# Studies on Genetic Variability in Open Pollinated Progenies of Vanilla.

Thesis submitted to
**University of Calicut**
for the award of **Doctor of Philosophy** in Botany

By
**Vimala Jose**

**University of Calicut**
**Kerala, India**
**2005**

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# Certificate

This is to certify that the thesis entitled "**Studies on genetic variability in open pollinated progenies of Vanilla**" is a bonafide research work carried out by Ms. Vimala Jose under my supervision and guidance in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Botany at Indian Institute of Spices Research (IISR), Calicut -673 012. The work presented in this thesis has not been submitted by her for any other degree or diploma earlier.

10/5/2005

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# Declaration

I hereby declare that the thesis entitled "**Studies on genetic variability in open pollinated progenies of Vanilla**" is a bonafide research work carried out by me at the Indian Institute of Spices Research (IISR), Calicut under the supervision and guidance of **Dr. K. Nirmal Babu**. No part of this thesis has been presented for the award of any degree or other diploma of any other university or institute earlier.

(Vimala Jose)

Calicut,
11-05-2005.

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Finally, I dedicate this work to Almighty God with humility and reverence.

**Vimala Jose**

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LIST OF ABBREVIATIONS

| **Abbreviations** | **Expansion** |
|---|---|
| cm | Centimeter |
| cm$^2$ | Centimeter square |
| g | Gram |
| hr | Hour |
| l | Litre |
| M | Molar |
| m | metre |
| µm | Micromole |
| mg | Milligram |
| ml | Milliliter |
| mm | Millimeter |
| mm$^2$ | Millimeter square |
| µl | Microlitre |
| min | Minute |
| ng | Nanogram |
| rpm | Revolutions per minute |
| W/V | Weight per volume |
| $^0$C | Degree Celcious |
| pH | Negative logarithm of hydrogen ion concentration |
| % | Percent |
| ~ | Approximately |
| ♂ | Male |
| ♀ | Female |

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# Introduction

*Outstanding contribution of the West to World's flavor*

The genus *Vanilla* Mill. (Plum. Ex. L) belonging to family Orchidaceae comprises more than 100 species, distributed in tropical parts of the world (Dressler, 1993). These are climbers with fleshy leaves and thin velamen. Plants are leafy or leafless with branching vines.

There are only three important tropical spices indigenous to the western hemisphere: the chilli peppers, pimento and vanilla. Vanilla may be said to be the outstanding contribution of the Western hemisphere to the flavoring substances or spices of the world (Ridley, 1912). Vanilla is the only member now in the orchid family which is a highly valued crop and hence the name "*Orchid of commerce*".

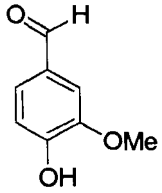
Vanilla pods are the cured fruits obtained from three different species, namely, *V. Planifolia* Andrews (Mexican vanilla), *V.tahitensis* Moore (Tahiti vanilla) and *V. pompona* Scheiede (West Indian vanilla). The most important is *V. planifolia* from which almost all vanilla comes from. Etymologically, the word vanilla came from the Spanish word "vanilla" which means a small pod due to the great similarity between this fruit and a true pod.

*Vanilla planifolia* Andrews (syn *V. fragrans*) (Salisb.) (Ames) is native to the humid tropical rain forests of Southeastern Mexico, Central America, the West Indies and northern part of South America. Amongst the five species reported from India, *V.pilifera* Holt. is endemic to Assam while, *V.andamanica* Rolfe.is reported only from Andaman Islands, *V.aphylla* Blume., previously known from Thailand, Laos, Vietnam, Malaya and Java is found occurring in Kerala and Tamilnadu, *V.walkeriae* Wight. from Travancore and Trivandrum and *V.wightiana* Lindl. from the states of Andhra Pradesh, Karnataka and Kerala (Kumar and Manilal, 1993).

The highly specialized floral structure of Vanilla renders self-pollination impossible. Even though vanilla flowers are bisexual in nature, because of the presence of a special structure inside the flower, called "rostellum", the natural pollination is almost impossible. In its native home, cross – pollination is effected by the bees of the genus *Melipona* and also by humming birds. Therefore, hand pollination must be used to get an economic crop.

Vanilla *(Vanilla planifolia)* is the most expensive spice traded in the global market after Saffron (*Crocus sativus L*). Vanilla essence is largely used in the preparations of ice creams, chocolates, bakery products, puddings, pharmaceuticals, liquors and perfumes. During 2000 the global trade of vanilla accounted for 103.17 million US $ giving direct and indirect employment to many thousands of persons both in the producing and consuming countries. Vanilla beans constitutes nearly 0.75 per cent of world import of spices in volume and in terms of value its share is six to seven per cent of nearly 1500 million US $ of global spice trade.

Vanilla is one of the world's most labor-intensive agricultural crops, which is why it is so expensive. Vanillin, the most abundant volatile aromatic constituent of vanilla beans was first isolated from vanilla by Gobley (1858) by macerating the cured beans in alcohol. Vanillin (4- hydroxy- 3 methoxy benzaldehyde) is classified as a phenolic aldehyde.



**Vanillin** Mol. Wt.-152.14

In fact, 97% of vanillin used is synthetic. It is synthesized from other compounds such as eugenol, and as byproduct from the breakdown of lignin in the manufacture of paper. This vanillin is much cheaper than nature vanilla flavor. However, there is a great demand for the natural vanillin in the international market obtained from the vanilla beans. The flavor of vanilla beans from *V. planifolia* is far superior to that of synthetic vanillin due to the presence of other flavor compounds in the natural product.

Mexico had the monopoly of growing vanilla, until the late 19th century. Now, Madagascar and Indonesia produce 90% of the world's vanilla bean crop. In India, it is mainly grown in states like Karnataka, Kerala and Tamilnadu.

The basic chromosome number for the genus *vanilla* is X = 16 and *Vanilla planifolia* is a diploid with 2n = 32 (Eftimiu-Heim, 1950; Hoffmann, 1929; Martin, 1963). Ravindran (1979) reported abnormalities in pollen grain mitosis in *Vanilla planifolia.* This included fusion between chromosomes, reduction in chromatin content, chromosome number less than the normal gametic etc. Such abnormalities were combined with comparatively high (nearly 65%) pollen sterility percentage. Nair and Ravindran (1994) reported somatic associations of non-homologous chromosomes in the root tip cells undergoing mitosis.

Vanilla is propagated by cuttings. Natural seed germination is possible only in association with mycorrhiza. This crop is suspected to be highly heterozygous with extensive genetic variations because of its cross-pollinated nature. As much of the vanilla in the Old World, were the introduced material; is propagated vegetatively is of clonal origin, there is little variation. Continuous clonal propagation of the limited introduced gene pool leads to evolutionary stagnation. This is one of the most important limiting factors in varietal improvement of vanilla.

This work was conducted with the broad objective of improvement of vanilla by generating variability in the available gene pool. The present investigation was undertaken to study the scope of varietal improvement in vanilla through the use of mutagens, colchicine and interspecific hybridization. In this study, seedling progenies, interspecific hybrids, colchicine treated and EMS treated progenies were analyzed for variation as expressed by morphological, cytological, anatomical and RAPD markers.

The present work was carried out with the following specific objectives:

- To generate large number of open pollinated seedling of vanilla through ovule culture and to study the variability among segregating progenies as reflected in morphology and cytology
- Interspecific hybridization between cultivated (*V.planifolia* and *V. tahetensis*) and the wild (*V.aphylla*) vanilla, to produce fertile progeny which may carry economically important genes
- To enhance the spectrum of variability by *in vitro* polyploidy / mutagenesis and to study the effect of different doses of mutagen on the different explants
- To provide preliminary chromosomal data towards the species used as parents in hybridization, and to study the effect of hybridization and colchicine treatment on the chromosome counts of the progenies
- To study the RAPD polymorphism among the seedling progenies, hybrids, colchiploids and mutants

- To study the vegetative anatomy among different species of Vanilla and use it as a means to identify species and
- To investigate the anatomical features of the colchiploids / hybrids and to confirm polyploidy / hybridity of the plantlets developed.

The study was conducted with the objective of improving the gene pool of vanilla by generating variability and to evaluate the variants produced by using morphological, cytological, anatomical and molecular parameters. An attempt has also been made for the classification of different species in its vegetative condition based on anatomy. A review regarding the evolutionary trends in this genus was also undertaken.

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# Review of Literature

*The cultivated Tahitian Vanilla*

## Origin

*Vanilla planifolia* Andrews (syn *V. fragrans*) (Salisb.) (Ames) is native to the humid tropical rain forests of Southeastern Mexico, Central America, the West Indies and northern part of South America. The generic name *Vanilla* L. is derived from the Spanish *vainilla*, a diminutive of vaina, a pod; its specific epithet, *planifolia* refers to the broad, flat leaf of the plant (Swartz, 1800). Vanilla is the most important spice from the west. The cultivation of vanilla spread after the discovery of America by Columbus and now it accounts for about 0.75 per cent of the total world trade in spices. The major vanilla growing countries are Indonesia, Madagascar, Mexico, Tahiti, The Comoros and Reunion (Madhusoodanan *et al.*, 2003).

## Morphology

The members of the genus are stout, terrestrial climbing, branched herbs; branches emitting adventitious roots; leafy or leafless. Leaves when present coriaceous or fleshy. Recemes usually axillary, sub sessile or peduncled. Flowers large, sepals and petals sub equal, spreading. Lip adnate by a claw to the base of the column and embracing it in its concave limb, entire or 3-lobed. Column elongated; foot 0. Anther incumbent, cells separate; pollen granular. Capsule long fleshy, 1-celled (Fischer in Gamble,1928).

In *V.planifolia* the inflorescence is axillary, simple, only rarely branched, 5-8 cm long, up to 20-30 flowers, opening from base upwards, generally with only 3 open at a time, each lasting one day. The rachis is stout, often curved, and 4 -10 mm diameter. The bracts are rigid, concave, persistent, 5 -15 mm long.

Flowers are large, waxy, fragrant, pale greenish yellow 10cm diameter; pedicel very short. The interior cylindrical, tricarpellary ovary often curved, 4 -7cm long and 3 -5 mm diameter. Three oblong lanceolate sepals, obtuse to sub acute, slightly reflexed at the apex, 4-7 cm long. The two upper petals resembles sepals in shape, but slightly smaller. The lower petal is modified as a trumpet - shaped labellum or lip, which is shorter than the other perianth lobes and is 4-5 cm long and 1.5-3 cm broad. It is attached to the column which it envelops. The tip of the lip is obscurely 3-lobe and is irregularly toothed on its revolute margin. Stamen containing the pollen masses or pollinia covered by a cap, and below is the concave sticky stigma, which is separated from the stamen by the thin flap like rostellum (Purseglove, 1981).

**Habit and habitat.**

The *Orchidaceae* comprises a group of fast evolving angiosperm species (Dressler and Dodson, 1960) with a high incidence of gene exchange within and between genera through interspecific hybridization. The wide range of morphological variation found between orchid species is attributed to gene mutation; random genetic drift (Sanford, 1974) and adaptive changes in response to intense natural selection. The *Orchidaceae* with about 20,000 species classified into 750 genera, is one of the larger families among flowering plants, occurring widely in the tropical and temperate regions of the world. The Indo-Malaysian belt ranging from the Himalayas to New Guinea and Columbia in the South America is a major center of Orchid distribution. Based on phytogeographical evidences India was suggested as the primary or secondary centre of origin for orchids (Jain, 1986).

The *Orchidaceae* is an extremely variable group, as has been made obvious by emphasis of the tremendously high species number (Sanford, 1974). Variants resulting from plastic response of identical genotypes will not be of taxonomic significance. Genotypic variants arise by gene and chromosomal mutation and by recombination of genes, through cross over, and recombination of chromosomes through hybridization and introgression (Sanford, 1974).

Garay (1960) lists 11 pan tropical genera in Orchidaceae family. Of these *Vanilla* Mill. is normally epiphytic and considered primitive. Orchid stems are of two main types, sympodial and monopodial. The sympodial habit is ancestral and follows the general habit of many monocots (Holttum, 1955). The monopodial types are characterized by a uniform axis producing leaves and stem at the apex in indeterminate fashion; flowers produced axillary and roots produced at all nodes successively, and are never deciduous. There are monopodial types, however, that are "leafless", the buds being protected by minute scales. Vanilla although ostensibly fitting the description of a monopodial orchid, must be considered by a class by itself, with its vine habit (Withner, 1974).

Vanilla is the only orchid that behaves like a vine, and though its seedling stages may pass on the ground, it eventually achieves epiphytic status as the bases of the stems die and terminal growth and axillary branching occur (Withner *et al*, 1974). The epiphytic habitat appears to be equivalent to the disturbed habitat in that there are few competitors. And, in fact epiphytic orchids have been called pioneers (Pittendrigh, 1948) The success of the epiphytic evolutionary leap was in finding a habitat where competition was at a minimum. The preceding adoption of mycorrhiza, by means of which cellulose and other complex organic materials could be broken down and utilized, made life on the bark surface of trees possible.

Vanilla plants are represented in two growth forms: green vines with leaves and those without leaves, or with reduced leaves. Some species may produce nodal scales, which soon fall off, or more expanded leaves, which never the less are still caduceus. The vines produce two kinds of roots, short, unbranched, aerial ones (variously termed clasping and anchoring) which clasp the supporting structure and are usually limited in extension growth and long, branched, terrestrial or absorbing roots which penetrate the substratum and are presumably of unlimited extension growth. Both root forms originate at the nodes of the same plant, usually one root at each node (Stern and Judd, 1999).

In its original habitat, vanilla is seen wild as a climber. Vanilla requires warm and mist conditions of humid tropical and thrives well between 10° N and 20° S latitudes having a well distributed rainfall of 150-300 cm with a temperature range of 25-32° C and comes up well up to 1500 m above msl. It prefers land with gentle slope and light porous soil with adequate drainage. Forest soil rich in humus is ideal but the plants can thrive well in sandy loam to even laterite soils. A moderate slope and good drainage aids in its easy establishment. The crop requires a short dry spell for flowering. Water stagnation, strong wind and arid conditions are detrimental to this crop.

## History

Vanilla was found in 1520 by soldiers of the Spanish conquistador Heman Cortes, while on military reconnaissance in the moist, shady rain forests of southeastern Mexico. The Spanish were much impressed by vanilla and took it back to Spain where by the end of the 16th century factories were established to manufacture chocolate with vanilla flavoring. This use of vanilla spread to other parts of Europe, notably England and France. For more than 3 centuries after the time of Cortes, Mexico was the leading vanilla producing country of the world enjoying complete and lucrative monopoly (Rosengarten, 1972).

**Pollination**

Although vanilla plants grew well in the Old World tropics, fruits were not produced because of the absence of natural pollinators (Purseglove *et al.*, 1981). This baffling mystery was not solved until 1836 when the Belgian botanist Charles Morren found the answer by hand-pollinating the flowers (Rosengarten, 1972). In 1841 Edmond Albins, a former slave on the French Island of Reunion perfected a quick method of pollinating with the pointed tip of a small bamboo stick. This same method of artificial pollination of is still used commercially today (Corell, 1953)

The vanilla flower is so constructed that self-pollination of the individual flower is impossible, unless hand pollinated, due to the separation of the stamen from the stigma by the rostellum (Purseglove *et al.*, 1981). In *V.wightiana*, a wild species from Andhra Pradesh, natural fruit production is reported (Rao *et al.*, 1994).

The plants could not produce fruit naturally unless the Mexican and Central American pollinating insects were present. In the vanilla growing areas of Mexico the *Melipona* bees and certain species of humming birds, not present in tropical Asia, were found to be the pollinating agents (Rosengarten, 1972).

Orchid species have evolved through the development of pollinator specificity as opposed to the incompatibility mechanisms that are usual in other plants (van Steenis, 1969). Pollination is a pivotal process in the reproduction of flowering plants. It has led to more or less specialized interactions between flowers and their pollinators. This in turn has resulted in the huge diversity of flowers and consequently in the tremendous actual biodiversity of flowering plants. In *Vanilla planifolia* it is much cheaper to use the matching pollinator instead of the expensive “assisted pollination” (Westerkamp *et al.*, 2001).

Thus if the breeding barrier that segregates the population and so maintains the species, is removed by hand pollination, it is not surprising that seed is formed. The relevance of this to speciation and environmental adaptability under natural circumstances hinges on whether or not the barrier of pollinator specificity can be bridged in nature (Sanford, 1974).

The effect of pollen load on growth and development of vanilla pods was studied by Bhatt and Sudharshan (2000). Hand pollination was made using different quantities of pollen (12.5 to 100%) and the growth and development of fruits were studied at fortnightly intervals; the results showed that length of the bean was maximum when pollen load was 100 percent and such beans attained 17.18 cm length in 75 days as against 12.77 cm and 14.22 cm length in 12.5 and 25 percent pollen load, respectively. The results indicated that to get best growth of fruits, more than 50% of the pollen grains have to be transferred to the stigma.

**Distribution**

Vanilla is an interesting curious tropical genus of leafy or leafless climbing terrestrial shrub. It has about 110 species distributed in the tropics of both the Old and New World (Bouriquet, 1954). Purseglove (1981) describes the three species viz. *Vanilla* *planifolia* Andrews, *V.pompona* Schiede and *V. tahetensis* J.W Moore which are commercially exploited and cultivated through out the tropics.

Abraham and Vatsala (1976) reported only one species from Kerala - *V. wightiana* Lindl. In the IUCN Red Data book of Indian vascular flora Nayar & Sastry (1978) recorded *V. wightiana* as the only threatened species of this genus endemic to the Southern most part of the Western Ghats.

Hooker (1885) and Fischer (1928) reported two species from South India – *V.walkeriae* Wt. and *V. wightiana* Lindl. Karthikeyan *et al* (1989) in the Flora of India, reported five species from India -*V. andamanica* Rolfe, *V.parishii* Reichb., *V. pilifera* Holtt., *V.walkeriae* Wt. and *V. wightiana* Lindl. Amongst the five species reported from India, *V.pilifera* Holt. is endemic to Assam while, *V.andamanica* Rolfe.is reported only from Andaman Islands, *V.aphylla* Blume., previously known from Thailand, Laos, Vietnam, Malaya and Java is found occurring in Kerala and Tamilnadu, *V.walkeriae* Wight. from Travancore and Trivandrum and *V.wightiana* Lindl. from the states of Andhra Pradesh, Karnataka and Kerala (Kumar and Manilal, 1993).

Backer and Brink (1968) in the Flora of Java reported three species from Andaman Islands – *V.aphylla, V.albida* and *V.mexicana. V.andamanica* is known only from the Andaman group of islands. Seidenfaden (1978) believes this to be the same as *V. albida* Blume. Dassanayake and Fosberg (1972) in the Flora of Ceylon described only two species endemic to Sreelanka – *V.walkeriae* and *V.moonii.*

Moreover, *Vanilla planifolia* Andr. native to Mexico, the capsules of which yield the Vanilla of commerce, has been experimentally introduced into India more than a hundred Years ago (Anonymous, 1976).

**Floristic description**

The floristic description of the five Indian species is as given below:

- *Stem leafless:*

1. *V.walkeriae, Wight Ic. t.* 932. Stem very stout, flowers 2 in. long, sepals oblanceolate, petals broader spathulately obovate acute margins undulate, epichile of lip ovate, acute margins undulate, disc with two ridges below the middle.

Hook. F., *Fl. Brit. India* 6:90. 1890; Fischer in Gamble; *Fl. Presi. Madras:*1451 (1015), 1928.

Distribution: South India.

2. *V.wightiana, Lindl. In Wight Cat.* 2091; Stem green 1.5 cm across; leaves scale like, 1 cm long; roots fleshy at each node; inflorescence axillary, 2-3 cm long and 3-5 flowers per inflorescence; flowers 1in. long, sepals linear-oblong, petals lanceolate, epichile of lip ovate, its disc fringed with long hairs.

Hook. F., *Fl. Brit. India* 6:90. 1890; Fischer in Gamble; *Fl. Presi. Madras*:1451 (1015), 1928.

Distribution: Deccan peninsula.

3. *V.aphylla, Lindl.* Leaves reduced to green, narrowly triangular, acute, 3-1 cm long, deciduous scales. Racemes 3 flowered; flowers widely expanded, sepals and petals much recurved at the tip, linear lanceolate, light green; c. 2 ¾ cm long; lip at the base adnate to the base of the column into a wide, over 1 cm long tube, above it with a much intruded densely violet-hairy median fold, on either side of it with 3-4 red streaks c. 2 ½ cm. long and broad.

*V.aphylla Lindl., Gen. Sp. Orch.* 436, 1840; Wt. Ic. t. 932, 1844-45. Backer and Bakhuizen (1968) in the *Fl. Java* (vol. III).

Distribution: South India.

- *Stem leafy*

4. *V. andamanica, Rolfe.* Base of the inner side of the lip and of the anterior side of the column glabrous, racemes c. 3 cm long, 6-12 flowered, bracts c. 3 mm sepals and petals light yellowish green, with a light yellow tip, claw of the lip on the inner side dark violet, ventricose in the anterior part; lamina pale margined, on the inner side at the base of the longitudinal thickening with a batch of reverse fimbriately incised curved, thin transversal lamellar lip in total 4 ¾ cm by 3 ¾ cm; rostellum very large and white.

*V. andamanica Rolfe. In Kew Bull.* 1918: 237.

Distribution: Andaman and Nicobar Islands.

5. *V. pilifera, Holtt.* Leaves subsessile, oblong to elliptic; leaf size 3-7 x 1-2.5; leaf apex acute to shortly acuminate.

*V. pilifera Holtt. In Gard. Bull. Sing.* 13; 251, 1951; Bothakur and Hajra in *Bull. Bot. Surv. India* 18: 228. (1976) 1979.

Distribution: N-E India.

**Systematic studies**

Vanilla belongs to the family *Orchidaceae*, an advanced group of monocotyledons. The family is the largest one of the flowering plants with about 700 genera and 20,000 species. One hundred and ten species of vanilla are reported (Purseglove *et al.*, 1981) consisting of terrestrial, climbing, epiphytic and saprophytic species. Apart from *V. planifolia* Andrews, two other cultivated species are *V. pompona*

Schiede (West Indian vanilla) and *V. tahitensis* J. W. Moore (Tahitian vanilla). The basic chromosome number for the genus vanilla is x = 16 and *V. planifolia* is a diploid with 2n = 32. In countries where vanilla has been introduced, variability is likely to be highly limited. The material is propagated vegetatively, hence of clonal origin.

Few systematic studies of the *Orchidaceae* have employed molecular techniques and only one (Chase *et.al.*, 1994) has addressed higher order relationships of this large and floristically important family. Floral characters, especially those relating to anther configuration and pollinarium structure, have been the primary basis for classification of orchids (Dodson, 1962; Romero, 1990). These floral features are hypothesized to be especially prone to selective pressure from pollinators and, hence, are likely to display high levels of convergence or parallelism (Dodson, 1962; Atwood, 1986).

The most recent treatment of Orchidaceae is that of Dressler (1993). This system originated 40 years ago (Dressler and Dodson, 1960) and has been altered and modified periodically by Dressler as basic knowledge of orchid morphology, anatomy, and genetics has expanded (Dressler, 1979, 1981, 1986, 1993). In the large intrafamilial analysis, tribe *Vanilleae sensu* Dressler (1990), together with Pogoniinae, appear as sister to the remainder of the monandrous orchids. Sequence divergence within the monophyletic subtribe *Vanilleae* is extraordinarily high, and consists of strongly supported monophyletic genus *Vanilla.* A cladistic parsimony analysis of rbcL nucleotide sequence data from 171 taxa of *Orchidaceae* revealed that *Vanilleae* are clearly an isolated group of monandrous orchids, and suggested the elevation of the vanilloid orchids to subfamilial status (Kenneth *et al.*, 1999). Lindley (1835) was the first to recognize the uniqueness of the vanilloid orchids and actually proposed a distinct family for them (*Vanillaceae*). Classification of orchids based on embryogeny was attempted by Schlechter (1926). According to this system, vanilla comes under the tribe *Polychondreae*.

### Production and trade

In certain crops, the wide gap between production and demand in the international market can be exploited with our favorable agro-climate. Vanilla is one of such item highly suited for cultivation in Kerala, Karnataka and Tamilnadu. The annual world production of vanilla is estimated to be 3000-4000 metric tones against the world demand of 32,000 metric tones (Peter *et al.*, 2004).

### World Scenario

The total area under vanilla cultivation in the world during 2002 was 38066 hectares with the production of around 4956 metric tones (FAO Rome). The major vanilla producing countries are Madagascar, Comoro, Indonesia, Mexico and Reunion (Table: 1)

As on today around 16-20 countries are producing vanilla in the world. Among these countries, Madagascar holds the prominent position having a cultivated area of 25900 hectares. Of late, Indonesia has enhanced production level to meet growing global demand and reduced supply from Madagascar.

### Shift in Production base

Madagascar used to meet 70 per cent of the world supply during 1970's. Now the position has changed and Madagascar stands next to Indonesia in its production. Indonesia was an insignificant producer with about 24 Mt production during late 1940's. Later it increased the production and become the world's second largest producer during

1980's. Production went up in subsequent years and it stands number one in production figures, now it is having a production of 1800 Mt during 2002 (FAO, Rome, 2003). These two countries together contribute about 76 per cent of the world production. The center of maximum world production of vanilla is getting shifted in course of time, from the native Mexico it got shifted to Sub-Saharan Africa during 1950's and from they're to countries in pacific basin during late nineties. Unstable production and a very small number of countries that enjoy large market power characterize the supply side.

**Table 1: Country wise production of vanilla**

(Metric Tonnes)

| Country | **1998** | **1999** | **2000** | **2001** | **2002** | **Average** |
|---|---|---|---|---|---|---|
| **Madagascar** | 1650 | 1650 | 1815 | 1815 | 1518 | 1690 |
| **Indonesia** | 1900 | 2102 | 2102 | 2102 | 1800 | 2001 |
| **India*** |  | 10 | NA | 60 | 92 | 54 |
| **Mexico** | 564 | 300 | 550 | 550 | 300 | 453 |
| **The Comoros** | 160 | 150 | 180 | 180 | 140 | 162 |
| **Reunion** | 30 | 30 | 28 | 28 | 35 | 30 |
| **Tonga** | 100 | 50 | 130 | 130 | 130 | 108 |
| **French Polynesia** | 30 | 34 | 40 | 40 | 35 | 36 |
| **Guadeloupe** | 8 | 40 | 8 | 8 | 8 | 14 |
| **Zimbabwe** | 10 | 10 | 10 | 10 | 10 | 10 |
| **China** | 550 | NA | NA | NA | 650 | 600 |
| **Kenya** | 10 | NA | NA | NA | 8 | 9 |
| **Malawi** | 22 | NA | NA | NA | 20 | 21 |
| **Uganda** | 60 | NA | NA | NA | 40 | 50 |
| **Turkey** | 100 | NA | NA | NA | 170 | 135 |
| **Cook Islands** | **2** | NA | NA | NA |  | **2** |
| **World** | **5196** | **4376** | **4863** | **4923** | **4956** | **4863** |

Analysis of time series data on country wise production indicates that no country could maintain its hold on vanilla production for as long period. Leadership in production went from Mexico to Madagascar and there to Indonesia in 1990's. Now there is a decreasing trend in Indonesia's production for the last few years, mainly due to unforeseen agro-climatological factors (Madan *et al.*, 2004).

### Supply, Demand gap analysis

Increasing demand for vanilla has resulted in increased production of low cost substitutes, which have taken over the use of vanilla beans, the source of natural vanillin. It is estimated that the world production of synthetic vanillin, which was 12000 tonnes in 1988, has grew up to 16000 tonnes in 1991 and the present production and use is estimated to be 30000 tonnes.

### Vanillin derivatives

Vanilla flavor is an important food additive and the present world wide annual demand exceeds 30 metric tones. Biosynthesis of natural vanillin and other related compounds follow the phenyl propanoid pathway. All the enzyme system required for the biosynthesis of natural flavors from precursor is present in cultured cells. These cells could function as biocatalysts if cultures are provided with the precursors.

Plant tissue cultures are potentially valuable for studying the biosynthesis of secondary metabolites. In view of the current consumer demand for natural foods and plant-derived medications, *in vitro* production of valuable secondary products has become an industrially promising venture. The production of flavor compounds with cultured plant cells is based upon their unique biochemical and genetic capacity, and the totipotency of plant cells (Schleiden, 1838; Schwan, 1939). The potential advantages of plant tissue culture systems include; independence from environmental factors, geographical indications, uniformity and controlled production in response to demand.

The synthetic vanillin currently in wide spread use is in the process of loosing demand as it contains only a single compound made from plant lignin (Nabard,1998). The natural vanilla flavor is far superior due to the presence of pmore than 130 organoleptic compounds.

The possibility of a bioprocess for the production of natural vanillin from a ferulic acid precursor with aerial roots as the biocatalysts and charcoal as a product reservoir has been attempted by Westcott *et al.*, (1994).

## IN VITRO TECHNOLOGY

### Embryo Culture

The purpose of embryo culture, in most applications, is to recover plants (embryos) during attempts at wide hybridization by sexual crosses between distantly related plants (Williams *et al.,* 1982). By 'rescuing' an embryo and growing it into an appropriate medium, a plant can be grown to maturity (Collins and Grosser, 1984). The primary reason for attempting wide crosses is the transfer of desired traits (*e g.* disease resistence, stress tolerence) from distantly related species to cultivated varieties.

In vanilla, the aseptic method ensures nearly cent percent germination of seeds and has largely been used for breeding fusarium root rot resistant varieties (Knudson, 1950; Withner, 1955). However, seeds rarely germinate under natural conditions, apparently because of the hard integument, or possibly because of other factors like inadequate, reservoir of food for the development and growth of the young plant until it is capable of manufacturing its own food (Childers and Ciber,1948).

## Somatic embryogenesis

*In vitro* propagation is normally based on the stimulation of multiple-shoot growth from cultured shoot-tip and nodal explants (Murashige, 1974; Brown and Thorpe, 1995). It has not always been possible to adapt this approach to certain plant taxa like orchids where the proliferation rate is too low (Litz and Gray, 1995). One of the most important prerequisites for genetic manipulation of plants *in vitro* has been the ability to grow somatic cells in sterile plant growth medium and to regenerate plants from these cultures (Litz and Gray, 1995). The regeneration pathways of plants from somatic cell cultures have been defined as either organogenesis (Christianson, 1987) or somatic embryogenesis (Ammirato, 1985, 1987). Somatic embryogenesis refers to the development of embryo like structures from cells of the somatic (non-sexual) origin. It is sometimes regarded as an advanced micro propagation technique but there are several advantages of recovery of plants from cells via somatic embryogenesis compared with micro propagation (Smith and Drew, 1990).

Protocorm like bodies (PLBs), which appear on the vegetative bud in culture, are adventive embryos analogous to the gametic embryos. There were morphological and physiological similarities between the protocorm like bodies and the protocorms of orchids (Huang *et al,* 1990). Orchid PLBs, which originates from vegetative tissues, were considered to be true somatic embryos by Moral (1974). Vegetative bud of an adult plant rejuvenates to from PLB, which has close similarities with the protocorms produced by seed germination. Ironically, the earliest commercialization of micropropagation involved the mass propagation of orchids, which involved the large-scale production of protocorms

(Rao 1977). According to Peterson (1975), bud regeneration in the region of root apical meristem could be considered a relatively rare process in higher plants.

In the orchid family, the direct conversion of root tip into PLBs *in vitro* was described in *Catasetum* (Kerbauy, 1984a) and *Cyrtopodium* (Sanchez, 1988). Techniques for the rapid mass propagation of orchids via PLB is reported in orchids like *Geodorum densiflorum* (Biswajit and Datta, 2000), *Dendrobium* (Yu *et al.*, 2001), Vanda (Munu *et al.*, 2000), *Oncidium* (Chen and Chang, 2000), *Cattleya intermedia* (Mello-e-silva *et al.*, 2000), *Ipsea malabarica* (Gangaprasad, 1999), *Pongonia japonica* (Takahashi and Kondo, 1998), *Catasetum fibriatum* (Kerbauy and Colli, 1997), *Cymbidium* (Kirdmanee *et al.*, 1992).

PLB formation may occur from yellow white embryonic calli in *Oncidium* (Chen and Chang, 2000), friable calli in *Phalaenopsis*, *Doritaenopsis* and *Neofinetia* (Islam and Ichihashi, 1999), root apex conversion to PLB as in *Catasetum fimbriatum* (Kerbauy and Colli, 1997), rhizome derived PLB (Takahashi and Kondo, 1998), or from nodal explants (Vij *et al.*, 1994).

PLB iduction is promoted by various media additives like peptone (Chen *et al.*, 2000, Biswajit *et al*, 2000), casein acid hydrolysate (Gangaprasad and Decruse, 1999), maltose and sorbitol (Islam and Ichihashi, 1999), potato juice (Kimura and Kurihara, 1991), p-coumaric acid (Colli and Kerbauy, 1993), peptone and tryptone (Amaki and Higuchi, 1989). Kerbauy (1993a) studied the effect of nitrogen sources, auxins and cytokinins on PLB induction and suggested that NAA and ammonium ions were the most effective substances to overcome the intrinsically low rate of regeneration of the root tip protocorm like bodies. Relatively low concentrations of sucrose and agar favored the formation of PLB (Kerbauy, 1993b). Park *et al.* (1996) investigated the multiplication of PLB of *Phalaenopsis* in liquid culture and concluded that Vacin and Went (VW) liquid medium was the most suitable for PLB multiplication.

Begum *et al.* (1994) did histological studies on the developing protocorm like bodies of *Cymbidium* and traced the PLB producing initials to a group of cells found just below the surface layer of epidermis. The histological details of the development of protocorms and buds as observed in culture are described by Kerbauy and Estelita (1996).

The effect of colchicine on the PLBs of *Cattleya intermedia* was studied by Mello-e-Silva *et al.* (2000).They could recover mixoploids and tetraploids from the treatment.

### Micro propagation

Traditionally vanilla is propagated from cuttings of mature vines. This method is not economical since the collection of stem cuttings leads to arrest of growth and developments of the mother plant (Ayyappan, 1990). Vanilla, being a monopodial orchid, yields only a small number of planting material (Tessy, 1995). More over the market demand for the propagules is hardly met with such cuttings. As the growers are looking for alternate sources, micro propagated plantlets serve the purpose and are popularly used (Geetha and Shetty, 2000).

Micropropagation of vanilla using stem nodes for large-scale multiplication was standardized by Nirmal Babu *et al* (1997). Protocols on micro propagation of vanilla have been reported using nodal explants (Kononowicz and Janick, 1984; George and Revishankar, 1997; Geetha and Shetty, 2000), and aerial root tips (Philip and Nainar, 1986) and through the culture of callus masses (Gu *et.al.* 1987; Davidonis and Knorr, 1991). Callus formation is good in MS medium containing, in addition to NAA and BA, 5 mg thiamine, 500 mg casein hydrolysate and 1000 mg inositol / litre. Geetha and Shetty (2000) observed that the axillary proliferation was initiated only when giving a longitudinal bisection injures the shoot tip. This might be due to the strong apical dominance exerted by the shoot tip meristem with the consequent inhibition of axillary buds, as has been previously reported by Lakshmanan *et. al.* (1997). Seed culture of *V. planifolia* in different combinations of MS media was standardized by Minoo (2002). Mary *et. al.*, (1999) in her experiments on *in vitro* seed culture of *V. planifolia* found that best germination was observed on half-strength MS medium supplemented with NAA and BAP.

Mass propagation for commercial cultivation requires a simple, economical, rapidly multiplying and highly reproductive protocol (Geetha and Shetty, 2000). High rate of multiplication of *Vanilla planifolia* clonal propagules was obtained from axillary bud explants using semi-solid medium (MS supplemented with BA 2 mg / l and NAA 1 mg / l) successively (George and Revishankar, 1997). Study undertaken to determine the efficiency of *in vitro* axillary bud cultures of *V. planifolia* proved that the growth of newly formed shoots was more vigorous on MS medium comprising of NAA and Kinetin as growth hormones (Rao *et. al.* 1993).

Attempts have been made to propagate vanilla from parts of the plant other than the shoot apex (Philip and Nainar, 1986, 1988; Philip and Jose, 1989). Since the constituent cells of the root apical meristems are genetically stable, less differentiated and permits plant regeneration in high frequency; they form an ideal material for the long-term preservation of germplasm. An endogenous level of auxin in the explant is important in determining the course of development of the root meristem *in vitro*. The organogenisis of young roots requires a relatively low concentration of IAA, (preferably between 1-5 mg / l) supplied from the medium. Scanning the root tip extracts for IAA using UV, TLC, GLC and GC-MS showed higher levels of auxin in old aerial roots and also in young cultured root tips in which the root meristem had transformed to shoots (Philip and Jose, 1989).

