Novel approaches for Molecular Analyses, Micropropagation and Curing of Vanilla (*Vanilla planifolia*)

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By

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Affectionately Dedicated to

"PRUTHVI"

– The Mother EARTH, she who nurtures you and me!

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DECLARATION

I, **Sreedhar R.V.**, declare that this thesis entitled **"Novel approaches for Molecular Analyses, Micropropagation and Curing of Vanilla (*Vanilla planifolia*)"** is the result of research work done by me under the supervision of **Dr. Bhagyalakshmi Neelwarne** at Plant Cell Biotechnology Department of Central Food Technological Research Institute, Mysore- 570 020, India during the period of January 2004 to February 2009. I am submitting this thesis for the award of **Doctor of Philosophy (Ph.D.)** degree in **BIOTECHNOLOGY** of the University of Mysore.

I further declare that this thesis has not been submitted by me for the award of any other degree / diploma of this or any other University.

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CERTIFICATE

This is to certify that the thesis entitled **"Novel approaches for Molecular Analyses, Micropropagation and Curing of Vanilla (*Vanilla planifolia*)"** submitted by **Mr. Sreedhar R.V.** to the **University of Mysore** for the award of the degree of **Doctor of Philosophy** in **Biotechnology** is the result of research work carried out by him under my guidance in **Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore** during the period of January 2004 to February 2009.

Place: Mysore
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Abstract

Vanilla is the most popular flavor both by monitory and tonnage basis finding vast applications in food, pharmaceutical, beverage and cosmetic industries. Natural vanilla flavor is obtained from careful curing of vanilla beans and it is the second most expensive spice traded in the world market. In a study for assessing the diversity among Indian vanilla clones and to create a database for the available germplasm, analysis of isozymes as markers was found to be inappropriate due to inconsistencies in zymograms of enzymes. On the other hand, genetic markers such as RAPD and ISSR were found highly suitable. The genetic diversity among 25 accessions collected from 13 major locations was studied. The PCR-amplification patterns within accessions were similar for a given primer indicating that the morphological difference observed had no genetic background. Molecular analysis among different accessions also yielded identical PCR band profiles in both RAPD and ISSR analyses. These results clearly indicate that *V. planifolia* cultivated in India appears to share the same genetic background and therefore, the genetic diversity is either extremely low or non-existing.

Occurrence of genetic variants during micropropagation is occasionally encountered when the cultures are maintained *in vitro* for long period. Through an investigation using RAPD and ISSR markers, micropropagated multiple shoots of vanilla established and multiplied *in vitro* for over 10 years were assessed which established that micropropagation protocol used in this study can be carried out for a considerable length of time without any risk of genetic instability. Vanilla shoot multiplication in semi-solid (SS), complete immersion system (CIS) and partial immersion system (PIS) were evaluated for biomass, shoot multiplication and elongation aiming at developing an improved micropropagation protocol. Growtek^{TM} bioreactor, functioning on the principle of PIS, was found to be most suitable for micropropagation of vanilla. For automation of the micropropagation system, different medium contact periods were provided to shoot cultures in CIS in which the 30 min contact three times a day appeared most congenial. Similarly a bioreactor developed with intermittent bathing of the cultures with nutrient medium was congenial for shoot multiplication of vanilla. A combination of red soil: sand: vermicompost in equi-proportion was found to be the best for greenhouse hardening. Field-evaluation showed that the micropropagated plants were early to flower and high yielders than the conventionally propagated ones.

The vanilla shoots cultured under completely immersed condition showed hyperhydricity syndrome (HHS). A study focusing on unraveling the major structural, biochemical and molecular changes occurring during HHS was carried out. The HHS was associated with severe damages at cellular and sub-cellular levels, increase in free polyamines and accumulation of water, and decrease in quantities of chlorophyll, protein and drastic changes in reducing and non-reducing sugars. The onset and progression towards hyperhydricity (HH) showed higher activities of antioxidant enzymes, indicative of shoots' defensive efforts against oxidative stress. Thirty one HH-associated cDNAs identified by DDRT-PCR were cloned and sequenced whose electronic homology searches using BLASTX analysis resulted in the identification of 23 cDNA clones showing homology with various stress, apoptosis, DNA repair and carbohydrate breakdown related proteins expressed differentially during HHS. BLASTN analysis yielded 18 fragments having homology with different stress linked cDNA clones. A partially characterized transcriptome of hyperhydric condition in *V. planifolia* has been developed which paves the way for a better insight into gene expression during this common physiological disorder.

Vanilla beans derived from the micropropagated plants along with the beans available commercially were cured following different biotechnological approaches for development of an efficient curing technique. In this study, effects of pre-treatments on the flavor formation during accelerated curing at 38 ^{o}C for 40 days were studied. Use of naphthalene acetic acid (5 mg/L) or ethrel (1%) with blanching pre-treatment resulted in 3-fold higher vanillin on 10th day. All major quality parameters analyzed were found comparable to commercial sample. In another experiment, food-grade elicitors were used in combination with pre-treatments for the accelerated curing of beans. When acetone dried red beet elicitor - a rich source of peroxidase was used, 2.65% vanillin was formed in 10 days, which was 1.7-fold higher than in control beans of this study and 3.2-fold higher than the conventional curing. HPLC analysis of elicitor-treated samples showed the formation of almost all the major compounds found in the conventionally cured beans (cured for 3-6 months) with better sensorial properties. These observations appear useful for developing a rapid process for the curing of vanilla beans.

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LIST OF ABBREVIATIONS

AFLP	:	Amplified Fragment Length Polymorphism
APS	:	Ammonium persulphate
BAP	:	Benzylaminopurine
BLAST	:	Basic Local Alignment Search Tool
bp	:	base pairs
BSA	:	Bovine Serum Albumin
BSR	:	Beet Seedling Root
º C	:	Degree Centigrade
CAT	:	Catalase
cDNA	:	Complementary Deoxyribonucleic Acid
CES	:	Cellulase
CIS	:	Complete Immersion System
cm	:	Centimeter
CMC	:	Carboxymethyl cellulose
DAC	:	Days After Curing
DCP	:	Dry Cell Powder
DD	:	Differential Display
DDRT-PCR	:	Differrential Display Reverse Transcription- PCR
DIG	:	Digoxigenin
DMF	:	Dimethyl formamide
DNA	:	Deoxyribonucleic Acid
dNTP	:	Deoxynucleotide triphosphate
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid
IPTG	:	Isopropyl-β–D–thiogalactopyranoside
kb	:	Kilobase
3’	:	Hydroxyl- terminus of DNA molecule
5’	:	Phosphate-terminus of DNA molecule
d	:	day
DNase I	:	Deoxyribonuclease I
EST	:	Expressed Sequence Tag
FW	:	Fresh Weight
g	:	gram
GANP	:	Germinal center associated nuclear protein
GLUC	:	Glucosidase
H	:	Hour
ha	:	hectare
HH	:	Hyperhydricity
HHS	:	Hyperhydricity syndrome
HPLC	:	High Performance Liquid Chromatography
ISSR	:	Inter Simple Sequence Repeats
kg	:	kilogram
LB	:	Luria- Bertani (medium)
L	:	Litre
M	:	Molar
MAP	:	Microtubule associated protein

MAPK	:	Mitogen activated protein kinase
mg	:	milli gram
min	:	minute
mL	:	milli Liter
mM	:	milli Molar
mm	:	milli meter
MOPS	:	4-Morpholinepropanesulfonic acid
mRNA	:	messenger RNA
MS	:	Murashige and Skoog
mS	:	milli Siemen
N	:	Normal
NAA	:	Naphthalene acetic acid
nm	:	nanometer
ng	:	Nano gram
nt	:	nucleotide
NCBI	:	National Centre for Biotechnology Information
NBT	:	Nitroblue tetrazolium
OD	:	Optical density
OPA	:	Operon A
PAs	:	Polyamines
PAGE	:	Polyacrylamide gel electrophoresis
PAL	:	Phenylalanine ammonium lyase
PCA	:	Principal Component Analysis
PCR	:	Polymerase Chain Reaction
PEG	:	Polyethylene glycol
pG1	:	Polygalacturonase
POD	:	Peroxidase
PP	:	Phenyl propanoid
QDA	:	Quantitative Descriptive Analysis
qRT-PCR	:	quantitative Reverse Transcriptase Polymerase Chain Reaction
RAPD	:	Rapid Amplification of Polymorphic DNA
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rRNA	:	ribosomal RNA
rpm	:	revolution per minute
RT	:	Room Temperature
RT-PCR	:	Reverse Transcriptase Polymerase Chain Reaction
SA	:	Salicylic acid
SAGE	:	Serial Analysis of Gene Expression
SD	:	Standard Deviation
SDS	:	Sodium dodecyl sulphate
Sec	:	Second
SEM	:	Scanning Electron Microscopy
SOD	:	Superoxide dismutase
SS	:	Semi-solid
SSR	:	Simple Sequence Repeat
TAE	:	Tris-acetate-EDTA
Taq	:	*Thermus aquaticus*
TBE	:	Tris-Borate-EDTA

TE	:	Tris-EDTA buffer
TIS	:	Temporary Immersion System
Tris	:	Tris (hydroxymethyl) amino methane
X-GAL	:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
U	:	Unit enzyme
UBC	:	University of British Columbia
UTM	:	Universal Texture Measurement
UV	:	Ultra Violet
V	:	Volt
v/v	:	Volume per volume
WAI	:	Week after inoculation
w/v	:	Weight per volume
α	:	Alpha
β	:	Beta
µg	:	Micro gram
µM	:	Micro molar
µL	:	Micro liter
%	:	Percent

G1. General Introduction

Vanilla is the most popular and widely used flavor by both monitory and tonnage basis. Being the second most expensive spice traded in world market only after saffron (Minoo et al. 2008), natural vanilla flavor is obtained as an extract from cured vanilla beans and is universally used as aromatic flavoring in food, pharmaceutical, beverage and cosmetic industries. The top category beans classified as "gourmet" grade are above 15 cm or more in length and are selected from A grade beans having over 1.75% vanillin (the chief flavouring compound) and 25-30% moisture content. Approximately 20-25% of the total production from well maintained farms often account for this grade. Natural vanilla consumption in India at present is only about 5% of a total consumption of 400-500 tonnes per year (Indian Food Industry, 2008). Export of Indian vanilla has steeply gone up from 125 tonnes in 2006-2007 season to 200 tonnes in 2007-2008 season showing 133% increase from the target of 150 tonnes (Spice India, 2008).

The major vanilla producing countries and their market sizes are presented in **Table G1**. Madagascar being the largest producer of vanilla accounts for 67% of market share, followed by Papua New Guinea with 9% share. Both Uganda and Indonesia produce 7% each of world vanilla whereas India is 4th major producer with a production of nearly 120 MT.

Table G1. World Vanilla Market (2007) (Gassenmeier et al. 2008)

Country	Production (MT)	Proportion of market (%)
Madagascar	1500	67
Papua New Guinea	200	9
Uganda	150	7
Indonesia	150	7
India	120	5
Comores Island/Tahiti	70	3
Mexico	40	2

G1.1 The Plant

Vanilla in wild is found as a climber. It is an herbaceous perennial succulent vine, climbing up trees or other supports by means of adventitious roots. During cultivation vines are trained to a height that facilitates hand pollination and harvesting. The long cylindrical succulent green stem is 1-2 cm in diameter and the leaves are large, flat, fleshy, subsessile, alternate and oblong-elliptical to lanceonate. The stout racemose inflorescence is axillary, normally simple but rarely branched (**Figure G1**). Flowering is seen usually from December to May and it takes 45-60 days from initiation of inflorescence to flowering. The tip of the column of the flower bears a single stamen with two masses (pollinia) covered by a cap or hood like structure called 'rostellum', which separates stamen from stigma and prevents natural pollination. The flower is so constructed that self-pollination is rather difficult unless hand-pollinated. Stingless bees of the genus Melipona (*Apoidea*) and humming birds are known to pollinate some flowers in Mexico and Central America and elsewhere hand-pollination is practiced.

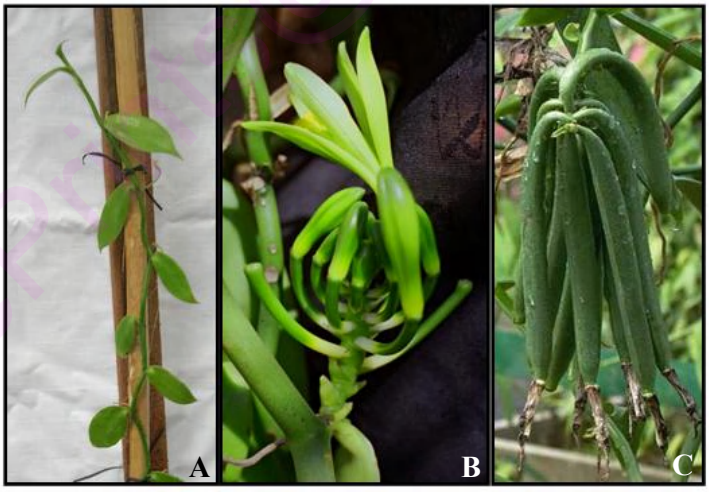

Figure G1. A: Vanilla plant; B: Inflorescence; C: Beans

G 1.2 The Bean

The syncarpous fruit of *Vanilla planifolia* develops from an inferior ovary and splits open along the three lines at maturity becoming a capsule. There are two principal regions in the fruit: The fruit wall or the 'green' region which includes epidermis, ground and vascular tissues. The 'white' region composed of three parietal placentae and the three bands of glandular hairs between them. The glandular hairs play a role in the biosynthesis of the flavouring compounds. The most important for the vanilla bean are the unusual glandular hairs that begin to develop quickly in the regions between the placentae. Vanillin and related intermediates of vanillin biosynthetic pathway accumulate in the inner white tissue of a developing vanilla pod, around placental hairs. This information may be important for understanding the rationale for the control of the curing process.

G 1.3 Chemistry of vanilla bean

The distinct aroma imparted by cured vanilla beans is due to a group of phenolic aromatic compounds accounting for over 170 flavoring compounds, of which the major ones are vanillin, vanillic acid, para-hydroxybenzoic acid and para-hydroxybenzaldehyde. The constituents responsible for the aroma and flavor are the volatiles such as aromatic carbonyls, aromatic esters, aromatic alcohols, aromatic acids, phenols, phenol ethers, aliphatic alcohols, carbonyls, acids, esters and lactones, aromatic hydrocarbons, terpenoids, aliphatic hydrocarbons and heterocyclics. The nonvolatile constituents are the tannins, polyphenols, resins and free amino acids. All these constituents together produce the delicate, rich and mellow aroma with spicy, woody and balsamic notes. The highest quantity of about 2-2.5% of the dry matter of cured pod is due to vanillin, the cost of latter is about a hundred-fold higher than the synthetic one. Concentrations of major volatile compounds in cured vanilla bean are presented in **Table G2**. Earlier studies have noted that vanillin or its glycoside do not accumulate in the interior of cells may be to avert the reactivity and possible toxicity of the carbonyl group. Therefore, vanillin biosynthesis in *Vanilla* species occurs in specialized cells where vanillin is glycosylated and expelled from the cellular interiors. This suggests that biological cells not equipped to deal with the cellular turnover of

vanillin might be ill equipped to accomplish vanillin production (Havkin-Frenkel and Belanger 2008).

Table G2. Concentrations of major volatile compounds in cured vanilla bean (Perez-silva et al. 2006)

Sl.No.	Compound	Concentration (ppm)
	Phenols	
1	Vanillin	19118±1124.71
2	Vanillic acid	1315±77.78
3	p-Hydroxybenzaldehyde	873.3±55.6
4	p-Hydroxybenzoicacid	255±13.44
5	Vanillyl alcohol	83.8±2.97
6	p-Hydroxybenzyl alcohol	65.1±4.88
	Aliphatic acids	
1	Linoleic acid	225.6±17.25
2	Hexadecanoic acid	126.6±5.94
3	Acetic acid	124.3±11.1
4	Oleic acid	16.3±1.56
5	Nonanoic acid	15.7±1.73
	Aromatic acids	
1	Cinnamic acid (isomer 2)	9.5±1.13
2	Benzene propanoic acid	3.9±0.28
3	Cinnamic acid (isomer 1)	3.4±0.57
4	Benzoic acid	2.6±0.35
	Alcohols	
1	2,3-Butanediol (isomer 1)	16.5±1.77
2	2,3-Butanediol (isomer 2)	8±0.14
3	Benzyl alcohol	2.7±0.21
4	1-Octanol	1.1±008
	Aldehydes	
1	2-Heptenal	2.1±0.28
2	2-Decenal	1.8±0.16
3	2,4-Decadienal	1.4±0.11
	Esters	
1	Ethyl linolenate	13.5±0.35
2	Anisyl formate	2.3±0.35
3	Methyl cinnamate	1.1±0.07
	Hydrocarbons	
1	Pentacosine	19.9±1.48
2	Tricosane	15.9±2.19

This brings to the fore the particular suitability of green vanilla beans for vanillin production by direct enzymatic treatments. Proper agronomic practices and

selection of lines producing high levels of vanillin could result in high quality vanilla pods since the present cultivation practices show that glucovanillin can accumulate to levels of up to 20% of the dry weight (Havkin-Frenkel and Belanger 2008). This being the case, with proper control of curing process, it might be possible to obtain cured beans with 10% vanillin (Havkin-Frenkel and Belanger 2008).

G1.4 Qualitative variations among vanilla species

Based on qualitative variations in the aroma and flavor of beans of vanilla various species and their geographical origins are as follows:

Bourbon vanilla (*V. planifolia* Andrews) - It is the collective term used for the beans of Madagascar, Reunion, the Comoro islands and the Seychelles origin. Bourbon vanilla is characterized by its sweet, creamy, rich, full-bodied, tobacco-like, woody and animal, and deep balsamic, sweet spicy flavor back notes.

Mexican vanilla (*V. planifolia* Andrews) - The flavor notes in this are described as sharp, slightly pungent and sweet spicy notes lacking body compared to Bourbon vanilla.

Java vanilla (*V. planifolia* Andrews) - This is from Indonesian islands and is less sweet and creamy than Bourbon type. It lacks bouquet note and has a strong woody and slightly smoky character with a freshly sharpened pencil note.

Indian vanilla (*V. planifolia* Andrews) - The flavor of carefully cured Indian vanilla is full-bodied but less sweet and creamy than Bourbon vanilla. It lacks balsamic note but has slightly spicy and pungent sour notes.

Uganda vanilla (*V. planifolia* Andrews) - These beans almost have aroma and flavor similar to Bourbon vanilla but less creamy and sweet.

Thaitian vanilla (*V. tahitiensis*) - This type has a distinctly perfumey and flowery, fragrant, helitropine-like, with a rather shallow vanilla character.

Papua New Guinea (PNG) vanilla - (*V. tahitiensis*, *V. hapape* or *V. politi*) – It has a weak flowery and perfumey note and possess anisic notes. It is weak in flavor and aroma character.

Guadeloup vanilla (*V. pompana*) - This species of Central America possesses a sweet anisic holiotropine-like fragrance.

Vanilla species, growing conditions, soil nutrition, harvest maturity of the beans and the curing method are known to determine the ratio of various flavor constituents of cured beans. Vanillin is the major abundant flavor and aroma chemical present in cured vanilla beans (normally varies between 0.3-3%). The other major aromatic constituents are p-hydroxybenzaldehyde (0.12-0.15%), vanillic acid (nearly 0.1%), p-hydroxybenzoic acid (nearly 0.02%), p-hydroxy benzyl methyl ether (nearly 0.02%) and acetic acid (nearly 0.02%).

G2. Vanillin

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the most widely appreciated flavor compound with an odor threshold of 11.8 × 10-14 M for humans (Buccellato 2005). It has the unique characteristic that even at a very high dose, the flavor is perceivable. Vanillin is found to have antimicrobial, antioxidant, antimutagenic, anticlastogenic and anticancerous activities. Its antimicrobial property on fungi *Aspergillus flavus*, *Bacillus subtilus* and *Staphylococcus aureus* makes it a potential food preservative. It is also of general interest because of its biogenetic relationship to the phenylpropanoid pathway and other molecules of physiological significance, notably salicylate.

G2.1 Natural occurrence of Vanillin

It is found in traces in many plants. Essential oils like clove, cinnamon and mace contain vanillin. Plants from the genus *Vanilla* have large amounts of vanillin. *Vanilla planifolia* (syn. *V. fragrans*), *V. tahitensis* and *V. pompona* of the family Orchidaceae are the commercially cultivated species for the production of natural vanilla flavor where *V. planifolia* is the most preferred one.

G2.2 Site of vanillin synthesis in vanilla beans

Some early studies indicated vanillin secretion 'in tissue around seeds'. A comprehensive localization study in developing fruit using catechin-HCl which binds to various phenolic compounds including vanillin as a staining agent revealed that endocarp parenchymatic cells contained vanillin and intermediates in the biosynthetic pathway. A descending staining gradient from endocarp in the fruit cavity outwards was observed indicating the site of synthesis. The study also showed that vanillin accumulation begins after 3 to 4 months of fruit development. As vanillin is sparingly soluble in water, particularly in acidic plant vacuoles, glycosylation of vanillin to glucovanillin is a likely mechanism for increasing the hydrophilicity of the compound, thus aiding in the sequestering and storage of the compound in aqueous extracellular regions. Special cells in the pod interior are thought to be dedicated to vanillin biosynthesis (Havkin-Frenkel and Belanger 2008).

G2.3 Biosynthesis of vanilla flavor compounds:

Though extensive studies have been made on the biosynthesis of vanillin and allied flavor compounds in plant and cell cultures, several questions remain unanswered. A common observation in all studies is the involvement of the shikimate pathway, and phenylalanine (phenylpropanoid) pathway in this. The stage of oxidation of C_3 side chain to yield aldehydes function could be before or after the formation of 3-methoxy, 4-hydroxy substitution pattern in the aromatic ring. It is not yet clear whether vanillin is derived from the lignin precursors having an alcohol function in the C_3 part of phenylpropanoid, or from the cinnamic acid type (acid group). Kanisaw et al. (1994) proposed that major pathway would go via 4-coumaric acid glucoside, which is the precursor for para-hydroxybenzaldehyde glucoside, the central intermediate for the biosynthesis of the glucosides A and B as well as vanillin. Various experiments with vanilla cell cultures give different results which might be due to the fact that different biosynthetic pathways operate in the beans and cell cultures. **Figure G2** shows the formation of vanillin through different ways in a complex network of compounds.

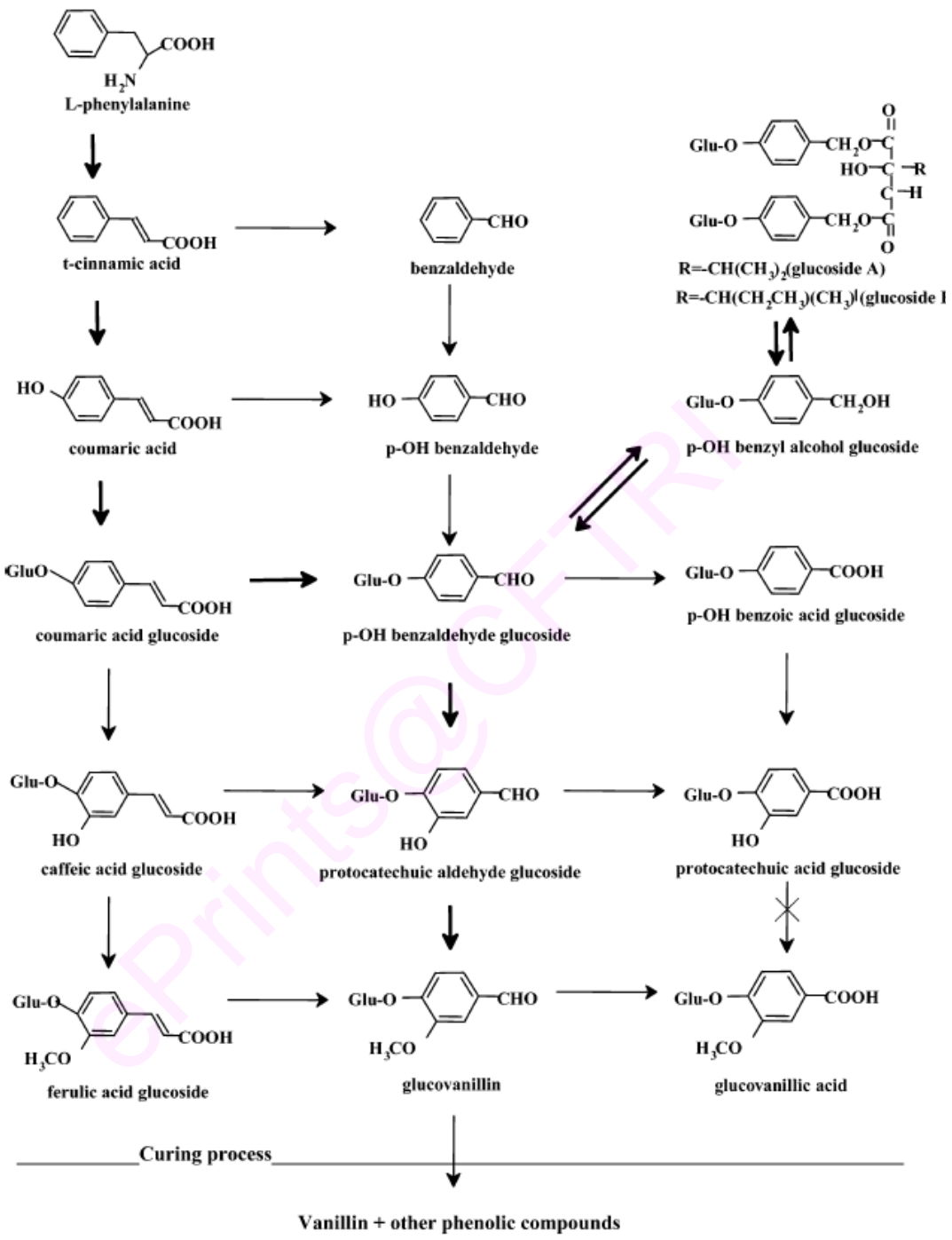

Figure G2. Shikimic acid pathway showing the formation of vanilla flavour compounds

G2.4 Other sources of Vanillin

Though several plant species produce vanillin in traces (**Table G3**), committed biosynthetic route has been identified only in *Vanilla* species and the biosynthetic pathways in others are still not clear. Various biotechnological approaches have been explored to produce natural vanillin at a lower price through various microorganisms, cell-free systems using enzymatic degradation and plant tissue culture.

Table G3. Vanillin content in different plants

Species	Plant part	% Dry weight
Unicorn plant (*Proboscidae cuisianica*)	Roots and pods	0.01
Potato (*Solanum tuberosum*)	Tuber skin	0.01
Clove (*Syzygium aromaticum*)	Dry flower buds	Traces
Narcissus (*Triandrus narcissi*, *Tazetta arsissi*)	Roots and basal plate	0.01-0.60
Hyacinth (*Hyacinhus orientalis*)	Roots and basal plate	0.20-0.50
Vanilla planifolia	Pod (cured)	1.00-8.00
Vanilla tahitensis	Pod (cured)	0.05-2.00
Vanilla pompana	Pod (cured)	0.01-2.00

G3. The Vanilla 'CRISIS'

The worldwide consumption of vanilla exceeded production throughout 1990's. This reduced the shortfall continuously reducing the surplus of earlier years which lead to surplus exhaust in 2000. Recognizing this shortfall, the extractors began heavy buying in late 1999 and the producers predictably increased the price seeing the growing demand. The ill-timed Cyclone 'Hudah' arrived shortly after and the initial reports showed 80% damage to vanilla crop in Madagascar though the actual damage was modest. Buyers quickly protected themselves against perceived shortage and this doubled the price overnight. Higher prices encouraged growers to plant more vanilla and on the other hand manufacturers of food and beverages reacted predictably by searching for alternatives for natural vanilla. Both these activities took time and so the for the next three years, vanilla prices spiraled upward. By the end of 2003, worldwide consumption of approximately 1100 metric tons was roughly half of what it had been in 1999 and the prices exceeded $500 per kg cured beans, nearly 15 times higher than they were four years ago (Rick 2006).

Presently, vanilla industry is experiencing a remarkable resurgence in growth and popularity and will probably take a decade to reach the level of consumption which existed before the crisis. Like all who experience and survive crisis, the industry is expected to emerge stronger and healthier. Production is likely to exceed consumption keeping prices low and stable. This rebound to natural vanilla has already started primarily in the retail (home) and food service (restaurant) segments but yet to get initiated in the large food and beverage manufacturers as they are adamant to reformulate their product to the use of natural vanilla again. Ultimately, consumer will decide what to use and the manufacturers need to rebound.

One major factor impacting vanilla consumption, post-crisis is the demand for healthier natural products. Vanilla is one of the only a handful of flavors to achieve organic certification. Natural vanillas are GMO-free and the Fair Trade vanilla will be another opportunity for the food and beverage manufacturers to position their products for todays environmentally and socially conscious consumers.

G4. Origin and Dissemination of Vanilla

The Vanilla genus contains more than 800 genera distributed in more than 25,000 species (Govaerts et al. 2006). The basic chromosome number of genus vanilla is X=16 and *V. planifolia* is a diploid with 2n=32. The genus Vanilla belongs to the Orchidaceae family (largest plant family) and *V. planifolia* is probably endemic from eastern Mexico tropical forests and its natural habitat roughly follows a straight line between the Oaxaca state towards Guatemala and Belize **(Figure G3**) (Soto Arenas 1999a). Another Vanilla species, *V. tahitensis* J.W. Moore is cultivated in several Pacific countries. Some other aromatic species grown locally or harvested in the wild having no economical importance are *V. pompona* Schiede in the West Indies, *V. chamissonis* Klotzsch in Brazil, *V. odorata* C. Presl in America, *V. claviculata* (W. Wright) Sw., *V. griffithii* Rchb. f. and *V. abundiflora* J.J.Sm. in the West Indies and in Asia (Soto Arenas 2003). The genus Vanilla is widely distributed throughout tropical and subtropical regions around the world (Indonesia, South and Central America, Mexico and Africa), and this distribution supports the theory that it is very old genus. Three species have economic value and are the *Vanilla planifolia* Andrews (earlier known as *V. planifolia*), *Vanilla pompana* Schiede and *Vanilla tahitensis* J.W. Moore. *V. planifolia* is the most cultivated species whereas the *V. pompana* is more resistant to diseases. *V. tahitensis* yields a distinctly different flavor and the beans are more expensive compared to the other two.

G4.1 *V. planifolia* in its area of origin and introduction

G4.1.1 The history of *V. planifolia* in Mexico

The pre-Columbian history of vanilla in Mexico is poorly documented. Stehle´ (1952) refers to archive documents dating from 1427 and mentions the gathering of vanilla pods by Aztecs. Vanilla belongs to a group of native plants in the Maya Lowlands that could have been subjected to human selection before 3400 B.C. The pods were used for medicinal purposes, as mentioned in a 1552 Aztec herbal, as well as to flavour the hot chocolate drinks valued among the Aztec nobles. The importance of vanilla since early times in Mexico, is evident by the mention of offering vanilla as a medicinal beverage as part of a tribute during reign of

Itzco´atl (Aztec Emperor) in 1427, and citing vanilla as a remedy for fatigue in Badianus manuscript in 1552 (Lubinsky 2004). Establishment of first vanilla plantation was by the Totonac Indians (in the Veracruz region), particularly in the Papantla and Misantla areas (**Figure G3**), from 1767 which marks the start of vanilla cultivation. According to Ecott (2004), Soto Arenas considers that Totonac Indians did not use manual pollination to produce vanilla pods. There is no evidence of manual pollination before the 19th century. From 1841, the technique of manual pollination discovered in Europe was transferred to Mexico and Totonac Indians became the world most important producers, until the supremacy of Madagascar in 1924 (Lucas 1990).

Figure G3. Geographical localization of Mexican and Meso-American places (adopted from Bory et al. 2008)

V. planifolia in Mexico is cultivated mainly in two areas: northern Veracruz and northern Oaxaca (**Figure G3**). Plants in Oaxaca were established by the end of the 1980's from regional spontaneous specimens and those cultivated in Veracruzs were established two centuries ago whose origin is not known. They are less variable and very different from the cultivated populations of Oaxaca.

G4.1.2 *V. planifolia* in introduced areas

After C. Colombus discovered the New World in 1492, the first record of dissemination of vanilla from Mexico is by Father Labat who imported three *V. planifolia* vines into Martinique in 1697 and from there to Guadeloupe in 1701. It was then introduced in Reunion Island in 1793. Early in the 19th century, one major event is *V. planifolia* introduction by Marquis of Blandford into the collection of C. Greville at Paddington where it flowered in 1807. Greville then supposedly sent some cuttings to the botanical gardens of Antwerp (Belgium) and from there to Paris (Correll 1953). From the Botanical Garden of Antwerp, it was introduced into Buitenzorg in Java in 1819 by Marchal (Purseglove et al. 1981) and in Reunion Island from the Jardin du Roi in Paris in 1822 by the ordinance officer of Bourbon, Marchant.

Vanilla was introduced to Europe from Mexico, in about 1,500 numbers and its reputation of being an aphrodisiac followed it to countries where it was introduced. An introduction event is documented in India in 1835 but the plant died after flowering (Correll 1953). Lack of natural pollinators in the areas of introduction prevented sexual reproduction and pod production until the first half of the 19th century. The British introduced *V. planifolia* into India about 200 years ago where five other species are native viz, *V. pilifera* Holtt., *V. andamanica* Rolfe, *V. aphylla* Blume, *V. walkeriae* Wight and *V. wightiana* Lindl. *V. pilifera* originally described from Malaya, recorded in Thailand is found in the Mikir hills of North East India.

It is commonly agreed that *V. planifolia* cultivation started from a very narrow genetic pool (Soto Arenas 1999b; Lubinsky 2003), and that the extensive vegetative reproduction that followed might be responsible for the relatively high genetic uniformity observed to date in vanilla crops. Soto Arenas even suggested that most *V. planifolia* cultivated specimens around the world came from a unique clone (of the 'Mansa' type) exported from Mexico (Ecott 2004) to the C. Greville collection at Paddington and disseminated by cuttings via the botanical gardens of Paris and Antwerp, from where it would have been disseminated worldwide (Correll 1953). However, in many areas of introduction, some phenotypic

variations are described. Some hypotheses are proposed to explain the morphological variability in cultivation areas like the existence of different introduction events, accumulation of somatic mutations, the possible role of sexual recombination, and the existence of epigenetic phenomena.

G4.2 Diversity analysis

Apart from responding to the challenges of improving food security and fostering socio-economic development, Biotechnological applications must also promote the conservation, diversification and sustainable use of plant genetic resources for food and agriculture. Currently the Biotechnology toolbox available to plant breeders offers several new possibilities for increasing productivity, crop diversification and production, while developing a more sustainable agriculture. Molecular markers have already played a major role in the genetic characterization and improvement of many crop species. They have also contributed to and greatly expanded our abilities to assess biodiversity, reconstruct accurate phylogenetic relationships, and understand the structure, evolution and interaction of plant and microbial populations. Molecular markers are now routinely used for characterization of genetic diversity, DNA fingerprinting, genome mapping, genome evolution, ecology, taxonomy, and plant breeding.

DNA-based markers are abundant, highly polymorphic and independent of environment or tissue type. Most DNA-based markers can be classified into three categories based on the technique used: Hybridization-based DNA markers, arbitrarily primed Polymerase Chain Reaction (PCR)-based markers, and sequence targeted and single locus DNA markers. Restriction fragment length polymorphism (RFLP) markers are based on hybridization in which DNA polymorphism is detected by digesting DNA with restriction enzymes followed by DNA blotting and hybridizations with probes. Arbitrarily primed PCR-based markers are employed in organisms for which no genome sequence is available.

Molecular markers have been successfully used to study introduced species and proven to be more useful tools in identifying the source of introduction and variability due to a new environment. Some widely used markers and their applications are given in **Table G4**. PCR-based random amplified

polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) have been widely used to survey genetic structure of populations. Among various molecular markers, the RAPD technique is simple, rapid, and requires only a few nanograms of DNA, has no requirement of prior information of the DNA sequence and has feasibility of automation with higher frequency of polymorphism, which makes it suitable for routine application for the analysis of genetic diversity (Babu et al. 2007). It is also proven to be quite efficient in detecting genetic variations, even in closely related organisms like two near isogenic lines of tomato (Martin et al. 1991). For the use of ISSRs, primers are not proprietary as in Microsatellites or Simple Sequence Repeats (SSRs) and can be synthesized by anyone and also allow the production of a high number of reproducible polymorphic bands. ISSR is found to be very simple, quick, cost-effective, highly discriminative and most reliable method which combines most of the advantages of SSRs and Amplified Fragment Length Polymorphism (AFLP) to the universality of RAPD (Reddy et al. 2002). ISSRs though considered mostly as dominant markers; they are shown to segregate co-dominantly in some cases (Sankar and Moore 2001) thus enabling distinction between homozygotes and heterozygotes. They are found to be more useful and reproducible than isozymes, RAPD and RFLP (Fang et al. 1997) and are also known to give more polymorphism than any other assay procedure (Virk et al. 2000). Detection of additional polymorphism could be done by the use of RAPD in combination with ISSRs (Joshi et al. 2000). Martins et al. (2004) suggest the use of a combination of two types of markers that amplify different regions of the genome and hence a better analysis of genetic variation can be made.

Table G4. Widely used markers and their applications

Markers	Applications
PCR Based markers	
AFLP: Amplified Fragment Length Polymorphism (D)	Fingerprinting, mapping, F1, Varietal identification, Gene tagging, Marker-assisted selection, Map-based gene cloning
CAPS: Cleaved Amplified Polymorphic Sequences (CD) **SCAR:** Sequence Characterized Amplified Region (CD)	Framework mapping, Can be converted to allele-specific probes, F1 identification, Gene tagging, Bulk segregant analysis, Diversity studies, Marker-assisted selection, Map-based cloning
EST: Expressed Sequence Tag (D/CD) **STS:** Sequence Tagged Site (D/CD)	Fingerprinting, Varietal identification, Genetic maps, F1 identification, Gene tagging and identification, Bulk segregant analysis, Diversity studies, Marker-assisted selection, Novel allele detection, High-resolution mapping, Map-based cloning
IPCR: Inverse Polymerase Chain Reaction (CD) **IRAP:** Inter-Retrotransposon Amplified Polymorphism (CD) **REMAP:** Retrotransposon-Microsatellite Amplified Polymorphism (CD)	Fingerprinting, Varietal identification F1 identification, Gene tagging Bulk segregant analysis, Diversity studies Marker-assisted selection, High-resolution mapping, Seed testing
ISSR: Inter-Simple Sequence Repeat amplification (D)	Fingerprinting, Varietal/line identification, Genetic maps, F1 identification, Gene tagging Breeding, Bulk segregant analysis, Diversity studies, Marker-assisted selection, High-resolution mapping, Seed testing
RAPD: Random Amplified Polymorphic DNA (D)	Genetic maps, F1 identification, Varietal/line identification, Breeding, Bulk segregant analysis, Diversity studies, Marker-assisted selection, Seed testing, Map-based gene cloning
SNP: Single Nucleotide Polymorphism (CD)	Genetic maps, F1 identification, Breeding, Gene tagging, Alien gene introduction, Bulk segregant analysis, Diversity studies, Novel allele detections, Marker-assisted selection, High resolution mapping
SSR: Simple Sequence Repeat (CD)	Fingerprinting, Varietal/line identification, Framework /region specific mapping, Genetic maps, F1 identification, Comparative mapping, Breeding, Bulk segregant analysis, Diversity studies, Novel allele detections, Marker-assisted selection, High-resolution mapping, Seed testing

Non PCR-based marker	
RFLP: Restriction Fragment Length Polymorphism (CD)	Comparative maps, Framework maps, Genetic maps, Breeding, Varietal/line identification, Marker-assisted selection, F1 identification, Diversity studies, Novel allele detections, Gene tagging, Bulk segregant analysis, Map-based gene cloning

D: Dominant, CD: Co-Dominant

G4.2.1 Genetic Diversity of Vanilla

The first molecular data on specimens from crops in northern Veracruz, Oaxaca and other Mexican regions obtained using iso-enzymes show low levels of total genetic variation. A molecular analysis using RAPD markers in Meso-America by Schlu¨ter (2002) differentiated *V. planifolia* from Costa Rica and Mexico. Among Mexican *V. planifolia*, two main groups were revealed: individuals from Oaxaca, Chiapas and Quintana Roo on one hand, and individuals from Veracruz, Federal District of Mexico, San Luis Potosi, Tabasco and Oaxaca on the other hand. The individuals from Oaxaca and Tabasco present in the second group most probably correspond to specimens that were collected from the Veracruz region at the time of the establishment of new crops. Attempts to study some hypervariable regions, such as introns in three protein coding genes (alcohol dehydrogenase, calmoduline and glyceraldehyde 3- phosphate dehydrogenase) in order to detect intraspecific sequence variations were unsuccessful due to lack in polymorphism (Soto Arenas 1999b). Vanilla is naturally distributed in Mexico and parts of Central America and the history of origin of cultivated vanilla suggests that the entire stock outside Mexico may be from a single genetic source. A study on genetic diversity of *V. planifolia* by Besse et al. (2004) using RAPD markers in vanilla cultivated areas of Reunion Island and Polynesia reveled a very low level of diversity.

G4.2.2 Diversity in Indian Vanilla

Vanilla cultivation was initiated in India, through the East India Company, nearly 250 years back in the spice garden at Kurtallam in Tamil Nadu (George 2005). Its organized cultivation started in 2001-2002 in 1600 ha yielding 60 tonnes of cured vanilla beans and steadily gained importance doubling its cultivation to 3427 ha in 2003-2004 resulting in the production of 131 tonnes

(http://www.foodindianews.com). In India, only a few cultivars have been recognized from the species *V. planifolia*. In the countries where vanilla has been introduced, variability is likely to be highly limited as the species is propagated only vegetatively. However, seed germination is also reported for vanilla (Havkin-Frenkel and Dorn 1997) indicating the possibility of bird dispersal leading to the chance for variations in the populations. The only study on genetic diversity of Indian vanilla using molecular markers is by Minoo et al. (2008). RAPD analysis of two indigenous collections of *V. planifolia* made from vanilla cultivating regions of India reveled that there is very limited variation within the collections indicative of its narrow genetic base. An estimation of genetic diversity in Indian vanilla is extremely essential to protect it from erosion due to epidemic diseases and pests apart from planning strategies for conservation of the genetic resources (secondary gene pools and cultivated resources).

G5. Micropropagation

Vanilla is generally propagated by stem cutting which is a slow, time consuming and labour intensive process. Removal of cuttings may also cause injury to the mother plant resulting in a set-back of growth and reduction in yield. Micropropagated plantlets have gained importance in the vanilla cultivating areas as the market demand for the propagules is hardly met through these cuttings. In vitro propagation of vanilla has been established by culturing axillary buds (George and Ravishankar 1997; Geetha and Shetty 2000; Giridhar et al. 2001, Kalimuthu et al. 2006), aerial root tips (Philip and Nainar 1988), through callus (Davidonis and Knorr 1991) and protocorms. Micropropagation using shoot tips or nodal segments has been found as an appropriate technique for clonal propagation of vanilla (George and Ravishankar 1997).

Mass propagation of plants by tissue culture is a costly and labour intensive technology. The gelling agents used are not inert medium components and do not enable easy automation for commercial mass propagation. Agar, the most commonly used non-nutrient gelling agent is one of the costliest ingredients of the culture medium. Use of liquid media, scale-up in bioreactors are some of the alternatives explored for minimization of the cost of production of micro-

propagules (Hvoslef-Eide and Melby 2000; Dey 2001). Low-cost culture vessels and minimization of contamination are the other options available. Techniques at present for the micropropagation need a large number of containers, gelled media and aseptic conditions and a complicated and costly production technology. It involves periodic transfer of plant material to fresh media, after subcultures, due to exhaustion of the nutrients in the medium, continuous proliferation of the plant material and limited size of the container. Though liquid culture systems are considered as advantageous in term of uniform culture conditions, ease for change of the medium and reduced manual labour requirement, it is usually associated with hyperhydricity disorder. An intermittent exposure of the culture to liquid medium rather than continuous was found to solve this problem. For this the bioreactors developed earlier do not suit as they are mainly adapted to bacterial culture and do not take in to consideration specific requirements of plant cultures like the shear force, mechanical damages or foam formation in bubble aerated bioreactors (Berthouly and Etienne 2005). High production cost limits the commercial use of micropropagation to markets with a high unit value, such as vanilla. It has been concluded for various species that extensive expansion of micropropagation would only take place if new technologies became available to automate procedures and acclimatization protocols were improved (Kitto 1997).

G6. Hyperhydricity syndrome

Switching over to submerged cultivation is the first step towards automation of shoot cultures in a micropropagation industry. Initial trials for this are normally conducted using shake-flask cultures, often known as complete immersion system (CIS) as the shoots are continuously bathed in liquid medium. During cultivation *in vitro*, the plantlets are exposed to a wide range of stress conditions caused by high relative humidity, gas accumulation in the headspace, altered nutrient/hormonal combinations and non-congenial osmoticity of the culture medium. Although most plant cultures adapt to changes in environmental conditions, some of them become abnormal with turgid, translucent, less green, watery, hypo-lignified, wrinkled and brittle appearance. This phenomenon, known as hyperhydricity syndrome (HHS), can lead to irreversible loss of multiplication as well as regenerative potential. HHS has also been a generic

problem in continuous cultivation or scale-up of plant organs *in vitro*. Such a shift towards hyperhydricity has been linked to various metabolic disorders, changed array of proteins and altered stress responsive pathways.

G6.1 Genetic analysis by Differential Display

There are many methods to analyze physiological disorders in plants. Differential display (DD) (Liang and Pardee 1992) and RNA arbitrary primed polymerase chain reaction (RAP-PCR) (McClelland et al. 1993) were simultaneously introduced in 1992 as a means to identify and compare genes expressed as mRNA in various cell populations under specified conditions (Liang and Pardee 1995). The DDRT-PCR technique only requires small amounts of RNA and it allows the comparison of several RNA populations simultaneously (Bosch and Lohmann 1997; Jorgensen et al. 1997). Nonetheless differential display has advantages over other methods which include: the use of small amounts of total RNA, the identification of mRNA species independent of prevalence (Wan et al. 1996), and the detection of rare and abundant transcripts of both known and novel genes (Matz and Lukyanov 1998). Though effective, the technique does have some drawbacks. The major limitation is the high incidence of false positive. However the single greatest contributor to false positives is arbitrarily primed PCR using short oligos (Matz and Lukyanov 1998), which can be solved with the use of longer arbitrary primers (Zhao et al. 1995). Classical differential display employs the use of a degenerate single base or two-base- anchored oligo-dT primer [$d(T)_{11}$ NN where N= G, A, T, C] to reverse transcribe a subset of the total mRNA population extracted from cell lines or tissues under various conditions (Liang and Pardee 1992). Amplification of the cDNA by PCR using the 3' anchored oligo dT primer and a 5' arbitrary decamer which anneals randomly along the length of the cDNA results in the amplification of a subset of the total cDNA population, which is resolved by denaturing polyacrylamide get electrophoresis (PAGE). In order to increase the cDNA amplified, it may be necessary to perform multiple amplifications with different primer combinations, although redundancy in cDNA amplification is likely as degenerative decamer may anneal upstream of a cDNA already represented in an amplified subpopulation of another primer set (Matz and Lukyanov 1998). Given that twenty different arbitrary decamers (priming as 6- to

7- mers) combined with twelve different combinations of oligo $d(T)_{11}$ MN primers are estimated to be required to display the approximately fifteen thousand individual mRNA species expressed in an average eukaryotic cell (Liang and Pardee 1992; 1995), which likely represents an underestimate if system redundancy is taken into account, many amplifications and gels would need to be run to display the whole set, a disadvantage of differential display.

