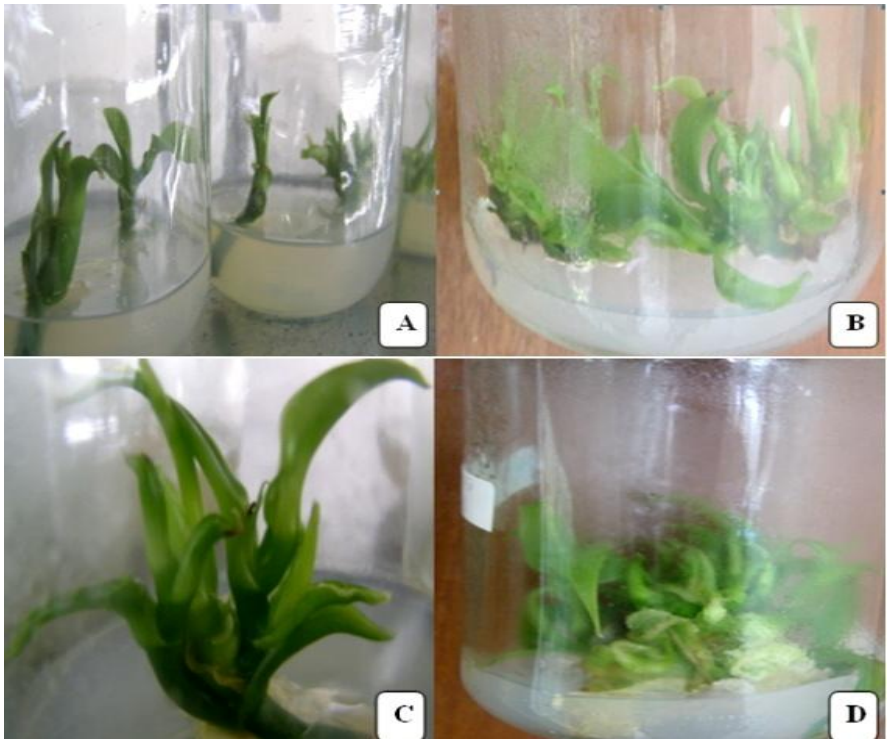

Fig. 1. Vanilla micro-shoots at different ages on multiplication media; (A) Vanilla explants a week after sub cultured from initiation to multiplication medium, (B) Vanilla plantlets after 3 weeks of cultured on shoot multiplication medium (C) Elongated shoots of vanilla at their fourth week of culture on a multiplication medium (D) Vanilla explants showing callus initiation, *i.e.* from the treatment involving MS medium added with 3 mg l^{-1} BAP

Table 1. Anova table for the effect of BAP concentrations combined with NAA concentrations on varied shoot growth parameters of vanilla plantlets *in vitro*

Source of variation	P values				
	df	Shoot number	Shoot length (cm)	Leaf number	Node number
BAP	3	***	***	***	***
NAA	3	***	***	***	***
BAP*NAA	9	***	***	***	***

*** = P < 0.001 BAP= 6-benzyl amino purine, NAA= α-Naphtalic Acetic Acid*

Table 2. The interaction effect of different levels of BAP and NAA treatments on *in vitro* shoot multiplication of vanilla after five weeks of culture

