Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Asian Journal of Plant Science and Research, 2015, 5(2):52-62

ISSN : 2249-7412
CODEN (USA): AJPSKY

Biotechnology for micropropagation and enhancing variations in *Vanilla*

Minoo Divakaran[1], K. Nirmal Babu*[2], P.N. Ravindran[3] and K.V. Peter[4]

[1]Department of Botany, Providence Women's College, Calicut - 673009, Kerala, India
[2] Project Coordinator, All India Coordinated Research Project on Spices, Indian Institute of Spices Research, Calicut – 673 012, Kerala, India.
[3] Consultant, Tata Tea Research Foundation, Bangalore, India.
[4] Director, World Noni Research Foundation, Chennai.

ABSTRACT

Vanilla (Vanilla planifolia), a tropical orchid cultivated for its pleasant flavour, is a native of Mexico, and an introduced crop to other countries. Continuous vegetative propagation and lack of sufficient variations in the gene pool hampers crop improvement programmes. Introduction of somaclonal variation through callus cultures has been attempted to broaden the narrow genetic base. A callus induction and in vitro plant regeneration system has been optimized from both vegetative and reproductive tissues. The best results were obtained using vegetative tissues and over 80% callusing was achieved in Murashige and Skoog's medium supplemented with 4.44µM BA and 2.68µM NAA. Callus differentiated into shoots which could be multiplied successfully in 1: 12 ratio in a combination of 4.44µM BA and 2.46µM IBA, when supplemented to MS medium. In vitro rooting was induced with an efficiency of 100% in basal MS media devoid of any growth regulators. This ability of dedifferentiated tissue to regenerate is a crucial prerequisite for future genetic transformation experiments. The protocol was successfully extended to the endangered wild species, V. aphylla, offering the potential of applying the protocol for mass multiplication as well as induction of variations in Vanilla species, in a limited time. Preliminary studies on the callus regenerants indicated variations in morphology and RAPD profiles.

Key Words: Callus, callus regeneration, *Vanilla planifolia*, *V.aphylla*, micropropagation, RAPD.

Abbreviations :
MS : Murashige and Skoog's medium (1962)
BA : 6- benzyl adenine
IBA : Indole-3-butyric acid
NAA : α-naphthalene – acetic-acid
RAPD : Randomly amplified polymorphic DNA

INTRODUCTION

Vanilla, *Vanilla planifolia* Andrews, (Syn. *Vanilla fragrans* Salisb.) Ames is cultivated for its pods which, when processed, yield vanilla extract making it the most economically important orchid. Three species of *Vanilla* are of commercial importance: *V. planifolia* Andrews [*V. fragrans* (Salisb.) Ames], *V. pompona* Schiede, and *V. tahitensis* J. W. Moore. About 5 million pounds of beans are produced in a year and half of the world's production is consumed by the US [1]. Vanilla is native to Mexico and Central America, but is now cultivated in other parts of the

tropics mainly in Madagascar and Indonesia [2]. The history of cultivated vanilla suggests that almost the entire stock of cultivated vanilla outside Mexico is a single genetic individual (clone) propagated by vegetative cuttings with practically no variability available for crop improvement. Continuous clonal propagation leads to monoculture making vanilla susceptible to diseases and pests [3]. The introduction of new genetic material is greatly constrained by factors like its asexual propagation, the fact that the flowers are mostly 'selfed' and the threatened wild populations of vanilla by land pressures [4]. Lack of sufficient variability in the gene pool, threat of destructive diseases that erase vanilla plantations as well as destruction of its natural habitats, makes the search for alternate methods to introduce variability in to the gene pool, vital.

Vanilla is suspected to be highly heterozygous with extensive inherent genetic variations because of its cross-pollinated nature and mitotic associations [5]. Seedling progenies of *V. planifolia* were developed earlier and isozyme analysis indicated the variability among them [6,7].

In the above context, utilization of biotechnological tools to introduce variability has been attempted. Reports on micropropagation concentrate on production of true-to-type plants, and few reports of callus regeneration [8, 9] are available. However protocols for induction and characterization of variations among callus regenerated plants have not been reported in vanilla. This study reports protocols to regenerate plants *via* callus from both vegetative and reproductive tissues, which was required to identify the most suitable physiological state for callus proliferation and regeneration and presence of variations among the regenerants. This can be utilized as a tool for increasing the variability available in vanilla germplasm since the variations were observed among few somaclones in morphology, tolerance to disease causing organisms and Randomly Amplified Polymorphic DNA (RAPD) profiles. An efficient micropropagation protocol optimized for *Vanilla* species, will not only be a powerful tool for commercial multiplication but will also open new avenues for future breeding programmes which were hitherto hampered.

MATERIALS AND METHODS

Explant source

Shoots tips and nodal segments, collected from field grown vines were established *in vitro*, after surface sterilization with 0.1% mercuric chloride solution for 5-7 minutes and subsequent washing off with 3-4 changes of sterile distilled water. Shoot tips and subsequent nodes from such established cultures of *V. planifolia* were used as explants for micropropagation and callus regeneration.

Pods were dipped in alcohol and flamed thrice before splitting them open longitudinally and transferring the numerous minute ex-albuminous seeds on to the culture medium for direct as well as indirect germination *via* callus phase.

