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Mass Multiplication of *Vanilla planifolia* with Pure Genetic Identity Confirmed by ISSR

Saikat Gantait[1*] • Nirmal Mandal[1] • Somnath Bhattacharyya[2] • Prakash Kanti Das[2] • Sanjib Nandy[3]

[1] Department of Biotechnology, Instrumentation and Environmental Science, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, W.B., India
[2] Department of Genetics, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, W.B., India
[3] Department of Plant Science, University of Saskatchewan, Saskatoon, Canada

Corresponding author: * **saikatgantait@yahoo.com**

ABSTRACT

A novel protocol was developed for *Vanilla planifolia* to enhance *in vitro* cloning through multiple shoot induction. Bud induction was recorded from nodal explants as compared to the shoot tip within 10 days in MS with 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} BAP. A maximum of 5 buds from a single explant appeared within 11 days after the first bud induction. MS with 2.0 mg l^{-1} BAP only proved best for multiple shoot proliferation resulting in 7 shoots per inoculated shoot bud within 55 days. Maximum roots per plantlet were observed in MS with 0.25 mg l^{-1} IAA and 2 g l^{-1} activated charcoal. Autoclaved sand and intermittent water spraying optimized the primary acclimatization period of 12 days and then larger pots filled with sand, soil, charcoal and coconut fibre ensured 86% acclimatization in next 20 days. Selected ISSR primers were used to ensure genetic clonality of these *in vitro*-generated propagules.

Keywords: acclimatization, *in vitro* cloning, ISSR, multiple shoot, sustainable culture, vanilla
Abbreviations: BAP, N^6-benzylamino purine; **DMRT**, Duncan's multiple range test; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **ISSR**, inter simple sequence repeat; **MS**, Murashige and Skoog; **NAA**, α-naphthalene acetic acid

INTRODUCTION

Vanilla is an important member of the Orchidaceae family. The genus *Vanilla* comprises almost 110 species in tropical parts of the globe (Purseglove *et al.* 1981). Commercial vanilla i.e. *Vanilla planifolia* Andrews, with a rich source of flavour as natural vanillin, is the most cultivated species in India (Bory *et al*. 2008). Vanillin or vanilla essence is extracted after curing of beans and widely used as a flavouring agent in cakes, sweets, chocolates, ice creams, beverage and condiments as well as in the cosmetics and perfumery industries (Giridhar *et al.* 2001). It is the only orchid genus that makes its position into commercial trade as world's most expensive spice after saffron (Verma *et al*. 2009). The Spanish and Portuguese explorers brought vanilla plant into Africa and Asia in the 16th century from the Gulf Coast of Mexico (Correll 1953). Vanilla offers a huge scope for its cultivation in several parts of southern India and other countries having tropical rainforests.

Though Mexico was the chief producer of vanilla until the mid-19th century, its annual production dropped from 500 to 10 tons in 2006 due to extreme weather, such as tropical cyclones and hurricanes. Moreover, deforestation, environmental conditions and political instability drove vanilla prices from US$20 to US$500 per kilo in 2004, which ultimately introduced new countries into the vanilla industry to meet the growing demand of natural vanilla flavour (Wikipedia; Vanilla Exchange 2009). However, its propagation method is slow, labour-intensive, time-consuming and non-economical. It is conventionally propagated through stem cuttings whereas, the collection of stem cuttings for further propagation causes growth arrest, lack of development and insufficient yield from the mother plant (Kalimuthu *et al.* 2006). So, different tissue culture techniques have appeared as an alternative to increase the production of vanilla plant in a short span of time using callus culture (Davidson and Knorr 1991), protocorms or root tips (Philip and Nainar 1986) and auxillary bud explants (George and Ravishankar 1997; Geetha and Shetty 2000; Giridhar *et al.* 2001). The present study aimed to generate *in vitro* plants directly from shoot tips and nodal segments at a large scale for its commercial application.

The study had the following objectives: to develop a novel protocol for *in vitro* cloning and establishment of an effective *ex vitro* acclimatization process; to enhance the multiplication rate through induction of multiple shoots; to raise and maintain a sustainable pool of propagules for further multiplication and constant supply; and to ensure the genetic clonality of the sustainable quality propagules through DNA fingerprinting.