George *et. al.* (1995) studied the long-term storage and subsequent regeneration of multiple shoot buds of vanilla. His experiments showed that among the various treatments used, those encapsulated in calcium alginate and incubated in distilled water at 25°C was most suitable for long-term storage.

Use of organic substances like d-biotin & calcium pantothenate has been reported to enhance multiplication in vanilla (Rao, *et al,* 1993). Presence of d-biotin & folic acid in the multiplication medium enhanced continued proliferation & elongation of axillary shoots (Geetha and Shetty, 2000).

Silver nitrate, an inhibitor of ethylene activity, has been reported to be responsible for inducing positive response not only on shoot initiation and growth but also increased root number length of *V. planifolia* when used in micro molar concentrations (Giridhar *et al,* 2001). Generally, silver nitrate inhibits ethylene action through the Ag2+ ions, by reducing the receptor capacity to bind ethylene (Yang, 1985). The investigation by Giridhar *et al* (2001) is of importance in demonstrating efficient multiplication of shoots along with root growth under the influence of silver nitrate during *in vitro* propagation of *V. planifolia.* Ganesh *et al.* (1996) investigated the effects of culture media type and BAP, GA or $AgNo_3$ on shoot proliferation *in vitro* of *V. planifolia;* good shoot proliferation was observed only in the presence of BAP.

Agrawal *et al.* (1992) devised a method of clonal propagation for *V. walkeriae* which is restricted to the tropical forests of Tamil Nadu and Kerala.

A commercially viable protocol for mass propagation of *V. tahetensis*, a cultivated species of vanilla was standardized by Mary *et. al* (2000). Among the various growth regulators tested, MS with BAP at 1 mg / l and NAA at 0.1 mg / l proved most suitable for efficient multiplication. In trials on the micropropagation of *V. tahetensis* and *V. planifolia,* it was found that explants obtained from the middle part of the donor plants exhibited the best growth in terms of length and no. of nodes, although explants from the base and tip produced more shoots (Pett *et al.*, 1997).

## INDUCTION OF VARIATION

### Seed culture

The vanilla bean contains thousands of seeds. Seeds are very minute black and globose in shape, about 0.3 mm in diameter.

Bernard (1909) discovered that fungi are important for the germination of orchid seeds. The symbiotic relationship between fungus and a root is known as a mycorrhiza. The fungal hyphae penetrate the protocorms and the roots of the plant and are 'digested' possibly making nutrients and other materials. Germinating or juvenile orchids are heterotrophic, reliant on fungi for food supply (Pierik, 1982)

Special conditions are necessary for the growth and development of orchid seeds and plantlets were obtained when sowing of seeds took place at the foot of the mother plant. Knowing the rudimentary state of minute orchid embryos, one can better understand that an exterior agent usually a *Rhyzoctonia*, can be useful in helping them through their first stages of development (Bernard, 1904). The orchid- fungal relationship although very broadly "symbiotic" may vary a great deal, as has been carefully reviewed by Arditti (1967). In nature this developmental stage involves a change over from almost completely mycorrhyzal nutrition to a partially auto tropic stages.

There are two different types of host cells in orchids – sub epidermal host cells, in which the fungus remain 'healthy' and more deeply lying host cells in which the fungus is 'digested' into an amorphous mass (Pierik, 1982). Once the fungal hyphae pierce through the epidermal cells of an orchid root, there is further penetration through passage cells of the exodermis. The passage cells in the vanilla root are found to be thin – walled and more active metabolically than the other cells of the exodermis (Alconero, 1969).

Alconero (1969) working with *Rhizoctonia solani* and vanilla found that a separation into a fungal – host cell layer and a digestion layer was not evident. Hyphal digestion occurred in cells scattered throughout the root, but it most frequently occurred in the peripheral cells.

Knudson (1922) showed that germination is possible in simple nutrient medium in the absence of fungus. After the juvenile phase, the plant becomes photosynthetic.

Mature seeds of tropical vanilla species and hybrids survive at least three years on synthetic media, germination occurring only where appropriate high temperature and low light levels are present (Knudson, 1950; Lugo, 1955) although these physical requirements are not present in immature seeds of vanilla (Withner, 1955). Immediate or delayed germination under laboratory conditions does not necessarily reflect the timing under natural conditions since we usually do not know what dormancy- breaking factors may be operative in the field (Warren, 1974).

Among Orchidaceae, subtribes *Vanillinae* and *Galeolinae* are found to possess most bizarre and diverse seeds. These are ovoid with a sclerotic, multilayered outer integument in vanilla (Cameron and Chase, 1998). The only orchid species producing sclerotic seed coats belong to the genera *Selenipedium, Vanilla, Galeola* and *Epistephium* (Garay, 1960; Beer, 1863). Of these species *Vanilla* (Knudson, 1950; Lugo, 1955; Withner, 1955) and Galeola has been experimentally germinated and the seed coats have not been strong barriers to germination (Warren, 1974).

Light inhibits the initial germination stages, but this mechanism is not operative once the protocorm is developing (Harvais and Hadley, 1967). Knudson (1950) working with vanilla was successful in growing seedlings when his cultures were kept in the dark during the first months of development. In vanilla, Withner (1955) found that the aseptic method ensures a nearly a 100 percent germination of the seeds and has used largely for breeding varieties resistant to fusarium root rot (Knudson, 1950; Withner, 1955; Childers and Cibes, 1948).

Withner (1955) demonstrated the beneficial effect of arginine (also lysine) on *Vanilla* seedlings. The nutrient requirements may change rapidly during the autotropic stages of germination and the relatively high sugar and mineral content of standard media may be toxic to such seedlings. Developing seedlings transferred to Knudson C containing only 10-25 % of the normal mineral and carbohydrate supply survive longer and death is delayed. Hardly 1-2 % of orchid seeds germinate under natural conditions even after an extensive dormancy period (Philip and Nainar, 1988). Bouriquest and Boiteau (1937) were the first to germinate vanilla seeds.

Growth curves to show the approximate growth of orchid seed pods in general (Duncan and Curtis, 1942) and vanilla in particular (Withner, 1955) illustrates the increments with the internal events of fertilization and ovule formation. Withner (1955) in his study of embryo development observed that seed from any particular pod was not homogeneous in its growth characteristics; natural variation existed with in the individuals. He traced the reason for this variation as the pollen grains with different potentialities and the time it took to fertilize the ovules at the base of the pod. There is considerable delay before fertilization of the ovules takes place after pollination, and after that, the limited development of the vanilla seed takes place. Seed maturity, precedes the pod maturity by a fairly wide margin (Withner, 1955).

The surface sterilization step for the aseptic inoculation of vanilla seeds is done by treating the pods with surface sterilents (Philip and Nainar, 1988) or dipping the pod in alcohol and flaming (Minoo *et al,* 1997); and then taking out the seeds by splitting apart the pods. Alternatively, Tonnier (1952) used a procedure of directly treating the seeds with disinfectants and surface sterilants for more than one hour. The impermeability and hardness of the sclerotic seed coats of vanilla (Garay, 1960) is arrested by this treatment, as the seeds still showed germination. At the same time, the procedure enabled him to get rid of the oleoresins gummy placental debris, which made planting of these seeds so difficult, and which possibly prevented their germination.

Withner (1955) observed that vanilla seeds which were collected from pods about nine months old; *i e.,* in its full maturity failed to germinate. This germination inhibition may be due to the vanillate compounds in the placental tissues and glandular hairs. As the seeds and pods mature, these materials form in increasing amounts, in sufficient quantity to reduce germination by chemical means (Withner, 1955). Embryo culture is an indispensable procedure when the endosperm is defective (Jensen, 1976). This can be successfully utilized in hybridization programme for rescuing embryos of artificial hybrid, thereby creating improved variant lines (Madhusoodanan *et al.*, 2003).

### ***Embryo development.***

The first important works related to orchid embryology were due to the Dutch Scientist Treub, in 1879, whose work offered a suggestive glimpse of the appearance, and some times the segmentation, of the embryo in diverse orchid group.

When the pod is ready to dehisce, the internal integument of the ovule as well as the deepest layers of the external integument is generally found to be degenerated. The cuticle of the epidermis of the inner integument of the ovule persists, and this seems to have the effect of impeding the hydration of the seeds and thus hindering their germination *in vitro* (Veyret, 1969).

The ovules are deprived directly of any vascular system, which would be unusual of other flowering plants. This fact made Withner *et al* (1974) to postulate that the orchid seeds are so minute because they were never afforded an ample food supply via developed vascular traces.

The embryo in the absence of *Rhyzoctonia* may be in limited contact with the seed coat or may be more or less isolated in the center according to the importance of the development of the external integument in the course of the embryogenesis. The cells of the coat are dead, empty and very thick in *Vanilleae*. The orchid embryo has developed in a sac without benefit of endosperm, and this has generally been interpreted as the cause of the rudimentary state of the embryo. The endosperm does not ordinarily form in the orchids, either from a lack of fusion of the second sperm nucleus with the endosperm nuclei, or from an immediate degeneracy of the nucleus of the endosperm if the double fertilization does takes place. Among a few species, however, the segmentation of the nucleus of the endosperm does take place, but it never leads to the production of a normal endosperm (Veyret, 1974). The ovule of *V. planifolia* is an example where the endosperm forms twelve nuclei (Swamy, 1947).

Polyembryony is more frequent in *Orchidaceae*, and has been reviewed by Wirth and Withner (1959). The orchid embryo at the shedding stage has been described as a mass of 10-100 similar undifferentiated cells, with organogenisis being initiated only after the seeds have been shed and brought under favorable conditions of germination (Philip and Nainar, 1988).

The germination of orchid seeds in general (Bernard, 1889) and vanilla in particular (Philip and Nainar, 1988) follows a common pattern: formation of protocorm, which is covered with rhizoids on about two – thirds of its basal part, lack of formation of the radicle; generally late development of a first root, while several leaves begin developing from the apex. Among the *Polychondreae*, the tribe to which vanilla belongs (Schlechter, 1926), the young protocorm is generally elongated and much less thick than in the other taxa and its tip is often curved (Bouriquet, 1947). During seedling development of vanilla, it was observed that the morphology of the first pair of leaf was comparable to those at the nodes of the adult vanilla vines (Philip and Nainar, 1988). Among the vanillas (Bouriquet, 1947; Knudson, 1950; Tonnier, 1952; Philip and Nainar, 1988), the root meristem originates as a belated structure, only after a minimum number of leaves have formed. The *in vitro* studies suggest that the development of roots in vanilla seedlings may require a higher level of endogenous auxin at the organogenitic center of the meristem (Philip and Nainar, 1988).

The first root is originated exogenously (Bouriquet, 1947) or endogenously (Philip and Nainar, 1988) while the subsequent ones originate more superficially. Further more the formation of a pair of roots was accompanied by the necrosis of the parenchymatous tissue at the basal end, which terminated the protocorm stage and resulted in the establishment of the seedling (Philip and Nainar, 1988).

Starch grains already present in the embryos of vanilla show an increased level of accumulation in the parenchymatous basal region of the protocorm compared to the mature embryo (Philip and Nainar, 1988). Accumulation of starch has been suggested to be a readily available source of energy for the organogenitic process and for the building up of wall materials in tissue cultures (Thorpe, 1977, 1980; Thorpe and Murashige, 1968, 1970).

In vanilla, the abundance of protein bodies in the embryo and their break down from the basal parenchymatous region during protocorm formation indicate that stored protein in the embryo is mobilized and utilized.

Vanilla is the only plant, which disturb the general pattern of embryology in the tribe *Polychondreae* to which it belongs. The specific chain of events of the embryo leading to seedling formation has no parallel amongst other reported studies in angiosperms (Philip and Nainar, 1988).

**Interspecific hybridization**

The transfer of genes from one genome to another is of great importance in many breeding projects, especially where only a few genes or a few traits are desired from one species. The remarkable ease of orchid hybridization even at the genus level might have as reasons (1) the huge number of ovules and pollen tubes present in each flower; (2) the rapidity of orchid evolution, which has resulted in the formation of a number of species not yet diverged far enough to be incompatible; (3) faulty definition of species and genera, which has resulted in a situation where so-called hybridization is not hybridization at all; or that orchids are 'just different' (Wallbrunn, 1969).

All the cultivated orchids, being complex hybrids, are just heterozygous, and there is very little hope of breeding pure lines out of them in the near future. Orchidaceae are well known as a family in which wide crosses are possible; interspecific and intergeneric hybrids are the basis for a thriving commercial market. This reputation is based on the great ease and frequency of artificial crosses, but because of mechanical barriers and pollinator specificity, no parallel exists in nature. If one examines orchid floras, such as that of North America (Luer, 1975), documented hybrids at either the generic or specific level are not particularly frequent. No data exists to substantiate the claim that natural hybrids are more frequent in *Orchidaceae* than in other families. Hybridization is unlikely to be a factor at higher taxonomic categories because natural hybridization occurs only between closely related species (Kenneth *et al.*, 1999).

Minoo (2002) reports interspecific hybridization between *V.planifolia* and V. *aphylla* and the characterization of the progenies using morphological and molecular markers. Natural hybridization between *V. claviculata* and *V. barbellata* is detailed by Nielsen (2000) and is supported by genetic, morphological and pollination experimental data. Electrophoresis with seven polymorphic enzymes supported the finding of Nielson and Siegismund (1999) about the chance of natural hybridization in localities where *V. claviculata* and *V. barbellata* coexists.

### *Hybridization and embryo culture*

The purpose of embryo culture, in most applications, is to recover plants (embryos) during attempts at wide hybridizations by sexual crosses between distantly related plants (Williams *et al*, 1982) by rescuing an embryo and growing it on an appropriate medium, a plant can be grown to maturity (Collins and Grosser, 1984). The primary reason for attempting wide crosses is the transfer of desired traits (*e.g.* disease-resistance, stress tolerance) from distantly related species to cultivated varieties.

Embryo culture is also used to break dormancy in seeds, thereby, shortening the breeding cycle by months or even years. It can also be used when important seed lots have lost viability during storage and have poor germination. (Biggs *et al.*, 1986).

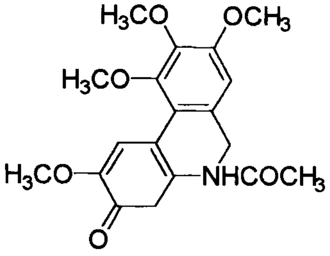
### In vitro polyploidy

Kihara and Ono (1926) introduced the terms auto- and allopolyploids. They state that doubling of the same chromosome set leads to autoploids, where as alloploidy is the result of interspecific hybridization followed by chromosome doubling. Most natural polyploids are not truly auto or allo but range in genome constitution from true autoploids to true alloploids. This situation has been described by the term segmental allopolyploidy (Stebbins, 1950), which may occur to different degrees between homoeologous chromosomes apart from homologous pairing.

Gene exchange between species in nature is restricted or completely absent. Useful genes derived from related species are predominantly those for resistance to diseases and insects, but adaptability, quality traits and yield also could be improved by including wild species and primitive forms in breeding.

Very early in the history of polyploidy breeding, Leven *et al.*,(1945) concluded that crops most amenable to improvement through chromosome doubling should have perineal habit and vegetative reproduction, having a bearing on the success of polyploidy breeding by reducing the crop's dependence on seed production. In angiosperms, polyploidy aids in the success of apomixes by increasing the size of the gene pool, while hybridization increases its diversity. Polyploidy seems to occur in about 60% of the species of *Orchidaceae*. Climatic vicissitudes have placed greater environmental pressure on plants so that adaptive tolerances, which may accompany polyploidy (Barber, 1970), have been strongly selected for in certain regions. Polyploidy was long believed to originate in nature mainly from spontaneous somatic doubling of the chromosome number in zygotes or meristems in response to stress conditions (Hermsen, 1984). Polyploids are frequently desired for genetic studies and other purposes in connection with many crop plants, since naturally evolved colchicine strains are usually unavailable, it is necessary to produce them through some artificial process. Generally, the first step in their production is to induce the desired genetic modification in diploid plants and most commonly, this is accomplished by treatment with colchicine.

Colchicine is an alkaloid derived from *Colchicum autumnale*, the autumn crocus, more specifically from the crocus bulb.



**Colchicine**

Colchicine was first used in cytogenetic studies during the 1950's. Colchicine binds to the primary molecules of the cell spindle, tubulin. Once inactivated by the binding of colchicine, tubulin cannot assemble into microtubules and therefore can no longer function as a control mechanism for chromosomes with the net result that nuclear division is halted. Colchicine can be introduced into cell cultures and then washed out after an appropriate period. The net result of this is to produce colchicine cells, which can be a valuable tool in investigation of cell division. The induction of polyploidy is less dependent on the genotype of the material, in contrast to the induction of mutations (Clark and Wall, 1996).

When somatic chromosomes are to be doubled for experimental as well as breeding purposes, there are a few conventional methods of colchicine treatment. These methods involve the treatment of either seeds (Frandsen, 1967; Hermsen and Deboer, 1971) or axillary buds (Ross *et. al.*, 1967) or protocorm like bodies (Mello - e - Silva, 2000) or callus tissue (Nagatomi *et al.*, 1998).

*Callus as explants for colchicine treatment:*

Polyploidy induction by colchicine treatment is reported in shoot apex derived calli in garlic (Jang *et al.*, 2000), leaf derived calli in *Lycium colchic* (Wang *et al.*, 1998), stem derived calli in potato (Maine and Simpson, 1999) and banana (Nagatomi *et al.*, 1998), floral petal derived calli in pineapple (Nagatomi *et al.*, 1997), ovule derived callus in *Gerbera jasmesonii* (Misoshi and Asakura, 1996), cotyledon and stem derived calli in *Cucumis sativus* (Zhang *et al.*, 1995), thin sections of calli in *Lycium barbarum* (Li-Jian *et al.*, 1999) and regenerating callus from F1 hybrid of *Allium fistulosum* x *A. cepa* (Song-ping *et al.*, 1997). Production of doubled haploids in Asiatic hybrid lily 'Connecticut king' was attained with treatment of haploid callus with colchicine (Han-dong, 1999). It was shown that the addition of colchicine retarded callus differentiation (Zhang *et al.*, 1995).

*Seed as explants for colchicine treatment:*

Chromosome doubling via colchicine treatment of seed explants was attempted in *Sesamum indicum* (Zhang *et al.*, 2001), grass pea (Dibyendu *et al*, 2001), and ryegrass (Pasakinskiene, 2000). Mehra *et al.* (1999) studied the effect of EMS and colchicine alone and in combination on the seeds of chilli and found that combined treatments yielded higher numbers of morphological and quantitative mutations than individual treatments. Pasakinskiene (2000) could attain a polyploid production rate of 65.8% by using a high concentration of sucrose (100g/l) and vacuum (0.14 kg/cm $^2$) to infiltrate colchicine solution in germinating embryos of ryegrass. Levites *et al.* (2000) suggested that colchicine treatment of the apozygotic seeds increases the proportion of the cells with high level of chromosome endo reduplication resulting in embryogenesis through sporophytic agamospermy. Increased guard-cell size is a consistent indicator of a doubling of the chromosome number (Watrous and Wimber, 1988).

*Hybrids for colchicine treatment:*

Polyploidization of interspecific hybrids proved the most practical in breeding work (Mar'-yakhina and Polumordvinova, 1989). Chromosome doubling utilizing colchicine was carried out on several sterile interspecific hybrids (Gangadevi *et al.*, 1988). Pollen fertility was restored by inducing tetraploidy through colchicine treatment of the F1 hybrids (Tuyl *et al.*, 1989). Ishikawa *et al.* (1999) produced colchicine-induced amphidiploids (2n=32) by treating the ovules from the interspecific hybrids *of Alstroemeria ligtu* (2n=16) and *A. pelegrina* var. *rosea* (2n=16). This colchicine induced amphidiploids showed larger flowers than the hybrids and grew more vigorously. Mousset *et al* (1989) used *in vitro* chromosome doubling method to solve sterility problems of interspecific hybrids in *Trifolium.*

Singsit and Ozias (1992) used chloroplast number in guard cells and pollen grain size to distinguish different levels of ploids from each other produced by the interspecific crosses of *Arachis*. Colchicine treatment of the vegetative axillary meristems of clover hybrids (*Trifolium* species) resulted in chromosome doubling among the different genotypes (Anderson *et al.*, 1991). Griesbach (1990) used chromosome doubling by colchicine to produce fertile hybrid between *Anigozanthos humilis* and *A. flavidus*. Pollen fertility of the *Lilium longiflorum* cv. *Mont Blanc* was restored by inducing tetraploidy through colchicine treatment (Tuyl *et al*, 1989).

**In vitro mutagenesis**

The extreme dispersibility of orchids has placed them in such a variety of habitats that mutations not only would be selected for through great variety of environmental pressures but also often could be isolated easily from the parent forms and so preserved. This would superficially give the appearance of a higher mutation rate (Sanford, 1974).

Mutation breeding is one of the methods available to the plant breeders when the crop is amenable to vegetative propagation. The generally high degree of heterozygosity, which causes a complex inheritance of genetic factors as well as a frequent polyploidy, both serious handicaps in the conventional methods of breeding, are advantageous in mutation breeding, as large variations can often be observed in the mutated populations. The most promising aspect of mutation induction in the vegetatively propagated plants, compared to the cross breeding methods is the ability to change only a very few characters of an otherwise good cultivar without altering significantly the remaining and often unique genotype (Broertjes, 1977).

Induced mutations have played a significant role in the development of many crop varieties and are instrumental in enhancing genetic variability (Micke, 1988). Induced mutation serves as an important tool for creating usable genetic variability in crop plants and significant achievements in crop improvement have been made through the mutation approach. It also serves as a supplement to conventional breeding programmes to improve one or two specific characters in a well-adapted and acceptable elite cultivar (Sarma *et. al.*, 2001).

Mutation breeding is used to increase the variability in those crops where the existing variability is limited. Induced mutation breeding is attempted on account of the narrow genetic base of the crop (Sareen and Koul, 1999; Lal *et al*, 1999). The main advantage of mutation breeding in vegetatively propagated plants is the possibility of changing 1-2 traits in and otherwise outstanding variety without altering the rest of the genotype (Przybyla, 1994). The progressive development and adoption of tissue culture and mutation induction techniques in support of traditional cross breeding programs has proved to be successful in generating new improved varieties (Morpurgo *et al,* 1997). The major shortcomings of mutation breeding are its random nature and tendency to produce somatic mutation thus leading to chimeras (Geier, 1989). Tissue culture increases the efficiency of mutagenic treatments for variation induction, handling of large populations, and use of ready selection methods and rapid cloning of selected variants. Molecular techniques can provide a better understanding of the potential and limitations of mutation breeding. e.g., molecular marker assisted selection, which can lead to early identification of useful variants (Predieri, 2002).

The use of chemicals for inducing mutation began since 1960 (Heslot, 1964) following the introduction of ethyl methane sulphonate (EMS). According to Heslot (1977), EMS is the most efficient member of alkylating agents. Alkylating agents react with DNA by alkylating the phosphate group as well as the purine and pyrimidine bases. Krishnaswamy (1968) observed that the chemical mutagens like EMS are capable of causing functional alterations in the genes in polyploidy plants. The treatment with chemical mutagen should be long enough to permit hydration through infusion (Konzak *et al.,* 1965). Amirov (1974), Dryagina and Limberger (1974) claimed that chemical mutagens had a higher efficiency and output of mutations, if the duration of the treatment and the concentrations were well adjusted.

In vegetatively propagated species, colchicine combined with *in vitro* culture techniques may be the only method to improve existing cultivars. Tissue culture and colchicine were applied to increase variation in sugar cane by N- nitroso-N- methyl urea (MNH) treatment of callus culture and subsequent *in vitro* development of plants (Khan *et.al.,* 1999). Swaminathan (1965) observed that alkylating agents are more efficient than radiations for inducing point mutations; but less efficient for inducing chromosome aberrations. Ethyl methane sulphonate has been successfully used in vegetatively propagated apple (Broertjes and Van Harten, 1978).

Various physical mutagens, such as X- rays, gamma rays, beta rays, neutrons, lasers, electron beams and ion beams, have been used in mutation breeding either individually or in combination with chemical mutagens, such as EMS, DES, $NaN_3$ etc. Not only the genotype of the initial plant material but also the treatment techniques play important roles in mutation induction. On the other hand, the role the genetic background of the initial materials plays in mutation induction has been studied in depth (Wen and Qu, 1996).

Experimental mutation is an integrated tool in genetics and plant breeding. Haploid cells provide ideal targets for mutation selection and transformation. Screening for both dominant and recessive mutations in the first generation after mutagenic treatment and lack of chimeras are the most important advantages of applying mutation and selection in the haploid system (Laib *et. al.*, 1996).

Induced mutations have been used effectively for identifying genes having a major phenotypic affecting certain pathways. Most mutagen induced DNA changes are genetic lesions. In most cases one has to assume that the damaged gene can no longer properly code for the original or a related enzyme and has therefore lost its function (non-sense mutation). A mutation leading to a non-functioning gene can be classified as recessive, although in most cases the term 'deleted' would be more appropriate. A dominant or a homozygous - recessive mutational lesion in an essential gene could not easily be tolerated by the plant and would probably have a lethal effect, unless the plant's genome contains intact copies of the affected gene elsewhere. Thus, it seems that the colchicine has only little chances to create usable genetic variation in essential genes (Micke, 1996).

The application of mutation breeding method was aimed at identification of mutants which posses positive agro-morphological and quality characteristics (Kwon ndung and Ifenkuri, 2000). Increasing radiation dose led to an increase in the percentage of cells with chromosome aberrations in *Trichosanthes anguina* (Dutta, 1995). There was an inverse relationship between germination percentage and mutagen concentration / dose (Buddnnyi and Naumov, 1994).

Mutation breeding have found to be significant in raising *Fusarium* tolerant lines in banana (Bhagwat and Duncan, 1998), *Phytopthora* tolerant lines in tomato (Yudhvir Singh, 1995) and sesame (Pathirana, 1992), *Alternaria* resistence in mustard (Rajbir *et al.*, 2001), *Sclerotium* resistence in garlic (Al-safedi *et al.*, 2000); induced dwarf mutants were identified in celery (Choudhary and Kaul, 1993), yam bean (Nair and Abraham, 1990), *Triticale* (Reddy and Gupta, 1988) and apple (Paprstein, 1988). Chlorophyll mutations were obtained in ground nut (Perumal *et al.*, 1999), *Trigonella* (Raisinghani and Mahna, 1996) and chrysanthemum (Banerji *et al.*, 1996).

In the case of *in vitro* mutagenesis of Musa species, Domingues *et al.*, (1994) reported that four *in vitro* subcultures is the minimum number of vegetative generations needed prior to screening for mutants.

RAPD analysis of induced mutants in groundnut was done by Anjali *et al.*, (1997); the analysis revealed characteristic band differences among the mutants and the parent.

The effect of EMS on plant regeneration and variations were studied using callus cultures in finger millet (Pius *et. al.*, 1994); sugar cane (Gahukar and Jambhale, 2000); rape (Zhao-Yun *et al.*, 1996); phragmites (Chen *et al.*, 1994); citrus (Deng *et al.*, 1989); *Glycyrrhiza glabra* (Tailang *et al.*, 1997). Cytological studies of mutagen treated calluses of *Phragmites* revealed that the variant was mixoploid with a chromosome number ranging from 33 to 100 (Chen *et al.*, 1994). Salt tolerant lines of citrus were recovered by treating habituated callus cells with EMS (Deng *et al.*, 1989); fusarium toxin resistant lines could be selected by treating wheat calli with crude toxin extract of *Fusarium graminaearum* (Li and Hang, 1992).

## CHARACTERIZATION OF VARIATION

### Anatomical characterization

Although the genus vanilla is easy to recognize, identification at the species level is difficult for a number of reasons including the rarity of finding plants in flower, the difficulty of drying specimens and the length and time required by plants in cultivation before they flower (Christenson, 1995). An anatomical study on the stems of *V. planifolia* and *V. siamensis* was made by Zhao and Wei (1999) and the results showed that the arrangement and structure of the stem tissues of these two species were different.

Two growth forms represent vanilla plants: green vines with leaves and those without leaves, or with reduced leaves. Some species may produce nodal scales which soon fall off or more expanded leaves which nevertheless are still caduceus .The vines produce two kinds of roots, short unbranched, aerial ones which clasp the supporting structure and are usually limited in extension growth and long, branched, terrestrial or absorbing roots which penetrate the substratum and are presumably of unlimited extension growth. Both root forms originate at the nodes of the same plant, usually one root at each node.

Hafliger (1901) studied *V.phalaenopsis* Rchb. F., and *V.planifolia* a leafy species. Roux's anatomical study (Roux, 1954) involved *Vanilla fragrans* (Salisb.) Ames (a synonym of *V.planifolia*) and *V. pompona*, both leafy species. Roux based the study on Pompilian's (1881) classification where by stems were organized into two large groups, those which fibrous sheath separating cortex and ground tissue and those lacking this sheath. In the latter case cortical cells merged directly with cells of the ground tissue. In Roux's (1954) general description of stem, however, both leafy species *Vanilla fragrans* and *V. pompona* had the sclerenchymatous sheath and there were not much difference between the two species. Epidermal cells were polygonal and bore tetragonal crystals of calcium oxalate, except for subsidiary cells. Leaves were hypostomatous and stomata superficial. Sub epidermal cells were of small volume and mesophyll cells increased in size between the epidermises toward the middle of the leaf where they were the largest. Mesophyll was undifferentiated and 18 to 26 cells deep. The outer parts of the mesophyll have long sacs filled with mucilage and raphides of calcium oxalate permeating the outer parts of the mesophyll. A sclerenchymatous ring of lignified fibers with large lumina surrounded each. Xylem was uppermost phloem lowermost. Vessels possessed annular, spiral and reticulate wall thickenings (Roux, 1954).

Heckel (1899) concluded that in stems of leafy vanillas a sclerified endodermis was present and that this layer was absent from leafless vanillas. The pith cells in leafy plants were provided with bands (Heckel, 1899). Holm (1915) did not agree that the fibrous sheath in stems constituted an endodermis. In *V.planifolia* there was no principal difference in anatomical or morphological structure between short roots (aerial) and long (terrestrial) roots. The main distinction depended upon the limited growth and rapid maturity of the 'root tendrils' compared with the unlimited growth of the long roots (Neubauer, 1961). In the root cortex of *V.planifolia* there is direct continuity between raphide cell protoplasts. This is due to disintrigation of transverse walls of contiguous raphide-bearing cells (Mollenhauer and Larson, 1966). The raphide-bearing cells may loose their end walls forming unbranched tubes that 'weave among the cortical parenchyma in roots. There were cortical lysigenous lacunae of varying sizes opposite phloem and endodermal cell wall thickenings were heaviest in aerial roots (Alconcero, 1968). For roots of *V.planifolia* the transverse cell walls between crystals idioblasts were thin and attenuated progressively during idioblast maturation so that cortical syncytia developed via schizo-lysigenous breakdown of the cell walls (Kausch and Horner, 1983).

The leaves of *V.planifolia* were hypostomatous with crystals in all epidermal cells, except subsidiary and guard cells. The stomatal apparatus were largely tetracytic with some anomocytic (Nayar *et al.,* 1976).

The evolutionary leap of the ancient, earth-growing orchid ancestors on to the trees where competition was minimum, made life possible because of the micorrhiza, by means of which cellulose and other complex organic materials could be broken down and utilized. The water conservation problem of the epiphytic orchids was accomplished by the water conserving modifications which were selected for, and this includes the xerophytic modifications of the present day epiphytes like thick epidermis, heavy cuticle and sunken stomata (Sanford, 1974).

## Cytological characterization

Somatic chromosome numbers and basic numbers show wide variation in the *Orchidaceae*, as was indicated by Duncan (1959). The basic chromosome number for the genus *vanilla* is X = 16 and *Vanilla planifolia* is a diploid with 2n = 32 (Eftimiu-Heim, 1950; Hoffmann, 1929; Martin, 1963). However, aneuploids having chromosome number 28-31 are reported (Hurel Py, 1938). The somatic chromosome number of the other two cultivated species, viz, *V. tahetensis* and *V. pompona* is also 2n = 32 (Eftimiu-Heim, 1950). Out of the 15 species of Vanilla studied so far (Table 2), twelve have 32 somatic chromosomes; one species- *V.roscheri* has 36 chromosomes and two species- *V.haapape* and *V.aphylla* has 2n=64.

**Table 2: Chromosome list of the different species of Vanilla**

| **Name of the species** | **Meristem chromosome no:** | **Reference** |
|---|---|---|
| *V.aphylla* | 64 | Vatsala,1964 |
| *V.aromatica* | 32 | Eftimiu-Heim, 1950 |
| *V.barbellata* | 32 | Martin, 1963 |
| *V.dilloniana* | 32 | Martin, 1963 |
| *V.fragrans* | 30-32 | Eftimiu-Heim, 1950 |
| *V.haapape* | 64 | Tonnier, 1951 |
| *V.hartii* | 32 | Eftimiu-Heim, 1950 |
| *V.imperialis* | 32 | Eftimiu-Heim, 1950 |
| *V.moonii* | 32 | Eftimiu-Heim, 1950 |
| *V.papeno* | 32 | Eftimiu-Heim, 1950 |
| *V.phaeantha* | 32 | Martin, 1963 |
| *V.planifolia* | 32 | Eftimiu-Heim, 1950; Hoffmann, 1929 |
|  | 28-32 | Hurel Py, 1938 |
| *V.pompona* | 32 | Eftimiu-Heim, 1950; Martin, 1963 |
| *V.roscheri* | 36 | Krupko *et al*, 1954 |
| *V.thaitii* | 32 | Eftimiu-Heim, 1950 |

Ravindran (1979) reported abnormalities in pollen grain mitosis and a pollen sterility percentage of about 65% in *Vanilla planifolia*. Nair and Ravindran (1994) reported somatic associations of non-homologous chromosomes in the root tip cells undergoing mitosis.

In the orchids, basic numbers are relatively high when compared with most other flowering plants. It has been suggested, therefore, that the orchids are ancient polyploids, but during the course of evolution there has been loss or gain of individual chromosomes coupled with structural alteration of sets, which has led to their now being effectively diploid (Keith, 1974). Thus, basic numbers are not only useful in providing an indication of existence of polyploidy; indeed, they are more valuable in suggesting evolutionary relationships and taxonomic classifications.

### *Somatic association*

It has been recognized by some investigators that homologous chromosomes in somatic cells lay closer together than would be expected by random placement. This phenomenon known as somatic association, has been observed in the root tip cells of wheat (Feldmann *et.al.*, 1966), oats (Sadasivaiah *et al.*, 1969; Thomas, 1973), and barley (Fedak and Helgason, 1970). Sticky chromosomes were first described in the 1930s in maize, the term referring to the sticky appearance of chromosomes in cells involved by a mutation provoked by a recessive gene. Many other reports have been published since then concerning different plant species in which stickiness was attributed to genetic (Rao *et al.*, 1990, Zanella *et al*, 1991) or environmental factors (Ericksson, 1968; Schwartz, 1958).

Somatic association of homologous chromosomes has been reported in various plants (Metz, 1916; Brown and Stack, 1968; Mc Guire, 1992). Brown and Stack (1968) examined *Haplopappus gracilis* and *Rhoeo discolour* and found somatic association not only in somatic cells but also during premeiotic pairing in the sporogenous cells. Mc Guire (1992) showed that homologous chromosomes appear to be closely paired side by side at the metaphase in sporogenous tissue of maize anthers. The chromosomes in the premeiotic cell manifest itself as chromocenters (prochromosomes), a term coined by Hyde (1953) for a condensed heterochromatic region situated on both sides of each centromere. Premeiotic pairing is used to refer to all homologous chromosome pairing which occurs in cells destined to undergo meiosis (Stack and Brown, 1969). Sadasivaiah *et al.*, (1969) have reported that homologous chromosomes are not distributed at random in root tip cells of *Avena sativa*.

Chromosome stickiness, caused by the intense chromosome clustering during any phase of the cell cycle, may be caused by genetic or environmental factors. Several external agents have been reported to cause sticky chromosomes. Among them are X-rays (Steffensen, 1955), gamma rays (Al Achkar *et al,* 1989), temperature (Ericksson, 1968) and herbicides (Badr and Ibrahim, 1987).