Culture medium

MS medium [10] fortified with 87.64mM sucrose and gelled with 0.65% agar was used as basal medium. Growth regulators *viz*., cytokinins - Benzyl adenine (BA) at 2.22 – 4.44 µM, kinetin at 2.32 - 4.64 µM and auxins - Indole-3-butyric acid (IBA) at 2.46 - 4.92 µM, α-naphthalene acetic acid (NAA) at 2.68 - 5.37 µM were supplemented to MS medium singly or in combinations to induce multiple shoots, rooting, callus and regeneration of plants from callus.

Culture conditions

The pH of the medium was adjusted to 5.8 in all cases prior to autoclaving at 15 lbs pressure and 120^0C temperature for 20 min. All cultures were incubated at 25± 2^0C with a photoperiod of 14 hrs and a light intensity of 2500 lux.

Hardening

In vitro regenerated plantlets were transferred to polybags with sterilized sand, garden soil and vermiculite in equal proportions and kept in humid chamber for 30 days for hardening.

Characterization of variability

Observations on the variations in plant characters at the nursery stage among a few callus-regenerated progenies in comparison to the cultivated parent were made. Anatomical and cytological studies were made to study the regenerative pathway.

Virulent strains of *Phytophthora meadii* and *Fusarium oxysporum* isolates from the Division of Crop Protection, Indian Institute of Spices Research were used for screening the somaclones for resistance to these major diseases affecting vanilla. Infection was affected by placing discs of these isolates in the axils and development of disease symptoms was recorded.

DNA amplification, band separation and RAPD profiles were also developed [11. 12].Genomic DNA was isolated from shoots at the beginning of the experiment and after retrieval from callus to compare the differences.

The dNTPs, Taq polymerases and other chemicals were procured from Amersham Pharmacia Biotech, Sweden. Sixteen arbitrary primers with 60% -70% GC content and no self-complementary ends, from Operon Technologies Inc. Alameda, California, were used for screening. Primers that exhibited high polymorphism and showed best readability were chosen for the study. The amplicons, named by primer and molecular mass in base pairs (bp), were scored as presence (1) or absence (0) of homologous bands and a binary matrix of the different phenotypes were put together. Paired Affinity Indices (PAI) was calculated to estimate the extent of variation between each of these somaclones.

Statistical analysis
All experiments were set up in a completely randomized design. Differences between means were scored with Duncan's Multiple Range Test. Analysis of samples from each treatment was statistically evaluated by analysis of variance (ANOVA, $p \leq 0.05\%$) using MSTAT-C software.

RESULTS

Callus induction
The vegetative and reproductive tissues *viz*., shoot and seed explants showed varied responses in different combinations of media (Table 1). Among the auxins tried, NAA supplemented at 2.68µM and 5.37µM induced callus from shoot explants whereas no callus was produced in IBA supplemented media, which induced rooting. The callus was hard, organogenic and white in colour initially which proliferated and turned green in colour on transfer to the same medium and small shoot buds initiated after 45 to 60 days in culture. In media supplemented with cytokinins, BA and kinetin, at 2.22µM and 4.44µM, singly, only multiple shoots were produced and no callus induction was noticed. When auxins and cytokinins were tried in combination, Interaction of 2.68µM NAA and 4.44µM BA gave the most favourable response. In this medium callus was induced at the base of the shoot explants and in root tip explants (Fig. 1a) after 30days of incubation. Further subculture into MS media with 4.44µM BA and 2.68µM NAA, regeneration of shoot buds occurred from these calli (Fig. 1b). About 5-20 shoot primordia were formed from each mass and each of them was capable of regenerating into individual shoot and plantlet, which were studied in comparison with parent (control), *Vanilla planifolia*.

Induction of callus from protocorms
The initial stages of seed germination were typical of most orchids, involving swelling of the embryo followed by rupturing of the seed testa and emergence of protocorm. In treatments with BA, most of the protocorms remained the same with the scale like leaf primordial developing into shoots whereas treatments with auxin supplements showed gradual disorganization of the protocorms into callus (Fig. 1c). In medium supplemented with NAA, it was found that sudden disorganization of the protocorms occurred to form a callus tissue, which was of yellowish colour initially. On culture in medium supplemented with BA and NAA, regeneration of protocorm like bodies (PLBs) occurred from these calli (Fig. 1d). About 5-20 PLBs were formed from each mass and each of them were capable of regenerating into individual shoots and plantlets. Media supplemented with BA alone, prevented formation of callus and seeds germinated directly into plantlets (Table 1).

Plant regeneration from callus
Among the various media combinations tried for callus induction and regeneration, the most suitable medium was MS medium fortified with 2.68µM NAA and 4.44µM BA (Fig. 3) in which 75% of the cultures developed callus and an average of 10 plants could be regenerated from them (Table 1). The regenerated plants induced from callus tissue produced roots in growth regulator free medium, in contrast to the BA supplemented media. NAA supplemented medium was found to induce thick fleshy velamen like roots, which were of a disadvantage in the hardening stages [7, 13].

The somaclones regenerated also exhibited significant variations *in vitro* (Fig. 4), in the rate of multiple shoots formation and also leaf size and internodal distance (Table 3).