MATERIALS AND METHODS

Plant materials

30 days-old single noded nodal segments and shoot tips with 3-4 cm length were collected from 6 months-old healthy vines of *V. planifolia* Andrews. The source plants were conventionally generated through stem cuttings in the experimental garden of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, India. Collected explants were surface sterilized with Teepol (Glaxo India Ltd., India), washed three times for 3 min with sterile water and treated with 25% Cetrimide (Nicholas Piramal India Ltd., India) as an antifungal solution for 5 min. They were then treated with 10% NaOCl for 5 min, washed three times with sterile water for 2 min and treated again with 0.1% (w/v) $HgCl_2$ for 5 min. Again the explants were then thoroughly washed 4-5 times in sterile water for 2 min, and finally trimmed to 1.5-2 cm. The whole process was performed under strict aseptic conditions.

Multiple bud induction

To induce bud break from shoot tips and nodal segments the explants were inoculated in MS basal medium (Murashige and

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Skoog 1962) plus 3% (w/v) sucrose supplemented with nine different levels of α-naphthalene acetic acid (NAA) and N^6-benzylamino purine (BAP) (**Table 1**). MS without NAA or BAP served as the control in this experiment. Excised explants were inserted in the media approximately 0.5 cm for good anchorage. The cultures were incubated under artificial growth conditions until fresh multiple greenish buds appeared. Best composition was identified in terms of the number of responding explants to bud break, days to bud induction and number of induced buds.

Proliferation of multiple shoots

The induced buds were separated and cultured on MS with nine different concentrations of BAP and NAA again for multiple shoot proliferation where MS devoid of plant growth regulators (PGRs) served as the control, as above (**Table 2**). During shoot multiplication and proliferation the cultures were incubated for 55 days. Meanwhile one subculture was performed at 25th day of multiple shoot culture and the best resulting media formulation was identified based on the response to multiple shoot formation, number and length of shoots.

Rooting *in vitro*

For root induction and elongation MS basal medium was supplemented with 11 different concentrations of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and activated charcoal (AC) as well (**Table 3**). Multiple shoots were separated and then transferred individually to rooting media. The efficiency of IAA and IBA including AC were tested by assessing the response of shoots to root induction, days to root induction, number and length of roots which ultimately determined the most successful media composition.

Culture conditions

The MS basal media (consisting of salts, vitamins and 3% sucrose) was solidified with 0.7% (w/v) agar. The PGRs NAA, BAP, IAA and IBA were added to the medium before pH adjustment to 5.7 (with 0.5 N NaOH and 0.5 N HCl) and autoclaving at 1.06 kg. cm^{-2}, 121°C for 15 min. MS basal salts, sucrose, agar, vitamins and PGRs were obtained from SRL, India. Incubation of *in vitro* cultures was done at 25 ± 2°C with 60% RH under a 16-hr photoperiod (using white fluorescent tubes, Phillips Maxlife, Phillips, India) and 3000 lux light intensity.

Acclimatization

Two steps were used to acclimatize selected healthy plantlets. At first plantlets were transferred into small plastic pots containing autoclaved sand with sprayed water and covered with a transparent polythene sheet to ensure high RH. These plantlets were allowed to grow for 12 days. Then, the partially-acclimatized plantlets with 4-5 primary leaves were transferred to large pots containing sand, soil, charcoal and coconut fibre mixture (1:1:1:1). Water was sprayed intermittently to ensure a high RH for plant survival. The entire procedure was done in the shade and well acclimatized plants were then recovered for further clonal fidelity study.

Clonal fidelity

Genetic purity of 90 days old *in vitro* generated and 40 days acclimatized plantlets was estimated through DNA fingerprinting using 10 selected ISSR primers (Prakash *et al.* 2009) (**Table 5**). ISSR primers were synthesized based on di-nucleotide GA, GT or CT as core sequence and terminated with a single nucleotide for 3′ anchoring. The di-nucleotide core sequence is based on its relative abundance in the plant genome (Chin *et al*. 1996; Liu *et al*. 1996; Briyan *et al*. 1997; Liu *et al*. 2001). DNA was extracted from fresh leaves according to the procedure described by Bhattacharyya and Mandal (1999). In the PCR amplification reactions a 25 µl reaction volume contained 2 µl (40 ng) DNA, 2.5 µl 10X PCR buffer, 3.5 µl 2.5 mM dNTPs, 2 µl (200 ng) primer, 14.5 µl double distilled water and 0.5 U *Taq* DNA polymerase (Chromous Biotech, Bangalore, India). PCR consisted of initial denaturation at 94°C for 5 min followed by 35 cycles of 45 s at 94°C, 45 s at annealing temperature, 90 s at 72°C and final extension at 72°C for 7 min, 4°C for 5 min. The annealing temperature was adjusted according to the Tm of the primer used in the reaction. Banding patterns were visualized after the PCR products and 50 bp ladder were resolved by electrophoresis on 1.5% agarose gels (Genei, Bangalore, India). The gel electrophoresis was run in 1X TBE buffer stained with ethidium bromide (10 µg l^{-1}) and documented on a Gel Logic 200 trans-illuminator (Kodak, USA). Similarly, shoots from each remaining subculture were also examined for their clonal fidelity.