Association of somatic chromosomes has been reported in root tip cells of *V.planifolia*. Association of morphologically dissimilar chromosomes is an indication of the existence of homoeologous pairing and the somatic association in vanilla may be due to the attraction between similar heterochromatic regions (Nair and Ravindran, 1994). This may lead to variation in chromosome number and subsequent variation in morphological and reproductive features of this crop. Thus, somatic pairing is considered as a possible source of variability in vanilla.

**Molecular characterization**

The use of DNA based markers has ushered in a new era of technological achievements. Based on the specific requirement, different types of marker systems detecting polymorphisms / variability in different regions of DNA evolving at different rates have been used. For assessing clonal fidelity in tissue cultured plants, DNA based markers mostly in use include Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Variable Number of Tandom Repeats (VNTR) or Simple Sequence Repeats (SSR) or microsatellites etc. Among these, the Polymerase Chain Reaction (PCR) based Random Amplified Polymorphic DNA method has the advantages such as ease of use, low cost, accessibility by non-specialists, potential for automation, requirement of extremely small amount of DNA, which can be of low quality, and there is no need for DNA blotting and the use of radioactivity.

***Molecular basis of RAPD polymorphisms***

The modification of the basic PCR technique which allows RAPDs to be generated is remarkably simple instead of using a pair of carefully designed and fairly long oligonucleotide primers to amplify a specific target sequence, a single short oligonucleotide primer which binds to many different loci is used to amplify random sequences from a complex DNA template such as a plant genome. Theoretically, the number of amplified fragments generated by PCR depends on the length of the primer and the size of the target genome and is based on the probability that a given DNA sequence (complimentary to that of the primer) will occur in the genome on opposite DNA strands in opposite orientation within a distance that is readily amplifiable by PCR. For most plants, primers that are 9-10 nucleotides longer predicted to generate on an average 2-10 amplification products. The primers are generally of random sequences biased to contain at least 50% Cytosine (C) and Guanine (G) and to lack internal inverted repeats. The products are easily separated by standard electrophoretic techniques and visualized by ultraviolet illumination of ethidium bromide stained gels. Polymorphism result from changes in either the sequence of the primer binding side (*e.g.* Point mutations), which prevent stable association with the primer or from changes, which alter size or prevent successful amplification of a target DNA (*e.g.* insertions, deletions, inversions). As a rule, size variants are only rarely detected and the individual amplification products represent one allele per locus in inheritance studies the amplification products are transmitted as dominant markers.

It is necessary to identify RAPD primers that identify polymorphisms between the parents of the mapping populations being analyzed. Large numbers of primers (several hundred to a few thousand) are usually screened, until a satisfactory number of polymorphisms are found. This number will vary with the intended application. Typically, 10mers are used for RAPD mapping experiments, and collections of suitable short oligonucleotides are available commercially (for example, from Operon Technologies). Finally, primers identified as producing polymorphisms are used for amplifications from all individuals in the population of interest.

### ***RAPDs –A breif history***

Two groups developed RAPD assay like the classical Hardy-Weinberg model of population genetics, independently and simultaneously during the year 1989-1990. Williams *et al.* (1990) described the use of single short oligonucleotide primers of arbitrary sequence for the amplification of rapidly distributed segments of genomic DNA.

The RAPD and AP-PCR techniques are based on the amplification of the DNA segments between pairs of small inverted DNA sequences scattered throughout the genome and provide an innovative technology for DNA mapping fingerprinting and related research (Waugh and Powell, 1992). This advance has resulted in a DNA marker technology that of the DNA can be readily employed because of the wide availability of synthetic oligonucleotides. RAPD polymorphisms result from mutations or rearrangements at or between oligonucleotide primer binding sites in a genome. These polymorphisms can be analyzed on either agarose or acrylamide gels, and manifest themselves in the presence or absence of an amplification products. RAPDs are visualized as dominant markers. They require extremely small amounts of genomic DNA, which can be of low quality but also eliminate the need of DNA blotting and the use of radioactivity.

### ***Various applications of RAPD polymorphism***

Differentiation of identical germplasm accessions by a combination of molecular and morphologic analysis by Waycott and Fort in 1994. In 1980, Bolstein and colleagues constructed a genetic map in man using Restriction Fragment Length Polymorphism (RFLP). High resolution DNA amplification fingerprinting – A strategy for genome analysis was done by Cactans *et al* (1991). Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations was reported by Giovannoni *et al.* in 1991.

In 1990, Williams *et al*, found that DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Sosinski and Douches (1996) reported using polymerase chain reaction based DNA amplification to fingerprint North American potato cultivars.

Genetic relationships and origins of six grape cultivars of *Galicia* using RAPD markers were done by Vidal and colleagues in 1999. Use of RAPDs for identification of rice accessions was reported by Shuichi *et al.* (1992). Molecular characterization of *Piper nigrum* L using RAPD markers was succeeded by Pradeep Kumar and colleagues (2001).

Phenotypic variation of the mutants of mulberry *(M. alba)* diploid varieties were analyzed at the molecular level using random amplified polymorphic DNA (RAPD) (Jiao *et al*, 2001). Randomly amplified polymorphic DNA (RAPD) and sequence characterized amplified region (SCAR) methods were used to tag the white rot resistant genes in 17 individuals of F1 progeny between Tangwei (*Vitis davidii*, white rot-resistant) and Ugni Blanc (*V. vinifera*, white rot-sensitive) and this proved that among the 155 random primers that gave distinct band patterns, one RAPD marker (OPP09-760) was tightly linked to a major gene resistant to *Coniothyrium diplodiella* (Xu-Yan *et al*, 2003).

Zhuang (2003) studied the genetic relationship in cultivated cucumber and its wild relative (*C. hystrix*) and melon cultivars using RAPD markers. The results from the UPGMA cluster analysis proved that the 23 cultigens could be classified into four groups.

The potential of randomly amplified polymorphic DNA (RAPD) markers in varietal identification and genetic purity test of hybrid varieties of *Capsicum annuum* was evaluated by Ilbi (2003). It was concluded that RAPD markers might be useful for cultivar identification and hybrid purity test in *Capsicum annuum*, especially for routine seed quality control programme.

Somatic hybrids between the cultivated potato diploid hybrid clone, ZEL-1136, and hexaploid non-tuber-bearing wild species *Solanum nigrum* L. exhibiting resistance to *Phytophthora infestans* were regenerated after PEG-mediated fusion of mesophyll protoplasts. The RAPD analysis of nuclear DNA confirmed the hybrid nature of 29 clones (Szczerbakowa *et al,* 2003). Matsumoto *et al* (2002) conducted somatic hybridization between triploid and diploid bananas by using protoplast electrofusion and nurse-culture techniques and identified the hybrids by using random amplified polymorphic DNA (RAPD) markers.

The importance of RAPD technique in parentage, cultivar and germplasm identification of three tea hybrids using random amplified polymorphic DNA (RAPD) technique was confirmed by Luo (2002) by identifying parental RAPD bands in 94.90, 97.92 and 98.64% of the progenies.

Generation of hybrids from crosses of Mexican diploid 2x (1EBN) species x 2x (2EBN) *Solanum tuberosum* haploids via embryo rescue and double pollination were conducted and the hybridity was confirmed through morphology, chromosome number and RAPD analysis. The results suggested the transfer of valuable characters from *S. pinnatisectum* to *S. tuberosum.* (Ramon and Hanneman, 2002)

Randomly amplified polymorphic DNA (RAPD) analysis was carried out to develop sufficient numbers of PCR-based genetic markers in cultivated and wild species of *Allium* Species-specific RAPD markers proved the hybrid origin of the plant (Shigyo *et al,* 2002).

Chen *et al* (2002) constructed a genetic linkage map of *B. oleracea* based on the segregation of 96 RAPD polymorphic loci in one F2 population of *B. oleracea* var. capitata x *B. oleracea* var. alboglabra. The genetic markers defined nine linkage groups, covering 555.7 recombination units. DNA from an F1 hybrid *Brassica oleracea* var. italica and its parental lines was subjected to RAPD-PCR analysis, which produced suitable male and female specific markers. The study clearly demonstrates that RAPD-PCR is a useful tool for genetic purity testing of commercial F1-hybrid broccoli seeds.

Randomly amplified polymorphic DNA (RAPD) markers were used to verify hybridity in the genus Clethra and to compare hybrids to their parents. In all cases, the hybrids had more RAPD markers in common with *C. alnifolia* than with their other parent (Reed *et al.*, 2002).

Random amplified polymorphic DNA markers were used to confirm the mutability of gladiolus *cv*. Eurovision mutants. Out of seven random primers used for PCR amplification, one primer OPX 02 (5'-TTCCGCCACC-3') produced polymorphic banding pattern (Pathania and Misra, 2001). Nabulsi *et al* (2001) used random amplified polymorphic DNA (RAPD) analysis to evaluate genetic diversity among eight garlic mutants resistant to white rot disease. Moreover, the results indicated that the banding patterns produced by primer OPB-15 (GGAGGGTGTT) with highly resistant mutants may by used as genetic markers for early selection of resistant plants.

Low-density RAPD markers of sweet potatoes were constructed from 76 pseudo testcross progenies by Ukoskit and Thompson (1997). The type of polyploidy in *I. batatas* was investigated using the ratio of non-simplex to simplex RAPD markers and the ratio of simplex RAPD marker pairs linked in repulsion to coupling and this suggested autopolyploidy. The level of selfing was evaluated in eight Australian sugarcane crosses using RAPD markers and selfed progeny were identified in each cross on the basis of absence of male-specific RAPD bands (McIntyre and Jackson, 2001). RAPD markers were used to calculate similarity values for *Prunus domestica* progenies (Heinkel *et al*, 1999).

# Studies on Genetic Variability in Open Pollinated Progenies of Vanilla.

Thesis submitted to
**University of Calicut**
for the award of **Doctor of Philosophy** in Botany

By
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**2005**

# Materials and Methods

*Endemic to Andaman group of islands*

The present study was carried out at the Indian Institute of Spices Research, Calicut during the period from 2000 – 2004.

The studies were conducted with the following two broad objectives:

1. **Induction of variations in open pollinated progenies of vanilla and,**
2. **Characterization of variations by using different markers.**

## IN VITRO CULTURE

### *Objective 1 -To find out the effect of age of fruit on seed germination in vitro*

In order to shorten the reproductive cycle and to speed up the breeding procedures, a method for finding out the earliest time to rescue and grow *V. planifolia* seeds *in vitro* has been optimized.

Selected inflorescences of 4-year-old field grown plants of *V. planifolia* were utilized in this experiment. The flowers were hand pollinated between 9 am and 11 am and the date of pollination was recorded along with the ovary size. Fruits were collected starting from the 2nd day after pollination. The length and girth of the pod was measured at the time or harvest and the embryos from each pod were cultured separately in bottles of 5.5 cm. diameter with 100 ml. of medium. The medium used was MS (1962) supplemented with 1 mg $l^{-1}$ BA, 0.5 mg $l^{-1}$ NAA, 2 g $l^{-1}$ tryptone and 2 % sucrose and adjusted the pH to 5.8 and solidified with 7 g $l^{-1}$ agar. Plating on the medium was done uniformly, and the bottles were scored for germination percentage. The cultured ovules were maintained in an incubator for one week in the dark (25 $\pm$ 2 °C) and after wards transferred to light under 8 hr. photoperiod. Scoring for germination was done by marking 1 $cm^2$ of the agar plate after maintaining 10 replicates in each and approximate percentage of germination was estimated.

***Objective: 2 - to standardize suitable culture media for seed culture of Vanilla***

Plants were selected and inflorescences from the plants were labeled. The flowers were hand pollinated on the day of flower opening. The pods were harvested starting from the third month of pollination and washed in tap water, dipped in 70% ethyl alcohol for two minutes for surface sterilization. The ovary wall was split open, and then ovules with placenta were excised and transferred to culture medium in 500 ml flasks or bottles of 5.5 cm. diameter with 100 ml. of medium. The medium combinations tried are given below:

1. MS (hormone free)
2. MS + BA 0.5 mg/l
3. MS + BA 1 mg/l + NAA 0.5 mg/l
4. MS + BA 1 mg/l + IBA 0.5 mg/l
5. MS + BA 1 mg/l + 2, 4 D 0.5 mg/l
6. MS + BA 1 mg/l + IBA 0.5 mg/l + 2ip 2g/l
7. MS + BA 1 mg/l + IBA 0.5 mg/l + peptone 2g/l
8. MS + BA 1 mg/l + IBA 0.5 mg/l + tryptone 2g/l
9. MS + BA 1 mg/l + IBA 0.5 mg/l + casein hydrolysate 2g/l

In all the combinations 2 % sucrose was used, pH was adjusted to 5.8, solidified with 7 g $l^{-1}$ agar and autoclaved at 120 ° C for 20 minutes. The cultures were first kept in dark at 25 °C and after one week transferred to a culture room under an 8 hr. light period of 2000 lux. The responses of the cultures were carefully monitored. Plantlets derived from ovule culture was transferred to culture tubes and numbered separately each of them being considered as an accession. Sub culturing was carried in the same media combinations.

## INDUCTION OF VARIATION

a. Development of segregating seedling progenies,

b. Interspecific hybridization,

c. *In vitro* polyploidy, and

d. *In vitro* mutation.

### Development of segregating seedling progenies

In the first set of experiment, vanilla pods of different ages were used for ovule culture in order to determine the feasibility of ovule cultures in the different media combinations and to select out an optimal media combination for the further studies. The second set of experiment was planed to find out the minimum time required to rescue and grow the *Vanilla planifolia* embryo *in vitro* so that this knowledge could be further used in the hybridization experiments where there is more chance of embryo abortion.

### *Culture medium*

MS (Murashige and Skooge, 1962) basal medium, the most extensively used medium for plant tissue culture was used in the present study. MS medium in full strength was used in all tissue culture experiments (Table-3). The chemicals used for micro and macronutrients were obtained from 'Hi-Media', Bombay, while the vitamins and growth regulators were from 'Sigma', USA.

## Table 3: Composition of Murashige and Skoog* basal medium

| **Composition** | | **Concentration ( mg $l^{-1}$ )** |
|---|---|---|
| **Macronutrients** | | |
| Ammonium nitrate | $NH_4NO_3$ | 1650.00 |
| Potassium nitrate | $KNO_3$ | 1900.00 |
| Calcium chloride | $CaCl_2 . 2H_2O$ | 440.00 |
| Potassium orthophosphate | $KH_2PO_4$ | 170.00 |
| Magnesium Sulphate | $MgSO_4 .7H_2O$ | 370.00 |
| **Micronutrients** | | |
| Sodium EDTA | $Na_2EDTA$ | 37.30 |
| Ferrous Sulphate | $FeSO_4 . 7H_2O$ | 27.80 |
| Boric acid | $H_3BO_3$ | 6.20 |
| Manganese Sulphate | $MnSO_4 .4H_2O$ | 22.30 |
| Potassium iodide | KI | 0.83 |
| Zinc Sulphate | $ZnSO_4 . 7H_2O$ | 8.60 |
| Sodium molybdate | $Na_2MoO_4 . 2H_2O$ | 0.25 |
| Copper Sulphate | $CuSO_4 . 5H_2O$ | 0.025 |
| Cobalt chloride | $CoCl_2 . 6H_2O$ | 0.025 |
| **Vitamins** | | |
| Myo-inositol | $C_6H_{12}0_6$ | 100.00 |
| Thiamine HCl | $C_{12}H_{17}ClN_4OS$. HCl | 0.10 |
| Nicotinic acid | $C_6H_5NO_2$ | 0.50 |
| Pyridoxine HCl | $C_8H_{11}NO_3$. HCl | 0.50 |
| **Amino acid** | | |
| Glycine | $C_2H_5NO_2$ | 2.00 |

Murashige and Skoog, 1962.

### *Carbon source*

Sucrose, (Qualigens, Bombay) was used as the carbon source at the concentration of 30g/L. in the experiments.

### *Growth regulators*

Auxins: Three major auxins namely, $\alpha$ naphthalene acetic acid (NAA), indole buteric acid (IBA) and 2, 4 dichlorophenoxy acetic acid (2, 4-D) were used in this study at various concentrations.

Cytokinins: Two cytokinins namely, 6 Benzyl amino purine (BAP) and Thidiazuron (TDZ) were used.

### *Gelling agent*

For solidifying the culture medium, Qualigens bacteriological grade agar agar was used at the concentration of 7g/L.

### *Glassware*

500 ml flasks and bottles were used for seed culture initiation and Borosil culture tubes for multiplication.

### *Sterilization of culture medium*

The medium and glass wares were sterilized by autoclaving at $121^0$ C for 20 minutes at 16 psi.

### *Plugging the culture vessel*

The culture vessels, tubes, bottles and flasks were closed with cotton plugs made of non-absorbent cotton, covered with cheesecloth, aluminium foil or polypropylene caps.

### *Incubation conditions*

The cultures were incubated at 22 $^0$ C and were given a photoperiod of 8 hours with a light intensity of 2000 lux, provided by 'Philips' cool white fluorescent tubes.

### *Distilled water*

Double glass-distilled water was used for preparation of stocks, media, buffers etc.

### *Instruments*

Inoculations under aseptic conditions were done in 'Klenzaids' horizontal laminar flow. Nat Steel horizontal autoclave was used for sterilizing the culture media, stock solutions and other instruments like blades, forceps, needles etc.

### *Preparation of nutrient medium*

MS (Murashige and Skooge, 1962) medium was used as nutrient medium. Separate stocks were prepared for macro nutrients, micro nutrients, vitamins and aminoacids. Stocks of calcium chloride, ferrous sulphate, $Na_2$ EDTA and glycine were prepared separately (Table-4).

## Table-4: Details of various stock solutions for MS medium.

| Stock | Composition | Stock strength | Quality / L medium |
|---|---|---|---|
| A | **Macronutrients** | x 20 | 50 ml |
| | $NH_4NO_3$ | | |
| | $KNO_3$ | | |
| | $CaCl_2.\ 2H_2O$* | | |
| | $KH_2PO_4$ | | |
| | $MgSO_4.\ 7H_2O$ | | |
| B | **Micronutrients** | x 100 | 10 ml |
| | $H_3BO_3$ | | |
| | $MnSO_4.\ 4H_2O$ | | |
| | KI | | |
| | $ZnSO_4.\ 7H_2O$ | | |
| | $Na_2MoO_4.\ 2H_2O$ | | |
| | $CuSO_4.\ 5H_2O$* | | |
| | $CoCl_2.\ 6H_2O$* | | |
| C | **Micronutrients** | x 100 | 10 ml |
| | $Na_2EDTA$* | | |
| | $FeSO_4.\ 7H_2O$* | | |
| D | **Vitamins** | x 100 | 10 ml |
| | Thiamine HCl | | |
| | Nicotinic acid | | |
| | Pyridoxine HCl | | |
| E | **Amino acid** | x 100 | 10 ml |
| | Glycine | | |
| F | Myo-inositol | x 100 | 10 ml |

Separate stocks prepared for each of the growth regulators used. Sucrose and agar agar, at concentration of 20 g/L and 7 g/L respectively were added directly to the culture medium. PH was adjusted before adding agar. The agar was melted to ensure uniform distribution in the medium. The media was autoclaved at $121^0$ C at 16 psi for 20 minutes.

### *Growth regulators*

For ovule culture, NAA / IBA and BAP were used at concentrations of 0.5 mg/L and 1mg/L respectively. For callus induction 2, 4-D in combination with BAP / TDZ was used at various concentrations and for PLB induction BAP was used in combination with NAA / IBA / 2, 4 D.

### *Incubation*

Cultures once initiated were placed in incubators or growth rooms where lighting, temperature and humidity can be controlled. The rate of growth of a culture will depend on the temperature (and sometimes the lighting) regime adopted. The cultures were incubated at 22 $^0$ C and were given a photoperiod of 8 hours.

### Hardening:

The rooted plants (10 –15 cm. long) were washed under running water to remove traces of agar and transferred to plastic cups containing sand. The cups were kept covered with polythene bags and kept in a shade house. The plants were watered once a week. The polythene cover was removed after one month and the plants were transplanted to polythene bags containing sterile potting mixture.

The selfed progenies of the other two species, viz, *V. tahetensis* and *V. aphylla* were raised by the same method. The morphological, cytological and molecular characters of the seedling progenies of *V.planifolia* were examined.

**Interspecific hybridization**

***Objective:***

Interspecific hybridization between cultivated vanilla (*V. planifolia)* and wild vanilla (*V. aphyll*a) was done to produce fertile progeny which may carry economically important genes.

***Materials used:***

Flowers of the female parent were emasculated before anthesis. Immediately after emasculation, the pollinia from the male parent were deposited on the stigmatic surface of female parent.

The following crosses were done with the intention to bring cultivated *Vanilla,* to Indian conditions:

*V. planifolia* (♀) X V. *aphylla* (♂).
*V.aphylla* (♀) X *V.planifolia* (♂)
*V.aphylla* (♀) X *V.tahetensis* (♂)
*V.tahetensis* (♀) X V. *aphylla* (♂).

***Methods:***

Green seed capsules of the hybridized vanilla were collected 2 months after pollination and *in vitro* seed culture was done onto a gelled (7 gm $l^{-1}$ agar) MS medium (Murashige and Skoog, 1962) supplemented with 1mg $l^{-1}$ BA, 0.5mg $l^{-1}$ NAA, 2 g $l^{-1}$ tryptone and 2% sucrose and adjusted the pH to 5.8. The cultures were first kept in dark at 25°C and after one week transferred to a culture room under an 8 hr. light period of 2000 lux. Plantlets derived from ovule culture was transferred to culture tubes and numbered separately each of them being considered as an accession. Sub culturing was carried in MS (1962) medium supplemented with 1mg $l^{-1}$ BAP, 0.5mg $l^{-1}$ IBA and 3% sucrose. There after the plantlets were hardened under green house conditions.

Eight different progenies (VH1, VH4, VH5, VH6, VH7, VH9, and VH15 & VH19) obtained from the cross between *V. planifolia* (♀) and V. *aphylla* (♂), six progenies (VH60, VH62, VH63, VH71, VH97 & VH99) from the cross *V.aphylla* (♀) *X V.planifolia* (♂) and four progenies ($VH_2$-1, $VH_2$-2, $VH_2$-3 & $VH_2$-4) from the cross *V.aphylla* (♀) *X V.tahetensis* (♂) were used for morphological, anatomical and cytological characterization. Nine lines (VH4, VH5, VH7, VH9, VH10, VH13, VH14, VH15 and VH19) of hybrid progenies of the cross between *V. planifolia* (♀) and *V. aphylla* (♂); and six hybrids ($VH_2$ 1, $VH_2$ 2, $VH_2$ 4, $VH_2$ 7, $VH_2$ 8 and $VH_2$ 11) from the cross between *V.aphylla* (♀) *X V.tahetensis* (♂) were used for molecular characterization in the present study.

### In vitro polyploidy

#### *Objective of polyploidy breeding:*

This research was carried out in order to determine the best method of autotetraploid induction in an attempt to exploit the characters associated with chromosome duplication in this crop.

***Starting materials for polyploidy breeding:***

Five groups of explants were used for this experiment.

1. Germinating seeds derived from selfed *V. planifolia*
2. Somatic embryos (PLBs)
3. Proliferating callus.
4. Axillary bud explants from different seed derived progenies of *V. planifolia*.
5. Axillary bud explants from *V. planifolia* (♀) X *V. aphylla* (♂) interspecific hybrids.

***Preparation of explants:***

*1. Germinating seeds derived from selfed V. planifolia:*

Green seed capsules of artificially self-pollinated *V.planifolia* were collected and sterilized by rubbing the capsule surface with 70 % (v/v) ethanol and dipping them in a 1% $HgCl_2$ solution for 10 minutes, and thoroughly rinsed with sterile distilled water. Capsules were cut open and seeds were sprinkled onto a gelled (7gm $l^{-1}$ agar) basal MS medium (Murashige and Skoog, 1962) supplemented with1mg $l^{-1}$ BA, 0.5 mg $l^{-1}$ IBA and 20 g $l^{-1}$ sucrose. The seed cultures illuminated at 2000 lux at 25 ± 1 °C for two months (were used for the study.

*2. Somatic embryos PLBs):*

The nodal segments obtained from seed derived progenies of *V.planifolia* were used as explants for inducing protocorm like bodies (PLBs). The steps for PLB production are given below:

*Production of seedlings:* seedlings were obtained from seeds germinated *in vitro* on MS medium supplemented with1mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ IBA. The pH was adjusted to 5.8 before adding 0.8 % agar.

*Axillary bud culture for PLB induction*: The basal medium used for axillary bud culture was MS. The media combinations used are:

1. MS+BA 0.5mg/l
2. MS+BA 1mg/l+IBA 0.5mg/l
3. MS+BA 1mg/l+2,4 D 0.5mg/l
4. MS+ BA 1mg/l+NAA 0.5mg/l
5. MS+BA 1mg/l+IBA 0.5mg/l+Tryptone2g/l
6. MS+BA 1mg/l+IBA 0.5mg/l+Peptone2g/l

*Mass multiplication of PLB via the thin cell layer (TCL) procedure:* In order to obtain a ready source of PLB s for polyploidy / mutational studies, the PLBs produced were cut into small bits and cultured separately to allow multiplication and growth. For this, thin cell layers (TCL) of explants were excised transversly from PLBs and cultured in MS medium containing BA 1mg $l^{-1}$ and IBA 0.5mg $l^{-1}$ the culture medium was adjusted to pH 5.8 before autoclaving and solidified with 0.7 % agar. The cultures were incubated at 25 ± 1°C with a light intensity of 2000 lux and a photoperiod of 8 hours.

Thus, a ready source of PLB as explants for colchicine treatment and mutational studies were ensured by this system.

*3. Proliferating callus*:

This experiment was carried out to obtain a ready source of callus from axillary bud of seedling to be used as explants for polyploidy / mutation studies. Axillary buds obtained from seedlings of *V.planifolia* were used as explants for callus induction.

*Production of seedlings*: seedlings were obtained from seeds germinated *in vitro* on MS medium supplemented with 1mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ IBA. The pH was adjusted to 5.8 before adding 0.8 % agar.

*Axillary bud culture for callus induction*: calli were induced by culturing the axillary buds in MS medium supplemented with different combinations of BA and TDZ alone and in combinations with 2, 4 D. The media combinations used are:

1. MS (growth regulator free)
2. MS + BA 0.5mg / l
3. MS + BA 1mg / l + 2,4 D 0.5mg / l
4. MS + BA 1mg / l + 2,4 D 1mg / l
5. MS + BA 1mg / l + 2,4 D 1.5mg / l
6. MS + BA 1mg / l + 2,4 D 2mg / l
7. MS + BA 1mg / l + 2,4 D 2.5mg / l
8. MS + BA 1mg / l + 2,4 D 3mg / l
9. MS + TDZ 0.1mg / l
10. MS + TDZ 0.1mg / l + 2,4 D 0.5mg / l
11. MS + TDZ 0.1mg / l + 2,4 D 1mg / l

12. MS + TDZ 0. 1mg / l + 2,4 D 1.5mg / l

13. MS + TDZ 0.1mg / l + 2,4 D 2mg / l

14. MS + TDZ 0.1mg/l + 2,4 D 2.5mg / l

15. MS + TDZ 0.1mg / l + 2,4 D 3mg / l

The pH of the culture medium was adjusted to 5.8 before autoclaving and solidified with 0.7% agar. The cultures were incubated at 25 ± 1°C with a light intensity of 2000 lux and a photoperiod of 8 hours. Each treatment had 5 replicates. They were later statistically analyzed.

*Maintenance of callus cultures:* the same medium for callus induction was tried for this also.

*4. Axillary bud explants*:

Axillary bud explants from different seed derived progenies of *V. planifolia* and from *V. planifolia* (♀) X *V. aphylla* (♂) interspecific hybrids were used for treatment. The nodal explants from 10 seed derived accessions were used for the experiment. The accessions included V56, V89, V92, V77, V113, V120, V124, V145, V161 and V260. The different accessions were used to study the responses of different seedling progenies to EMS and colchicine.The nodal explants from four different interspecific hybrids (VH1, VH4, VH5 and VH6) were used for the study. The interspecific hybrids were used in this experiment with the intention to produce fertile hybrid by chromosome doubling.

***Treatment method:***

*Colchicine treatment via media supplementation method:*

Under media supplementation half strength Murashige and Skoog (1962) medium supplemented with BAP (0.1 mg $l^{-1}$), NAA (0.01 mg $l^{-1}$) and sucrose (30 g $l^{-1}$) was autoclaved and shifted to laminar airflow chamber. After cooling about 55 °C the medium was shifted to 5 conical flasks and required volume of filter sterilized freshly prepared solution of colchicine was added in 4 of these 5 flasks holding above medium to make 4 different concentrations of medium, i.e., 0.05, 0.1, 0.2 and 0.4 percent leaving one flask for control (medium without colchicine). Sufficient replications were maintained in test tubes by pouring different concentrations of media. Different types of explants (seeds, PLBs, callus and nodal segments) were inoculated on to the cooled media and were maintained at 25 ± 2 °C temperature under 8 / 16 h (light / dark) photoperiod. First sub culturing for shoot proliferation was done after 21 days of inoculation on the same medium without colchicine. Proliferated shoots were transferred to full strength MS containing NAA (1 mg $l^{-1}$), BAP (0.5 mg $l^{-1}$) and sucrose (30 g $l^{-1}$). In each case, the pH was adjusted to 5.8 before adding agar (0.8 percent w / v). The well-rooted plants (10 – 15 cm. long) were hardened under green house conditions and the morphological characters were recorded.

### In vitro mutation

Breeding objective:

Induced mutation breeding was initiated to augment the spectrum of variation and select desirable mutants.

***Starting materials for mutation breeding:***

Five groups of explants were used for this experiment.

1. Germinating seeds derived from selfed *V. planifolia*
2. Somatic embryos (PLBs)
3. Callus.

4. Axillary bud explants from different seed derived progenies of *V. planifolia*. .
5. Axillary bud explants from *V. planifolia* (♀) X *V. aphylla* (♂) interspecific hybrids.

The explants were prepared as in the above experiment.

***Mutagen used:***

Ethyl methane sulphonate (EMS) from Sisco Research Laboratories, Bombay at four different concentrations - 0.25, 0.5, 0.75 and 1 percent was the mutagen used.

***Treatment method:***

*Explant agitation method*:

In this method, nodal explants with at least one viable bud were used for treatment with EMS. In the case where seed and / or PLB are used as explant they were used as such. In the case where calli is used as explant, they were cut into small pieces (~ 1cm. diameter) and then used for treatment. These explants were soaked in different concentrations of aqueous solution of EMS, *viz,* 0.25, 0.5, 0.75 and 1 percent for 6-8 hours at room temperature (30 ± 2°C) with intermittent stirring and shaking, all the operations being done under sterile condition. A sample of untreated explants was soaked in water for same period to serve as control. Later the treated explants were thoroughly washed in sterile distilled water; air dried and cultured on same establishment medium as for previous experiment, without mutagen along with control and incubated under the same conditions.

The *in vitro* mutants after 4 vegetative generations (Domingues *et al.*, 1994) were screened for chlorophyll mutations and morphological variants. The plants were hardened under green house conditions and the morphological characters were recorded. A study of chromosomes in root tip squashes of the control and the EMS treated variants was also undertaken.

## CHARACTERIZATION OF PUTATIVE VARIANTS

### Morphological characterization

***Selfed progenies:***

Vegetative morphological characters of 30 numbers of two year old *V.planifolia* seedling progenies were documented as per the descriptor for vanilla (Kuruvilla *et al.*, 2000). The characters used included both qualitative and quantitative values and are given below:

*Stem characters:*

1. Stem type: simple, branched
2. Stem shape: cylindrical, slender
3. Vine colour: green, pale green
4. Vertical groove: present, absent
5. Stem length: in cm.
6. Stem girth: in cm.
7. Internodal length: 5 th leaf from the apex

*Leaf characters:*

1. Leaf nature: sessile, petiolate
2. Leaf shape: oblong, elliptic, lanceolate, ovate
3. Leaf tip nature: acute, acuminate
4. No. of leaves / 15 cm.
5. Lamina length in cm.

6. Lamina width: in cm

*Root characters:*

1. No. of roots /15 cm.
2. Length of aerial roots: in cm
3. Colour of aerial roots: green, yellow, brown

### *Interspecific hybrids:*

The characters used for morphological analysis included leaf type, stem colour, internodal length, leaf length and leaf width. The hybrids were compared for the following traits (1) colour of the stem (2) shape of leaf (3) internodal length (4) leaf length (5) leaf width etc. Mean and SD were calculated for quantitative traits and the data was also analyzed by means of univariate ANOVA, which is a non metric technique. The F value for each character and the significance level was also determined.

### *Colchicine / EMS treated progenies:*

Observations were taken to study the effect of EMS/ colchicine on morphological characters of the plant.

Data on various characters were recorded for each accession *in vitro* and compared with the control. Observations were recorded monthly on survival percentage, number of sprouts, height of sprouts, number of leaves per sprout, number of roots, length of root, internodal length etc. The observations recorded at *in vitro* stages included:

1. Survival percentage: The number of explants that survived the mutagen treatment under different concentrations in each of the accessions 30 days after treatment was recorded and expressed as percentage of the control. Retention of green colour and emergence of sprouts was taken as the criterion for sprouting.
2. Number of sprouts and height of sprout: Number of sprouts per explant was noted and the sprout / plant height was measured from base of stem to the base of terminal leaf – and expressed as percentage of the control.
3. Number of leaves per plant: The number of leaves per sprout / plant was recorded on each month and expressed as percentage of the control.
4. Lamina length and width: Lamina length and width was recorded approximately and the second leaf from the apex was used as the standard.
5. Internodal length: The length of the Internode from the second node from the apex was measured approximately.
6. Number of roots per plant: The roots arising from the stem base or the lowest node were counted and expressed as percentage of the control.
7. Length of root: The average length of absorbing root was recorded and expressed as percentage of the control.

The vegetative morphological characters of the plants were recorded one year after planting as per the minimum descriptor for vanilla (Kuruvilla *et al.*, 2000).

*Statistical analysis:* The statistical analysis involved univariate computer programmes described in SPSS (1986) program of Roskam and Lingoes (1970). The univariate pattern of the data was analyzed by means of ANOVA, which is a non metric technique. The F values for different morphological characters and the significance level was also determined.

## Anatomical characterization

### *i. Species and Interspecific hybrids:*

*Materials used:*

Anatomical characters of 7 species were studied and this information was put to analyze the segregation pattern of the hybrids.

Plant materials for anatomical work were collected from the field gene bank of IISR, Calicut. Samples from healthy plants grown in pots under uniform conditions were used for the study. Plant parts of almost the same maturity were used and both young and mature plants were used for the study. The species used for anatomical examination included: *V. planifolia* Andr., *V. aphylla* Blume., *V. andamanica* Rolfe., *V. pilifera* Holt., *V.tahitensis* Moore., *V. whightiana* Lindl. *and V. walkierie* Wight. Eight different progenies (VH1, VH4, VH5, VH6, VH7, VH9, and VH15 & VH19) obtained from the cross between *V. planifolia* (♀) and V. *aphylla* (♂), six progenies (VH60, VH62, VH63, VH71, VH97 & VH99) from the cross *V.aphylla* (♀) X *V.planifolia* (♂) and four progenies ($VH_2$-1, $VH_2$-2, $VH_2$-3 & $VH_2$-4) from the cross *V.aphylla* (♀) X *V.tahetensis* (♂) were the interspecific hybrids used for anatomical charecterization.

*Methods:*

Methods for the study of stem and leaves were those of Johansen (1950). The plant parts were cut into 2 cm. bits and preserved in formalin-acetic acid-alcohol mixtures, washed in distilled water and sectioned. They were later stained with 1 % Safranine '0' and fast green, and observed under light microscope.

***Epidermal study***

Isolation of epidermis

I. For fresh shoot tissues (O'Brien and Mc Cully, 1981)

a. Use a sharp razor blade to make shallow cuts 2-5 mm apart in two directions at right angles. Do not cut deeply so that the pieces become separated from one another.

b. Using fine forceps gently peel of the epidermis and float cuticle side up on water till enough material has been accumulated.

c. Stain by floating cuticle side up on 0.05 % Toluidine blue 0 in water. Rinse in water for one or two minituts. Mount in water or blot dry gently on paper towel, filter paper or tissue and mount in immersion oil.

d. Cover the sections with 1% aqueous safranin for 5 minutes, wash with 3 changes of distilled water and observe under light microscope.

Staining of calcium oxalate crystals

Presence or absence of calcium oxalate crystals in the specimen were analyzed by:

Tests for insoluble calcium salts (Kashiwa and House, 1964)

Reagents: 3.4% caustic soda in 75% alcohol;

90% alcohol saturated with sodium carbonate.