Micropropagation
In order to generate sufficient material for further studies, shoot tips and nodal explants of *V. planifolia*, and the regenerants were initiated for bud break in MS medium with 2.22µM BA and the sprouting shoots. Was used for micropropagation experiments. Of the combinations tested, BA when used alone or in combination with IBA induced multiple shoots. Interaction of 4.44µM BA and 2.46 µM IBA enhanced the proliferation of shoots (Table 2). In this medium, an average of 15 multiple shoots were induced in 60 days after establishment, in more than 90% of the cultures (Fig. 1b) and was conducive for proliferation as well as elongation of shoots. Nodal segments gave better response with a mean of 15 shoots per culture compared to the shoot tips (mean of 7 shoots per culture), due to absence of apical dominance.

In the present study kinetin had no effect of the induction of multiple shoots and gave rise to single shoots whereas NAA supplemented media led to induction of 'callusy' roots or velamen roots which were unfavorable during transplantation and hardening. When BA was added singly, it induced multiple shoots which had very short internodes (i.e., no elongation) and total absence of roots. These problems were overcome by supplementing a combination of auxin and cytokinin.

On transfer to growth regulator free MS medium, the shoots elongated, developed good root system, and could be harvested for further experimental studies, proliferation or establishment in soil. This method was effectively used throughout the study to multiply different genotypes / morphotypes generated.

Hardening and planting out
The rooted shoots were carefully removed from culture vessels, washed thoroughly to remove any traces of nutrient medium, treated with 0.2% Indofil and transferred to polybags containing potting mixture (sand, soil and vermiculite). They were hardened for 30 days under controlled conditions, before transferring them to pots. The plantlets hardened with over 80% success and the plants were field planted (Fig. 1g) with *Glyrcidia* standards for proper shade and support.

Characterization of callus regenerated plants
Morphological characters:
The callus regenerated progenies exhibited high variations among themselves with plant and leaf characters, shapes varying from ovate to lanceolate and internodal length. Sc40 showed highest plant growth of 19.64 cm after six months of planting out (Table 3). In general, Sc24 was characterized by good vegetative growth in terms of plant height (18.4 cm), leaf size (length of 3.10 cm and breadth of 2.5 cm) and internodal length (2.55 cm), when compared to *V. planifolia* (plant height of 12.3 cm).

Sc32 was characterized by comparatively shorter internodes (Fig. 1g), which can be of immense agronomic importance since the number of nodes per meter of the vine in increased thereby increasing the number of inflorescences obtained at every node, making a notable yield impact per meter of the vine.

A few attempts to index these progenies cytologically were made and one of the callus regenerated progenies, V 8.1 did not show any cell with the normal somatic compliment of 2n = 32. It instead had variations in chromosome numbers *viz*., 2n= 24, 26, 28 and aberrations like lagging and clumping of chromosomes were observed, indicative of the high degree of variations existing in these plants (Fig. 1i). Reports of mitotic associations in vanilla and its potential in developing cytotypes have been discussed [5].

Anatomical changes observed were similar for all genotypes. BA induced proliferation of vascular tissue and callus parenchyma cells within the callus. The differentiating meristematic tissue was characterized by cells filled with dense cytoplasm and prominent nuclei.

Screening of somaclones for disease resistance
The somaclones were screened against the infection of *Phytophthora meadii* and *Fusarium oxysporum*, the causal agents of foot rot and wilt diseases in vanilla. The disease progression was manifested as browning and water soaked patches at the axil spreading out to either side of the internode. It was observed that the somaclones exhibited high

degree of variability in tolerance to the pathogens, the spread ranging from no infection, superficial infection to complete infection, when *V.planifolia* was susceptible. In the absence of resistant lines in *Vanilla planifolia*, further studies for identification of tolerant line would be important for development of disease resistant varieties.

RAPD profiles of callus regenerated progenies of Vanilla

The few regenerated somaclones were selected based on preliminary morphological differences observed at nursery stage. Operon primers OPB20, OPA10 and OPD03 gave good polymorphism (Fig. 1h) between the progenies. The somaclones tested are variable (70% to 47.5%) when compared to each other, indicative of somaclonal variation as the possible cause for this variation. They showed 40% dissimilarity with its parent, *V.planifolia* (Fig. 5). A few seedling progenies were analyzed with their respective callus regenerated plants, a variability ranging from 15 – 30% was observed. V7.1 and its root callus regenerated progeny V7.1R showed 85% similarity, 70% similarity in V179 and its callus regenerated progeny V179.1, 72.5% similarity among V112 and its callus regenerated progeny V112.1 and 77.5% similarity among V101 and its callus regenerated progeny V101.1.

RAPD profiles from shoots regenerated from callus were compared to control and few variations were detected. The number of polymorphic bands ranged from 12.5 to 40% and the match percentage between mother microcutting and newly sprouted callus regenerating shoots were 76.25 %, sharing values of similarity between 60 to 87.5%.