Sustained culturing of multiple shoots

In this scheme each individual shoot from 55 days old multiple shoot proliferation medium was cultured for further shoot multiplication on MS supplemented with similar PGR combination. Five subcultures were performed at two-months intervals over a total period of 8 months. Performance of these subcultures was assured on the basis of multiple shoot formation, multiple nodes and shoot length on the 50th day of inoculation (**Table 4**). Well developed shoots were used for further root induction and acclimatization. The clonal fidelity of shoots cultured for such a long period was assessed through ISSR markers.

Statistical analysis

All experiments were repeated in triplicate using 20 explants per replication in a Completely Randomized Design. Each explant was considered as an experimental unit. Data were subjected to ANOVA and significant differences among the treatments were tested by Duncan's multiple range test (DMRT) (Duncan 1955) at the 5% level using WINDOWSTAT 7.5 (Indostat services, Hyderabad, India) software package (Uttar Banga Krishi Viswavidyalaya, India).

RESULTS

Multiple bud induction

The bud induction from nodal explants was observed on the 10th day after inoculation (**Fig. 1A**) which compared to proliferate better in all respects than that of shoot tip explants. 18 inoculants out of 20 showed bud proliferation from nodal explants in a MS media with 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} BAP, whereas only 15 inoculants responded to bud proliferation in shoot tip explants. Only ~4 greenish shoot buds appeared after ~21 days of prolonged culture (**Table 1; Fig. 1B**). Presence of these PGRs in prolonged culture of 21 days helped to establish dark green buds.

Multiplication and proliferation of shoot

Each shoot bud generated from nodal explants was inoculated separately in MS basal medium with various combinations of NAA and BAP in different concentrations. On average 19.67 buds out of 20 shoot inoculum responded at 15 days incubation of culture on MS with only 2.0 mg l^{-1} BAP (**Table 2**). Under this media formulation around 7 shoots each with approximately 4 nodes per plantlet were recorded on the 55th day of culture (**Fig. 1C**) with an average healthy shoot length was around 6 cm.

Induction and elongation of roots *in vitro*

54 selected shoots with (at least) 2 nodes were transferred to MS with varying concentrations of IAA, IBA and AC for induction of roots. MS supplemented with 0.25 mg l^{-1} IAA and 2 g l^{-1} AC induced roots from 93% of shoots within 10 days after inoculation (**Fig. 1D**). However, the average number of roots per shoot was 7.33 and average root length was 5.8 cm on 28th days after culturing (**Fig. 1E**). During this time average shoot length was 7.53 cm (**Table 3**). Out of 54 plantlets, 51 well-rooted and healthy plantlets were acclimatized.