Differentially expressed genes are represented by bands that are present on the gel in one sample but not in another, or at different intensities indicating varying levels of abundance between samples (Matz and Lukyanov 1998). Thus specific advantages of differential display include the simultaneous detection of both up- regulated and down-regulated genes and the comparison of more than two RNA populations; in fact limitations to the number of comparisons that can be done are only imposed by the size of the gel.

Some of the notable advantages of differential display are:

1. A minimal amount of starting material is required. (Differential Display can be performed with as little as 200 μg of total RNA per sample)
2. Multiple samples can be analyzed simultaneously. (Alternative methods are limited to comparing two samples, whereas Differential Display is limited only by the number of lanes available on the gel apparatus)
3. Sensitivity (As Differential Display is a PCR- based procedure, transcripts present at very low copy numbers are included in the analysis)
4. Speed

Genes involved in physiological events, stress responses, signal transduction and secondary metabolism have been isolated and characterized. Some of the isolated genes encode transcription factors, membrane proteins and rare enzymes that were previously difficult to purify. These results suggest that differential display is a powerful tool used to investigate the rare genes involved in the plant life cycle without using information from proteins. The stress response genes affected by environmental factors such as ultraviolet (UV) light exposure, extreme temperatures, oxygen, salt and desiccation was isolated using by DD and characterized (Yamazaki and Saito 2002). A well focused study unraveling the major structural, biochemical and molecular changes occurring during

hydrophonic cultivation of shoot cultures is needed for a better understanding of the hyperhydricity syndrome.

G7. Curing of Vanilla

Natural 'vanilla flavor' comprises of a large array of aromatic compounds formed after systematic curing of the beans of *Vanilla planifolia*. The major compound vanillin is the most preferred flavouring compound among the universally used aromas and has a great market potential in food, beverage, cosmetic and pharmaceutical industries. Sixty five volatiles and 26 odour-active compounds are identified in the extract of cured vanilla beans (Perez-Silva et al. 2006); the major ones after vanillin are vanillic acid, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde (**Figure G4**). In green vanilla beans, these phenolic aromatic compounds are present as their respective glucosides major being glucovanillin (**Figure G4**) synthesized from phenylalanine of shikimic acid pathway and curing process is meant to release the aglycones as the free aroma compounds. Curing also induces the formation of many other compounds that complement to the delicate aroma of natural vanilla flavour. In fact, it is the presence of these minor compounds in large numbers that fetch high price for natural "vanilla extract".

Botanical study of vanilla beans reveals that the flavour precursors are found in the bean interior, i.e., placental region around the seeds, whereas the hydrolytic and other degenerative enzymes that are known to catalyze the reactions for the release of flavour compounds are localized mostly in the outer fruit wall (Havkin-Frenkel et al. 2005). The purpose of curing is to create contact between the flavour precursors and the enzymes that catalyze the hydrolysis of precursor compounds (Havkin-Frenkel et al. 2004).

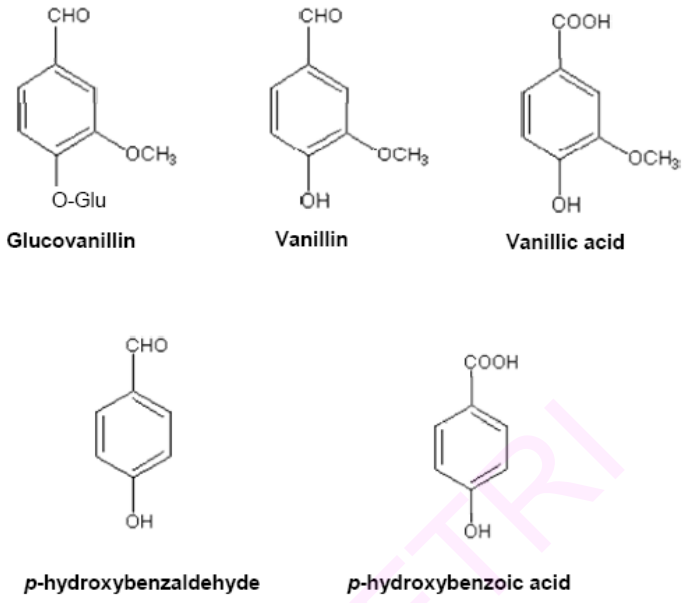

Figure G4. Chemical structures of the major flavor compounds found in cured beans of *Vanilla planifolia*

Curing of vanilla beans is a traditionally well-established process which is laborious and takes 3 to 6 months depending on different curing procedures adopted in different vanilla-producing regions (Havkin-Frenkel et al. 2004). Despite the long time required for the curing process, the enzymatic transformation of the glycosides to flavouring compounds is not very efficient. Only a fraction of the vanillin is produced by systematic curing of green beans of which a part may also be lost during exposure to sun as well as during extraction (Frenkel and Havkin-Frenkel 2006). Finally, the total flavour yield depends upon the quality of starting material where the best quality beans range in length from 15-20 cm.

The conventional curing depends on weather conditions as it involves intermittent exposure to sun and sweating followed by conditioning (Dignum et al. 2001). Therefore, it is lengthy and cumbersome involving several months, may often fail to completely hydrolyze glucosides resulting in only fractions of flavour compounds (Frenkel and Havkin-Frenkel 2006).

To overcome the above problems, earlier workers used methods such as curing of the cut beans, covering beans in plastic sheets and heating them at 60 °C along with high humidity, freeze curing, treatment of green beans with various enzymes like β-glucosidase, pectinase and hemi-cellulase, hot-air drying and solar drying (Dignum et al. 2001; Ruiz-Teran 2001). The development of vanilla flavour during these treatments is partly due to the hydrolysis of glycosylated precursors occurring in the green bean (Arana 1943). The most important step in vanilla curing has been found to be the scalding followed by incubation at 45 ^{o}C (Jones and Vincente 1949). An almost the same process wherein scalding in hot water of 63 ^{o}C for 2-3 minutes, followed by cutting the beans into pieces and incubation in an oven at 38 ^{o}C for 48 h followed by further curing in closed containers at 38 ^{o}C for 2-3 months resulting in pleasing aroma was also suggested (Broderick 1956). From then onwards, blanching in hot water has been an essential step traditionally followed before curing of vanilla beans. This has been a convention for several decades in various vanilla-growing countries of the world. For imparting the mild temperature treatment, sunning of the fruits has also been a regular practice, which invariably leads to losses at each exposure.

Thus, there is a need for a process, which is simple and effective. Therefore, the present study aimed at developing a novel biotechnological curing process that reduces the processing time and increases the flavouring compounds in cured vanilla beans.

With this background, present research work was conducted with the following objectives

- To collect elite clones of Vanilla from different areas of Karnataka and Tamil Nadu and develop biochemical and genetic markers to identify varietal differences
- To study the kinetic parameters that affect shoot proliferation in shake flask and to standardize conditions for high rate of shoot multiplication in a bioreactor
- To study the pattern of hardening of plantlets derived from shoots multiplied in bioreactor and their field performance
- To study the novel differentially expressed mRNA transcripts in hyperhydric shoot cultures of *Vanilla planifolia* as depicted by Differential Display analysis
- To study the quality of green vanilla beans and their curing using various elicitors for increasing flavor production as well as reduction in curing period

The following chapters deal with the results obtained on experiments on genetic diversity of Indian vanilla, micropropagation, hyperhydricity syndrome of vanilla shoot cultures and novel improved methods for curing vanilla beans. The information thus obtained aids overall improvement of vanilla cultivation, flavor production and allied industries.

Summary

In the present study, use of isozymic markers to study genetic diversity was found to be inappropriate due to variation in zymogram of peroxidase enzyme within the plants. Therefore the usefulness of genetic markers such as RAPD and ISSR for assessing the diversity among clones of *V. planifolia* cultivated in India was evaluated. The genetic diversity among 25 accessions collected from 13 major locations was studied. Forty random amplified polymorphic DNA (RAPD) and 11 inter-simple sequence repeats (ISSR) primers resulted in 326 scorable bands ranging in size from 200 bp to 2800 bp and 83 scorable bands from 200 bp to 2500 bp, respectively. Banding pattern among the different samples collected within accessions was similar indicating that the morphological difference observed within accession had no genetic background. On the other hand, molecular analysis among different accessions from different locations also yielded identical PCR band profiles in both RAPD and ISSR analysis. These results clearly indicate that *V. planifolia* cultivated in India appears to share the same genetic background and therefore, the genetic diversity is extremely low. Hence other biotechnological approaches may be considered to induce genetic variations to improve genetic diversity in vanilla.

Publication

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1.1 Introduction

Vanilla planifolia (syn. *V. fragrans*), *V. tahitensis* and *V. pompona* of the family Orchidaceae are the commercially cultivated species for the production of natural vanilla flavour where *V. planifolia* is the most preferred one. Vanilla is cultivated in an area of 37,000 ha with a production of 2230 tonnes of cured beans globally (Gassenmeier et al. 2008). Natural vanilla flavour, extracted from cured vanilla beans, is one of the most important and universally used aromatic flavours in food, pharmaceutical, beverage and cosmetic industries. Vanilla was indigenous to Mexico and was introduced to Europe by the Spanish Conquistadores in 1520 (Dignum et al. 2001). Vanilla cultivation was initiated in India, through the East India Company, nearly 250 years back in the spice garden at Kurtallam in Tamil Nadu (George 2005). Its organized cultivation started in 2001-2002 in 1600 ha yielding 60 tonnes of cured vanilla beans and steadily gained importance doubling its cultivation to 3427 ha in 2003-2004 resulting in the production of 131 tonnes. Indian vanilla occupies 5% of International vanilla market with a production volume of nearly 120 tonnes (Gassenmeier et al. 2008). In India, only a few cultivars have been recognized from the species *V. planifolia*. In the countries where vanilla has been introduced, variability is likely to be highly limited as the species is propagated only vegetatively. However, seed germination is also reported for vanilla (Havkin-Frenkel and Dorn 1997) indicating the possibility of bird dispersal leading to the chance for variations in the populations. Therefore, it is necessary to analyze the extent of variations in *V. planifolia* plants collected from various locations in India.

Molecular markers have been successfully used to study introduced species and proven to be more useful tools in identifying the source of introduction and variability due to a new environment. Traditionally researchers employ isozymic markers to understand the genetic structure or variation in various plant species. Leaf isozymes were successfully utilized to study the genetic structure of various walnut cultivars (Vyas et al. 2003). Allozymic variation was studied in *Castilleja grisea* herb which led to conclusion that there existed a moderately high genetic diversity and that the herb might have not lost substantial genetic variation during 150 years of overgrazing (Helenurm et al. 2005). A combination of isozyme technique coupled with molecular markers such

as RAPD and AFLP have been used to study genetic diversity in various plants like Wyethia (Ayres and Ryan 1999), sunflower inbreds (Popov et al. 2002) and Lentil (Sultana and Ghafoor 2008). PCR-based random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) have been widely used to survey genetic structure of populations. Among various molecular markers, the RAPD technique is simple, rapid, and requires only a few nanograms of DNA, has no requirement of prior information of the DNA sequence and has feasibility of automation with higher frequency of polymorphism, which makes it suitable for routine application for the analysis of genetic diversity (Babu et al. 2007). It is also proven to be quite efficient in detecting genetic variations, even in closely related organisms like two near isogenic lines of tomato (Martin et al. 1991). For the use of ISSRs, primers are not proprietary as in Microsatellites or Simple Sequence Repeats (SSRs) and can be synthesized by anyone and also allow the production of a high number of reproducible polymorphic bands. ISSR is found to be very simple, quick, cost-effective, highly discriminative and most reliable method which combines most of the advantages of SSRs and Amplified Fragment Length Polymorphism (AFLP) to the universality of RAPD (Reddy et al. 2002). ISSRs though considered mostly as dominant markers; they are shown to segregate co-dominantly in some cases (Sankar and Moore 2001) thus enabling distinction between homozygotes and heterozygotes. They are found to be more useful and reproducible than isozymes, RAPD and RFLP (Fang et al. 1997) and are also known to give more polymorphism than any other assay procedure (Virk et al. 2000). Detection of additional polymorphism could be done by the use of RAPD in combination with ISSRs (Joshi et al. 2000). Martins et al. (2004) suggest the use of a combination of two types of markers that amplify different regions of the genome and hence a better analysis of genetic variation can be made.

1.1.1 Isozymic analysis

Isozyme or isoenzyme refers to any two distinguishable proteins catalyzing the same biochemical reaction. They are separated most commonly by horizontal gel electrophoresis. Characterization is primarily based on the basis of charge and size, and the visualization of the enzymatic activity is with specific substrates. These variants are extensively used as genetic markers in many crops. The technique involves simple inexpensive laboratory procedure and consumes less of time and money. Proteins from seeds or leaf tissue serve the purpose, avoiding

growing plant to maturity. Maize, tomato, *Brassica*, wheat, barley, soybean and sugar-beet are the major crops where this technique has been routinely applied.

1.1.2 RAPD

RAPD technique uses any DNA segment that is amplified using short oligo-deoxy-nucleotide primers of arbitrary nucleotide sequence (amplifiers) and polymerase chain reaction procedures (PCR). Random amplified polymorphic DNAs (RAPDs) are produced by PCR using genomic DNA and arbitrary primers. PCR is typically carried out using two random oligonucleotide primers that flank the DNA fragment to be amplified. These primers hybridise to complementary strands of the target sequence and are oriented so that DNA synthesis by the polymerase proceeds across the region between the primers. The result is an exponential accumulation of the specific target fragment by the *de novo* synthesis of the region of DNA flanked by the two primers. Any variation in the DNA sequence representing the genetic variation among different individuals leads to variation in the size or presence/absence of PCR product when amplified by RAPD primers. This is displayed as variation in the banding pattern when separated on agarose or acrylamide gel.

1.1.3 ISSR

This technique is a variant of the PCR that uses simple sequence repeat primers (eg. $[AC]_n$) to amplify regions between their target sequences. The technique exploits the abundant and random distribution of SSRs in plant genomes by amplifying DNA sequences between closely linked SSRs. More complex banding patterns can be achieved using 5'-anchored primers that incorporate the SSR regions in their amplification. ISSR technique is nearly identical to RAPD technique except that the primer sequences are designed from microsatellite or SSR regions and the annealing temperatures used are higher than those used for RAPD. Any variation variation in the DNA sequence is depicted as change in the banding pattern after gel electrophoresis.

1.1.4 Genetic diversity analyses in vanilla and other orchids

Molecular markers have been successfully used for various studies in orchids. For instance, Pillon et al. (2006) studied genetic diversity in the genus *Dactylorhiza* using molecular markers and concluded that the diversity was greatest in the

Mediterranean basin and the Caucasus, which were then considered to be the major targets for conservation based on their results. They also showed that the results of phylogenetic analyses and genetic data obtained with molecular tools could offer an alternative measure of biodiversity that is not sensitive to taxonomic inflation. Study of genetic diversity and phylogenetic relationships among and within species of Cymbidiums using RAPD analysis showed full agreement with the groups identified by morphological, physiological and ecological characteristics. RAPD markers have also been successfully employed to reveal relationships and classifications in Cymbidiums at cultivar levels (Obara-Okeyo and Kako 1998; Ok et al. 2004). Chung et al. in 2006 successfully differentiated *Paphiopedilum* and *Phragmipedium* using RAPD which were in good agreement with morphologically-based classification. Schlu¨ter (2002) in Meso-America successfully used RAPD markers to differentiate *V. planifolia* from Costa Rica and Mexico. A study on genetic diversity of *V. planifolia* by Besse et al. (2004) using RAPD markers in vanilla cultivated areas of Reunion Island and Polynesia reveled that there exists a very low level of genetic diversity. The only study on genetic diversity of Indian vanilla using molecular markers is by Minoo et al. (2008). RAPD analysis of two indigenous collections of *V. planifolia* made from vanilla cultivating regions of India reveled that there is very limited variation within the collections indicative of its narrow genetic base.

In the present study, both the PCR based techniques, RAPD and ISSR, were adopted for the evaluation of genetic variation in *V. planifolia*. Therefore, the objectives of the present study were to assess the usefulness of genetic markers for assessing the diversity among clones of vanilla cultivated in India and to create a database for the available germplasm.

1.2 Materials and Methods

1.2.1 Plant sampling

Since commercial cultivation of vanilla in India is mainly concentrated in the states of Karnataka, Kerala and Tamil Nadu, leaf samples were collected from plantations of these states (**Figure 1.1; Table 1.1**). Standard procedures were followed for the collection of samples (Li et al. 2006) and preparation of DNA extract. Briefly, leaf samples from six different vines per accession were collected

by looking at the morphological variations. Samples were stored in plastic bags, which were kept at -20 ºC until DNA was extracted.

Table 1.1 List of accessions of *V. planifolia* and their geographical origin used in this study

Sl. No.	Population localities	No. of accessions*
1	Mysore, Karnataka	5
2	Hassan, Karnataka	1
3	Madikeri, Karnataka	2
4	Dakshina Kannada, Karnataka	3
5	Shimoga, Karnataka	6
6	Udupi, Karnataka	1
7	Coimbatore, Tamil Nadu	1
8	Kanyakumari, Tamil Nadu	1
9	Dindigul, Tamil Nadu	1
10	Ooty, Tamil Nadu	1
11	Mallapuram, Kerala	1
12	Kasargud, Kerala	1
13	Pallakad, Kerala	1

* Refer to Figure 1.1 for location of accessions.

1.2.2 Isozymic analysis of peroxidase (POD)

Native-PAGE analysis

For analyzing the isozymes of POD from different clones of vanilla, standard protocol was used. An initial experiment was conducted to check the variation in zymogram pattern within same clone. For this, leaf material obtained from different stages of leaves (unopened, first and fourth leaf pairs from shoot tip) from the same plant was used for extraction of the proteins. Protein extract was made in sodium phosphate buffer (pH 6.0) containing 1 mM Dithiothreitol and 0.1 mM Phenyl Methyl Sulfonyl Fluoride. Zymogram was prepared by polyacrylamide gel electrophoresis (7.2% (w/v)) (PAGE) carried out at 120 V for 4 h using 12 × 14 × 0.5 cm gel without SDS. The gel was stained for POD activity with a 100 ml solution of sodium phosphate buffer containing 10 ml of 0.25%

(w/v) orthodianisidine dihydrochloride and 10 ml of 1% (v/v) hydrogen peroxide and immediately photographed (Thimmaraju et al. 2007).

1.2.3 DNA extraction and quantification

Approximately 100 mg of young leaf tissue was ground into fine powder in liquid nitrogen and total genomic DNA was extracted using the GenElute^{TM} Plant Genomic DNA Mini prep Kit (Sigma Aldrich, India). Quality and quantity of DNA preparations were checked by standard spectrophotometry and the samples were diluted to 25 ng μL^{-1} in TE buffer and stored at 4 °C.

1.2.4 Primer selection

Various RAPD and ISSR primers were selected, based on specific relevance to family Orchidaceae to which vanilla belongs, from the studies of Besse et al. (2004), Tsai et al. (2002) and NCBI-database. Others were those for monocots that were successfully used in our earlier study in banana (Venkatachalam et al. 2007). Out of the 60 RAPD 10-mer primers and 20 ISSR primers, 40 RAPD and 11 ISSR primers were selected depending on their consistency in amplification (**Table 1.2**).

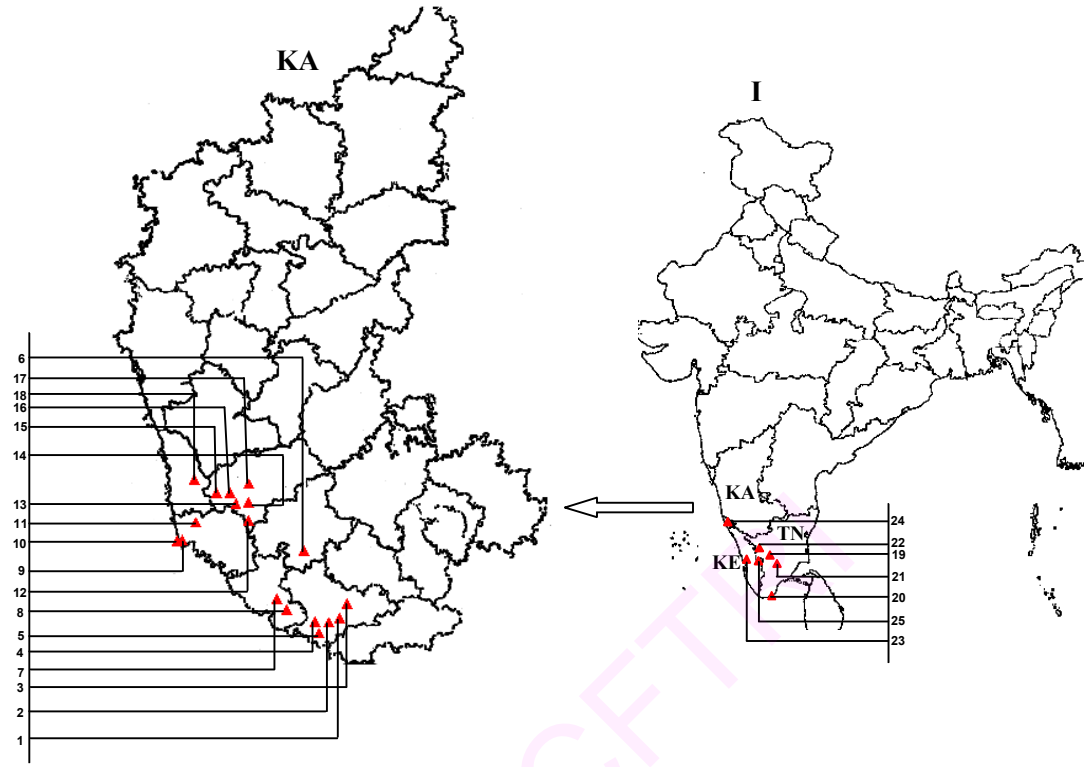

Figure 1.1 Map showing sampling locations (filled triangles) in India (I) and Karnataka (K)

Table 1.2 List of selected RAPD and ISSR primers

RAPD primers	
Kit OPA	A-03; A-04; A-11; A-14; A-20
Kit OPC	C-01; C-02; C-04; C-05; C-06; C-07; C-08; C-09; C-10; C-12
Kit OPD	D-04; D-11; D-16
Kit OPF	F-12
Kit OPJ	J-07; J-08; J-09; J-10; J-11; J-12; J-13; J-15; J-16; J-17; J-18; J-19
Kit OPM	M-16; M-18; M-20
Kit OPN	N-03; N-04; N-06; N-09; N-10; N-14
ISSR primers	
Kit UBC	809; 810; 811; 813; 823; 824; 826; 834; 836; 840; 848

1.2.5 DNA amplification

Optimum PCR conditions for both RAPD and ISSR were standardized with different quantities of template DNA (12.5, 25 and 50 ng), dNTPs (100, 200 and 300µM) and $MgCl_2$ (1, 2 and 3 mM). Based on the results, RAPD amplifications

were performed routinely using a PCR mixture (25 µL) which contained 25 ng of genomic DNA as template, 1X PCR buffer (Fermentas GMBH, Germany), 200 µM dNTPs (Fermentas GMBH, Germany), 1 unit (U) of *Taq* DNA polymerase (Bangalore Genei, India), 0.5 µM of each primer (Operon Technologies, Alameda, California, USA) with varied concentration of $MgCl_2$ (Fermentas GMBH, Germany) depending on the primer (**Table 1.3**). PCR was performed at initial denaturation at 93 °C for 4 min followed by 36 cycles of 1 min denaturation at 94 °C, 1 min annealing at 36 °C and 2 min extension at 72 °C with a final extension of 72 °C for 10 min using a thermal cycler (Eppendorf thermal cycler 5332, Germany).

For ISSR primers, optimal annealing temperature was found to vary according to the base compositions of the primers. PCR mixture (25 µL) contained 25 ng of genomic DNA as template, 1X PCR buffer, 200 µM dNTPs, 1 unit (U) of *Taq* DNA polymerase, 0.5 µM of each primer with varied concentration of $MgCl_2$ depending on the primer (**Table 1.4**). PCR was performed at initial denaturation at 94 °C for 4 min followed by 40 cycles of 1 min denaturation at 94 °C, 1 min at 2 °C lower than the specified annealing temperature for each primer and 2 min extension at 72 °C with a final extension also at 72 °C for 10 min using a thermal cycler.

1.2.6 Analysis of PCR product by agarose gel electrophoresis

The PCR products obtained were separated by gel electrophoresis on 2% agarose gel (ICN, USA), in 0.5X TBE buffer and stained with ethidium bromide (0.001%) and visualized by image analysis software documented in a gel documentation system (Lab works software, version 3.00,UVP Hero-Lab GMBH, Germany). The size of the amplification products was estimated from 100 bp DNA ladder (Fermentas GMBH, Germany).

1.2.7 Data analysis

Well-resolved and consistently reproducible fragments ranging form 200 bp-2.8 kb were scored as present or absent for both RAPD and ISSR markers for each sample. Comparison of RAPD and ISSR profiles of all the 21 DNA samples were done for the analysis. Genetic distances could not be established since there was not a single band differing from others in RAPD and ISSR reactions.

Table 1.3 List of selected primers used in RAPD analysis and number of scorable bands

Sl. No.	Primer	Primer sequence (5′–3′)	$MgCl_2$ concentration (mM)	No. of scorable bands
1	OPA 03	AGTCAGCCAC	0	11
2	OPA 04	AATCGGGCTG	1	5
3	OPA 11	CAATCGCCGT	0	10
4	OPA 14	CTCGTGCTGG	1	7
5	OPA 20	GTTGCGATCC	0	8
6	OPC 01	TTCGAGCCAG	0	7
7	OPC 02	GTGAGGGCTC	2	6
8	OPC 04	CCGCATCTAC	1	8
9	OPC 05	GATGACCGCC	1	8
10	OPC 06	GAACGGACTC	0	6
11	OPC 07	GTCCCGACGA	0	8
12	OPC 08	TGGACCGGTG	1	7
13	OPC 09	CTCACCGTCC	0	9
14	OPC 10	TCTCTGGGTG	0	3
15	OPC 12	TCTCATCCCC	1	7
16	OPD 04	TCTGGTGAGG	1	6
17	OPD 11	AGCGCCATTG	0	9
18	OPD 16	AGGGCGTAAG	0	8
19	OPF 12	ACGGTACCAG	1	8
20	OPJ 07	CCTCTCGACA	0	9
21	OPJ 08	CATACCGTGG	2	4
22	OPJ 09	TGAGCCTCAC	1	7
23	OPJ 10	AAGCCCGAGG	2	8
24	OPJ 11	ACTCCTGCGA	1	5
25	OPJ 12	GTCCCGTGGT	0	7
26	OPJ 13	CCACACTACC	1	6
27	OPJ 15	TGTAGCAGGG	0	5
28	OPJ 16	CTGCTTAGGG	1	8
29	OPJ 17	ACGCCAGTTC	2	11
30	OPJ 18	TGGTCGCAGA	2	6
31	OPJ 19	GGACACCACT	1	10
32	OPM 16	GTAACCAGCC	0	15
33	OPM 18	CACCATCCGC	2	6
34	OPM 20	AGGTCTTGGG	2	10
35	OPN 03	GGTACTCCCC	0	12
36	OPN 04	GACCGACCCA	1	10
37	OPN 06	GAGACGCACA	0	11
38	OPN 09	TGCCGGCTTG	2	11
39	OPN 10	ACAACTGGGG	1	14
40	OPN 14	TCGTGCGGGT	1	10

Table 1.4 List of selected primers used in ISSR analysis and number of scorable bands

Sl. No.	Primer name	Primer sequence (5′–3′)	$MgCl_2$ concentration (mM)	No. of scorable bands
1	UBC 809	$(AG)_8G$	0	4
2	UBC 810	$(GA)_8T$	0	9
3	UBC 811	$(GA)_8C$	0	10
4	UBC 813	$(CT)_8T$	1	5
5	UBC 823	$(TC)_8C$	0	4
6	UBC 824	$(TC)_8G$	0	6
7	UBC 826	$(AC)_8C$	1	15
8	UBC 834	$(AG)_8YT$	0	8
9	UBC 836	$(AG)_8YA$	0	5
10	UBC 840	$(GA)_8YT$	1	11
11	UBC 848	$(CA)_8RG$	0	6

Y : C or T

1.3 Results

A total of five isozymes of POD were observed in the zymogram out of which only three were found in the protein extracts from unopened and first leaf pair. Protein extract from the fourth leaf pair displayed all the five isozymes in the gel (**Figure 1.2)**. Standardization of conditions for PCR amplification such as concentrations of magnesium chloride, *Taq*-polymerase, template DNA and annealing temperature were initially varied to arrive at a most effective combination to obtain highly intense distinctly separated bands without smearing effects. Forty RAPD primers (**Table 1.2**) were selected from 60 arbitrary primers (having 60-70% GC content) based on their amplification products. Each RAPD primer generated a unique set of amplification products ranging in size from 200 bp to 2800 bp where the number of bands for each primer varied from 4 in OPJ 08 and 15 in OPM 16 (**Table 1.3**). The 40 primers used in this analysis yielded 326 scorable bands with an average of 8.15 bands per primer. Screening with the 20 ISSR primers generated 83 scorable bands in 11 primers (**Table 1.2**) ranging from 200 bp to 2500 bp. An average of 7.54 bands per ISSR primer was obtained ranging from 4 to 15 (**Table 1.4**). Banding pattern among the different samples collected within an accession was similar indicating that the morphological difference observed within accessions had no genetic background. On the other hand, molecular analysis among different accessions from different locations also

yielded an identical PCR band profile in both RAPD and ISSR analysis (**Figures 1.3, 1.4**).

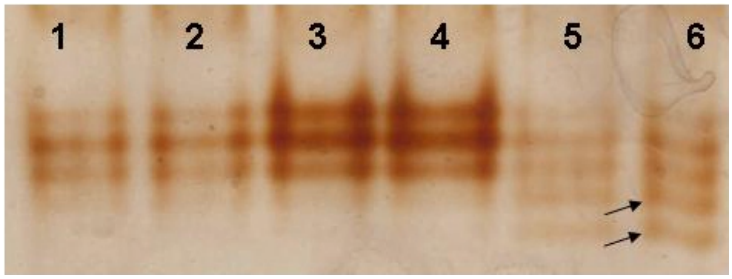

Figure 1.2 Isozymic pattern of peroxidase enzyme from leaves at different stages of development in vanilla. (1,2: un-opened leaf; 3,4:first leaf pair; 5,6:fourth leaf pair)

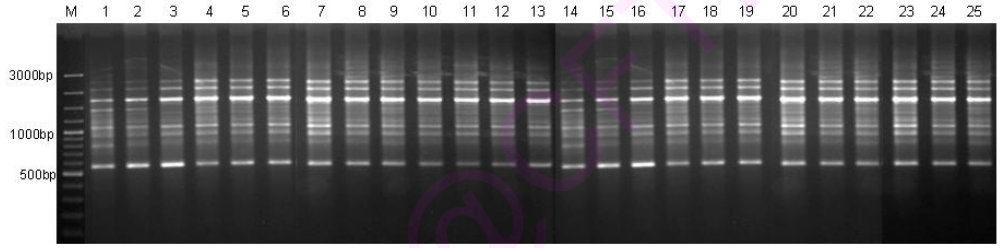

Figure 1.3 Randomly Amplified Polymorphic DNA (RAPD) banding pattern generated by primer OPJ 10. Lanes 1-25 designate accessions. Lane M: GeneRuler^{TM} 100 bp DNA Ladder Plus

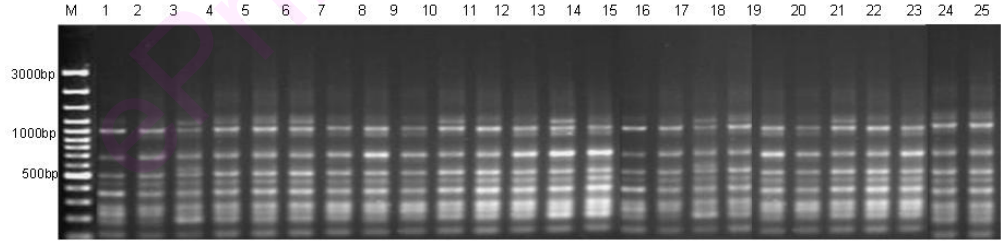

Figure 1.4 Inter Simple Sequence Repeats (ISSR) of DNA showing amplification pattern generated by primer UBC 810. Lanes 1-25 designate accessions. Lane M: GeneRuler^{TM} 100 bp DNA Ladder Plus

1.4 Discussion

Isozymic analysis of POD enzyme for its possible use in analysis of genetic diversity among various clones of vanilla showed that there was variation in the zymogram pattern within same clone between protein extracts of leaf material from different stage of development. This difference within same plant/clone may be due to variation in isozymes synthesized during various stages of growth and development of the plant depending on the biochemical status of the plant tissue. These observations suggested exclusion of isozymic analysis for diversity study in vanilla clones collected which otherwise lead to erroneous conclusions. Of the various biochemical and molecular techniques used to resolve genetic diversity in lentil, isozymes and seed proteins gave low levels of genetic diversity. RAPD was found to be the best option for determining inter- and intra-accession variation (Sultana and Ghafoor 2008). A population genetic study of *Goodyera procera* with allozyme and RAPD markers supported that RAPD can detect higher levels of genetic variation than allozyme (Wong and Sun 1999). In *Brassica oleracea*, high variability in the banding pattern within and among cultivars was observed (Arus et al. 1985). RAPD analysis proved to be more informative and effective approach for estimation of genetic diversity in inbred sunflower lines (Popov et al. 2002). The use of isozymes in limited by the lower number of polymorphic isozyme markers in many populations.

The present study involved two types of efficient genetic markers involving a large number of primers for marker-based genetic analyses of 25 accessions collected from different locations of India (**Figure 1.1; Table 1.1**). This study has clearly showed the absence of genetic variation within and among *V. planifolia* populations. A very low level of genetic diversity was detected in *V. planifolia* in geographical areas such as Mexico (Soto and Arenas 1996; Cibrian 1999), Reunion Island (Besse et al. 2004) and Polynesia (Pacific Ocean) (Besse et al. 2004), which is in accordance with the vegetative mode of dispersion as stem cuttings and the history (introduced plant species) of recent introduction in these regions. A thorough analysis of different species (such as *V. planifolia, V. tahitensis* and *V. pompona*) and clones within the species of Vanilla cultivated in Reunion and Central America (Besse et al. 2004) showed no variation in the introduced locations.

It is well known that population genetic variation is influenced by factors such as historical events, genetic drift, breeding systems and natural selection (Barrett 1992). History of introduction determines the genetic variation within and among populations to some extent. Introductions usually consist of a small number of founders, resulting in lower genetic variability in introduced populations (Nei et al. 1975). Multiple introduced populations have higher genetic diversity than those that have been introduced only a few times (Li et al. 2006). Low genetic variation may also be a result of a few well-adopted genotypes that might have established successfully and expanded rapidly even in the case of multiple introductions. During the time of the introduction, a super aggressive genotype among the introduced ones might have been fixed by some biotic and abiotic factors (Li et al. 2006). The introduced plant need not accumulate enough genetic variation to adapt to its newer environment in its spread phase if its phenotypic plasticity is stronger and could buffer against the selection pressure (Weber and Schmid 1998). The degree to which introductions are accompanied by genetic bottlenecks depends on the species breeding systems and is expected to be lowest in highly selfing species or those that reproduce vegetatively (Nei et al. 1975).

Vanilla, being an introduced crop in most countries where it has been cultivated, much of the planting material is originated from limited clonal propagation, which largely limits the genetic variability in the crop (Divakaran 2006b). Vanilla might have been introduced to India through a "stepping-stone" process resulting in all cultivars sharing the same genetic background. Vanilla cultivation, having begun in India by the East India Company nearly 250 years back in the spice garden at Kurtallam in Tamil Nadu indicates that a few vines belonging to same mother plant or plantation might have been introduced and further expansion to all other parts is from these plants, which are of a similar genetic background. Reports indicate that plantations of Reunion, Mauritius, Seychelles and Malagasy Republic can all be traced back to a single clone (Madhusoodanan et al. 2003). Besse et al. (2004) while studying genetic diversity of *V. planifolia* by using RAPD markers in vanilla cultivated areas of Reunion Island and Polynesia reported a very low level of diversity. The only study on genetic diversity of Indian vanilla using molecular markers is by Minoo et al. (2008) by RAPD analysis of two indigenous collections of *V. planifolia* made

from vanilla cultivating regions of India reveled that there is very limited variation within the collections indicative of its narrow genetic base.

The development of strong adaptability of a plant species to its current environments is more important for its survival than the accumulation of rich genetic diversity, which usually takes a long time to achieve (Xu et al. 2003). Obviously, the shortcomings of low genetic diversity in a plant species can be highly compensated by the development of its strong adaptability, at least for a temporal period of time. However, it is difficult to predict the long-term effect caused by the low genetic variation of the clonal species. Through rapid and massive expansion, a few successful clones with favorable genotypes might be the essential component in all individuals in its new colonies, although overall genetic variability in these clones might appear to be low.

1.5 Conclusion

This preliminary investigation has determined the absence of genetic variations in introduced and then commercially cultivated *V. planifolia* in India indicating a threat of extinction due to pest and environment vagaries. These observations indicate the need to increase the number of introductions and broaden the gene pool of cultivated vanilla in India to reduce its vulnerability to diseases and insect pests apart from its genetic improvement for other attributes. Genetic variability is required to permit an adaptive response of the introduced species to the new selective regime imposed by the environment. A recent study by Divakaran et al. (2006a) involved inter-specific hybridization in vanilla and molecular characterization of hybrids and selfed progenies using RAPD and AFLP markers indicated the possibility of taking up breeding work in vanilla. In order to broaden the genetic base of vanilla cultivars, further efforts such as seed germination, mutation breeding, genetic engineering and induction of somaclonal variants are to be urgently pursued.

Summary

Occurrence of genetic variants during micropropagation is occasionally encountered when the cultures are maintained *in vitro* for long period. Therefore, the micropropagated multiple shoots of vanilla developed from axillary bud explants that were established 10 years ago were used to determine somaclonal variation using Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeats markers (ISSR). One thousand micro-plants were established in soil of which 95 plantlets (consisting of 4 phenotypes) along with the mother plant were subjected to genetic analyses using RAPD and ISSR markers. Out of the 45 RAPD and 20 ISSR primers screened, 30 RAPD and 7 ISSR primers showed 317 clear, distinct and reproducible band classes resulting in a total of 30,115 bands. However, no difference was observed in banding patterns of all the samples for a particular primer indicating the absence of variation among the micropropagated plants. The results suggest that the micropropagation protocol used for *in vitro* proliferation of vanilla plantlets for the last 10 years might be applicable for the production of clonal plants over a considerable period of time.

To study the possibility of up-scaling the protocol, vanilla shoot multiplication in semi-solid (SS), complete immersion system (CIS) and partial immersion system (PIS) were evaluated keeping track of kinetics of growth and nutrient uptake. Significant reduction in osmolarity due to high uptake of sucrose was higher in CIS than in PIS with no difference in the conductivity pattern, indicating that the mineral uptake was probably similar in both. The rate of shoot multiplication was although marginally higher in SS than in CIS and PIS, by the end of five-weeks culture period, the biomass production and shoot elongation were significantly higher in PIS than in SS and CIS. Shoot cultivation in CIS was associated with hyperhydric shoots (>80%) having poor ability to establish in fresh medium or soil. Growtek^{TM} bioreactor, the cheaper version of bioreactor, functioning on the principle of PIS enabling constant supply of the nutrients and aeration to the plants was found to perform better than SS and CIS and can be an efficient liquid culture system for shoot cultivation of vanilla. To obtain preliminary data on the conditions required for the shoot multiplication in bioreactor where the shoots are intermittently bathed with liquid medium, different medium contact periods were provided to shoot cultures in CIS where in

the 30 min contact three times a day appeared most congenial for best shoot multiplication and elongation. An equi-proportion of red soil: sand: vermicompost was found to be the best hardening soil medium for vanilla. The shoots sub-cultured in PIS were robust with good elongation producing both geotropic and aerial roots resulting in highest survival (90.5%) on transfer to this soil mixture. When a comparison was made on the growth and yield performance of plants derived from micropropagation and stem cuttings, plants form micropropagation flowered early and were higher yielders than those from the stem cuttings.

Publication

Sreedhar RV, Venkatachalam L, Bhagyalakshmi N (2007) Genetic fidelity of long - term micropropagated shoot cultures of Vanilla (*Vanilla planifolia* Andrews) as assessed by molecular markers. **Biotechnology Journal 2: 1007-1013**

2.1 Introduction

Genus *Vanilla* Swartz belongs to the family Orchidaceae which consists of 110 species, of which 15 are known to yield aromatic pods. The seeds of vanilla do not generally germinate and hence the plants are propagated by vegetative means through stem cuttings that result in slow rate of multiplication and non-uniformity in planting material. Removal of cuttings may also cause injury to the mother plant resulting in a set-back of growth and reduction in yield. Market demand for the propagules is hardly met through these cuttings; therefore, micropropagated plantlets have gained importance in the vanilla cultivating areas. *In vitro* propagation of vanilla by culturing axillary buds (George and Ravishankar 1997; Giridhar et al. 2001), aerial root tips (Phillip and Nainar 1988), through callus (Davidonis and Knorr 1991) and protocorms has been reported earlier. Micropropagation using shoot tips or nodal segments has been found to be an appropriate technique wherein about one lakh plantlets may be obtained in fifteen sub cultures (George and Ravishankar 1997).

True-to-type clonal fidelity is important for utilizing the advantages of micropropagation. A major problem encountered with *in vitro* culture is the occurrence of somaclonal variation amongst sub-clones of one parental line, arising as a direct consequence of *in vitro* culture condition itself (Gould 1986). Micropropagated plants obtained from preformed organs, especially from axillary buds and shoot tips have been reported to maintain clonal fidelity as organized meristems are more resistant to genetic changes compared to unorganized callus under *in vitro* culture (Ostry et al. 1994). However, the possibility of occurrence of somaclonal variants even in such cultures cannot be ruled out (Devarumath et al. 2002). The exact causes for such variations are still unknown, although it is believed to be induced by alterations in the supply of nutrients, auxin-cytokinin concentrations and their ratio, *in vitro* stress due to unnatural conditions and disturbed diurnal rhythm. Cultured plant tissues are also known to undergo high levels of oxidative stress and are exposed to reactive oxygen species (ROS), the latter being known to cause DNA damage including microsatellite instability (Jackson et al. 1998). Thus, the array of variations is often heritable and undesirable, challenging the very "clonal" nature of micropropagated plants. Reliable assays to assess the genetic stability of a genotype throughout *in vitro*

culture period are highly desirable in micropropagation as well as genetic engineering experiments, especially in the latter case wherein the starting material itself should not contribute to the variation. Hence, it is important to detect genetic variations as early as possible to prevent further tricky situations.

Tissue and environmental independent expression of DNA based markers have made them more reliable than morphological and isozymic markers. Among various nucleic acid markers, Restriction Fragment Length Polymorphism (RFLP) though can be used for screening genetic stability of tissue cultured plants, the method involves high cost, radioactivity and is laborious. Amplified Fragment Length Polymorphism (AFLP), also being of high cost, is not suitable for routine application for tissue cultured plants. Microsatellites or Simple Sequence Repeats (SSRs) consist of short tandem repeats of 2 to 5 base pair motifs, distributed throughout eukaryotic genomes and hence are highly informative. However, for their efficient use, flanking regions must be known so that polymerase chain reaction (PCR) primers may be generated. On the other hand, Random Amplified Polymorphic DNA (RAPD) is simpler and has proven to be quite efficient in detecting genetic variations, even in closely related organisms (Martin et al. 1991). With Inter-Simple Sequence Repeats (ISSRs), primers are not proprietary as in SSRs and can be synthesized by anyone and also allow production of a high number of reproducible polymorphic bands. ISSR is a very simple, quick, cost-effective, highly discriminative and reliable method which combines most of the advantages of Simple Sequence Repeats and Amplified Fragment Length Polymorphism with the universality of RAPD (Reddy et al. 2002). They are found to be more useful and reproducible than isozymes and RAPD; less cumbersome and cost-effective for routine application than RFLP (Fang et al. 1997). In addition, the ISSRs are also found to give more polymorphism than any other assay procedure (Virk et al. 2000). Thus, detection of additional polymorphism could be done by the use of RAPD in combination with ISSRs. At present, RAPD and ISSR markers have been widely utilized to detect the genetic similarities and dissimilarities in micropropagated material in various plants (Devarumath et al. 2002; Martins et al. 2004; Venkatachalam et al. 2007).

Mass propagation of plants by tissue culture is a costly and labour intensive technology. The gelling agents used are not inert medium components

and do not enable easy automation for commercial mass propagation. Agar, the most commonly used non-nutrient gelling agent is one of the costliest ingredients of the culture medium. High production costs limit the commercial use of micropropagation to markets with a high unit value for crops such as vanilla. It has been concluded for various species that extensive expansion of micropropagation would only take place if improved technologies became available for automation and acclimatization (Kitto 1997). Liquid culturing has been considered as an ideal technique for mass propagation as it reduces manual labor and facilitates better control over medium manipulation apart from rendering automation opportunities of the entire process. Moreover, plant tissues from numerous species are known to perform better when cultured in liquid medium rather than on agar medium. It is also considered to be an ideal solution for reducing plantlet production costs and the system provides more uniform culturing conditions. Use of liquid medium allows scale-up in bioreactors minimizing the number of operations with the advantage of medium manipulation, thereby reducing the cost of production of micro-propagules (Hvoslef-Eide and Melby 2000; Dey 2001). The available bioreactors are basically the modified microbial bioreactors and are un-suitable for higher plants due to their high sensitivities to shear force leading to mechanical damages. Alternatively, the aerated type exhibit foam formation, particularly in bubble aerated bioreactors (Berthouly and Etienne 2005).

Vanilla shoots when grown in liquid medium undergo hyperhydricity or vitrification (Sreedhar et al. 2009) which is a major problem encountered during submerged cultivation. To compensate this problem, it would be judicious to expose the culture to the liquid medium intermittently rather than continuously. It has been shown that increased aeration and intermittent contact between the plant material and the medium could reduce hyperhydricity (Aitken-Christie and Jones 1987) and these two characteristics are combined in temporary immersion systems (TIS). Medium may thus be easily renewed without changing the container and sterilization is possible by ultrafiltration (Berthouly and Etienne 2005). Recently, a type of bioreactor which allows partial immersion of culture has been shown to offer numerous advantages compared to semi-solid and completely immersed liquid medium system in improving the productivity and reducing the time taken

to multiply commercially-important material. PIS combine the advantages of gelled and liquid medium having intermittent to total availability of nutrients with an added advantage of allowing the plants to grow in an air space. Such a system is also known to reduce hyperhydricity in other plants by avoiding submergence of the entire plant material. Combining all these parameters, a low-cost bioreactor working on the principle of PIS has been designed, patented and commercialized under the name Growtek^{TM} with unique features like floating and rotating explant holder with an option for perforated explant support and a side tube for medium changing and content monitoring. Its usefulness in terms of enhanced multiplication rates, reduced bioreactor costs, saving in terms of incubation time, the minimization of contamination and plantlet transfer without root injury have been well documented (Dey 2005).

Reports on *in vitro* propagation of vanilla indicate the usefulness of culturing axillary buds (George and Ravishankar 1997; Geetha and Shetty 2000; Giridhar et al. 2001, Kalimuthu et al. 2006) and aerial root tips (Phillip and Nainar 1988). Reports on the plantlet formation through callus cultures (Davidonis and Knorr 1991) and protocorms are sporadic. Micropropagation using shoot tips or nodal segments appears to be an appropriate technique for clonal propagation of vanilla.

An *in vitro* clonal propagation protocol for *V. planifolia* developed by George and Ravishankar 1997 has been adopted as an initial step for establishment and maintenance of shoot cultures in the present study. While developing a protocol for micropropagation of vanilla, George and Ravishankar (1997) emphasized on the effects of various growth regulators as well as different culture conditions, where a maximum number of shoots was formed in liquid medium with a further higher number occurring in two-phase (solid/liquid) culture system.