DISCUSSION

Callus can be used as target tissues for genetic transformation experiments which, is the next step to bring in desirable traits into this commercially important species. Since the regeneration protocol standardized in this system is simple, and of high frequency, it can shorten the length of such experiments. Though NAA alone induced callus, they could not be directed to regenerate in to plants. Low cytokinins have been reported to enhance callusing [14]. Addition of low cytokinin especially BA, was essential in dedifferentiation of the explant into regenerative callus in the present study. Regeneration potential of vanilla callus here is comparable to micropropagation indicating that changes in auxin supplementation do not influence the regenerative potential of the tissue. Unfavorable ratio between total cell population and cells capable of stably expressing the foreign genes has been the most serious hindrance to monocotyledonous genetic manipulation [15].

Factors affecting callus regeneration included explant and tissue type. Vegetative tissues produced longer shoots which were harvestable and directly utilized for further studies however shoots regenerated from reproductive tissue needed to be excised and cultured for further elongation before they could be multiplied (Fig. 2). Using callus tissue, from embryos/ reproductive tissues does put forth a concern since Vanilla is basically a cross pollinated crop in its native country. Hence its use as target tissue for transformation may not be able to reveal whether the variations were inherent seed variability or transgenic effect.

Embryogenic calli with regeneration potential were observed in culture of early immature embryos (3 DAP) and 7-14 DAP and shoot base of young seedlings of *Stenotaphrum secundatum* (Li et al. 2006). Successful plant regeneration from callus has been reported in many orchids viz., *Cymbidium* [16], *Phalaenopsis* [17], *Paphiopedilium* [18] and these morphogenesis systems are being used in some to explore the control process on reducing juvenility and precocious flowering *in vitro*. Earlier studies in *Vanilla planifolia* [8] indicated that shoot sections from first nodes has best callus initiation and proliferation rates and callus from cytokinin containing Linsmaeir and Skoog medium and its subsequent regeneration from stem and leaf sections [9]. The potential of protocorm derived calli to regenerate PLBs in developing a callus associated micropropagation system in *Geodorum densiflorum* has been discussed by [13]. The present protocol offers the possibility of its application to both cultivated and wild species of *Vanilla*.

Micropropagation of vanilla has been reported earlier [19, 20, 7]. I*n vitro* transformation of root meristem from aerial roots into shoot and plantlets in *V. planifolia* as an efficient method for clonal propagation has been reported [21]. Micropropagation and *in vitro* conservation of five diverse species of vanilla *viz.*, *V.planifolia, V.andamanica, V. wightiana, V.aphylla* and *V. pilifera* have been reported [22] as an effective alternative to conserve this important species in laboratories or *in vitro* gene banks.

In the present study, BA alone suppressed rooting while inducing multiple shoots and IBA/NAA alone suppressed multiple shoot induction. BA is known to induce multiple shoots in a wide range of crops. However, its addition in

concentration higher than 4.4 µM led to fasciated shoots. This may be a feedback inhibition of multiplication at higher concentration of BA due to which proliferation at vigorous rate is promoted at lower concentration of BA as has been observed in bamboo [23]. Inhibitory effects of many cytokinins on seedling development have been reported in angiospermic plants [24, 25]. Other stimuli to change the environmental conditions have been reported to improve shoot regenerative potential. Use of phenylacetic acid as primary factor to induce multiple shoots from axillary buds of *V. planifolia* and silver nitrate at 10-40 µM for shoot and root formation. me genotypes performed better indicating that a process of selection and cloning can identify and multiply lines of faster multiplying lines which may suitably meet the demand for planting materials without hampering the natural resources, have been reported [26, 27].

Reports on variability among callus regenerated plants in vanilla are not available, except for studies among indigenous collections of vanilla, through polyacrylamide electrophoretic (PAGE) studies [28]. The present study comprising of randomly selected callus regenerated progenies, shows variability among the callus regenerants in general and also between a parent and its own callus regenerated plant. This is the first report of characterization of plants regenerated through callus in vanilla, revealing important information on the amount of variability that can be generated. This study can be utilized for developing variants with desirable agronomic characters like short internode and tolerance to disease for utilizing them in vanilla improvement programmes, and broadening the narrow genetic base. Earlier attempts have been made to increase the spectrum of variability by effective wide crossing between cultivated *V. planifolia* and *V.aphylla*, their molecular profiles indicating introgression of male and female characters into the hybrids [12] and isolation and fusion of protoplasts of *V.planifolia* and *V.andamanica*, a species indicating possibilities of natural seed set [11]. *In vitro* regeneration systems are being used for conservation of endangered orchid species too [29].

In future attempts to genetically transform vanilla, the ability of transformed tissue to regenerate is a crucial prerequisite. The regeneration protocol optimized here is very simple and not time consuming, hence could shorten the length of any genetic transformation experiments while inducing a high frequency of regeneration. *In vitro* roots could be developed in growth regulator free medium, so that effect of exogenous hormones would not affect the hardening or planting out of plantlets. The rare natural seed germination hampers conventional propagation and harvesting natural variability through seed germination. This is overcome by *in vitro* seed germination and the protocol to multiply plants in large numbers. The media conducive to induce responses *viz*., multiple shoots, roots as well as plant regeneration from callus was similar irrespective of the maturity / juvenility of the tissue, indicating the non-specificity of the optimized conditions to different genotypes, explants or species. The protocol thus optimized a highly coordinated pathway led by the interaction of auxin and cytokinin (Fig. 3) into plant regeneration directly and *via* callus phase in *Vanilla* while indicating the amount of variability that can be generated through this system. Thus it is the first report in *Vanilla* to establish an efficient indirect plant regeneration system *via* callus applicable to both cultivated *V.planifolia* and wild and endangered, *V.aphylla*.