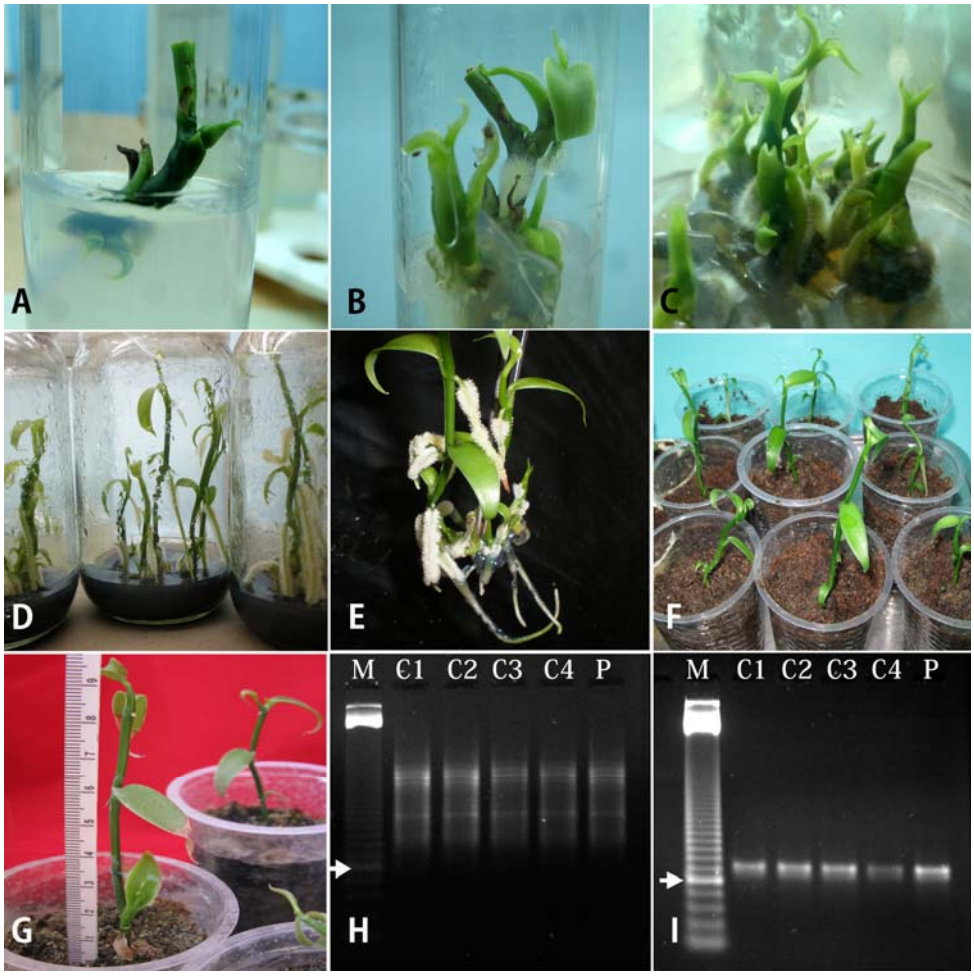

Fig. 1 (**A**) Bud break from nodal segment, (**B**) Multiple bud formation, (**C**) Multiple shoot proliferation, (**D**) Rooting of shoots, (**E**) Plantlet with stout and healthy roots, (**F**) Primary acclimatization on autoclave sand, (**G**) Final acclimatization on sand, soil, charcoal and coconut fibre mixture, (**H**) and (**I**) Agarose gel electrophoresis of ISSR fragments of *in vitro* regenerated clones (C_1-C_4) with their mother (P) showing monomorphic bands generated by primer IS-7 and IS-63. Lane M – 50 bp ladder (white arrow indicates 250 bp).

Table 1 Standardization of explants on bud induction in vanilla.

Growth regulators (mg l^{-1})		Nodal segments			Shoot tip		
NAA	**BAP**	**Response to bud induction (out of 20)**	**Days to bud induction**	**No. of buds**	**Response to bud induction (out of 20)**	**Days to bud induction**	**No. of buds**
0	0	1.33 ± 0.58 g	44.33 ± 1.54 a	1.00 ± 0.00 e	1.00 ± 0.00 f	43.00 ± 2.00 a	1.00 ± 0.00 c
0.1	0.5	7.33 ± 1.55 ef	27.33 ± 2.52 b	1.33 ± 0.58 de	4.33 ± 0.58 e	30.67 ± 1.53 b	1.33 ± 0.58 bc
0.1	1.0	12.67 ± 1.55 c	20.33 ± 1.53 de	1.67 ± 0.58 cde	10.67 ± 0.58 c	24.00 ± 1.73d e	1.67 ± 0.58 bc
0.1	1.5	10.00 ± 1.73 d	13.67 ± 0.58 g	2.00 ± 0.00 bcd	7.67 ± 1.55 d	17.33 ± 0.58 g	2.00 ± 0.00 ab
0.1	2.0	8.67 ± 0.58 de	23.67 ± 1.53 c	2.33 ± 0.58 bc	5.33 ± 0.58 e	27.00 ± 0.00 c	2.00 ± 0.00 ab
0.5	0.5	6.33 ± 0.58 f	21.67 ± 0.58 d	1.33 ± 0.58 de	4.33 ± 0.58 e	25.67 ± 1.55 cd	1.33 ± 0.58 bc
0.5	1.0	18.33 ± 0.58 a	9.67 ± 1.55 h	4.67 ± 0.58 a	15.33 ± 0.58 a	15.671.53 g	2.67 ± 0.58 a
0.5	1.5	14.33 ± 1.53 b	16.33 ± 1.55 f	2.67 ± 0.58 b	12.33 ± 1.53 b	21.00 ± 1.00 f	2.00 ± 0.00 ab
0.5	2.0	9.67 ± 1.55 d	19.00 ± 1.73 e	2.00 ± 0.00 bcd	7.67 ± 1.55 d	22.67 ± 2.08 ef	2.00 ± 0.00 ab
Overall mean		9.85	21.78	2.11	7.63	25.22	1.78
SE (±)		0.5514	0.6419	0.2635	0.5077	0.8051	0.2257
CD at 5%		1.653	1.924	0.790	1.522	2.414	0.677