For addressing the problems associated with the continuous production of high quality planting material of vanilla, a study was undertaken with four major objectives:

a. To assess the micropropagation protocol on long-term culture of *V. planifolia* for possible occurrence of genetic variants
b. To study the kinetic parameters that affect shoot proliferation

c. To standardize conditions for high rate of shoot multiplication which leads to development of an efficient method for clonal propagation in large scale
d. To study on hardening of plantlets derived from shoots multiplied in bioreactor and their field performance

2.2 Material and Methods

2.2.1 Genetic fidelity

2.2.1.1 Plant material and establishment of shoot cultures

A vanilla clone collected from Burliar plantation, Nilgiris, India served as the mother plant from which shoot cultures had been established as described earlier (George and Ravishankar 1997). These shoots were routinely multiplied and maintained for about 10 years at 25±2 ^{o}C under 16 h photoperiod having illumination of 37.5µE m^{-2} s^{-1} provided by fluorescent lamps. Over 1000 plantlets from such cultures were established as rooted plantlets, of which four groups of plants were selected where group one had 26 normal-looking and the other three groups consisted of 23 plantlets each showing unusual phenotypes such as pale green leaves, hyper-hydric, multiple-apiced and stunted growth (**Figure 2.1**). The plantlets from these four groups were used for genetic analysis along with the leaf material from the mother plant (maintained in the departmental garden). Thus a total number of 96 samples (including mother plant) were subjected for genetic marker analyses.

Normal Hyper-hydric Multiple apiced Stunted

Figure 2.1 Different phenotypes developed during long-term micropropagation of shoot cultures of *Vanilla planifolia*

2.2.1.2 Extraction of DNA, PCR analysis, Primer selection, DNA amplification, Analysis of PCR product by agarose gel electrophoresis and Data analysis were done as explained earlier in Chapter I

2.2.2 Micropropagation

2.2.2.1 Plant material

The plant material, establishment and maintenance of shoot cultures were as in section 2.2.1.1. Shoot bud clusters (**Figure 2.6A**) were trimmed to have an average of five buds per cluster and were used as initial inoculum for all the studies. Each cluster weighed approximately 1 gm and each bud was 0.5-1 cm in length.

2.2.2.2 Culturing conditions

The medium used for all the studies was modified MS medium (supplemented with 30 g of sucrose, 8.87 µM BAP and 2.69 µM NAA). Medium gelled with 7.2 g L^{-1} of agar (HiMedia, India) served as SS system. As a CIS, Erlenmeyer's flasks (150 mL) with 40 mL of medium (**Figure 2.6B**) were used. For PIS, Growtek^{TM} bioreactors (100×150 mm (Φ×h), Tarsons Products, India) (**Figure 2.2**) filled with 200 mL of liquid medium having unique features like floating, rotating, non-absorbing explant holder with perforated explant support matrix; side-tube with silicon rubber septum for changing media and online monitoring of medium environment were used. For larger-scale PIS, a bubble column bioreactor (BIOFLO 111, New Brunswick Scientific, Edison, N.J., U.S.A.) with 1.75 liter of medium was used. The bubble column bioreactor was made of a glass column (Corning glass, height 22 cm and diameter 14 cm) of 3 liters capacity with a working volume of 1.75 liter. The upper lid had provision for air inlet/outlet for sparger and ports for inoculation and sampling. Air was supplied through a glass sparger of 45 cm height and 7 cm diameter, molded into a circular shape at the bottom having pores of size 1 mm. An autoclavable basket made of stainless steel wire mesh of 10.5 cm height and 8.5 cm diameter was used to provide anchorage for the biomass. This anchorage had pores of 0.5 cm diameter at the bottom as well as sides and was placed at a height of 7 cm from the bottom of the bioreactor vessel supported by a stainless steel stand. The distance between sparger and the

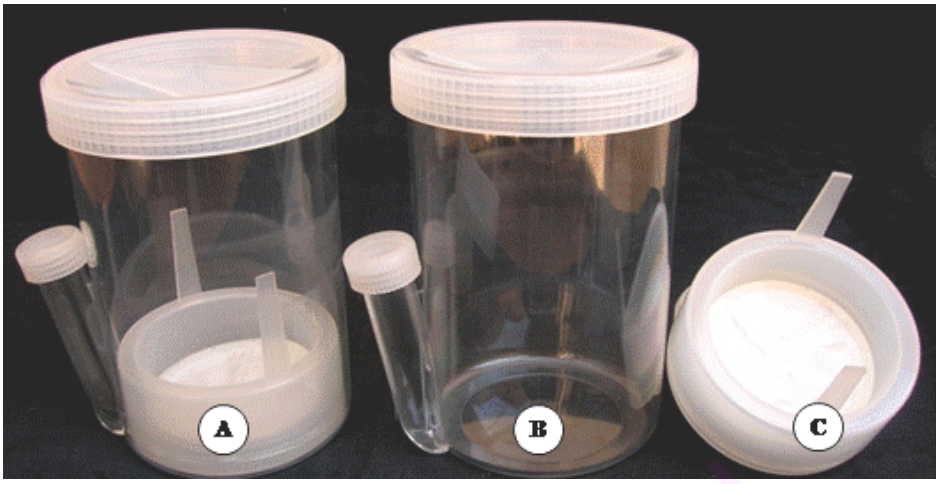

Figure 2.2 Growtek™ bioreactor used in the present study. A: The entire setup; B: Container with lid and side-tube with silicon rubber septum, C: Explant holder with perforated explant support matrix

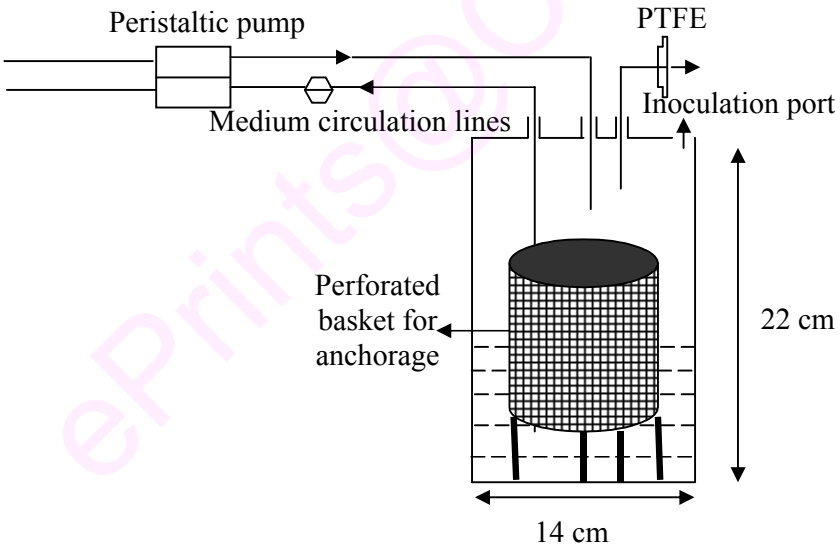

Figure 2.3 Diagrammatic representation of modified bioreactor developed for automation of submerged cultivation of vanilla (as TIS)

anchorage was kept at 4 cm. The bioreactor was operated at a temperature of 25±2 °C with 2000-lux illumination (provided from outside the jar) for a period of 16h a day for 3 weeks with a continuous air sparging at a rate of 33.4 cc sec^{-1}. As a Temporary Immersion System (TIS), a modified bioreactor was developed which was made up of a column glass container (Corning glass, height 22 cm and diameter 14 cm) of 3 liters capacity with a working volume of 1.75 liter (**Figure 2.3**). The upper lid was provided with openings for air inlet/outlet and ports for inoculation and medium pumping. The basket used to provide anchorage for the biomass was similar to that in bubble column bioreactor. The bioreactor was operated at a temperature of 25±2 °C with 2000-lux illumination (provided from outside the jar). Peristaltic pump was fitted to medium circulation lines for pumping liquid medium in and out to provide required period and number of contacts by the medium. Effect of different contact periods on the shoot multiplication, elongation and morphology were studied for a total period of five weeks.

Clusters of shoot buds (shoot bud length 0.5-1 cm) were used as initial inoculums. Erlenmeyer's flasks and Growtek^{TM} bioreactors were maintained at 25±2^{o}C under 16h photoperiod having illumination of 37.5µE $m^{-2}s^{-1}$on a gyratory shaker set at 80 rpm throughout the culturing period. Approximately 2 g of the plant material was inoculated in to Erlenmeyer's flasks, 50 g in bubble column bioreactor and modified TIS bioreactor and 15 g in to the Growtek^{TM} bioreactor. Enough number of replicates were used so that periodically a set of cultures could be removed for recording data.

2.2.2.3 Kinetic parameters

The pH of the medium was adjusted to 5.8 prior to autoclaving. The changes in the pH of the medium were recorded using digital pH meter (Control Dynamics, India). The nutrient depletion in the medium was examined by measuring the electrical conductivity using a conductivity meter of Wiss-teelm-werkstalten model LF-54 (Wielhalm, Germany). Conductivity was expressed in the units of mS (milli Siemen). Osmolarity of the medium was measured in order to determine the level of total solutes, both charged and neutral compounds in the medium by using an automatic cryoscopic osmometer (Osmomat 030-D

Gonotech, GmbH, Germany). The calibration of the instrument was done using triple distilled water. Osmolarity was expressed as Osmol kg^{-1}.

2.2.2.4 Growth parameters

To check the total biomass accumulation, culture from each type of container was removed, gently rolled on blotters and weighed using electronic balance. Fresh weight served as a basis for the rate of biomass accumulation per gram biomass inoculated. Shoot multiplication rate was calculated as rate of multiplication per bud inoculated and shoot length was recorded in centimeters in all the systems. All the observations were made at weekly intervals for five weeks during culturing.

2.2.2.5 Rooting, hardening and green house cultivation

Rooting of the elongated shoots from Growtek^{TM} bioreactor was achieved on MS medium with 1/2 strength NH_4NO_3 supplemented with 5.37 µM NAA and 15 g L^{-1} sucrose which was found to be most suitable by earlier observations. Well rooted plantlets of 8-10 cm length were then planted in soil mixture containing varied proportion of red soil, sand and vermicompost and observations on percent survival and growth response were made four weeks after planting.

2.2.3 Planting material for field performance study

2.2.3.1 Stem cuttings

Stem cuttings of nearly one foot length were derived from 5 years old mother plant maintained in the garden of Plant Cell Biotechnology Department of CFTRI, Mysore. Each cutting had a minimum of 2 buds and initial bud germination was made in greenhouse conditions in a soil mixture having equi-proportion of red soil, sand and vermicompost. These sprouted cuttings having shoots of 2 to 3 inches length with at least a pair of leaves were then planted in pits following standard agronomical practices for vanilla.

2.2.3.2 Micropropagated plantlets

Well rooted and greenhouse hardened plantlets (**Figure 2.10A, B**) of at least 15 cm length derived from PIS bioreactor system were selected and planted in pits following standard agronomical practices. Plants were grown under shade-net (providing 50% shade) and were trained to metal rod or bamboo stick support

(**Figure 2.10C, D**). Vines were trained longitudinally instead of horizontally to have an induce more number of flowers which was as per earlier visual observation that more number of flowers were induced in longitudinally trained vines compared to horizontally trained ones. Pollination of all the bloomed flowers was done using a thin bamboo splinter between 7.00 and 8.00 AM and a mist of water spray was given to the plant to improve pollination and fertilization rate. Observations on various growth and yield parameters were made at regular intervals in both the cases.

2.2.4 Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel XP (Microsoft Corporation, Washington) and the data are presented as an average of mean±error bars ($p \leq 0.05$).

2.3 Results

2.3.1 Genetic fidelity

Table 2.1 lists the RAPD primers used in the present study, their sequence, the additional levels of $MgCl_2$ needed for proper amplification, the number of scorable bands formed for each primer and the range of fragment sizes. A comparison of RAPD and ISSR profiles of all the DNA samples extracted was done for analyzing the variations, if any. Out of the 45 arbitrary primers (having 60-70% GC content) tested for RAPD, 30 primers were selected based on their amplification products. Each RAPD primer generated a unique set of amplification products ranging in size from 200 bp to 2,800 bp where the number of bands for each primer varied from 4 in OPJ 08 and 15 in OPM 16. The 30 primers used in this analysis yielded 258 scorable bands with an average of 8.60 bands per primer. Out of 20 ISSR primers, 7 were selected and they produced 59 scorable bands which ranged from 200 bp to 2,500 bp (**Table 2.2**). An average of 8.43 bands per ISSR primer was obtained which ranged from 4 to 15. A total of 30,115 bands (no. of plantlets analysed × no. of band classes with all primers) were generated by the RAPD and ISSR techniques giving rise to monomorphic band classes for all the 95 DNA samples of micropropagated plants analyzed. Both micropropagated plants and the mother plant showed identical banding

pattern. An example of the monomorphic band classes obtained is shown in **Figure 2.4 and Figure 2.5** for RAPD and ISSR markers respectively.

Table 2.1 List of selected primers used in RAPD analysis, the respective additional levels of $MgCl_2$, the number of scorable bands obtained and the range of fragment size for each primer

Sl. No.	Primer	Primer sequence (5'-3')	Additional $MgCl_2$ (mM)	Number of scorable bands	Fragment size range (bp)
01	OPA 03	AGTCAGCCAC	0	11	700-2800
02	OPA 04	AATCGGGCTG	1	5	400-1200
03	OPA 11	CAATCGCCGT	0	10	200-2800
04	OPA 14	CTCGTGCTGG	1	7	500-1000
05	OPA 20	GTTGCGATCC	0	8	400-1200
06	OPC 01	TTCGAGCCAG	0	7	450-1300
07	OPC 02	GTGAGGGCTC	2	6	300-1200
08	OPC 04	CCGCATCTAC	1	8	450-1500
09	OPC 05	GATGACCGCC	1	8	300-1700
10	OPC 06	GAACGGACTC	0	6	900-2500
11	OPC 07	GTCCCGACGA	0	8	400-2800
12	OPC 09	CTCACCGTCC	1	9	400-2500
13	OPC 08	TGGACCGGTG	0	7	300-1500
14	OPD 04	TCTGGTGAGG	0	6	900-2300
15	OPD 11	AGCGCCATTG	1	9	300-2800
16	OPD 16	AGGGCGTAAG	1	8	400-1500
17	OPF 12	ACGGTACCAG	0	8	300-2000
18	OPJ 07	CCTCTCGACA	0	9	300-2000
19	OPJ 08	CATACCGTGG	1	4	400-1000
20	OPJ 09	TGAGCCTCAC	0	7	850-2200
21	OPJ 10	AAGCCCGAGG	2	8	650-2800
22	OPM 16	GTAACCAGCC	1	15	200-2000
23	OPM 18	CACCATCCGC	2	6	950-2500
24	OPM 20	AGGTCTTGGG	2	10	300-1200
25	OPN 03	GGTACTCCCC	1	12	200-1500
26	OPN 04	GACCGACCCA	0	10	350-1500
27	OPN 06	GAGACGCACA	2	11	300-1000
28	OPN 09	TGCCGGCTTG	2	11	200-1900
29	OPN 10	ACAACTGGGG	0	14	200-2000
30	OPN 14	TCGTGCGGGT	1	10	300-2800

Table 2.2 List of selected primers used in ISSR analysis, the respective additional levels of $MgCl_2$, the number of scorable bands obtained and the range of fragment size for each primer

Sl. No.	Name of the primer	Primer sequence (5'-3')	Tm (^oC)	Additional $MgCl_2$ concentration (mM)	Number of scorable bands	Fragment size range (bp)
1.	UBC 809	$(AG)_8G$	52	0	4	200-400
2.	UBC 810	$(GA)_8T$	50	0	9	200-1000
3.	UBC 811	$(GA)_8C$	52	0	10	300-2500
4.	UBC 823	$(TC)_8C$	52	1	4	200-600
5.	UBC 824	$(TC)_8G$	52	0	6	200-900
6.	UBC 826	$(AC)_8C$	52	0	15	200-1500
7.	UBC 840	$(GA)_8YT$	53	1	11	200-1000

Y = C or T

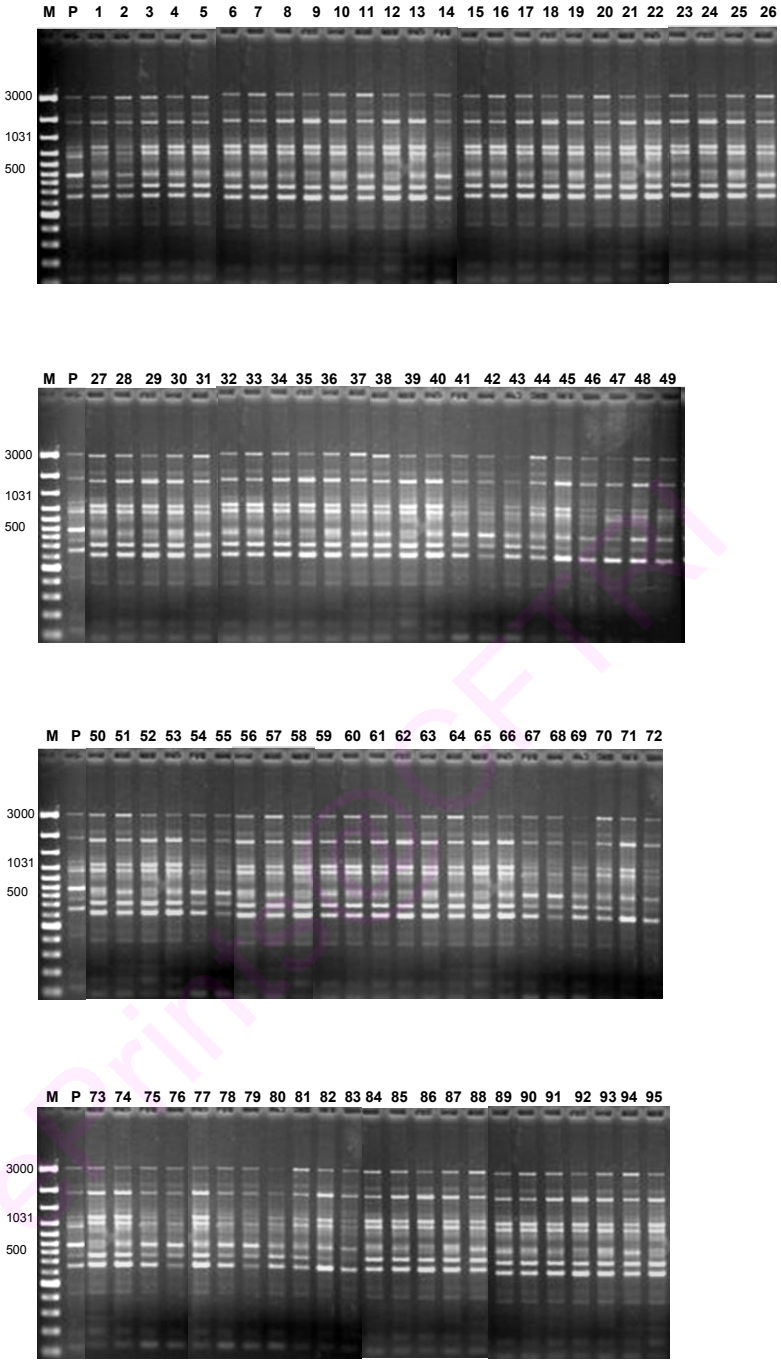

Figure 2.4 A representative Randomly Amplified Polymorphic DNA (RAPD) amplification pattern obtained for mother plant (P) and for long-term micropropagated shoot cultures (lanes 1-26: normal-looking; 27-49: hyperhydric; 50-72: multiple-apiced; 73-95: stunted) generated by primer OPJ 10. M: GeneRuler^{TM} 100 bp DNA Ladder Plus (Fermentas GmbH, Germany)

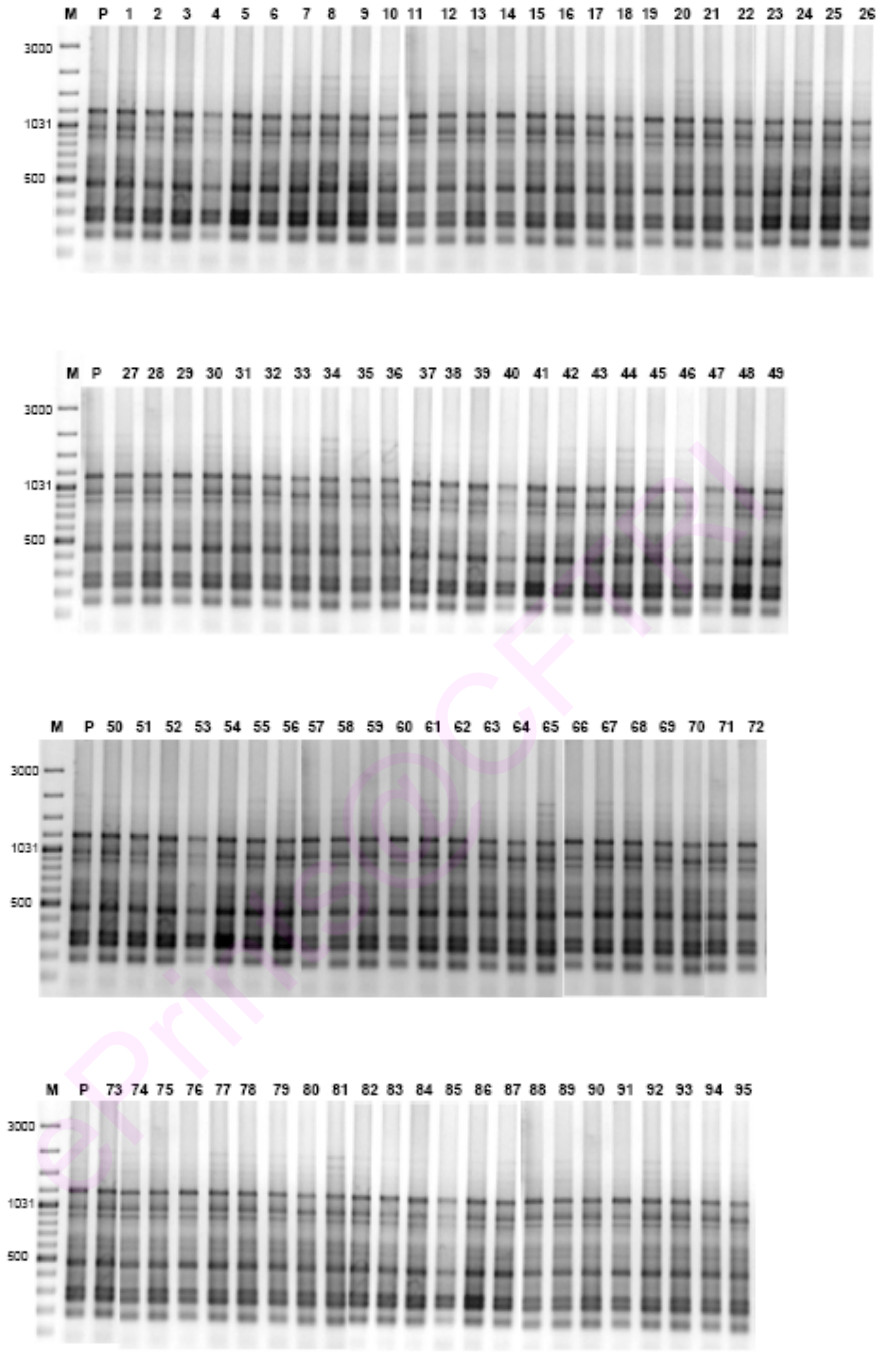

Figure 2.5 A representative Inter Simple Sequence Repeat (ISSR) amplification pattern obtained for mother plant (P) and for long-term micropropagated shoot cultures (lanes 1-26: normal-looking; 27-49: hyperhydric; 50-72: multiple-apiced; 73-95: stunted) generated by primer UBC 826. M: GeneRuler^{TM} 100 bp DNA Ladder Plus (Fermentas GmbH, Germany)

2.3.2 Micropropagation

While the cultures in agar-gelled medium showed a steady rate of multiplication with an average of 6.2 shoots per bud (**Figure 2.6A**), those in liquid medium showed higher multiplication rate. Due to high multiplication rate as well as to initiate the scale-up process, studies in liquid medium were considered. For this shake flask culture, Growtek^{TM} bioreactor and bubble column bioreactors were used. Cultivation in shake flask invariably resulted in abnormal shoots with collapsed and decayed tips (**Figure 2.6B**). The shoot bud clusters inoculated into the bubble column bioreactor developed hyperhydric syndrome and became turgid, translucent, watery, wrinkled and brittle (**Figure 2.6E**). Bleaching of the shoots and shoot bud death was observed which led to death of the culture within 2 weeks after inoculation (WAI). Hence, the cultivation was terminated and Growtek^{TM} bioreactor (**Figure 2.6C, D**) which showed a good response was considered as PIS for further studies. In order to evaluate the effectiveness of PIS, it was compared with semi-solid and CIS for various parameters like pH, conductivity, osmolarity and their effects on the rate of biomass accumulation, shoot multiplication and elongation.

The pH of the medium which had been adjusted to 5.8 during preparation, dropped to 5.7 after autoclaving, with a steep drop further to 4.3 after inoculation. It later stabilized at around 4.7 throughout the culture period from 1 WAI. The pH changes were almost similar for both PIS and CIS (**Figure 2.7**). The medium conductivity depends on the electrolyte concentration and ignores the changes in sugars which are present in higher concentration (Madhusudhan et al. 1995). The trend of uptake was almost similar in both CIS and PIS with a slight higher rate of uptake in CIS 4 WAI. A decrease in conductivity from an initial value of 6.4 mS to 4.2 mS in PIS and 4.05 mS in CIS was notable (**Figure 2.7**). The Osmolarity takes into account the number of moles of all solutes present in the medium. Initial osmolarity of the medium before inoculation was 0.225 Osmol kg^{-1} which gradually decreased to 0.086 Osmol kg^{-1} in PIS and 0.069 Osmol kg^{-1} in CIS indicating all solutes in the media are probably exhausted by 5 weeks of culture (**Figure 2.7**). Though there was a similar trend of uptake of solutes in both PIS and CIS in the first week, a higher uptake of nutrients in case of CIS between 1st and 4th week was apparent from the osmolarity data.

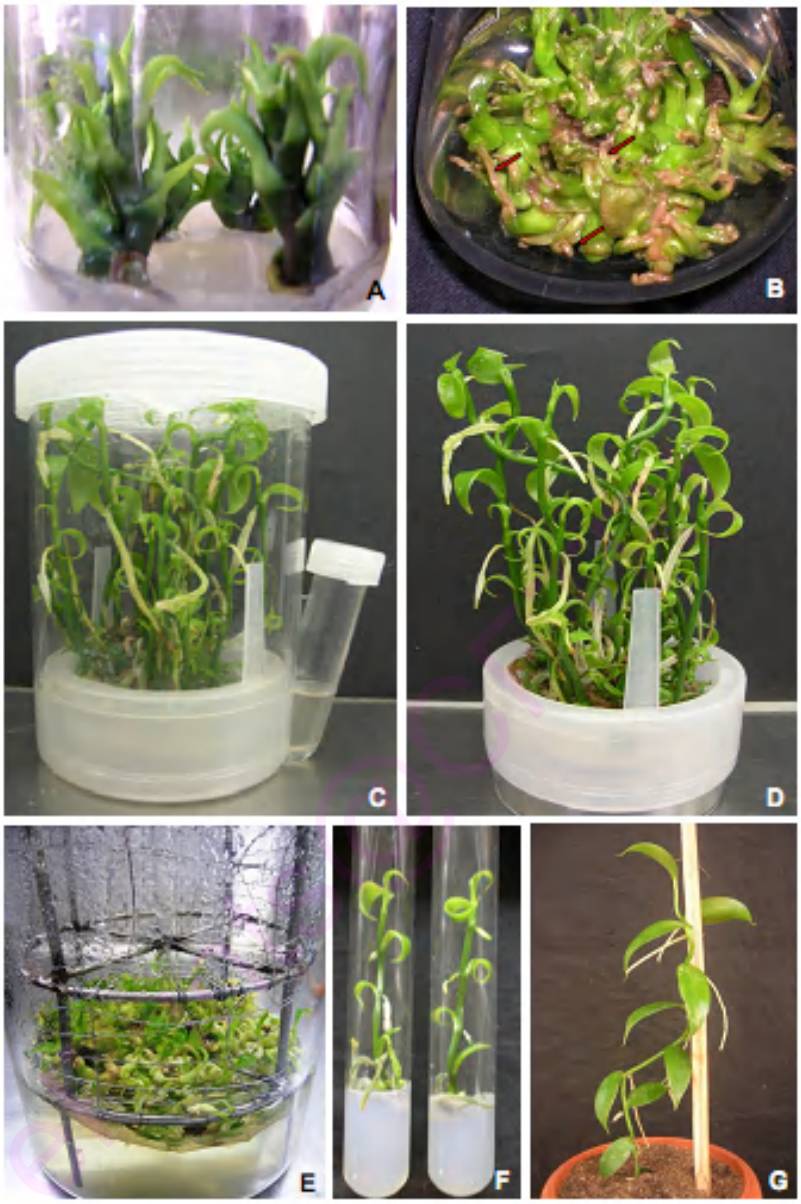

Figure 2.6 Various micropropagation systems used in the present study. A: Shoot buds cluster cultured on solid medium; B: Erlenmeyer's flasks (used as CIS) showing malformed hyperhydric shoots and bud death; C: Growtek^{TM} bioreactor used (as PIS); D: Explant holder with uniformly elongated shoots 5WAI; E: Bubble column bioreactor used in the present study; F: Rooting of shoots in MS medium with 1/2 strength NH_4NO_3 supplemented with NAA (5.37 µM) and 15 gm L^{-1} of sucrose; G: Growth and acclimatization of plantlets derived from Growtek^{TM} bioreactor in greenhouse conditions in equi proportion of red soil, sand and vermicompost.

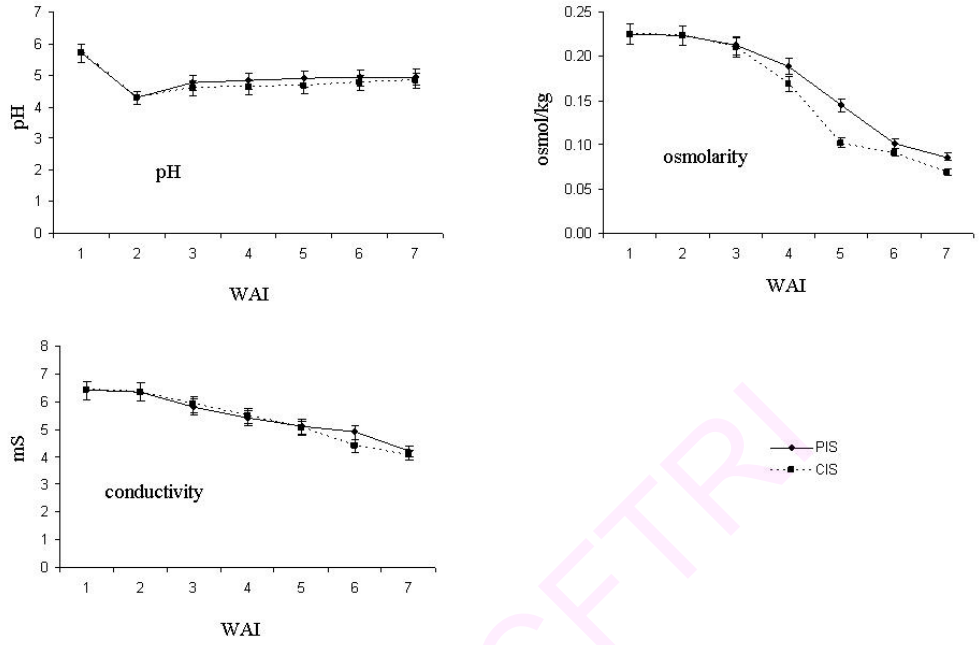

Figure 2.7 pH, osmolarity and conductivity of the medium in Erlenmeyer's flasks (CIS) and Growtek^{TM} bioreactor (PIS) used at various stages of culturing shoot cultures. WAI: weeks after inoculation. All treatments had at least fifteen replicates and the data are presented as an average of mean±error bar ($p \leq 0.05$) of replicates of two separate experiments.

The rate of shoot multiplication was 2.71 in SS and 2.46 in PIS 5WAI which did not differ significantly (**Figure 2.8**). However, there was a significant difference in shoot multiplication rate between SS, CIS and PIS from 2 WAI to 4 WAI. Cultures in SS showed higher rate of multiplication than their counterparts. Average shoot length was 9.56 cms in PIS 5WAI which was significantly very high compared to that in SS (4.55 cms) and CIS (2.89 cms) (**Figure 2.8**). There was a rapid increase in shoot elongation from 3 WAI in PIS which was significantly higher than SS and CIS.

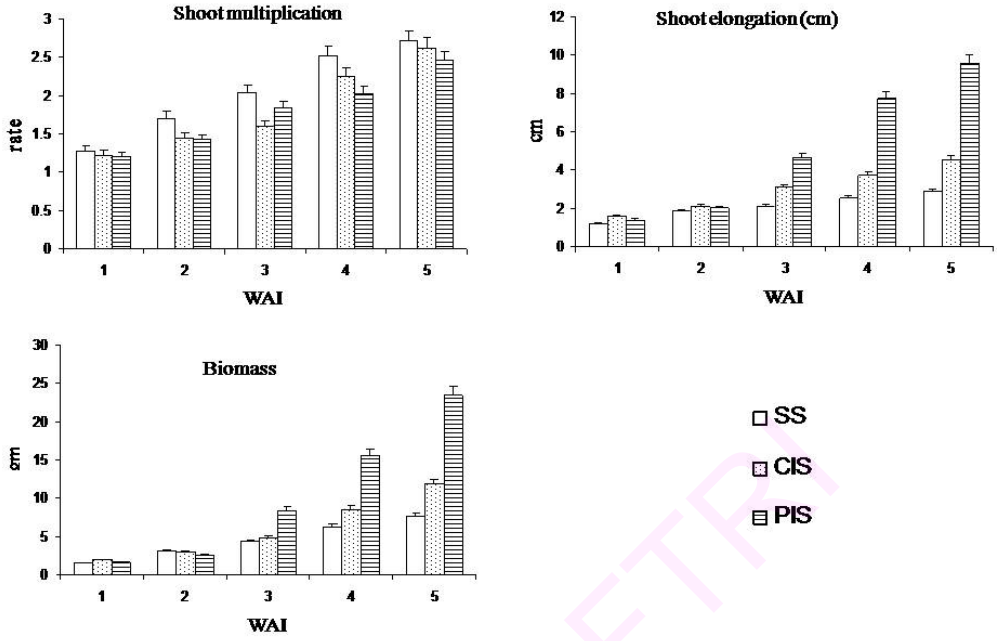

Figure 2.8 Shoot multiplication, shoot elongation and biomass production in semi-solid medium (SS), Erlenmeyer's flasks (CIS) and Growtek™ bioreactor (PIS) at various stages of culturing shoot cultures. WAI: weeks after inoculation. All treatments had at least fifteen replicates and the data are presented as an average of mean±error bar ($p \leq 0.05$) of replicates of two separate experiments.

There was no significant difference in shoot length during first and second week of culturing. Highest biomass accumulation was found in PIS 5 WAI which was 23.45-fold (**Figure 2.8**). It was significantly higher compared to CIS (11.89-fold) and SS (7.78-fold). A significantly higher increase in biomass production was found in PIS from 3 WAI. No significant variation in biomass accumulation was found between SS, CIS and PIS during the first two weeks of culturing. However shoot cultivation in CIS was associated with hyperhydricity syndrome wherein more than 80% of shoots appeared hyperhydric (**Figure 2.6B**).

The shoots derived from PIS (Growtek™ bioreactor) appeared better and well- grown than those from CIS or SS (**Figure 2.6D**). Development of root initials was a notable feature in the Growtek™ bioreactor grown shoot clusters. Each shoot in the cluster developed 2-3 root initials 5 WAI. All the shoots were then separated and were inoculated into rooting medium. Uniform rooting (3-4

roots per plantlet) in MS medium with 1/2 strength NH_4NO_3 supplemented with 5.37 µM NAA and 15 gm L^{-1} of sucrose was observed (**Figure 2.6F**).

In case of Temporary Immersion System, a contact period of 30 min three times a day was found to be the best compared to the other treatments for automation of vanilla shoot cultivation. Shoot multiplication rate was 2.42 which was significantly higher and 1.25-fold higher than the 15 min treatment (**Table 2.3**). Although the treatment with 45 min contact period showed significantly higher number of shoots with 1.19-fold higher than 30 min treatment, the shoots were stunted and associated with hyperhydricity syndrome which started by 3rd week of inoculation. Highest shoot elongation was observed in 30 min contact treatment which was 9.45 cm by the end of 5 week cultivation (**Table 2.3**). It was 1.43-fold higher than the 15 min treatment and 2-fold higher compared to 45 min contact treatment. Total biomass accumulation was found to be highest in 30 min contact period treatment which was around 654.1 g and was 3.04-fold higher than the 45 min contact treatment and 1.53-fold higher than 15 min treatment (**Table 2.3**). The shoot cultures which got a 15 min contact period three times a day appeared dried and less vigorous whereas the ones which received 30 min contact period treatment were more vigorous and dark green. Shoots which received a 45 min contact treatment were pale and translucent probably due to hyperhydricity which started by the third week of culture.

Further, scale-up of shoot cultures was successfully achieved in modified bioreactor developed for automation of submerged cultivation of vanilla (**Figure 2.9**) with a medium contact period of 30 min three times a day. This resulted in a biomass accumulation of more than 663.5 g with shoot multiplication rate of nearly 2.55 fold and shoot elongation of around 9.55 cm five weeks after inoculation.

Table 2.3 Effect of different contact periods on growth and development of vanilla shoot cultures (5 weeks after inoculation). Initial inoculums was 50 g per container and all treatments had at least three replicates and the data are presented as an average of mean±error bar ($p \leq 0.05$) of replicates of two separate experiments.

Contact period (minutes)	Contacts per day	Shoot multiplication (fold)	Shoot elongation (cm)	Biomass accumulation (gm)	Shoot morphology
15	3	1.93±0.21	6.63±0.57	426.36±15.8	Green and dried appearance
30	3	2.42±0.30	9.45±0.87	654.1±26.1	Dark green and vigorous
45	3	2.88±0.32	4.71±0.29	215.2±11.8	Pale green and hyperhydric (by 3 weeks)

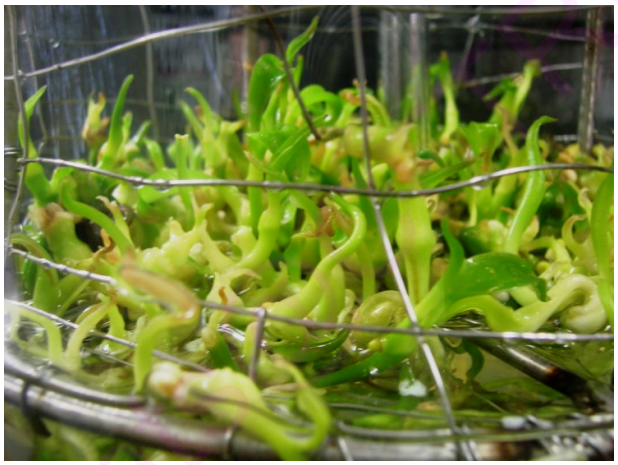

Figure 2.9 Scale-up of shoot cultivation in a modified bioreactor with a contact period of 30 min three times a day period (2-week-old-culture)

2.3.3 Greenhouse hardening and field performance studies

Study on the effect of different combinations of soil mixture for hardening plants under green house conditions showed that soil mixture of red soil: sand: vermicompost in a ratio of 1: 1: 1 was found to be the best with percent survival of 90.5 and an excellent growth response. It was closely followed by a ratio of 2: 1: 1 and 2: 1: 2 both showing nearly 80% shoot survival and good growth response (**Table 2.4, Figure 2.6G**).

Table 2.4 Effect of different combinations of soil mixtures used for hardening the plants under green house conditions (Average of 25 plantlets)

Soil Combination (Red soil: Sand: Vermicompost)	% Survival	Growth Response
3:1:0	69.5	Poor
3:2:1	73.9	Fair
3:1:2	71.2	Good
2:1:1	83.5	Good
1:1:1	90.5	Excellent
2:1:2	80.6	Good

A study was conducted to compare the growth and yield performance of vanilla plants propagated through conventional and micropropagation techniques. Germination of the shoot buds in the cuttings took a period of 30-45 days compared to 15-20 days taken by those in micropropagation. Shoots of stem cuttings reached a height of 13-15 cm in 16-18 weeks which was comparatively lesser in the case of micropropagated ones which took just 12-14 weeks in the greenhouse conditions (**Table 2.5**). After a six month period, the conventionally propagated plants reached a height of 30-35 cm which was significantly higher in micropropagated ones which reached a height of more than 50 cm. Micropropagated plants started flowering from 2 years of planting while flowering in the stem cutting propagated plants was after a period of 3 years.

Table 2.5 Field performance of Conventionally propagated and Micropropagated vanilla (Average of 25 plants each)

Parameter	Conventional propagation (Stem cuttings)	Micropropagation (Partial Immersion System)
Shoot bud germination	30-45 days	15-20 days (*in vitro*)
Plant of 13-15 cm length	16-18 weeks (4 months)	12-14 weeks (3 months)
6 months after planting	30-35 cm shoot length	50-60 cm shoot length
2 years after planting	No flowering	Flowering and fruit set
3 years after planting	Flowering and fruit set	Flowering and fruit set (higher)
Yield (3 years old)	342 ± 12.3 gm/plant	472 ± 21.4 gm/plant
% Fertilization	80-85	80-85

An average yield of conventionally propagated plant was around 342 gm of beans per plant which was much higher in the micropropagated ones with an average yield of 472 gm of beans per plant (**Table 2.5**). However, there was no variation in the flower fertilization percentage between the plants.

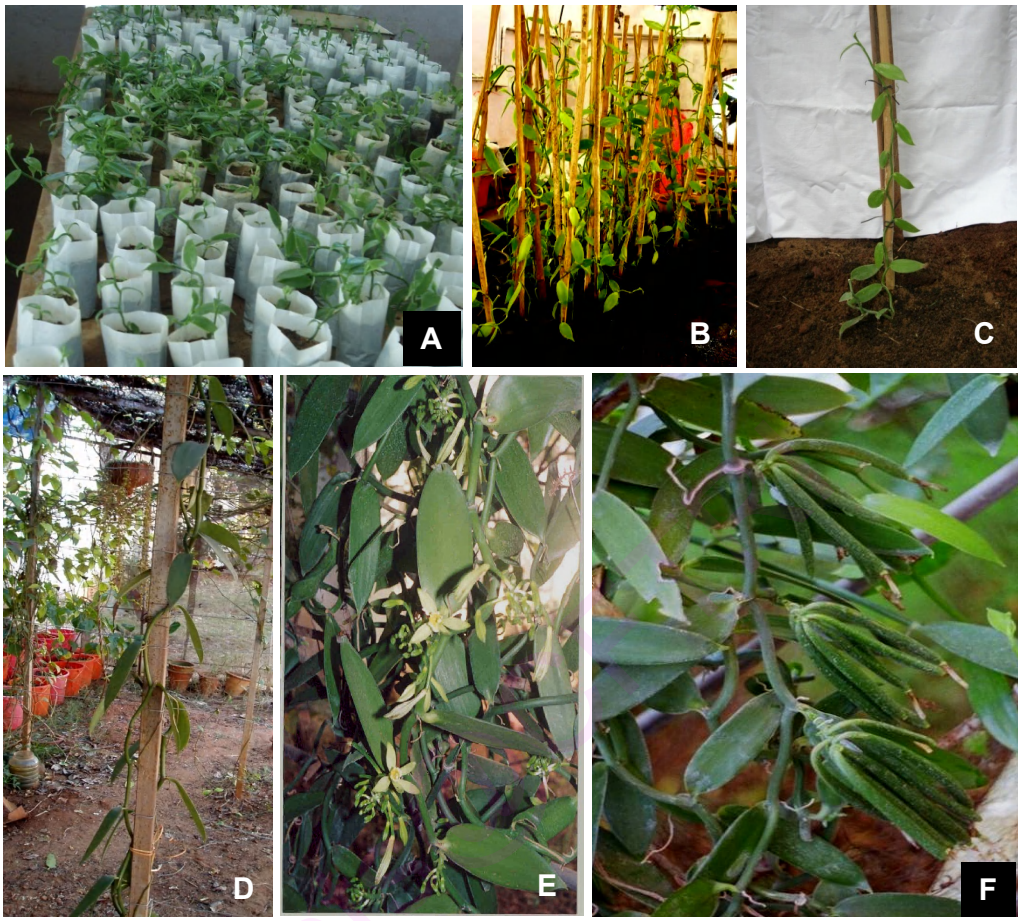

Figure 2.10 Greenhouse hardening and field cultivation of vanilla. A: Hardening in greenhouse conditions in equi-proportional soil mixture of red soil, sand and vermicompost. B: Vanilla plants in greenhouse ready for field planting. C: Tissue culture (PIS bioreactor) derived plant planted in field trained to a bamboo support. D: One year old plant grown under shade net condition. E: Profuse flowering in vertically trained branch of vanilla of two year old tissue culture derived plant. F: Fruiting in plant of tissue culture origin.

2.4 Discussion

2.4.1 Genetic fidelity

The present study involved two types of efficient genetic markers involving a large number of primers for marker-based genetic analyses of 95 micropropagated plants. This study has clearly shown the absence of genetic variation among the plantlets of *V. planifolia.* By using a combination of two types of markers which amplify different regions of the genome a better analysis of genetic stability of plantlets can be made (Martins et al. 2004). It is (Palombi and Damiano 2002)

suggested that the use of more than one DNA amplification technique proves to be advantageous in evaluating somaclonal variation while screening the micropropagated plants of kiwi fruit for any genetic variation. Hence, in the present study, two PCR based techniques, RAPD and ISSR were adopted for the evaluation of clonal fidelity of micropropagated *V. planifolia* plantlets.

The large number of bands produced by RAPD and ISSR primers indicates that several sites were effectively amplified by these primers and hence they appear appropriate for genetic evaluation of *Vanilla planifolia*. Absence of genetic variation using RAPD has been reported in several cases like in micropropagated shoots of *Pinus thunbergii* Pral. (Goto et al. 1998), axillary bud proliferations of chestnut root stock hybrids (Carvalho et al. 2004) and almond plantlets (Martins et al. 2004). In contrast, somaclonal variations were reported in micropropagated plants of *Populus tremuloides* (Rahmann and Rajora 2001), cauliflower (Leroy et al. 2001) and *Actinidia deliciosa* (Palombi and Damiano 2002). In the present study, no variation in the banding pattern was observed among the micropropagated plants in any of the RAPD or ISSR profiles, indicating the absence of variation in DNA sequence and therefore the absence of somaclonal variation.

The vanilla shoots cultured *in vitro* for several subcultures appeared to have developed into three unusual phenotypes (**Figure 2.1**). Development of different phenotypes during prolonged *in vitro* culturing was also observed while working on micropropagated phenotypes of *Pinus thunbergii* Pral. (Goto et al. 1998) and *P. radiate* (Ishii et al. 1987). In these studies, the use of cytokinins, especially the exposure to BAP (6-Benzylaminopurine), has been noted to induce hyper-hydricity. The latter phenomenon has been prevalent in xerophytic plants. Since vanilla plants are also partially xerophytic in nature one can expect high morphological variability and concomitant genetic changes, especially when shoot cultures are exposed to cytokinins for a long-period. Morphological changes such as stunted growth of shoots and unusual shapes of leaves observed in the present study have also been commonly known to occur during micropropagation in many plants either due to direct effect of plant growth regulators or due to re-juvenilization during long-term culture (George 1996). Formation of callusy shoot apices in some of the cultures during prolonged multiplication could be due

to repeated apical suppression with concomitant lateral dominance resulting in the development of clusters of meristems at their growing points, which is a well-known effect of continuous exposure to cytokinins (Anderson et al. 1982). The minor variations in the band intensities for certain fragments observed in RAPD and ISSR studies are, however, not quantitative as recorded in other such studies (Ryynanen and Aronen 2005).