Table 1. Effect of auxins on callus induction and plant regeneration in *Vanilla planifolia*

Explant type	Media@	Callus induction rate (%)*	No. of shoots regenerated
Immature seeds	Control	0.40 ± 0.89 e	0.0 c
	+ NAA (2.68)	79.4 ± 4.88 ab	0.0 c
	+ IBA (2.46)	0.0 e	0.0 c
	+ BA (4.44) + NAA (2.68)	76.0 ± 4.30 b	4.80 ± 3.70 b
	+ BA (4.44) + IBA (2.46)	0.60 ± 1.34 e	0.0 c
Mature seeds	Control	1.0 ± 1.73 e	0.20 ± 0.45 c
	+ NAA (2.68)	82.0 ± 2.55 a	1.40 ± 2.61 c
	+ IBA (2.46)	0.2 ± 0.45 e	0.0 c
	+ BA (4.44) + NAA (2.68)	80.4 ± 2.97 a	11.0 ± 2.74a
	+ BA (4.44) + IBA (2.46)	10.8 ± 2.77 c	1.0 ± 1.22 c
Shoot base	Control	0.60 ± 0.89 e	0 c
	+ NAA (2.68)	5.80 ± 6.06 d	0.2 ± 0.45 c
	+ IBA (2.46)	0.0 e	0 c
	+ BA (4.44) + NAA (2.68)	81.60 ± 5.46 a	11.8 ± 2.77 a
	+ BA (4.44) + IBA (2.46)	6.60 ± 4.98 d	1.20 ± 1.10 c

@ Concentrations of growth regulators are in µM

**Mean ± S.D. Means in the same column followed by different letters are significantly different at 0.05% probability level*

Table 2. Effect of growth regulators on multiple shoot and root induction from shoot explants of *Vanilla planifolia* on MS medium*

Growth regulators (µM)				Multiple shoots frequency (%)*	Average no. of shoots/culture±SD*	Roots development /culture	
Kin	BA	NAA	IBA			No	Type
2.32	-	-	-	0.0		-	-
4.64				0.0		-	-
-	2.22	-	-	71 ± 3.45	4.18 ± 0.30	-	-
	4.44			20 ± 3.63	1.0	-	-
-	-	2.68	-	0.0	1.0	1	Velamen
		5.37		0.0	1.0	1	Velamen
-	-	-	2.46	0.0	1.0	1	Long roots
			4.92	0.0	1.0	1	Long roots
2.32	2.22			0.0	1.0	-	-
4.64	4.44			0.0	1.0	-	-
2.32	4.44			0.0	1.0	-	-
2.32		2.68		0.0	1.0	1	velamen
4.64		2.68		0.0	1.0	1	branching
2.32		5.37		0.0	1.0	1	velamen
2.32			2.46	0.0	1.0	1	-
4.64			2.46	0.0	1.0	1	-
2.32			4.92	0.0	1.0	1	-
	2.22		2.46	0.0	1.0	1	
	4.44		2.46	97 ± 6.5	15.15 ± 3.63	-	-
	2.22		4.92	65 ± 11.4	10.35 ± 3.45	-	-
0.0	0.0	0.0	0.0	0.0	1.0	1	Healthy roots

**Mean of 20 replicates*

Table 3. Variations in plant characters observed at the nursery stage among a few callus regenerated progenies of *V. planifolia* (six months after planting out)

No.	Progenies	Plant height (cm) ±S.D	Internodal length (cm) ±S.D	Leaf size (cm)	
				Length ±S.D	Breadth ±S.D
1	Sc21	14.35±1.00	1.05±0.29	**3.86**±0.30	1.25±0.23
2	Sc24	18.40±2.03	**2.55**±0.40	3.10±0.72	**2.50**±0.24
3	Sc32	9.80±1.27	0.86±0.52	1.54±0.34	2.24±0.35
4	Sc39	12.03±1.80	1.82±0.45	2.02±0.42	1.72±0.41
5	Sc40	**19.64**±1.45	2.21±0.39	1.82±0.36	1.70±0.41
6	Sc43	14.19±2.06	2.07±0.34	2.02±0.31	1.62±0.34
7	Sc47	7.30±1.01	1.66±0.37	1.22±0.42	1.23±0.33
8	Sc3.1	11.60±1.26	1.57±0.38	2.82±0.70	0.75±0.23
9	*V.planifolia*	12.30±1.44	2.03±0.29	3.39±0.30	2.11±0.29