Mean ± SD of 20 clones per treatment in three repeated experiments
Different letters indicate significant difference at $P < 0.05$ according to DMRT.

Acclimatization

Profusely rooted plantlets were first washed thoroughly in running tap water for removing traces of culture medium to prevent any type of bacterial or fungal contamination as suggested by Sharry and Teixeira da Silva (2006). Before acclimatization, pruning of elongated roots was done for easy *ex vitro* regeneration of fresh roots. Each single plantlet was then transferred to small plastic pots of 4 cm diameter containing autoclaved sand (**Fig. 1F**) with intermittent water spraying to maintain mist condition, kept under shade and covered with transparent polyethylene sheet. These plantlets were transferred after 12 days of acclimatization into larger pots (6 cm diameter) containing sand, soil, charcoal and coconut fibre mixture (1:1:1:1) (**Fig. 1G**). Forty four healthy plants were recovered after 20 days of acclimatization.

Propagule maintenance

Regenerated shoots were subcultured at an interval of 2 months which were further maintained over a period of more than 8 months on MS with 2.0 mg l^{-1} BAP (**Table 4**). The performance index was methodically monitored for

Table 2 Multiple shoot proliferation in vanilla (using Nodal segment derived buds)

Growth regulators (mg l^{-1})		Response to shoot multiplication (out of 20)	No. of shoots on 55th DAI	No. of nodes on 55th DAI	Shoot length (cm) on 55th DAI	Remarks
NAA	BAP					
0	0	1.33 ± 0.58 h	1.00 ± 0.00 f	0.67 ± 0.58 d	1.17 ± 0.35 f	Abnormal
0.5	1.0	18.33 ± 0.58 b	4.33 ± 0.58 b	2.00 ± 0.00 c	2.60 ± 0.62 de	Lanky, pale green
0.5	2.0	9.67 ± 1.55 e	2.00 ± 0.00 de	2.33 ± 0.58 bc	2.17 ± 0.25 e	Thin, aerial roots
0.5	3.0	9.00 ± 0.00 ef	1.67 ± 0.58 ef	2.33 ± 0.58 bc	2.10 ± 0.20 e	Thin, aerial roots
0.5	4.0	6.33 ± 0.58 g	1.67 ± 0.58 ef	2.00 ± 0.00 c	2.00 ± 0.36 e	Healthy, aerial roots
0	1.0	13.33 ± 0.58 d	2.33 ± 0.58 de	2.33 ± 0.58 bc	3.73 ± 0.47 c	Normal, green
0	2.0	19.67 ± 0.58 a	7.33 ± 0.58 a	4.67 ± 0.58 a	5.67 ± 0.45 a	Healthy, stout, green
0	3.0	15.33 ± 0.58 c	3.33 ± 0.58 c	3.00 ± 0.00 b	4.50 ± 0.53 b	Normal, callus lump at base
0	4.0	8.00 ± 1.73 f	2.67 ± 0.58 cd	2.33 ± 0.58 bc	3.10 ± 0.17 cd	Normal, callus lump at base
Overall mean		11.22	2.93	2.41	3.00	
SE (±)		0.4082	0.2940	0.2515	0.2395	
CD at 5%		1.224	0.881	0.754	0.718	

Mean ± SD of 20 clones per treatment in three repeated experiments
Different letters indicate significant difference at $P < 0.05$ according to DMRT.