Growth regulator types and levels are known to influence genomic dynamics during *in vitro* culture as observed in *Foeniculum vulgare* Mill. (Bennici et al. 2004). Culture media with high concentrations of different growth regulators like NAA (α-Naphthalene Acetic Acid), BAP and 2, 4-D (2, 4-dichlorophenoxyacetic acid) are known to contribute significantly to the somaclonal variation (George 1996). Though a high concentration of BAP and NAA have been continuously used in the medium for sub culturing over a period of 10 years, in the present study, no somaclonal variation could be detected within the micropropagated vanilla shoots. The morphological changes that occurred seem to be purely due to varied physiological and/or developmental states with no effect on genetic composition of the plantlets. Almond plantlets multiplied through axillary branching maintained their genetic integrity even after 4 and 6 years of *in vitro* multiplication (Martins et al. 2004). Similar was the case in long-term (70 months) tissue cultured silver birch (Ryynanen and Aronen 2005).

Most of the organized cultures, especially the shoot-tips are known to maintain strict genotypic and phenotypic stability under tissue culture conditions (Bennici et al. 2004) which appears applicable even to vanilla since no genetic variations were observed. Somaclonal variation has often been linked to the source of explant and the method of culture. In the present study, axillary buds have been used for micropropagation of vanilla and were found to be appropriate explants for initiation of *in vitro* shoot cultures. Such explants are considered to be a low-risk material, as organized meristems are proven to be more resistant to genetic changes than un-organized callus (Ostry et al. 1994).

From the results obtained in the present study using RAPD and ISSR markers it is apparent that there exists genetic homogeneity of vanilla plantlets and therefore the present micropropagation system for vanilla appears practically feasible and can be carried out for a considerable length of time (for at least 10

years) without any risk of genetic instability. The present study of evaluating different primers (RAPD and ISSR) for amplification of genomic DNA in vanilla can also form a basis for the use of these primers for other genetic studies like DNA fingerprinting.

2.4.2 Micropropagation

The culture medium kinetics with reference to pH shows that the pH drops down after inoculation and stabilizes after first week and remains constant throughout. The narrow change in pH due to autoclaving the medium is known and the change in presence of explant (Escalona et al. 1999). Although vanilla shoots were obtained from previous cultures, the trimming of unwanted tissues creating cut ends probably results in leaching of sap. The initial sudden drop in pH may be due to the inoculation with acidic plant material. This would lead to a sudden drop in the pH of the medium to 4.3, and its improvement and stabilization later at 4.7 suggests that the ionic status is maintained and hence there could not be a catastrophic effect of medium pH on the development of hyperhydricity syndrome (HHS). The later lower pH can be attributed to the preferential uptake of ammonium ions in the medium in exchange to protons. In the medium containing both $NO_3^-$and NH_4^+ ions, preferential uptake of NH_4^+ ions leads to drop in pH during early growth stage which increases NO_3^- utilization and a gradual increase and stabilization of the pH.

A rapid fall of the osmolarity of the medium noted between the 1st and 3rd week of culture indicates high uptake of minerals as well as sugars and the steady decline in conductance reflects the progressive uptake of ions (minerals). These trends inversely correlate with the increase in shoot growth and accumulation of biomass. The lesser rate of change in osmolarity and conductance from 3rd week onwards indicates poor utilization of nutrients and another possibility could be saturation of the water/nutrient uptake system. A constant immersion of the plant material in CIS may lead to an increased rate of uptake compared to PIS.

V. planifolia with xerophytic characteristics is expected to pose difficulty in growing in submerged (hydrophytic) conditions. But, as in most of the other such shoot cultures, which easily adapt to submerged conditions, the vanilla shoots were also expected to grow and multiply better in CIS. Cultivation of plants in SS culture medium allows good amount of explant air exchange but the

nutrient uptake is limited to the explant basal region only. CIS works the other way where in the entire explant is well covered with liquid medium providing more surface for nutrient uptake and less area for gas exchange and hence is usually associated with hyperhydricity. PIS which provide enough exposure of the explant to the nutrient medium along with good exposure to air for better O_2 exchange invariably resulting in good elongation of shoots promoting pronounced growth of aerial roots.

Vanilla micropropagation using nodal explants requires a period of 5-6 months to obtain plantlets in good numbers. Use of an intervening liquid medium also requires at least 5 months for moderate rate of shoot multiplication (George and Ravishankar 1997). Use of a single culture medium for initiation, multiplication, elongation and rooting was proposed by Kalimuthu et al. (2006) and the entire protocol takes more than 15 weeks to obtain plantlets of 8-9 cms. Culturing of shoot tip and nodal explants for 10-12 weeks in MS medium supplemented with BAP and coconut water yielded 9-10 shoots and 2-3 vigorously growing shoots which elongated with expanded leaves and root initials. Further culturing of such shoots in fresh medium with similar composition for another 3 weeks produced elongated shoots with roots (Kalimuthu et al. 2006). The proposed protocol developed in the present study needs 4-5 weeks for shoot bud proliferation after initiation of shoot cultures from nodal explants and another 5 weeks for elongation of the shoots to a length of 9-10 cms. Rooting could be achieved in 2-3 weeks and the total period required is 12-14 weeks to obtain plantlets ready for greenhouse planting. A uniform elongation of the shoot cultures could be achieved in this system which was lacking in the earlier protocol. The initiation of aerial roots in the shoots in the Growtek^{TM} bioreactor offers a great advantage in faster rooting and acclimatization. Additionally the use of Growtek^{TM} bioreactors is extremely useful for large scale multiplication of the plantlets allowing minimum contamination due to reduction in the number of handlings. It also allows monitoring of various kinetic parameters of the liquid medium and replacement of fresh medium when the cultures are still growing in the container.

Various plant species are found to perform well in liquid cultures compared to semi-solid medium (Etienne and Berthouly, 2002). A combination

of liquid and semi-solid medium in the same culture vessel enhanced axillary bud and shoot production in many plants (Molnar 1987). Use of successive semi-solid and liquid media for establishing propagation systems have been reported for *Lilium* hybrids (Simmonds and Cumming 1976). Shoots of vanilla cultivated in CIS started developing HHS by the end of 2 WAI and were completely hyperhydric by the end of 3 weeks. A similar observation was noted wherein stunted growth and hyperhydricity of shoots occured in cultures liquid medium as early as within 21 days after sub-culture (George and Ravishankar, 1997).

It is known that continuous immersion in the medium leads to inadequate oxygen supply to the immersed buds and cause bud death. Rotation at higher speed exposes plant material to shear forces causing mechanical damage, affecting normal growth and morphogenesis in CIS. On the other hand, in Growtek^{TM} bioreactor, circular floating explant holder provides unique advantage to use it in agitated mode apart from providing support for the shoot culture for partial immersion which allows better gaseous exchange compared to CIS. Perforations in the explant holder permits free access to nutrient medium. It was observed that plant material propagated by temporary immersion can perform better during the acclimatization phase than those grown on semi-solid or in liquid media (Etienne and Berthouly 2002). There are other similar reports on the advantages of Growtek^{TM} bioreactor (Dey 2005). Lesser physical stress seems to improve overall growth and development of healthier plantlets in vanilla as found in PIS (Growtek^{TM}) compared to CIS. In the latter case, continuous agitation leads to mechanical damages with higher hydrodynamic force and physiological stress, as observed by Liu et al. 2003. Root initiation and root health is known to be seriously affected by ethylene accumulation in the vessels with inadequate gas exchange (Zobayed et al. 2001; Klerk 2002). Growtek^{TM} bioreactor without this problem aided initiation and development of healthy roots. The present study which compared the solid, liquid (referred as CIS) and PIS substantiates that Growtek^{TM} (as PIS) proves to be an efficient culture system in all respects.

Temporary Immersion System (TIS) enables contact between all parts of the explant and the medium along with complete renewal of the culture atmosphere by forced ventilation. The positive effect of temporary immersion on shoot elongation has also been reasoned due to larger volume of the container

(Krueger 1991). Shoots of radiate pine derived from temporary immersion system were longer and of better quality than those obtained on agar medium (Aitken-Christie and Jones 1987). Krueger et al. in 1991 reported that cultures that grew in intermittent contact with the culture medium gave higher values for the shoot multiplication rate, shoot weight and shoot length. Work on sugarcane with twin flask system immersion system showed that it clearly stimulates shoot formation and length (Lorenzo et al. 1998). Hyperhydricity in outer leaf sheaths of stem portion immersed continuously in liquid medium with continuous bubble-aeration in banana was reported by Alvard et al. 1993 which was successfully prevented when cultured in temporary immersion system.

Immersion or continuous/intermittent contact period was reported to have a major influence on the quality of shoots. Longer contact periods and complete immersion resulted in hyperhydricity development in coffee and rubber. Several immersions for shorter time (1 min) were found to be more appropriate in case of coffee somatic embryos whereas several immersions with an immersion time of 15 min resulted in glassy embryos (Berthouly and Etienne 2005). In case of rubber, excessive time of immersion (15 min every 6 hours) induced hyperhydricity in the somatic embryos (Etienne et al. 1997). Temporary immersions of 5 min every hour were found to prevent hyperhydricity in serviceberry shoots but more frequent immersion of 5 min every 30 min resulted in hyperhydricity with highly translucent shoots with curled and thickened leaves (Krueger et al. 1991). A similar observation has been made in the present study wherein immersion of the shoot cultures for lengthier period of time (45 min per day) resulted in hyperhydricity problem which initiated in a period of 3 weeks after inoculation. An immersion of microcuttings of *Coffee arabica* for a period of 15 min every 6 hours showed no symptoms of hyperhydricity whereas the same was not true for *Coffee canephora* which reveled hyperhydricity symptoms for the same treatment. The sensitivity to the duration and frequency of immersion in liquid medium may vary with species of same genera (Berthouly et al. 1995). Immersion or contact period of 30 min for 3 times per day was found to be most appropriate for vanilla shoot cultures which yielded dark green vigorous shoots with better shoot elongation and biomass accumulation than the other treatments. This can form basis for development of automated large scale micropropagation

technique for vanilla. Based on the above results, the bioreactor used earlier (**Figure 2.9**) was re-considered for a study with intermittent bathing of shoot cultures. Immersion or contact period of 30 min for 3 times per day which was found to be most congenial was provided and the shoot cultures were successfully propagated with lesser hyperhydricity and excellent shoot multiplication and elongation. Thus, an automated micropropagation system for large scale multiplication of vanilla shoot cultures was achieved by developing a modified bioreactor (**Figure 2.3**) for intermittent submergence of the shoot cultures. Apart from the positive effects of TIS on multiplication and plant material quality, it also provides an opportunity to reduce cost of production, thereby making the whole process of micropropagation simpler.

2.4.3 Greenhouse hardening and field performance studies

An equi-proportion of red soil: sand: vermicompost was found to be the best hardening soil medium for vanilla by the present study. Vanilla is reported to thrive well in the soil with good amount of humus and sand and needs soil which provides good drainage for the excess water to drain down. The plants are sensitive to water logged conditions. A combination of red soil, sand and vermicompost in equal proportion in perforated poly-bags provides an excellent growing substrate for the vanilla plantlets and aids maximum survival (90.5%) and growth (**Figure 2.6G**).

When a comparison was made on the growth and yield performance of plants derived from micropropagation and stem cuttings, plants from micropropagation were found to perform better than those from the stem cuttings. They were early and high yielders. A comparative study using morphological and biochemical tests of field established micropropagated and conventionally cutting-derived plants of mulberry genotypes conducted reveled that the micropropagated mulberry plants showed significantly better vigour than plants raised through cuttings (Zaman et al. 1997). A similar observation was made even in this study wherein the micropropagation derived plants were found to be more vigorous than the stem cutting derived ones. In a study on field performance of blueberries derived from softwood cuttings and from micropropagation, propagation methods were found to exert significant influence on nursery and field performance. Cutting-derived plants grew more slowly, produced significantly less and shorter

shoots and were more variable than micropropagated plants. However, the majority of cutting-derived plants developed flowers one year earlier, flowered more abundantly, bore significantly larger berries than tissue cultured plants. Plants obtained through *in vitro* culture were more uniform than cutting-derived plants for the number of inflorescences per plant. On the contrary, better yielding of tissue cultured plants without deterioration of berry quality was reported for half-highbush blueberry (El-Shiekh et al., 1996), and lingonberry (Gustavsson 1999). Thus such differences may be attributed to different effects of micropropagation protocol on different plant species (Litwin´czuk et al. 2005). The conventionally vegetatively propagated plants are known to carry latent systemic infections, leading to low yields. Higher plant size with more pseudostem height and higher uniformity in fruit characteristics were observed in banana plants derived from micropropagation compared to those from conventional method. Bunches from plants derived from micropropagation could be harvested earlier by nearly a month than the conventionally cultivated ones (Msogoya et al. 2006). In the present study, micropropagation derived plants started yielding earlier than the cutting derived ones which might be due to continuous illumination during *in vitro* cultivation and low temperature incubation that are known to alter the vernalization in plants. Fast growth of micropropagated plants may also be due to the well developed roots and leaves in them when planted in fields.

2.5 Conclusion

The results from this study clearly indicated that the micropropagation protocol followed for establishment and maintenance of shoot cultures is appropriate and does not induce any genetic instability during long-term cultivation *in vitro*. Through the use of partial immersion system in the form of Growtek^{TM} bioreactor for micropropagation, plantlets for greenhouse hardening could be obtained within a period of 12-14 weeks compared to 20-24 weeks required in conventional methods with the advantage of cutting the cost of laborious sub-culturing in solid medium. Moreover the plants derived from this micropropagation system were found to outperform those from stem cuttings in both growth and yield parameters. Operating conditions for a bioreactor with net support and temporary

immersion of shoot cultures in liquid medium demonstrated through the present study forms a basis for developing improved bioreactor models.

Summary

Shoot cultures of Vanilla (*Vanilla planifolia*) when transferred to liquid medium from gelled solid medium showed a progressive increase in their watery appearance resulting in hyperhydricity syndrome (HHS) which caused necrosis of shoot buds. HHS in *in vitro* cultures is a major constraint and a generic problem in cultured shoots. HHS hinders automation and large-scale production of plantlets. HHS was also associated with severe damages at cellular and sub-cellular levels, increase in free polyamines (PAs) and accumulation of water, and decrease in quantities of chlorophyll, protein and drastic changes in reducing and non-reducing sugars. Spermine was far the major polyamine in all the analyzed cultures. The onset and progression towards HHS showed higher activities of antioxidant enzymes, indicative of shoots' defensive efforts against oxidative stress. The specific enzyme activities of normal and H2 stages respectively were 342.6 and 350.35 U mg $^{-1}$ protein for peroxidase (POD, EC 1.11.1.11), 38.4 and 30.38 U mg $^{-1}$ protein for superoxide dismutase (SOD, EC 1.15.1.1) and 71.3 and 82.75 U mg $^{-1}$ protein for catalase (CAT, EC 1.11.1.6). The kinetic parameters of culture medium, suggested that nutrient utilization being normal in HHS, the severe biochemical alterations and cellular damages mainly occur due to oxidative stress. There is also a paucity of information on the genetic control of this phenomenon. In the present study, Differential Display Reverse Transcription-Polymerase Chain Reaction was employed to have information about the up/down regulated genes during this disorder. Of the 114 HH-associated cDNAs identified, 31 were cloned in T/A cloning vector and sequenced using M13 forward and reverse primers. Electronic homology searches using BLASTX analysis resulted in identification of 23 cDNA clones showing homology with various stress related proteins like Zinc-finger like protein, gag-pol polyprotein, apoptosis related MAP kinase activating protein, DNA replication related GANP protein, DNA repair related DNA-binding SAP zinc finger. Endopolygalacturonase, pG1 protein, Triglycerol lipase and Enolase responsible for carbohydrate breakdown, Biotin-carboxylase having a role in fatty acid biosynthesis were also found to be differentially expressed during HHS. BLASTN analysis yielded 18 fragments having homology with different stress linked cDNA clones whilst the remainder did not show any significant homology to known sequences. Quantitative reverse

transcriptase polymerase chain reaction analysis of selected genes indicated that there was a more than 20-fold increase in the relative expression levels of genes having homology to Zinc finger family protein, Enolase and MAP kinase activating protein during the course of HHS. The relative transcript abundance level of clone having homology with putative GANP protein showed a drastic reduction to a 0.06-fold in hyperhydric shoots compared to normal shoots indicating down-regulation of this particular gene during HHS. A partially characterized transcriptome of hyperhydric condition in *Vanilla planifolia* has been developed which paves the way for a better insight into gene expression during this common physiological disorder.

Publication

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3.1 Introduction

Switching over to submerged cultivation is the first step towards automation of shoot cultures production in a micropropagation industry. Initial trials for this are normally conducted using shake-flask cultures, often known as complete immersion system (CIS) as the shoots are continuously bathed in liquid medium. During cultivation *in vitro*, the plantlets are exposed to a wide range of stress conditions caused by high relative humidity, gas accumulation in the headspace, altered nutrient/hormonal combinations and non-congenial osmoticity of the culture medium. Although most plant cultures adapt to changes in environmental conditions, some of them become abnormal with turgid, translucent, less green, watery, hypo-lignified, wrinkled and brittle appearance. This phenomenon, known as hyperhydricity syndrome (HHS), can lead to irreversible loss of multiplication as well as regenerative potential. HHS has also been a generic problem in continuous cultivation or scale-up of plant organs *in vitro*. Such a shift towards hyperhydricity has been linked to various metabolic disorders, changed array of proteins and altered stress responsive pathways.

Under stressful conditions cells undergo a surge of reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The generation of ROS has been associated with oxidases in plasma membrane and the electron transport of chloroplast and mitochondrion (Laloi et al. 2004, Ye et al. 2006) and known to affect photosynthetic pigments, membranes and cell ultra-structure (Xu et al. 2008). A higher concentration of intracellular ROS has been shown to create cytotoxic conditions, including oxidative damage to lipids, proteins, and nucleic acids and disruption of cellular functions, finally leading to cell death.

Plants exhibit several antioxidant enzymes functioning as scavengers of ROS. In addition, non-enzymatic antioxidants including polyamines have also been observed engaged in the prevention and alleviation of ROS effects (Tasxgin et al. 2006). Among the enzymes, peroxidases, (POD) ubiquitously found in higher plants with various isoforms, function as rapid detoxifiers of stress-induced H_2O_2. Superoxide dismutases (SOD) are metallo-enzymes playing a key role in plants rendering protection against oxidative stress. Catalases (CAT) are tetrameric homoproteins which are among the main antioxidant defense in plants.

Polyamines (PA) affect membrane fluidity, act as ROS scavengers and control many functions associated with DNA, RNA, and protein turnover (Tiburcio et al. 1993). In few cases involving experiments with herbicides, oxidative injury occurring in hyperhydric tissues was directly related to changes in PA levels (Zheleva et al. 1993; Ye et al. 1997). Possible role of the PAs in the HHS is rarely studied, although some reports establish similarities between the symptoms of hyperhydricity and other physiological situations that enhance PA synthesis (Piqueras et al. 2002).

Comprehensive expression analysis is an important tool for understanding gross genetic changes. Isolating differentially expressed and functionally important stress-regulated genes is expected to reveal the genetic networks within the cells. This can be achieved by applying different techniques, such as RT-PCR differential display, serial analysis of gene expression (SAGE), subtractive hybridization, and cDNA microarray. RT-PCR differential display (Liang and Pardee, 1992, 1995) has been widely used to isolate genes whose expression profiles have been altered under different abiotic or biotic cues because of its technical simplicity and lack of requirement for previous genomic information of the species of interest (Kuno et al. 2000; Carginale et al. 2004; Basse 2005; Lang et al. 2005).

Genes involved in physiological events, stress responses, signal transduction and secondary metabolism have been isolated and characterized. Some of the isolated genes encode transcription factors, membrane proteins and rare enzymes that were previously difficult to purify. These results suggest that differential display is a powerful tool used to investigate the rare genes involved in the plant life cycle without using information from proteins. The stress response genes affected by environmental factors such as ultraviolet (UV) light exposure, extreme temperatures, oxygen, salt and desiccation was isolated using by DD and characterized (Yamazaki and Saitom 2002).

Transcriptome analyses in apricot (Grimplet et al. 2005) and tomato (Alba et al. 2005) have also been performed in an effort to get an insight into the vast array of genes that may be involved in ripening. These studies have been successful in identifying several novel genes related to fruit ripening whose action in the ripening process was not obvious earlier. The isolation and characterization

of the promoters of these novel genes could provide important *cis*-elements useful for delayed ripening through targeted repression of ripening related genes in fruit or for the expression of target proteins for value addition.

Vanilla planifolia is a member of Orchidaceae, and vanilla is popular for its natural flavour prepared from the extract of carefully cured vanilla beans. Although vanilla plants are propagated by cuttings, there is a large demand for elite planting material produced via micropropagation technique. While developing a protocol for micropropagation of vanilla, George and Ravishankar (1997) studied the effects of various growth regulators as well as different culture conditions, where a maximum number of shoots was formed in liquid medium, and a higher number of shoots than in solid medium occurred in two-phase culture system. Long-term culturing of shoots *in vitro* and the use of genetic markers for testing the clonal nature of plantlets thus produced have also been evaluated (Sreedhar et al. 2007a). Further for scale-up of shoot cultures in bioreactor, initial trials in fully submerged cultivation of vanilla shoots in air-lift bioreactor (2-L capacity) resulted in rapid death and leaching of white exudates into the medium. Therefore, a thorough investigation was considered aiming at acclimatizing the shoot cultures to liquid medium. The present study focuses on unraveling the major structural, biochemical and molecular changes occurring in vanilla shoots grown in liquid medium. The study also records the changes in kinetics of nutrient utilization from the medium, measured in terms of pH, osmolarity and conductance. To have a better insight on the molecular regulation of hyperhydricity process, Differential Display Reverse Transcription-Polymerase Chain Reaction (DD RT-PCR) technique coupled with silver-staining was applied to identify and isolate cDNAs representing transcripts differentially expressed.

3.2 Materials and Methods

3.2.1 Morphological and Biochemical Changes

3.2.1.1 Plant material and *in vitro* culturing conditions

Vanilla clone collected from Burliar plantation, Nilgiris, India served as the mother plant from which shoot cultures had been established under conditions described in earlier chapter. These shoots were routinely multiplied (Sreedhar et al. 2007a) in gelled Murashige and Skoog's (MS) (Murashige and Skoog 1962)

culture medium supplemented with 8.87 µM BAP and 2.69 µM NAA and maintained at 25±2 ^{o}C under 16-h photoperiod having illumination of 37.5 µmol photons $m^{-2}s^{-1}$ provided by fluorescent lamps.

Transferring to liquid medium involved the culturing of shoots in Erlenmeyer flasks (150 ml) containing 40 ml of MS based medium supplemented with 8.87 µM BAP and 2.69 µM NAA. Approximately 2.5 g of the plant material was used as initial inoculum, which included 1 to 2 cluster(s) having 5 to 6 shoot buds (of length 0.5-1 cm) each. The culture vessels were maintained at 25±2 ^{o}C under 16-h photoperiod, having illumination of 37.5 µmol photons $m^{-2}s^{-1}$ on a gyratory shaker set at 90 rpm throughout the culturing period of 5 weeks. The cultures thus established were continuously bathed in liquid medium and termed as continuous immersion system. The shoots cultured on a solid medium having composition as above but gelled with 7.2 g L^{-1} of agar (HiMedia, India) served as control. Observations in terms of morphological and biochemical changes were made at various stages of HHS for 5 weeks.

3.2.1.2 Morphological changes

While the shoot multiplication was better in complete immersion system (CIS), compared to solid system (**Figure 3.1**), the shoots in CIS displayed HHS towards the end of the culture period of 5 weeks and the four stages towards HHS are as shown in **Figure 3.2**. The other drastic morphological changes occurring progressively towards HHS were recorded by light microscopy, followed by scanning electron microscopy (SEM). Observations were made in terms of leaf morphology and changes at tissue level. For latter purpose freehand sections of about 0.5 to 1 mm thickness were made using new stainless steel razor blade. The sections were mounted on a slide and observed under an inverted light microscope (Leitz, LABOVERT, Ernst Leitz GmbH, Wetzlar, Germany) at 320× magnification and the responses were documented through photomicrographs. For SEM, the normal and hyperhydric cultures were processed according to Fowke et al. (1994). The samples were fixed in 2% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 6.8)

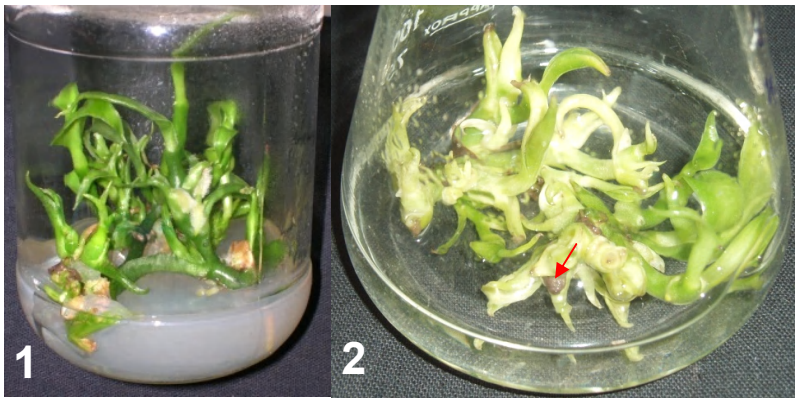


Figure 3.1 Shoot cultures of *Vanilla planifolia* cultured on (1) solid and (2) complete immersion system (CIS) (liquid media) after 5 weeks of culture showing hyperhydricity and bud death

Figure 3.2 Shoots cultivated in normal and liquid medium showing various degrees of hyperhydricity. (1) Shoots cultivated on solid medium; (2) Shoots from CIS 1 week after inoculation (H1 stage); (3) Shoots from CIS 3 weeks after inoculation (H2 stage); (4) Shoots from CIS 5 weeks after inoculation (H3 stage)

for 6 h, dried in alcohol series up to 100% (v/v), sputter coated with gold and examined in a LEO Scanning Electron Microscope 435 VP (Leo Electron Microscopy Ltd., Cambridge, UK).

3.2.1.3 Measurement of pH, conductance and osmolarity

The pH of the medium was adjusted to 5.8 prior to autoclaving and the changes in the pH after autoclaving and after the placement of shoot bud inoculum were monitored using digital pH meter (Control Dynamics, India) pre-calibrated with buffer standards.

Osmolarity was measured in order to determine the level of total solutes, both charged and neutral compounds in the medium, by using an automatic

cryoscopic osmometer (Osmomat 030-D Gonotech GmbH, Berlin, Germany). The calibration of the instrument was done using triple distilled water as well as sodium chloride standard. The osmolarity was expressed as Osmol kg^{-1}. The depletion of nutrients from the medium was examined by measuring the electrical conductance using a conductance meter of Wiss-teelm-werkstalten model LF-54 (Wielhalm, Germany), pre-calibrated with triple distilled water. The conductance was expressed in the units of μS (micro Siemen). The pH, osmolarity and conductance of the media were recorded before inoculation, just after inoculation and every week during five weeks of culture.

3.2.1.4 Chlorophyll content

The chlorophyll content was calculated using Lichtenthaler equations (Lichtenthaler 1987) after measuring the absorbance of the acetone extracts at 645 and 661.5 nm.

3.2.1.5 Carbohydrate content

Samples were repeatedly crushed in absolute ethanol and the alcohol solubles and insolubles were separated by filtration and vacuum evaporation of alcohol. Total carbohydrate in each fraction was estimated by phenol sulphuric acid method (Dubois et al. 1956); reducing sugars by dinitrosalicylic acid method (Miller 1959) and non-reducing sugars were quantified by subtracting the reducing sugar values from the total sugar values. The alcohol insoluble material was dried and after recording the weight, a known weight was hydrolyzed using 72% (v/v) H_2SO_4 keeping samples in ice bath. The mixture was appropriately diluted and used for estimating reducing or non-reducing sugars as above.

3.2.1.6 Analysis of polyamines

The extraction of polyamines and their HPLC analyses were conducted according to the method of Flores and Galston (1982). Authentic standards of Putrescine (*Put*), Spermidine (*Spd*) and Spermine (*Spm*) (Sigma, St. Louis, USA) were benzoylated before analyzing by HPLC. Free polyamines were extracted by homogenizing the plant materials (1 g of tissue) in 10 ml of 5% (v/v) ice-cold perchloric acid using a pestle and mortar. The homogenate was then centrifuged for 30 min at 20000×g. Free polyamines in the supernatant were benzoylated and analyzed by HPLC (Shimadzu LC6A, Tokyo, Japan). The elution system consisted of methanol:water (64:36) solvent, running isocratically with a flow rate

of 1.0 ml min^{-1}. The benzoyl-polyamines were eluted through a C_{18} column (300 × 4.6 mm i.d., with pore size of 5 μM), an SLC-6A system controller, and a CR4A data processor was used. Detection of eluted compounds was done by a UV detector SPD-AV set at a sensitivity of 0.04 AUFC and absorbance at 254 nm. A relative calibration procedure was used to determine the polyamines in the samples, using data of standards – *Put*, *Spd* and *Spm* by comparing peak areas and retention times. Results were expressed as nanomoles per gram of fresh weight. Extractions from three different samples were made independently and each extract was quantified in triplicate.

3.2.1.7 Protein content and antioxidant enzyme activity

Protein content of the plant material was estimated by Lowry's method (Lowry et al. 1951). The plant material (1g each) for enzyme assay was extracted by crushing using pestle and mortar with 10 ml of respective buffer and after homogenization, the homogenate was centrifuged at 12,000 rpm twice and the supernatant was used.

POD enzyme was estimated following the method explained earlier (Sreedhar et al. 2007b). The material was extracted in sodium phosphate buffer (pH 6.0) containing 1 mM Dithiothreitol and 0.1 mM Phenyl Methyl Sulfonyl Fluoride. **SOD** activity was measured spectrophotometrically based on the reduction of nitro blue tetrazolium (NBT) to water insoluble blue formazan as described by Murthy et al. (2002). Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT to 50%. The specific activity was expressed in terms of units mg^{-1} protein. **CAT** activity was determined by adding sample extract in a 50 mM phosphate buffer (pH 7) containing 18 mM H_2O_2 in a total volume of 3 mL. The consumption of H_2O_2 by CAT was measured by the decrease in absorbance at 240 nm (ε = 39.4 mM cm^{-1}) at 25 ^{o}C (Beers and Sizer 1952).

Chlorophyll, carbohydrates, free polyamines and protein contents along with enzyme activities were recorded initially, 1st, 3rd and 5th week after inoculation in both normal(solid) and liquid cultures at respective moisture contents.

3.2.1.8 Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel XP (Microsoft Corporation, Washington), and post-hoc mean separations were performed by Duncan's Multiple Range Test at $p \leq 0.05$ (Harter 1960) by using the statistical program SPASS7.0

3.2.1.9 Isozymic analysis of POD

Native-PAGE analysis: For analyzing the isozymes of POD from normal and hyperhydric stage (stage H2) standard protocol was used. Zymogram was prepared by polyacrylamide gel electrophoresis (7.2% (w/v)) (PAGE) carried out at 120 V for 4 h using 12 × 14 × 0.5 cm gel without SDS. The gel was stained for POD activity with a 100 ml solution of sodium phosphate buffer containing 10 ml of 0.25% (w/v) orthodianisidine dihydrochloride and 10 ml of 1% (v/v) hydrogen peroxide and immediately photographed (Thimmaraju et al. 2007).

3.2.2 Molecular Changes

3.2.2.1 RNA isolation

Total RNA was extracted from shoot cultures of vanilla at four stages of HHS (**Figure 3.3**) for 5 weeks using RNAqueous® kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol.

3.2.2.2 mRNA Differential Display

The experimental model adopted for this study is given in **Figure 3.3**. The mRNA Differential Display was performed using the RNAimage kit® (GenHunter Corporation, Nashville, TN, USA) according to the manufacturer's protocol provided. The analysis was conducted in four RNA samples using 0.2 µg of total RNA from each independent sample. Briefly, poly(A)$^+$–RNA (0.2 µg) was heated at 65 °C for 10 min and chilled on ice immediately. First-strand cDNA synthesis was performed in a reaction mixture containing 50 mM Tris–HCl (pH 8.5), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 1mM each dNTP, 40 units of RiboLock™ Ribonuclease inhibitor (MBI (Fermentas GmbH, St. Leon-Rot) and 40 units of H-minus M-MuLV Reverse Transcriptase (MBI Fermentas) with 50 mM of different anchor primer (**Table 3.1**) for 1 h at 42 °C. The reaction was stopped by heating the mixture at 70 °C for 10 minutes and chilling immediately. Enzymes were then

heat-denatured at 95 °C for 5 min. Product thus obtained was used for PCR amplification of the cDNAs.

Table 3.1 List of anchor primers and arbitrary primers used for Differential Display

Anchor primers			Arbitrary primers
Sl. No.	Name of the primer	Sequence (5'-3')	Operon Primers:
1	DD1	$(T)_{11}A$	OPA-03, OPC-08, OPD-11, OPJ-09, OPM-16, OPN-04, OPN-06, OPN-10
2	DD2	$(T)_{11}G$	
3	DD3	$(T)_{11}C$	

3.2.2.2.1 PCR amplification

PCR mixture was prepared as described in Chapter I using a mixture of anchor primers and each arbitrary primer in all possible combinations (**Table 3.1**). PCR was performed using a thermal cycler (MWG peqlab, Germany) and the reaction parameters were as follows:

a. Initial Denaturation	: 94 °C for 4 min	
b. Denaturation	: 94 °C for 60 sec	10 cycles
c. Annealing	: 36 °C for 45 sec	
d. Extension	: 72 °C for 2 min	
e. Denaturation	: 94 °C for 60 sec	10 cycles
f. Annealing	: 38 °C for 45 sec	
g. Extension	: 72 °C for 2 min	
h. Denaturation	: 94 °C for 60 sec	10 cycles
i. Annealing	: 40 °C for 45 sec	
j. Extension	: 72 °C for 2 min	
k. Final extension	: 72 °C for 30 min	

3.2.2.2.2 Urea formamide denaturing polyacrylamide gel electrophoresis and staining

After selective amplification, a 6 µL aliquot of PCR amplified product was mixed with equal volume of denaturing loading buffer (98% formamide, 10 mM EDTA, 0.05% Xylene-cyanol, 0.05% Bromo-phenol blue), denatured at 90 ^{o}C for 3 min

and immediately cooled on ice. Samples (12 µL) were loaded on pre-warmed denaturing 6% polyacrylamide standard sequencing gel of 28:2 ratio acrylamide:bisacrylamide, 7.5 M urea, 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.0), then electrophoresed in 1X TBE buffer at 50W constant power for about 2 h and 30 min; until the loading dye reached the bottom of the gel. The gel was fixed by incubating the gel slab in fixation solution (2% ethanol and 0.1% acetic acid) for 10 min with gentle shaking. Then the gel was rinsed with distilled water for twice for 5 min each and incubated in staining solution (chilled 0.2% $AgNO_3$ prepared in fixation solution) for 20 min followed by brief wash in double distilled water for 10 sec. The gel was developed with developing solution (0.6% NaOH and 0.2% of 37% formaldehyde). The developing solution was discarded as soon as it turned yellow and was replaced with a fresh portion. When a sufficient degree of staining has been obtained, developing solution was replaced with 5% acetic acid and the gel was washed with distilled water.

3.2.2.3 Elution of differentially expressed amplicons

The gel slice containing differential DNA bands was excised using an aseptic surgical sharp scalpel blade (no.11) fitted to handle. DNA was extracted using QIAEX II Polyacrylamide Gel Extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

3.2.2.4 Re-amplification

For re-amplification, 2 µL of the supernatant solution was used in standard PCR reaction using the same primer pair that was used in the Differential Display of the correspondent reaction. PCR reaction mixture was prepared to a final volume of 20 µL containing 1X *Taq* buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 9.0), 0.25 mM dNTPs, 0.5 µM of each primer and 1 U of *Taq* DNA polymerase enzyme (Sigma Aldrich, St. Louis MO). The PCR cycle program was with an initial step of 4 min at 94 °C, 35 cycles of 30 sec at 94 °C, 60 sec at 40 °C and 45 sec at 72 °C, followed by a final extension step of 8 min at 72 °C.

3.2.2.5.1 T/A cloning of isolated differential amplicons

A-tailing: The PCR product was heated at 95 °C for 20 min and 1µL of 2 mM dATP per 10 µL of PCR product was added. This was incubated at 70 °C for 15

min. The mixture was purified using HiPura PCR clean-up kit (HiMedia, India) before ligation.

3.2.2.5.2 Ligation of A-tailed PCR product to T-tailed vector

The A-tailed purified PCR product was cloned after ligating it to pKRXT vector using T/A Clone PCR Product Cloning Kit (SBS Genotech, Beijing, China) and transforming it into competent cells of *Escherichia coli* strain DH5α.

Ligation was carried out using the following components in a thin-walled 0.2 mL PCR reaction tube:

Plasmid vector pKRX-T DNA	:	1.0 µL
Purified PCR fragment	:	4.0 µL
10X Ligase Buffer	:	1.0 µL
T4 DNA Ligase, 5 U μL^{-1}	:	0.5 µL
Deionized water	:	vol. made up to 10.0 µL

The reaction components were mixed by pipetting or a brief spin and the mixture was incubated at 16 °C for 4 hours. The enzyme was then inactivated by heating the reaction mixture to 65 °C for 10 min.

3.2.2.5.3 Transformation of *E. coli* with the ligation reaction mix

Reagents

Luria-Bertani broth (LB) (components used per litre)

Bacto-tryptone	:	10 g
Bacto-yeast extract	:	5 g
Sodium chloride	:	10 g

The pH was adjusted to 7.0 with 2 N NaOH and the total volume was made up to 1 litre with deionized water.

SOB (components used per litre)

Bacto-tryptone	:	20.0 g
Bacto-yeast extract	:	5.0 g
Sodium chloride	:	0.6 g
Potassium chloride	:	0.19 g
Magnesium sulphate	:	10.0 mM (added from 1.0 M stock)
Magnesium chloride	:	10.0 mM (added from 1.0 M stock)

The first four components and the magnesium salts were autoclaved separately and mixed to constitute the SOB medium.

SOC (per 100 mL): To 1.0 mL of SOB, 7 µL of filter-sterilized (Millipore, 0.4 µm) glucose solution (50% w/v) was added to prepare SOC medium.

0.1 M $CaCl_2$ stock solution: 1.47 g of $CaCl_2$ was dissolved in 100 mL of deionized water. The solution was sterilized by filtration and stored as 20 mL aliquots at -20 $^{\circ}$C.

Ampicillin stock solution: 100 mg of Ampicillin (Ranbaxy, India) was dissolved in 1.0 mL of deionized water and the solution was sterilized by filtration. It was stored at 4 $^{\circ}$C and used at a working concentration of 100 µg mL^{-1}.

0.1 M IPTG stock solution: 0.12 g of IPTG was dissolved in 5 mL of deionized water and the solution was filter-sterilized and stored as aliquots at -20 $^{\circ}$C.

X-Gal stock solution: 100 mg of X-Gal was dissolved in 2 mL of N, N'-dimethylformamide (DMF) and the solution was stored in micro-centrifuge tube wrapped in aluminium foil at -20°C.

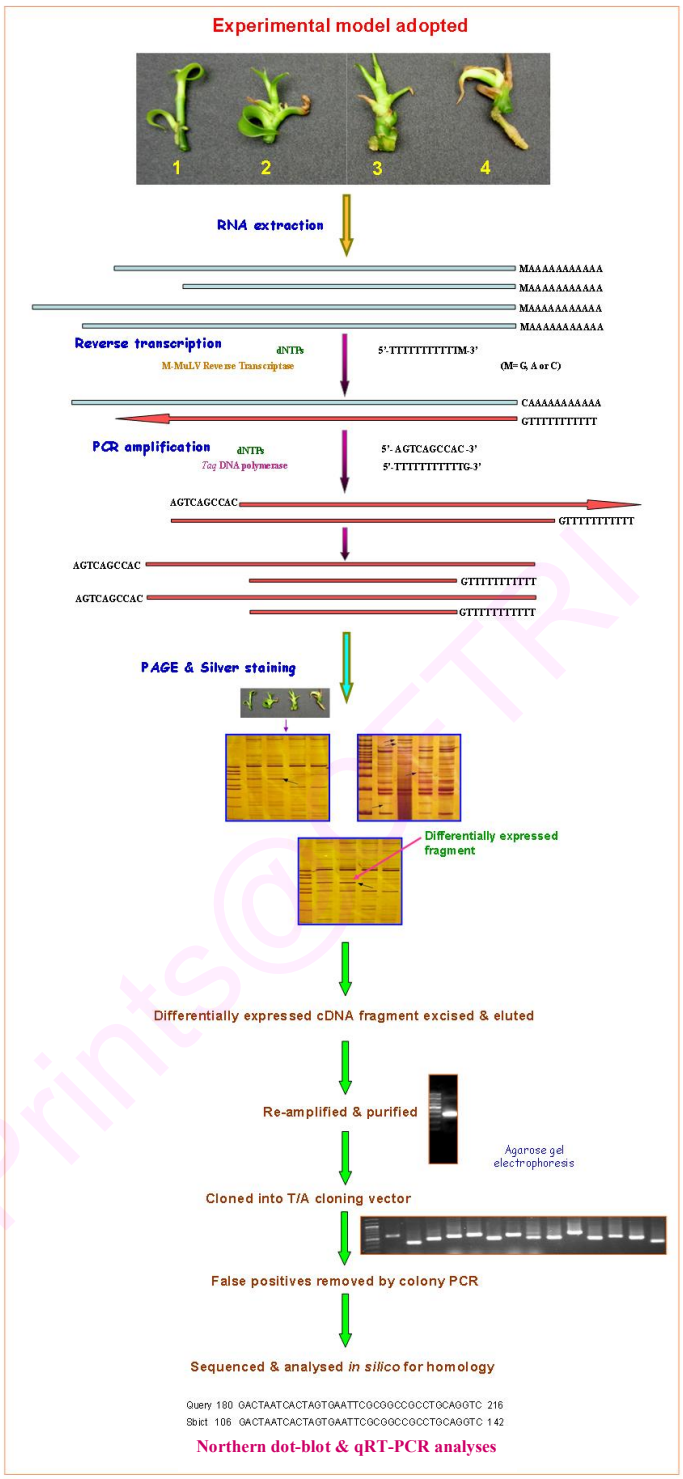


Figure 3.3 The experimental model adopted for the differential display study

3.2.2.5.4 Preparation of competent cells using $CaCl_2$

A single colony of *E. coli* (DH5α strain) from freshly grown culture (at 37 °C for 16-20 h) was picked and transferred into 50 mL of LB broth in a 250 mL conical flask. The culture was incubated at 37 °C with rigorous shaking. The OD_{600} of the culture was determined periodically to monitor cell growth. When the OD_{600} reached 0.4-0.5, the cells were transferred aseptically to 50 mL sterile polypropylene tube. The culture was cooled by storing the tube on ice for 10 min and the cells were recovered by centrifugation at 4000 rpm for 8 min at 4 °C. The medium was decanted and the pellet was re-suspended in 10 mL of ice-cold 0.1 M $CaCl_2$ and was stored on ice. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4 °C. The fluid from the cell pellet was decanted and the tubes were kept in an inverted position for 1 min to allow the last traces of fluid to drain away. The cell pellet was re-suspended in 10 mL of ice-cold 0.1 M $CaCl_2$ and cells were stored at 4 °C for 4-8 hours.

3.2.2.5.5 Transformation of competent cells

About 200 µL of the suspension of competent cells was added to sterile micro-centrifuge tubes along with the Plasmid DNA (~50 ng) or 4 µL of ligation mixture. The contents of the tubes were mixed by swirling gently and the tubes were stored on ice for 30 min. The samples represented (a) competent cells that received standard supercoiled plasmid DNA and (b) competent cells that received no plasmid DNA. The tubes were transferred to water-bath set at 42 °C for 90 sec to subject the cells to heat shock. The tubes were then rapidly transferred to ice and the cells were allowed to chill for 45 sec. An 800 µL of SOC medium was added to each tube and the cultures were incubated at 37 °C for 45 min.

3.2.2.5.6 Selection of transformants/recombinants and PCR confirmation of the transformation

About 100 µL of transformation mix was plated onto LB agar plates containing 100 µg mL^{-1} ampicillin, 0.5 mM IPTG and 80 µg mL^{-1} X-Gal. The plates were incubated overnight at 37 °C for the colonies to grow.

Colonies harbouring the recombinant plasmid have a disrupted lacZ gene and appeared white while non-recombinant vectors were blue. White colonies were selected from the LB plate and screened by PCR for the presence of insert

using the T3 and T7 primers flanking the cloning site. PCR amplifications were performed using PCR mixture (250 µL) containing 25 µL of 10X PCR buffer, 5 µL of 10 mM dNTPs, 10 U of *Taq* DNA polymerase and 5 µM of each primer (T7 and T3 primers) where volume was made up to 250 µL using nuclease free water. Transformed cells from white colonies were picked by sterile tips and were mixed to the PCR mixture. PCR was performed at initial denaturation at 94 °C for 5 min, 30 cycles of: 30 sec at 94 °C; 30 sec 42 °C; 50 sec at 72 °C, and final elongation for 10 min at 72 °C using a thermal cycler according to the manufacturers protocol for pKRXT vector cloning and detection of transformants (SBS Genotech, Beijing, China). The PCR products obtained were separated on 1.8% agarose gel, stained with ethidium bromide (0.001%), and documented. The size of the amplification products was estimated from the 100-bp DNA ladder (Fermentas GmbH).

3.2.2.5.7 Isolation of plasmid DNA from the transformed colonies

Plasmids from recombinant clones were isolated with the Qiagen mini prep Plasmid Mini Kit according to the manufacturers' protocol.

3.2.2.6 Sequencing of the clones

DNA sequencing was carried out by dideoxy chain termination method (Sanger et al. 1977). The reaction was carried out in an automatic DNA sequencer (ABI prism, Applied Biosystems, USA) with fluorescent dideoxy chain terminators at the Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India.

3.2.2.7 Validation by RNA dot blot

The observed differential display expression levels need to be re-confirmed to eliminate possible false positive differential display results. There are several techniques that allow fast corroboration of gene expression. Northern dot blot hybridizing was used to verify differential expression to verify the changes in gene expression. The cloned differential display products were used to probe with total RNA isolated from different stages of hyperhydricity. DNA-dig labeling was done according to manufacturer's protocol using DIG High Prime DNA labeling and detection starter kit I (Roche Diagnostics GmbH, Mannheim, Germany). High prime DNA-dig labeled probes effectively incorporated the digitoxin and provided sufficient sensitivity for detecting their target mRNAs. The total RNA was precipitated overnight at 4 °C by the addition of 0.3 volumes of 10 mM LiCl

and then precipitated in ethanol. One µL of total RNA (RNA from the stock 1µg µL $^{-1}$) was denatured with 2 volumes of denaturing buffer (1X MOPS containing formamide and formaldehyde) by boiling for 5 minutes and immediately chilled on ice and spotted to a Hybond-N nylon membranes (Ambion Inc., Austin, TX). The RNA was fixed to the membrane by using a 302 nm ultraviolet cross linker for 45 min. Then the membranes were pre-hybridized for more than 1 h in ULTRAHyb buffer (Ambion Inc., Austin, TX) and hybridization was then performed overnight with the same buffer containing the gene specific DIG-labeled probe at 42 °C. Two selected clones VP2 (zinc finger family protein) and VP9 (Putative GANP protein) one representing up-regulation and the other down-regulation were assessed for regulation by RNA blots using total RNA isolated from four stages of hyperhydricity. The clone 18*S* (18*S* ribosomal RNA gene of *Vanilla planifolia*) was used as a control. The hybridization, post-hybridization treatments and detection were done as follows:

Pre-hybridization

The membranes with RNA blots were placed in a polythene bag containing 15 mL pre-warmed (65 °C) hybridization buffer and were sealed and incubated at 42 °C overnight with mild agitation.