values are an average of 20 observations; S.D: Standard Deviation

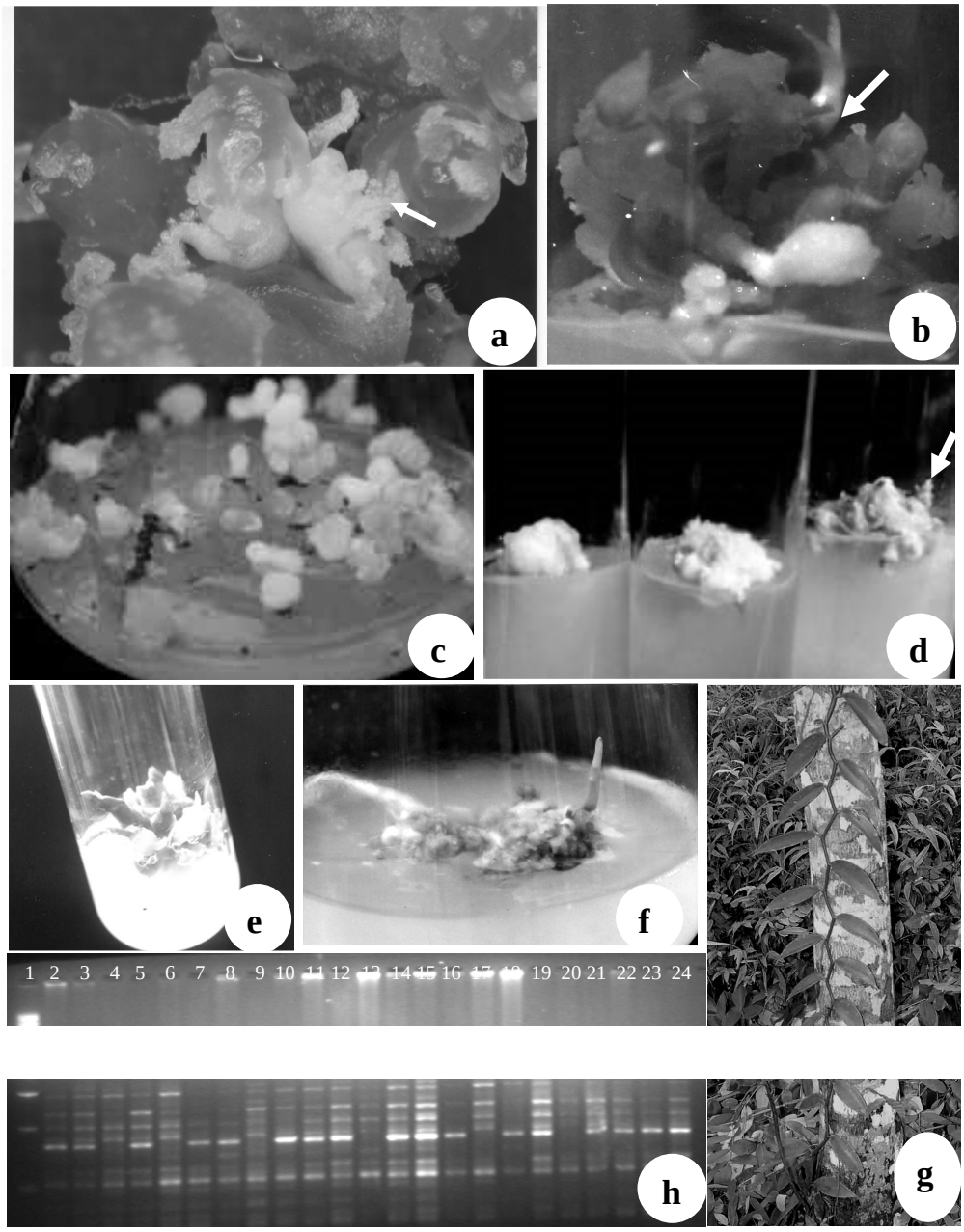

Fig. 1. Plant regeneration from callus cultures in Vanilla

a. Organogenic callus induced in shoot cultures of *V.planifolia*, b. Shoot regeneration from callus, c. Callus induction from seeds, d. Plant regeneration from seed callus, e. Induction of callus from shoot bases of *V.aphylla*, f. Plant regeneration from callus cultures of *V.aphylla*, g. Somaclone with small internode, h. RAPD profiles of **callus regenerated plants of vanilla using OPERON primer** OPA 20 Lanes 1 . 1 kb ladder 2 :V8.1; 3 : V56.1; 4 : V92; 5 : V92.1; 6 : V98.1; 7 : V101; 8 : V101.1; 9 : V112; 10: V112.1; 11 ; 115.1; 12 : V124.1; 13 : V142.1; 14 : V156.1; 15 ; V161.1; 16 : V179; 17 : V179.1; 18 : V258.1; 19 : V1.1R; 20 : V2R; 21 : V7.1; 22 : V7.1R; 23 : V53R ; 24: *V.planifolia* (Control), i. Variations in chromosome numbers in adjacent cells in a callus regenerated variant