95% alcohol containing 0.1% fast green

Working technique:

1. Cover the sections with 2 ml. of caustic soda solution in which 0.1g of glyoxal bis (2-hydroxyanyl) is dissolved at the moment of use, and leave to act for 5 minutes;
2. Rinse in 70% alcohol and then in two bathes of 95% alcohol;

3. Treat for 15 minutes for alcohol saturated with sodium carbonate;
4. Mount in glycerine and observe under light microscope

A positive result shows up as an intense red, granular stain; the insoluble calcium salts react strongly.

Starch (O'Brien and Mc Cully, 1981)

The presence or absence of reserve food as starch on the plant parts was verified using this test.

Iodine / potassium iodine (KI) solution was used for staining of starch. 0.2 g of iodine was dissolved in KI solution to prepare the working solution.

***ii. Colchicine treated putative polyploids:***

*Stomatal study* (Cutler, 1978)

An area of about 1cm square was removed from a central / standard position, always midway between the base and apex, of the mature and fresh leaves of the different species. Stomatal preparation was made by boiling these collected specimens of each of these nine taxa in different test tubes containing 70% ethanol for 10 minutes. These were allowed to cool and later bleached in 8% sodium hypochlorite solution (NaOCl) for 5 minutes. Epidermal peels were stained with 1% ethanol safranin and temporarily mounted in aqueous glycerol solution (Cutler, 1978). Photomicrographs of the epidermal features were taken from the slides using an Olympus microscope fitted with an automatic camera. Thirty randomly selected stomata were selected using a micrometer eyepiece.

Stomatal size and density was scored in the lower part of the middle part of the third leaf. The lower part was used due to its greater stomata (Stern and Judd, 1999), which facilitates the measurements. The stomata size was measured in 10 guard cells and stomata density in 10 high power fields of a light microscope.

Procedure developed by Cutler, 1978 to obtain epidermal peel from abaxial surface was used. Length and breath of 10 random stomata were measured using ocular and stage micrometers. Average dimensions were worked out for the control and the putative hybrids / polyploids. For determining the frequency of the stomata 5 random microscopic fields were scored and mean values worked out. Stomatal index, which is a measure of relative abundance of stomata is derived from the formula: Stomatal index = No. of stomata in a microscopic field of view divided by the number of stomata plus the no. of epidermal cells in the same microscopic field of view multiplied by hundred.

*Stomatal Index and Stomatal Density (Stomatal Frequency)*

Calculation: (Salisbury, 1932)

Stomatal Index (SI):

$$SI = \frac{S}{E+S} \times 100$$

Where E= no. of epidermal cells, S = no. of stomata in the field.

Stomatal Density (SD):

$$SD / mm = \frac{XY^2 x 10^6}{S}$$

X = no. of stomata in the field,

Y = magnification,

S =square area of the field in mm $^2$

*Measurements:*

The size of stomata, epidermal cells etc. were measured using ocular micrometer. The stomatal size and stomatal density were scored for the putative polyploids and their control. All the observations were based on an average of 10 readings and the results were statistically analyzed.

*Chloroplast number studies*:

Number of chloroplasts per stomata apparatus was determined by the method suggested by Jambhale and Nerkar (1980).

Adaxial epidermis of leaves exposed to sunlight for 4 hours was stripped off and placed on a glass slide. A drop of 2% silver nitrate solution was added on the strip for a minute. The strip was then washed thoroughly with distilled water and fixed for 5 minutes in a few drops of hypo solution (25 g. sodium thio sulphate and 0.1 g. potassium metabisulphate dissolved in 200 ml. of distilled water) and observed. The chloroplast number of the stomatal guard cells of the putative polyploids and their controls were undertaken by the above method.

*Statistical analysis:*

Correlation and regression analysis were performed to determine linear relationships between ploidy and stomatal traits. This analysis was done using the SPSS (1986) programme of Roskam and Lingoes (1970).

*Palynological study:*

Palynological study of *V.planifolia* and *V.aphylla* were done following acetolysis method of Nair (1976).

*Acetolysis method of pollen preparation* :

Fresh pollen + 70 % alcohol
↓
Crush with a glass rod
↓
Filter through muslin cloth
↓
Sediment + glacial acetic acid
↓
Sediment + acetolysis mixture (acetic anhydride and conc. $H_2SO_4$ in 9:1 ratio)
↓
Keep it in boiling water bath (70 – 100° C)
↓
Cool and centrifuge at 3000 rpm
↓
Dispersion + glacial acetic acid
↓
Wash sediment in distilled water 2-3 times
↓
Mount sediment in glycerine and observe under light microscope

### Cytological characterization

#### *i. Selfed progenies:*

*Root-tip chromosome studies:*

Chromosome number of the seedling progenies and their parents were determined by chromosome counting of root-tip cells following the methodology of Tijo and Levan (1950).

In this study of somatic chromosomes, young healthy root tips were selected and split longitudinally into 2-6 parts; according to their thickness, for penetration of the pretreating chemicals and clarification of both the cytoplasmic background and individual chromosome morphology. The young velamen tissues were removed. The root tips were pretreated with a mixture of saturated aqueous solutions of para dichloro benzene (PDB) and / or 8- hydroxy quinoline (0.002 M) (OQ). In certain cases PDB along with 2-4 drops of 0.25% colchicine was used. Pretreatment was carried out at 10-15 °C for 1-3 hours (table-5). The root tips were then washed in distilled water and then kept in acetic acid ethanol mixture (1:3) for 24 hours at 4°C and then transferred to 1 N HCl for maceration. The optimum pretreatment schedules tried are given below:

**Table 5: The different pretreatment combinations used in the root tip mitosis**

| Pretreatment chemicals | Temperature (°C) | Duration (hour) |
|---|---|---|
| PDB + colchicine(2-4 drops) | 10-12 | 1-2 |
| PDB+OQ (2:1) | 10-12 | 1 |
| PDB+OQ (4:1) | 10-12 | 3 |
| PDB+OQ (1:1) | 10-12 | 2 |
| PDB+OQ (2:1) | 10-12 | 2 |
| PDB+OQ (3:1) | 10-12 | 2 |
| PDB (Saturated) | 10-12 | 2 |

The procedure for Orcein staining is as under:

1. Prefixation: about 0.5 cm. of root tip of plantlets were removed and pretreated.

2. Fixation: the root tips were then transferred to Carnoy's fluid (glacial acetic acid and absolute ethyl alcohol in 1:3 ratio) and kept in a refrigerator for 24 hours.

3. Maceration: the root tips were warmed with 1N HCl at 60° C for 5 minutes.

4. Staining: the root tips were thoroughly washed with distilled water and stained with 2% lacto-propionic orcein (2 g of orcein dissolved in100 ml of a mixture of equal parts of lactic acid and propionic acid). The root tips were kept immersed in the stain for 3 hours.

5. Mounting: The root tips were placed over a drop of 40% acetic acid in a clean slide, the older parts cut and removed, and squashed with uniform pressure over the cover glass. Subsequently the slides were sealed and observed.

Chromosome numbers and structural aberrations of the selfed progenies were worked out.

*Pollen studies:*

Fresh (or fixed) pieces of pollinia were squashed in a 1% solution of acetocarmine and are gently heated over a spirit flame to aid differentiation. The pollinia with different divisional stages were used to study meiosis.

Photography:

Photomicrographs were taken with Olympus microscope having a camera system. Kodak 100 ASA colour films were used for taking photographs.

***ii. Interspecific hybrids:***

The interspecific hybrids obtained from the three different crosses were subjected to root tip chromosome counting to study the effect of hybridization on the chromosome number of the progenies.

***iii. Colchicine treated progenies:***

The root tip chromosome counting of the putative polyploids and their controls were determined by the method of Tijo and Levan (1950). Those treatments which were confirmed as polyploids by chromosome counting were designated as 'VCP'.

***iv. EMS treated progenies:***

The EMS treated putative mutants were subjected to root tip chromosome examination for determining any structural or numerical alterations.

**Molecular characterization**

*PCR techniques*

A PCR machine from 'M J Research' was used. The primers were obtained from Operon technologies. DNTP's were obtained from Amersham Pharmasica. Taq polymerase, magnesium chloride and PCR assay buffer were obtained from Bangalore Genei. The 1 kb ladder was from SIGMA, USA. For gel electrophoresis, agarose was obtained from 'Biogene'. The centrifuges used for DNA isolation and PCR purposes were from Hitachi.

*Isolation of DNA:*

***Principle***

Isolation and purification of high molecular weight DNA is a fundamental requirement for the identification and isolation of genes for plant genetic engineering as well as for the study of gene organization and gene expression at the molecular level.

Fresh and healthy leaves from vigorously growing vanilla plants were used for isolation of DNA. DNA was isolated from the accessions given in table-6 using modified method of Ausubel *et al.,* (1995). Apart from these accessions, vegetative propagated

*V.planifolia* was treated as control for the seedling progenies and *V.planifolia, V.aphylla* and *V.tahetensis* mother plants as controls for the hybrid progenies.

**Table-6: Different accessions of vanilla used to detect RAPD polymorphisms.**

| Sl.No: | Seedling progenies | Interspecific hybrids | Colchicine treated * | EMS treated* |
|---|---|---|---|---|
| 1 | V77 | VH4 | V56 (0.05%) | V56 (0.75%) |
| 2 | V89 | VH5 | V89 (0.2 %) | V77 (0.75%) |
| 3 | V92 | VH7 | V92 (0.2 %) | V89 (0.75%) |
| 4 | V113 | VH9 | V113 (0.2 %) | V92 (0.25%) |
| 5 | V150 | VH10 | V124 (0.1 %) | V113 (0.25%) |
| 6 | V218 | VH13 | V145 (0.4 %) | V113 (0.25%) |
| 7 | V225 | VH14 | V161 (0.05 %) | V113 (0.5%) |
| 8 | V233 | VH15 | V260 (0.1 %) | V113 (0.75%) |
| 9 | V259 | VH19 | VCP2 (0.4 %) | V124 (1%) |
| 10 | V260 | VH$_2$1 | VCP3 (0.4 %) | V153 (0.25%) |
| 11 | V334 | VH$_2$2 | VCP4 (0.4 %) | V153 (0.75%) |
| 12 | V335 | VH$_2$4 | VCP5 (0.4 %) | V161 (0.75%) |
| 13 | V358 | VH$_2$7 | V seed 2 (0.1 %) | V260 (1%) |
| 14 | V408 | VH$_2$8 | Vseed 4 (0.4 %) | Vseed 1 (0.25%) |
| 15 | -- | VH$_2$ 11 | Vseed 5 (0.2 %) | V seed 3 (0.5%) |

*** Concentration of the mutagen in parenthesis**

**V77 toV408: seed derived progenies of *V.planifolia*, VH1 to VH19: hybrids derived from** *V. planifolia* (♀) X V. *aphylla* (♂) cross, **VH$_2$ 1 to VH$_2$ 11: hybrids derived from** *V.aphylla* (♀) X *V.tahetensis* (♂) cross, **VCP 1 to VCP 5: polyploids obtained by colchicine treatment, V seed 1 to V seed 5: plants derived from seeds treated with colchicine / EMS**

The study was to obtain the RAPD profiles and to use them as an index for the extent of variation among and between the seedling progenies, hybrids, colchicine treated progenies and EMS treated progenies.

**Table 7: Details of various buffers used for DNA extraction**

| | Buffer | Method of preparation | Comments |
|---|---|---|---|
| 1 | CTAB Extraction Buffer(1 litre)100mM Tris HCl (pH 8.0) 20mM EDTA (pH 8.0) 1.4M NaCl, 2%CTAB (w/v) 0.2%B-mercapto ethanol(v/v) | Measure 100ml Tris (1M), 280ml of NaCl, 40ml of EDTA (0.5M). Mix with about 400ml of hot distilled water ,add 20g of CTAB to this. Adjust final volume to 1 litre. Dispense to reagent bottles and autoclave.Just before use, add 0.2% β-mercaptoethanol. | CTAB will take time to dissolve<br>avoid foaming. |
| 2 | TE (0.1mM) buffer 100ml.<br>100mM Tris HCl (pH 8.0)<br>0.1mM EDTA (pH 8.0) | Take 1ml of Tris HCl (1M), 20µl of EDTA (0.5M).made up to 100 ml with double distilled water taken in a reagent bottle, mixed thoroughly, autoclave. | TE (0.1mM) is written since there is TE with 1mM EDTA also |
| 3 | TAE buffer 10x :1 litre | Weigh 48.4g of Tris base ;add 20ml of EDTA (0.5M);11.42ml of Glacial acetic acid and dissolved in 150ml double distilled water. Adjust the volume to 1 litre. Autoclave | It will dissolve much easily in 500ml solution |
| 4 | Gel loading buffer (6x) 100 ml.<br>0.25% Bromophenol blue (Sigma )<br>30% Glycerol (Merck) | Dissolve 0.25g of BPB in 99ml of 30% Glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense to reagent bottles and keep in 4 °C | Strong dye, handle carefully. |

(Sambrook *et al* 1989)

*Quality analysis of DNA in agarose gels:*

The quality of DNA extracted was visualized on 0.8% agarose gel in the following way:

1. Prepared 0.8% agarose gel in 1x TAE buffer, adding Ethidium bromide (10mg/ml) and casted the gel
2. Loaded 3ul of DNA sample mixed with 2ul of gel loading buffer (6x)
3. Gel was run at 60 volts, for 2 hours
4. Visualised the image under UV Transilluminator in Biorad Gel Doc 1000 system.

**Table 8: Details of various stock solutions used for DNA extraction**

| **Solutions** | **Method of preparation** | **Comments** |
|---|---|---|
| **1M Tris (pH 8.0,)** 500ml | Dissolve 60.55gm Tris base (sigma) in 300 ml distilled water. Adjust ph to 8 by adding concentrated HCl. Adjust volume to 500ml. Dispense to reagent bottles and sterilize by autoclaving. | *pH of Tris solution is temperature dependent.<br>*Tris will completely dissolve only when pH becomes<br>* Initial pH will be around 11 |
| **0.5M EDTA pH 8.0** | Dissolve 93.05g of EDTA-disodium salt (sigma) in 300ml of water. Adjust pH to 8 by adding NaOH pellets. Adjust volume to 500 ml. Dispense in to reagent bottles and autoclave. | *The salt will take time to dissolve |
| **5M NaCl 500 ml.** | Weigh 146.1g NaCl (Merck) add 200ml of water and mix well. When the salts get completely dissolved, adjust the final volume to 500ml. Dispense in to reagent bottles and autoclave. | *The salt will take much time to dissolve. |
| **3M Sodium acetate (pH5.2) 250 ml.** | Dissolve 61.523g of anhydrous sodium acetate (Qualigens) in 200 ml of water and mix well. When dissolved completely adjust the pH of the solution to 5.2 with glacial acetic acid (99- 100%). Dispense to reagent bottles and autoclave. | *The salt will take much time to dissolve. |

| | | |
|---|---|---|
| **Ethidium Bromide 10mg/ml, 100mL** | Add 1g. Ethidium Bromide to 100 ml of distilled water. Keep on magnetic stirrer to ensure that the dye has dissolved completely. Dispense to amber coloured reagent bottle and store at 4°C. | *Ethidium Bromide is a powerful mutagen and is moderately toxic. So handle carefully. |
| **70% ethanol, 500 ml** | Take 360 ml. of ethanol; mix with 140 ml of distilled water. Dispense to reagent bottle and store at 4°C. | Stock ethanol is 98% that is why 360 ml is taken instead of 350 ml. |
| **Chloroform: isoamyl alcohol (24:1),500 ml** | Measure 450 ml of chloroform and 20 ml of isoamyl alcohol. Mix well and store in reagent bottle in room temperature | Chloroform will evaporate, so close the cap tightly. |
| **1M $MgCl_2$, 100ml** | Weigh 20.33g of Mg $Cl_2$, dissolve in double distilled water, make up to 100ml, dispense in to reagent bottles and autoclave. | |

(Sambrook *et al,* 1989)

*Quantification of DNA:*

DNA quantity measurements were done on 0.8% agarose gel by loading samples along with the λ DNA standard (Bangalore Genei 250ng/µl). Then comparing the bands in the sample lines with that of the standard, the quantity of DNA in each sample (ng/µl) was quantified.

*Polymerase chain reaction*

*PCR Reaction Components*

1. Template DNA concentration: One of the most appealing features of PCR is that the quantity and quality of the DNA sample to be subjected to amplification do not need to be high. When DNA of known concentration is available, amounts of 50-100 ng are typically used for amplifications of single copy loci (Innis *et al*, 1990). Genomic DNA diluted to a concentration of 30 ng was used in this study.

2. Buffer: The standard buffer contains 50 mM KCl, 10 mM TrisHCl (pH 8.4), 1.5 mM $MgCl_2$, and 100 µl / ml of gelatin will be adequate for the majority of genomic PCR (Innis *et al,* 1990). 10x buffer was used, from which we directly added 2µl for each reaction

3. Deoxy Nucleotide Triphosphates: The four dNTPs (dATP, dTTP, dCTP and dGTP) are usually used in concentrations between 20-200 µM each result in the optimal balance among yield, specificity and fidelity. All the four dNTPs were used at equivalent concentrations to minimize incorporation errors (Innis *et al,* 1990). In the present study, a ready-made stock of 10 mM dNTPs was used.

4. Enzyme: The optimal concentration of *Taq* DNA polymerase is about 2U/100 µl. As a rule, concentrations in excess of 4U tend to result in the accumulation of non-specific amplification products, whereas amounts less than 1U usually reduce the yield of the desired product (Innis *et al,* 1990). *Taq* polymerase (Bangalore Genei) having a concentration of 3U/µl, were used in the study.

5. Primers: Primer concentrations between 0.1 – 0.5 µM are generally optimal. Higher primer concentrations may promote mispriming and accumulation of non-specific product and may increase the probability of generating a template independent artifact termed primer-dimer. Non specific products and primer dimer artifacts are themselves substrates for PCR and compete with the desired product for enzyme, dNTPs and primers resulting in a low yield of the desired product (Innis *et al,* 1990).

*Primer Screening*

After optimization of PCR conditions and variables, twenty-four, 10 mer primers

(Operon Technologies Inc. Alameda, California) were screened using *V.planifolia* (control) for amplification and polymorphism. The list of primers used for primer screening is given in Table-9.

**Table-9: Operon primers screened for RAPD analysis and their base sequences**

| Sl. No | Primer number | Base sequence |
|---|---|---|
| 1 | OPA-05 | 5'AGGGGTCTTG3' |
| 2 | OPA-08 | 5'GTGACGTAGG3' |
| 3 | OPB-01 | 5'CTTTCGCTCC3' |
| 4 | OPB-03 | 5'CATCCCCCTG3' |
| 5 | OPB-05 | 5'TGCGCCCTTC 3' |
| 6 | OPB-06 | 5'TGCTCTGCCC3' |
| 7 | OPB-12 | 5'CCTTGACGCA3' |
| 8 | OPB-19 | 5'ACCCCCGAAG3' |
| 9 | OPC-09 | 5'CTCACCGTCC3' |
| 10 | OPC-11 | 5'AAAGCTGCGG3' |
| 11 | OPC-16 | 5'CACACTCCAG3' |
| 12 | OPD-01 | 5'ACCGCGAAGG3' |
| 13 | OPD-06 | 5'ACCTGAACGG3' |
| 14 | OPD-08 | 5'GTGTGCCCCA3' |
| 15 | OPD-18 | 5'GAGAGCCAAC3' |
| 16 | OPE-03 | 5'CCAGATGCAC3' |
| 17 | OPE-07 | 5'AGATGCAGCC3' |
| 18 | OPE-12 | 5'TTATCGCCCC3' |
| 19 | OPE-13 | 5'CCCGATTCGG3' |
| 20 | OPE-15 | 5'ACGCACAACC3' |
| 21 | OPE-18 | 5'GGACTGCAGA3' |
| 22 | OPE-19 | 5'ACGGCGTATG3' |
| 23 | OPF-10 | 5'GGAAGCTTGG3' |
| 24 | OPF-18 | 5'TTCCCGGGTT3' |

The reaction mixture was prepared as follows.

| | |
|---|---|
| Assay buffer | - 2.5 0 μl (10 X) |
| *Taq* DNA polymerase | - 0.33 μl (1U/μl) |
| d NTP's | - 3.75 μl (150μM) |
| $MgCl_2$ | - 1.50 μl (0.5 mM) |
| DNA | - 3.50 μl (30 ng / μl) |
| Primer | - 2.00 μl (10 picomoles) |
| Sterile distilled water | - 11.42 μl |
| TOTAL | - 25.00 μl |

*Randomly amplified polymorphic DNA:*

Procedure:

Good quality high molecular weight DNA from the isolated samples were selected for the study. The PCR reaction mix was prepared as mentioned earlier, containing 10x PCR buffer, dNTPs, *Taq* DNA polymerase, $MgCl_2$, and sterile distilled water. This mixture was dispensed into the PCR tubes and the required concentration of the selected primers and template DNA was added.

*Optimisation of PCR condition:*

Enzyme concentration

The requirement of *Taq* DNA polymerase may vary with respect to individual target templates or primers. When optimizing a PCR, recommended enzyme concentrations ranges from 0.5-5-units/25μl and the results were assayed by gel electrophoresis.

### Deoxynucleotide Triphosphates

Primary dNTP stocks are diluted to 10mM, aliquoted and stored at – 20 $^0$C. A working stock containing 1mM each dNTP is recommended. Deoxynucleotide concentrations between 20-200 µM, each result in the optimal balance among yield, specificity, and fidelity. The four dNTPs should be used in equal concentrations to minimize misincorporation of errors. Low dNTP concentrations minimize mispriming at non target sites and reduce the likelihood of extending misincorporated nucleotides (Innis *et al.* 1988).

### Magnesium Concentration

Magnesium concentration affects primer annealing, strand dissociation temperatures of both template and PCR product specificity, formation of primer- dimmer artifacts, and enzyme activity and fidelity. PCR should contain 0.5-2.5mM, magnesium over the total dNTP concentration. The presence of EDTA or other chelators in the primer stocks or template DNA may disturb the apparent magnesium optimum.

### Denaturation time and temperature:

The most likely cause for failure of a PCR is incomplete denaturation of the target-template and/or the PCR product. Incomplete denaturation reduce the product yield, while denaturation steps that are too high and/or too long lead to unnecessary loss of enzyme activity The typical denaturation conditions are 95 °C for 30 seconds / 97 °C for 15seconds. However, higher temperature may be appropriate especially for G+C rich targets.

Only 94 °C for 2 minutes in the first cycle and 94 °C for 1 minute in the second and third cycles were tested for denaturation.

Primer annealing:

The temperature and length of time required for primer annealing depend upon the base composition, length and concentration of amplification primers. Primer annealing was done at 40 °C for 1minute except for the second cycle where it is 40 °C for 30 seconds.

Primer extension:

Extension time depends upon the length and concentration of the target sequence and upon temperature. Primer extensions are traditionally performed at 72 °C because this temperature was near optimal for extending primers on an M-13 based model template (Innis *et al* 1990). However, longer extension times may be helpful in early cycles if the substrate concentration is very low and at late cycles when product concentration exceeds enzyme concentration. Primer extension was done at 72 °C for 1 minute for first and second cycles except for the final cycle, the extension time was increased to15 minutes.

Cycle number:

The optimum number of cycles will depend mainly upon the starting concentration of target DNA when other parameters are optimized .Too many cycles can increase the amount and complexity of non specific background products while too few cycles give low product yield (Innis *et al*, 1990). Here 32 cycles were used.

The PCR reaction mixes was prepared using all the seven components and in each tube the final reaction volume was made up to 25µl, the vials were loaded in the wells in the PCR machine, and the instrument was made to run according to the loaded program. The total time taken by the PCR machine to complete the reaction was around 3 hours.

The PCR reaction profile tested are given below:

| | | | |
|---|---|---|---|
| Cycles: 3 | Ist cycle | 94°C 2 minutes | |
| | | 40 °C 1 minute | |
| | | 72 °C 1 minute | Cycle repeats: 1 |
| | IInd cycle: | 94 °C for 2 minutes | |
| | | 40 °C for 30 seconds | |
| | | 72 °C for 1 minute | Cycle repeats: 30 |
| | IIIrd cycle : | 94 °C for 1 minute | |
| | | 40 °C for 1 minute | |
| | | 72 °C for 15 minutes | Cycle repeats: 1 |

### ***Assessment of variability***

The RAPD profiles were compared between the seedling progenies; hybrids, colchicine treated and EMS treated progenies to estimate the extent of variability. Similarity indices were estimated between different hybrids and between the EMS treated progenies and dendrograms were drawn indicating interrelationship between the hybrid progenies and between EMS treated progenies using NTSYS software.

# Studies on Genetic Variability in Open Pollinated Progenies of Vanilla.

Thesis submitted to
**University of Calicut**
for the award of **Doctor of Philosophy** in Botany

By
**Vimala Jose**

**University of Calicut**
**Kerala, India**
**2005**

# Results

*Old World species which links 'leafy' and 'leafless' Vanilla*

Results obtained from this investigation are presented under different heads.

## IN VITRO CULTURE

### Effect of age of the embryo on germination

The mature pod size was attained by the time the pod was 6 weeks of age and maximum percentage of germination was obtained 56 days after pollination (Fig 1). The rate of germination seemed definitely to decline after about 27 weeks until no germination after 30 weeks (Table-10 & 11).

**Table-10: Effect of size and maturity of bean on germination *in vitro*.**

| Sl. No | DAP* | Length of bean (in cm.) on: Pollination day | Length of bean (in cm.) on: Inoculation day | Width of bean (in cm.) on: Pollination day | Width of bean (in cm.) on: Inoculation day | Intensity of germination** |
|---|---|---|---|---|---|---|
| 1 | 2 | 5 ± 0.4 | 5.4 ± 0.2 | 1.4 | 1.6 ± 0.07 | 0 |
| 2 | 4 | 4.9 ± 0.2 | 5.5 ± 0.3 | 1.4 | 1.5 ± 0.06 | 0 |
| 3 | 6 | 5 ± 0.1 | 5.9 ± 0.5 | 1.4 | 1.7 ± 0.1 | 0 |
| 4 | 8 | 4.8 ± 0.2 | 5.8 ± 0.4 | 1.4 | 1.9 ± 0.3 | 0 |
| 5 | 10 | 5.1 ± 0.2 | 6.3 ± 0.35 | 1.4 | 2 ± 0.3 | 0 |
| 6 | 12 | 5 ± 0.25 | 6.7 ± 0.4 | 1.4 | 2.2 ± 0.1 | 0 |
| 7 | 14 | 5.2 ± 0.3 | 7.1 ± 0.9 | 1.4 | 2.3 ± 0.09 | 0 |
| 8 | 16 | 5.2 ± 0.15 | 7.3 ± 0.7 | 1.4 | 2.3 ± 0.09 | 0 |
| 9 | 18 | 5 ± 0.3 | 8.6 ± 1.1 | 1.4 | 2.6 ± 0.2 | 0 |
| 10 | 20 | 4.8 ± 0.3 | 9.1 ± 1.2 | 1.4 | 2.9 ± 0.15 | 0 |
| 11 | 22 | 5 ± 0.2 | 12.7 ± 1.3 | 1.4 | 3.8 ± 0.3 | + |
| 12 | 24 | 4.9 ± 0.2 | 12.3 ± 1.2 | 1.4 | 3.6 ± 0.3 | + |
| 13 | 26 | 5 ± 0.1 | 11.8 ± 1.5 | 1.4 | 3.6 ± 0.25 | ++ |
| 14 | 28 | 5.1 ± 0.3 | 13 ± 1 | 1.4 | 3.7 ± 0.3 | ++ |
| 15 | 30 | 5 ± 0.2 | 15.5 ± 1.2 | 1.4 | 3.9 ± 0.2 | +++ |

***DAP : Days after pollination.** **** 0, nil; +, low; ++, moderate; +++, high**

**Data scored at the end of 6 months in culture.**

**Table-11**: **Effect of size and maturity of bean on germination *in vitro.***

| Sl. No | Age of Pod * | Length of bean (in cm.) on: |  | Width of bean (in cm.) on: |  | Intensity of germination |
|---|---|---|---|---|---|---|
|  |  | Pollination day | Inoculation day | Pollination day | Inoculation day |  |
| 1 | 2 | 5.2 ± 0.2 | 7.1 ± 0.5 | 1.4 | 2.3 ± 0.09 | 0 |
| 2 | 4 | 5.1 ± 0.25 | 13 ± 1 | 1.4 | 3.7 ± 0.3 | ++ |
| 3 | 6 | 5 ± 0.1 | 13.9 ±1.2 | 1.4 | 3.7 ± 0.25 | +++ |
| 4 | 8 | 5.1 ± 0.3 | 14.2 ± 1.2 | 1.4 | 3.9 ± 0.2 | ++++ |
| 5 | 10 | 5 ± 0.15 | 13.4 ± 1.1 | 1.4 | 3.5 ± 0.2 | ++++ |
| 6 | 12 | 5 ± 0.4 | 13 ± 1.3 | 1.4 | 3.2 ± 0.25 | ++++ |
| 7 | 14 | 5.2 ± 0.3 | 12.9 ± 1.5 | 1.4 | 3.5 ± 0.2 | ++++ |
| 8 | 16 | 5 ± 0.15 | 14.2 ± 1.2 | 1.4 | 3.9 ± 0.15 | ++++ |
| 9 | 18 | 5.1 ± 0.4 | 13.1 ± 1.1 | 1.4 | 3.4 ± 0.3 | ++++ |
| 10 | 20 | 4.9 ± 0.3 | 14 ± 1 | 1.4 | 3.8 ±0.1 | ++++ |
| 11 | 22 | 5 ± 0.15 | 13.5 ± 0.8 | 1.4 | 3.6 ± 0.15 | +++ |
| 12 | 24 | 5 ± 0.1 | 12.5 ± 1.1 | 1.4 | 3.3 ± 0.15 | +++ |
| 13 | 26 | 5.2 ± 0.1 | 12.9 ±1.1 | 1.4 | 3.6 ± 0.2 | ++ |
| 14 | 28 | 5.1 ± 0.25 | 13.3 ± 1.1 | 1.4 | 3.5 ± 0.1 | + |
| 15 | 30 | 5 ± 0.3 | 14 ± 1.1 | 1.4 | 3.7 ± 0.2 | 0 |
| 16 | 32 | 5.1 ± 0.3 | 15.3 ± 1.2 | 1.4 | 3.9 ± 0.3 | 0 |
| 17 | 34 | 5 ± 0.2 | 13.9 ± 1 | 1.4 | 3.7 ±0.2 | 0 |

***Age of the pod in weeks** **** 0, nil; +, low; ++, moderate; +++, high; ++++, intense.**

**Data scored at the end of 6 months**

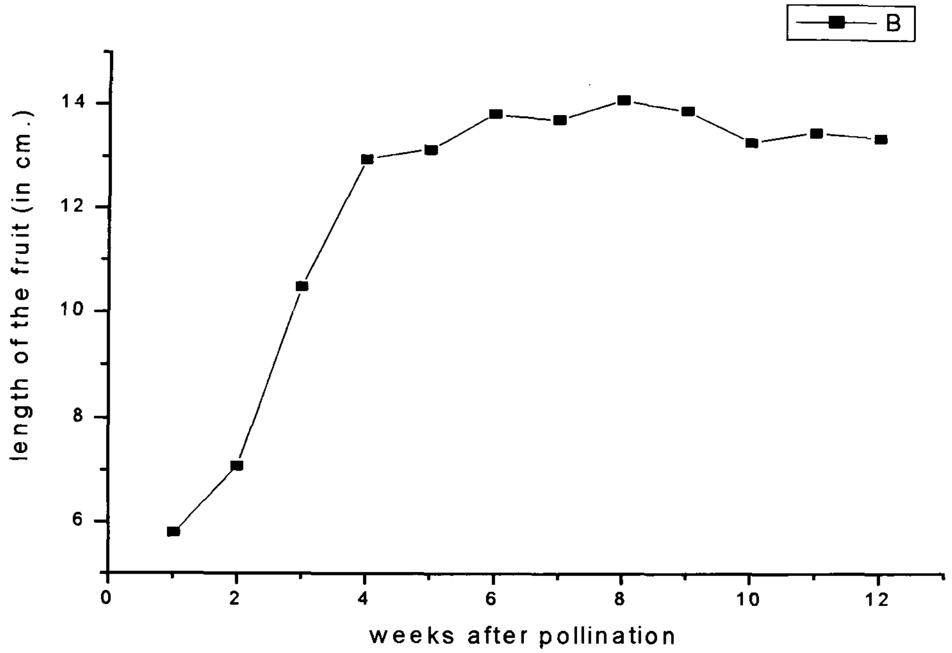

Fig 1: **Graph showing fruit size increment with time**

**Effect of different media combinations on seed germination**

The seeds were cultured on various combinations of nutrient media (Table-12). Growth of the seedlings in the bottles of various media combinations was variable and not consistent. Although the seeds started germinating in the media with 2, 4 D they ultimately turned brown and died. Among the ten treatments, the combination MS + BA 1mg/L + IBA 0.5mg/L + Tryptone 2g/L resulted in a relatively higher embryo germinating percentages (>80%) when compared to the control treatment (MS media without hormones). The protocorms proliferating rate was highest in the media supplemented with Tryptone / peptone. There was considerable variation in growth pattern of the seedlings in the media with various organic additives (Table-12). The germinating seeds of *V. planifolia* failed to develop chlorophyll in the initial stages (Fig 2a, 2d), while those of *V.aphylla* developed green colour from the initial stage itself (Fig

2 b, 2e), the protocorms developed from seeds also developed chlorophyll (Fig 2g). The germinated seeds of *V. planifolia, V.aphylla* and *V.tahetensis* showed characteristic differences in their developmental pattern (Fig 2f).

**Table-12: The morphogenetic response of seeds to different media supplements.**

| Sl.No. | Media | Morphogenetic response * |
|---|---|---|
| 1 | MS (hormone free) | Multiplication and growth rate low. |
| 2 | MS + BA 0.5 mg/l | More uniform growth |
| 3 | MS + BA 1 mg/l + NAA 0.5 mg/l | Shiny white elongated protocorms |
| 4 | MS + BA 1 mg/l + IBA 0.5 mg/l | White clumps of protocorms from germinating seeds |
| 5 | MS + BA 1 mg/l + 2,4 D 0.5 mg/l | Callusing of germinated seeds; but failed to survive. |
| 6 | MS + BA 1 mg/l + IBA 0.5 mg/l + 2ip 2g/l | Fine hairy structures spreading out from surface of germinating seeds |
| 7 | MS + BA 1 mg/l + IBA 0.5 mg/l + peptone 2g/l | Multiplying protocorms appearing as thick stout clumps. |
| 8 | MS + BA 1 mg/l + IBA 0.5 mg/l + tryptone 2g/l | Yellowish white multiplying protocorms. |
| 9 | MS + BA 1 mg/l + IBA 0.5 mg/l + casein hydrolysate 2g/l | Yellowish white protocorms; thick and stout |

- **based on visual observations at the end of 6 weeks**

## INDUCTION OF VARIATION

### Development of segregating seedling progenies

The seeds were germinated by the above method and separately transferred to culture tubes, each of them being considered as an accession. Renewal of growth could be brought about by sub culturing the seedlings to fresh medium (Fig 2 h, i, j). Data indicates that growth of explants could be enhanced by their culture on MS medium with 0.5 mg $l^{-1}$ BA. This media resulted in greater number of uniform and vigorous shoots per explant (table-13). In general, shoots from explants grown on MS media containing BA and IBA resulted in PLB formation than those that developed on plant growth regulator free media or containing BA only.

**Table-13: The morphogenetic response of seedlings to different supplements in multiplication media.**

| Sl.No. | Media | Morphogenetic response * |
|---|---|---|
| 1 | MS (hormone free) | Multiplication and growth rate slow. |
| 2 | MS + BA 0.5 mg/l | High growth rate, more uniform shoots. |
| 3 | MS + BA 1 mg/l + NAA 0.5 mg/l | Multiple shoot formation; rooting rate high |
| 4 | MS + BA 1 mg/l + IBA 0.5 mg/l | Multiplication rate high; PLB formed |
| 5 | MS + BA 1 mg/l + 2,4 D 0.5 mg/l | Callusing; recovery rate low. |
| 6 | MS + BA 1 mg/l + IBA 0.5 mg/l + 2ip 2g/l | High rate of shoot initiation. |
| 7 | MS + BA 1 mg/l + IBA 0.5 mg/l + peptone 2g/l | More vigorous root and shoot formation; PLBs formed. |
| 8 | MS + BA 1 mg/l + IBA 0.5 mg/l + tryptone 2g/l | More vigorous root and shoot formation; PLBs formed. |

***based on visual observations at the end of 6 weeks.**

The plants were transferred to well watered river sand taken in plastic cups and covered with ventilated polythene bags to prevent excess loss of water due to transpiration (Fig 2k). The cups were maintained in nursery conditions (Fig 2m) and after one month transferred to polybags with potting mixture (Fig 2n).