Hybridization

A 5 µL aliquot of probe for respective gene was heat-denatured by incubating in boiling water for 5 min, followed by snap cooling on ice and 2 µL aliquot of denatured probe was added to 5 mL of pre-warmed (65 °C) hybridization buffer. Hybridization buffer containing the denatured probe was added to the polythene bag containing the membrane and sealed and it was incubated at 42 °C for 6 h with mild agitation in a water bath.

Post-hybridization washes

The hybridization buffer was discarded and the membrane was washed twice in 50 mL of post hybridization washing buffer-I for 5 min at room temperature under mild agitation. The membrane was again washed twice in 50 mL of post hybridization washing buffer-II for 15 min at 65 °C under mild agitation.

Detection

1. The membrane was rinsed briefly at room temperature in maleic acid buffer and incubated in 50 mL 1X blocking solution for 30 min.

2. The blocking solution was discarded and the membrane was incubated in 10 mL of antibody solution (1:5000 Anti-DIG-AP conjugate in 1X blocking solution) at room temperature for 45 min under mild agitation.
3. The membrane was then washed in 50 mL maleic acid buffer twice for 15 min and incubated in 20 mL detection buffer for 5 min.
4. The detection buffer was discarded and the membrane was incubated overnight in 10 mL freshly prepared color solution.
5. The membrane was kept in dark for color development.
6. The reaction was stopped by washing the membrane for 5 min with 50 mL of deionized water. The results were documented by photography of the wet membrane and the membrane was stored at 4 °C.

3.2.2.8 Quantitative (relative) reverse transcriptase polymerase chain reaction (qRT-PCR)

Quantitative relative RT-PCR was used to confirm the differential expression of DNA fragments isolated from normal and hyperhydric shoot cultures. The sample selection and RNA isolation was done as mentioned earlier. Specific oligonucleotide primers were designed for each DD product for RT-PCR (**Table 3.2**) and synthesized (Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India). For amplification of the 18*S* rRNA sequence, primers from banana were used with success as there was non-availability of 18*S* rRNA sequence in the GenBank database for *Vanilla planifolia*. Sequence analysis of the amplified product showed a very high degree of homology with 18*S* rRNA of plants like *Vanilla aphylla* (*E* score; identities: 0.0; 97%) and *Lecanorchis multiflora* (*E* score; identities: 0.0; 96%). The sequence of the *Vanilla planifolia* 18*S* rRNA obtained is presented in the appendix. First-strand complementary DNAs were synthesized from 1.5 µg of total RNA using M-MuLV reverse transcriptase and oligo-dT (18-mer) primer (Fermentas GmbH, Germany). PCR amplifications were performed using PCR mixture (15 µL) containing 1 µL of RT reaction product as template, 1× PCR buffer, 200 µM dNTPs, 1 U of *Taq* DNA polymerase and 0.1 µM of each primer depending on the gene. PCR was performed at initial denaturation at 94 °C for 4 min, 30 cycles of: 1 min at 94 °C; 1 min at 60-62 °C (depending on primer); 1 min at 72 °C, and final elongation for 10 min at 72 °C using a thermal cycler. The optimal PCR annealing temperatures are shown in **Table 3.2**. The PCR

products obtained were separated on 1.8% agarose gel, stained with ethidium bromide (0.001%), and documented. The size of the amplification products was estimated from the 100-bp DNA ladder (Fermentas GmbH). The band intensity of each gel was checked using the Herolab E.A.S.Y Win 32 software. The transcript levels of each gene in control shoots were taken for comparison in calculating the transcript abundance of respective genes during hyperhydricity. Five genes (**Table 3.2**) were selected based on their vital role played during various stress responses for the qRT-PCR study.

Table 3.2 Gene specific primers and annealing temperatures used for RT-PCR

Primers	Primer sequence (5'-3')	Annealing temperature (°C)
VP2 *Forward*	GACCGTCGAGTCGCTAAGAG	60
VP2 *Reverse*	GGTCGGTGCCTGTGTGTAT	
VP9 *Forward*	AGCCACCTCCTCCAGGTACT	61
VP9 *Reverse*	CAATGTGATCGCAAGGTGAG	
VP11 *Forward*	CGTTTAAGGAGGCCATGAAG	61
VP11 *Reverse*	TCCAATTACAACCTTGCCAGT	
VP12 *Forward*	CGGGAATTCGATTAGTCAGC	60
VP12 *Reverse*	ATCATTCCGGATAACGCTTG	
VP30 *Forward*	GGGAGAGGTGCATTCGTAAC	61
VP30 *Reverse*	GCTTTTCTCTTGCGCATTTC	
18*S Forward*	GGCAGCTATGTGTTTGTCCA	60
18*S Reverse*	TGTGGAATTGTGAGCGGATA	

3.3 Results

3.3.1 Morphological and Biochemical changes

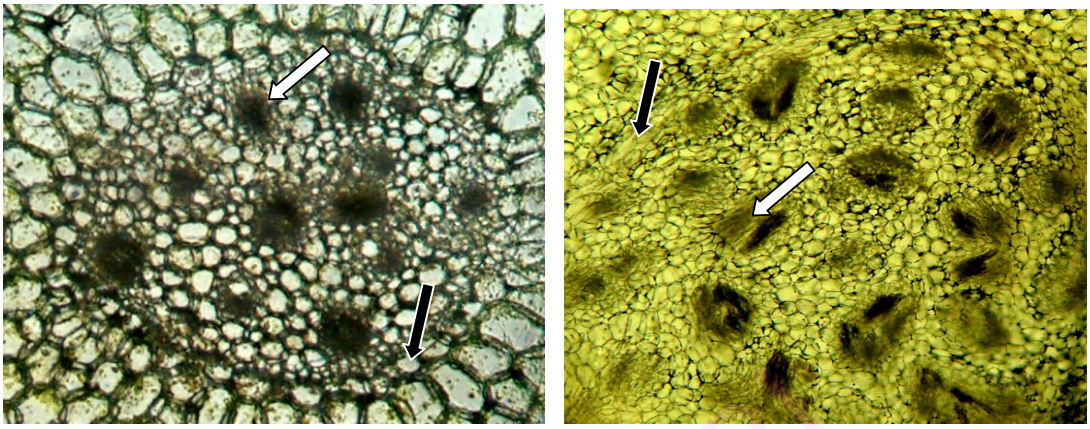
3.3.1.1 Morphological and ultra structural changes

Normal and HHS shoots of vanilla displayed significant differences, when leaves and stem portions were observed by optical and scanning electron microscopy (SEM). Normal vanilla shoots cultured on solid medium displayed xerophytic morphology, with succulent waxy leaves. The moisture content was 88.8% in normal shoot cultures, 90.3% in H1 stage cultures, 93.7% in H2 stage and 96.4% in H3 stage cultures. Light microscopic observations of stem sections of H2 cultures revealed distinct degradation of vascular tissues and lesser degradation of cortical tissues, reduction in size of the cortical cells and the hypertrophy of tissues inside the vascular cylinder. Degradation of the endodermal cells was noted in the hyperhydric shoots (**Figure 3.4**), whereas the leaf sections showed higher degradation of vascular bundles, loss of compactness of palisade parenchyma with abnormal enlargement and more intercellular space (**Figure 3.5**). The scanning electron microscopy of stem surface revealed uniform cuticular ridges in normal cultures and scattering in hyperhydric cultures (**Figure 3.6A**). Vascular tissue was severely collapsed in hyperhydric shoot cultures (**Figure 3.6B & 3.6C**). Stomata showed regular structure in normal leaf and lacked closure mechanism in hyperhydric leaf (**Figure 3.6D**).

3.3.1.2 Changes in medium kinetics in CIS

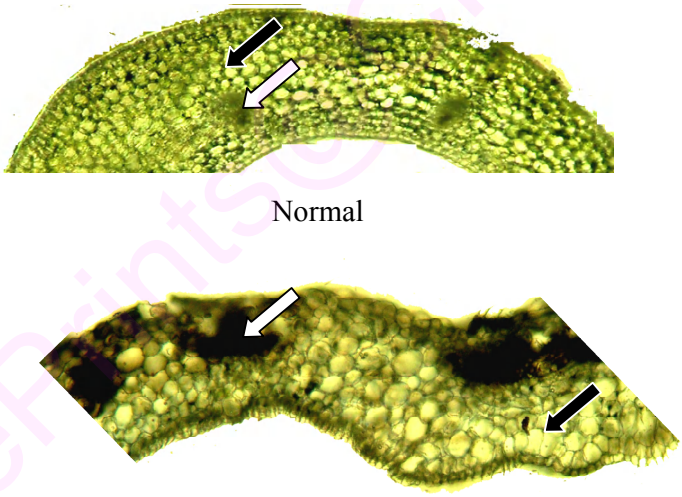
The pattern of variation in key media characteristics like pH, osmolarity and conductance during the course of HHS development is presented in **Figure 3.7**. A sudden fall in the pH of the medium from 5.7 to 4.3 was observed just after inoculation (JAI) of the shoot cultures, which stabilized within a week attaining an equilibrium pH of 4.7 throughout the culturing period of five weeks (**Figure 3.7**). Omolarity of the medium, which was initially 0.225 Osmol kg^{-1}, showed a steady decline to a level of 0.069 Osmol kg^{-1} by the end of culturing period with a steep fall between 2^{nd} and 3^{rd} week (**Figure 3.7**). The medium conductance, which reflects the electrolyte concentration, showed an initial value of 6.4 mS. There was a steady decline to a level of 4.05 mS by the end of culturing period

indicating a slow and continuous uptake of the nutrients by the shoot cultures (**Figure 3.7**).



Normal Hyperhydric

Figure 3.4 Cross-sections of stem in normal and hyperhydric (stage H2) shoot cultures of vanilla. Vascular tissue (white arrows) and endodermis (black arrows) of stem



Normal

Hyperhydric

Figure 3.5 Cross-sections of leaf in normal and hyperhydric (stage H2) shoot cultures of vanilla. Vascular tissue (white arrows) and palisade parenchyma (black arrows) of leaf

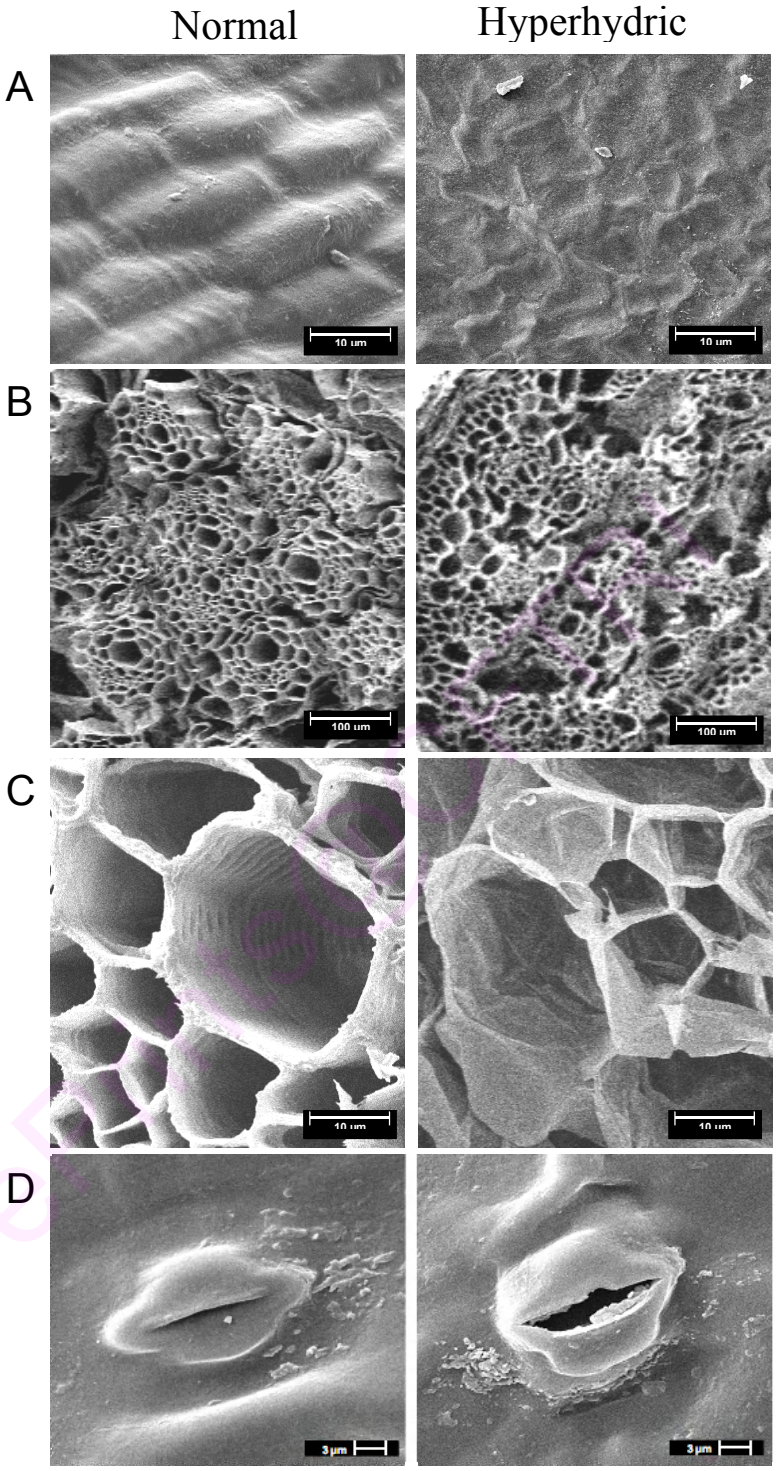


Figure 3.6 Scanning electron micrographs of normal and hyperhydric shoot cultures (stage H2) of vanilla. A: Stem surface. B: Vascular tissue. C: An enlarged view of B showing secondary wall thickening of the vascular tissue. D: Stomatal structure

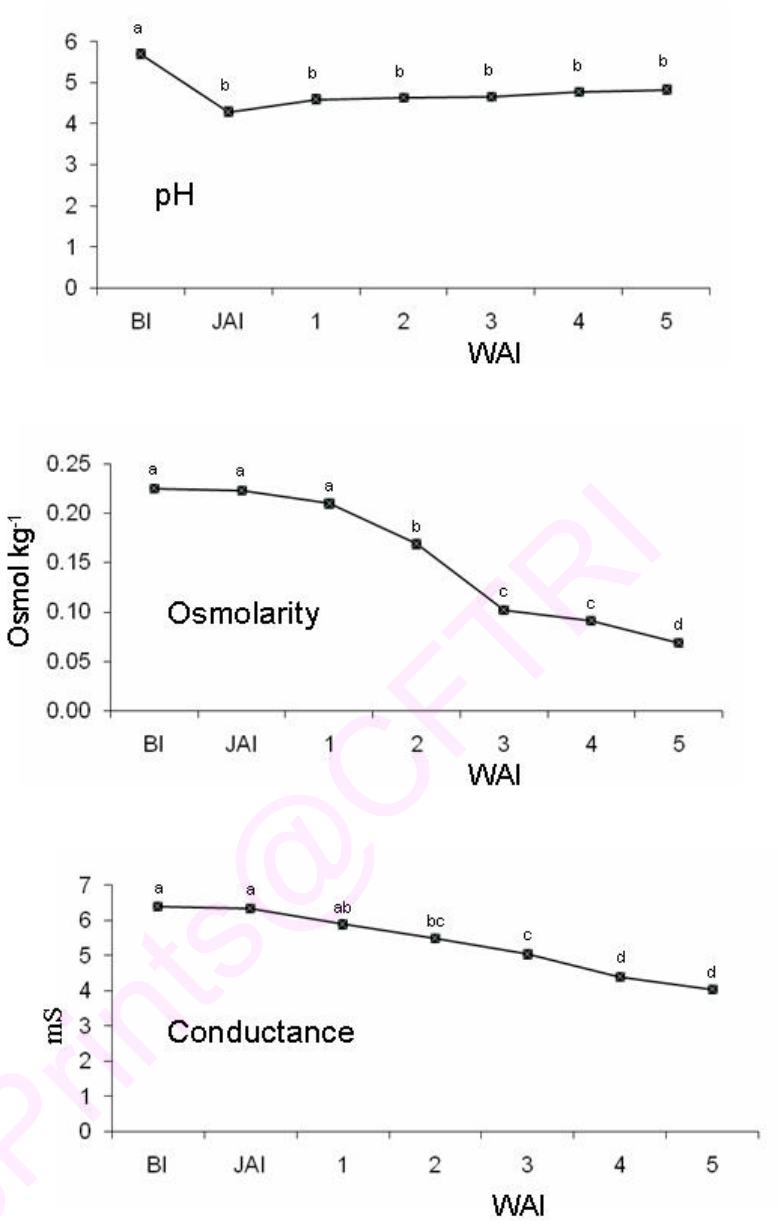


Figure 3.7 Kinetic parameters of the medium used for culture of vanilla shoots. pH, osmolarity, and conductance in complete immersion system (CIS). BI: before inoculation; JAI: just after inoculation; WAI: weeks after inoculation. Data presented as mean of five replicates. Means with different letters are significantly different at $p \leq 0.05$, according to Duncan's multiple-range test

3.3.1.3 Changes in chlorophyll and carbohydrate contents

At H1 stage, shoot cultures exhibited much lesser variations in levels of total chlorophyll and chlorophyll-b, compared to normal shoots (**Table 3.3**). However, at subsequent periods there was a significant loss of chlorophyll at H2 and H3 stages. The loss of chlorophyll-a was about 72.5% and the loss in chlorophyll-b was over 64% from normal to H2 stage, with a loss of 70% of total chlorophyll. H3 stage had almost lost the major amount of chlorophyll, which is also evident from the **Figure 3.2**.

An estimation of total carbohydrates, taking into account the reducing and non-reducing sugars from both soluble and insoluble fractions, was carried out in the normal and hyperhydric shoot cultures (**Table 3.3**). The total soluble sugar was highest in normal shoots whereas the total insoluble sugar was highest in both normal and stage H1 cultures. Hyperhydric shoot cultures had significantly lower concentrations of soluble reducing sugars compared to the normal shoots. Total soluble and soluble reducing sugars were very low in stage H3 cultures. Highest amount of reducing sugars was found in the insoluble fraction in H1 stage. However, normal cultures showed highest content of non-reducing sugars in the insoluble fraction. Regarding non-reducing sugars in insoluble fractions, there was a steady decline in their levels from normal to H2 stage, with an abnormal high level at the H3 stage.

3.3.1.4 Polyamines content

The change in the content of free polyamines is presented in **Table 3.4**. A significantly sharp increase in the concentration of all the three PAs was noted in stage H2 cultures. A sudden increase in the concentration of *Put* was found in cultures of stage H2 from base level in normal and H1 stage cultures. However, it showed a pitfall in H3 stage cultures.

Almost a 1.8-fold increase in *Spd* content was observed in H2 compared to normal and H1 stage, which dropped in H3 stage. *Spm* was high in the normal cultures, with a significant decline at H1 and steeply increased at H2 stage. The increase in *Spm* concentration was to an extent of 1.6-fold in H2 stage cultures compared to H1 stage shoots. It again declined to nearly half of its concentration from H2 to H3 stage.

Table 3.3 Changes in the levels of chlorophyll and carbohydrates in normal (N) (shoots cultivated on solid-medium) and hyperhydric shoot cultures of vanilla

	Chlorophyll (% FW)			Soluble sugars (mg g^{-1} FW)			Insoluble sugars (mg g^{-1} FW)		
Stage	Total	Chlorophyll-a	Chlorophyll-b	Total	Reducing sugars	Non-reducing sugars	Total	Reducing sugars	Non-reducing sugars
N†	0.0057±0.0005a	0.0040±0.0003a	0.0017±0.0002a	13.45±1.24a	8.86±0.34a	4.58±0.13a	4.55±0.2a	2.94±0.18b	1.6±0.04a
H1	0.0051±0.0005a	0.0034±0.0003b	0.0018±0.0002a	10.04±0.5b	6.01±0.22b	4.04±0.3a	4.84±0.21a	4.11±0.2a	0.73±0.07b
H2	0.0017±0.0017b	0.0011±0.0001c	0.0006±0b	6.92±0.34c	3.79±0.17c	3.13±0.18b	2.63±0.12b	2.54±0.11b	0.09±0d
H3	0.0003±0c	0.0002±0d	0.0001±0c	2.78±0.21d	2.15±0.15d	0.63±0.06c	1.46±0.08c	1.1±0.1c	0.37±0.02c

Hyperhydric shoots: 1 week after inoculation (H1), 3 weeks after inoculation (H2) and 5 weeks after inoculation (H3)
† No significant differences in the chlorophyll and carbohydrate contents were noticed among solid-medium cultivated shoot cultures of initial, 1st, 3rd and 5th weeks of culturing.
Data presented as mean±SD of five replicates. Means with different letters are significantly different at $p \leq 0.05$, according to Duncan's Multiple Range Test

Table 3.4 Levels of free polyamines (Putrescine (*Put*), Spermidine (*Spd*) and Spermine (*Spm*)) in solid-medium and CIS cultured shoots

	***Put* (nmol g^{-1} FW)**		***Spd* (nmol g^{-1} FW)**		***Spm* (nmol g^{-1} FW)**	
	Solid	CIS	Solid	CIS	Solid	CIS
1.	12.92±1.09ab	12.92±.09b	18.1±0.81a	18.1±0.81b	2074.44±199.12a	2074.44±199.12a
2.	16.9±1.91a	9.05±0.91b	17.93±0.99a	18.64±1.76b	1985.4±89.12a	1291.77±122.34b
3.	16.34±2.03a	595.07±57.95a	19.96±0.12a	32.00±3.01a	2100.3±34.19a	2121.27±211.1a
4.	14.68±1.17a	64.5±6.22b	20.1±1.99a	8.48±0.88c	1947.36±34.88a	1087.63±104.7b

Solid-medium cultured shoots: (1) 0 weeks after inoculation (N), (2) 1 week after inoculation, (3) 3 weeks after inoculation and (4) 5 weeks after inoculation; CIS cultured shoots: (1) 0 weeks after inoculation (N), (2) 1 week after inoculation (H1 stage), (3) 3 weeks after inoculation (H2 stage) and (4) 5 weeks after inoculation (H3 stage).

Data presented as mean±SD of triplicates. Means with different letters are significantly different at $p \leq 0.05$, according to Duncan's Multiple Range Test

3.3.1.5 Protein content and activities of antioxidant enzymes

Vanilla shoot cultures displayed wide variations in total protein concentrations, with significant loss towards the progression of HHS (**Figure 3.8**). Lowest protein content of 8.14 mg g^{-1} FW was found in H3 stage, which was nearly 77% lesser than that in normal shoots.

POD: A high activity of POD was maintained in normal, H1 and H2 stage cultures (**Figure 3.8**). Highest enzyme activity (356.93 U mg^{-1} protein) was noticed in H1 stage which was on par with the activity in H2 stage cultures (350.35 U mg^{-1} protein). However, HHS stage H3 showed a significant loss in the enzyme activity (59.93 U mg^{-1} protein) (**Figure 3.8**). In the zymogram (**Figure 3.9**) POD activity showed three isoforms in both normal and HH shoot cultures with a significant difference between the two, i.e., the bands of H2 stage cultures showed high intensity indicative of high activity whereas those of normal shoots (N) showed faint activity.

SOD: The activity of SOD was highest in normal and H1 stage cultures with a significant loss in H2 stage cultures. A substantial drop in the SOD activity was noted (nearly 6-fold) (6.01 U mg^{-1} protein) in H3 stage cultures compared to normal and H1 stage cultures (**Figure 3.8**).

CAT: A significantly higher activity of CAT was noticed in H2 stage cultures compared to H1 stage and normal cultures. The enzyme displayed highest activity of 82.75 U mg^{-1} protein in H2 stage cultures with more than 1.4-fold increase in the activity compared to H1 stage (58.04 U mg^{-1} protein). However, there was a steep fall (more than 2-fold) in its activity in H3 stage cultures (38.48 U mg^{-1} protein) compared to H2 stage cultures (**Figure 3.8**).

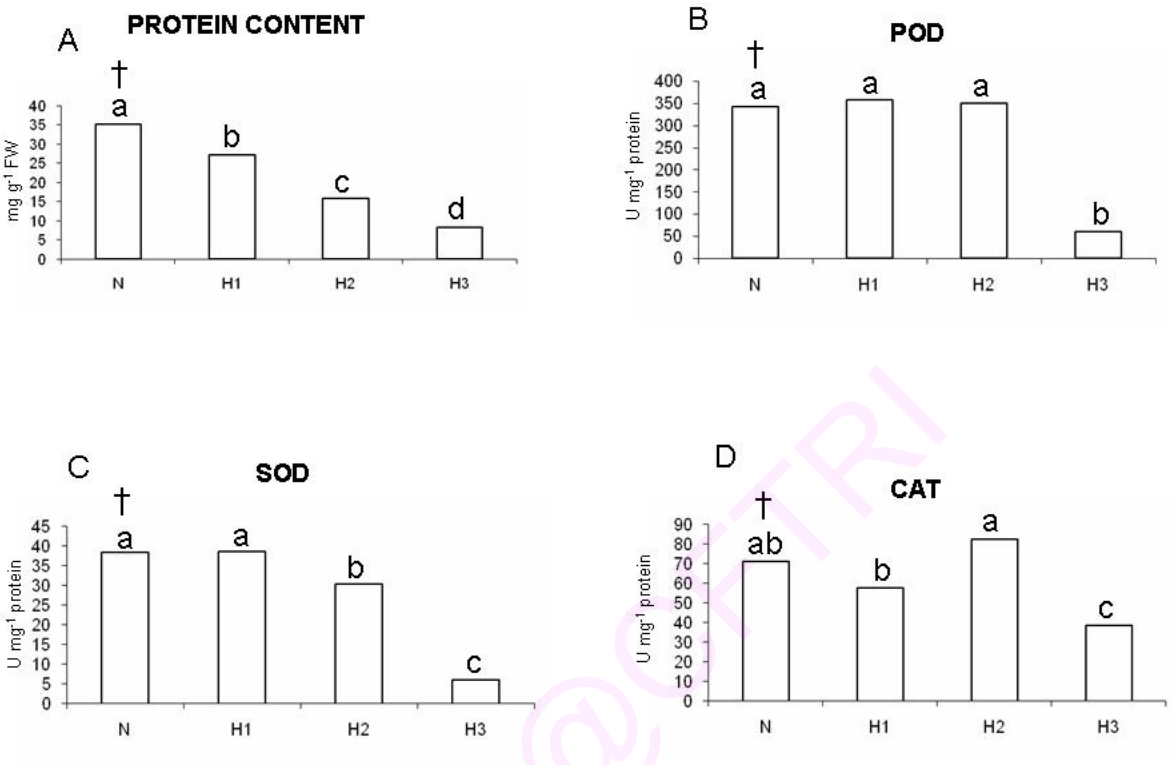


Figure 3.8 Protein content and activity of antioxidant enzymes in normal (shoots cultured in solid medium) and hyperhydric shoot cultures: shoots from CIS 1 week after inoculation (H1 stage), shoots from CIS 3 weeks after inoculation (H2 stage), and shoots from CIS 5 weeks after inoculation (H3 stage). Data presented as mean of five replicates. Means with different letters are significantly different at $p \leq 0.05$, according to Duncan's multiple-range test. † No significant differences in the protein content and activity of antioxidant enzymes were noticed among solid-medium cultivated shoot cultures of initial, first, third, and fifth week of culturing.

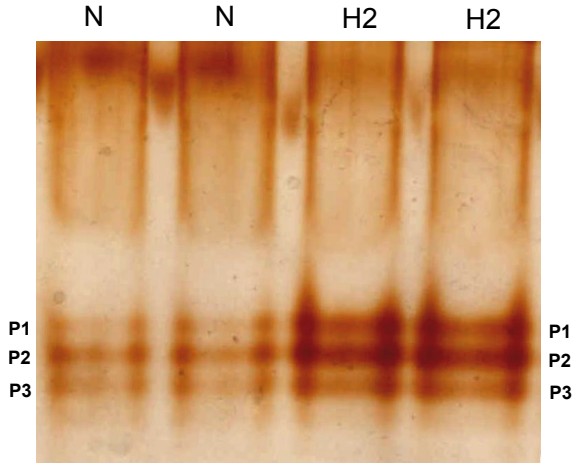


Figure 3.9 Zymogram on denaturing gel of PODs after activity staining. N: of normal shoots and H2: of hyperhydric (stage H2, shoots from complete immersion system 3 weeks after inoculation) shoot cultures. P1, P2, and P3 represent various isoforms of PODs

3.3.2 Molecular Changes

In the present study, mRNA Differential Display technique was employed by the means of RNAimage kit® (GenHunter Corporation, Nashville, TN, USA) as a general strategy to obtain fragments of genes differentially expressed under hyperhydric stress condition. This technique amplifies cDNA sequences from subsets of mRNAs by reverse transcription and PCR. Total RNA was isolated from four different stages of shoot cultures and was used for cDNA synthesis. The use of 27 primer combinations (**Table 3.1**) in the four RNA populations resulted in 114 distinct scorable fragments in the silver-stained polyacrylamide gels within the scorable area of 100 to 1000 bp. Thirty one fragments were selected based on differential display pattern of either present/absent or of higher/lower intensity (**Figure 3.10**). These were eluted from the gel and were re-amplified using the same primer combinations as used earlier for amplification of respective cDNA fragment after reverse transcription. Most of the candidate bands generated single target PCR product which corresponded to the size observed in the original DD gel. The remaining candidate bands produced more than one PCR product from which the target PCR product appeared more intense than the non target one. These single bands and the intense bands were selected for cloning and sequence analysis. Initially, the up-regulation and down-regulation (differential expression) of the genes was screened and

documented based on the variation in intensity levels of the selected candidate bands, which were eluted from the DD gel (**Table 3.5**). Later Northern dot-blot analysis was performed for selected clones to confirm the differential expression which was followed by analysis of the expression levels of important genes by qRT-PCR.

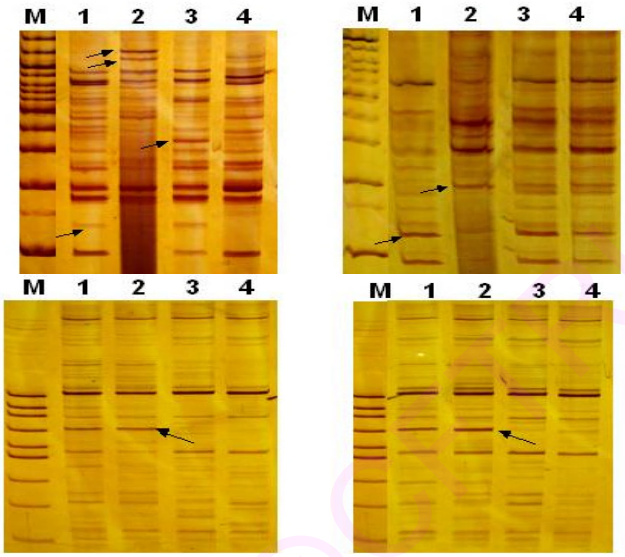


Figure 3.10 Differentially expressed bands visualized on polyacrylamide gel (M: Protein marker; 1, 2, 3, 4: PCR amplified products of different cDNAs)

Table 3.5 List of the isolated bands, their size and expression based on their expression in PAGE gel electrophoresis

Sl. No.	Fragment ID	Size (bp)	Expression	Sl. No.	Fragment ID	Size (bp)	Expression
01	VP1	250	Down regulated	17	VP17	241	Down regulated
02	VP2	392	Up regulated	18	VP18	218	Up regulated
03	VP3	239	Down regulated	19	VP19	361	Up regulated
04	VP4	285	Up regulated	20	VP20	771	Up regulated
05	VP5	254	Down regulated	21	VP21	684	Down regulated
06	VP6	147	Down regulated	22	VP22	615	Down regulated
07	VP7	115	Up regulated	23	VP23	506	Down regulated
08	VP8	358	Up regulated	24	VP24	729	Down regulated
09	VP9	313	Down regulated	25	VP25	420	Up regulated
10	VP10	333	Down regulated	26	VP26	506	Down regulated
11	VP11	264	Up regulated	27	VP27	557	Up regulated
12	VP12	345	Down regulated	28	VP28	537	Down regulated
13	VP13	216	Up regulated	29	VP29	533	Down regulated
14	VP14	309	Up regulated	30	VP30	416	Up regulated
15	VP15	175	Down regulated	31	VP31	376	Down regulated
16	VP16	324	Up regulated				

3.3.2.1 Cloning

All the selected re-amplified amplicons were cloned into pKRX-T cloning system which takes advantage of the ability of *Taq* polymerase to add a terminal 3'deoxyadenosine during the PCR. These vectors contain a 3'T overhang at the cloning site, allowing sticky end ligation which is more successful than blunt end ligation. However, the resolution of the re-amplified PCR products on a 1.5% agarose gel was insufficient to detect 5-20 bp differences and multiple products were not observed until cloned fragments were amplified using the universal sequencing T3 and T7 primers.

3.3.2.2 PCR analysis to confirm cloning

Both the T3 and T7 primers anneal to sites in the pKRXT vector 60 bp upstream or downstream respectively from the cloning site. Therefore, colonies that contained plasmids with inserts gave a band of 160 plus the size of the insert. Based on the PCR results, clones with the correct insert size were selected. All the 31 cloning and transformation reactions yielded recombinant clones from which the plasmid DNA was isolated for sequencing.

3.3.2.3 Sequence analyses of cDNA fragments

Clones from all the 31 cDNA fragments were sequenced using both T3 forward as well as T7 reverse primers which produced readable sequences. Sequence length varied from 115 bp to 771 bp. The size of these cDNA fragments were relatively close to the estimated size from the original display from which these fragments were obtained. All the cDNA fragments were amplified with their respective arbitrary primers on both the 5' and 3' ends as determined by sequencing. Nucelotide sequences of these cloned cDNA fragments are presented in the Appendix.

3.3.2.4 BLASTN analysis

Analysis of the 31 sequences of the cloned products for homology with the EST sequences available in the genetic data base resulted in four groups on their functional similarity (**Table 3.6**). They are as follows:

Group I. Abiotic Stress

This particular group consists of thirteen sequences which are potentially involved in the event of abiotic stress. Three of these cDNA fragments were found related to salt stress cDNA libraries of *Oryza sativa* and *Fragaria vesca*. Three of the fragments were found to have homology with ESTs of nutrient starvation like phosphate (VP1) and nitrogen or glucose (VP22). The other was found to have homology with cDNA library of nutrient deprived roots in tomato (VP24). Two of the fragments had homology with ESTs of etiolated *Panicum virgatum* seedlings (VP2) and light stressed *Haematococcus pluvialis*

(VP6). The other fragments had homology with the cDNA libraries of heat, water and other biotic and abiotic stress.

Group II. Biotic Stress

The five cloned sequences of this group were detected to share homologies with the genes expressed during various biotic stresses. While three of them had sequence similarity with the cDNA library of the wild leaf response of *Zingiber* species to *Phythium aphanidermatum* (VP14, VP18 and VP19), the other two had homology with the cDNA library of the soybean disease resistance (VP13) and *Citrus sinensis* affected by *Xylella fastidiosa*.

Group III. Ungrouped sequences

The third group included seven clones homolog to different cDNA libraries. Two of them (VP9 and VP10) were found related to genes expressed during tillering stage of *Oryza sativa*. Another two sequences (VP16 and VP30) showed homology with cDNA library associated with callus induction in oil palm and *Citrus sinensis*. The clone VP3 showed significant homology with genes encoding non-stressed condition in *Musa* while the clone VP7 showed homology with cDNA induced in banana by ethylene treatment. Clone VP27 had homology with EST sequence of developing shoot buds of *Actinidia deliciosa*.

Group IV. Novel sequences

This group is constituted by the sequences which were novel and had no homology with the genetic database available. A total of six clones were found in this category.

3.3.2.5 BLASTX analysis

The BLASTX analysis of all the 31 sequences resulted in five groups of clones which are mainly associated with stress, metabolism and DNA replication and repair. Between the other two groups, one had unclassified clones and novel sequences in the other (**Table 3.7**).

Group I. Stress

This group consists of three sequences having protein homology with zinc finger family protein (VP2) which is known to express as a response to oxidative stress in living systems, a truncated disease resistance protein of *Musa* (VP14) and gag-pol protein (VP16) which offers stress resistance.

Group II. Metabolism

Eight sequences were found to make this group in which three clones (VP5, VP12 and VP26) showed homology with pG1 protein having endopolygalacturonase activity. The clone VP11 had homology with gene coding for enoloase which has a major role in the glycolytic pathway. Clone VP15 showed homology to gene encoding triacylglycerol lipase enzyme involved in carbohydrate breakdown. The clone VP17 showed similarity to alpha-gliadin protein encoding gene while VP22 had sequence similarity for Golgi complex component Cog3 gene. VP28 displayed sequence homology with biotin-carboxylase encoding gene having a role in fatty acid biosynthesis.

Group III. DNA replication and repair

The third group constitutes of sequences which showed homology with the genes coding for proteins and enzymes having a major role in DNA replication and repair. While the first two (VP9 and VP10) sequences shared similarity with putative GNAP protein encoding genes involved in DNA replication, the next two (VP18 and VP19) had homology with the genes coding for transposase which is involved in DNA transposition. The other clone (VP21) displayed similarity with genes coding DNA binding SAP related to chromosomal organization and DNA repair. The clone VP24 was closely related to spliceosome-associated factor gene which splices hnRNA and clone VP29 had good homology with transcriptional regulator coding gene.

Group IV. Ungrouped sequences

This particular group consists of three sequences associated with proteins with varied functions. Clone VP1 had homology with phi-1 like protein coding gene which helps in phosphorylation and cell division. Another clone VP20 showed sequence similarity to gene encoding oxidoreductase domain protein involved in oxidation-reduction reactions. Clone VP30 had homology with sequence coding MAP kinase activating protein which has a major role in cell death or apoptosis.

Group V. Novel sequences

Ten sequences makeup this category which have no functional homology attached in the genetic base. These were treated as novel sequences of the products whose functions are yet to be arrived at.

Table 3.6 Homology search results for differentially expressed cDNAs during hyperhydricity syndrome of vanilla: BLASTN analysis

Fragment ID	Accession No.	Size (bp)	Homologue with highest homology in the genetic database		Significance *E* score; identities (%)
			EST-cDNA library	Acc. No.	
Group I. Abiotic Stress					
VP1	GE746280	250	*Emiliania huxleyi* grown on phosphate starved media	GE205735.1	0.006; 75%
VP2	GE746281	392	Ethiolated *Panicum virgatum* seedlings	FL901957.1	3e-16; 68%
VP5	GE746284	254	*Tamarix hispida* leaves at different stress times	EH055866.1	6e-117; 97%
VP6	GE746285	147	Light stressed *Haematococcus pluvialis*	GE649945.1	5.6; 100%
VP8	GE746287	358	Wheat during abiotic stress	CK163419.1	0.37; 86%
VP11	GE746290	264	Salt stressed *Fragaria vesca*	EX663966.1	8e-64; 85%
VP12	GE746291	345	Salt stressed *Oryza sativa*	EX451515.1	1e-121; 97%
VP21	GE746300	684	Abiotic and biotic stressed *Panicum virgatum*	GD019023.1	2e-13; 83%
VP22	GE746301	615	Nitrogen deprived *Neurospora crassa*	GH152236.1	8.2; 93%
			Glucose deprived *Neurospora crassa*	GH122179.1	8.2; 93%
VP24	GE746303	729	Nutrient deprived roots of Tomato	BF096278.1	9.8; 84%
VP25	GE746304	420	Salt stressed *Fragaria vesca*	EX657176	0; 96%
VP26	GE746305	506	Heat stressed *Fragaria vesca*	EX673998.1	0; 96%
VP31	GE746310	376	Water stressed *Glycine max*	CX705303.1	5e-06; 66%
Group II. Biotic Stress					
VP13	GE746292	216	Soybean disease resistance cDNA library	CV998036.1	6e-08; 97%
VP14	GE746293	309	*Zingiber* species wild leaf response to *Phythium aphanidermatum*	ES560490.1	5e-17; 98%
VP18	GE746297	218	*Zingiber* species wild leaf response to *Phythium aphanidermatum*	ES560490.1	7e-19; 98%
VP19	GE746298	361	*Zingiber* species wild leaf response to *Phythium aphanidermatum*	ES560490.1	6e-17; 98%
VP28	GE746307	537	*Citrus sinensis* leaf affected by *Xylella fastidiosa*	EY665891.1	1e-09; 76%

Group III. Ungrouped sequences					
VP3	GE746282	239	Nonstressed *Musa*	FL658728.1	2e-09; 78%
VP7	GE746286	115	Ethylene induced cDNA in banana	GE470242.1	0.027; 93%
VP9	GE746288	313	*Oryza sativa* tillering stage	CK036789.1	2e-04; 85%
VP10	GE746289	333	*Oryza sativa* tillering stage	CK036789.1	2e-04; 85%
VP16	GE746295	324	Non-embryogenic callus of oil palm	EY400192.1	4e-19; 69%
VP27	GE746306	557	Developing shoot buds of *Actinidia deliciosa*	FG411204.1	0.05; 88%
VP30	GE746309	416	Callus of *Citrus sinensis*	CV717662.1	4e-40; 90%
Group III. Novel sequences					
VP4	GE746283	285		NA	
VP15	GE746294	175		NA	
VP17	GE746296	241		NA	
VP20	GE746299	771		NA	
VP23	GE746302	506		NA	
VP29	GE746308	533		NA	

Table 3.7 Homology search results for differentially expressed cDNAs during hyperhydricity syndrome of vanilla: BLASTX analysis

Fragment ID	Accession No.	Size (bp)	Homologue with highest homology in the genetic database			Significance *E* score; identities (%)
			Protein homologue	Putative function	Acc. No.	
Group I. Stress						
VP2	GE746281	392	Zinc finger family protein	Oxidative stress response	NP850020.1	6e-19; 50%
VP14	GE746293	309	Truncated disease resistance protein of *Musa*	Disease resistance	CAP66362.1	3.4; 100%
VP16	GE746295	324	Gag-pol polyprotein	Stress resistance	AAR13317.1	4e-35; 62%
Group II. Metabolism						
VP5	GE746284	254	pG1 protein	Endopolygalactu -ronase	AA066461.1	3e-14; 61%
VP11	GE746290	264	Enolase	Glycolytic pathway	AAQ77241.1	8e-37; 91%
VP15	GE746294	175	Triacylglycerol lipase	Carbohydrate breakdown	ZP03388601.1	1e-07; 96%
VP17	GE746296	241	Alpha-gliadin protein		AAY45928.1	0.081; 33%
VP22	GE746301	615	Golgi complex component Cog3	Glycosylation	XP2143969.1	4e-07; 37%
VP26	GE746305	506	pG1 protein	Endopolygalactu -ronase	AA066461.1	6e-1; 57%
VP28	GE746307	537	Biotin-carboxylase	Fatty acid biosynthesis	YP1707481.1	1e-84; 98%
Group III. DNA Replication and repair						
VP9	GE746288	313	Putative GANP protein	DNA replication	AAT39189.1	0.4; 70%
VP10	GE746289	333	Putative GANP protein	DNA replication	AAT39189.1	0.4; 70%
VP18	GE746297	218	Transposase	Transposition of DNA segmants	CAL47051.1	0.18; 100%
VP19	GE746298	361	Transposase	Transposition of	CAL47051.1	0.18; 100%

VP21	GE746300	684	DNA binding SAP	DNA segmants Chromosomal organization, DNA repair	ABD33066.1	3e-08; 34%
VP24	GE746303	729	Spliceosome-associated factor	Splice hnRNA	XP954790.1	0.1; 28%
VP29	GE746308	533	Transcriptional regulator	Transcription regulation	ZP02620321.1	5e-04; 22%
Group IV. Ungrouped sequences						
VP1	GE746280	250	phi-1 like protein	Phosphorylation, cell division	AAM08535.1	8e-05; 56%
VP20	GE746299	771	Oxidoreductase domain protein	Oxidation-reduction	YP821867.1	9e-81; 57%
VP30	GE746309	416	MAP kinase activating protein	Apoptosis	BAD61807.1	1e-19; 45%
Group IV. Novel sequences						
VP3	GE746282	239		NA		
VP4	GE746283	285		NA		
VP6	GE746285	147		NA		
VP7	GE746286	115		NA		
VP8	GE746287	358		NA		
VP13	GE746292	216		NA		
VP23	GE746302	506		NA		
VP25	GE746304	420		NA		
VP27	GE746306	557		NA		
VP31	GE746310	376		NA		

3.3.2.6 Verification of the DD cDNAs expression by RNA blot analysis

Results of Dot blot hybridization confirmed that clone VP2 and VP9 (**Figure 3.11**) were differentially expressed during hyperhydricity syndrome where there was increased accumulation of VP2 mRNA (Zinc finger family protein) and fall in accumulation of VP9 mRNA (Putative GANP protein). The clone 18*S* (18*S* ribosomal RNA gene of *Vanilla planifolia*) was used as a control which had uniform expression.

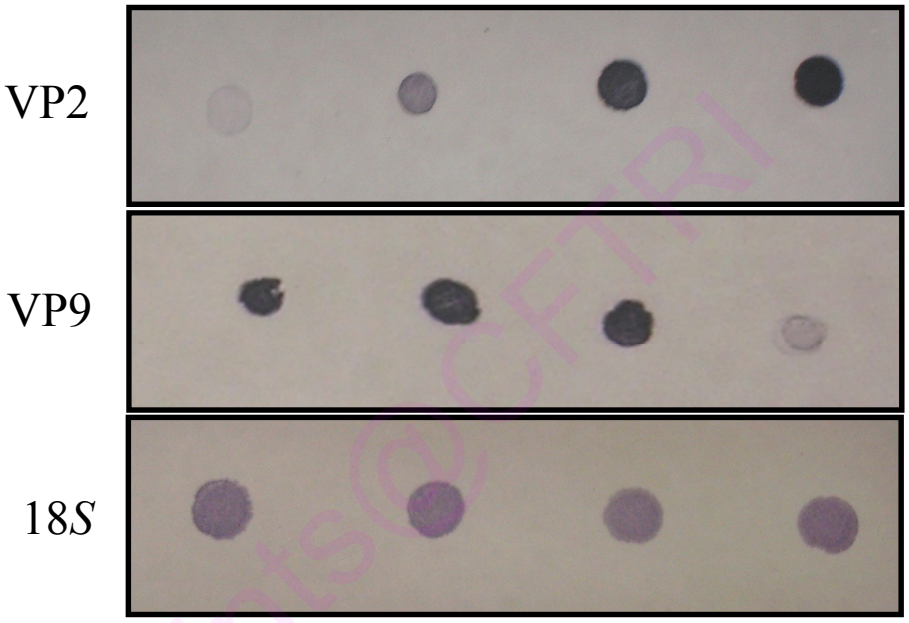


Figure 3.11 Formation of blots of different intensities confirming differential accumulation of gene products of VP2 and VP9. Total RNA was used for dot blot analysis and was hybridized with Dig-labeled probes

3.3.2.7 Quantitative (relative) reverse transcriptase polymerase chain reaction (qRT-PCR) of selected genes

Each band was normalized against the intensity obtained with the same cDNA using the internal 18*S* primers of *Vanilla planifolia*. All the comparisons of the abundance for the transcripts were made with the abundance in normal shoot cultures. There was a more that 20-fold increase in the relative transcript abundance level of VP2 clone corresponding to zinc finger family protein in the H2 stage of HHS and a relatively higher level of 19-fold was maintained in the H3 stage compared to the level in normal shoots (**Figure 3.12A**). The relative transcript abundance level of

VP9 (putative GANP protein) was highest to an extent of 1.46-fold in the H1 stage which showed a drastic reduction to a 0.06-fold compared to normal shoots indicating down-regulation of this particular gene during HHS (**Figure 3.12B**). A slight but significant up-regulation of clone VP12 (pG1 protein) was observed as HHS progressed (**Figure 3.12C**). A near 20-fold increase in the relative expression level of VP11 (Enolase) in the H2 and H3 stage cultures was seen compared to normal shoots (**Figure 3.12D**). Clone VP30 (MAP kinase activating protein) showed an increase of nearly 22-fold in H2 stage and 17.5-fold in H3 stage cultures (**Figure 3.12E**).