Treatment*		Shoot number	Shoot length (cm)	Leaf number	Node number
BAP	NAA				
0.0	0.0	2.33^{h}	3.63^{c}	1.78^{e}	1.92^{bcd}
0.0	0.5	1.50^{ijk}	2.86^{e}	1.33^{g}	1.55^{e}
0.0	1.0	1.33^{jk}	2.43^{f}	1.22^{g}	1.11^{f}
0.0	2.0	1.00^{k}	1.90^{g}	1.00^{h}	1.00^{f}
1.0	0.0	2.50^{gh}	2.96^{e}	1.93^{de}	1.86^{bcd}
1.0	0.5	3.17^{ef}	3.20^{ef}	2.16^{bcd}	1.99^{bc}
1.0	1.0	2.00^{hi}	3.50^{cd}	2.00^{cde}	2.05^{ab}
1.0	2.0	1.66^{ij}	3.23^{cde}	1.88^{e}	1.69^{de}
2.0	0.0	3.50^{def}	4.50^{b}	2.14^{bcd}	2.09^{ab}
2.0	0.5	5.33^{a}	4.90^{a}	2.41^{a}	2.31^{a}
2.0	1.0	3.66^{cde}	4.20^{b}	2.28^{ab}	2.14^{ab}
2.0	2.0	4.00^{bc}	3.23^{cde}	2.21^{abc}	2.16^{ab}
3.0	0.0	3.00^{fg}	2.46^{f}	1.55^{f}	1.50^{e}
3.0	0.5	4.17^{bc}	3.40^{cd}	2.00^{cde}	1.95^{bcd}
3.0	1.0	3.83^{bcd}	2.93^{e}	2.13^{cd}	1.91^{bcd}
3.0	2.0	4.33^{b}	3.40^{cd}	1.79^{e}	1.73^{cde}
CV (%)		7.3	4.5	4.6	5.8

CV=Coefficient of variation treatment means within a column followed by the same letter are not significantly different from each other using REGWQ at P = 0.05

In addition to these, the correlation between shoot length and leaf number, as well as number of leaves and nodes were observed to be highly significant (P< 0.001) and positive, with coefficients of 0.77 and 0.92, respectively. The strong positive correlation between the number of leaves and nodes could be ascribed to the inherent anatomy of the vanilla plant, whereby a leaf is always produced at an alternate position on each node.

The enhanced shoot proliferation recorded in the present study from the combined use of BAP and NAA was in agreement with the reports of several authors (12, 16, and 17). In all these cases, the beneficial effects of using BAP in combination with either NAA or other cytokinins to attain shoot proliferation in the course of vanilla *in vitro* culture had been well established.

The result obtained in this investigation for shoot multiplication and elongation of *V. planifolia* is highly comparable with the previous works done by Giridhar et al. [6] in India. Moreover, the present finding is also in agreement with the study conducted in Indonesia by Hapsoro and Yusnita [16], whereby apical meristems were used as explant sources. However the present finding is in contrast with the findings of [17] who used indirect organogenesis from leaf using MS medium supplemented with 1 mg l^{-1} 2,4 D and 0.5 mg l^{-1} BAP for callusing and 3 mg l^{-1} BAP and 2.5 mg l^{-1} NAA for subsequent shoot regeneration with highest average (14 shoots per explant) shoot number. This may be ascribed due to the utilization of different steps and methods of shoot regeneration.

As exemplified in this study, in cases when the concentration of BAP exceeds the optimal level (i.e. 2 mg l^{-1}) with devoid of NAA, the cultures will be liable to produce calli that would obstruct subsequent shoot multiplication (Fig. 1D). Besides, the use of sole NAA in the shoot

multiplication medium would also affect vanilla shoot development negatively, since NAA alone doesn't initiate shoot development. In most of the cases, the combined use of BAP and NAA had proved to be the best in enhancing shoot multiplication, beside its benefits in giving rise to superior responses in most of the shoot parameters considered in this experiment (Table 2).

Plant growth regulators do not function in isolation within the plant body; instead they also react with each other, as well; consequently, the balance of PGRs is apparently more important in the course of any plant growth and development than the absolute concentration of any one PGR. In other words, the balance between cytokinin and auxin plays a detrimental role in the overall growth of plant tissues [18]. Hence the PGR differentials within each medium composition could have different effects on the growth and development of the cultured explants [19].

The present result is much better than the protocol reported by Giridhar et al. [20], which stated MS medium added with BAP (5 mg l^{-1}) and Phenyl Acetic Acid (PAA) (1 mg l^{-1}) to be the best giving rise to an average of 2-3 shoots with 2.32 cm length from nodal explants within one month time. This could be associated to the weak chemical nature of the auxin (PAA) used in their study to exert a synergistic effect on vanilla shoot proliferation [21]. On the other hand, it could also be ascribed to the relatively higher concentration of BAP used in their case that is thought to be more than enough for enhancing shoot proliferation, which instead might have played an inhibitory role. Moreover, the observed differences in response could also be associated with the differences in the prevailing physiological status of the explants cultured.