***Morphological characterization***

Most of the progenies resembled their parent and among themselves. They showed the characteristic differences in leaf shape and internodal length. The results indicated that the progenies were not homogeneous in its growth pattern (Table-14).

**Table-14: Morphological characters of seedling progenies of *V.planifolia***

| Sl No | Accn. No. | Morphological characters* | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Stem | | | | | | | Leaf | | | | | | Root | | |
| | | St | Ss | Vc | Vg | Sl | Sg | In.l | Ln | Ls | Lt | Ln. | L. l | Lw | Rn | R. l | Rc |
| 1 | Control | s | c | g | ab | 23 | 2 | 5.8 | pe | ob | acc | 3 | 8.1 | 2.3 | 3 | 3.1 | gr |
| 2 | vc77 | s | c | g | ab | 62 | 1 | 3.8 | pe | la | acc | 5 | 5.5 | 1.9 | 3 | 4.4 | gr |
| 3 | v89 | s | c | g | ab | 75 | 1 | 5.5 | pe | la | acc | 3 | 6.7 | 4 | 2 | 3.8 | gr |
| 4 | v113 | s | sl | g | ab | 22 | 1 | 2.8 | pe | la | a | 7 | 5.1 | 2.1 | 6 | 1.2 | gr |
| 5 | v161 | s | c | g | ab | 56 | 1 | 3.4 | pe | la | acc | 7 | 5.2 | 2.3 | 6 | 9 | gr |
| 6 | v260 | s | c | g | ab | 18 | 1 | 2.7 | pe | la | acc | 6 | 3.5 | 2.3 | 2 | 0.9 | gr |
| 7 | v56 | s | c | g | ab | 19 | 1 | 2 | pe | la | acc | 7 | 2.6 | 1.5 | 5 | 1.8 | gr |
| 8 | v92 | s | c | g | ab | 70 | 1 | 3.2 | pe | la | a | 6 | 4.8 | 1.9 | 6 | 2.5 | gr |
| 9 | v153 | s | c | g | ab | 36 | 1 | 3 | pe | ob | acc | 8 | 3.4 | 2 | 3 | 3.5 | gr |
| 10 | v24 | s | sl | g | ab | 14 | 1 | 1.7 | pe | la | a | 8 | 2.1 | 0.9 | 4 | 0.8 | gr |
| 11 | v67 | s | sl | g | ab | 16 | 1 | 2 | pe | la | a | 7 | 2.5 | 1.3 | 5 | 2.6 | y |
| 12 | v8 | s | c | g | ab | 21 | 1 | 3.4 | pe | ob | acc | 5 | 3.4 | 2 | 4 | 4.3 | gr |
| 13 | v11 | s | c | g | ab | 45 | 1 | 6.1 | pe | ov | acc | 3 | 5.7 | 2.3 | 3 | 5.8 | gr |
| 14 | v32 | s | c | g | ab | 31 | 1 | 3.8 | pe | la | acc | 6 | 4.8 | 2.4 | 3 | 1.2 | gr |
| 15 | v53 | s | c | g | ab | 18 | 1 | 5.4 | pe | la | acc | 3 | 8.5 | 3.5 | 3 | 2.1 | gr |
| 16 | v65 | s | c | g | ab | 21 | 1 | 3.6 | pe | ob | acc | 6 | 4.1 | 2 | 5 | 2.6 | gr |
| 17 | v108 | s | sl | g | ab | 18 | 1 | 2.3 | pe | la | acc | 8 | 3.5 | 2 | 2 | 1 | gr |
| 18 | v138 | s | c | g | ab | 58 | 1 | 2.9 | pe | la | acc | 7 | 3.1 | 2 | 5 | 2 | gr |
| 19 | v140 | s | c | g | ab | 63 | 1 | 3.1 | se | la | acc | 7 | 4.5 | 3.6 | 4 | 2.1 | y |
| 20 | v150 | s | c | g | ab | 62 | 1 | 5.1 | pe | la | a | 4 | 6 | 2.1 | 4 | 3.5 | y |
| 21 | v162 | s | c | g | ab | 68 | 1 | 4.2 | pe | la | acc | 5 | 5.1 | 3.2 | 3 | 2.5 | y |
| 22 | v163 | s | c | g | ab | 40 | 1 | 4.4 | pe | ob | acc | 4 | 8 | 2.5 | 2 | 3 | gr |
| 23 | v218 | b | c | g | ab | 54 | 1 | 3.8 | pe | ob | acc | 4 | 4.7 | 1.2 | 4 | 2.1 | gr |
| 24 | v259 | b | c | g | ab | 78 | 1 | 5 | pe | la | acc | 5 | 5.2 | 2.2 | 4 | 2 | gr |
| 25 | v334 | s | sl | g | ab | 32 | 1 | 2.9 | pe | la | acc | 6 | 3.8 | 2 | 6 | 1.3 | gr |
| 26 | v335 | b | c | g | ab | 61 | 1 | 2.5 | pe | la | acc | 6 | 4.1 | 1.8 | 5 | 2.1 | y |
| 27 | v352 | b | c | g | ab | 61 | 1 | 3.9 | pe | ov | acc | 4 | 4.8 | 2.1 | 4 | 2.6 | gr |
| 28 | v358 | s | sl | g | ab | 62 | 1 | 3.2 | pe | la | acc | 6 | 4.2 | 2 | 4 | 4.3 | gr |
| 29 | v377 | b | c | g | ab | 3 | 1 | 3.2 | se | ob | acc | 6 | 5 | 1.5 | 6 | 4.8 | gr |
| 30 | v434 | s | c | g | ab | 18 | 1 | 2.4 | pe | la | acc | 7 | 4 | 2.2 | 5 | 2.3 | y |

***St – stem type** (s - simple, b – branched); **Ss – stem shape** (c -cylindrical, sl – slender ); **Vc – vine colour** (g – green); **Vg – vertical groove** (a – absent); **Sl – stem length** (in cm.); **Sg – stem girth** (in cm.); **In. l – internodal length** (in cm.); **Ln – leaf nature** (pe – petiolate, se – sessile); **Ls – leaf shape** (la - lanceolate, ov – ovate, ob – oblong); **Lt – leaf tip nature** (acc - accuminate, a – acute); **L n. – leaf number; Ll – leaf length** (in cm.); **Lw – leaf width** (in cm.); **Rn – root number; Rl – root length** (in cm.); **Rc – root colour** (gr – green, y – yellow). * Vegetative morphological charecters recorded as per the descriptor for Vanilla (Kuruvilla *et al*, 2000) two years after planting.

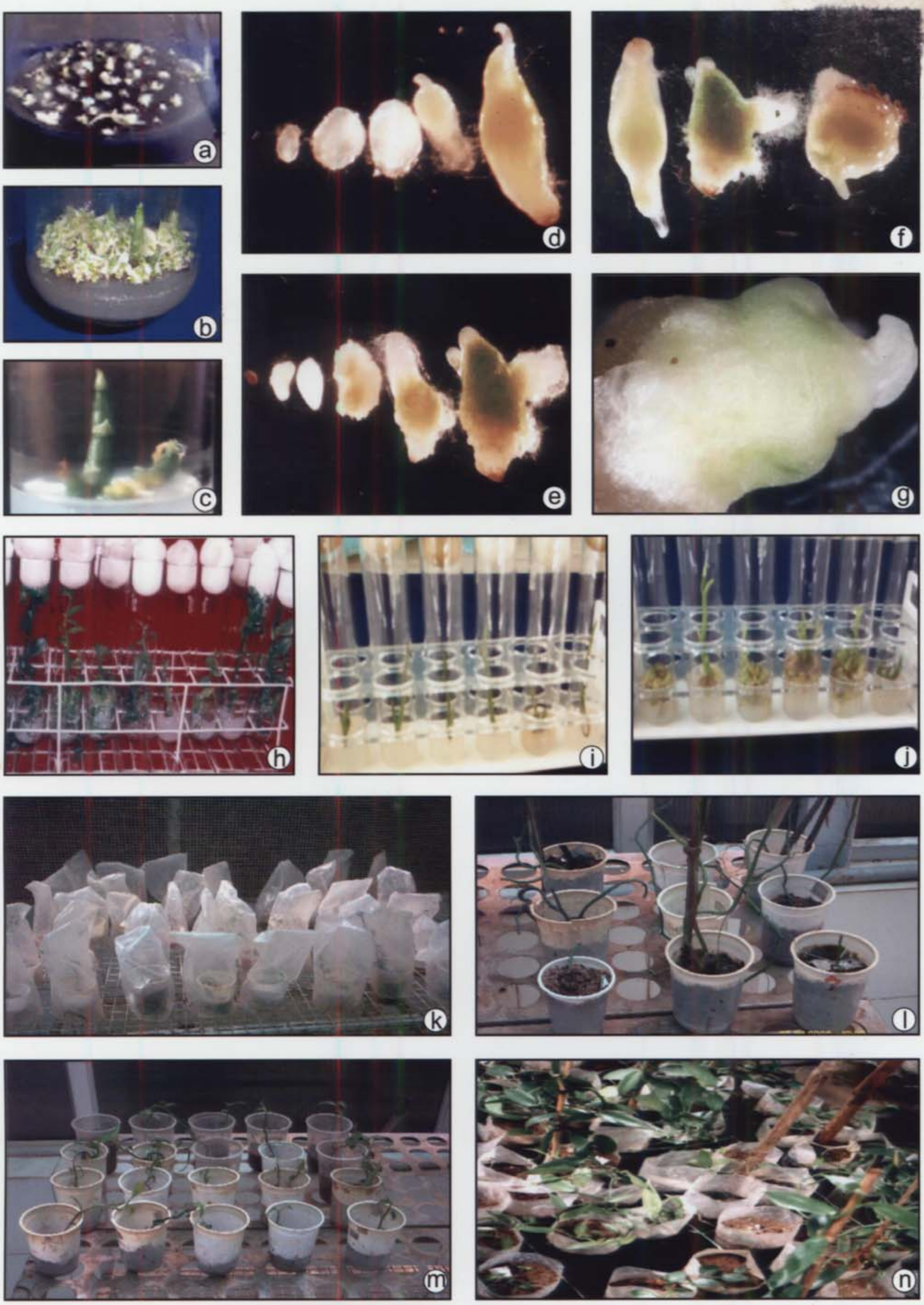

**Fig 2**: **Ovule culture and plant regeneration in selfed plants: a:** *V.planifolia* – seed culture; **b,c:** *V.aphylla* – seedculture, **d-f: Stages of germination**: **d:** *V.planifolia*; **e:** *V.aphylla*: **f:** germinated seeds of *V.planifolia*, *V.aphylla* & *V.tahetensis* in order; **g:** seed protocorm of *V.planifolia;* **h-j: seed derived progenies**: **h:** *V.planifolia*; **i:** *V.aphylla*; **j:** *V.tahetensis*; **k-m: hardening stages**: **k,m:** *V.planifolia*; **l:** *V.aphylla*; **n:** established progenies of *V. planifolia*

The segregation of the qualitative characters among the seedling progenies are represented in the form of Pie charts (Fig 33).

### ***Cytological characterization***

*Meiotic studies*:

Heterochromatin is distinctly visible as chromocenters in the inter phase nuclei of *V.planifolia*. Early diplotene stages showed even and thin chromatin interrupted by heterochromatic region, which seem to lie in pairs (Fig 3a). Interphase nuclei preceding diplotene showed darkly stained, compact and thick chromocenters whose number equals the diploid number of chromosomes (Fig 3b). Sporogenous cells during the early stages of meiosis almost showed 32 distinct and deeply stained chromocenters. The pairing exhibited by chromocenters is progressive through time as meiosis approached. This is evident from the intact nucleolus in the earlier stages and gradually disappearing in the later stages. The nearer the anthers were to having their sporogenous cells in meiosis, the larger the no. of cells having chromocenters. In the later stages, larger prochromosomes really represent two prochromosomes in close association making the total no. of chromosomes less than 32 (Fig 3c).

Early metaphase stage was characterized by the presence of bivalents and multivalents laying together, the number of pairs often less than 16 (Fig 3 d). During late metaphase homology between the bivalents makes the pairing units to stick together and appear as a single entity (Fig 3 e). The PMC of *V.aphylla* also showed multivalent formation during early and late metaphase (4a, b) and laggards during anaphase movement (Fig 4 c).

These studies showed that *V. aphylla* at the synchronized stage of pollen mitosis confirmed the chromosome number of n=32 (Fig 4d) and *V. planifolia* showed n=16 (Fig 3g). This in turn supported the chromosome number observed in the mitotic preparations (Fig 4g, k). Some of the pollen grains were characterized by chromosome numbers less than (Fig 3i) or greater than (Fig 3h, 4e) the normal gametic number, and a few chromosomes of pollen exhibited sticky nature (Fig 3j) and multivalents during different stages (Fig 3d, 4b).

Pollen stainability of *V. planifolia* showed low viability when stain absorption is taken as the criterion of viability. There were sterile pollen grains intermingled with fertile ones (Fig 3 k).

*Mitotic studies*:

Pretreatment in PDB along with 2-4 drops of 0.25% colchicine was found to be effective for the study of root tip mitosis

. The cytological examination of the progenies produced by selfing showed the expected number of 2n=32 in most of the *V planifolia* progenies (fig 4g). Apart from the diploid chromosome number some aneuploids with chromosome number less than 2n =32 were also observed (fig 4i). Cells showing mitotic associations were common in most of the preparations (fig 4h). Some plants were unstable with haploid cells intermingled with diploid cells (fig 4j).

Cytological study of the root tip cells of *V.aphylla* and *V.tahetensis* showed 2n = 60 + and 2n = 32 respectively (Fig 4k, 4l).

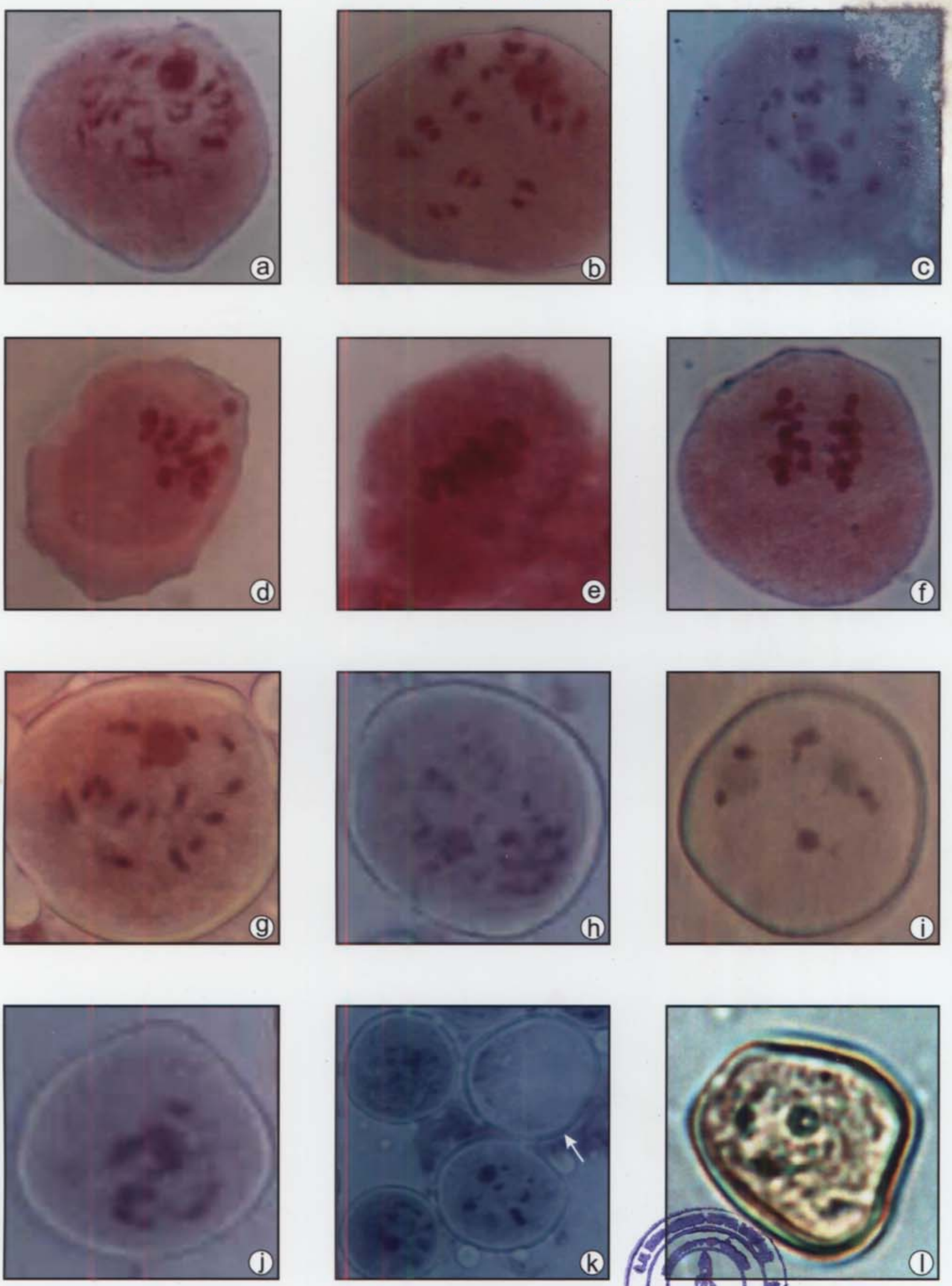

**Fig 3 :Cytological behavior of *V.planifolia* during meiosis of pollen mother cells**; **a:** diplotene bivalents sticking togather due to homology; **b:** diakinesis- chromocenters of homologous chromosomes in pairs; **c,d:** multivalent formation during early metaphase; **e**: late metaphase chromosomes undergoing pairing; **f:** anaphase movement; **g**: pollen 1st mitosis stage with normal 'n' number (n=16) of chromosomes; **h:** pollen chromosome number greater than normal gametic; **i:** pollen chromosome number less than the normal gametic; **j:** sticky chromosomes of pollen; **k:** sterile pollen; **l**: acetolysed pollen of *V. planifolia* showing porate aperture.

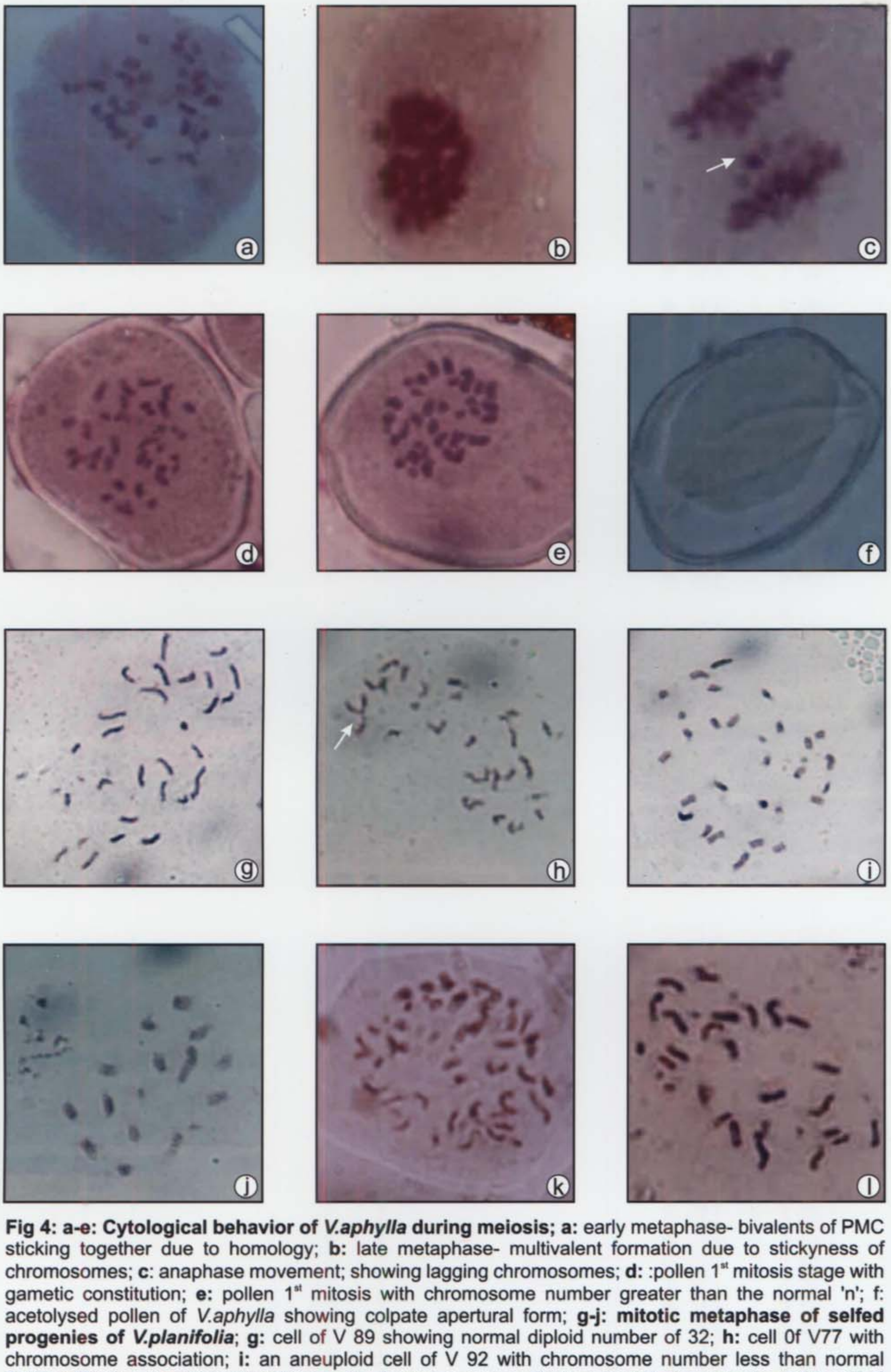

**Fig 4: a-e: Cytological behavior of *V.aphylla* during meiosis; a:** early metaphase- bivalents of PMC sticking together due to homology; **b:** late metaphase- multivalent formation due to stickyness of chromosomes; **c**: anaphase movement; showing lagging chromosomes; **d:** :pollen 1st mitosis stage with gametic constitution; **e:** pollen 1st mitosis with chromosome number greater than the normal 'n'; f: acetolysed pollen of *V.aphylla* showing colpate apertural form; **g-j: mitotic metaphase of selfed progenies of *V.planifolia***; **g:** cell of V 89 showing normal diploid number of 32; **h:** cell 0f V77 with chromosome association; **i:** an aneuploid cell of V 92 with chromosome number less than normal '2n'(2n=30); **j:** a haploid cell with gametic chromosome constitution; **k: mitotic metaphase in selfed progeny of *V.aphylla (2n=64)***; **l: mitotic metaphase in selfed progeny of *V.tahetensis* (2n=32).**

### ***Molecular characterization***

#### *Genotypes Used*

Fourteen lines from seed derived plants of *V. planifolia* were used in the present study. The parent line *V. planifolia* was used as control. V77, V89, V92, V113, V150, V218, V225, V233, V259, V260, V334, V335, V358 and V408 were the accessions used.

#### *DNA Isolation and Quantification*

Genomic DNA was successfully isolated from young and fresh leaves of vanilla plants using modified CTAB method of Ausubel *et al.*, (1995). High molecular weight, high quality DNA was obtained from spooled samples and these were used for RAPD studies. There were considerable amount of RNA contamination in the isolated samples.

#### *Purification of DNA*

To remove RNA contamination from the extracted samples, they are further purified by RNase treatment followed by phenol: chloroform : isoamyl alcohol (25:24:1) extraction and precipitation of DNA with isopropanol. Finally, it was resuspended in TE buffer and tested for quality and quantity of DNA. It was found that after purification, the RNA contamination was reduced substantially.

#### *Optimization of PCR Programming*

The optimized reaction conditions for PCR that gave good amplification are as follows.

Cycles: 3 I$^{st}$ cycle 94°C 2 minutes

40 °C 1 minute

72 °C 1 minute Cycle repeats: 1

II$^{nd}$ cycle: 94 °C for 2 minutes

40 °C for 30 seconds

72 °C for 1 minute Cycle repeats: 30

III$^{rd}$ cycle: 94 °C for 1 minute

40 °C for 1 minute

72 °C for 15 minutes Cycle repeats: 1

*Primer screening*

Twenty-four Operon decamer primers were tested for random amplification using genotype *V. planifolia* (Fig 5) with optimized PCR components. Most of them gave good amplification.

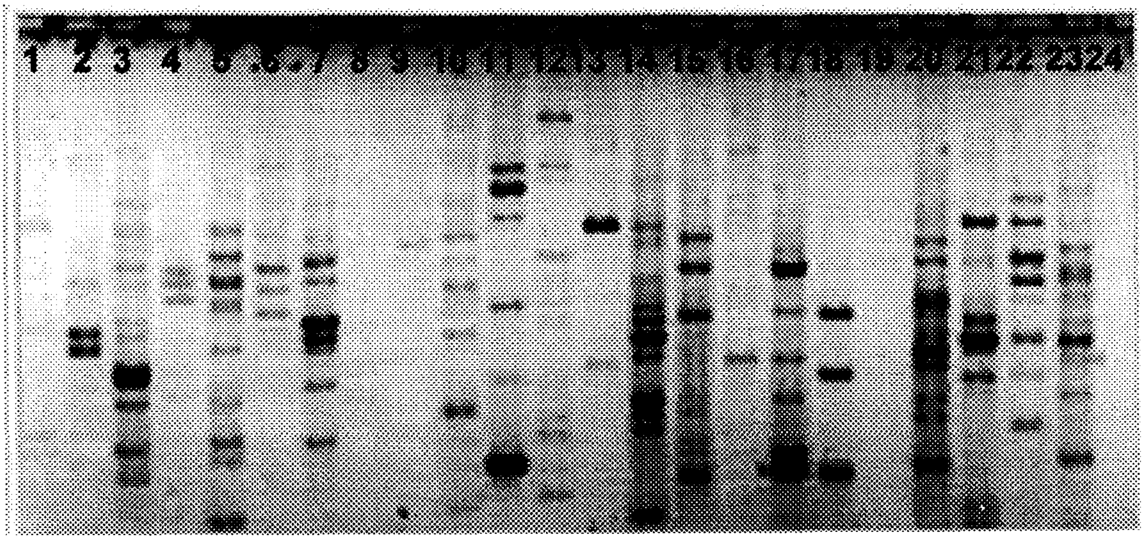

**Fig 5: RAPD amplification expressed by different OPERON primers in *Vanilla planifolia*.** 1.OPA 05 2.OPA 08 3. OPB 01 4.OPB 03 5. OPB 05 6.OPB 06 7. OPB 12 8. OPB 19 9. OPC 9 10. OPC 11 11. OPC 16 12. OPD 01 13. OPD 06 14. OPD 08 15. OPD 18 16. OPE 03 17. OPE 07 18. OPE 12 19. OPE 13 20. OPE 15 21. OPE 18 22. OPE 19, 23. OPF 10, 24. OPF 18.

*RAPD profiles of the seedling progenies using different primers*

The results of the six different primers are given below.

OPB 01: The primer gave good amplification of most of the genotypes (fig 6). The pattern shows absence of certain bands in a few genotypes. Four of the genotypes (V92, V218, V225, and V358) showed absence of clear band at 1 Kb region, indicating variations among the seed regenerated progenies.

OPB 05: This primer gave good amplification in all the genotypes studied (Fig 7). The RAPD profiles did show some amount of polymorphism at 0.5 Kb regions and 0.75 Kb regions. Two of the genotypes, V89 and V334 clearly showed distinct polymorphism from rest of the genotypes at 0.5 Kb regions.

OPE 07: This primer also amplified all the genotypes and gave fairly well RAPD profiles. However, most of the bands are exactly similar in all the genotypes except the band at 1 Kb region. This band was absent / less intense in V92, V218 and V225 (Fig 8).

OPE 12: This primer amplified the genotypes and developed detectable bands only at 0.5 Kb region, 1 Kb region and 1.5 Kb region but could not detect clear-cut polymorphism in any of the genotypes (Fig 9).

OPE 18: This primer gave excellent amplification in all the genotypes tested among the seed derived plants (Fig 10) and was characterized by the presence of more number of minor bands. The RAPD profiles indicated that genotypes V113 and V408 have different profiles with the absence of clear-cut bands at 0.75 Kb region which was present in other genotypes.

OPE 19: The primer could gave good amplification products in seed derived plants (Fig 11). However, major polymorphisms were not detected among the RAPD profiles developed using this primer in any of the genotypes.

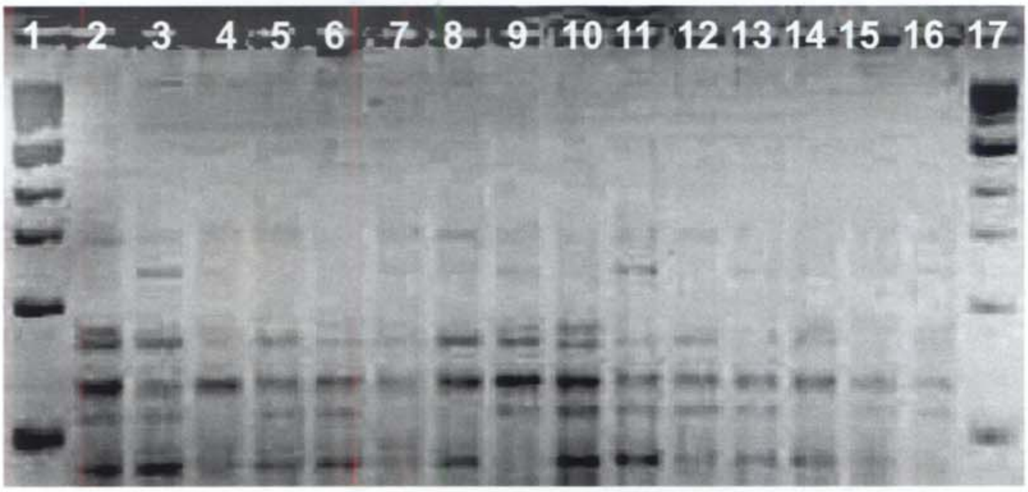

**Fig 6: RAPD polymorphism expressed by OPB 01 in seed derived progenies**

1. 1Kb ladder
2. V77
3. V89
4. V92
5. V113
6. V150
7. V218
8. V225
9. V233
10. V259
11. V260
12. V334
13. V335
14. V358
15. V408
16. *V. planifolia* (control)
17. 1Kb ladder

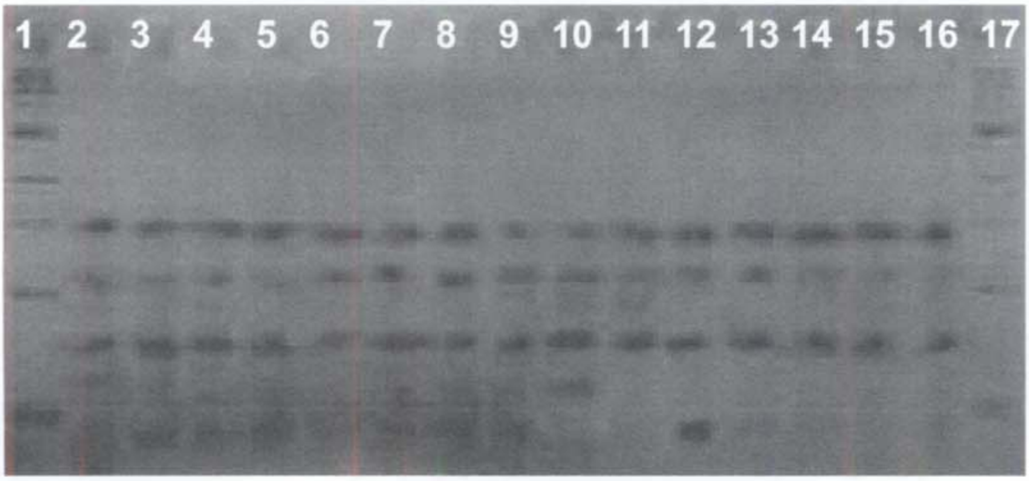

**Fig 7: RAPD polymorphism expressed by OPB 05 in seed derived progenies**

1. 1Kb ladder
2. V77
3. V89
4. V92
5. V113
6. V150
7. V218
8. V225
9. V233
10. V259
11. V260
12. V334
13. V335
14. V358
15. V408
16. V. *planifolia* (control)
17. 1Kb ladder

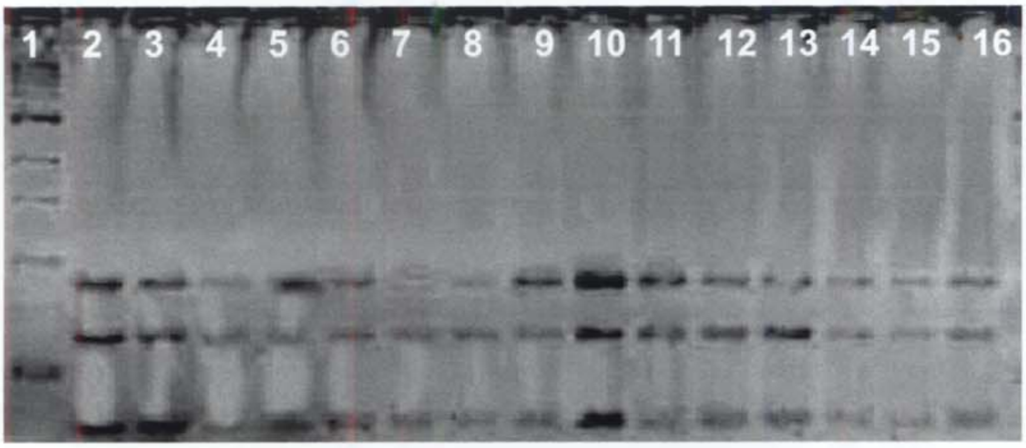

**Fig 8: RAPD polymorphism expressed by OPE 12 in seed derived progenies**

**1. 1Kb ladder**
**2. V77**
**3. V89**
**4. V92**
**5. V113**
**6. V150**
**7. V218**
**8. V225**
**9. V233**
**10. V259**
**11. V260**
**12. V334**
**13. V335**
**14. V358**
**15. V408**
**16. *V. planifolia* (control)**

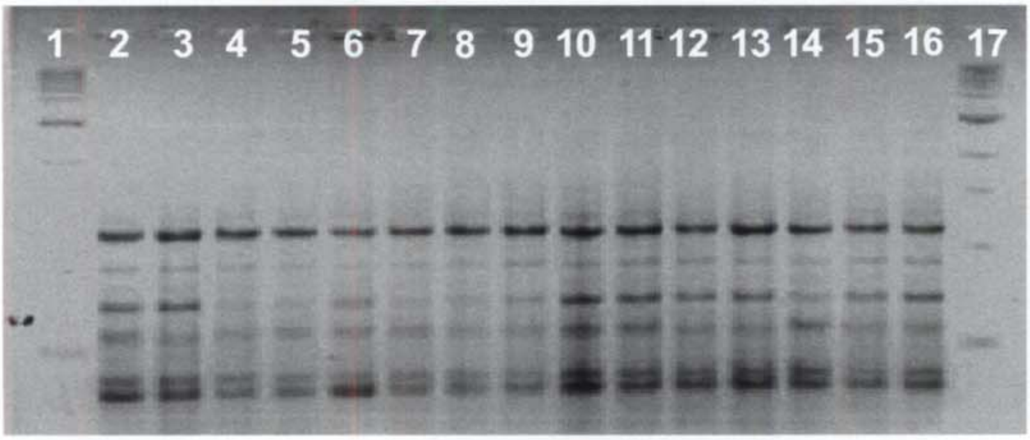

**Fig 9: RAPD polymorphism expressed by OPE 07 in seed derived progenies**

**1. 1Kb ladder**
**2. V77**
**3. V89**
**4. V92**
**5. V113**
**6. V150**
**7. V218**
**8. V225**
**9. V233**
**10. V259**
**11. V260**
**12. V334**
**13. V335**
**14. V358**
**15. V408**
**16. *V. planifolia* (control)**
**17. 1Kb ladder**

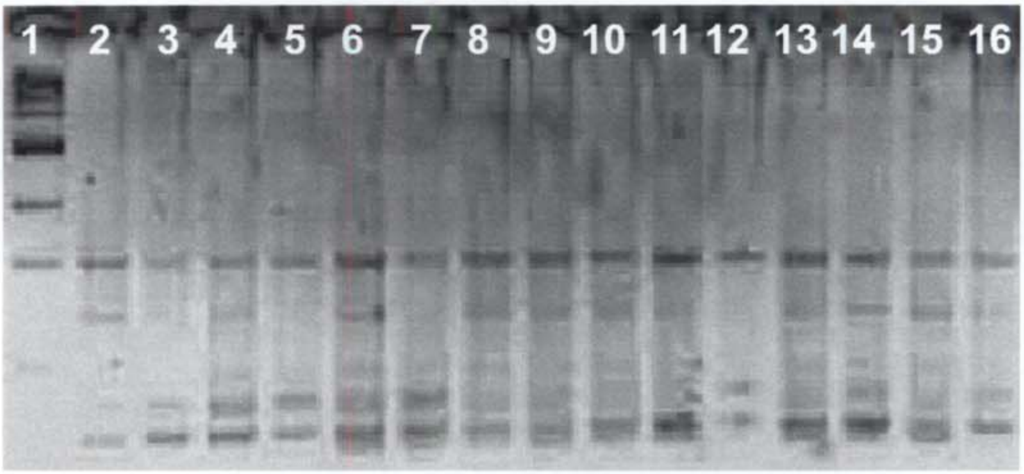

**Fig 10: RAPD polymorphism expressed by OPE 18 in seed derived progenies**

**1. 1Kb ladder**
**2. V77**
**3. V89**
**4. V92**
**5. V113**
**6. V150**
**7. V218**
**8. V225**
**9. V233**
**10. V259**
**11. V260**
**12. V334**
**13. V335**
**14. V358**
**15. V408**
**16. *V. planifolia* (control)**

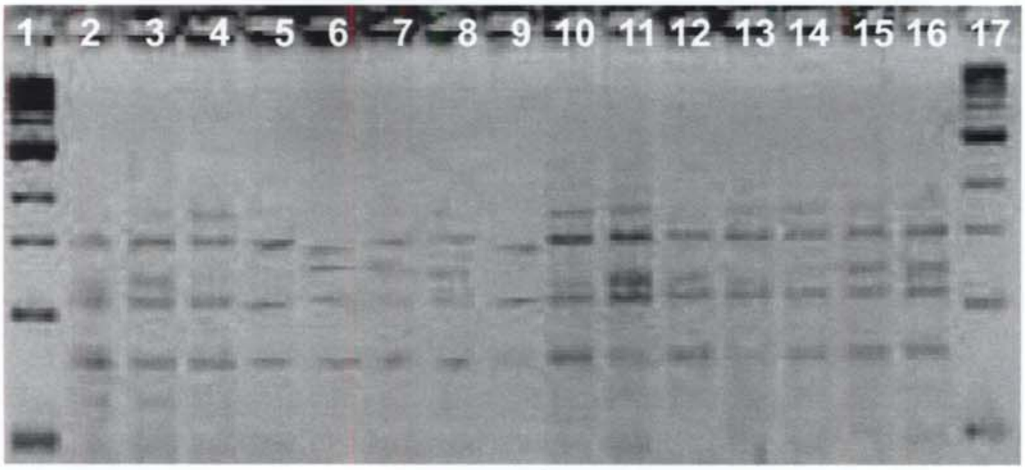

**Fig 11: RAPD polymorphism expressed by OPE 19 in seed derived progenies**

**1. 1Kb ladder**
**2. V77**
**3. V89**
**4. V92**
**5. V113**
**6. V150**
**7. V218**
**8. V225**
**9. V233**
**10. V259**
**11. V260**
**12. V334**
**13. V335**
**14. V358**
**15. V408**
**16. *V. planifolia* (control)**
**17. 1Kb ladder**

**Interspecific hybridization**

Embryo culture was done in the medium combination (MS + BA 1mg / l + IBA 0.5mg / l + Tryptone 2g / l), which gave good germination in the selfing experiment.