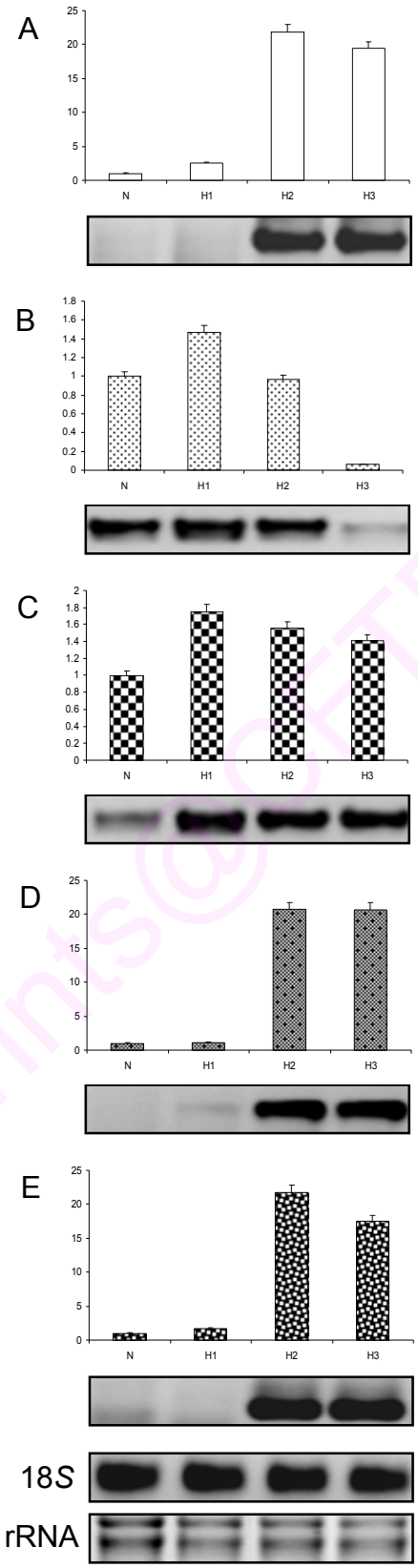


Figure 3.12 Expression and relative transcript abundance of different hyperhydricity related genes. A: Zinc finger family protein (VP2); B: Putative GANP protein (VP9); C: pG1 protein (VP12); D: Enolase (VP11); E: MAP kinase activating protein (VP30)

3.4 Discussion

3.4.1 Morphological and Biochemical changes

Vanilla planifolia normally displays xerophytic characteristics and therefore, one can expect the difficulty in growing this plant in submerged (hydrophytic) conditions. However, as in most of the other shoot cultures, which easily adapt to agitated medium, the vanilla shoots were also expected to grow and multiply better in CIS. Although shoot number increased in CIS as expected (**Figure 3.1**), the shoots underwent severe hyperhydricity conditions leading to HHS (**Figure 3.2**), where progressive accumulation of water was evident. In other plants where hyperhydricity conditions are reported, anatomically there was hypertrophy of the cortex and pith, and parenchyma displayed large intercellular spaces (Hazarika 2006). Even in case of vanilla with HHS, there was a general loss of cellular integrity and pronounced degradation of vascular tissues. Severe structural damage was observed at the surface of the leaves. The endodermis, palisade and vascular tissues were found severely collapsed (**Figure 3.4 & 3.5**). The conducting vessels, particularly xylem and tracheids in normal plants displayed beautiful architecture of secondary walls in xylary tissues whereas the vasculatures of HHS appeared decomposed (**Figure 3.4, 3.5 & 3.6**). Vascular bundles lacked the typical arrangement as found in normal shoots, indicative of lack of lignin synthesis and degradation of existing secondary wall structures (George 1996). It has been a general observation that most stomata of *in vitro* plantlets do not have a closure mechanism, a cause linked to water loss and death of plantlets during acclimatization under low relative humidity (Hazarika 2006). Contrarily, vanilla plants *in vitro* displayed structurally normal stomata (**Figure 3.6D**). Regarding the induction of HHS stage, the significant changes in the carbohydrates indicate the structural degradation of cell walls.

With respect to the medium kinetics, the drop in pH upon introduction of cultures into medium is due to the acidic nature of the plant material (Escalona et al. 1999). Although vanilla shoots were obtained from previous cultures, the trimming of unwanted tissues creating cut ends probably results in leaching of sap. This would lead to a sudden drop in the pH of the medium to 4.3, and its improvement and

stabilization later at 4.7 suggests that the ionic status is maintained and hence there could not be a catastrophic effect of medium pH on the development of HHS. A rapid fall of the osmolarity of the medium noted between the 1st and 3rd week of culture indicates high uptake of minerals as well as sugars and the steady decline in conductance reflects the progressive uptake of ions (minerals). These trends inversely correlate with the increase in shoot growth and accumulation of biomass (data not shown). The lesser rate of change in osmolarity and conductance from 3rd week onwards indicates poor utilization of nutrients. Another possibility could be saturation of the water/nutrient uptake system.

Polyamines (PAs), the chemically non-protein straight-chain aliphatic amines, are known to play important role in various cellular processes (Bais and Ravishankar 2002). PAs have been implicated in direct scavenging of free radicals, thereby reducing oxidative effects. They may also act indirectly by elevating the levels of antioxidants. *Put* is known to suppress the level of superoxide and H_2O_2 in leaf of stressed plants. The chilling induced H_2O_2 production was found inhibited in cucumber seedlings after *Spd* pretreatment (Shen et al. 2000). *Spm* (a tetramine), *Spd* (a triamine) and their precursor *Put* (a diamine) are known to play a major role in assisting the plants and their cells/tissues to adapt to stressful conditions through acting as antioxidants and rendering protection to biological membranes against peroxidation (Verma and Mishra 2005). In vanilla, *Spm* level was far higher than *Spd*, although both are derived from *Put* showing a particular dynamics of free PAs. Such stress-dependant increase in the levels of *Spm* has also been observed in other studies (Kumar and Rajam 2004; Silveira et al. 2006). A similar trend was observed even in the case of banana cultures with progressive subcultures (Venkatachalam and Bhagyalakshmi 2008). The control vanilla shoot cultures (in solid medium) did not show significant variations in the contents of PAs during the culture of 5 weeks; contrarily our study with banana cultures showed a slow accumulation of free PAs during 12 weeks period (Venkatachalam and Bhagyalakshmi 2008). In the present study, the cultures in CIS displayed the accumulation of free PAs, *Spm*, *Put* and *Spd* increasing substantially during HHS progression. The H3 stage where shoots had attained HHS, the levels of *Spd* and *Put* decreased and most of the proteins and

activities of antioxidant enzymes also decreased possibly due to proteolysis. Treatment with herbicide atrazine, which induces HHS in plants, showed higher accumulation of *Put*, *Spd*, and *Spm* in pea leaves (Piqueras et al. 2002) indicating that even in case of vanilla shoots, the increase in all the three PAs are possibly associated with HHS. The polycationic nature of PAs can help them strongly bind to nucleic acids, proteins and phospholipids. They also interact with membrane phospholipids to stabilize then under stress conditions. *Spm* is known to be prominently involved in free radical scavenging. While *Put* was enhanced by cold stress, *Spd* was enhanced by water stress in chickpea, although the accumulation of PAs was short-lived in the combined presence of both the stresses (Nayyar and Chander 2004). Nevertheless the oxidative injury to stressed tissue could be reduced by exogenous application of respective PAs leading to concomitant increase in the endogenous levels of respective PAs, suggesting their direct or indirect role as antioxidants.

Reduction in the chlorophyll content was one of the earliest symptoms observable with the onset of HHS (**Figure 3.2**), which probably serve as an early marker for HHS. The changes in the levels of reducing and non-reducing sugars are expected to occur due to hydrolysis of polysaccharides as well as inter-conversions of soluble sugars as happens in ripening fruits with higher water activity. The decrease in soluble sugars at H3 stage may be due to their further degradation forming uronides. However, since there is no drastic change either in the pH of the medium, its conductance or osmolarity, the degradative products may be held within the tissues, without leaching into the medium.

The capacity of antioxidant defense system often increases under stress conditions (Gressel and Galun 1994). Saher et al. (2004) observed that hyperhydric leaves of carnation suffering from oxidative stress accumulate H_2O_2 and the tissues try to reduce the damage via induction of the sub-cellular antioxidant systems, by increasing the activity of detoxifying enzymes like CAT and PODs. In many other studies higher levels of antioxidant enzymes have been correlated with the oxidative stress and hence are considered as molecular markers for such situations (Mishra et al. 2006, Arbona et al. 2008). Therefore, the significant increase in the activity of CAT and maintenance of a higher activity of POD associated with the progression of

HHS is indicative of oxidative stress in vanilla shoot cultures. Among the three enzymes, SOD appears the foremost one to defend against the injury caused by ROS, catalyzing the dismutation of O_2^- to H_2O_2 and molecular oxygen. The H_2O_2 produced is then scavenged by several classes of PODs, the activity of which is also high at H1 stage. PODs are homoproteins present as multiple isozymes in plant tissues and are distributed throughout the cell carrying the function of catalyzing the reduction of H_2O_2 to H_2O. They belong to a large family of enzymes, having the ability to oxidize/reduce different substrates in the presence of H_2O_2 and thus maintain redox status of the cell. While the activity of POD was highest in vanilla cultures, the simultaneous increase in the activity of this enzyme and of CAT in the hyperhydric cultures at H3 stage indicates their good orchestrated effects in scavenging H_2O_2 as well as other free radicals. It is also known that PODs exist as a large group of isoenzymes with an extreme range of isoelectric points, serving a multitude of functions. Each group is thought to have a different function in the cell. Acidic (anodic) isoenzymes of POD are known for their involvement in growth and differentiation of cells, the basic forms are assumed to provide H_2O_2 for other PODs (Gulen and Eris 2004). In the present study the zymograms of POD from normal and HH shoots showed no new isoform formation in response to HH stress. However, higher intensity bands are indicative of increase in their activities.

Earlier studies have indicated both physical and chemical methods for the reversal and control of hyperhydricity. Among the physical methods, measures like flushing out ethylene, CO_2 and other volatile components accumulated in the headspaces of culture vessels appear essential to contain HHS (Lai et al. 2005). Avoiding the complete submergence of shoots has also been suggested for which, the use of temporary immersion systems providing intermittent contact between the plant material and the liquid culture medium have been proposed (Etienne and Berthouly 2002). In case of vanilla, our earlier studies have shown that despite 10 years of maintenance in solid medium (Sreedhar et al. 2007a) or any increase or decrease of cytokinin concentration in solid medium (George and Ravishankar 1997), the induction of HHS did not occur. Our present study shows that liquid medium in particular induces HHS indicating that water potential plays an important role in the

induction of HHS. However, in case of *Vitis vinifera* (cv. Albarino) although the increase in cytokinin level enhanced HHS condition, the addition of liquid phase to solid medium further increased HHS (Couselo et al. 2006). It is worth noting that our present study does not involve any change in growth regulator, where the level of cytokinin is constant both in liquid and solid medium. Agar at a concentration of 0.8% was found to be the best gelling agent to avoid hyperhydricity as lower concentrations clearly induced hyperhydricity. A double-phase culture (using liquid and gelling agent solidified culture system) was also proposed to obtain higher biomass and shoot number compared to solid medium in Japanese pear (Kadota et al. 2001). However, as mentioned above, this aggravated HHS condition in *Vitis vinifera* (cv. Albarino) (Couselo et al. 2006). Among the chemicals, activated charcoal, phloridzin and paclobutrazol have been used for specific shoot cultures (George 1996).

3.4.2 Molecular Changes

To obtain comprehensive information on gene expression pattern a more effective and straightforward strategy would be the genomic approach where isolation of numerous genes and their characterization on the basis of homologies and similarities of expression patterns with genes previously studied. It would be possible to gain, in short time, a general framework of those genes involved, from which one can select appropriate exemplars for a more comprehensive analysis of the genes controlling hyperhydricity. Over the last 15 years, differential display has been used to isolate genes involved in physiological events, signal transduction, stress response and secondary metabolism. The present approach has led to the isolation, successful cloning, sequencing and bioinformatics analysis of 31 transcripts that were differentially expressed in DD gels in response to hyperhydricity in vanilla shoot cultures. This can be useful to assist future investigations to unravel the molecular mechanisms of hyperhydricity syndrome. Out of the 31 cDNA transcripts analyzed, 17 were found to be down-regulated and 14 up-regulated.

Results of sequence analysis of the 31 cDNAs are encapsulated in **Table 3.6 & 3.7**. BLASTN analysis showed that more than half of the hyperhydricity-induced cDNAs share homology with stress response expressed sequences in the GenBank

database. The abiotic stress seems to have developed as a result of higher availability of various salts and other nutrients in the liquid culture medium during cultivation of the shoot cultures as depicted by variation in the patterns of expression of genes involved in salt and nutrient stresses (**Table 3.6**). In view of the over expression of carbohydrate hydrolases, one may expect influx of salts causing ionic imbalance and expression of salt-stress-responsive genes. The development of callus in the hyperhydric shoot cultures was also observable which was complemented by an increase in expression levels of the genes related to growth response (VP27 and VP30, **Table 3.5 & 3.6**), especially re-induced type.

BLASTX analysis reveled that majority of cDNAs (10) did not share protein homology with any of the entries in the GenBank database with only three of them sharing homology with proteins associated with stress response.

Group I. Stress

Zinc-finger proteins have been reported to be involved in regulation of gene expression and RNA metabolism by direct associations with respective cognate target promoters. Some zinc-finger proteins have a role in defence or senescence regulation while many of other identified proteins have no function associated to them. A zinc-finger transcription factor is known to increase during tomato ripening together with other transcriptional factors, including MADS box genes, homebox genes and polycomb genes (Bartley and Ishida 2002). In pepper (*Capsicum annum* L.), a zinc-finger protein gene was found to express in red fruit but was undetectable in the green ones (Kim et al. 2004). This gene was also proposed to function as an early defense gene against pathogen (Kim et al. 2004). A zinc-finger identified by Luis and Cristina (2007) had high homology to proteins having putative role in responses to a variety of abiotic stresses.

The gag-pol poly protein which is a major protein imparting stress resistance in plants was found to be up-regulated during the course of development of hyperhydricity in vanilla. A similar observation was made by Maqbool et al. (2008) while studying drought stress responsive transcripts by differential display in cotton where two of the transcripts having homology with the gag-pol protein studied showed a significant increase in their expression levels. But in another study

involving identification of drought-related mRNAs in common bean roots, there was no regulation in the expression level of mRNA transcript having homology with gag-pol protein (Torres et al. 2006).

A near 20-fold increase in the relative transcript abundance of the gene related to zinc finger protein (**Figure 3.12A**) and up-regulation of the genes having homology with gag-pol protein in the hyperhydric shoot cultures of vanilla indicate their pivotal role in counteracting stress development due to HHS.

Group II. Metabolism

One of the most significant pectin-degrading enzymes in plants is polygalacturonase (PG). Its involvement in the softening of tomato during ripening by hydrolysis of the pectin of cell wall, causing loss of cell adhesion in the middle lamella is well established (Monic et al. 2007). Endopolygalacturonases have long been proposed to play an important role in fungal pathogenicity to plants by depolymerizing homogalacturonan, a major component of the plant cell wall. Besides acting as virulence factors, endoPGs may also function as avirulence determinants through release of oligogalacturonide inducers of plant defense and interaction with plant proteins that modulate PG activity (PGIPs) (Cervone et al. 1989; Antonio and Isabel 1998). Maintenance of a significant high level of relative transcript abundance of VP12 clone corresponding to pG1 protein during the development of severe collapse of the endodermis, palisade parenchyma and vascular tissue observed in an earlier study (Sreedhar et al. 2009) reveal that homogalacturonon in cell wall and which cause loss of cellular integrity during HHS.

Enolase is an integral enzyme involved in glycolytic pathway. It is involved in the dehydration of 2-phosphoglycerate to phosphoenolpyruvate. It has been identified as an anaerobic stress protein in *Echinochloa oryzoides* based on the homology of its interna1 amino acid sequence with those of enolases from other organisms, by immunological reactivity, and induction of catalytic activity during anaerobic stress (Theodore et al. 1995). An enolase cDNA clone (pENO2) from maize was successfully cloned and sequenced and its expression in maize roots under anaerobic stress was reported. Expression of ENO2 was found to be constitutive under aerobic conditions, whereas ENO1 levels were induced 10-fold in maize roots

after 24 h of anaerobic treatment (Shailesh et al. 1998). In a study during the induction of Crassulacean acid metabolism by environmental stresses in the common ice plant (*Mesembryanfhemum crysfallinum* L.) enzyme activities involved in glycolysis and gluconeogenesis, including enolase, increased significantly. Two nearly identical cDNA clones (Pghla and PghIb) encoding enolase from the common ice plant were described. Enolase activity increased more than 4-fold in leaves during salt stress. This increase was accompanied by a dramatic increase in Pghl transcription rate and the accumulation of enolase transcripts in leaves. Pghl transcript levels also increased in leaves in response to low temperature, drought, and anaerobic stress conditions and upon treatment of unstressed plants with the plant growth regulators abscisic acid and 6-benzylaminopurine. Development of anaerobic environment around the shoot cultures of vanilla during their submerged cultivation in liquid medium might play an important role in up-regulation of the gene controlling enolase (**Figure 3.12D**) and may play a vital role in HHS of vanilla. In roots of *Mesembryanthemum crystallinum*, enolase transcripts increased in abundance in response to salt, low and high temperature, and anaerobic stresses. Surprisingly, no increase in enolase protein levels was observed despite the increased levels of mRNA and enzyme activity during salt stress. The stress-induced increase in enolase activity was therefore found to be due to post-translational regulation of steady-state enzyme pools (Nancy et al. 1995).

Group III. DNA Replication and repair

Biological organisms are constantly exposed to environmental stimuli named stresses, and they are capable of establishing mechanisms of protection and adaptation. Stress has the most significant and mainly negative effect on organism growth, development, and reproduction and increases the genetic variability (Capy et al. 2000). Two mechanisms are frequently described: those involving the SOS response (the activation of mutagenic activity) or the MRS response (inhibition of an antimutagenic system, the mismatch repair system) (Taddei et al. 1997) and those involving transposable elements (TEs) (Capy et al. 1997). Transposase aids in the transposition of DNA segments or TEs. An increase in the expression levels of the DNA sequence having homology to Transposase enzyme (VP19) was observed with

the progression of hyperhydricity indicating the development of stress and the response of the shoot cultures to it (**Table 3.5**).

Induction or expression of defence genes has been observed as a short term response and transposable elements mobility as long term response of an organism to stressful environment (Capy et al. 2000). Increase in the expression levels of stress responsive DNA fragments like those having homology with zinc finger family protein (VP2) and gag-pol polyprotein (VP16) (**Table 3.5**) was observed as a short term response and increased expression of DNA sequence having similarity with Transposase enzyme for possible development of new genetic variability to adapt to new environment was observed in vanilla shoot cultures undergoing hyperhydricity stress. Down regulation of protein factors that are involved in replication, transcription and repair of DNA was an observable feature in the hyperhydric cultures.

Group IV. Ungrouped sequences

Plants respond to a variety of biotic and abiotic signals that influence growth and development. Although the responses of plants to these signals have been extensively studied at the physiological and the biochemical levels, the perception and the intracellular signal transmission mechanisms are largely unknown. Studies indicate that the MMK4 kinase pathway mediates drought and cold signaling independently of ABA. Extreme temperatures, drought, and salt stress induce a partially overlapping set of genes in different organisms. Incubation of tobacco leaf pieces in high salt medium induced a protein kinase with very similar properties to a MAP kinase. MAP kinases have been demonstrated to be activated upon injury such as cutting of leaves (Seo et al. 1995, Usami et al. 1995) and biotic stress such as exposure of cells to fungal elicitor (Suzuki and Shinshi 1995). Increased transcript levels of genes encoding a MAP kinase module have been taken as a key evidence for the involvement of a MAP kinase pathway in signaling response to touch, cold, salt, and water stress. It is quite evident that environmental stresses are mediated by post-translational activation of a specific MAP kinase in alfalfa. The MAP kinase pathway appears to mediate only specific forms of stress, because cold and drought, but not high temperature and osmotic stress, induce the activation of this pathway (Jonak et

al. 1996). Many MAPKs are activated by osmotic stress, cold, salt, drought, and wounding. All of these conditions are known to disturb the redox balance of plants. A role of the Arabidopsis MAPK module MEKK1-MKK2-MPK4/MPK6 was reported for cold and salt stress (Pitzschke and Hirt 2006).

3.5 Conclusion

The present study has given an insight into morphological and biochemical changes occurring during the progression of HHS in vanilla shoots. One of the main consequences of HHS appears to be the oxidative stress, which is evident from the increase in the levels of activities of antioxidant enzymes. The rapid loss of chlorophyll with concurrent increase in the content of free PAs serve as useful biochemical indicator of HHS in *V. planifolia.* On the basis of homology with known genes, the sequences which have been identified as preferentially expressed in vanilla shoot cultures during hyperhydricity would be involved in a wide range of basic metabolic functions and hence the data generated here would be highly valuable for understanding the regulatory cascades of hyperhydricity syndrome.

Summary

For developing an efficient rapid curing method, vanilla beans derived from the micropropagated plants as well as the commercial beans were subjected for curing following different biotechnological approaches. Aiming at reducing the curing period, effects of pre-treatments on the flavour formation in vanilla beans during accelerated curing at 38 ^{o}C for 40 days were studied. Moisture loss, change in texture, levels of flavouring compounds and activities of relevant enzymes were compared among various pre-treatments as well as the commercial sample. Use of naphthalene acetic acid (5 mg L^{-1}) or ethrel (1%) with blanching pre-treatment resulted in 3-fold higher vanillin on 10^{th} day. Other flavouring compounds - vanillic acid, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde fluctuated highly showing no correlation with the pre-treatments. Scarification of beans resulted in nearly 4-fold and 3.6-fold higher vanillin formation on 10^{th} day in NAA and ethrel-treated beans respectively as compared to control with a significant change in texture. When activities of major relevant enzymes were followed, addition of NAA or ethrel helped to retain higher levels of cellulase throughout the curing period and higher levels of β-glucosidase on 20^{th} day that correlated with higher vanillin content during and subsequent periods. Peroxidase, being highest throughout, did not correlate with the change in levels of major flavouring compounds. The pre-treatment methods of the present study may find importance for realizing higher flavour formation in a shorter period since the major quality parameters were found comparable to commercial sample. In another experiment, food-grade elicitors, selected after an initial screening, were used in combination with pre-treatments for the accelerated curing of beans. When acetone dried red beet elicitor-a rich source of peroxidase (98,500 U g^{-1} dry weight) was used, 2.65% vanillin was formed in 10 days, which was 1.7-fold higher than in control beans (blanched + scarified) of this study and 3.23-fold higher than the conventional curing (0.82%). HPLC analysis of elicitor-treated samples showed the formation of almost all the major compounds found in the conventionally cured beans (cured for 3-6 months) with better sensorial properties. These observations appear useful for developing a rapid process for the curing of vanilla beans.

Publications

1. **Sreedhar RV,** Roohie K, Venkatachalam L, Narayan MS, Bhagyalakshmi N (2007) Specific pre-treatments reduce curing period of Vanilla (*Vanilla planifolia*) beans. **Journal of Agricultural and Food Chemistry 55: 2947-2955**

2. **Sreedhar RV,** Roohie K, Maya P, Venkatachalam L, Narayan MS, Bhagyalakshmi N (2008) Biotic elicitors enhance flavour compounds during accelerated curing of vanilla beans. **Food Chemistry 112: 461-468**

4.1 Introduction

Natural vanilla flavour, obtained from cured beans of *Vanilla planifolia* forms the highest-priced flavour ingredient in food (60%), cosmetics (33%) and aromatherapy (7%) (Priefert et al. 2001). The major compound vanillin is the most preferred flavouring compound among the universally used aromas and has a great market potential in food, beverage, cosmetic and pharmaceutical industries. Sixty five volatiles and 26 odour-active compounds are identified in the extract of cured vanilla beans (Perez-Silva et al. 2006); the major ones after vanillin are vanillic acid, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde. Characteristic vanilla flavour in beans is formed only during a careful curing process resulting in 2% vanillin (on dry weight basis) and over 170 other compounds with delicate sweet fragrance. In green vanilla beans, these phenolic aromatic compounds are present as their respective glucosides major being glucovanillin synthesized from phenylalanine of shikimic acid pathway and curing process is meant to release the aglycones as the free aroma compounds. Curing also induces the formation of many other compounds that complement to the delicate aroma of natural vanilla flavour. In fact, it is the presence of these minor compounds in large numbers that fetch high price for natural "vanilla extract".

Botanical study of vanilla beans reveals that the flavour precursors are found in the bean interior, i.e., placental region around the seeds, whereas the hydrolytic and other degenerative enzymes that are known to catalyze the reactions for the release of flavour compounds are localized mostly in the outer fruit wall (Havkin-Frenkel et al. 2005). The purpose of curing is to create contact between the flavour precursors and the enzymes that catalyze the hydrolysis of precursor compounds (Havkin-Frenkel et al. 2004). Curing of vanilla beans is a traditionally well-established process which is laborious and takes 3 to 6 months depending on different curing procedures adopted in different vanilla-producing regions (Havkin-Frenkel et al. 2004). Despite the long time required for the curing process, the enzymatic transformation of the glycosides to flavouring compounds is not very efficient. Only a fraction of the vanillin is produced by systematic curing of green beans of which a part may also be lost during exposure to sun as well as during extraction (Frenkel and Havkin-Frenkel 2006). Finally,

the total flavour yield depends upon the quality of starting material where the best quality beans range in length from 15-20 cm.

The conventional curing depends on weather conditions as it involves intermittent exposure to sun and sweating followed by conditioning (Dignum et al. 2001). Therefore, it is lengthy and cumbersome involving several months, may often fail to completely hydrolyze glucosides resulting in only fractions of flavour compounds (Frenkel and Havkin-Frenkel 2006). The curing process has been conventionally developed over a few centuries in vanilla-growing countries, as an art rather than a science. Thus curing is the most crucial and laborious step in the entire process of natural vanilla production.

To overcome the above problems, earlier workers used methods such as curing of the cut beans, covering beans in plastic sheets and heating them at 60 °C along with high humidity, freeze curing, treatment of green beans with various enzymes like β-glucosidase, pectinase and hemi-cellulase, hot-air drying and solar drying (Dignum et al. 2001; Ruiz-Teran et al. 2001). The development of vanilla flavour during these treatments is partly due to the hydrolysis of glycosylated precursors occurring in the green bean (Arana 1943). The most important step in vanilla curing has been found to be the scalding followed by incubation at 45 ^{o}C (Jones and Vincente 1949). An almost the same process wherein scalding in hot water of 63 ^{o}C for 2-3 minutes, followed by cutting the beans into pieces and incubation in an oven at 38 ^{o}C for 48 h followed by further curing in closed containers at 38 ^{o}C for 2-3 months resulting in pleasing aroma was also suggested (Broderick 1956). From then onwards, blanching in hot water has been an essential step traditionally followed before curing of vanilla beans. This has been a convention for several decades in various vanilla-growing countries of the world. For imparting the mild temperature treatment, sunning of the fruits has also been a regular practice, which invariably leads to losses at each exposure. In the present study, vanilla beans derived from both micropropagated plants and those bought from the market were cured following different biotechnological approaches for development of an efficient curing technique.

4.1.1 Use of pre-treatments

A simple and an effective process is needed to cut short the long processing time and increases the flavouring compounds in cured vanilla beans. To do this, the present study was aimed at the use auxin and ethrel in combination with different pre-treatments. Auxins and ethrel are compounds having popularity in the area of horticulture to induce parthenocarpy and ripening/senescence respectively. Auxins are plant growth regulators that are known to act by loosening the cell wall via enhancement of β-glucanase enzyme (Felix and Meins 1985) as well as by acidification of cell walls (Schubert and Matzke 1985) probably via inducing the secretion of hydrogen ions into and through the cell wall. Such acidification of cell wall by auxin leads to lipid breakdown thereby increasing the extensibility of membranes enhancing the permeability. Such effects in the present context are expected to create appropriate physico-chemical conditions for the enzymes to come in contact with the substrates and act to release the flavouring compounds. A preliminary screening was done and among the auxins, naphthalene acetic acid (NAA) was selected due to its better efficacy over others in supporting the biosynthesis of extractable phenolics (Funk and Brodelius 1990a, 1990b). An earlier report on cell cultures of *Vanilla planifolia* showed that NAA increased the secondary metabolism, resulting in high phenylalanine ammonia lyase (PAL) activity and high concentration of extractable phenolics (Funk and Brodelius 1990c). Ethrel is widely used as a source of ethylene to induce ripening in fruits. Its involvement in the phenyl-propanoid pathway is also known and hence selected for pre-treatment. Curing temperature was chosen as 38 °C, which is congenial for the activities of key enzymes involved in bioconversions of flavour precursors.

Sensorial analysis of the final product is done in food industry to evaluate the product acceptance. Instrument like Electronic nose is commonly used to make a quick assessment of aroma quality for quality control and product development. Electronic nose, like human nose makes a global analysis of vapours emitted from the sample and performs a classification process by comparing the samples with a database. E-nose utilizes the data preprocessor which is analogous to the olfactory bulb in the human olfactory region. The E-nose generally consists of 12 metal oxide sensors and the sensors essentially

measure the change in voltage due to the presence of odorous volatile molecules. Sensor responses are then analyzed by the software built-in with the equipment to get an olfactive picture of the product. E-nose has been successfully used for analysing coffee aroma (Gretsch et al. 1998) tea quality (Lucas et al. 1998). It has been used by Ravi et al. (2007) for charactrization of coriander aroma. Evaluation of flavour quality of pepper was carrriedout with the help of E-nose by Mamatha et al. in 2008.

4.1.2 Use of elicitors

A large number of handlings for sunning and sweating generally result in a low quality product. Due to these reasons, there are various attempts to modernize the curing process, which involves solar drying, oven drying and enzyme treatment (Dignum et al. 2001). However, so far there has been no attempt to apply elicitors for curing vanilla beans. Elicitors are compounds that trigger the increased production of pigments, flavones, phytoalexins and other defence related compounds. Elicitation has been found as an effective strategy for the induction and enhancement of secondary metabolites at a commercial scale. For example, synthesis of shikonin and its derivatives by suspension cultures of *Lithospermum erythrorhizon* was by the use of elicitors – agaro-pectins (Tabata and Fujita 1985). In genetically transformed root cultures of red beet a significantly high productivity of 5-fold betalain was observed when pullulan was used and 4-fold higher pigment accumulated when cultures were treated with dry cell powder of *Penicillium notatum* (Savitha et al. 2006). Peroxidase is one of the key enzymes in phenyl-propanoid (PP) pathway through which defence-related compounds are biosynthesized in higher plants. Significant elicitation in the activity of peroxidase was accomplished by the addition of the dry cell powder of Candida versatalis (3.5-fold higher than the control) or glutathione (3.44-fold) or *Rhizophus oligosporus* (3.09-fold) (Thimmaraju et al. 2006). Rao and Ravishankar (2000) listed biotransformations of a number of PP compounds to vanillin/vanillic acid. Nevertheless, failure of an elicitor to trigger a particular metabolic pathway does not necessarily establish its total in-efficacy. An elicitor may be ineffective under a combination of inappropriate conditions as well as unsuitable concentration of an elicitor. For example, the PP pathway was not induced in cultures of *V. planifolia* by yeast extract whereas the same elicitor induced phytoalexin in other cultures; the PP pathway in *V. planifolia* could however be triggered by using

chitosan (Funk and Brodelius 1990c), indicating that a successful elicitation is a very challenging process requiring intense screening procedures. While processing vanilla beans, the low yield of flavour compounds is also due to inefficient enzymatic conversion of the phenolic glycosides (Ruiz-Teran et al. 2001). Although flavour substrates and relevant enzymes co-exist in vanilla beans, low yields of flavour have been attributed to the compartmentalization of substrate and the enzyme (Odoux et al. 2003). The total picture of chemical reactions occurring while curing vanilla beans is not fully understood. However, the enzymes playing important roles in the flavour formation have been identified. β-Glucosidase (β-GLUC) is known to hydrolyze the glucovanillin, the non-aromatic precursor, resulting in the release of the major flavouring component - vanillin (Odoux et al. 2003). Peroxidase (POD) activity in vanilla beans is found to be quite high even during curing (Sreedhar et al. 2007b) and hence may be implicated in oxidation/reduction of flavour-forming compounds. For vanilla flavour development one may also envisage the involvement of cell wall degrading enzymes like cellulase, hemicellulase and pectinase by way of breaking down the cell walls and making the flavour substrates available for the enzyme to act (Ranadive 1992). Accordingly, the treatment of vanilla beans with additional enzymes resulted in enhanced flavour formation. For example, successive treatment of green vanilla beans with pectinase and β-glucosidase followed by curing was found to result in 6% vanillin compared to 1.75% in traditionally cured beans (Mane and Zucca 1999). In one study it was observed that a two step enzymatic reaction system with Viscozyme (mixture of cellulase, arabinase, hemicellulase, xylanase and pectinase from *Aspergillus*) and Celluclast-(Novo)® (cellulase from *Trichoderma reseii*) resulted in an increase of glucovanillin extraction and its further conversion to vanillin to an extent of 3.13-folds (Ruiz-Teran et al. 2001). With this background, the present study was undertaken to find out first whether the elicitors are useful for hastening the process of flavour formation, and if so, whether the levels of flavour turnover are corroborative with the levels of endogenous enzymes of the beans or with those of the elicitors, or both. The present study also addresses whether the flavour compounds thus generated are comparable with the profile and sensorial properties of conventionally cured beans.

4.2 Materials and Methods

4.2.1 Chemicals

Authentic vanillin, vanillic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde used as standards and the auxin - naphthalene acetic acid (NAA) were obtained from Sigma Chemical Co. St. Louis, MO, USA. Ethephon (39% SL, Bayer Crop Science India Ltd., India) served as a source for ethrel. Solvents used were of HPLC-grade methanol (Ranbaxy, India), triple distilled water and AR grade glacial acetic acid (Qualigens, India). Ethanol was distilled before use. Horse-radish peroxidase was obtained from MP Biomedicals, USA.

4.2.2 Vanilla beans

Mature beans of vanilla ranging in length 10-13 cm (second quality) were obtained from the growers in "Western ghats" region of Karnataka, India during the second phase of harvesting season (early December) and transported within 24 h to the laboratory. In addition, the mature beans of similar lengths were harvested from the plants derived *via* micropropagation cultivated in the institute and mixed with the samples collected from the growers. For comparison with the commercial samples, the conventionally cured beans from market where the curing is done by intermittent sun-drying and sweating process for 3-6 months (Dignum et al. 2001) were used.

4.2.3 Measurement of moisture loss

Beans from each treatment were separately subjected for drying in an oven at 60 °C and the loss of water was recorded by gravimetric method throughout the experiment at required intervals.

4.2.4 Texture measurement

Texture of the beans at various stages of curing was measured using Instron 4301-UTM (Universal Texture Measuring system) by WB Shear at a speed of 100 mm min^{-1} and a load of 100 kg. Ten beans were removed from each treatment at 10 days intervals for a total curing period of 40 days and were used for the analysis. All the results were expressed as force in Newton (N).

4.2.5 Flavour analysis

4.2.5.1 Standards

Vanillin (1.2 g), vanillic acid (0.08 g), *p*-hydroxybenzoic acid (0.02 g) and *p*-hydroxybenzaldehyde (0.06 g) were separately weighed into a 100 mL volumetric flask and diluted to 100 mL with 95% pre-distilled ethanol. From these, 10 mL aliquot was further diluted to 100 mL with 40% ethanol separately and was used as standard.

4.2.5.2 Extraction of flavour compounds

The extraction of flavour components from vanilla beans was done by the method as described earlier (Ranadive 1992). Briefly, triplicate of 10 g cured vanilla beans were finely crushed in liquid nitrogen. The extraction was done with 75 mL of 44% aqueous ethanol for 48 h at 45 ^{o}C in stoppered conical flasks. The mixture was stirred occasionally, filtered and washed with 36% ethanol until the total volume of filtrate, along with washings, was 100 mL. An aliquot of the filtrate was taken in a syringe and passed through a membrane filter (Millipore, 0.45µm) to remove coarse particles and clear aliquot was used for HPLC analyses.

4.2.5.3 Analytical High Performance Liquid Chromatography (HPLC)

The levels of flavouring compounds formed at different stages of curing were analysed by HPLC. Shimadzu LC 6A model (Tokyo, Japan) with µ-Bondapack (Waters Corp., Miford, MA) C_{18} Column (300 x 4.6 mm i.d., with pore size of 5 micron) a SLC-6A system controller and CR4A data processor was used. For calibration purpose, solutions of vanillin, vanillic acid, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde at concentrations from 1-100 mg L^{-1} were separately prepared in 100% ethanol and injected into the HPLC system described above to build the calibration curve. Detection was done by a UV detector SPD-AV set at a sensitivity of 0.04 AUFC and a wavelength of 254 nm and flow rate of 1 mL min^{-1}. Mobile phase used was methanol-acidified water (10:90) where 800 mL of water was acidified with 10 mL glacial acetic acid. Samples were injected using a 20 µL sample loop injection system and the concentrations of major flavour components were calculated by peak proportioning using the standard curves.

4.2.6 Extraction and estimation of total protein content

Total protein was extracted from the beans and estimated by Macro-Kjeldahl method (AOAC, Official Methods of Analysis No. 984.13) since the phenolic compounds hindered the estimation by Lowry's method.

4.2.7 Activities of major enzymes

Earlier reports indicated the involvement of three major enzymes during curing of vanilla beans; the involvement of β-glucosidase (β-GLUC) for catalyzing the conversion of glucovanillin and other glycosides to vanillin and respective flavour compounds, cellulase (CSE) for cell-wall degradation assisting the permeabilization of β-GLUC from surface of the beans to the centre and peroxidase (POD) in various bio-conversions of phenyl-propanoid compounds (Dignum et al. 2001). Therefore, the activities of these three enzymes were followed throughout the curing period after different treatments.

4.2.7.1 Extraction of enzymes and assay

4.2.7.1.1 Extraction

The extraction involved chopping of beans into 1 cm pieces of which 5 g was extracted at 4 °C with 20 mL of respective buffers and centrifuged twice at 5000×g for 15 min. and the supernatant was used as the enzyme source.

4.2.7.1.2 β-glucosidase

The buffer used was 0.1 M sodium citrate (pH 5). The activity of β-GLUC was determined at 30 ºC in fresh extraction buffer (pH 5) using *p*-nitrophenol glucopyranoside as the substrate. The *p*-nitrophenol released after the glucose is hydrolysed by the enzyme is measured spectrophotometically. The reaction mixture contained 100 µL of 0.1 M sodium citrate buffer (pH 5), 100 µL of enzyme extract and 100 µL of 0.0055 M *p*-nitrophenol β-D glucopyranoside (Spagna et al. 2002). The hydrolysis of one micromole of substrate per minute was recorded as one unit of activity and quantified based on $\varepsilon = 18500\ mol^{-1}\ cm^{-1}$.

4.2.7.1.3 Cellulase

CSE activity was determined by measuring the reducing groups released from carboxymethyl cellulose (CMC, Sigma) by following the method explained elsewhere (Priya-Sethu et al. 1996). The reaction mixture contained 0.25 mL of crude enzyme, 0.5 mL of 0.1% (w/v) CMC and 0.25 mL of sodium citrate buffer (pH 5), incubated at 37 °C for 1 h. One unit is defined as amount of enzyme that catalyzed the formation of one micro molar reducing group per minute.

4.2.7.1.4 Peroxidase

POD was extracted in sodium phosphate buffer (pH 6) at 4 °C and the activity was determined following the procedure explained elsewhere (Agostini et al. 1997). Briefly, 1 mL assay mixture was prepared which consisted of 100 µL of 1% H_2O_2, 100 µL of 0.25% ortho-dianisidine dihydrochloride, 10 µL of enzyme extract and 790 µL of sodium phosphate buffer (pH 6). The change in absorbance at 460nm per minute (dA min^{-1}) at 27 °C was recorded using kinetic program in UV- visible spectrophotometer (Shimadzu UV-160A). Activity was quantified on the basis of standard curve, using the same substrate, of horseradish POD enzyme obtained from ICN-biochemicals. One unit of enzyme activity refers to the rate of change of absorbance by 1 unit per minute.

4.2.8 Tissue printing

Activities in the fresh beans were also checked by tissue printing method where cross sections of beans were obtained from distal end (away from the petiole), middle region and proximal end (near the petiole) and immediately and carefully placed on nitrocellulose paper supported on sterile blotter sheets. After the sap from the sections imbibed into nitrocellulose, the latter was processed at 4 ^oC in buffer solutions containing substrates for the enzyme in question. For peroxidase, 100 mL of (at 4 ^oC) sodium phosphate buffer (pH 6.0) containing 10 mL of 0.25% o-dianisidine dihydrochloride and 10 mL of 1% H_2O_2 was used. For β-glucosidase, 100 mL of reaction solution containing per ml concentration of 100µL of 0.1 M sodium citrate buffer (pH 5) and 100 µL of 0.0055 M p-nitrophenol β -D-glucopyranoside (Spagna et al. 2002) was used. For control, the prints of bean slices were processed in respective buffers without substrate. After 10 min, each nitrocellulose sheet was kept on multilayered sterile blotters and the colour developed was photographed. Localization of enzymes in bean tissues was

re-checked by keeping the thin sections on a glass slide and directly adding the respective buffer solutions with or without substrate. The enzymatic reactions were immediately photographed.

4.2.9 Sensorial properties

Sensory evaluations were carried out in seven separate booths maintained at a temperature of 22±2 ^{o}C with 45±5% of relative humidity with fluorescent lights, which was equivalent to day light illumination. The Quantitative Descriptive Analysis (QDA) method (Stone and Sidel 1998) used for profiling sensory attributes consisted of 15 cm line scale wherein 1.25 cm was anchored as low and 13.75 cm as high.

4.2.9.1 Odour profile analysis of vanilla pods

One gram of vanilla pod was taken in 250 mL conical flask with stopper. Panelists were trained to sniff the headspace and mark the intensity of odour notes in the scorecard. The scorecards were decoded and mean values of the attributes were calculated from three separate analyses. The profiles were generated as spider web diagram by plotting attributes versus mean scores.

4.2.9.2 Panel training

A group of 12-15 panelists were trained over three sessions for descriptive sensory analysis. The members of the staff were familiar with sensory analysis techniques used in plantation products and flavour technology and related fields. The training included development of a common lexicon of the sensory attributes in evaluation. For this, a vanilla flavour lexicon wheel procedure was used (Hariom et al. 2006). The common descriptors selected by at least one-third of the panel and few important descriptors cited in the literature were utilized in the development of the scorecard. In order to assist panelists in the selection of descriptors, dominant flavour notes of vanilla and appropriately diluted reference compounds corresponding to the flavour notes (Hariom et al. 2006) were provided. The panelist evaluated the vanilla extracts in a group and recorded the perceived attributes individually. Following this, an open discussion was held to reach to an agreement on appropriate descriptors and the threshold levels were decided for plotting the graph.

4.2.9.3 E-nose analysis

An E-nose (Alpha Fox 3000, Alpha M.O.S. SA, Toulouse, France) equipped with six doped and six undoped metal-oxide semiconducting sensors was used in the present study. The samples that were subjected for the analysis were commercial sample (1); green beans (2); blanched and cured beans (3) and beans cured after scarification and pre-treatment with 5 mg L^{-1} NAA (4). Two grams of the vanilla pods from each group were placed separately in the sample vials and the volatiles were allowed to accumulate in the headspace by holding the vials at 25 ^{o}C for 120 sec. Then, the volatiles were carried by a stream of zero air (flow rate 150 ml min^{-1}) to the sensor chamber. Injection and acquisition times were 60 and 120 sec, respectively.

4.2.10 Statistical analyses

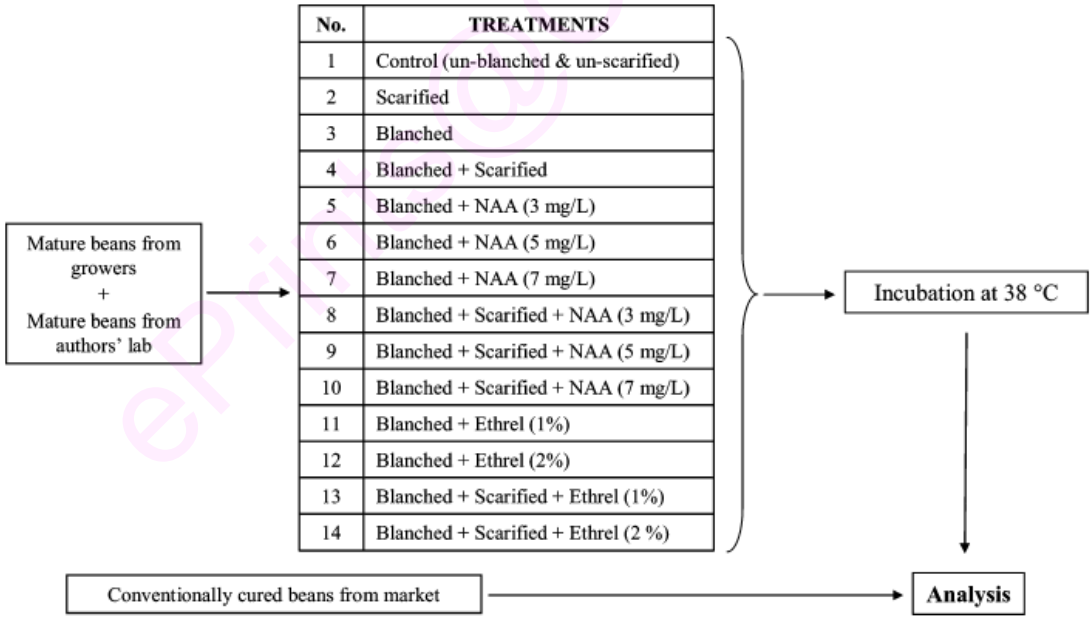
Each treatment had at least 150 beans and thirty beans were randomly picked, at a known point of time, for physical, chemical and sensorial analyses. The entire experiment was repeated in the subsequent year. Student '*t*' test has been used to compare the mean values and the tests were considered statistically significant at $p<0.05$. The data was analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel XP® (Microsoft Corporation, Washington), and post-hoc mean separations were performed by Duncan's Multiple Range Test at $p\leq0.05$ (Harter 1960).