Table 3 Root induction and elongation in vanilla

Growth regulators (mg l^{-1})		Additive AC (g l^{-1})	Response to root induction (0ut of 20)	Days to root induction	No. of roots on 28th DAI	Root length (cm) on 28th DAI	Shoot length (cm) on 28th DAI	Remarks
IAA	IBA							
0	0	0	1.33 ± 0.58 g	38.33 ± 1.53 a	1.33 ± 0.58 e	1.40 ± 0.62 fg	1.40 ± 1.17 g	Abnormal rooting
0	0	1	5.67 ± 1.55 f	28.00 ± 1.00 c	2.00 ± 0.00 de	1.33 ± 0.25 fg	2.00 ± 0.10 f	Very thin, abnormal
0	0	2	8.00 ± 1.00 de	26.33 ± 1.55 c	2.33 ± 0.58 d	1.60 ± 0.36 fg	2.53 ± 0.35 e	Thin root
0	0.1	2	6.33 ± 0.58 ef	33.67 ± 2.31 b	1.67 ± 0.58 de	1.00 ± 0.10 g	3.30 ± 0.10 d	Thin root
0	0.25	2	8.33 ± 1.55 d	28.00 ± 1.73 c	2.00 ± 0.00 de	1.33 ± 0.25 fg	2.73 ± 0.43 e	Thin, short root
0	0.5	2	9.33 ± 0.58 cd	26.67 ± 1.523 c	2.00 ± 0.00 de	1.63 ± 0.31 f	3.47 ± 0.38 d	Thin, short root
0	1.0	2	11.67 ± 2.08 b	23.00 ± 1.73 d	2.33 ± 0.58 d	2.40 ± 0.44 e	4.80 ± 0.44 b	Healthy root
0.1	0	2	10.67 ± 1.53 bc	17.67 ± 0.58 e	3.33 ± 0.58 c	3.87 ± 0.25 d	3.90 ± 0.20 c	Thin short root
0.25	0	2	18.67 ± 0.58 a	10.33 ± 1.53 g	7.33 ± 0.58 a	5.80 ± 0.56 b	7.53 ± 0.35 a	Robust root
0.5	0	2	17.67 ± 1.55 a	13.67 ± 1.55 f	5.67 ± 0.58 b	6.43 ± 0.31 a	5.07 ± 0.15 b	Robust root
1.0	0	2	17.00 ± 1.00 a	15.33 ± 1.53 ef	5.00 ± 0.00 b	4.70 ± 0.44 c	4.80 ± 0.36 b	Robust root
Overall mean			10.42	23.73	3.18	2.86	3.78	
SE (±)			0.6122	0.8710	0.2621	0.2141	0.1283	
CD at 5%			1.806	2.569	0.773	0.632	0.378	

Mean ± SD of 20 clones per treatment in three repeated experiments
Different letters indicate significant difference at $P < 0.05$ according to DMRT.

Table 4 Performance of sustainable subcultures on shoot multiplication on vanilla.

Treatments	Responding shoots (0ut of 20)	No. of shoots on 50th DAI	No. of nodes on 50th DAI	Shoot length (cm) on 50th DAI	Remarks
Subculture I	19.67 ± 0.52 ab	7.33 ± 0.52 a	4.67 ± 0.55 a	5.58 ± 0.34 a	Healthy, stout, green
Subculture II	19.67 ± 0.52 ab	7.50 ± 0.55 a	4.50 ± 0.55 a	5.60 ± 0.38 a	Healthy, stout, green
Subculture III	20.00 ± 0.00 a	7.67 ± 0.82 a	4.83 ± 0.75 a	5.73 ± 0.19 a	Healthy, stout, dark green
Subculture IV	19.67 ± 0.52 ab	7.50 ± 0.55 a	4.50 ± 0.55 a	5.62 ± 0.21 a	Healthy, stout, dark green
Subculture V	19.33 ± 0.52 b	7.17 ± 0.41 a	4.33 ± 0.52 a	5.57 ± 0.19 a	Healthy, stout, green
Overall mean	19.67	7.43	4.57	5.62	
SE (±)	0.1491	0.2506	0.2068	0.1075	
CD at 5%	0.440	0.739	0.610	0.317	

Mean ± SD of 20 clones per treatment in three repeated experiments
Different letters indicate significant difference at $P < 0.05$ according to DMRT.

multiple shoot proliferation which revealed that no significant variation in performance was observed. Morphogenetic efficiency continued to remain unaltered with sustained subculturing. DNA fingerprinting was performed to ascertain the genetic purity using ISSR primers.