In the cross where *Vanilla tahetensis* was used as female parent, most of the seeds were shriveled, and none of them germinated. In the other three crosses (Fig 12 d, 12e, 12f) seed germination and further growth of the seedlings could be attained.

Eight different progenies (VH1, VH4, VH5, VH6, VH7, VH9, and VH15 & VH19) obtained from the cross between *V. planifolia* (♀) and V. *aphylla* (♂), six progenies (VH60, VH62, VH63, VH71, VH97 & VH99) from the cross *V.aphylla* (♀) X *V.planifolia* (♂) and four progenies ($VH_2$-1, $VH_2$-2, $VH_2$-3 & $VH_2$-4) from the cross *V.aphylla* (♀) X *V.tahetensis* (♂) were subjected to morphological, cytological and anatomical characterization.

***Morphological characterization***

Most of the hybrid plants showed a phenotypic similarity to the maternal parent (Table15, 16). Some progenies segregated into broad leaf type and narrow leaf type (Fig 12g, 12i). In the cross between *Vanilla aphylla (♀)* and *Vanilla planifolia (♂)*, maternal inheritance was obvious and all of them except one turned out to be albinos in different degrees (Fig 12h). *Vanilla aphylla* and the hybrids of the second cross come under the scale leaf morphotype (Fig 12j). In the other two crosses, the foliage leaf type and scale leaf type segregated in the progenies (Fig 12k, 12l).

## Table-15: Foliar morphology of interspecific hybrids and their parents

| **Lines** | **Leaf nature** | **Leaf shape** | **Leaf length** | **Leaf breadth** |
|---|---|---|---|---|
| **Parents** | | | | |
| *V.planifolia* | subsessile,succulant | elliptic, acuminate | 2.1 | 0.9 |
| *V.aphylla* | sessile, scaly | elliptic, acute | 0.5 | 0.3 |
| *V.tahetensis* | subsessile,succulant | oblong, acuminate | 1.6 | 0.5 |
| **Hybrids** | | | | |
| *V.planifolia* x *V.aphylla* | | | | |
| VH1 | subsessile,succulent | elliptic, acuminate | 2.27 | 1.13 |
| VH4 | sessile, scaly | oblong, acuminate | 0.6 | 0.3 |
| VH5 | sessile, scaly | oblong, acuminate | 0.5 | 0.2 |
| VH6 | subsessile,succulant | elliptic, acuminate | 2.3 | 1.16 |
| VH7 | sessile, succulant | lanceolate, acuminate | 1.8 | 0.3 |
| VH9 | sessile,succulant | lanceolate,acuminate | 1.2 | 0.35 |
| VH15 | subsessile,succulant | elliptic, acuminate | 0.9 | 0.5 |
| VH19 | subsessile,succulant | elliptic, acuminate | 0.3 | 0.3 |
| *V.aphylla* x *V.planifolia* | | | | |
| VH60 | sessile, scaly | oblong, acuminate | 0.2 | 0.2 |
| VH62 | sessile, scaly | elliptic, acute | 0.5 | 0.2 |
| VH63 | sessile, scaly | oblong, acuminate | 0.5 | 0.2 |
| VH71 | sessile, scaly | oblong, acute | 0.5 | 0.2 |
| VH97 | sessile, scaly | oblong, acuminate | 0.4 | 0.2 |
| VH99 | sessile, scaly | oblong, acuminate | 0.5 | 0.2 |
| *V.aphylla* x *V.tahetensis* | | | | |
| VH$_2$-1 | sessile, succulent | lanceolate, acuminate | 1.2 | 0.3 |
| VH$_2$-2 | sessile,succulant | lanceolate, acuminate | 0.7 | 0.2 |
| VH$_2$-3 | sessile, scaly | oblong, acuminate | 0.2 | 0.2 |
| VH$_2$-4 | sessile, scaly | oblong, acuminate | 0.4 | 0.3 |

***5th leaf from apex of *in vitro* plants (in cm).**

**Table-16: Cauline morphology of interspecific hybrids and their parents**

| **Lines** | **Stem colour*** | **Length of internode (in cm.)**** | |
|---|---|---|---|
| | | **Mean** | **Range** |
| **Parents** | | | |
| *V.planifolia* | green | 2.1 | 1.6 – 2.4 |
| *V.aphylla* | green | 1.5 | 1.2 – 2.3 |
| *V.tahetensis* | green | 1.3 | 1.1 – 1.5 |
| **Hybrids** | | | |
| *V.planifolia x V.aphylla* | | | |
| VH1 | green | 1.6 | 1.2 – 1.7 |
| VH4 | yellow | 1.6 | 1.4 – 1.7 |
| VH5 | yellow | 1.5 | 1.2 – 1.6 |
| VH6 | green | 1.4 | 1.2 – 1.7 |
| VH7 | green | 2 | 1.8 – 2.3 |
| VH9 | light green | 1.7 | 1.4 – 1.9 |
| VH15 | green | 1.4 | 1.3 – 1.7 |
| VH19 | green | 0.3 | 0.35 – 0.4 |
| *V.aphylla x V.planifolia* | | | |
| VH60 | dark green | 1.6 | 1.35 – 1.8 |
| VH62 | pale green | 1.2 | 1.09 – 1.4 |
| VH63 | yellow | 1.1 | 0.9 – 1.4 |
| VH71 | pale green | 2.1 | 1.8 – 2.2 |
| VH97 | pale green | 0.9 | 0.77 – 1.2 |
| VH99 | pale green | 1.2 | 1.0 – 1.36 |
| *V.aphylla x V.tahetensis* | | | |
| VH$_2$-1 | green | 1 | 0.8 – 1.1 |
| VH$_2$-2 | green | 0.5 | 0.22 – 0.76 |
| VH$_2$-3 | pale green | 0.4 | 0.25 – 0.5 |
| VH$_2$-4 | pale green | 0.4 | 0.3 – 0.55 |

***Based on visual observation  **5th node from apex of *in vitro* plants**

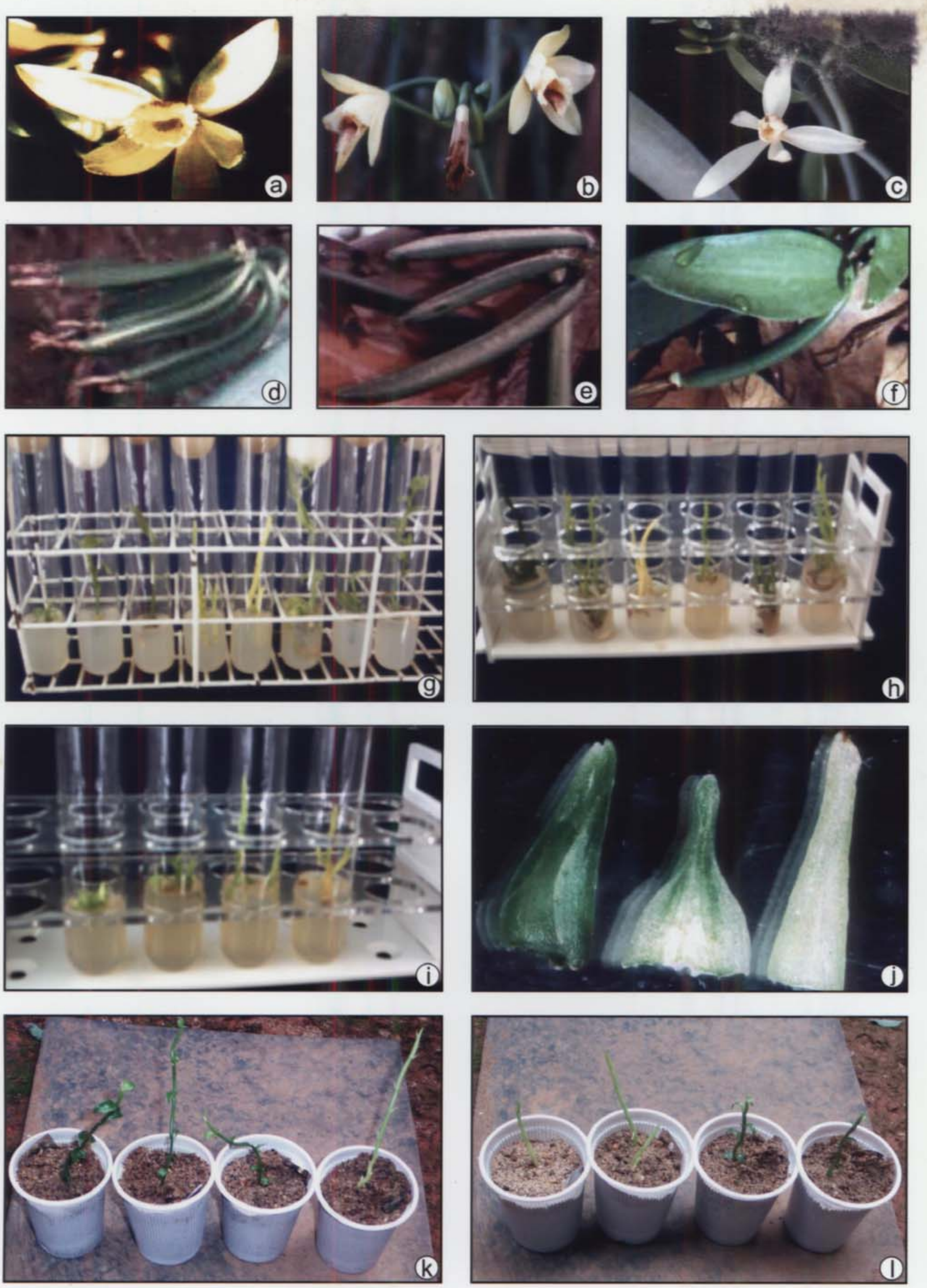

**Fig 12: Hybridization and plant regeneration**: **a:** *V.planifolia* flower; **b:** *V.aphylla* flower; **c:** *V.tahetensis* flower; **d:** V.planifolia fruits; **e:** *V.aphylla* fruits; **f:** *V.tahetensis* fruit; **g-i: interspecific hybrids: g:** *V.planifolia* x *V.aphylla*; **h:** *V.aphylla* x *V.planifolia*; **i:** *V.aphylla x V.tahetensis*; **j:** leaf variation among *V.aphylla x V.planifolia* hybrids; **k:** hardening of *V. planifolia* x *V.aphylla* hybrids showing segregation of maternal and paternal characters ; **l:** hardening of *V.aphylla x V.tahetensis* hybrids showing segregation of maternal and paternal characters.

***Cytological characterization***

*Mitotic studies*: The somatic chromosome number was determined for the parents and the hybrids. Somatic associations of chromosomes were evident in most of the preparations. The chromosome number of the parents *V planifolia* (Fig 4g) and *V aphylla* (Fig 4 k) were 32 and 64 respectively and that of *V.tahetensis* was 2n=32 (Fig 4 l).

The chromosomal number of some of the progenies derived from the cross *Vanilla planifolia* (♀) x *Vanilla aphylla* (♂) was 2n = 48 (Fig 13 c, d); they included VH7, VH6 and VH19. The other progenies of this cross had a chromosome number of 2n=32 (Fig 13 a, b) and 2n=64; VH1, VH9 and H15 had 32 chromosomes as their maternal parent and VH4 and VH5 had 64 chromosomes each resembling *Vanilla aphylla.*

The progenies recovered from the cross *Vanilla aphylla* (♀) x *Vanilla planifolia* (♂) had around 60 chromosome (Fig 13 e, f). Among the progenies of the cross between *V. aphylla* (♀) and *V.tahetensis* (♂), $VH_2$-1 and $VH_2$-2 showed 2n=32 and $VH_2$-3 and $VH_2$-4 showed 2n > 60 as their diploid number.

***Anatomical characterization***

*i. Species*

The results of the anatomical study of the 7 species are given below:

There are two morphoforms in *Vanilla*; Based on gross morphological features, the following groups can be recognized: Group 1- those with persistent foliage leaves; group II -those with scale leaves (Table-17, Fig 14 a-h).

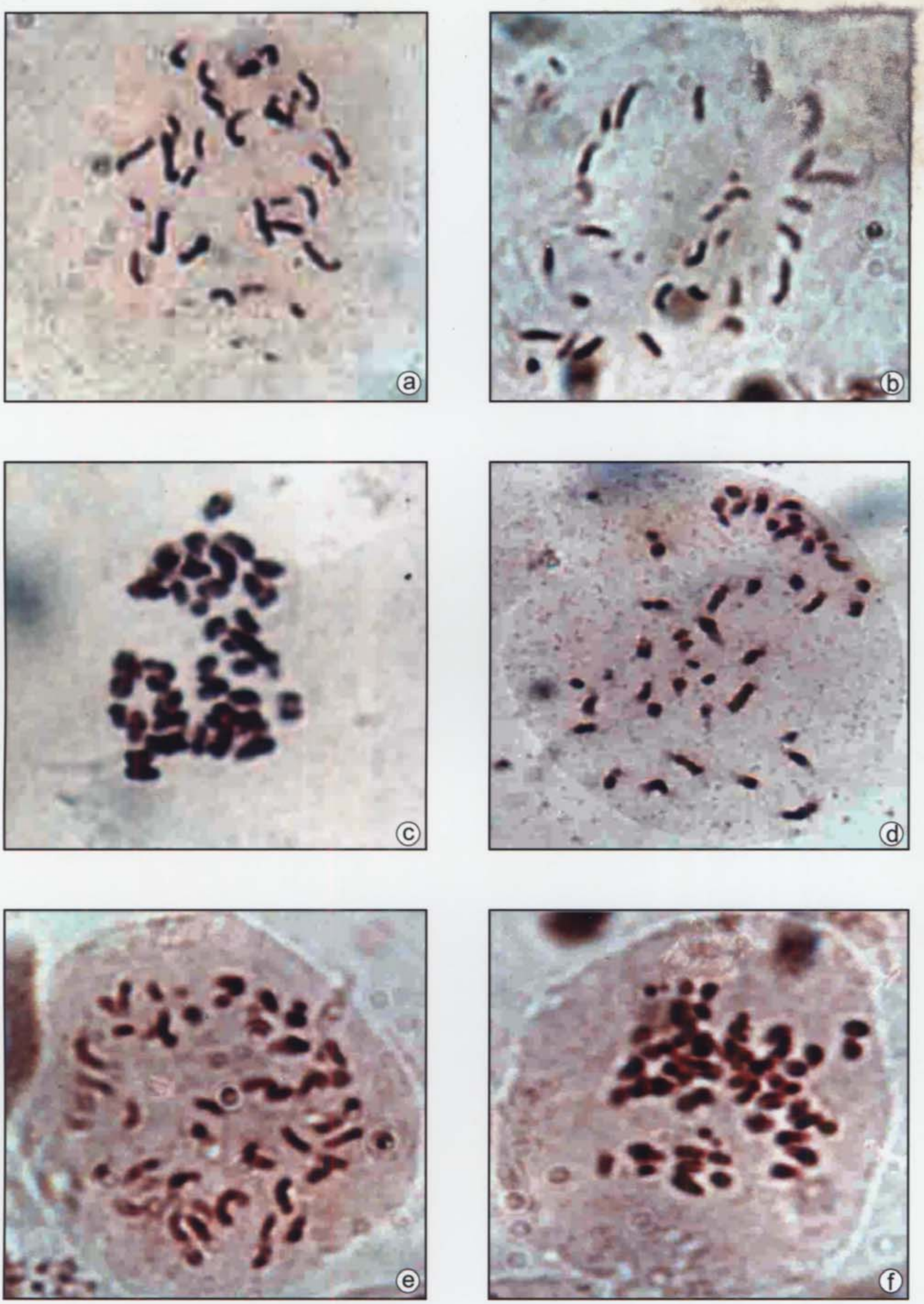

**Fig 13: a-f: Mitotic metaphase in interspecific hybrids; a:** VH 9 showing 32 chromosomes; **b:** VH 15 showing 32 chromosomes; **c:** V H 1 with 48 chromosomes; **d**: VH 7 with 48 chromosomes; e: VH 60 with 64 chromosomes; **f:** VH5 with 60+ chromosomes.

Leaf:

In outline, scale leaf is dorsiventral and broadly V- shaped on adaxial side in the mid-rib region, edges of the scale tapering. Both sides of the leaves have a surface covered with cutinous layers. The upper surface is covered with a thicker cutin layer in all the leafy species. The thick adaxial cuticle is present in varying degrees. In scale leaf, type the cuticle is equally thin on both surfaces.

**Table-17: List of species used in anatomical investigation:**

| **Species studied** | **Centre of distribution** | **Morphological type** | **Reference** |
|---|---|---|---|
| *V. planifolia* Andr. | Mexico, C America | Group I * | Purseglove,1974 |
| *V. aphylla* Blume. | Malaya, Kerala, Tamil Nadu | Group II ** | Backer & Bakhuizen,1968 |
| *V. andamanica* Rolfe. | Andaman Islands | Group I | Rolfe, 1918 |
| *V. pilifera* Holt. | Malaya, Tailand and Assam | Group I | Borthakur & Hajra, 1976 |
| *V.tahitensis* Moore. | Tahiti and Hawaiian Islands | Group I | Purseglove, 1974 |
| *V. whightiana* Lindl. | Andhra Pradesh, Karnataka | Group II | Hooker, 1890 |
| *V. walkierie* Wight. | South India to Sreelanka | Group II | Hooker, 1890 |

***Group 1**- those with persistent foliage leaves, ** **Group II** - those with scale leaves.

Stomata basically anomotetracytic and abaxial, *i.e.*, hypostomatic except in *Vanilla aphylla* where they are seen on both the surfaces. In *Vanilla pilifera* the characteristic 'floating stomata' in mature leaves is due to the partial dissolution of the anticlanal walls of the subsidiary cells.

The stomata are oval, with the long dimension running parallel to the length of the leaf. Substomatal chambers are present and vary in size. The leaves in general are hypostomatic, but in some scale leaf types the stomata is present on both sides. The guard cells usually lie at the level of the adjacent epidermal cells. However, in *Vanilla tahetensis* they are slightly sunken (Fig 14n). The guard cells are of equal size and they are unevenly thickened, the thickenings being heavy along the aperture. Stomatal measurements show considerable interspecific variability (Table-18). The smallest stomata were observed in *V.pilifera* (29 x 26 µm) and the largest in *V. tahetensis* (51 x 43 µm). Table-19 contains stomatal index.

**Table-18: Quantitative data on foliar stomatal size.**

| Sl# | Species | Stomatal size (in µm). | | |
|---|---|---|---|---|
| | | **Average (l x b)*** | **l / b** | **Range (l x b)** |
| 1 | *V. planifolia* Andr. | 45 x 30 | 1.5 | 42-48 x 28-31 |
| 2 | *V. aphylla* Blume. | 33 x 25 | 1.3 | 30-35 x 23-27 |
| 3 | *V. andamanica* Rolfe. | 42 x 37 | 1.1 | 37-46 x 33-41 |
| 4 | *V. pilifera* Holt. | 29 x 26 | 1.1 | 25-31 x 23-29 |
| 5 | *V. tahitensis* Moore. | 51 x 43 | 1.2 | 47-53 x 40-46 |
| 6 | *V. whightiana* Lindl. | 37 x 30 | 1.2 | 32-39 x 27-34 |
| 7 | *V. walkierie* Wight. | 31 x 27 | 1.1 | 27-34 x 24-30 |

* (l x b): length x width

**Table-19 : Quantitative data on foliar stomatal index**

| Sl# | Species | Stomatal index ( S I ) * |
|---|---|---|
| 1 | *V. planifolia* Andr. | 3.9 |
| 2 | *V. aphylla* Blume. | 4.6 |
| 3 | *V. andamanica* Rolfe. | 2 |
| 4 | *V. pilifera* Holt. | 5.9 |
| 5 | *V. tahitensis* Moore. | 3.7 |
| 6 | *V. whightiana* Lindl. | 7 |
| 7 | *V. walkierie* Wight. | 5.3 |

- S I of abaxial leaf surface

The epidermal cells are thick walled and sclerified. The cells of the upper surface tend to be somewhat larger than those of the lower surface. A uniseriate suberised hypodermal layer underlies the epidermis (Fig 14n). Uniseriate hypodermis present in most of the species except for *V.aphylla* where it is present only on abaxial surface. Chloroplasts are generally higher in young cells than in more mature ones. The concentration of chloroplasts in the epidermis is somewhat less than in cells from the interior of the leaf.

Mesophyll cells are mostly rounded; and are homogeneous; *i.e.* not differentiated into palisade and spongy parenchyma (Fig 14 m). Mesophyll 8-15 layered, and rich in chloroplasts. Mesophyll cells are polygonal to oval with small intercellular spaces, a few of them have needle-like raphide bundles (crystalliferous idioblasts) and such cells are devoid of chloroplasts. In scale - leaf types the mesophyll cells are irregular. Chloroplasts are present in greater concentration towards the adaxial side of the leaf.

Vascular bundles travel parallel to the leaf for its entire length and smaller bundles alternates with the larger ones (Fig 14 m). Their shape ranged from oval to elliptic – oblong. In scale leaf types the bundles are closer towards adaxial side. In each vascular bundle xylem is placed towards the adaxial surface and the phloem towards the abaxial surface of the leaf (Fig 14 m), The individual bundle is composed of xylem and phloem surrounded by and often separated from each other by a layer of sclerenchyma fibers. A sclerenchymatous sheath surrounds the bundles and is more towards the phloem side. In most cases a sclerenchymatous girder encircles the bundles except for few very small bundles where they may be seen on the phloic side as a patch; they are completely absent in *Vanilla wightiana*. In all the species studied, alternate vascular bundles are smaller and the smaller ones are more remote from the adaxial side and sclerenchyma does not encircle them completely. The size of the vascular bundles and the supporting sclerenchyma girders seem to show difference between the different species (Table-20).

**Table-20: Quantitative data on foliar vascular bundle size**

| Species | Vascular bundle size ( in µm ) | | | |
| --- | --- | --- | --- | --- |
| | Small bundle | | Large bundle | |
| | Mean (l x w) | Range (l x w)* | Mean (l x w) | Range (l x w) |
| *V. planifolia* Andr. | 24 x 90 | 21-26 x 81-93 | 192 x 384 | 181-203 x 371-392 |
| *V. aphylla* Blume. | 75 x 94 | 70-87 x 87-99 | 135 x 270 | 128-142 x 265-281 |
| *V.andamanica* Rolfe. | 85 x 108 | 81-92 x 101-116 | 90 x 115 | 82-98 x 147-160 |
| *V. pilifera* Holt. | 86 x 105 | 79-88 x 98-111 | 111 x 117 | 106-122 x 96-128 |
| *V. tahitensis* Moore. | 22 x 86 | 18-27 x 78-89 | 152 x 329 | 143-166 x 315-340 |
| *V. whightiana* Lindl. | 69 x 88 | 63-75 x 83-95 | 120 x 243 | 113-127 x 226-257 |
| *V. walkierie* Wight. | 61 x 77 | 53-67 x 70-82 | 132 x 255 | 119-143 x 241-270 |

- (l x w): length x width

The feature that seems to differentiate the mature foliage leaves from the scale leaves besides thicker epidermal walls and greater cutinous covering is the presence of a series of transverse bundles running through the mesophyll. These bundles are composed of sclerenchyma fibers and vascular elements.

### Stem

Stems are photosynthetic, terete or angled. In outline stem is almost circular in the leafy species, bilobed with two grooves in *Vanilla wightiana* and *Vanilla walkeriae.* Stomata anomotetracytic; hypodermis single layered isodiametric cells without intercellular spaces. Cortex 10-16 layered, and composed of circular to polygonal, thin walled parenchymatous cells. The outer cortical cells are comparatively smaller than those of the middle region (Fig 15 d). Stems of some species have 1-3 layered, continuous, thick walled sclerenchyma cells at the extreme innermost region of the cortex, which enclosed the ground tissue (Fig 15 a). Some species lack the sclerenchyma band (Table-21, Fig 15 b), and these species have large sized hyaline water storage cells in the ground tissue (Fig 15 e).

**Table-21: Qualitative data on cauline sclerenchyma band**

| Sl# | Species | Sclerenchyma band |
|---|---|---|
| 1 | *V. planifolia* Andr. | present |
| 2 | *V. aphylla* Blume. | absent |
| 3 | *V. andamanica* Rolfe. | absent |
| 4 | *V. pilifera* Holt. | absent |
| 5 | *V. tahitensis* Moore. | present |
| 6 | *V. whightiana* Lindl. | absent |
| 7 | *V. walkierie* Wight. | absent |

Ground tissues among the stems falls under three categories; consisting solely of assimilatory cells, mixed assimilatory and water storage cells and assimilatory cells in the center of the ground tissue surrounded by water storage cells (Table-22, Fig 15 d,e,f). Assimilatory cells are isodiametric and characterized by the presence of chloroplasts and test positively for starch (Fig 15 c). Water storage cells are bigger in size, are hyaline with their characteristic-pleated cell walls (Fig 15 e).

**Table-22: Qualitative data on cauline ground tissue pattern**

| Sl# | Species | Type I * | Type II ** | Type III *** |
|---|---|---|---|---|
| 1 | *V. planifolia* Andr. | + | -- | -- |
| 2 | *V. aphylla* Blume. | -- | + | -- |
| 3 | *V. andamanica* Rolfe. | -- | -- | + |
| 4 | *V. pilifera* Holt. | -- | -- | + |
| 5 | *V. tahitensis* Moore. | + | -- | -- |
| 6 | *V. whightiana* Lindl. | -- | + | -- |
| 7 | *V. walkierie* Wight. | -- | + | -- |

+: present; --: absent

**Type I** *: Ground tissue completely filled with assimilatory cells,

**Type II** ** Ground tissue with peripheral water storage cells and central core of assimilatory cells,

**Type III** *** Ground tissue with mixed assimilatory and water storage cells.

Vascular bundles are collateral and closed, 15-25 in number which lie scattered in the ground tissue and they vary greatly in their size and shape (Fig 14 j, Table-23). Smaller bundles are towards the periphery and larger ones towards the center (Fig 14a) and most of the bundles are completely encircled by sclerenchyma (Fig 14 i). Stem of *V.planifolia* has the central two bundles lying adjacent to each other (Fig 14 k).

**Table-23: Quantitative data on cauline vascular bundle size**

| Species | Vascular bundle size ( in µ m ) | | | |
|---|---|---|---|---|
| | **Small bundle** | | **Large bundle** | |
| | **Mean (l x w)** | **Range (l x w)*** | **Mean (l x w)** | **Range (l x w)** |
| *V. planifolia* Andr. | 90 x 180 | 85-94 x 173-189 | 210 x 256 | 202-216 x 248-260 |
| *V. aphylla* Blume. | 53 x 60 | 49- 57 x 56-64 | 151 x 213 | 145-158 x 206-215 |
| *V.andamanica* Rolfe. | 136 x 264 | 131-146 x 258-269 | 227 x 274 | 224-236 x 265-279 |
| *V. pilifera* Holt. | 109 x 127 | 93-115 x 121-133 | 146 x 204 | 140-155 x 195-211 |
| *V. tahitensis* Moore. | 114 x 191 | 104-119 x 184-198 | 219 x 258 | 213-225 x 253-263 |
| *V. whightiana* Lindl. | 70 x 91 | 66-75 x 87-97 | 175 x 231 | 171-184 x 222-236 |
| *V. walkierie* Wight. | 67 x 79 | 62-71 x 74-88 | 133 x 188 | 127-138 x 182-197 |

* (l x w): length x width

## Root

The anatomy of the terrestrial and epiphytic types of roots shows significant differences. The epiphytic roots are characterized by the presence of uniseriate velamen tissue, the presence of root hairs only in points where it is attached to the substratum and the presence of chloroplasts in the root cortex. In the terrestrial roots the velamen layer and chloroplasts are lacking and they have root hairs (Fig 15 l).

Aerial roots circular in outline; terrestrial roots usually wavy in outline. Velamen uniseriate in all the species studied and consists of radially elongated rectangular cells with thick cutinized walls. Epivelamen generally gets ruptured in mature roots.

Epidermal hairs present in terrestrial roots. In aerial roots of *Vanilla tahetensis*, hairs seen in the surface attached to the support. Exodermis uniseriate and consists of polygonal to oval, elongated, thick walled cells except in the aerial roots of *Vanilla andamanica* and *Vanilla pilifera* where the walls remain thin. Cortex is 9-18 layered, comprising polygonal to oval, thin walled parenchymatous cells (Fig 15 j); cells of the outer few layers are relatively smaller and slightly lignified packed without intercellular spaces but the innermost layers are unlignified. In the aerial roots of some species (*e.g.*, *V. planifolia* ), the inner most cortical region shows circular to irregularly shaped air cavities around the stellar cylinder whose number is equal to the number of xylem rays (Fig 15 k). The cells of terrestrial roots of all the species lack chloroplasts while the aerial roots are with chloroplasts. Cortical cells are packed with starch grains. Endodermis uniseriate, cell walls thickened in the inner tangential walls and lateral walls in *Vanilla andamanica*, thickened throughout in *Vanilla planifolia* (Fig 15 k), and unthickened in *V.aphylla* (Fig 15 i). Intermittent thin walled passage cells are seen opposite to xylem rays. Pericycle cells completely thickened (Fig 15 j). The xylem rays may be multiarch with narrow pith as in *Vanilla aphylla* (Fig14i); or with wide pith as in *Vanilla planifolia* (Fig 15 k). The pith cells also showed interspecific differences, and may be either sclerenchymatous or parenchymatous (Fig 15 h, g ).

Anatomical characters of the different species were studied and the anatomical key (Table-24) to the species was prepared, which was used to distinguish species in vegetative condition.

**Table-24: Anatomical Key to vanilla species**

Stem cortex with assimilatory cells and / or large sized water storage cells; foliar vascular bundles characterized by alternate smaller and larger bundles which are encircled by sclerenchymatous girder; stomata anomocytic; calcium oxalate crystals; xylem vessels larger with scaleriform pitting; vascular tissue of root embedded in sclerenchymatous matrix........... ***Vanilla***

Stem cortex with sclerenchymatous ring; ground tissue with only assimilatory cells; ........... ***V. planifolia, V. tahitensis***

Inner cortex (of stem) with starch grains; vascular bundles with 3-5 vessels.... ...... ***V. planifolia***

Inner cortex (of stem) without starch grains; vascular bundles with 2-3 vessels.... ......... ***V.tahitensis***

Stem cortex without sclerenchymatous ring; ground tissue with water storage cells ... ***V. andamanica, V. pilifera, V. aphylla, V. walkierie, V .whigtiana***

Ground tissue (of stem) with mixed assimilatory and water storage cells...... ..... ***V. andamanica. V. pilifera***

Stem terete; vascular tissue of aerial roots embedded in sclerenchyma... ...... ***V. andamanica***

Stem bilobed; vascular tissue of aerial roots embedded in parenchyma... ....... ***V. pilifera***

Ground tissue (of stem) with water storage cells only........... ***V. aphylla, V. walkierie, V. whigtiana***

Pericycle cell walls of aerial roots unthickened; stomata lacking on adaxial leaf surface................ ***V. aphylla***

Pericycle cell walls of aerial roots thickened; stomata present on adaxial leaf surface ......... ***V. pilifera, V. walkierie, V. whigtiana***

Stomata(at maturity) floating type ... ***V. pilifera***

Stomata(at maturity) not floating type.... ***V.walkierie, V whigtiana***

Cauline hypodermis present ......... ***V. whigtiana***

Cauline hypodermis absent ......... ***V. walkierie.***

The results obtained from the present investigation revealed a number of interesting features that may be useful in classifying these plants, which are otherwise confusing. The species could be demarcated into two distinct classes - the New World Species and Old World species. The New World species (*V.planifolia* and *V.tahetensis*) share many common anatomical characters and the rest of the species, which belongs to the Old World; have their own common anatomical characters (Table-25).