4.2.11 Treatments

4.2.11.1 Pre-treatments experiment

In conventional curing, high flavour yields are achieved by way of blanching vanilla beans in hot water of 55 to 65 °C for 3 to 10 minutes (Dignum et al. 2001). In the present study, a preliminary screening of different temperatures of 55, 57, 59, 61, 62, 63, 65 and 65 °C versus time ranging from 2 to 10 minutes was done. Based on this study, the beans were blanched in hot water (tap water) at a temperature of 63 °C for 3 minutes and spread on blotter sheets and gently pressed with fresh sheets of blotters to remove water droplets adhering to the surface of beans. The beans were allowed to stay for 30 min at room temperature (RT, 28 to 30 °C) until the surface of each bean appeared free from water. Scarification treatments were given by way of individually picking up the beans and creating

length-wise scarification with a brush having fine stainless-steel bristles. Before arriving at the list of chemicals and their concentrations for pre-treatment, a preliminary study was done where both NAA (3, 5 & 7 mg L^{-1}) and ethrel (1 and 2%) showed highest induction of flavour (data not shown) and hence were included for the treatment. For treatment with auxin, appropriate levels of NAA was weighed to obtain the required final concentrations (3, 5 & 7 mg L^{-1}) where the compound was first dissolved in a few drops of NaOH (0.1 N) to which a required volume of lukewarm water was added and allowed to cool to RT. After blanching/non-blanching or scarification/non-scarification treatments, the beans were dipped for 5 minutes in NAA-containing or ethrel-containing water at RT. To obtain appropriate level (1-2%) of ethrel, Ethephon was diluted with water appropriately. For the entire experiment, tap-water washed untreated beans (without blanching/scarification and without NAA/ethrel-treatments) served as general control; thus all the treatments had respective controls (**Figure 4.1**).



No.	TREATMENTS
1	Control (un-blanched & un-scarified)
2	Scarified
3	Blanched
4	Blanched + Scarified
5	Blanched + NAA (3 mg/L)
6	Blanched + NAA (5 mg/L)
7	Blanched + NAA (7 mg/L)
8	Blanched + Scarified + NAA (3 mg/L)
9	Blanched + Scarified + NAA (5 mg/L)
10	Blanched + Scarified + NAA (7 mg/L)
11	Blanched + Ethrel (1%)
12	Blanched + Ethrel (2%)
13	Blanched + Scarified + Ethrel (1%)
14	Blanched + Scarified + Ethrel (2 %)

Figure 4.1 Experimental model adopted in the pre-treatment study showing various treatments. Each treatment had atleast 150 beans where as the market sample had 30 beans.

All the samples with different treatments as well as general control were separately wrapped in double-layered wax paper and gently tied with cotton thread. Incubation of the beans was carried out at 38 °C for a period of 40 days.

Since the moisture loss was very high initially, the wax wrappers were replaced with fresh ones on 3rd, 6th and 10th days. For each treatment, 150 beans were used. After every 10th day thirty randomly picked beans from each treatment were used for chemical and physical analyses. After different curing periods, the beans were re-bundled in fresh butter paper and allowed to condition at room temperature (28 to 30 °C) for 15 days, followed by storing in thermocole boxes at RT in self-sealable polythene bags.

4.2.11.2 Elicitation experiment

The vanilla beans were blanched at 63 °C for 3 min as explained earlier (Sreedhar et al. 2007b). To bring the elicitors in contact with the living cells of the beans, the beans were scarified lengthwise with a brush having fine stainless steel bristles (automation for blanching and scarification is also possible). Different sets, each having 20 scarified beans (×6), were kept on two-layered butter paper sheet and sprinkled with different elicitor powders at the rate of 5 mg DCP per bean. The treated beans (20 in each bundle) were wrapped and incubated in an oven at 38±1 °C as reported (Sreedhar et al. 2007b).

4.3 Results

4.3.1 Pre-treatments experiment

4.3.1.1 Changes in physical parameters

The pattern of water loss in vanilla beans during the curing period of 40 days at 38 °C is shown in **Figure 4.2**. The initial water content of over 70% was drastically reduced, in most of the treatments, to around 10% (w/w of the beans) by the 40th day except in control. The water loss was higher in treated beans than control, with more so in scarification treatments. The conventionally cured market samples were also subjected for this test and found to contain 25% moisture. The analysis of texture (**Figure 4.3**) showed progressively low values indicating increase in softness till the end of the curing period in most of the treatments. However, at the end of the curing period the control and scarified samples showed higher resistance due to more hardness than in other treatments. The conventionally cured commercial sample showed texture comparable to the 10 days cured samples of the present study indicating that 10th day is the right time to

terminate the incubation at 38 ^{o}C. The NAA-pretreated beans cured for 10 days and conditioned at RT retained their flexibility even after one year (**Figure 4.4**). Thus both texture and moisture levels of 10 day-cured beans and conditioned at RT are comparable to the commercial sample in almost all the treated beans except for un-blanched control.

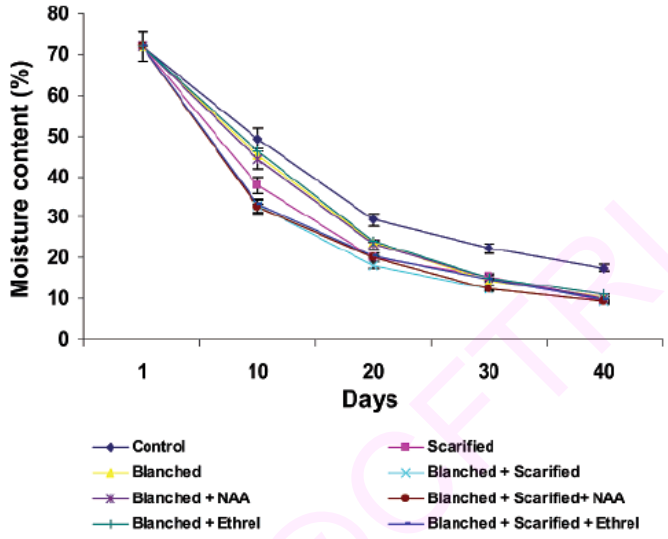


Figure 4.2 Moisture content at different periods of curing of vanilla beans after various pre-treatments. The values presented are averages of 10 replicates±SD.

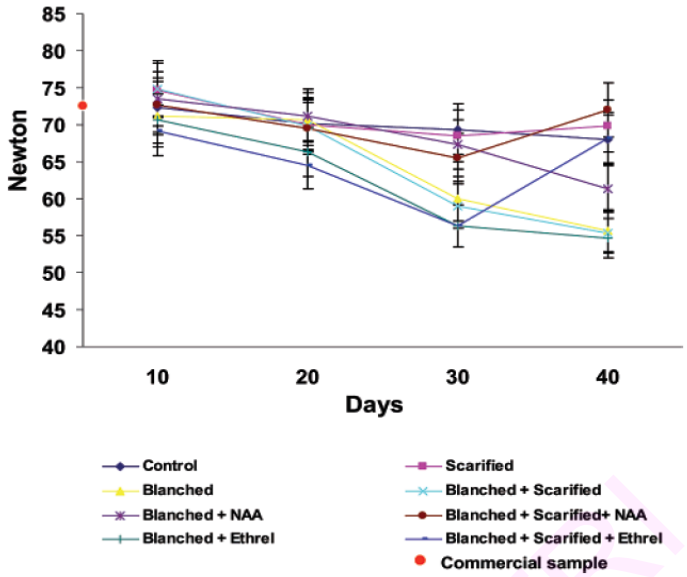


Figure 4.3 Texture analysis of vanilla beans during different stages of curing after various pre-treatments. The values presented are averages of 10 replicates±SD.

Figure 4.4 Flexibility of vanilla bean cured for 10 days after pre-treatment with NAA during blanching-pretreated beans. The figure shows the flexibility of the bean stored for one year at room temperature in air-tight pouches.

4.3.1.2 Formation of flavour compounds

HPLC profiles of standard flavouring compounds and those of variously cured beans are shown in **Figure 4.5**. Vanillin, the major flavouring compound, was formed to various degrees with respect to different treatments (**Table 4.1**). After 10 days of incubation, the control (un-blanched and un-scarified) showed substantial quantity of vanillin formed, which declined steadily up to 30th day followed by a negligible improvement on 40th day. Scarification of beans

enhanced vanillin content by nearly 40% on 10^{th} day, which decreased further during the entire curing period of 40 days showing 15-25% increase over the respective control, i.e., un-scarified beans (**Table 4.1**). Blanching, the conventional pre-treatment, showed an increase of nearly 1.5-fold (150% increase) in vanillin content when compared to un-blanched control after 10 days of curing. A very significant increase of vanillin content by nearly 2-fold was evinced when blanching was combined with scarification treatment compared to blanching alone. A similar 2-fold increase in vanillin was observed when NAA (5 mg L^{-1}) treatment was followed after blanching treatment when compared to the respective control i.e., blanching alone. Though all the treatments with NAA resulted in significant increase in the turn-over of vanillin and other flavouring compounds, the best concentration was 5 mg L^{-1}.

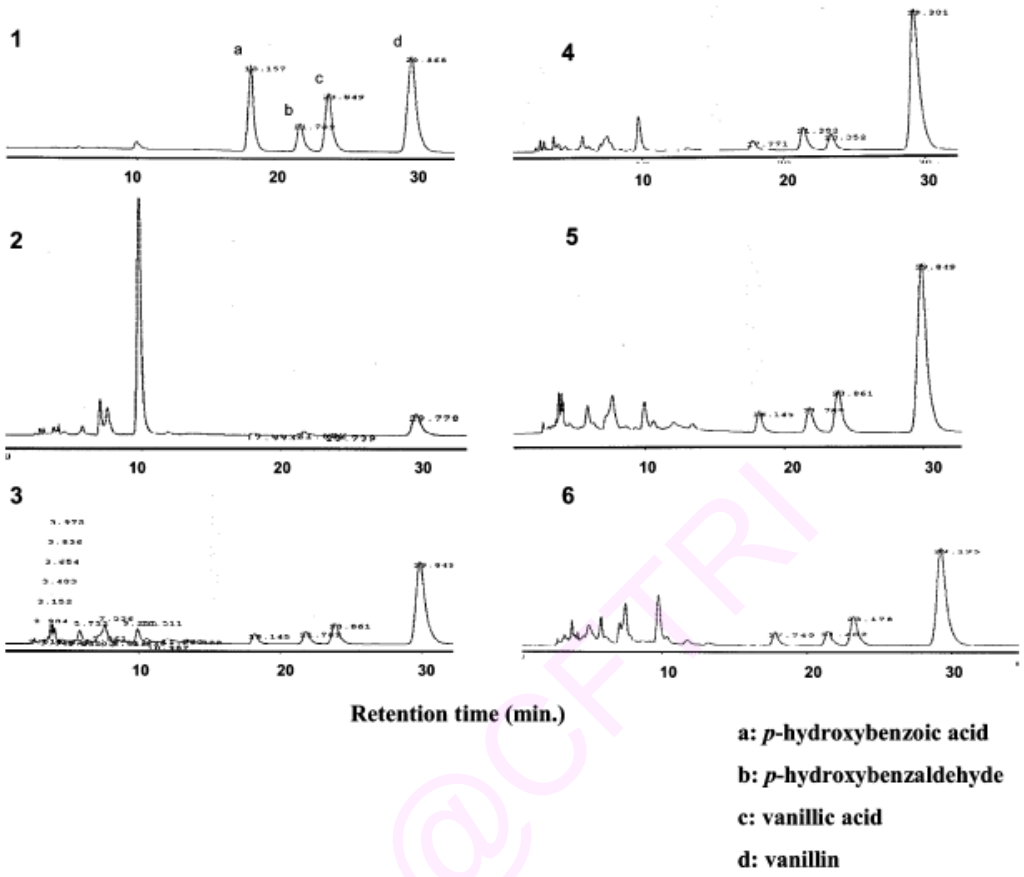


Figure 4.5 HPLC profiles of standard compounds (1), green bean (2), blanched bean (3), Ethrel-pre-treated bean (Blanched, scarified and treated with 1% Ethrel) (4), NAA-pre-treated bean (Blanched, scarified and treated with 5 mg L^{-1} of NAA) (5) and commercial bean sample (6) showing elution time for major vanilla flavour compounds detected at 254 nm. The HPLC profiles of (3), (4) and (5) are of beans incubated for 10 days at 38 ^{o}C.

Table 4.1 Vanillin content in pre-treated beans (% g^{-1} bean weight*)

Sl No.	Treatments	10 DAC	20 DAC	30 DAC	40 DAC
1	Control	0.546 ^{de}	0.518 ^{de}	0.421 ^{de}	0.463 ^{d}
2	Scarified	0.759 ^{d}	0.655 ^{d}	0.486 ^{de}	0.586 ^{d}
3	Blanched	**0.821** ^{d}	1.362 ^{bc}	1.151 ^{c}	1.287 ^{bc}
4	Blanched + Scarified	**1.583** ^{bc}	1.422 ^{bc}	1.584 ^{b}	1.988 ^{a}
5	Blanched + NAA (3 mg L^{-1})	1.29 ^{c}	1.671 ^{b}	1.235 ^{c}	1.612 ^{b}
6	Blanched + NAA (5 mg L^{-1})	**1.692** ^{ab}	2.137 ^{a}	2.071 ^{a}	2.022 ^{a}
7	Blanched + NAA (7 mg L^{-1})	1.41 ^{bc}	1.823 ^{ab}	1.461 ^{bc}	1.822 ^{ab}
8	Blanched + Scarified + NAA (3 mg L^{-1})	1.301 ^{c}	1.265 ^{c}	0.912 ^{cd}	1.218 ^{bc}
9	Blanched + Scarified + NAA (5 mg L^{-1})	**2.174** ^{a}	2.058 ^{a}	1.513 ^{b}	1.945 ^{a}
10	Blanched + Scarified + NAA (7 mg L^{-1})	1.541 ^{bc}	1.412 ^{bc}	1.216 ^{c}	1.564 ^{b}
11	Blanched + Ethrel (1%)	1.612 ^{ab}	1.911 ^{ab}	1.641 ^{b}	1.844 ^{ab}
12	Blanched + Ethrel (2%)	1.411 ^{bc}	1.713 ^{b}	1.414 ^{bc}	1.623 ^{b}
13	Blanched + Scarified + Ethrel (1%)	**1.983** ^{ab}	1.962 ^{ab}	1.72 ^{ab}	1.956 ^{a}
14	Blanched + Scarified + Ethrel (2%)	1.562 ^{bc}	1.642 ^{b}	1.513 ^{b}	1.428 ^{b}

* at 25% moisture level; DAC: Days After Curing

When blanching and scarification was combined with NAA treatment (5 mg L^{-1}), there was a very high increase in vanillin formation, accounting to nearly 4-fold increase as compared to untreated control and 2.6-fold higher than blanched control on 10th day of curing. In this treatment, the increase was to the tune of 4-fold over the control on 10th and 20th day and 40th compared to control (untreated). Other concentrations of NAA, i.e., 3 and 7 mg L^{-1}, were lesser efficient than 5 mg L^{-1}. Between the two ethrel treatments, use of 1% ethrel after blanching and scarification showed significantly higher vanillin content on 10th day than the respective control, i.e., blanching and scarification (**Table 4.1**).

Regarding the other major flavouring compounds, vanillic acid concentration showed a significant increase in control beans after 10 days of incubation, blanched + Scarified + NAA (5 mg L^{-1}) treated beans 20 days after incubation (**Table 4.2**). Concentration of *p*-hydroxybenzoic acid was higher in control on 10th and 20th day of curing than the pre-treated ones which had a fall after 20th day (**Table 4.3**). There was a significant increase (3-fold) in *p*-hydroxybenzaldehyde concentration when beans were blanched and incubated for 20 days. Blanching followed by scarification and NAA (5 mg L^{-1}) treatment showed an increase of 2.4-fold compared to control on 30th day (**Table 4.4**).

Table 4.2 Vanillic acid content in pre-treated beans (% g^{-1} bean weight*)

Sl No.	Treatments	10 DAC	20 DAC	30 DAC	40 DAC
1	Control	**0.009** [a]	0.002 [d]	**0.013** [a]	0.001 [cd]
2	Scarified	0.005 [bc]	0.001 [de]	0.004 [cd]	0.002 [cd]
3	Blanched	0.003 [bc]	0.003 [cd]	0.003 [cd]	0.008 [ab]
4	Blanched + Scarified	0.003 [bc]	0.001 [de]	0.004 [cd]	0.009 [a]
5	Blanched + NAA (3 mg L^{-1})	0.001 [de]	0.002 [d]	0 [e]	0 [d]
6	Blanched + NAA (5 mg L^{-1})	0.002 [d]	0.003 [cd]	0.002 [d]	0.002 [cd]
7	Blanched + NAA (7 mg L^{-1})	0 [e]	0.001 [de]	0.001 [de]	0 [d]
8	Blanched + Scarified + NAA (3 mg L^{-1})	0.003 [cd]	0.004 [bc]	0.006 [c]	0 [d]
9	Blanched + Scarified + NAA (5 mg L^{-1})	0.005 [bc]	**0.007** [a]	0.012 [ab]	0 [d]
10	Blanched + Scarified + NAA (7 mg L^{-1})	0.004 [c]	0.005 [b]	0.005 [c]	0 [d]
11	Blanched + Ethrel (1%)	0.004 [c]	0.005 [b]	0.009 [b]	0.004 [bc]
12	Blanched + Ethrel (2%)	0.002 [d]	0.003 [cd]	0.007 [bc]	0.001 [cd]
13	Blanched + Scarified + Ethrel (1%)	0.001 [de]	0.004 [bc]	0.007 [bc]	0.007 [ab]
14	Blanched + Scarified + Ethrel (2%)	0 [e]	0.003 [cd]	0.005 [c]	0.006 [b]

* at 25% moisture level; DAC: Days After Curing

Table 4.3 *p*-hydroxybenzoic acid content in pre-treated beans (% g^{-1} bean weight*)

Sl No.	Treatments	10 DAC	20 DAC	30 DAC	40 DAC
1	Control	**0.003** [a]	**0.006** [a]	0.003 [b]	0.001 [b]
2	Scarified	0.002 [b]	0.002 [c]	0 [e]	0.002 [a]
3	Blanched	0.001 [c]	0.003 [bc]	0.002 [c]	0.002 [a]
4	Blanched + Scarified	0.001[c]	0.001 [d]	0.001 [d]	0.002 [a]
5	Blanched + NAA (3 mg L^{-1})	0 [d]	0 [e]	0.001 [d]	0.001 [b]
6	Blanched + NAA (5 mg L^{-1})	0.001 [c]	0.001 [d]	0.001 [d]	0.001 [b]
7	Blanched + NAA (7 mg L^{-1})	0 [d]	0 [e]	0 [e]	0 [c]
8	Blanched + Scarified + NAA (3 mg L^{-1})	0 [d]	0 [e]	0.002 [c]	0.001 [b]
9	Blanched + Scarified + NAA (5 mg L^{-1})	0.001 [c]	0.001 [d]	0.004 [a]	0.002 [a]
10	Blanched + Scarified + NAA (7 mg L^{-1})	0 [d]	0 [e]	0.003 [b]	0.001 [b]
11	Blanched + Ethrel (1%)	0.002 [b]	0 [e]	0.003 [b]	0 [c]
12	Blanched + Ethrel (2%)	0.001 [c]	0 [e]	0.002 [c]	0 [c]
13	Blanched + Scarified + Ethrel (1%)	0.001 [c]	0.001 [d]	0.002 [c]	0.002 [a]
14	Blanched + Scarified + Ethrel (2%)	0 [d]	0.001 [d]	0.001 [d]	0.001 [b]

* at 25% moisture level; DAC: Days After Curing

Table 4.4 *p*-hydroxybenzaldehyde content in pre-treated beans (% g^{-1} bean weight*)

Sl No.	Treatments	10 DAC	20 DAC	30 DAC	40 DAC
1	Control	0.002 ^{d}	0.004 ^{d}	0.007 ^{c}	0.002 ^{c}
2	Scarified	0.008 ^{a}	0.003 ^{d}	0.002 ^{de}	0.002 ^{c}
3	Blanched	0.005 ^{bc}	**0.013** ^{a}	0.002 ^{de}	0.005 ^{a}
4	Blanched + Scarified	0.003 ^{cd}	0.003 ^{de}	0.003 ^{c}	**0.005** ^{a}
5	Blanched + NAA (3 mg L^{-1})	0.002 ^{d}	0.001 ^{e}	0 ^{e}	0.001 ^{cd}
6	Blanched + NAA (5 mg L^{-1})	0.004^{c}	0.003 ^{de}	0.002 ^{de}	0.002 ^{de}
7	Blanched + NAA (7 mg L^{-1})	0.001 ^{de}	0.002 ^{de}	0.001 ^{de}	0 ^{de}
8	Blanched + Scarified + NAA (3 mg L^{-1})	0 ^{e}	0.004 ^{d}	0.008 ^{c}	0 ^{e}
9	Blanched + Scarified + NAA (5 mg L^{-1})	0.001 ^{de}	0.009 ^{bc}	**0.017** ^{a}	0.002 ^{de}
10	Blanched + Scarified + NAA (7 mg L^{-1})	0 ^{e}	0.005 ^{cd}	0.006 ^{cd}	0.001 ^{de}
11	Blanched + Ethrel (1%)	0.001 ^{de}	0.002 ^{de}	0.009 ^{bc}	0.003 ^{b}
12	Blanched + Ethrel (2%)	0 ^{e}	0.001 ^{e}	0.001 ^{de}	0.001 ^{cd}
13	Blanched + Scarified + Ethrel (1%)	0.003 ^{cd}	0.007 ^{c}	0.004 ^{d}	0.003 ^{b}
14	Blanched + Scarified + Ethrel (2%)	0.002 ^{d}	0.004 ^{d}	0.002 ^{de}	0.001 ^{cd}

* at 25% moisture level; DAC: Days After Curing

4.3.1.3 Protein content

Total protein content estimated on the basis of nitrogen analysis showed the presence of 5.64% protein on 25% moisture basis. This value remained constant without any significant change throughout the curing process (**Figure 4.6**).

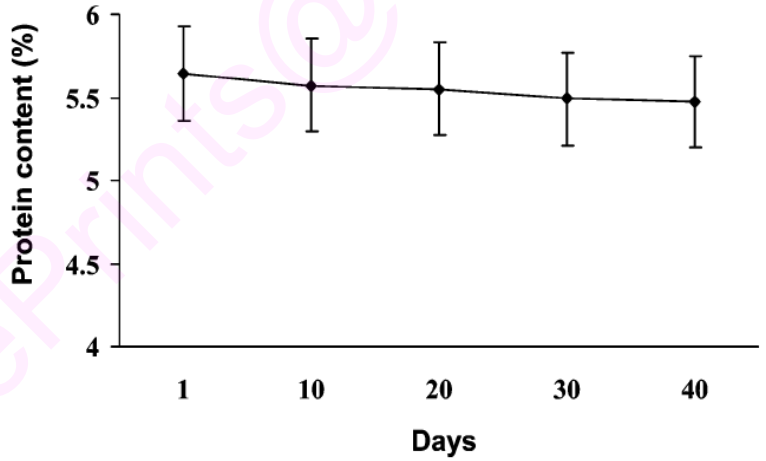


Figure 4.6 Total protein content of vanilla beans (at 25% moisture level) at different incubation periods as estimated by Macro-Kjeldahl method

4.3.1.4 Enzyme activities

4.3.1.4.1 β-Glucosidase

In general, incubation at 38 ^{o}C for 10 days showed a significant increase in the activity of β-GLUC compared to the initial activity in green beans (172.3 U g^{-1} tissue at 25% moisture level). Blanched beans exhibited a higher activity on 10th day while the activity declined significantly on 20th and 30th days of curing (**Table 4.5**). Beans blanched, scarified and treated with NAA (5 mg L^{-1}) as well as those blanched, scarified and treated with 1% ethrel showed an increased activity on 20th day. The enzyme activity declined upon further incubation.

Table 4.5 β -glucosidase activity in pre-treated beans (U g^{-1} tissue*)

Sl No.	Treatments	Zero Day Mean±SD	10 DAC	20 DAC	30 DAC	40 DAC
1	Control	172.3±9.3	188.094 ^{bc}	311.306 ^{bc}	141.133 ^{cd}	119.846 ^{cd}
2	Scarified		187.013 ^{bc}	315.898 ^{bc}	208.593 ^{bc}	204.085 ^{bc}
3	Blanched		**257.263** ^{a}	**243.352** ^{cd}	**126.931** ^{d}	201.480 ^{bc}
4	Blanched + Scarified		213.74 ^{b}	358.14 ^{bc}	318.660 ^{a}	246.639 ^{b}
5	Blanched + NAA (3 mg L^{-1})		105.90 ^{d}	174.9 ^{d}	101.21 ^{d}	108.55 ^{d}
6	Blanched + NAA (5 mg L^{-1})		211.876 ^{b}	349.875 ^{bc}	134.032 ^{cd}	217.112 ^{bc}
7	Blanched + NAA (7 mg L^{-1})		110.2 ^{d}	190.2 ^{d}	120.4 ^{d}	126.2 ^{cd}
8	Blanched + Scarified + NAA (3 mg L^{-1})		102.69 ^{de}	243.35 ^{cd}	100.24 ^{d}	106.22 ^{d}
9	Blanched + Scarified + NAA (5 mg L^{-1})		205.39 ^{b}	**486.703** ^{a}	192.616 ^{c}	183.242 ^{c}
10	Blanched + Scarified + NAA (7 mg L^{-1})		114.24 ^{d}	200.26 ^{d}	108.22 ^{d}	123.21 ^{cd}
11	Blanched + Ethrel (1%)		178.365 ^{bc}	417.830 ^{ab}	227.234 ^{bc}	274.429 ^{ab}
12	Blanched + Ethrel (2%)		156.21 ^{c}	316.24 ^{bc}	196.21 ^{c}	246.22 ^{b}
13	Blanched + Scarified + Ethrel (1%)		193.499 ^{bc}	**470.174** ^{a}	265.402 ^{ab}	308.299 ^{a}
14	Blanched + Scarified + Ethrel (2%)		176.22 ^{bc}	373.41 ^{b}	246.24 ^{b}	278.42 ^{ab}

* at 25% moisture level; DAC: Days After Curing

4.3.1.4.2 Cellulase

All the pre-treatments including blanching showed a significant increase in the CSE activity compared to initial activity (0.74 U g^{-1} tissue at 25% moisture level) in green beans. Though there was a higher activity on 20th day in all the treatments, a sudden fall was noticed upon further incubation (**Table 4.6**).

Table 4.6 Cellulase activity in pre-treated beans (U g^{-1} tissue*)

Sl No.	Treatments	Zero Day Mean±SD	10 DAC	20 DAC	30 DAC	40 DAC
1	Control	0.74±0.12	0.972 ^{cd}	3.007 ^{ab}	0.589 ^{d}	0.864 ^{ab}
2	Scarified		1.238 ^{cd}	2.103 ^{bc}	0.437 ^{de}	0.623 ^{c}
3	Blanched		2.637 ^{ab}	0.914 ^{d}	0.598 ^{d}	0.716 ^{bc}
4	Blanched + Scarified		2.684 ^{ab}	2.093 ^{bc}	1.567 ^{a}	0.976 ^{a}
5	Blanched + NAA (3 mg L^{-1})		1.342 ^{c}	1.682 ^{c}	0.304 ^{e}	0.464 ^{d}
6	Blanched + NAA (5 mg L^{-1})		2.695 ^{ab}	3.363 ^{a}	0.608 ^{d}	0.929 ^{ab}
7	Blanched + NAA (7 mg L^{-1})		1.462 ^{c}	1.841 ^{c}	0.421 ^{de}	0.562 ^{cd}
8	Blanched + Scarified + NAA (3 mg L^{-1})		1.324 ^{c}	1.415 ^{cd}	0.308 ^{e}	0.446 ^{d}
9	Blanched + Scarified + NAA (5 mg L^{-1})		2.648 ^{ab}	2.83 ^{b}	0.617 ^{d}	0.892 ^{ab}
10	Blanched + Scarified + NAA (7 mg L^{-1})		1.562 ^{c}	1.678 ^{c}	0.546 ^{d}	0.729 ^{bc}
11	Blanched + Ethrel (1%)		2.96 ^{a}	3.272 ^{ab}	0.665 ^{d}	0.819 ^{b}
12	Blanched + Ethrel (2%)		2.746 ^{ab}	2.944 ^{ab}	0.428 ^{de}	0.662 ^{c}
13	Blanched + Scarified + Ethrel (1%)		2.81 ^{a}	3.484 ^{a}	0.592 ^{d}	0.94 ^{a}
14	Blanched + Scarified + Ethrel (2%)		2.442 ^{ab}	2.991 ^{ab}	0.322 ^{e}	0.774 ^{b}

* at 25% moisture level; DAC: Days After Curing

4.3.1.4.3 Peroxidase

An initial POD activity of 1125.3 U g^{-1} tissue (at 25% moisture level) was found in the green beans which increased upon scarification treatment on 10th day and increased further on 20th day. Blanching generally retarded the activity compared to the un- blanched control, though uniformity in the activity was found in most of the treatments throughout the curing period (**Table 4.7**).

Table 4.7 Peroxidase activity in pre-treated beans (U g^{-1} tissue*)

Sl No.	Treatments	Zero Day Mean±SD	10 DAC	20 DAC	30 DAC	40 DAC
		1125.3±				
1	Control	29.4	1492.24 ^{ab}	1697.55 ^{bc}	1524.67 ^{a}	1517.48 ^{a}
2	Scarified		**1749.64 ^{a}**	**2541.84 ^{a}**	513.78 ^{d}	592.27 ^{d}
3	Blanched		1278.70 ^{bc}	2104.65 ^{ab}	1109.86 ^{b}	308.36 ^{e}
4	Blanched + Scarified		995.38 ^{c}	1038.63 ^{cd}	539.65 ^{d}	1010.81 ^{ab}
5	Blanched + NAA (3 mg L^{-1})		589.24 ^{de}	554.32 ^{de}	398.45 ^{de}	347.47 ^{de}
6	Blanched + NAA (5 mg L^{-1})		1178.48 ^{bc}	1108.65 ^{cd}	796.91 ^{cd}	694.94 ^{cd}
7	Blanched + NAA (7 mg L^{-1})		624.42 ^{d}	739.10 ^{d}	531.27 ^{d}	463.29 ^{de}
8	Blanched + Scarified + NAA (3 mg L^{-1})		634.45 ^{d}	559.45 ^{de}	332.87 ^{e}	352.31 ^{de}
9	Blanched + Scarified + NAA (5 mg L^{-1})		1268.9 ^{bc}	1118.90 ^{cd}	665.74 ^{cd}	704.62 ^{cd}
10	Blanched + Scarified + NAA (7 mg L^{-1})		845.9 ^{cd}	745.93 ^{d}	443.82 ^{de}	469.74 ^{de}
11	Blanched + Ethrel (1%)		1359.32 ^{b}	1182.91 ^{cd}	787.01 ^{cd}	753.05 ^{cd}
12	Blanched + Ethrel (2%)		906.21 ^{cd}	801.46 ^{d}	524.67 ^{d}	502.03 ^{d}
13	Blanched + Scarified + Ethrel (1%)		1404.53 ^{b}	1277.64 ^{c}	816.46 ^{c}	779.69 ^{cd}
14	Blanched + Scarified + Ethrel (2%)		936.35 ^{cd}	851.76 ^{d}	544.3 ^{d}	519.79 ^{d}

* at 25% moisture level; DAC: Days After Curing

4.3.1.5 E-nose analysis

The Electronic nose analysis of the vanilla pods is shown in the **Figure 4.7** as a two dimensional representation of Principal Component Analysis (PCA) with respect to axes. As seen in the graph, the data analysis was done based on the values of maximum change in resistance of the sensors by the Principal Component Analysis (PCA) using inbuilt software supplied by the manufacturers. The results are shown as a two dimensional representation of PCA with respect to two axes. Principal Component 1 (PC1) accounted for major differences (92.6%) in variance and Principal Component 2 (PC2) accounted for very minor differences of 6.7%. Beans cured by blanching pre-treatment (3) was placed in one quadrant while the green beans cured without any pre-treatment (2) was placed in a separate quadrant away from the others. Both the NAA pre-treated beans (4) and beans from commercial sample (1) were placed in a similar quadrant indicating that both have a similar odour profile.

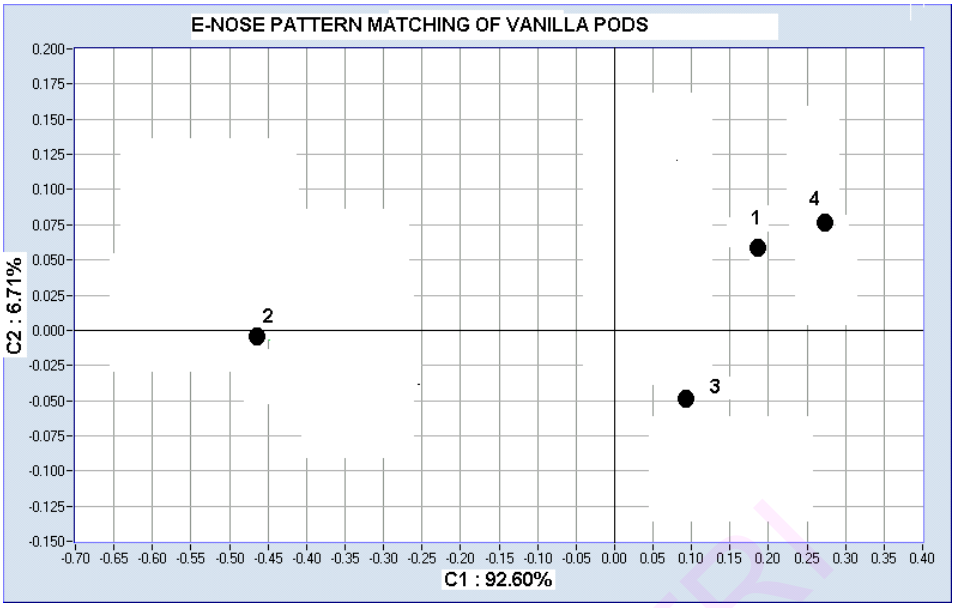


Figure 4.7 Electronic nose analysis pattern of vanilla pods from various sources. 1: commercial sample, 2: green beans, 3: blanched beans and 4: NAA pre-treated beans

4.3.2 Elicitation experiment

4.3.2.1 Total protein

There was no significant change in the level of protein during the treatment period in vanilla beans throughout the curing period. In all treatments, the initial protein content of 5.7% was found and the cured beans showed 5.55% protein indicating an insignificant loss during the treatment period of 40 days (data not shown separately).

4.3.2.2 The enzymes

The levels of activities of the three enzymes followed in the present study are summarized in **Table 4.8**. POD was dominant in all the elicitor powders, with a high level of activity (98,500±260 U g^{-1} DW) in acetone-dried powder of red beet seedling root (BSR) than in the lyophilized counterpart (92,800±280 U g mg L^{-1} DW). The other elicitors, *A. niger* and *S. cerevisiae*, showed considerably high activity of POD (over 1000 U g^{-1} DW), however, the values were significantly lower than those in BSR elicitors. The activity of β-GLUC was not traceable in BSR whereas some activities of CSE and POD were found in all the elicitors. Even in case of vanilla beans, the fresh ones showed high POD activity (1125±29 U g^{-1} tissue) distributed throughout the fruit, although the core and the distal region showed higher activity of POD than the surface and the proximal (petiolar)

region. Assay of nitrocellulose membranes on which tissue prints were obtained also revealed similar results with good intensity of colour developed when substrate for POD was used. In contrast, the activity of β-GLUC was feeble in tissue slices indicated by slight increase in yellow colour with no results in tissue prints. When assayed, the activity of β-GLUC was 172.3±9.3 U g^{-1} tissue (at 25% moisture level) (**Table 4.8**). Throughout the curing period, with and without elicitor treatment, the enzyme activities in beans showed large differences (**Table 4.9**). The level of β-GLUC, which was 172.3±9.3 U g^{-1} tissue at initial level, increased to 213.7 U g^{-1} tissue on the 10th day with a further increase to 358.1 U g^{-1} tissue on the 20th day. However, there was a steady decline towards the end of the curing period. POD activity significantly decreased to 995.4 U g^{-1} tissue from 1125±29 U g^{-1} tissue at initial level during curing with a narrow increase on the 20th day and a significant drop on the 30th day following a slight increase on 40th day (**Table 4.9**). The traces of cellulose activity present in the beans on 10th day of curing steadily declined towards the end. Upon elicitor treatment, there was a steady increase in the activity of β-GLUC, especially in the acetone dried *A. niger*-treated beans, with a highest activity of 513 U g^{-1} tissue on the 40th day of curing. A higher activity was recorded in acetone-dried elicitor treated material than that treated with lyophilized material (**Table 4.9**). Acetone-dried BSR (BSR-A) was also an efficient enhancer of β- GLUC activity in the beans as compared to the control. Yeast elicitors showed a narrow enhancement in β-GLUC only at initial stage with lower activities during further curing periods. However, significant increase as well as fluctuations in the activities of POD were observed when the beans were treated with *A. niger* and BSR-A, where the latter showed highest value on 20th day of curing with a steady decline later. The activity of CSE was very low (0.74±0.12 U g^{-1} tissue) in the fresh vanilla beans (**Table 4.8**). During curing a three to four fold increase in activity was observed on 10th day and *A. niger* (lyophilized) showed a slightly higher activity on 20th and 30th day of curing (**Table 4.9**).

Table 4.8 Activities (U g^{-1} Dry Weight) of enzymes in elicitor powders processed either by lyophilization (L) or acetone-wash (A) and in fresh vanilla beans

Enzyme→	Cellulase		*β*-glucosidase		Peroxidase	
Elicitor name	L	A	L	A	L	A
Aspergillus niger	3.5±0.13	15.7±0.42	18.7±0.45	5.8±0.67	1085.1± 40.56	1205.6±24.94
Saccharomyces cerevisiae (yeast)	19.0±1.64	14.0±0.68	Tr	Tr	1507.1± 36.86	1350.3±43.58
Red beet seedling root	3.5±0.1	3.2±0.12	0	0	92810.3± 282.51	98498.1± 260.95
Fresh vanilla beans[a]	0.74±0.12		172.3±9.3		1125±29.4	

[a] at 25% moisture level

Table 4.9 Levels of activities of different enzymes in control and elicitor-treated vanilla beans during various stages of curing [a]

Elicitor	10 DAC	20 DAC	30 DAC	40 DAC
		Peroxidase (U g^{-1} tissue[b])		
Control[c]	995.38 [b]	1038.63 [cd]	1004.21 [b]	1010.81 [bc]
A. niger-A	1045.33 [b]	1288.42 [b]	1096.91 [b]	1157.11 [ab]
A. niger-L	1010.40 [b]	1235.29 [b]	1239.33 [ab]	1043.64 [b]
BSR-A	1323.54 [a]	1504.48 [a]	1327.33 [a]	567.12 [d]
BSR-L	1235.05 [ab]	1407.95 [ab]	814.01 [c]	707.18 [cd]
Yeast-A	969.37 [b]	1051.91 [cd]	1080.97 [b]	1159.52 [ab]
Yeast-L	1000.66 [b]	1030.11 [cd]	1072.61 [b]	1362.81 [a]
		β-Glucosidase (U g^{-1} tissue[b])		
Control[c]	213.74 [c]	358.14 [ab]	318.66 [bc]	246.63 [cd]
A. niger-A	237.12 [b]	396.39 [a]	463.63 [a]	513.31 [a]
A. niger-L	219.33 [bc]	288.99 [bc]	381.63 [b]	316.59 [bc]
BSR-A	249.33 [a]	198.31 [d]	222.43 [cd]	234.30 [cd]
BSR-L	195.43 [b]	241.36 [cd]	210.28 [cd]	207.16 [cd]
Yeast-A	228.31 [b]	264.33 [c]	240.39 [cd]	180.01 [d]
Yeast-L	224.4 [bc]	259.21 [c]	238.81 [cd]	209.34 [cd]
		Cellulase (U g^{-1} tissue[b])		
Control[c]	2.69 [d]	2.09 [d]	1.57 [d]	0.98 [cd]
A. niger-A	2.97 [cd]	3.05 [bc]	2.54 [c]	1.07 [c]
A. niger-L	4.10 [a]	4.68 [a]	4.89 [a]	2.24 [a]
BSR-A	2.19 [de]	1.90 [d]	1.74 [d]	0.71 [d]
BSR-L	1.72 [e]	2.28 [cd]	2.00 [cd]	0.68 [d]
Yeast-A	2.60 [d]	2.82 [c]	2.43 [c]	0.97 [cd]
Yeast-L	2.43 [d]	2.94 [c]	1.82 [cd]	0.65 [d]

[a] Data are the mean value of three replicates
[b] At 25% moisture level
[c] Experiment-specific control where the beans were blanched and scarified
DAC, days after curing; A, Acetone-dried; L, Lyophilized; BSR, Beet Seedling Root
Data followed by different letters within each column are significantly different according to Duncan's multiple-range test at $p \leq 0.05$

4.3.2.3 Formation of flavour compounds

The control beans on zero day contained 0.38% vanillin, which may be formed mainly during extraction because vanilla beans do not impart any flavour before curing. Other compounds were not traceable by HPLC on day zero. **Figure 4.8** shows the HPLC patterns of standard compounds, commercial sample, green bean (uncured), as well as those obtained from elicitor-treated bean samples cured for 10 days. The major compound, vanillin, was formed to an extent of nearly 1.58% in control beans on the 10th day. However, in the samples treated with elicitor of red beet seedling root prepared by acetone-drying method, a very high level of 2.65% of vanillin was observed on the 10th day itself (**Table 4.10**). This level of vanillin formation is 1.7-fold higher than control beans of this study on the 10th day and much higher (3.23-fold) than the conventionally cured sample (0.82%) (Sreedhar et al. 2007b) and nearly 7-fold compared to zero day (0.38% in green bean). Nearly similar effects were observed in case of treatments with *A. niger* and BSR-L elicitors, all producing nearly 2% vanillin with 2-fold increase in vanillic acid and slight fluctuations in the levels of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid. In the control sample itself, there were significant fluctuations in the quantities of flavour compounds formed during the entire curing process, with a decline in vanillin after 20 days with a steady increase up to 40 days.

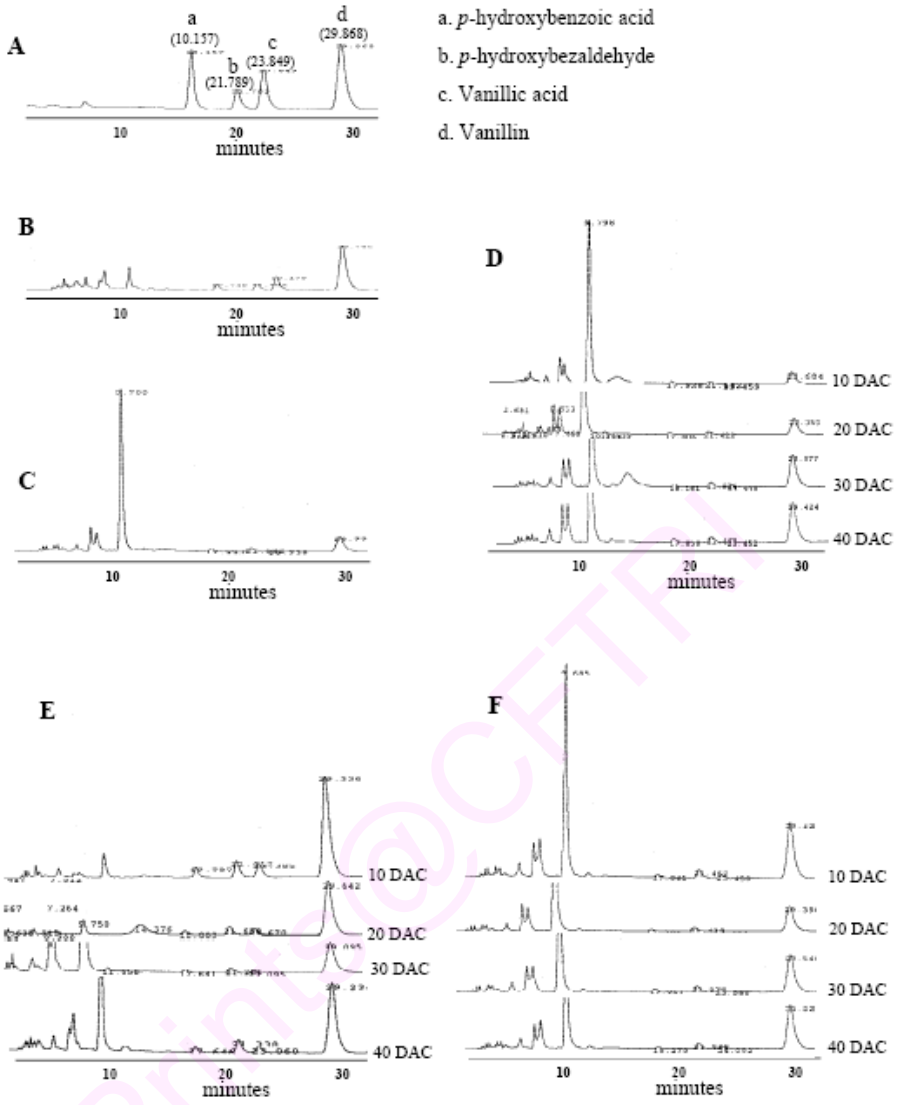


Figure 4.8 HPLC patterns showing profiles of vanilla flavour compounds formed after 10 days of curing at 38 ^{o}C in elicitor-treated vanilla beans as compared with standards and control beans. (A) Standard compounds, (B) commercial sample, (C) green bean (uncured), (D) control (blanched and scarified), (E) treated with red beet seedling root powder-acetone dried, (F) treated with *A. niger* powder-acetone dried, DAC: days after curing.

Table 4.10 Concentrations of different flavoring compounds in control and elicitor treated vanilla beans during different stages of curing [a]

Treatment	Vanillin	Vanillic acid	*p*-Hydroxy-benzaldehyde	*p*-Hydroxy-benzoic acid
		(Gram Percent of Bean Weight [b])		
		10 DAC		
Control[c]	1.58 [d]	0.003 [c]	0.003 [c]	0.001 [cd]
A. niger-A	2.02 [c]	0.006 [ab]	0.005 [bc]	0.004 [b]
A. niger-L	1.98 [c]	0.006 [ab]	0.003 [c]	0.006 [a]
BSR-A	**2.65** [a]	0.007 [a]	0.006 [b]	0.003 [bc]
BSR-L	1.98 [c]	0.005 [b]	0.005 [bc]	0.002 [c]
Yeast-A	1.67 [d]	0.004 [b]	0.009 [b]	0.001 [cd]
Yeast-L	1.80 [cd]	0.003 [c]	0.010 [a]	0.002 [c]
		20 DAC		
Control[c]	1.42 [d]	0.001 [d]	0.003 [bc]	0.001 [cd]
A. niger-A	1.65 [bc]	0.004 [c]	0.004 [b]	0.005 [ab]
A. niger-L	1.60 [c]	0.005 [bc]	0.000 [d]	0.004 [b]
BSR –A	1.95 [a]	0.005 [bc]	0.004 [b]	0.003 [bc]
BSR-L	1.89 [ab]	0.008 [a]	0.006 [a]	0.004 [b]
Yeast-A	1.58 [c]	0.006 [b]	0.002 [c]	0.00 [cd]
Yeast-L	1.58 [c]	0.005 [bc]	0.005 [ab]	0.006 [a]
		30 DAC		
Control[c]	1.59 [c]	0.004 [bc]	0.003 [c]	0.001 [c]
A. niger-A	1.80 [ab]	0.004 [bc]	0.004 [c]	0.000 [c]
A. niger-L	1.45 [d]	0.006 [ab]	0.002 [d]	0.007 [a]
BSR-A	1.87 [a]	0.006 [ab]	0.007 [b]	0.005 [bc]
BSR-L	1.70 [bc]	0.008 [a]	0.010 [a]	0.006 [ab]
Yeast-A	1.62 [c]	0.00 [c]	0.005 [bc]	0.006 [ab]
Yeast-L	1.85 [ab]	0.007 [ab]	0.008 [ab]	0.008 [a]
		40 DAC		
Control[c]	2.00 [ab]	0.009 [b]	0.005 [cd]	0.002 [d]
A. niger-A	1.85 [bc]	0.004 [c]	0.004 [e]	0.003 [cd]
A. niger-L	1.65 [d]	0.00 [d]	0.005 [cd]	0.005 [bc]
BSR-A	2.05 [a]	0.01 [a]	0.006 [c]	0.008 [a]
BSR-L	1.85 [bc]	0.008 [b]	0.008 [a]	0.004 [c]
Yeast-A	1.82 [c]	0.006 [bc]	0.007 [b]	0.005 [bc]
Yeast-L	1.75 [cd]	0.005 [bc]	0.005 [d]	0.006 [b]

[a] Data are the mean value of three replicates
[b] At 25% moisture level
[c] Experiment-specific control where the beans were blanched and scarified
DAC, days after curing; A, Acetone-dried; L, Lyophilized; BSR, Beet Seedling Root
Data followed by different letters within each column are significantly different according to Duncan's multiple-range test at $p \leq 0.05$

4.3.2.4 Sensory attributes

The observations made on odour profile of vanilla bean samples are shown in **Figure 4.9**. In case of unblanched and unscarified treatment, vanilla note differed significantly ($p \leq 0.05$) from blanched beans and BSR-A treated beans (**Figure 4.9**). Treatment with red beet seedling elicitor appeared to impart good sensory

profile to the beans with high notes of vanilla, sweet and floral odour and low intensity of woody, beany and smoky notes.