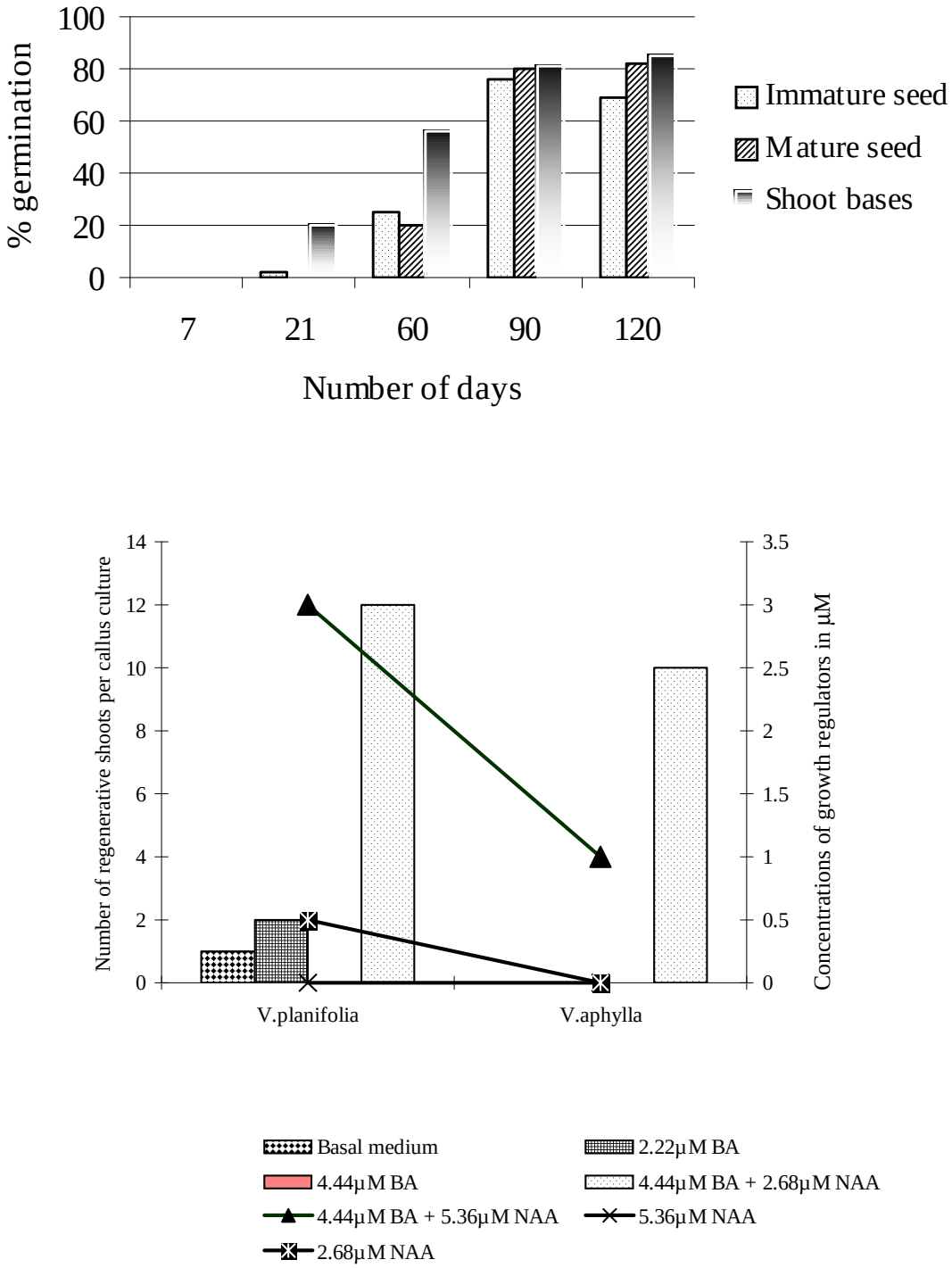

Fig. 3. Callus regenerative potentials influenced by auxin-cytokinin interaction in *V.planifolia* and *V.aphylla*