**Table-25: Classification of the 7 species into two major groups based on anatomical differences.**

| Sl# | **New World species** (*V.planifolia,V.tahetensis*) | **Old World species** (*V aphylla, V andamanica V pilifera, V whightiana, V walkierie)* |
|---|---|---|
| | STEM CHARACTERS | |
| 1 | Cortex with sclerenchymatous ring | sclerenchymatous ring abesent |
| 2 | Ground tissue filled with assimilatory cells | Water storage cells intermixed with few assimilatory cells |
| 3 | Sclerenchymatous bundle cap | Sclerenchymatous bundle sheath |
| 4 | Small size of vascular bundle | Vascular bundle comparatively larger. |

| | | |
|---|---|---|
| | compensated by its greater number. | |
| 5 | Pith narrow or lacking | Prominent pith |
| | ROOT CHARACTERS | |
| 6 | Inner cortex with lacunae whose no. is equal to the no. of radial bundles | Lacunae absent |
| | LEAF CHARACTERS | |
| 7 | Foliar stomatal size higher (~ 50 x 40 m) | Foliar stomatal size lower (~ 30 x 27 m) |
| 8 | Stomatal index lower (<4 ) | Stomatal index higher (<4) except in *V andamanica* |
| 9 | Vascular row is more towards the center of the mesophyll. | Vascular row is more towards adaxial surface.* |
| 10 | Mesophyll tissue circular to oval | Mesophyll tissue irregular |

*Transverse bundles present in the leaf mesophyll in *V. andamanica* which is composed of sclerenchyma fibers and vascular elements.

*ii. Interspecific hybrids:*

Based on the results given above, the segregation of the anatomical features among the hybrids was investigated and is as given below:

*V.planifolia*, *V.tahetensis* and the hybrids VH1, VH6, VH7, VH9, VH15, $VH_2$-1 and $VH_2$-2 come under the category with persistent foliage leaves (Fig 12g, 12h, 12i). All the other hybrids are with scale leaves very much resembling the parent *V. aphylla* (Table-26, Fig12j).

The presence of cauline sclerenchymatous band between cortex and ground tissue in *Vanilla tahetensis* and *Vanilla planifolia* and some of the hybrids can be contrasted with its absence in *Vanilla aphylla* and a few hybrids (Fig 17 a, 17b).

The mesophyll cells may be oval as in VH7 or irregular as in VH19 (Fig 17 g). Foliar vascular bundles may be towards abaxial side as in VH62 (Fig 17 e) or towards the centre of the leaf (Fig 17 f).

The water storage cells in the cauline ground tissue are characteristic of *Vanilla aphylla* in contrast to the assimilatory cells in *Vanilla planifolia* and *Vanilla tahetensis*. While a few hybrids showed an intermediate character of mixed water storage and assimilatory cells, most of the hybrid progenies resembled either of their parents (Table-27, Fig 17 d). However, they differed from the paternal parent in their triarch root stele (Fig 17 h), the parenchymatous pith (Fig 17 k) and the deformed chloroplasts (Fig 17 d).

The hybrids VH4 and VH5 differed from the other hybrids with regard to the area occupied by each tissue in the root, they possessing bigger proportions of cortex and lesser of stele (Fig 17 l). The hybrids differed from themselves in the shape and size of the pith; VH99 having interrupted and wavy pith (Fig 17 j) unlike $VH_2$ 4, which has continuous and circular pith (Fig 17 k).

Thus, many of the anatomical features of the parents segregated and a few showed intermediacy among the hybrids. Twenty unit characters (Table 26) of vegetative anatomy of the three species and eighteen hybrids were scored and given as a character matrix (Table-27) which was used as the basis for analyzing the segregation pattern among the hybrids. A dendrogram was constructed based on the scored values and used to study the interrelationships between the hybrids (Fig 16).

**Table-26: Characters used for analysis of segregation pattern among the hybrids.**

1. Vines with foliage leaves (0); vines with scale leaves (1)
2. Vines chlorotic (0); vines green (1)
3. Stomata lacking on adaxial leaf surface (0); stomata present (1)
4. Foliar epidermal crystals lacking (0); present (1)
5. Foliar adaxial hypodermis absent (0); present (1)
6. Foliar bundle sheath corona absent (0); present (1)
7. Mesophyll cells oval (0); irregularly shaped (1)
8. Foliar vascular bundles towards abaxial side (0); towards center (1)
9. Cauline sclerenchymatous ring lacking (0); present (1)
10. Water storage cells lacking in cauline ground tissue (0); present (1)
11. Stem more or less terete (0); stem bilobed (1)
12. Cauline hypodermis absent (0); present (1)
13. Root pith parenchymatous (0); sclerenchymatous (1)
14. Root pith discontinuous (0); continuous (1)
15. Root pith cells circular to oval (0); polygonal (1)
16. Chloroplasts lacking in pith cells of roots (0); chloroplasts present (1)
17. Pericycle cell walls of unthickened (0); thickened (1)
18. Endodermal cell walls of unthickened (0); thickened (1)
19. Root cortex without lacunae radiating from stele (0); lacunae present (1)
20. Root stele with primary xylem points <4 (0); ≥ 4 (1).

**Table-27: Character state values of taxa used for analysis of segregation pattern.**

| Taxon | Character |
|---|---|
| | 11 1111 11 1 12 |
| | 1 234 567 8901 2345 678 90 |
| *V.planifolia* | 0 1 0 1 1 0 1 0 1 0 0 1 0 1 1 1 1 1 1 1 |
| *V.aphylla* | 1 1 1 0 0 1 0 1 0 1 1 1 1 1 1 0 0 0 0 1 |
| *V.tahetensis* | 0 1 0 1 1 1 0 1 1 0 0 1 0 1 0 1 1 0 0 1 |
| VH1 | 0 1 0 0 1 1 1 0 1 0 0 1 0 1 1 1 0 0 1 1 |
| VH4 | 1 0 1 0 0 0 0 1 0 1 0 1 0 0 1 0 0 0 0 0 |
| VH5 | 1 0 1 0 1 0 0 1 0 1 1 1 0 0 1 0 0 0 0 0 |
| VH6 | 0 1 0 0 1 1 1 0 0 0 0 1 0 1 1 1 1 1 0 1 |
| VH7 | 0 1 0 1 1 0 0 0 1 0 0 1 0 1 1 1 0 0 0 1 |
| VH9 | 0 1 0 0 1 0 1 1 1 1 0 1 0 1 1 0 0 0 1 1 |
| VH15 | 1 1 0 0 1 0 1 0 1 0 0 1 0 1 1 1 1 0 1 1 |
| VH19 | 1 1 0 1 1 0 1 0 0 0 0 1 0 1 1 1 0 0 1 1 |
| VH60 | 1 1 0 0 0 1 0 0 0 1 0 1 1 1 1 1 0 0 0 1 |
| VH62 | 1 0 1 0 1 1 0 0 0 1 0 1 0 1 1 0 0 0 0 0 |
| VH63 | 1 0 1 0 0 1 0 1 0 1 0 0 1 0 1 0 0 0 0 0 |
| VH71 | 1 0 1 0 1 1 0 1 0 1 0 0 0 1 1 0 0 0 0 1 |
| VH97 | 1 0 1 0 0 0 0 1 0 1 0 1 0 0 1 0 0 0 0 0 |
| VH99 | 1 0 1 0 0 0 0 1 0 1 0 0 0 1 1 0 0 0 0 1 |
| VH$_2$-1 | 0 1 0 0 1 1 0 1 0 0 0 0 0 1 0 1 0 0 0 1 |
| VH$_2$-2 | 0 1 0 0 1 1 0 1 0 0 0 1 0 1 1 1 1 0 0 1 |
| VH$_2$-3 | 1 010 010 101 011 11 00001 |
| VH$_2$-4 | 1 0 1 0 1 1 0 1 0 1 1 1 1 1 1 0 0 0 0 1 |

The dendrogram (Fig 16) showed that *V.planifolia* and the hybrids VH7, VH9, VH10, VH13, VH14, VH15 and VH19 clustered together; *V.tahetensis*, VH$_2$-1 and VH$_2$-2 clustered together; while the hybrids VH4, VH5, VH$_2$-4, VH$_2$-7, VH$_2$-8 and VH$_2$-11 comes under the aphylla cluster .

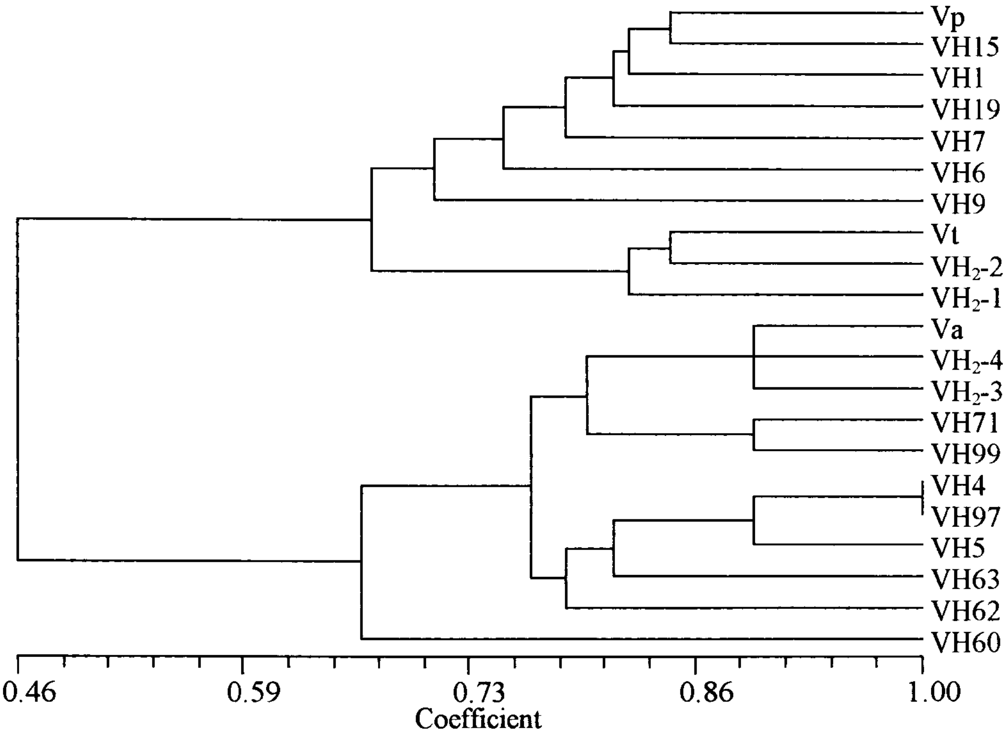

**Fig 16: Dendrogram of Vanilla showing the interrelationship between the 3 species and the hybrid progenies as expressed by anatomical parameters.**

**Vp : *Vanilla planifolia,* Vt :*Vanilla tahetensis,* Va : *Vanilla aphylla,* VH1 to VH19 : *Vanilla planifolia* (♀) x *Vanilla aphylla* (♂) hybrids, VH60 to VH 99 : *Vanilla aphylla* (♀) x *Vanilla planifolia* (♂) hybrids, VH$_2$-1 to VH$_2$-4 : *V. aphylla* (♀) x *V.tahetensis* (♂) hybrids.**

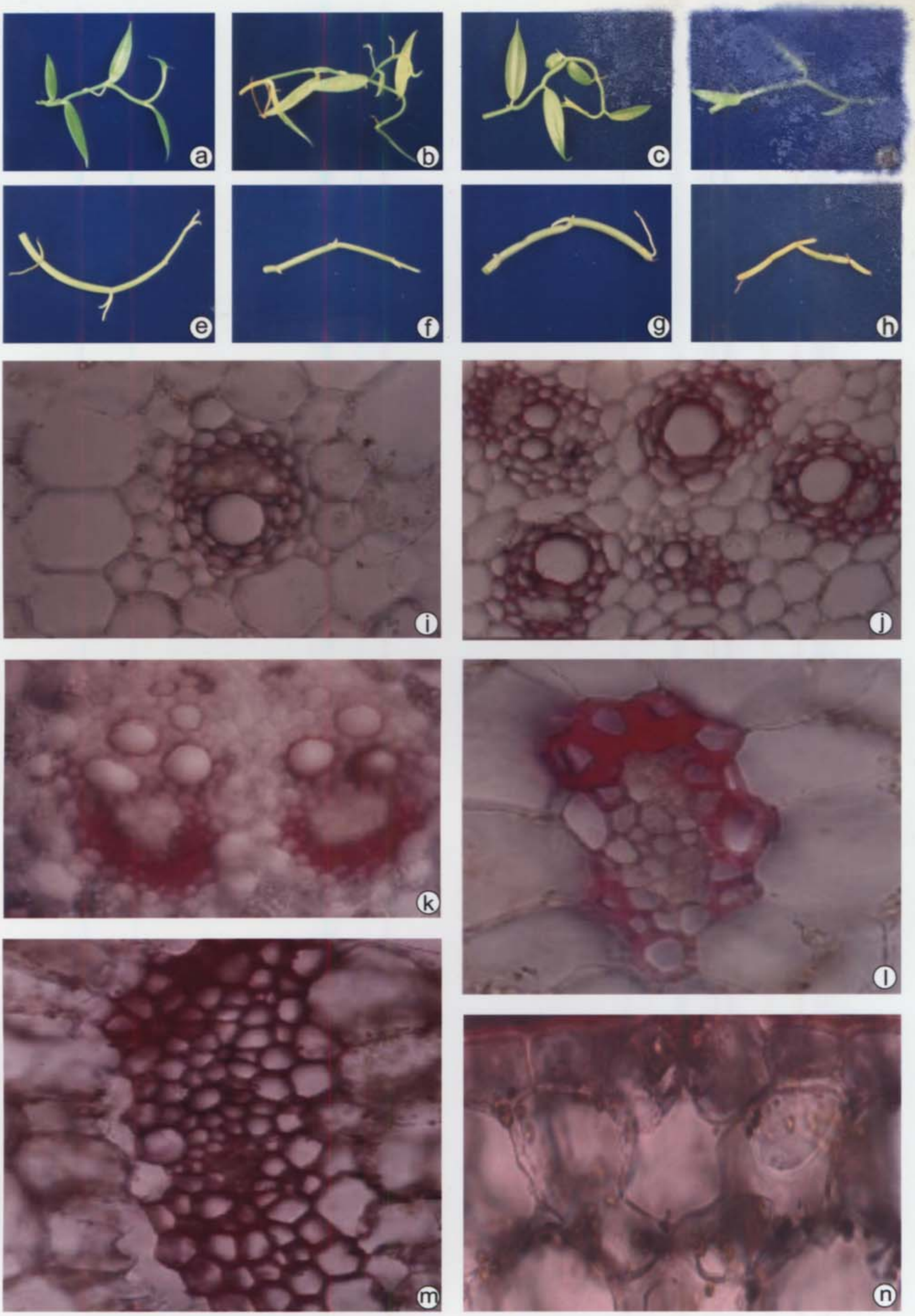

**Fig 14: a-h: Species included for anatomical study; a:** *V.planifolia*; **b:** *V.tahetensis*; **c:** *V.andamanica*; **d:** *V.pilifera*; **e:** *V.aphylla*; **f:** *V.vatsalana*; **g:** *V.walkieriae*; **h:** *V.wightiana*; **i-m: vascular bundle organization**; **i:** *V.tahetensis* stem; **j:** *V.andamanica* stem; **k:** *V.planifolia* stem; **l:** *V. planifolia* leaf; **m:** *V.andamanica* leaf; **n:** *V.tahetensis* stomata.

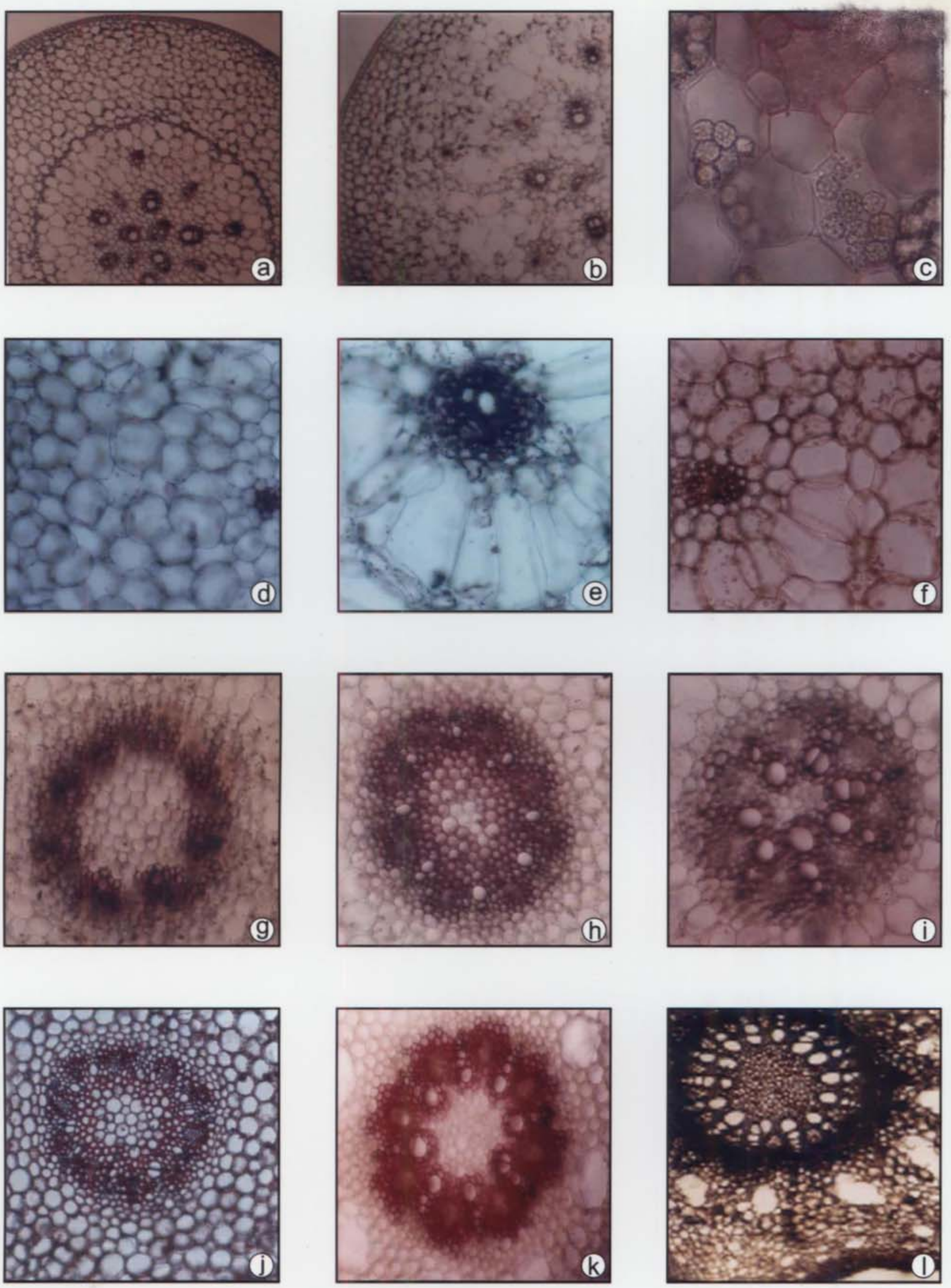

**Fig 15: Anatomical variations among the species; a-f: stem variations; a:** *V.tahetensis* with sclerenchymatous ring; **b:** *V.walkeriae* with out ring; **c:** *V.planifolia* with spiranthosomes containing starch grains; **d:** *V.tahetensis* cortex with assimilatory cells; **e:** *V.aphylla* cortex with water storage cells; **f:** *V.pilifera* cortex with mixed assimilatory and water storage cells; **g-l: root variations: g:** *V.wightiana* with parenchymatous pith; **h:** *V.andamanica* with sclerencymatous pith; **i:** *V.aphylla* with reduced parencymatous pith in mature root; **j:** *V.tahetensis* with multiarch xylem; **k:** *V.planifolia*- mature aerial root with cortical lacunae and parencymatous pith; **l:** *V.planifolia* cortical lacunae of mature terrestrial root.

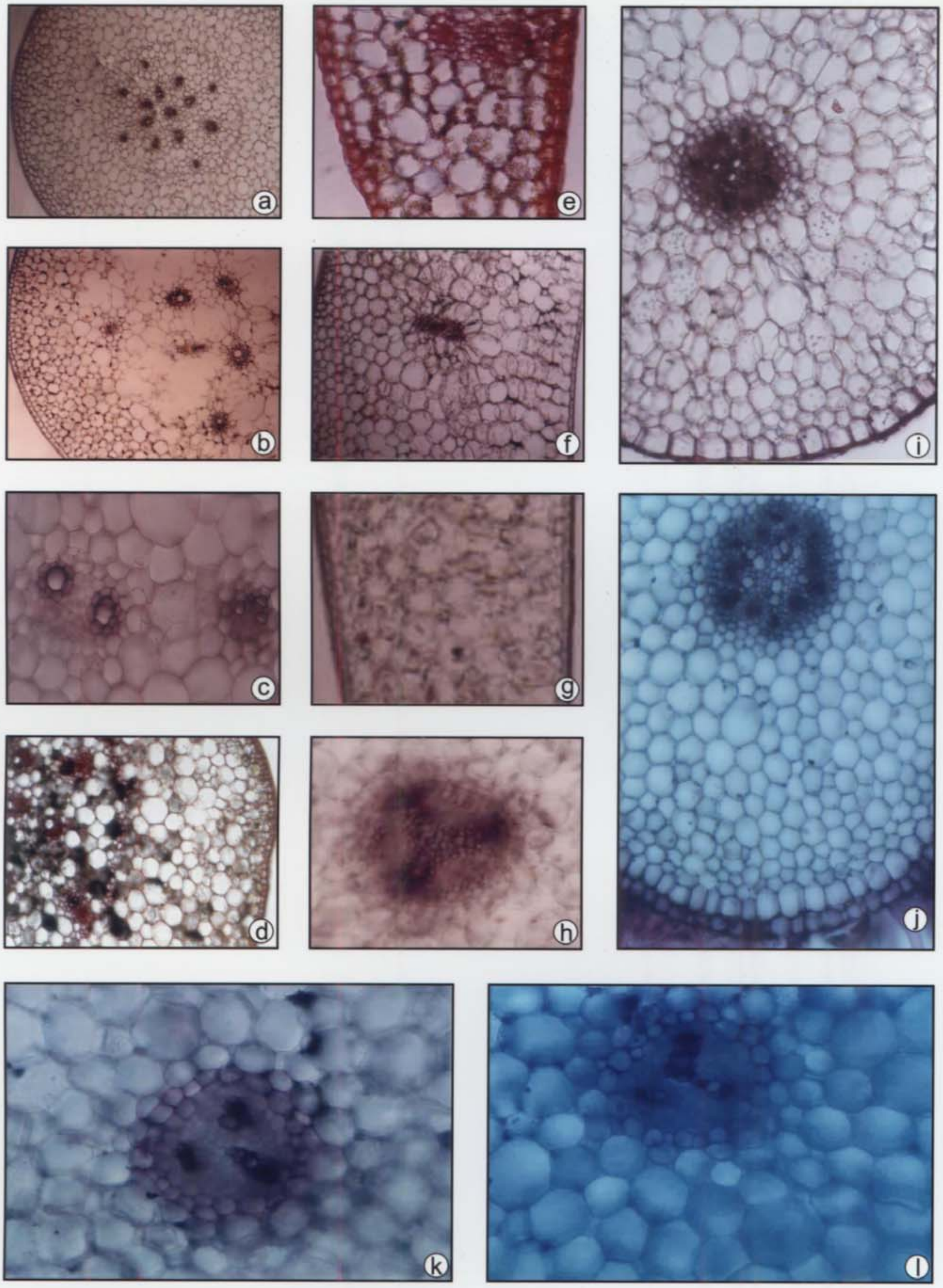

**Fig 17: Anatomical variations of hybrids; a-d: cauline variations; a:** VH18 stem with sclerenchymatous ring; **b:** VH5 stem with out ring and with water storage cells; **c:** VH17 stem with ground tissue having assimilatory cells; **d**. VH6 stem with ring and with starch grains **e-g: foliar variations; e:** VH62 leaf with abaxial vascular bundle; **f:** VH63 with central vascular bundle; **g**:VH19 with irregular mesophyll; **h-l**: **root variations**; **h**:VH63 with sclerenchymatous pith. **i:** tetrarch stele in VH62; **j:** multiarch stele in VH99; **k:** VH$_2$ 4 with continuous stele and endodermal thickening in the inner tangential and radial walls; **l**: VH4 with discontinuous stele and endodermal thickenings in the inner tangential walls.

### ***Molecular characterization***

*Genotypes Used:*

Nine lines of hybrid progenies of the cross between *V. planifolia* and *V.aphylla*; and six hybrids from the cross between *V.aphylla* and *V.tahetensis* were used in the present study. VH4, VH5, VH7, VH9, VH10, VH13, VH14, VH15 and VH19 were the progenies used from the first cross, and $VH_2$ 1, $VH_2$ 2, $VH_2$ 4, $VH_2$ 7, $VH_2$ 8 and $VH_2$ 11 were the progenies used from the second cross. The parents of the respective crosses were used as control.

*Test for polymorphism:*

Out of the twenty-four primers used for screening, six were selected and used in six separate reactions to detect polymorphism among the fifteen different hybrids.

Primer OPB 01 could amplify *V.planifolia*, *V.aphylla*, *V. tahetensis* and most of the hybrids (Fig 18). Primer OPB 05 could amplify specific products in their hybrids indicated by their distinct bands (Fig 19).

Primer OPE 07 gave significant amplification products in all the eighteen genotypes (Fig 20). The amplification products for the hybrids showed clear variation in the banding pattern, while *V. planifolia*, *V.aphylla* and *V. tahetensis* showed distinctly different banding pattern.

Primer OPE 12 and OPE 18 (Fig 21, 22) also showed segregating banding patterns compared to that of the parents indicating their hybridity. Primer OPE 19 gave amplification products for the parents and the hybrid genotypes (Fig 23).

Of the six primers tested, the results indicate OPE 07 and OPE19 are the best to study polymorphism between the hybrids.

**Fig 18: RAPD polymorphism expressed by OPB 01 in interspecific hybrids**

1. *V. planifolia*
2. *V.aphylla*
3. VH 4
4. VH 5
5. VH 7
6. VH 9
7. VH 10
8. VH 13
9. VH 14
10. VH 15
11. VH 19
12. *V. aphylla*
13. *V. tahetensis*
14. VH$_2$-1
15. VH$_2$-2
16. VH$_2$-4
17. VH$_2$-7
18. VH$_2$-8
19. VH$_2$-11
20. 1kb ladder

**Fig 19: RAPD polymorphism expressed by OPB 05 in interspecific hybrids**

1. 1kb ladder
2. *V. planifolia*
3. *V.aphylla*
4. VH 4
5. VH 5
6. VH 7
7. VH 9
8. VH 10
9. VH 13
10. VH 14
11. VH 15
12. VH 19
13. *V. aphylla*
14. *V. tahetensis*
15. VH$_2$-1
16. VH$_2$-2
17. VH$_2$-4
18. VH$_2$-7
19. VH$_2$-8
20. VH$_2$-11

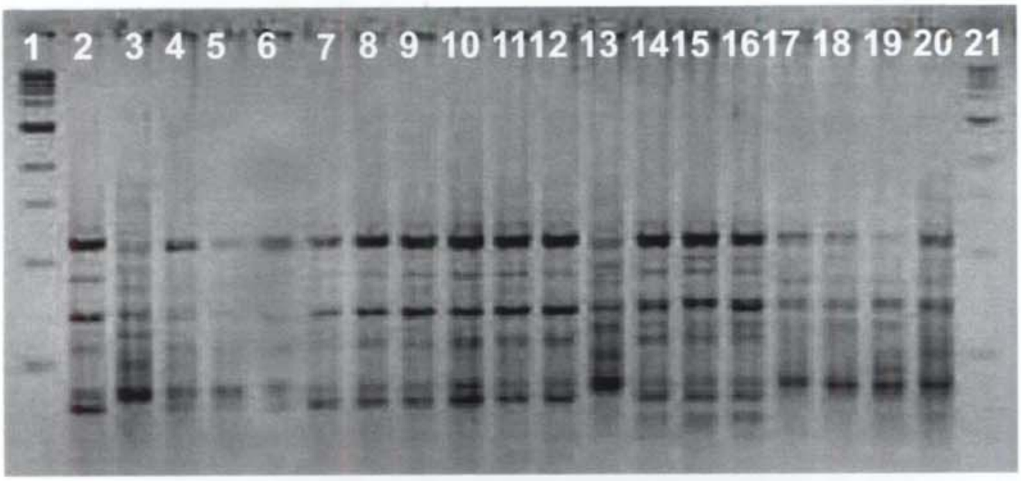

Fig 20: RAPD polymorphism expressed by OPE 07 in interspecific hybrids

**1. 1kb ladder**
**2. *V. planifolia***
**3. *V. aphylla***
**4. VH 4**
**5. VH 5**
**6. VH 7**
**7. VH 9**
**8. VH 10**
**9. VH 13**
**10. VH 14**
**11. VH 15**
**12. VH 19**
**13. *V. aphylla***
**14. *V. tahetensis***
**15. VH$_2$ -1**
**16. VH$_2$ -2**
**17. VH$_2$ - 4**
**18. VH$_2$ -7**
**19. VH$_2$ -8**
**20. VH$_2$ -11**
**21. 1kb ladder**

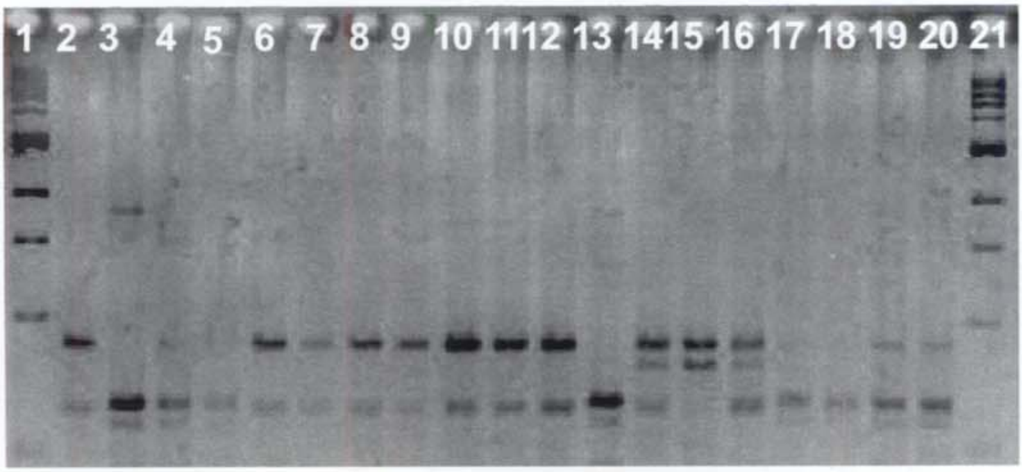

Fig 21: RAPD polymorphism expressed by OPE 12 in interspecific hybrids

**1. 1kb ladder**
**2. *V. planifolia***
**3. *V. aphylla***
**4. VH 4**
**5. VH 5**
**6. VH 7**
**7. VH 9**
**8. VH 10**
**9. VH 13**
**10. VH 14**
**11. VH 15**
**12. VH 19**
**13. *V. aphylla***
**14. *V. tahetensis***
**15. VH$_2$ -1**
**16. VH$_2$ -2**
**17. VH$_2$- 4**
**18. VH$_2$ -7**
**19. VH$_2$ -8**
**20. VH$_2$ -11**
**21. 1kb ladder**

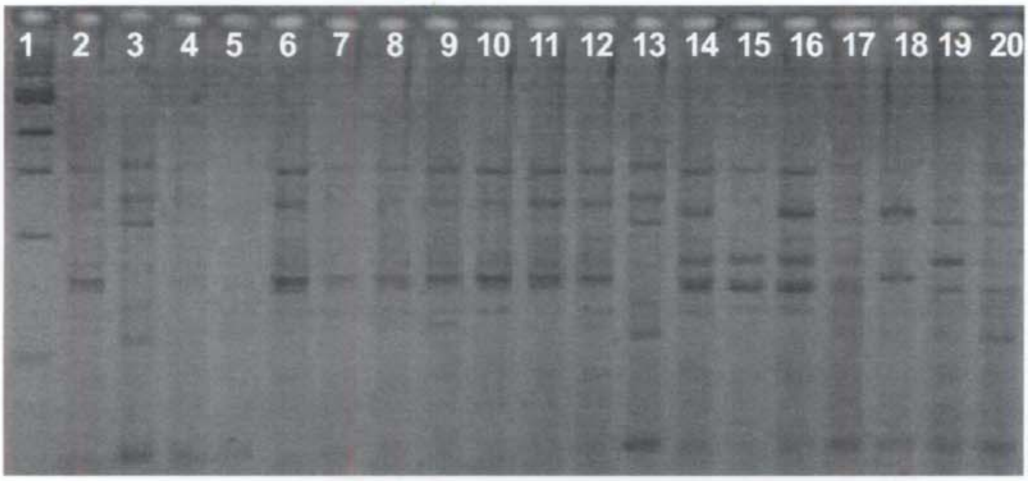

**Fig 22: RAPD polymorphism expressed by OPE 18 in interspecific hybrids**

1. 1kb ladder
2. *V. planifolia*
3. *V. aphylla*
4. VH 4
5. VH 5
6. VH 7
7. VH 9
8. VH 10
9. VH 13
10. VH 14
11. VH 15
12. VH 19
13. *V. aphylla*
14. *V. tahetensis*
15. VH$_2$-1
16. VH$_2$-2
17. VH$_2$-4
18. VH$_2$-7
19. VH$_2$-8
20. VH$_2$-11

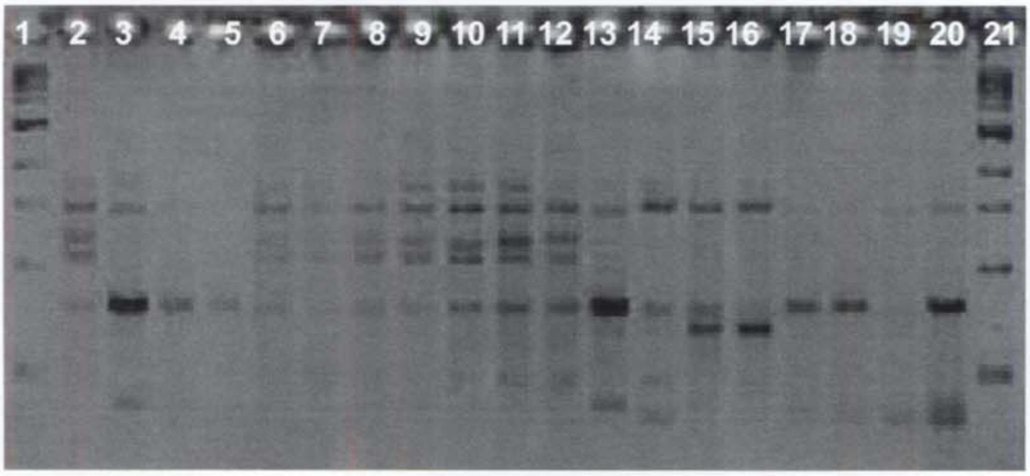

**Fig 23: RAPD polymorphism expressed by OPE 19 in interspecific hybrids**

1. 1kb ladder
2. *V. planifolia*
3. *V. aphylla*
4. VH 4
5. VH 5
6. VH 7
7. VH 9
8. VH 10
9. VH 13
10. VH 14
11. VH 15
12. VH 19
13. *V. aphylla*
14. *V. tahetensis*
15. VH$_2$-1
16. VH$_2$-2
17. VH$_2$-4
18. VH$_2$-7
19. VH$_2$-8
20. VH$_2$-11
21. 1kb ladder

The polymorphic bands were scored and the data is given in Table 28. The RAPD data was analyzed using Paired Affinity Indices and % similarity was calculated (Table-29).