3.2 The Effect of Different Concentrations of IAA and MS Basal Medium Strengths on the *In vitro* Root Initiation of Vanilla Shoots

In the course of *in vitro* rooting of vanilla plantlets, 100% rooting was attained within four weeks of culture on the different rooting media types evaluated in this study. The ANOVA test for the average number of initiated roots showed very highly significant differences (P< 0.001) among treatment combinations (Table 3).

This could be attributed to the differential combination effects of the auxin, IAA and the MS salt strengths evaluated in the present study. Consequently, the treatment combination containing ½ MS added with 0.5 mg l^{-1} IAA gave the highest mean number of roots (4.00) per explant, followed by the same MS salt strength supplemented with IAA at 1 mg l^{-1}(Table 4). However, the latter had not revealed any significant difference with the treatments involving a combination of IAA at 1mg l^{-1} with either ¼ or full strength MS basal medium. The average number of roots recorded in this experiment ranged from 1.50 to 4.00 per explant. In general, the medium with ¼ MS strength gave plantlets having roots that are so thin and fragile at any level of the IAA supplement. However, all the rest treatment combinations involving either full or ½ MS salt strengths developed thicker and good looking roots, irrespective of the level of IAA supplement. Meanwhile, the lowest rate of rooting was recorded from those treatments containing either full or ¼ MS basal salt strengths, but devoid of any PGR supplement (Table 4).

Table 3. Anova table for the effect of different concentrations of IAA & MS basal medium strengths on the *in vitro* root parameters of vanilla after four weeks of culture at JARC

Source of variations	df	P-values	
		Root number	Root length
MS strength	2	***	***
IAA	3	***	***
MS S * IAA	6	***	***

MS = Murashige and Skoog (1962) Medium Strength, IAA = Indiol-3- Acetic Acid
**** = P < 0.001*

Likewise, root length had also revealed very highly significant differences ($p < 0.001$) in the present study. In line with this, the longest average roots per plantlet (6.20 cm) were recorded from treatment full strength MS medium supplemented with IAA at 1.5 mg l^{-1} concentration. However, it has no any significant difference with the treatment involving ½ strength MS salts supplemented with 0.5 mg l^{-1} IAA. On the other hand, the shortest roots were recorded from those plantlets cultured on full strength MS salt medium added with 1 mg l^{-1} IAA (Table 4).

Along all levels of MS salt strengths evaluated in the present study, culture medium amended with the particular auxin used in this study (IAA) resulted in a better root development within four weeks of culture, unlike those treatments devoid of it. In this study, the use of sole IAA had given

rise to the formation of more roots. In substantiating the present results, other workers Gantait et al. [22] a Gopi et al. [23] had also reported the remarkable effect of IAA for *in vitro* root growth and development of vanilla plantlets.

In this experiment, a significant increase in the number of *in vitro* initiated roots of vanilla was observed when the MS basal salt strength was retained at half and the concentration of IAA was kept at its minimum (0.5 mg l^{-1}). Reducing the mineral salt concentrations within the MS basal medium to half had also been reported to enhance the root initiation, as well as other associated root growth and development parameters, together with qualities of the roots produced [24,25].

The results obtained in the current study from the use of ½ strength MS salts supplemented with 0.5 mg l^{-1} IAA are in line with the reports of Gopi et al. [23]. The present finding is also in agreement with the outcomes publicized by Giridhar et al. [6] from using Rimler-Shotts (1973), RS, medium. However, the current findings are in contrast to the reports of Giridhar et al. [20] who succeeded from using MS medium supplemented with 2 mg l^{-1} IBA. This could be attributed to the variation in the efficacy different rooting PGRs in relation to the specific vanilla genotype, as well as the interaction between genotype and MS salt strength used in each case.

Rooting response of micro-shoots is stated to be controlled by the type and concentration of PGRs used in the medium [26]. According to Mineo [19], auxin compounds generally stimulate cell expansion, particularly cell elongation, besides promoting adventitious root development.