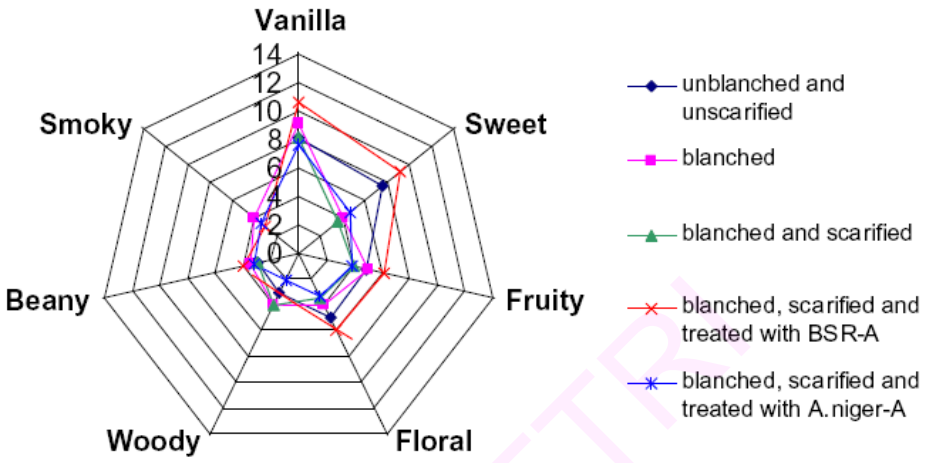


Figure 4.9 Sensory profile of vanilla beans cured for 10 days with or without elicitors and different pre-treatments. The data represents average of atleast 12 panelists in three separate analyses.

4.4 Discussion

4.4.1 Pre-treatments experiment

Traditionally the appearance, the flexibility and size characteristics have been of importance since there is a fairly close relationship between any two factors and the aroma/flavour quality. The moisture content is one of the several parameters, which is important for bean quality. It is therefore, very important to realize that moisture content is inter-dependent on other quality parameters and cannot be considered, by itself, as an index of quality. The presence of appropriate water content ranging from 25 to 30% has been noted by earlier workers, to result in the desirable texture. The present study has clearly shown that the use of NAA or ethrel coupled with bean scarification more quickly reduced the moisture content than with similar set of conditions with blanching treatment (**Figure 4.2**). Beans used for extraction have low moisture content while gourmet beans contain higher moisture content (Havkin-Frenkel et al. 2005). The moisture content is a major factor in the preservation of cured vanilla beans since low moisture content is essential to prevent microbial growth. Also, the water content of properly cured beans must be sufficiently low to prevent growth and activity of microorganisms, since low water, in combination with high phenolic content offer protection against spoilage in cured beans. Our observation of NAA/ethrel-treated 10-day cured beans, stored at RT for one year did not show spoilage due to any fungal/microbial infestation (**Figure 4.4**).

The present study has documented the texture measurement of vanilla beans for the first time. Since the desirable moisture content in vanilla beans and the texture comparable to commercial samples were achievable in a period of 10 days, the curing conditions (time and temperature) selected in the present study appears appropriate to accomplish faster curing. Supporting this is the data of HPLC-profile of flavours, which is also comparable to the commercial sample (**Figure 4.5**). Thus, the present study has clearly demonstrated the possibility of reducing the curing period of vanilla beans to as short as 10 days with almost similar array of flavouring compounds and texture compared to the conventionally cured ones. However, for the formation of age-related compounds, which takes place during the conditioning of the beans after the curing period, the cured beans need to be stored appropriately. Therefore, the cured beans that result after

NAA/ethrel-pre-treatment also need to be conditioned as per the traditional methods.

Though several reports indicate the absence of vanillin in the green beans, the results of the present study showed that some amount of vanillin and negligible amounts of other flavouring compounds were already formed in green beans before the onset of curing process (**Figure 4.5**). It appears that the glucosyl form of vanillin is largely predominant in green beans but it does not exclude the presence of a fraction in free form (Odoux 2006). This indicates that the glucolytic and other hydrolytic enzymes within the vanilla beans get activated immediately after harvest. Blanching, a traditionally followed curing process (Bourbon method) substantially enhanced the flavour formation from the respective glucosides, as compared to untreated beans, despite the well-documented fact that the specific β-GLUC gets arrested after blanching and missing during curing (Dignum et al. 2002). An extensive study of the thermal sensitivity of β-GLUC showed that the activity of the enzyme was lost within 24h after blanching (Dignum et al. 2002). However, in the present study, a much higher activity than reported elsewhere was observed throughout the curing period where most of the treatments significantly enhanced the enzyme activity on 20th day. Although direct correlation could not be made between the enzyme levels and the quantity of flavours formed at a given period of treatment, one can reason such fluctuation in turn-over of each compound due to either inter-conversions of various precursor compounds or variations in reaction kinetics. The biosynthetic pathway of vanillin and other flavour related compounds is shown in **Figure G2**. The formation of vanillin precursor, the glucovanillin originates from glucosides of ferulic acid or protocatechuic aldehyde, both the compounds being derived from coumaric acid glucoside of phenyl-propanoid pathway (**Figure G2**). In studying the biosynthesis of vanillin in vanilla beans, it was found that ferulic acid was incorporated to a greater extent into vanillin than vanillic acid indicating that ferulic acid is β-oxidized to vanillyl-Co-A, which either can be reduced to vanillin or de-acetylated to vanillic acid (Zenk 1965). Thus the native β-glucosidase plays a crucial role in the conversion of not only the glucovanillin into vanillin but also the other precursors to glucovanillin. This probably could be the reason for fluctuations in the levels of flavouring compounds during the curing period and hence not strictly corroborative with the enzyme levels. The limiting factor in

glycoside hydrolysis is more likely to be the cellular compartmentation than the enzyme activity level. Glucoside hydrolysis is complete if the treatment allows a total decomparmentation and if sufficient residual glucosidase activity can continue (Odoux 2006). Native peroxidase which was found to be thermo-stable and persisted with high activity throughout the curing process might be responsible for oxidation of vanillin to produce quinine bodies having a different aroma than vanillin, and thus be partially responsible for the overall loss of vanilla aroma and fluctuation in vanillin content. Also, the continuous escape of flavouring molecules into the air leading to erroneous quantifications cannot be ignored. This is also indicative of substantial flavour losses occurring in conventional curing where routine sunning is involved. The pre-treatments appear to enhance the flavour formation via up-regulation of the β-GLUC and CSE.

The E-nose analysis of vanilla beans from different sources complimented well with the hplc quantification of major flavour components. Sample from green beans which consists of least amount of vanillin and allied flavouring compounds was placed in a separate quadrant away from the other samples derived from cured beans. The PCA mapping clearly distinguished the samples based on the dominant volatiles from the beans. A similar trend was observed in a study on coriander oil samples aroma analysis (Ravi et al. 2007).

4.4.2 Elicitation experiment

Elicitors are known to interfere with plant phenyl-propanoid (PP) compounds catalyzing lignification of plant cells. Since the natural vanilla flavour from cured beans of *V. planifolia* comprises over a hundred flavour molecules derived from the PP pathway, the effects of elicitors were studied, assuming that they might hasten the curing process by catalyzing relevant enzyme activities.

The data presented in **Table 4.8** show significant differences in the activities of enzymes, indicating that the method used for elicitor preparation possibly influenced their characteristics. Peroxidase showed lesser sensitivity to processing conditions than other enzymes. Enzyme activity change due to processing conditions is widely known. The major enzyme present in both vanilla beans and elicitors was the POD. The formation of very high levels of vanillin was also noted in the treatment with high levels of POD, which is indicative of the

major role played by this enzyme in catalyzing the flavour pathway. Earlier studies have established that peroxidases (of Class III type) are widely distributed in higher plant cells. Plant PODs and their various isoenzymes have been proven responsible for a plethora of physiological functions where they preferentially use phenolics as electron donors resulting in the formation of oxidized phenolic compounds of brown colour (Hanum 1997). Since their isoenzymes are diversely regulated (Welinder and Gajhede 1993), one can expect wide variations as well as an array of the end product. Whereas the fungal peroxidases (Class II type) are known to act at extra-cellular level and are mainly involved in lignin degradation; often contributing for the re-formation of vanillin (Hanum 1997; Priefert et al. 2001). Ferulic acid (an iso-lignin) occurs abundantly in most of the plant cells including vanilla beans. Earlier labeling studies have established its preferential incorporation into vanillin over other closely related substrates leading to the higher turnover of vanillin (Zenk 1965). Thus, there is a good relationship between the levels of POD (in situ + that of elicitor) and the levels of vanilla flavour compounds where the enzyme could probably catalyze the inter-conversions of the flavour precursors resulting in the formation of appropriate substrates for the action of the other key enzyme-β-GLUC. The potential of *A. niger* to convert natural precursors of vanilla flavour, i.e., isoeugenol to vanillin (Abraham et al. 1988), ferulic acid into vanillic acid (Bonnin et al. 1999) and vanillic acid to vanillin (Priefert et al. 2001), is well known. *A. niger* has also been described as a ferulic acid-degrading organism (Labuda et al. 1992). Commercial exploitation of *A. niger* for the conversion of ferulic acid to vanillic acid has been described (Lesage- Meessen et al. 1999). The treatment with the dry cell powder of *A. niger*, particularly the acetone-dried powder, significantly enhanced vanillin on 10th day itself, which was nearly 1.3-fold higher than the blanched and scarified control, and 2.46-fold higher than the traditional method of blanching (Sreedhar et al. 2007b). Noteworthy improvements in the other flavour components were also observed during this period. Lyophilized *A. niger* powder was more effective in enhancing vanillic acid and benzoic acid steadily up to 30th day. A few studies have demonstrated the direct involvement of β-GLUC as the key enzyme for the de-glucosylation of flavour substrates, which is also controversial due to compartmentalization of the enzyme from the substrate

(Dignum et al. 2001; Odoux 2006; Odoux et al. 2003; Havken-Frenkel et al. 2004). β-GLUC is also expected to enhance flavour extractability by hydrolysis of cell wall components (Ruiz-Teran et al. 2001). Nevertheless the present study and our previous report (Sreedhar et al. 2007b) indicate that the high levels of POD of the beans and that in the elicitor may also be involved in the quantum turn over of the flavour molecules, although a deeper study is needed to unequivocally establish the same. It is interesting to note that there is a decline in the level of vanillin on 20th and 30th days. These changes may again be attributed to the catalytic activities of POD followed by β-GLUC, where the latter systematically catalyzes the de-glucosylation whereas the former may be implicated in a cascade of redox reactions. Plant PODs (POD class III), (both from vanilla beans and red beet seedling root-derived) having high redox potential may not only build up but also degrade vanilla flavour compounds. Therefore, in a mixture of phenolics, the radicals of phenolics that are good substrates for POD can oxidize a poor electron donor molecule for peroxidase, rapidly. Thus, even the un-preferred substrate is indirectly catalyzed by POD, bringing about fluctuations in the flavour profile as observed in the present study. However, the fungal PODs are known to work the other way, where they depolymerize lignin to form vanillin (Hammel et al. 1993; Kirk and Farrell 1987; Priefert et al. 2001; Ten Have et al. 1998). Thus the highest turnover of vanillin in the red beet seedling powder or *A. niger*-treatments (being rich in POD) may be attributed to the re-conversion of degraded vanillin and similar iso-lignin molecules. Beans treated with red beet seedling powder showed a better sensory profile with high notes of vanilla, sweet, and floral and low woody, beany and smoky notes which is a characteristic feature of good quality vanilla (Hariom et al. 2006). Attributes such as fruity, floral, woody and beany notes did not differ significantly from the others, which might be due to the use of the higher concentrations of the sample which might have masked the subtle differences.

4.5 Conclusion

The present study has clearly demonstrated that for developing a process for the production of vanilla flavour compounds from vanilla beans, one need to carefully control the process parameters with periodical monitoring for flavour compounds

and terminating the reaction as desired because the endogenous enzyme may divert the flavour molecules towards lignin biogenesis. The pre-treatment methods developed as a result of the present investigation may find importance for realizing higher flavour formation in a shorter period since the major quality parameters and sensorial analysis were found to be comparable and similar with conventionally processed commercial sample. This study has also shown that the HPLC profile as well as the sensorial properties of the beans cured just for 10 days with elicitor were characteristically similar to the conventionally cured beans indicating that the present finding holds great promise for further application in vanilla bean curing.

Summary and Conclusions

- ✓ The study on genetic diversity of Indian vanilla using RAPD and ISSR markers indicated that *V. planifolia* cultivated in India is likely to share the same genetic background and therefore, the genetic diversity is extremely low. This preliminary investigation has determined the absence of genetic variations in introduced and then commercially cultivated *V. planifolia* in India indicating a threat of extinction due to pest and environment vagaries.

- ✓ Through an investigation on the occurrence of genetic variants in micropropagated multiple shoots of vanilla developed from axillary bud explants established 10 years ago using RAPD and ISSR, it can be concluded that the micropropagation system for vanilla can be carried out for a considerable length of time (for at least 10 years) without any risk of genetic instability.

- ✓ Vanilla shoot multiplication in semi-solid (SS), complete immersion system (CIS) and partial immersion system (PIS) were evaluated for biomass, shoot multiplication and elongation aiming at developing an improved micropropagation protocol. Shoot cultivation in CIS was associated with hyperhydric shoots having poor ability to establish in fresh medium or soil. The shoots sub-cultured in PIS were healthy with good elongation producing both geotropic and aerial roots resulting in highest survival on transfer to soil. A bioreactor model was worked out where the cultures were bathed with aerated medium for different periods to arrive at a final set of operating conditions leading to improvement in the multiplication rate of shoots which were adequately elongated and capable of rooting on hardening medium.

- ✓ The present study compares the solid, liquid (referred as CIS) and partial immersion system (PIS) and substantiates that Growtek^{TM} bioreactor (as PIS) appears to be an efficient culture system in all respects. A combination of equal ratios of red soil: sand: vermicompost was found to

be the best for greenhouse hardening. Field evaluation showed that the micropropagated plants flowered early with higher bean yield than the conventionally propagated ones.

- ✓ A study focusing on unraveling the major structural, biochemical and molecular changes occurring during hyperhydric syndrome in vanilla shoots grown in liquid medium was carried out. The syndrome was associated with severe damages at cellular and sub-cellular levels, increase in free polyamines and accumulation of water, and decrease in quantities of chlorophyll, protein and drastic changes in reducing and non-reducing sugars. The onset and progression towards hyperhydricity showed higher activities of antioxidant enzymes, indicative of shoots' defensive efforts against oxidative stress.

- ✓ Thirty one hyperhydricity-associated cDNAs identified by DDRT-PCR were cloned and sequenced whose electronic homology searches using BLASTX analysis resulted in identification of 23 cDNA clones showing homology with various stress, apoptosis, DNA repair and carbohydrate breakdown related proteins to be differentially expressed during HHS. BLASTN analysis yielded twelve fragments having homology with different stress linked cDNA clones. A partially characterized transcriptome of hyperhydric condition in *V. planifolia* has been developed which paves the way for a better insight into gene expression during this common physiological disorder.

- ✓ Aiming at developing of an improved bean curing technique using biotechnological methods, effects of different pre-treatments on the flavour formation in vanilla beans during accelerated curing at 38 ^{o}C for 40 days were studied. Use of naphthalene acetic acid (5 mg/L) or ethrel (1%) with blanching pre-treatment resulted in 3-fold higher vanillin on 10th day. Other flavouring compounds - vanillic acid, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde fluctuated highly showing no correlation with the pre-treatments. The pre-treatment methods found through the present study appear important for realizing higher flavour formation in a

shorter period since at this stage the major quality parameters were found comparable to commercial sample.

- ✓ In another bean curing experiment, food-grade elicitors were used in combination with different pre-treatments and accelerated conditions. When acetone dried red beet elicitor - a rich source of peroxidase was used, 2.65% vanillin was formed in 10 days, which was 1.7-fold higher than that in control beans (blanched + scarified) of this study and 3.23-fold higher than the conventional curing. HPLC analysis of elicitor-treated samples showed the formation of almost all the major compounds found in the conventionally cured beans (cured for 3-6 months) and better sensorial properties. These observations appear useful for developing a rapid process for the curing of vanilla beans.

Future prospects

- Absence of genetic diversity of Indian vanilla population as reveled from the present study indicates the need to increase the number of introductions and broaden the gene pool of cultivated vanilla in India to reduce its vulnerability to diseases and insect pests apart from its genetic improvement for other attributes.

- In order to broaden the genetic base of vanilla cultivars, further efforts such as seed germination, mutation breeding, genetic engineering, induction of somaclonal variants and other biotechnological means are to be urgently pursued.

- The information on the use of different micropropagation systems for vanilla and identification of partial immersion system as an easy method helps to develop an efficient technology for large scale production of micropropagules.

- A partially characterized transcriptome documented for identification of various genes involved in development of hyperhydricity syndrome through DDRT-PCR technique and study of their expression at various stages of hyperhydricity opens-up the gate way for further investigation and development of molecular methods to control the syndrome not only in vanilla but probably in other plant systems also.

- The study of the use of pre-treatments and the use of food-grade elicitors in combination with pre-treatments for the accelerated curing of beans appear useful for developing a rapid process for the curing of vanilla beans which appears to offer economical advantage.

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List of the differential amplicons, accession numbers and their sequences

Frag. ID	Acc. No.	Sequence (5'-3')
VP1	GE746280	GACCGACCCAGAGGTAGGGGGCGCCGCCGGGGAGGCGGGAGTGGGTTCCACAT CGGGAGGAGCAGAAGCCCTCGACAGCGTCGTCGGCGGAGGTGATGATGAGGGC GATGGCGAAAGGGTTGGTGTTGTTGGAAAGGGCGACGGCGGCGAGGGCGGGAA GGGCCGAGGAGTGGAGGATGCGGCCGAGGGTGCTGGAAGGGAGGAGGACCTG AGGGCCGAGGGTGATGGACGGGGGGGATGTGGGTCGGTC
VP2	GE746281	ACAACTGGGGCAATGTGCTGAGAGGTGATGCTTCCCTTGCGAAACTGGCACAGCT GGAGAGGTTCTCAACCTGCTTGAGAAGAAGAATAGTAGCTCTTGAAGGGACCGTC GAGTCGCTAAGAGTAGAAGCCGAAGACGACACCTGCTGCTGCATCTGCTACGCCT CCGAGTCCGACACCCAATTCGAGCCTTGTCACCACCAGTCTTGCCTTGGCTGCATTA CCAGGCACCTGCTGAATAGTCGAAGATGCTTCTTTTGCAACGCAACTGTTACAGAA GTGGTTAGGGTTTGCAAAAATTCTAATCAGATTTCTGGAGGATGATTCTCTTGAAC CCACCTTTTCTGTTTTCTTGAATTGAAAGGTATGCAATATACACACAGGCACCGACC A
VP3	GE746282	AGTCAGCCACCAAATCTGTATAACGATGATTCTTATGTCCGAGGTCGTGGACGGG GCAGAGGAAGAGGAAGAAGTTGGGGTAGAGGCGCATATGGTGGCTACGGCGGA GGATACGAGCGTTATGGTGGATATAGTGGTTATGGAGGATATTCAAATGATCATG AAAATGGTGAATGGAATTATAATTGGAATCGAGGCAATGGCCGAGGCAGAGGAA ATTGGAGTTATCGTGGCTGACT
VP4	GE746283	AGTCAGCCACATGGACCAAGGCCACCAAGACAGACTGAGGTCAGGACGGGTGAA GGAGTGCTAGGATCATCTATCTGGATTTTACAATAAAGAAAAAGAAAGTTACTAAA GATTTTGATTGGTGCAGAAGGGTAAGTGAGCACAGCAGTGTTAGAGAATGACAA AAAGAAGGGGCTGAGGTGCTAGGGATGAAGTGGGTAGGGGACACTGGAGTGGG GGGAAACATCCCTCCATCCTTCACTACCCCAGGGCTCTCCTCCAGACCGGGTGCTA TGGTGGCTGACT
VP5	GE746284	AGTCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTATGGGAGGCAGCAGT GAGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATGCCGCGTGGAGGAA GAAGGCCCACGGGTTGTGAACTTCTTTTCTCGGAGAAGAAGCAATGACGGTATCT GAGGAATAAGCATCGGCTAACTCTGTGCCAGCAGCCGCGGTAAGACAGAGGATG CAAGCGTTATCCGGAATGATTGGGCGTAAAGCGTCTG
VP6	GE746285	TATGGTGGAGCACGACACTCTGATCAGATCAGAAGATTACCACTTGCCTCAGACG GAGCGATGATATACCATTGTACACTTGAACTTACCGATGATAAAGCATCATCACTG GGAAGAGGTCCCGTCTACCCACGACAGGCGGTATTC
VP7	GE746286	TGGTCGGTGCCTGTGTGTATTCTGTTTATCGATCCCCTGCTCTTTGTTCATCGATATC TATCTTTTTTTCTGTATCGGTCGGCATCTTCTTTCTCAACTCGTCCAGGGCAAGCTA
VP8	GE746287	TAGCGCCATTGCAACGAGGTGTGAGATTAAAAGTTACATCAAAAGGGTGAACAAA GTTGAAGGTAGAAACTTTGTCATATGATGAGTCATTTTGTAAATAGCATTAGATGG TACACCCATGTATTGAGATGATCCACTAATGAAACAATATGAAGATAACTGTAACC TAGTGCAAGGTGACCAAAATTAAAAAATCATTTGATACAATGATAAAGCATATTGT ATGTTTTGGTGAATGCTTGCTCATAGTCATTTATTTTGAATACTATTGTTTTGATCAT TATGTATGATTAGACTTTAGGTATCTTAAGATAGTCTAATAGACTTTCTTGCTTTTTC CATAGCTACAATGGCGCTA
VP9	GE746288	TAGTCAGCCACCTCCTCCAGGTACTACATCATGGAGAGGAAATTTGAGCTCTCCCC TCTTGCCTTCTCCAAAGGTTTGTAACTTTTCCGTTTTTTTTAATTTTTATGATTATTTT TTCAGTTTGAACCATTCTTTTTATCAACACTTCTTCTTTCAGCAAAGGCAAAACTAGC TTTCTTAGAGCTCACCTTGCGATCACATTGGTTCAACTAGTTGGGAGGACTATCATT

		ATATTTGGAAATTTTAGGTTATTAGGCAGGCATTTTGTTGTTTTCTTCAGCCATATC TGTATTATACACACAGGCACCGACCA
VP10	GE746289	TAGTCAGCCACCTCCTCCAGGTACTACATCATGGAGAGGAAATTTGAGCTCTCCCC TCTTGCCTTCTCCAAAGGTTTGTAACTTTTCCGTTTTTTTTAATTTTTATGATTATTTT TTCAGTTTGAACCATTTTTTTTATCAACACTTCTTCTTTCAGCAAAGGCAAAACTAGC TTTCTTAGAGCTCACCTTGCGATCACATTGGTTCAACTAGTTGGGAGGACTATCATT ATATTTGGAAATTTTAGGTTATTAGGCAGGCATTTTGTTGTTTTCTTCAGCCATATC TGTATTATACACACACACACACACACATACACACAGGCACCGACCA
VP11	GE746290	TAGCTTGCCCTGGTACGAGTTTATGATCCTTCCTGTTGGAGCAACCTCGTTTAAGG AGGCCATGAAGATGGGTGTTGAAGTATATCACAATCTAAAGGGTGTGATTAAGAA GAAGTATGGTCAGGATGCTACCAATGTTGGAGATGAAGGTGGCTTTGCACCTAAT ATTCAGGAGAACAAGGAGGGACTAGAATTGCTGAAGATTGCTATTTCTAAGGCTG GATATACTGGCAAGGTTGTAATTGGAATGGATGTGGCTGACTA
VP12	GE746291	ACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAGTCAGCCAC ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTC CGCAATGGGCGAAAGCCTGACGGAGCAATGCCGCGTGGAGGAAGAAGGCCCAC GGGTTGTGAACTTCTTTTCTCGGAGAAGAAGCAATGACGGTATCTGAGGAATAAG CATCGGCTAACTCTGTGCCAGCAGCCGCGGTAAGACAGAGGATGCAAGCGTTATC CGGAATGATTGGGCGTAAAGCGTCTGTAGGTGGCTGACTAATCACTAGTGAATT CGCGGCCGCCTGCAGGTC
VP13	GE746292	AACAACTTAACAATAATCAAATCTCACAAATGAAAAATACCAGAATACAAGTCCAA TACATAACGAACGAAATAAAGATATGAACAATAACACTGTAATTAGAACAAGTAC CCAAAACAACATATGTTGGAGAGCGAAATAAAGGGAGAGGAGGAATGGGGGGC CGTTGGGTGGTGGCTGACTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTC
VP14	GE746293	ACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAGTCAGCCAC AGACAAGACGGCAAAGCAAAGGGTGGACTTGTGGTTTTGGAGAGGTGAGTTGAT GGAATCTTGTAGATGAGATCAATAGGGAAGCTACATGTGATGATGATGATGATTG GGAGAACTGAGAAGCCGCATGCAAAATTGGTGGGATTGATGCAAGTATGGCTTGT GAAGAGAGGTGGCACGGTAAGGAGGGGAGGAGGATATTGAAGGCATGGTGGCT GACTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTC
VP15	GE746294	TAGTCAGCCACCGCAAACGTGAGTAGTTTGTGTGATTTCATACGAACCGTGTCCTT GGTGAGTAGCTACGTTCTAACTTTGATCGTTTTGGTCATTCGCTGAATGGCAACGG CGTGCACTGGATGGTGTGTGCGTGAGTCGGGTTGAGTTCGTTATCGACCATGTGG CTGACTAA
VP16	GE746295	TTGGACCGGTGCAGCAGAGACGAAGAAAACTCGGGCCAGAGCGATCCCAAACTG TGGAAGAGCAAGTGCAGGCACTGTTGGAAGCCGAATTTATAAGAGAAGTCAAAT ACCCACTATGGCTAGCCAACGTCGTTTTGGTGAAAAAATCAAATGGGAAGTGGCG GATGTGCACCGATTACACAGATCTCAACAAAGCTTGCCCAAAAGATCCATACCCAC TCCCGAGTATTGACGCCCTGGTAGATGCTTCCTCTGGGTATAAATATCTCTCGTTTA TGGATGCGTATTCGGGATACAACCAAATCCCATGTACCCACCGGTCCA
VP17	GE746296	TGGTCGGTGCCTGTGTGTAGTATGTACTTGGTTGGTTAAGGGTATAGGGAACTTG TGGGCGAACGTCAAGAGATGGAGGTGCAGGTTGTTGCAAAGGTAGCTCTTTGTAA GACTGGTTTTGATCGGGAGTTCTGGGTCGTGAGGTTGAAGAGGTTATTTGGTGAG GAACCAGCGCTAATTGCTGATTTGGGATTTCGGTGTAATTGCTCTGGGTTTTTGTTT CAGGAGGTGTGGCTGACTA
VP18	GE746297	GACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAGTCAGCCA CCAATAATAAAAAAGAAAATAGGAAAATAAAAAAAAAAAAAGCTAAACAAATGG AAAAATTACGTCGCACCGGTAATGCGTTGACTTCAACGGACGCTCAAATGTCCATC GTCGGCGGTCGTGGCTGACTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTC

VP19	GE746298	ACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAGTCAGCCAC AGCAGTATCAGCAGCCACAGCAGTATCAACAGTCACAACAGTATCACCAGCAGCC ACATCGACAGCCACCAAATCTGTATAACGATGGTGTGGCTTTGTTAGCTTGTGTTG ATTACTAGTGCTGTATAGAATATTGTGGTTATTTTTTCCTTTAGGTTATTGTTTCCCA CTCATTGGTATTATATATTGGTGTTAAGATTCTTATGTCCGAGGTCGTGGACGGGG CAGAGGAAGAGGAAGAAGTTGGGGTAGAGGTGCATATGGTGGCTGACTAATCAC TAGTGAATTCGCGGCCGCCTGCAGGTC
VP20	GE746299	AGCGCCATTGGGTATAGGCATAATGGGTTTTAACAACGTGGCAACAGCTTTAAAA GTACCGGGTGTTGAACTGGTAGCTGTGGCAGATTTATATACCGGAAGGCTTGAGA GAGCTAGGGAGTTATATGGTCAGCAGCTCTTCACGACGAAAGACTACGAAGAGAT TTTAAATAGAAAAGATATTGATGCAGTGATTGTTTCCACTTCAGATAATTGGCACG ATAGGATTGTAATTGAAGCTTTGAGAAAAGGCAAAGCAGTGTACTGCGAAAAGCC AATGGTTCATAAAATTGAACAAGGTTTGGATGTCGTTAAAGCGCAAAAAGCAAC GGCTGGCATACGCAAGTGGGTAGTCAGAGAGTGAGCAGCATTGTATACGCCAAA GCCAGAGAGCTTTATAAAACAGGAGAGATTGGTCAATTGAATTGTATTGAGGCTT CCTTTGACCGTCATTCTGCATTGGGTGCCTGGCAGTATACAATGCCAATGGATATTT CCGAAAAAACAGTGGCCTGGGAAAAATATGTGCGTTCGAAAAAAGATGTTCCCTT CGATCCGAAACAATTTTTCTGGTGGCGGAATTATAAAGAATTCGGAACAGGTGTA GCTGGCGATTTATTTGTTCATCTTTTATCGGGGATACATTTTATTACTGGTTCGCTT GGTCCTTCTCAGATATTTGCTACAGGTGATATCAGTTATTGGAAAGATGGCCGTAA CGTGCCTGATGTAATGACCGGTGTCATGCAATATCCCGCTACTAACCAGCAT
VP21	GE746300	AGCGCCATTGCCACAACAGATGCCTGCAGAATCATGACTGAATTAGGGGAACTTC TGCAGGAAAATAATGCAGAATATGCATATGATGCTGAAATGAATGGAAGCCTGGT TAATAACCCATTAGCATTTGGCTGTGATGACCCCTCCTTGCAAATTTTTCTTCCAACT CAACCGGATAGCCGAACTGTGCATGCTGGCTTGGGAGAACAATCAAAATTGACTA GTGTGTTTGAAGACGGTTGGATCTCTCTTGCTCCTGCTGCTGATGGAGTTCAAACT GCTGCAACTCGAGAATGTAGGCCAAATTCAAGAAACCATTTGTTTCTACATGAAAA CTGGCCAGAGAGCATGGAAAATGATTCTGGTTCTCCCCTCCTGTCTCTGAATAGAC AACATGAATTGAAAGCAACTTTGAATGGCCAAAAGCCTGATGCATCCTTGCGCCAT TCCCATCAGCAAAGGTCTATGAGATCCAGATTTATGTTTACACTTGATATAGACTCA GATAGCCAGTGAATGTAGTATCTGGTGATGAGATTTTGTGGGTCATTTCTATTTCA CCGTCCTGTTAATAACCTTGGTTACACGAAGAAAAGGCTAAAGAGGAGGTATAGA AGTATCTAGAAATGAACCTTGTTTGTTCATGTAAATTTTATCTTTCTTTTACCTTTCA ACAATGGCGCT
VP22	GE746301	AGCGCCATTGCTCAGGGCGAAGCTGGAGGAGTTTTTGGCTGACGATCGTCGTACG CTCGAGACGCTGGTCATGGCTGTGCGCGATCAGGTGGTTTTGGGGTACGAGGAGT TTTTCGATGGGTGGGTTGAGCAGCTGGGCGGTGAGAGGAAGGTTAGTCGGAAAG GCAAGGGCAGAGAGTCGGATGTACTTGGGCCGGAGGTGTTTGGCGATGTCGTTG GGCGGGTCTTTGAGACTAAGGCGCTTGAGGATGTTGATGATGGAGATAACTCTTG AGGTTGTGAGGTCCGTGTGGGAGGTTGCAGCGCCTGTGGGCGTGCTTTCTTGGGA TCTTCAAGAAACCCATGGGAAAATAGCATTTGAACATTTCCTTGGACGGTCCTACC ATGTGGTCCTTTCCCCCACGTCTGCGTGCTACTCGGCGTGTTTATCGCTGGTTTCTC TCATGACGTCGAGACGGACGATCTCATACCAAAGAGTTCAAACAGACCGCGTCCA CAACCCTGCCAGTCCGCGAACAGTTACTAGATGTCTGTGAACACATGTTTTGTTAT ATGTTCACTTTCAAACAATGACCCACGTTTTCTTGTTTTTCACGTTATTGCCACAATG GCGCT

VP23	GE746302	CCAGCATGGGCAACAAGAGTGAAACTCTATCTCAAAAAAAAAAAAAAAAAAAAG GAAACTCATTAATGCTGGCTCAGGAGTTCTGACAACATTACCTTTGTTTTTATAGGT TCTTACAGTAGAGATAAGTTTGCAAAGGCCAGTGTTTGCCTATTTTTCTAATAATTA ATTCCTTTCCTAATACCAGATGTGATCTCAATCATTCCATAATGCCACACATTTTATT AGCTTTGCCTCTCATATAGGGATTACTTATTTGTTTTTTAGAAGGGCTGAACGAAAT AGAAGTTTAATTCCTTCACACGTGGCCTGTAAGAGAGTCACCATTCTTACTGAAGC TGAGAAGGAAGTTCAAGAATGAGAAGGAAAACTATCTCTGATCTTCTCCAAAGAC GATAATTTATTTACAGCTGTCTCTTCTCTTGTCTTTACCTTTATTATTAAACACACAC AGGCACACGCACGCACACAGGCGCACACACACATATACACACAGGCACCGACC
VP24	GE746303	ACGGTTCCACCCTCCTTGGGGATCCTTTCCACCTCCGAGTGCTCGGATGGTAGTGT AATCTTCCGGTTCGGAGATGCTGCGGAGGAGTCCTGGAAGGATTCGGTTGGAGA GATGGAACCGACGGGGTCTAGTGAAGATGTTGGGATACTGGAGGAGAATGCAGA AGATAGCGGACTTGAGACAAGGAATCGAATTCTTGCGTCACTGTCGAGAGTGAAG CCGGCGTGTGCTCCGATTCTGAGTTTTTTGGTGCTGGAGATGACTTCACAACCGAC CGTTTTGGTTTTGGTATTGTCACAGAACAAGATTTACGTGATGAAGGAAGCCGGG GTGCGCAGGCTATTGATAAGATGTTGGGCAGCGATGGGATGGAGGATGTCCTTG AAGAGGAATTTTCTGAAACATGGTCAGGAGAGCTGCAATCGGAGGAATCTCACAA TGAAGATGGTTTGATTGCCGTGGAAGGCCGTGAAACTTCACCATATGGCGGGTAC AGTAGTTTTGTTTCAACTGAAGTTGGTATGCTGCAGTTGCCCGAGAGTGAGAGCA AGATTAAGGCAGATTCATTGCAGTCTGATGAGGTTTCATCAAACATTGTAGAAAAT GATTCCGATGTCGAACAAGATCAAAATGTTGGTTCATCTAACGAGCTTCTTCTTGG TTCCGAATCTTTATCAAATGCTGAAGCTGAAGCTCTTCAAGTGGAAGACACTCAAA ACCCAGAAGCAT
VP25	GE746304	GCTCGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCATTCTTGCGAACGTACACCCCA GGCGGGATACTTAACGCGTTAGCTACAGCACTGCACGGGTCGATACGCACAACAC CTAGTATCCATCGTTTACGGCTAGGACTACCGGGGTCTCTAATCCCATTTGCTCCCC TAGCTTTCGTCTCTCAGTGTCAGTGTCGGCCCAGCAGAGTGCTTTCGCCGTTGGTG TTCCTTCCGATCTCTACGCATTTCACCGCTCCACCGGAAATTCCCTCTGCCCCTACCG CACTCCAGCTTGATAGTTTCCACCGCCTGTCCAGGGTTGAGCCCTGGGATTTGAC GGAGGACTTGAAAAGCCACCTACAGACGCTTTACGCCCAATCATTCCGGATAACG CTTGCATCCTCTGTCTTACCGCGGCTG
VP26	GE746305	GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTCTCCGCAATGG GCGAAAGCCTGACGGAGCAATGCCGCGTGGAGGAAGAAGGCCCACGGGTTGTGA ACTTCTTTTCTCGGAGAAGAGGCAATGACGGTATCTGAGGAATAAGCATCGGCTA ACTCTGTGCCAGCAGCCGCGGTAAGACAGAGGATGCAAGCGTTATCCGGAATGAT TGGGCGTAAAGCGTCTGTAGGTGGCTTTTCAAGTCCTCCGTCAAATCCCAGGGCTC AACCCTGGACAGGCGGTGGAAACTATCAAGCTGGAGTGCGGTAGGGGCAGAGG GAATTTCCGGTGGAGCGGTGAAATGCGTAGAGATCGGAAGGAACACCAACGGCG AAAGCACTCTGCTGGGCCGACACTGACACTGAGAGGCGAAAGCTAGGGGAGCAA ATGGGATTAGAGACCCCGGTAGTCCTAGCCGTAAACGATGGATACTAGGTGTTGT GCGTATCGACCCGTGC
VP27	GE746306	CAGCCACCGCGCCGAGGTAGGGGTCGTCCGCCGAAGGCAAAGGTCTCTGGGACA GAAGGGGCGGGATCTTCGTTAGCCGTTACTAAATTTTGTTCTGAGATGCCCCGAAC CATGGTGAAGCGGGGACGAGGGCGGCCGCCGAAGGCCAAGATCTCTGGGACAG AAGGGGCGAGATCTTCCGCCCCCAAGGGTACAGAATCCTCCATTTCTGCCGGAGG AAAAGATCCCGGTATGTCTCCGAAGAAGAACCAAGATGGGAATGTTGCATCACCA TCTTCGTTAGCTGGTACTAGTTTTGGTTCTGAGATACCCCAGGCCGGGGTGAAGCG GGGACGAGGGCGGCCGCCGAAGGTGAAACCCTTTGGGGCGGATGTTGCCGGCAG

		GTGAATTGACTCCAAAGATTTTGTTTTTTTATTGGACTTGTAGTTCTTGGGAAATAT TTGTCTTGAGTTGGCGATGCGATTTATAAATCTGGGGAACTATAGCTTTGTTGCTG TTTCAGCTGTGTTTGCTCTTCGTGGTGCATTGTATATGATCTCTACTTGTTGTGGCT GACT
VP28	GE746307	ACTCTTCCGTTACCGCGCTTAATCACCTCGGGGATTCATAATAATGTTGCAAAAAGT TTTAATTGCAAACCGTGGTGAAATTGCCCTGCGCATCACCCGAGCTTGCAAAACTT TAGGAATTAAAACTGTCGGTGTCTATTCAGATGCTGACAAGGACTTGATGCATCTG CGTTTCTGCGATGAAGCAGTATGTATCGGCCCTGGCGCAAGCAGTGACAGTTACCT AAATATTCCAGCAATGATCACAGCAGCTGAGATTACTGGTGCGGATGCGATCCAC CCAGGTTATGGTTTCCTGTCTGAAAATGCTGAATTTGCTGAAATTGTTGAAAGTTCT GGCTTTATTTTTATTGGCCCACGTCCTGAACACATTCGCCTGATGGGTAAGAAGGT TTCTGCCATTATTGCCATGAAAAAAGCCGGCGTACCAACAGTACCAGGCTGTGATC ATGCAGTAACCATCCACAATGCCCTTGCTGAAGCCAAAGAAATCGGTTTCCCGCTG ATCGTAAAAGCTGCTGCGGGTGGTGGCTGACT
VP29	GE746308	CCAAAAAAGACGTTTCCGGTGCAGAATCACTACGATTAATCGCTTGAATAGAGTTC GTAAGCGCTTTCGTCTGGATATTGACAACAATGGCGTTTTTTAAACCAGCTGCAGT AGCCCCCGGTTCTAATAATATATACGAGCATACATTAGCCGATTGCGTTGCTTCATC TACAGATGCCACAATAGGACGGCCATGATACGCTGATACAAAAGCTTTGTCGGTT AACAACTTGGCAATCGCTTGATCTGAAAATGCAGATGCTGGTTGGCGCTCACCGCT CTGTGAGGAGTAAAACAAGTCCAAACCCGCATTGTAAAGATACACACTGTCGATA TCTTTCAGTGTCAGAAGATGCTTGTCAATGACTTTGCTTGTTAAGAGAGCCACGCG CTCATCATTTTTATTCATATTTAAAAATTCAATGATCTGATTGTCGCTATAAAGAGCC CTCTCTAATTTTTTTGCGGTCTCGTCAAGATAGGTGATACTGTAGTTCGACTGCGCA ATCAGTTTATCGTTATAGTGGCTGACT
VP30	GE746309	AGCCACGCCATATCGTCGAGGGGAGAGGTGCATTCGTAACGTCTCTGTTATGGAT GATTATGCAGCAATAGGTTCTCCACTTGATCCAGAGGAAGTCTCAGAAGGTGGAA TGTTATCCAGGATGCGCATTGCTCGTTGCGATGACAACCAAAGGCGGCCGAGCTTT CCAGAGAGTGTTTGTGATGTTGTTTTTGGAGGAAGTGAAATCATGGTCGATGACG TGAAGTTTACTGTTTCGCACGTAGTGGAGCCAATGGAAAGAGGAATCGCTAGGCT AAACAAAACGATTCATCAGATCTCAGAAAACATAAAGCAGCATGAAATGCGCAAG AGAAAAGCCAAGGATGATTCTGATCTAATCCCTTTGCGTACTTGGACAAAAGAGTT TTCTGAAGCACGCGACCATGTGGCTGACT
VP31	GE746310	TAGTCAGCCACTCGGATGGAACTATTAAGGTTTGGGATATTGGGAAGAGGGGTTG GCAGTTAGTTCAGGAAGTTCAAGAACATCTGAAGGCTGTTACGGGTCTTTACATTC CTCTGTCAAGTGATAGATTATACAGTTGTTCTCTTGATAAAACAATTCGCACTTGGA CTATTGAACCGGAGATTCGTGGCCTTCAGATTCATGACATGAAGGAACCTGTGCAC TGTTTGGCTGCGAATGCCAATATTGGGTGTTTCTCAACTCAAGGCACTGGTGCAAA GGTCTCTACCTGGGGTGGACTTACAAAGCACATTAATTTCAACAAAAATGTGAAA TGCATATCCATGACAGATGCAAATCTATATTGTGGCTGACT
18*S*		CTCCCGGCCGCCATGGCGGCGCGGGAATTCGATTAACGGCTACCACATCCAAGGA AGGCAGCAGGCGCGCAAATTACCCAATCCTGACACGGGGAGGTAGTGACAATAA ATAACAATACCGGGCTCCACGAGTCTGGTAATTGGAATGAGTACAATCTAAATCCC TTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAG CTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGACCTTGG TTTGGGTCGGTCGGTCCGCCTTTTGGTGTGCACCGCCCGCCCTGATCCTTTTGTCGA CGATGCGGTCTGGCCTTAGCTGGCCGGGTCGTGCCCTCGGCGTTGTTACTTTGAAG AAATTAGAGTGCTCAAAGCAAGCCCACGCTCTGGATACATTAGCATGGGATAACA TCACAGGATTTCGATCCTATTGTGTTGGCCTTCGGGATCGGAGTAATGAAATCACT AGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTG GATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGT

	CATAGCTGTTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGA GCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACAT TATTTGCGTTGGCGCTCACTTGCCGCTTTCAGTCGGGGAAACTGTCGTGCCAGCTG CATTAATAATTCGGCACGCCCGGGAAAAGGGGTTGGTTTTGGGGCTCTTCCTTTCC CCTCCACAATCCTCCCGGCT

Publications

Papers published

1. **Sreedhar RV,** Venkatachalam L, Bhagyalakshmi N (2009) Hyperhydricity-related morphological and biochemical changes in Vanilla (*Vanilla planifolia*). **Journal of Plant Growth Regulation DOI 10.1007/s00344-008-9073-4**

2. **Sreedhar RV,** Venkatachalam L, Bhagyalakshmi N (2007) Genetic fidelity of long -term micropropagated shoot cultures of Vanilla (*Vanilla planifolia* Andrews) as assessed by molecular markers. **Biotechnology Journal 2: 1007-1013**

3. **Sreedhar RV,** Roohie K, Maya P, Venkatachalam L, Narayan MS, Bhagyalakshmi N (2008) Biotic elicitors enhance flavour compounds during accelerated curing of vanilla beans. **Food Chemistry 112: 461-468**

4. **Sreedhar RV,** Roohie K, Venkatachalam L, Narayan MS, Bhagyalakshmi N (2007) Specific pre-treatments reduce curing period of Vanilla (*Vanilla planifolia*) beans. **Journal of Agricultural and Food Chemistry 55: 2947-2955**

5. **Sreedhar RV,** Venkatachalam L, Roohie K, Bhagyalakshmi N (2007) Molecular Analyses of *Vanilla planifolia* Cultivated in India using RAPD and ISSR Markers. **Orchid Science and Biotechnology 1(1): 29-33**

Papers for publication

1. **Sreedhar RV,** Bhagyalakshmi N (2008) A Partial Immersion System for micropropagation of vanilla.

2. **Sreedhar RV,** Venkatachalam L, Bhagyalakshmi N (2008) Gene expression in hyperhydric shoot cultures of *Vanilla planifolia* as depicted by mRNA differential display.

3. **Sreedhar RV,** Maya Prakash, Bhagyalakshmi N (2008) Flavor and Sensorial profiling of Indian and Madagascar vanilla beans.

Papers presented in Symposia/ Conferences

1. **Sreedhar RV**, Venkatachalam L, Kaunain Roohie and Bhagyalakshmi (2007) Molecular analyses of genetic diversity in Indian vanilla and genetic fidelity of long -term micropropagated vanilla shoot cultures. Poster presented at **Asia Pacific Conference on Plant Tissue Culture and Agri-Biotechnology (APaCPA) 2007** held between 17th-21st June 2007 at Putra World Trade Center (PWTC), **Kuala Lumpur, Malaysia**

2. **Sreedhar RV**, Venkatachalam L and Bhagyalakshmi (2007) Novel differentially expressed mRNA transcripts in hyperhydric shoot cultures of *Vanilla planifolia*. Poster presented at the symposium **Novel Approaches for Food and Nutritional Security** held between 6th-8th Dec 2007 at CFTRI, Mysore, India

3. **Sreedhar RV**, Venkatachalam L, Kaunain Roohie, Thimmaraju R and Bhagyalakshmi N (2005) Molecular analysis of genetic fidelity in long-term micropropagated shoots of vanilla (*Vanilla planifolia*). Poster presented at **National symposium on Plant Biotechnology: New Frontiers**, held between 18-20th November at Central Institute of Medicinal and Aromatic Plants, Lucknow, India