Clonal fidelity test

Ten ISSR primers (**Table 5**) were used for checking the genetic purity of *in vitro* generated propagules. Three ISSR primers *viz.* IS-6, IS-9 and IS-10 could not amplify vanilla DNA. The remaining primers (i.e. IS-7, IS-8, IS-11, IS-12, IS-61, IS-63 and IS-65) displayed a positive interaction but among these IS-7 and IS-63 were reproducible. A total number of 40 reproducible monomorphic bands were scored from the clones including their parental lines. In our study the primers amplified distinct bands between 250 bp to 1250 bp molecular size range. None of the primers showed any polymorphism in banding pattern (**Fig. 1H, 1I**) proving that the purity of the *in vitro* regenerated clones was maintained. On the other hand, a similar result was displayed in case of sustained multiple shoot culture also, as major reproducible bands among the prolonged cultured clones were monomorphic.

DISCUSSION

The present study revealed some interesting and positive outcomes from the *in vitro* establishment of explants and maintenance of sustained culture to assessment of clonality. A flow-diagram is presented to illustrate the entire protocol (**Fig. 2**). Fresh shoot bud induction from nodal segment proved itself superior to the apical shoot tip in all types of media formulations. Low concentrations of BAP and NAA combination proved effective for induction and establishment of shoot buds from both shoot tip and nodal segments. The superiority of nodal segment as an explant source (Giridhar *et al.* 2001) and the positive role of low concentration of cytokinin and auxin in shoot bud initiation is well established in vanilla (George and Ravishankar 1997). MS

Table 5 ISSR primers used for the test of fidelity of the *in vitro* generated clones.

Oligo-name	Tm (°C)	Sequence (5′-3′)	Reaction to vanilla DNA
IS-6	52	GAG AGA GAG AGA GAG AC	Negative
IS-7	50	GTG TGT GTG TGT GTG TA	Positive, reproducible and monomorphic
IS-8	52	AGA GAG AGA GAG AGA GC	Positive but not reproducible
IS-9	46	TGT GTG TGT GTG TGT A	Negative
IS-10	52	CGA GAG AGA GAG AGA GA	Negative
IS-11	52	CAC ACA CAC ACA CAG	Positive but not reproducible
IS-12	52	GTG TGT GTG TGT GTG TC	Positive but not reproducible
IS-61	50	GAG AGA GAG AGA GAG AT	Positive but not reproducible
IS-63	52	AGA GAG AGA GAG AGA GC	Positive, reproducible and monomorphic
IS-65	50	AGA GAG AGA GAG AGA GT	Positive but not reproducible

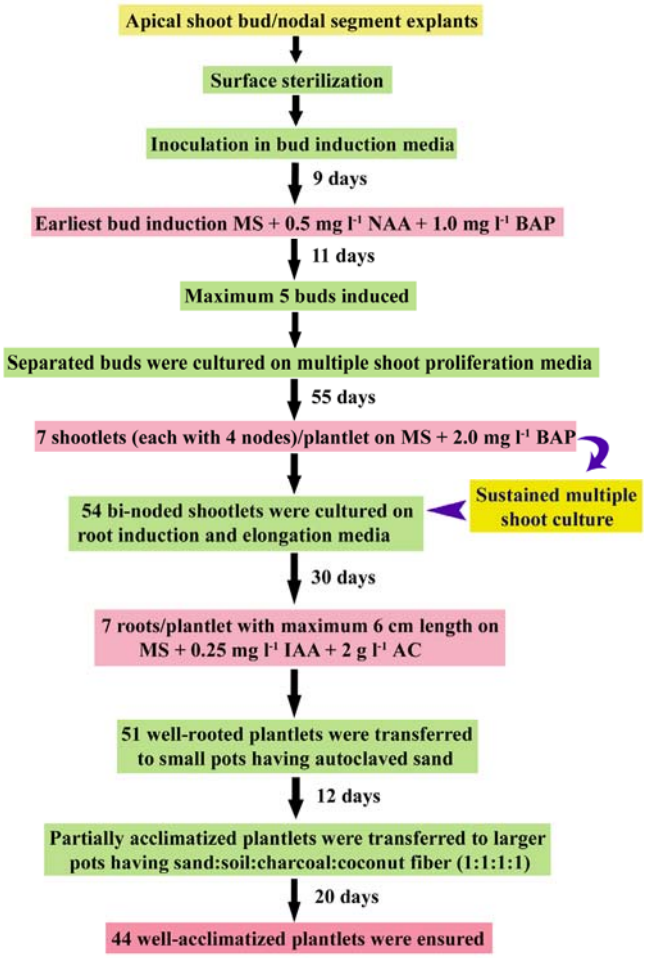

Fig. 2 Flow diagram illustrating *in vitro* cloning to *ex vitro* acclimatization.