In the current investigation, reducing MS salt strength to half accompanied with the use of a lower level of IAA supplement had found to be superior in enhancing *in vitro* rooting of *V. planifolia*. Similarly, the promoting effect of reducing the salt concentration to ½ MS in the course of rooting of *in vitro* growing plantlets had been stressed by Baskaran and Jayabalan [27] and Hossain [28], which substantiates the findings from the current study. However, when the MS salt concentration is reduced significantly to the level of a quarter, the quality of the roots initiated was reduced considerably irrespective of the level of IAA supplement. This could be associated with the insufficiency of the medium salt composition to provide the required nutrient supply for the growth and development of quality roots of vanilla *in vitro*. Successful root initiation in the course of *in vitro* culture is so essential, since it largely determines subsequent *ex vitro* acclimatization and field survival of tissue culture derived plantlets.

3.3 Acclimatization of *in vitro* Derived Vanilla Plantlets

In this study, successful acclimatization of tissue culture derived *V. planifolia* plantlets with pre-initiated *in vitro* roots was attained after completing all the *ex vitro* associated steps (Fig. 2). Consequently, the plantlets had recorded

Table 4. The interaction effect of different levels of IAA and MS salt strengths on the *in vitro* rooting of vanilla plantlets after four weeks of culture

Treatments		Root number	Root length (cm)
MS strength	**IAA (mg/l)**		
MS	0.0	1.50±0.11^{e}	3.80± 0.09^{h}
MS	0.5	2.50±0.11^{c}	5.30± 0.09^{b}
MS	1.0	3.16±0.11^{b}	3.40± 0.09^{i}
MS	1.5	1.83±0.11^{e}	6.20±0.09^{a}
½ MS	0.0	2.00±0.11^{de}	4.40± 0.09^{ef}
½ MS	0.5	4.00± 0.11^{a}	6.10±0.09^{a}
½ MS	1.0	3.50±0.11^{b}	4.70±0.09^{de}
½ MS	1.5	2.33±0.11^{cd}	5.03±0.09^{bc}
¼ MS	0.0	1.50±0.11^{e}	3.86±0.09^{gh}
¼ MS	0.5	2.66±0.11^{c}	4.23± 0.09^{fg}
¼ MS	1.0	3.33±0.11^{b}	3.93±0.09^{gh}
¼ MS	1.5	2.00±0.11^{de}	4.83±0.09^{cd}
CV (%)		7.37	3.28

** MS = Murashige and Skoog (1962) Medium, IAA = Indiol-3- Acetic Acid, CV=Coefficient of variation. Treatment means within a column followed by the same letter are not significantly different from each other using REGWQ at P≤ 0.05*

Fig. 2. Acclimatization of *in vitro* raised vanilla plantlets; (A) Vanilla plantlets extracted from the rooting medium (B) Vanilla rooted plantlets being washed with lukewarm water, prior to fungicidal treatment (C) Vanilla *in vitro* derived plantlets planted on appropriate soil mix, four plantlets in each polytube pot (D) Survived vanilla plantlets from hardening stresses (E) Survived vanilla plantlets with in single polytube pot after one month of hardening

a mean survival rate of 83.4%. In the course of this, *in vitro* derived vanilla plantlets having broader and well developed leaves were the best in their *ex vitro* survival than those with reduced and under developed leaves (Fig. 2).

In the present study, a step wise acclimatization of vanilla *in vitro* derived plantlets helped to overcome the *ex vivo* condition and hence showed good survival rate in the green house and external condition as well (Fig. 2 C-E). Since the plantlets got special care to adapt the external environment and could develop functional stomata and leaf cuticle, thereby start to photosynthesize [29]. After one month of acclimatization the vanilla plantlets transferred into single polytube pot soil mix, so as to ease transportation of seedlings to its ideal agroecological area in Ethiopia (Fig. 2E).

4. CONCLUSION

Since this particular clone is represented with only few plants at the Tepi Research Center, the research system is still unable to respond to the prevailing huge demands of vanilla planting material. Particularly, when one considers the limited number of this newly introduced commercial clone, its supply to the users seems farfetched making use of the conventional vegetative propagation. The use of stem cuttings for large scale production and dissemination of planting materials is less efficient, very slow, labor intensive and hence costly. So the present findings contributed enhanced protocol for the rapid multiplication of vanilla propagules and enable us to respond to the demand at national level using the modern plat tissue culture technique.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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