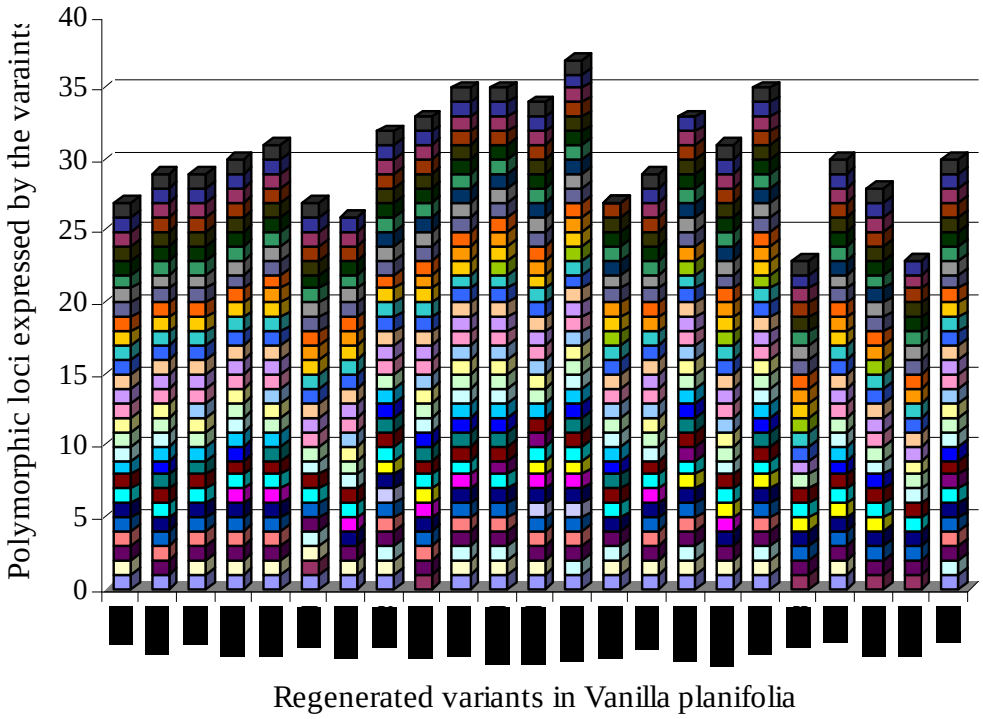

Fig. 5 Graphic representation of the polymorphism expressed by callus regenerated progenies of vanilla in pooled RAPD data

Acknowledgement
The authors are thankful to the scientists and staff of Indian Institute of Spices Research, India for help during the course of the study.

REFERENCES

[1] Prevost B A. Vanilla is appreciated the world over. In Proc. Vanilla 2003. 1st Int. Congress, Nov. 11-12, Princeton, NJ 08540, New Jersey, USA, **2003**.
[2] Purseglove JW, Brown EG, Green CL, Robbins SRJ . Spices. Vol. 2. Tropical Agricultural Series. Longman Inc., New York. **1981**, p 644-735.
[3] Gopinath C Secret of vanilla – Farmer's notebook on vanilla. Indian Spice Associates, Puttur, Karnataka. **1994**, p.31.
[4] Lubinsky P, Conservation of wild vanilla. In Proc. Vanilla 2003. 1st International Congress, Nov. 11-12, Princeton, NJ 08540, New Jersey, USA. **2003**.
[5] Nair RR and Ravindran P N *Caryologia* , **1994**, 47, 65-74.
[6] Nirmal Babu K, Ravindran PN, Peter KV. Protocols for Micropropagation of Spices and Aromatic Crops. Indian Institute of Spices Research, India. **1997**,35 p.
[7] Minoo D, Sajina A, Nirmal Babu K, Ravindran P N Ovule culture of vanilla and its potential in crop improvement. In. Edison S, Ramana KV, Sasikumar B, Nirmal Babu K & Santhosh J. Eapen (eds.). Biotechnology of Spices, Medicinal and Aromatic Plants, Indian Society for Spices, Calicut, India, **1997**, p. 112-118,.
[8] Davidonis G, Knorr D *Food Biotechnology*, **1991**, 5, 59-66.
[9] Gu Z, Arditti J, Nyman LT,. *Lindleyana*,**1987**, 2, 48-52.
[10] Murashige T, Skoog F. *Physiol Plant*, **1962**, 15, 473-497.
[11] Minoo D Somaclonal variation and their characterization in *Vanilla*. Ph.D Thesis, University of Calicut, **2002**, India.
[12] Minoo D, Nirmal Babu K, Ravindran PN, Peter KV. *Scientia Horticulturae*, **2006b**, 108 (4), 414-422.
[13] Roy J, Banerjee N *Ind. J. Exp. Biol.* **2001**, 39, 1041-1047.
[14] Bai Y, Qu R *Plant Breeding*, **2000**, 120, 239 – 242.
[15] Delporte F, Li S, Jacquemin JM *Plant Cell Tiss Org Cult*, **2005**, 80, 139-149.
[16] Chang C, Chang WC *Plant Cell Rep*, **1998**, 17, 251-255.
[17] Chen Y, Chang, C C, Chang WC *In Vitro/Plant*, **2000**, 36, 420-423.
[18] Lin YH, Chang C, Chang W C *Plant Cell Tiss Org Cult*, **2000**, 62,21-25.
[19] Philip V J, Nainar *J. Plant Physiol*. **1986**, 122, 211-215.
[20] George PS, Ravishankar GA *Plant Cell Rep*, **1997**, 16, 490-494.
[21] Philip V.J, Nainar SAZ *Annals of Bot*, **1988**, 61, 193-199.
[22] Minoo D, Nirmal Babu K, Peter KV *Scientia Horticulturae*, **2006a**, 110 (2): 175-180.
[23] Kapoor P, Rao UI *Plant Cell Tissue Org Cult*, **2006**, 85, 211 – 217.
[24] Nielsen JM, Brandt K, Hansen J *Plant Cell Tiss Org Cult*, **1993**, 35, 173.
[25] Sutter EG General laboratory requirements, media and sterilization methods. In. Plant Tissue Culture concepts and laboratory exercises. (Trigiano RN & Gray DJ (Eds.). CRC press, Inc., New York. **1996**, p.11.
[26] Giridhar P, Ravishankar GA *Indian J. Biotechnol*, **2004**, 3 : 113-118.
[27] Giridhar P, Vijaya Ramu D, Ravishankar GA *Tropical Sci.*, **2003**, 43, 92-95. DOI: 10.1002/ts.96.
[28] Rao YS, Madhosoodanan K.J, Naidu R *J. Plantation Crops*, **1993**, 21, 363-365.
[29] Myo Ma Ma Than, Amita Pal and Sumita Jha *Propagation of Ornamental Plants*, **2012**, 12(2), 102-108.