with 2.0 mg l^{-1} BAP was most effective for multiple shoot formation and proliferation than any other combinations. The results of multiple shoot proliferation demonstrated that cytokinin alone without auxin successfully induced maximum multiple shoots (Bhojwani 1981; Sugla *et al.* 2007). After successful shoot multiplication, profuse *in vitro* rooting was recorded in MS basal media with 0.25 mg l^{-1} IAA and 2 g l^{-1} AC. A very low concentration of IAA and the presence of AC proved most efficient in inducing as well as promoting root growth for vanilla. AC plays an effective role in enhancement of root induction because it absorbs the polyphenols produced through chemical processes within the media which may act as growth inhibitors during morphogenesis (George and Ravishankar 1997; Madhusudhanan and Rahiman 2000). Additionally, AC helps to eliminate light and provides a reasonable physical environment for the rhizosphere (Nissen and Sutter 1990).

Development of good number of healthy roots on each shoot is necessary for better acclimatization and 51 selected plants were subjected to two-step acclimatization process. First, washing the plants to remove residual media was very effective to restrict any kind of microbial contamination and second, pruning of roots at the time of planting facilitated *ex vitro* establishment of plants for root regeneration. Root pruning also helped to reduce variability arising due to the difference in the number and length of *in vitro*-generated roots (Thomas and Ravindra 1997). The maximum credit of this successful acclimatization was gained by the retention of high humidity using coconut fibre, other than the intermittent water spraying and covering with transparent polyethylene sheet (Thomas and Ravindra 1997). Out of 51 regenerated plantlets from a single nodal segment 44 healthy growing plants were ready for field transfer. Overall 86% success was achieved following the above-mentioned acclimatization protocol. This technique efficiently produced a high frequency of plants within a limited time span. It is clear from the activity chart that it would take around 138 days or almost 20 weeks (from proliferation of bud to complete acclimatization of plantlets) to raise as many well acclimatized plants from a single nodal segment.

There are two distinct advantages to maintain a steady supply of propagules from regenerated shoots under identified MS media. First, there was no need to start with a fresh nodal segment. As a result, the time lag would be curtailed by at least 3 weeks and secondly, an unbroken propagule supply can be sustained over a period of time without genetic damage. In the present study morphological parameters like shoot number and shoot length determined the performance of *in vitro* propagules, which did not differ significantly with further sustained subcultures. Naik *et al.* (2003), in contrast, observed a decrease in performance with an increased passage of subculturing in *Gmelina arborea*, a tree species.

The genetic purity of regenerated vanilla clones was also examined by ISSR markers. ISSR is a PCR-based method to amplify DNA fragments present at an amplifiable distance in between two identical microsatellite repeats with opposite orientation (Reddy *et al*. 2002). The test of clonal fidelity using ISSR primers was successfully attempted in different crops like *Swertia chirayita* (Joshi and Dhawan 2007), banana (Lakshmanan *et al.* 2007) and gerbera (Bhatia *et al.* 2009). In these study two ISSR primers (IS-7 and IS-63) revealed discreet monomorphic bands. It was proven from ISSR experiment that clones developed form *in vitro* direct organogenesis are true to its genetic identity and safest mode of micropropagation to have true-to-type progeny (Shu *et al*. 2003; Carvalho *et al*. 2004; Martins *et al.* 2004). Clones of the prolonged cultures of micropropagated vanilla plants equally maintained their genetic purity (Sreedhar *et al.* 2007a). This uniformity would help to maintain sustained activity for *in vitro* propagation of vanilla.

CONCLUSION

The source of explant and proper formulation of auxin-cytokinin have significant effect on large scale micropropagation of vanilla to maintain its genetic purity. Even the propagules of vanilla can be maintained through subculture at long intervals without altering their clonality for constant

supply to meet up the growing demand. So the present exercise by and large offers a promising approach to engage in commercial activity for aromatic plants like vanilla through micropropagation.

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