**Table-28: various RAPD markers showing polymorphism in *V.planifolia*, *V. aphylla*, *V. tahetensis* and their interspecific hybrids ***

| S.N | Primer | A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | A11 | A12 | A13 | A14 | A15 | A16 | A17 | A18 | A19 | Kb. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | OPB 01-1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0.5 |
| 2 | OPB 01-2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0.6 |
| 3 | OPB 01-3 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0.75 |
| 4 | OPB 05-1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| 5 | OPB 05-2 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1.8 |
| 6 | OPB 05-3 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 2.3 |
| 7 | OPE 07-1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0.5 |
| 8 | OPE 07-2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.7 |
| 9 | OPE 07-3 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 10 | OPE 07-4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.75 |
| 11 | OPE 07-5 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0.85 |
| 12 | OPE 07-6 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1.2 |
| 13 | OPE 12-1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 2 |
| 14 | OPE 12-2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0.6 |
| 15 | OPE 12-3 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0.8 |
| 16 | OPE 18-1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1.3 |
| 17 | OPE 18-2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0.65 |
| 18 | OPE 18-3 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.8 |
| 19 | OPE 19-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0.9 |
| 20 | OPE 19-2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0.5 |
| 21 | OPE 19-3 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.6 |
| 22 | OPE 19-4 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1.2 |

0 : Band absent; 1: band present;

**A1*: V.planifolia*, A2: *V. aphylla*, A3: VH4, A4: VH5, A5: VH7, A6: VH9, A7: VH10, A8: VH13, A9: VH14, A10: VH15, A11: VH19, A12: *V. aphylla*, A13: *V. tahetensis*, A14: $VH_2$ 1, A15: $VH_2$ 2, A16: $VH_2$ 4, A17: $VH_2$ 7, A18: $VH_2$ 8 and A19: $VH_2$ 11**

**Table-29: Paired Affinity Indices (PAI; % similarity) of the 3 species and interspecific hybrids of Vanilla**

| | A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | A11 | A12 | A13 | A14 | A15 | A16 | A17 | A18 | A19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A1** | 100 | | | | | | | | | | | | | | | | | | |
| **A2** | 36 | 100 | | | | | | | | | | | | | | | | | |
| **A3** | 54 | 72 | 100 | | | | | | | | | | | | | | | | |
| **A4** | 54 | 72 | 90 | 100 | | | | | | | | | | | | | | | |
| **A5** | 86 | 50 | 59 | 68 | 100 | | | | | | | | | | | | | | |
| **A6** | 95 | 40 | 59 | 59 | 81 | 100 | | | | | | | | | | | | | |
| **A7** | 95 | 40 | 59 | 59 | 81 | 90 | 100 | | | | | | | | | | | | |
| **A8** | 100 | 36 | 54 | 54 | 86 | 95 | 95 | 100 | | | | | | | | | | | |
| **A9** | 100 | 36 | 54 | 54 | 86 | 95 | 95 | 100 | 100 | | | | | | | | | | |
| **A10** | 95 | 40 | 59 | 59 | 81 | 90 | 100 | 95 | 95 | 100 | | | | | | | | | |
| **A11** | 100 | 36 | 54 | 54 | 86 | 95 | 95 | 100 | 100 | 95 | 100 | | | | | | | | |
| **A12** | 36 | 100 | 72 | 72 | 50 | 40 | 40 | 36 | 36 | 40 | 36 | 100 | | | | | | | |
| **A13** | 72 | 27 | 36 | 36 | 59 | 68 | 68 | 72 | 72 | 68 | 72 | 27 | 100 | | | | | | |
| **A14** | 68 | 22 | 31 | 31 | 54 | 63 | 63 | 68 | 68 | 63 | 68 | 22 | 95 | 100 | | | | | |
| **A15** | 68 | 31 | 40 | 40 | 54 | 63 | 72 | 68 | 68 | 72 | 68 | 31 | 86 | 90 | 100 | | | | |
| **A16** | 40 | 86 | 86 | 86 | 54 | 45 | 45 | 40 | 40 | 45 | 40 | 86 | 31 | 27 | 36 | 100 | | | |
| **A17** | 36 | 81 | 81 | 81 | 50 | 40 | 40 | 36 | 36 | 40 | 36 | 81 | 36 | 31 | 31 | 95 | 100 | | |
| **A18** | 40 | 86 | 77 | 77 | 54 | 45 | 45 | 40 | 40 | 45 | 40 | 86 | 40 | 36 | 45 | 81 | 77 | 100 | |
| **A19** | 40 | 95 | 77 | 77 | 54 | 45 | 45 | 40 | 40 | 45 | 40 | 95 | 31 | 27 | 36 | 90 | 86 | 90 | 100 |

**A1***:* *V.planifolia*, **A2:** *V. aphylla*, **A3:** VH4, **A4:** VH5, **A5:** VH7, **A6:** VH9, **A7:** VH10, **A8:** VH13, **A9:** VH14, **A10:** VH15, **A11:** VH19, **A12:** *V. aphylla*, **A13:** *V. tahetensis*, **A14:** $VH_2$ 1, **A15:** $VH_2$ 2, **A16:** $VH_2$ 4, **A17:** $VH_2$ 7, **A18:** $VH_2$ 8 and **A19:** $VH_2$ 11

A dendrogram was constructed based on the paired values to show the relationship between the hybrids and their parents (Fig 24).

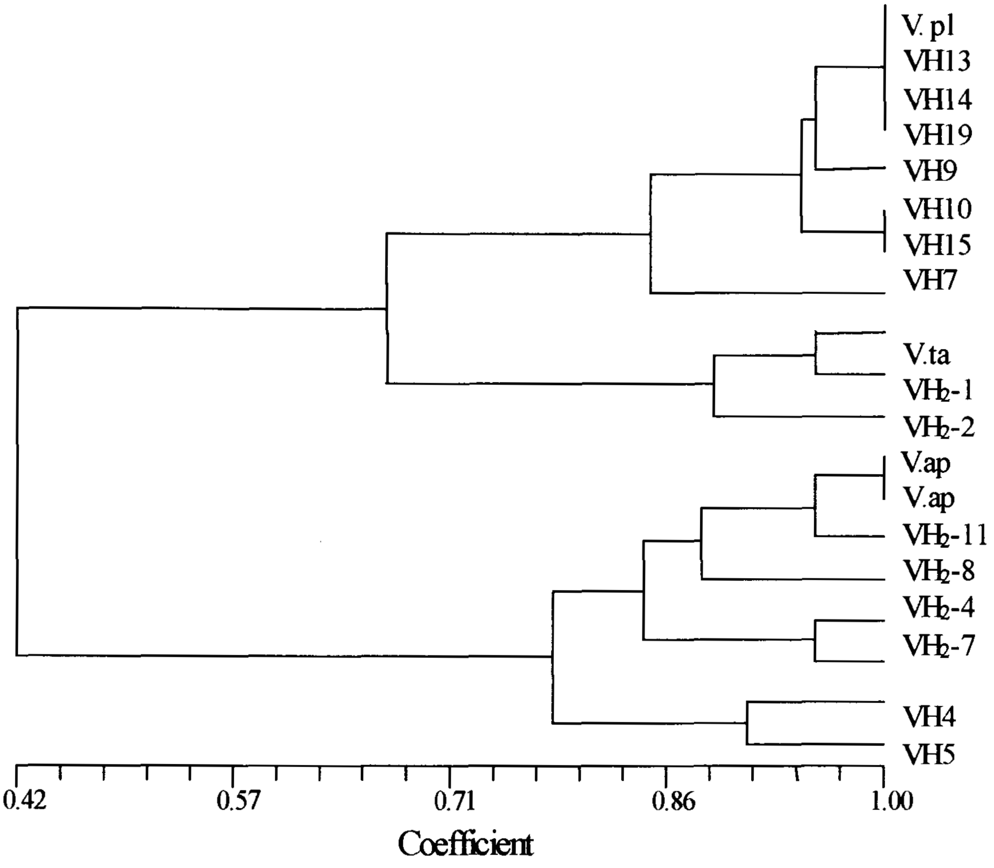

**Fig 24: Dendrogram of Vanilla showing the interrelationship between the 3 species and interspecific hybrids with respect to RAPD polymorphism. V.pl : *Vanilla planifolia,* V.ta :*Vanilla tahetensis,* V.ap : *Vanilla aphylla,* VH4 to VH19 : *Vanilla planifolia* (♀) x *Vanilla aphylla* (♂) hybrids, $VH_2$-1 to $VH_2$-11: *V. aphylla* (♀) x *V.tahetensis* (♂) hybrids.**

## In vitro polyploidy

### *Preparation of explants for colchicine treatment:*

*i. Callus induction:*

A technology for production, multiplication and maintenance of callus was developed. For callus induction 2, 4 D in combination with BAP or Thidiazuron (TDZ) were tried (Table-30). In all cases, nodal segments were used as explants. 2, 4 D (1 mg /l) in combination with BA (1 mg /l) produced maximum amount of friable callus (fig 25 a). 2, 4 D when combined with TDZ produced callus but the percentage of response was low. With higher concentrations of 2, 4 D, the callus ultimately turned brown and lost its regeneration capacity.

**Table-30: Reponses of explants to various concentrations of 2,4 D, TDZ and BAP.**

| Sl.No. | 2,4 D (mg/l) | BAP (mg/l) | TDZ (mg/l) | Intensity of callus development |
|---|---|---|---|---|
| 1 | -- | -- | -- | 0 |
| 2 | -- | 1 | -- | 0 |
| 3 | 0.5 | 1 | -- | +++ |
| 4 | 1 | 1 | -- | ++++ |
| 5 | 1.5 | 1 | -- | + |
| 6 | 2 | 1 | -- | ++ |
| 7 | 2.5 | 1 | -- | 0 |
| 8 | 3 | 1 | -- | 0 |
| 9 | -- | -- | 0.1 | 0 |
| 10 | 0.5 | -- | 0.1 | + |
| 11 | 1 | -- | 0.1 | ++ |
| 12 | 1.5 | -- | 0.1 | ++ |
| 13 | 2 | -- | 0.1 | + |
| 14 | 2.5 | -- | 0.1 | + |
| 15 | 3 | -- | 0.1 | 0 |

**0, nil; +, low; ++, moderate; +++, high; ++++, intense.; Data scored at the end of 4 weeks in culture.**

*ii. Somatic embryo (PLB) induction:*

For PLB induction BAP in combination with different auxins were tried (Table-31). Among the three auxins tried, only the combination of BAP with IBA could induce PLB development in nodal explants. When this medium was supplemented with tryptone / peptone, the intensity of PLB development was found to be enhanced. The types of responses included root tip / shoot apex conversion into PLB and further proliferation and multiplication of PLB (Fig 25 g,h,i ).

**Table-31: The intensity of PLB development in the axillary bud explants to different media supplements.**

| Sl.No. | Media | Intensity of PLB development |
|---|---|---|
| 1 | MS + BA 0.5 mg/l | 0 |
| 2 | MS + BA 1 mg/l + 2,4 D 0.5 mg/l | 0 |
| 3 | MS + BA 1 mg/l + NAA 0.5 mg/l | 0 |
| 4 | MS + BA 1 mg/l + IBA 0.5 mg/l | ++ |
| 5 | MS + BA 1 mg/l + IBA 0.5 mg/l + peptone 2g/l | +++ |
| 6 | MS + BA 1 mg/l + IBA 0.5 mg/l + tryptone 2g/l | +++ |

**0, nil; ++, moderate; +++, high.**

**Data scored at the end of 4 weeks in culture.**

**(10 replicates per treatment).**

*iii. Germinating seeds:*

Growth of the seedlings in the bottles of various media combinations was variable and not consistent (Table-13). Although the seeds started germinating in the media with 2, 4 D they ultimately turned brown and died. Among the ten treatments, the combination MS + BA 1mg/L + IBA 0.5mg/L was used to obtain the seeds for this experiment. The germinated seeds were used for colchicine treatment (Fig 25 b).

*iv. Nodal explants:*

The nodal explants from 10 different seedling progenies (V56, V89, V92, V77, V113, V120, V124, V145, V161 and V260) and 4 interspecific hybrids (VH1, VH4, VH5, and VH6) were used for further treatment (Fig 25 c). The interspecific hybrids were used in this experiment with the intention to produce fertile hybrid by chromosome doubling.

***Responses of explants after colchicine treatment:***

The immediate effects noticed in the suspected polyploids of all the treatments were swelling of the root / shoot bud tips (Fig 30 a, b). Regeneration of plantlets following *in vitro* colchicine treatment decreased with increasing colchicine concentration (Table 32). Survival rate was lowest using colchicine concentration of 0.4% (Fig 27); however, at this concentration the chromosome-doubling rate was highest (Table 32, Fig 28). The chromosome doubling seldom occurred when the concentration of colchicine was lower than 0.2 % (Table-33). Callus tissue failed to produce any polyploids after treatment (Table-34).

***Morphological characterization***

Thirty days after treatment, none of the seedlings of a plant height of 1.6 cm and above was chromosome doubled. Height of the plant tend to decrease with increase in concentration of colchicine (Table 33, Fig 26). Reductions in plant height in the early stages (Fig 30c), thicker and greener leaves in the later stages (Fig 30 e, 30 h) were the other effects. Similarly, leaf length and width tend to vary with change in ploidy (Fig 29 a, 29 b). Polyploid leaves were thicker with more narrow and entire leaf blade than diploids (Fig 30 g).

## Table 32: Influence of colchicine in induction of polyploidy

| Treatment * | Colchicine % | Survival %* | Plant height (in cm.)* | Tetraploids (no) | Tetraploids (% of surviving cultures) | Tetraploids (% of initial cultures) |
|---|---|---|---|---|---|---|
| Germinating seeds | | | | | | |
| T1 | 0 (control) | 100 | 2.3 | 0 | 0 | 0 |
| T2 | 0.05 | 80 | 2.3 | 0 | 0 | 0 |
| T3 | 0.1 | 60 | 2.1 | 0 | 0 | 0 |
| T4 | 0.2 | 50 | 0.6 | 3 | 60 | 30 |
| T5 | 0.4 | 40 | 0.7 | 3 | 75 | 30 |
| PLBs | | | | | | |
| T6 | 0(control) | 100 | 2.6 | 0 | 0 | 0 |
| T7 | 0.05 | 100 | 2.0 | 0 | 0 | 0 |
| T8 | 0.1 | 100 | 2.3 | 0 | 0 | 0 |
| T9 | 0.2 | 60 | 0.7 | 0 | 0 | 0 |
| T10 | 0.4 | 60 | 1 | 1 | 8 | 5 |
| Callus | | | | | | |
| T11 | 0(control) | 60 | 2 | 0 | 0 | 0 |
| T12 | 0.05 | 20 | 1.8 | 0 | 0 | 0 |
| T13 | 0.1 | 40 | 1.2 | 0 | 0 | 0 |
| T14 | 0.2 | 20 | 0.9 | 0 | 0 | 0 |
| T15 | 0.4 | 0 | 0 | 0 | 0 | 0 |
| Axillary bud | | | | | | |
| T16 | 0(control) | 100 | 2.8 | 0 | 0 | 0 |
| T17 | 0.05 | 100 | 3 | 0 | 0 | 0 |
| T18 | 0.1 | 66 | 2.5 | 0 | 0 | 0 |
| T19 | 0.2 | 60 | 1.6 | 0 | 0 | 0 |
| T20 | 0.4 | 56 | 1.1 | 2 | 13 | 8 |

*** Data taken 30 days after treatment**

**Table-33: Effect of colchicine concentration on tetraploidy and plant height.***

| **Sl No** | **Characters*** | | **Colchicine concentration (%)** | | | | | **SE** | **F value** |
|---|---|---|---|---|---|---|---|---|---|
| | | | **0** | **0.05** | **0.1** | **0.2** | **0.4** | | |
| 1 | Plant height | Mean | 2.48 | 2.22 | 2.04 | 1.02 | 0.86 | **0.15** | **12.6** |
| | | SD | 0.32 | 0.47 | 0.49 | 0.42 | 0.55 | | |
| 2 | Tetraploid number | Mean | 0 | 0 | 0 | 0.8 | 1.4 | **0.2** | **2.5** |
| | | SD | 0 | 0 | 0 | 1.3 | 1.5 | | |
| 3 | Tetraploid percent | Mean | 0 | 0 | 0 | 12 | 19.2 | **3.8** | **1.2** |
| | | SD | 0 | 0 | 0 | 26.6 | 31.6 | | |

**SD: Standard Deviation; SE: Standard Error *Data taken 30 days after treatment**

**Table-34: Effect of colchicine on tetraploidy and plant height in different explants.***

| **Sl No** | **Characters *** | | **Explant type** | | | | | **SE** | **F value** |
|---|---|---|---|---|---|---|---|---|---|
| | | | **Seed** | **PLB** | **Callus** | **Node** | **Hybrid** | | |
| 1 | Plant height | Mean | 1.43 | 1.5 | 0.98 | 2.05 | 1.73 | **0.17** | **1.10** |
| | | SD | 0.89 | 0.77 | 0.75 | 0.85 | 0.38 | | |
| 2 | Tetraploid number | Mean | 1.5 | 0.25 | 0 | 0.75 | 0 | **0.24** | **1.47** |
| | | SD | 1.7 | 0.5 | 0 | 1.5 | 0 | | |
| 3 | Tetraploid percent | Mean | 33.8 | 2 | 0 | 3.25 | 0 | **4.65** | **2.6** |
| | | SD | 39.4 | 4 | 0 | 6.5 | 0 | | |

**SD: Standard Deviation; SE: Standard Error * Data taken 30 days after colchicine treatment.**

### *Cytological characterization*

Somatic chromosomes were studied from the root tip squashes of control and tetraploid plants. Chromosome number of some plantlets regenerated from *in vitro* colchicine treated seeds was doubled (Fig 30 i, j, k).

Eighteen mature plants were obtained from the germinating seeds treated with colchicine, and among them six plants were found to be tetraploids. Tetraploid plants were produced at the frequency of 30% for the concentration of 0.4% when seeds were used for treatment. Seeds treated with 0.4% of colchicine yielded the highest number of tetraploid plants. The doubling seldom occurred when the concentration of colchicine was lower than 0.2%. PLB treatment was also effective in inducing polyploidy. The PLB derived from the accession no. V153 (0.4 % colchicine) turned out to be tetraploid. This polyploidy is designated as VCP 4. Callus treatment was ineffective in polyploidy induction (Fig 27, Table 34), and is due to the loss of regeneration capacity following colchicine treatment. Nodal explant treatment was effective only to a very low extent (0.8 %) at the concentration of 0.4 % colchicine. The nodal explant derived polyploids were VCP 5 (from V120 at 0.4 % colchicine) and VCP 6 (from V113 at 0.4 % colchicine). Hybrids failed to produce any polyploids. The list of promising variants obtained by colchicine treatment are given in Table 35. Present investigation indicated that seed treatment was most effective inducing polyploidy (Table-34).

**Table 35: List of variants obtained by colchicine treatment.**

| **Name** | **Explant used** | **Colchicine %** | **Morphological type** | **Ploidy** |
|---|---|---|---|---|
| VCP 1 | Seed | 0.4 % | Dark green stem, narrow leaves | 4n |
| VCP 2 | Seed | 0.4 % | Dark green stem, narrow leaves | 4n |
| VCP 3 | Seed | 0.4 % | Dark green stem, narrow leaves | 4n |
| VCP 4 | PLB (V153) | 0.4 % | Slender stem, narrow leaves | 4n |
| VCP 5 | Node (V120) | 0.2 % | Dark green stem, narrow leaves | 4n |
| VCP 6 | Node (V113) | 0.4 % | Slender stem, narrow leaves | 4n |
| VCP 7 | Seed | 0.4 % | Dark green stem, narrow leaves | 4n |
| V145 | Node (V145) | 0.1% | Very stout stem, broad leaves | 2n |

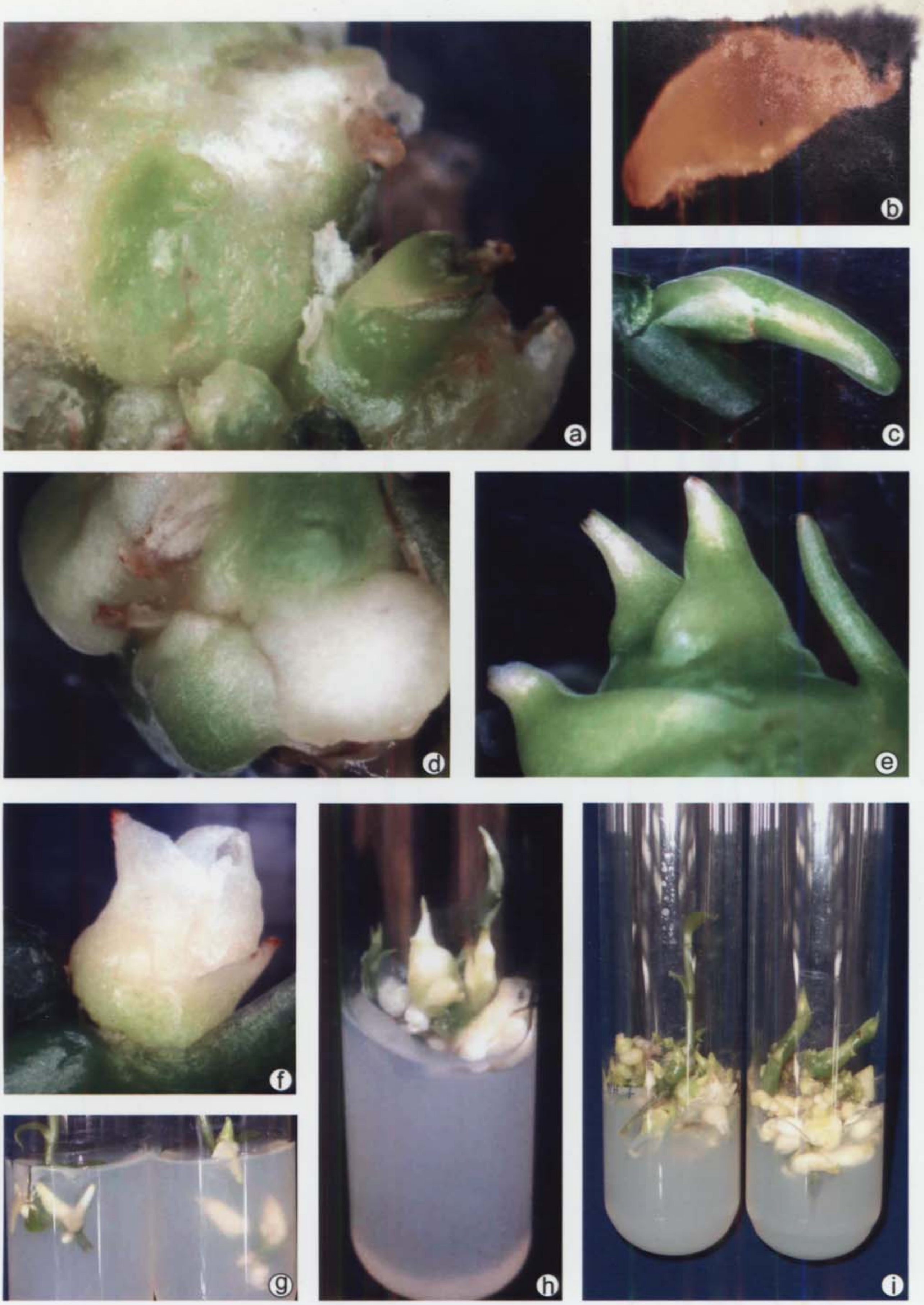

**Fig 25: Explants used for colchicine / EMS treatment: a:** regenerating callus; **b:** germinating seed; **c:** axillary bud; **d-i: stages of PLB induction and multiplication**; **d:**multiple PLB; **e:**conversion of PLB to shoot; **f:**PLB initiation in the leaf axil; **g:** *invitro* root tip conversion into PLB; **h:** shoot bud conversion into PLB; **i:** PLB multiplication and proliferation.

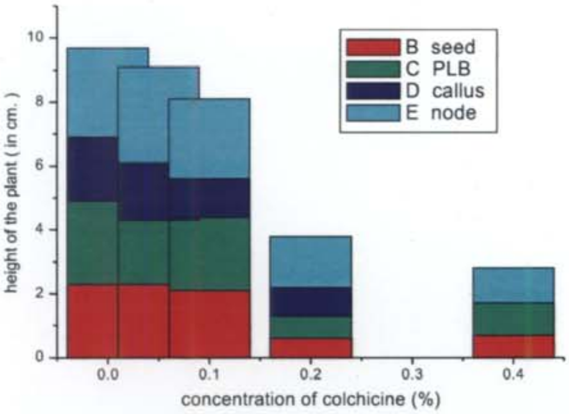

**Fig 26: Graph showing effect of colchicine on plant height in different types of explants**

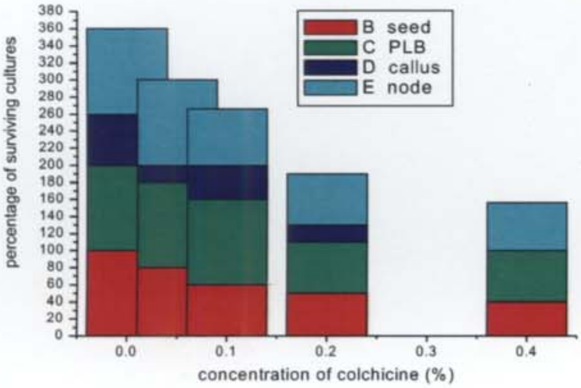

**Fig 27: Graph showing effect of colchicine on percentage of surviving cultures in different types of explants.**

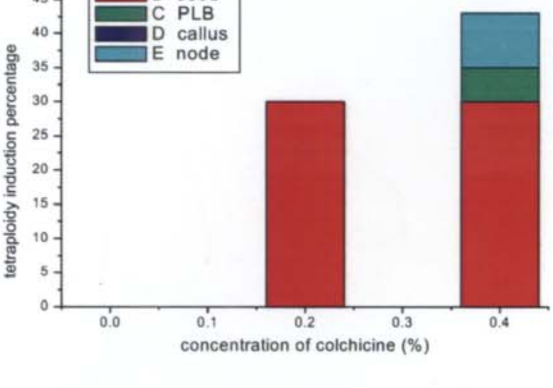

**Fig 28: Graph showing effect of colchicine on tetraploidy induction percentage in different types of explants.**

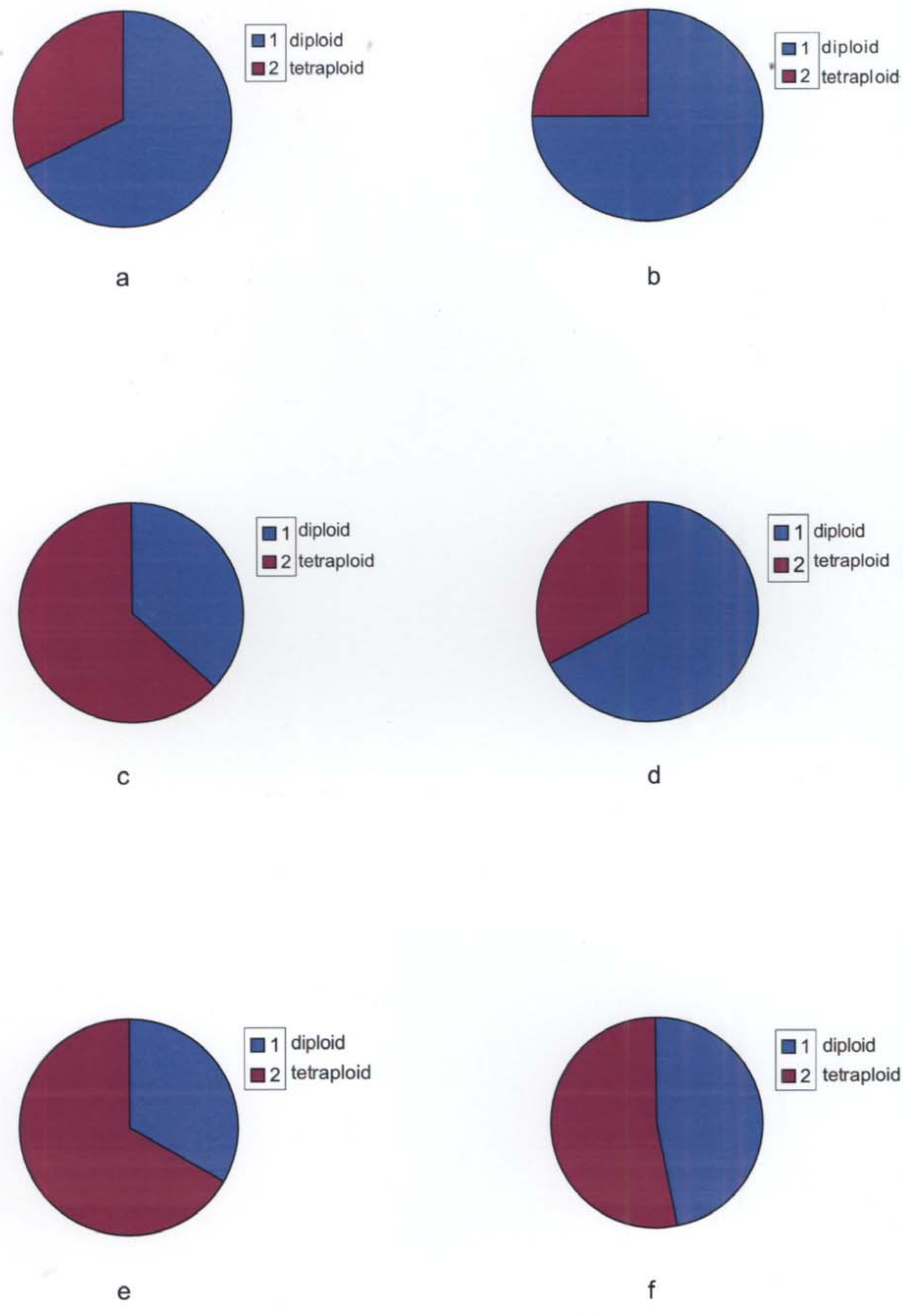

**Fig-29: Pie charts showing comparative morphology of diploids and induced autotetraploids with regard to (a): leaf length, (b): leaf breadth, (c): stomatal size, (d): stomatal density, (e): guard cell chloroplast number, (f): stem perimeter.**

### *Anatomical characterization*

For histological analysis, only the leaves were considered. Samples of the lamina of expanding as well as mature leaves were sectioned. Cells of the tetraploid plants were much larger, and there were at least two additional layers of mesophyll. Chloroplasts were distinctly more abundant (Fig 29e, 30g).

The stomatal parameters used are the stomatal size, stomatal density and the number of chloroplasts per guard cell. Those, which showed maximum stomatal size, showed least stomatal density (Fig 29 d). The number of chloroplasts in the guard cell of the polyploids was almost double the number in the control. Stomata of regenerated plants were smallest in the diploid genotype followed by tetraploid (Fig 29 c). Diploids had, on average 29 stomata per sq.mm; where as tetraploids had an average 15 stomata per sq. mm (table-36).

The comparative morphology of diploids and autotetraploids is represented in the form of pie charts (fig 29).

**Table-36: Comparative morphology of diploids and induced autotetraploids:**

| **Characters studied** | **Diploid genotype (2n=32)** | **Tetraploid genotype (2n=64)** |
|---|---|---|
| Stem perimeter (cm) | 0.7 ± 0.05 | 0.8 ± 0.32 |
| Leaf length (cm) | 2.7 ± 0.2 | 1.3 ± 0.25 |
| Leaf breadth (cm) | 1.2 ± 0.11 | 0.4 ± 0.08 |
| Stomatal size(μm) | 40 ± 2.2 x 36.1 ± 1.6 | 69 ± 2.8 x 48 ± 2.4 |
| Stomatal density | 28.7 ± 1.4 | 14.8 ± 1.75 |
| Guard cell chloroplast no. | 6 ± 0.3 | 12 ± 1.02 |

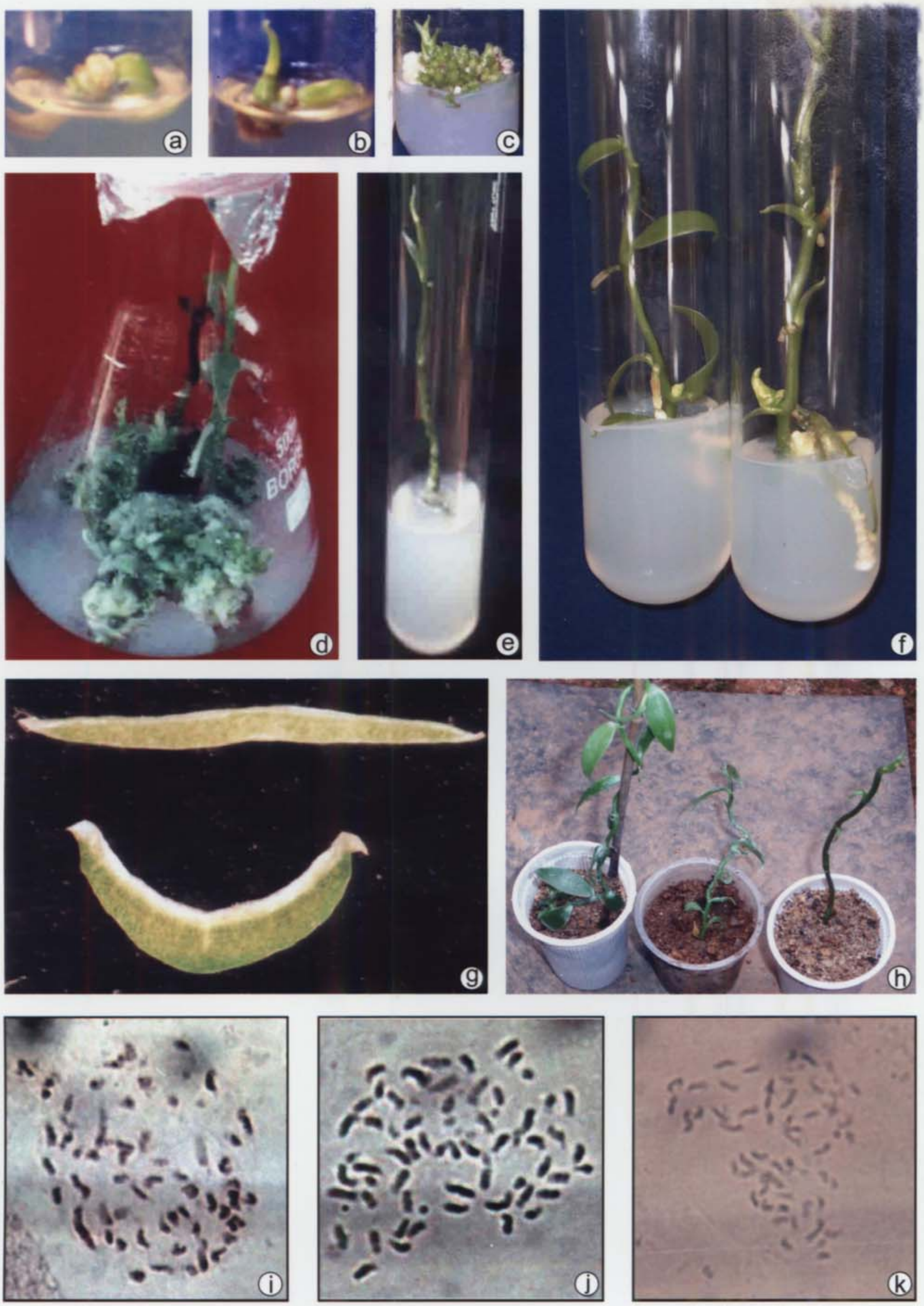

**Fig 30**: **a-c**: **Stages of production of colchiploids; a,b:** characteristic swelling after treatment; **c:** characteristic multiplication of shoot; **d:** *invitro* response of a colchicine treated plant; **e:** single polyploid plant (VCP2); . **f:** polyploid and its control; **g:** foliar transverse cuts of polyploid and its control; **h:** hardening stage of polyploid (VCP5) and its control; **i - k: cytological behavior of colchiploids of *V.planifolia*** showing 4n chromosome constitution during root tip mitosis; **i:** VCP2; **j:**VCP5; **k:** VCP6.

### *Molecular Characterization*

#### *Genotypes Used*

Fifteen lines from colchicine treated plants of *V. planifolia* were used in the present study. V56 (0.05% colchicine), V89 (0.2 % colchicine), V92 (0.2 % colchicine), V113 (0.2 % colchicine), V124 (0.1 % colchicine), V145 (0.4 % colchicine), V161 (0.05 % colchicine), V260 (0.1 % colchicine), VCP2 (0.4 % colchicine), VCP3 (0.4 % colchicine), VCP4 (0.4 % colchicine), VCP5 (0.4 % colchicine), V seed 2 (0.1 % colchicine), Vseed 4 (0.4 % colchicine) and Vseed 5 (0.2 % colchicine) were the accessions used. The seed derived progeny V260 (0 % colchicine)was used as control.

#### *DNA Isolation and Quantification*

Genomic DNA was successfully isolated from young and fresh leaves of vanilla plants using modified CTAB method of Ausubel *et al*., (1995).

#### *Primer screening*

Twenty four Operon decamer primers were tested for random amplification using genotype *V. planifolia* (Fig 5) with optimized PCR components.

Of the 24 primers tested, three primers i.e., OPB-01, OPB-05 and OPE-07 that showed amplification were used for RAPD to detect polymorphism among the colchicine treated progenies.

#### *RAPD profiles of different genotypes using different primers*

The results of different primers showed that the banding pattern were not homogenous in all the genotypes studied (Fig 31, 32, 33). Thus, the primers identified minor polymorphism between the colchicine treated progenies.

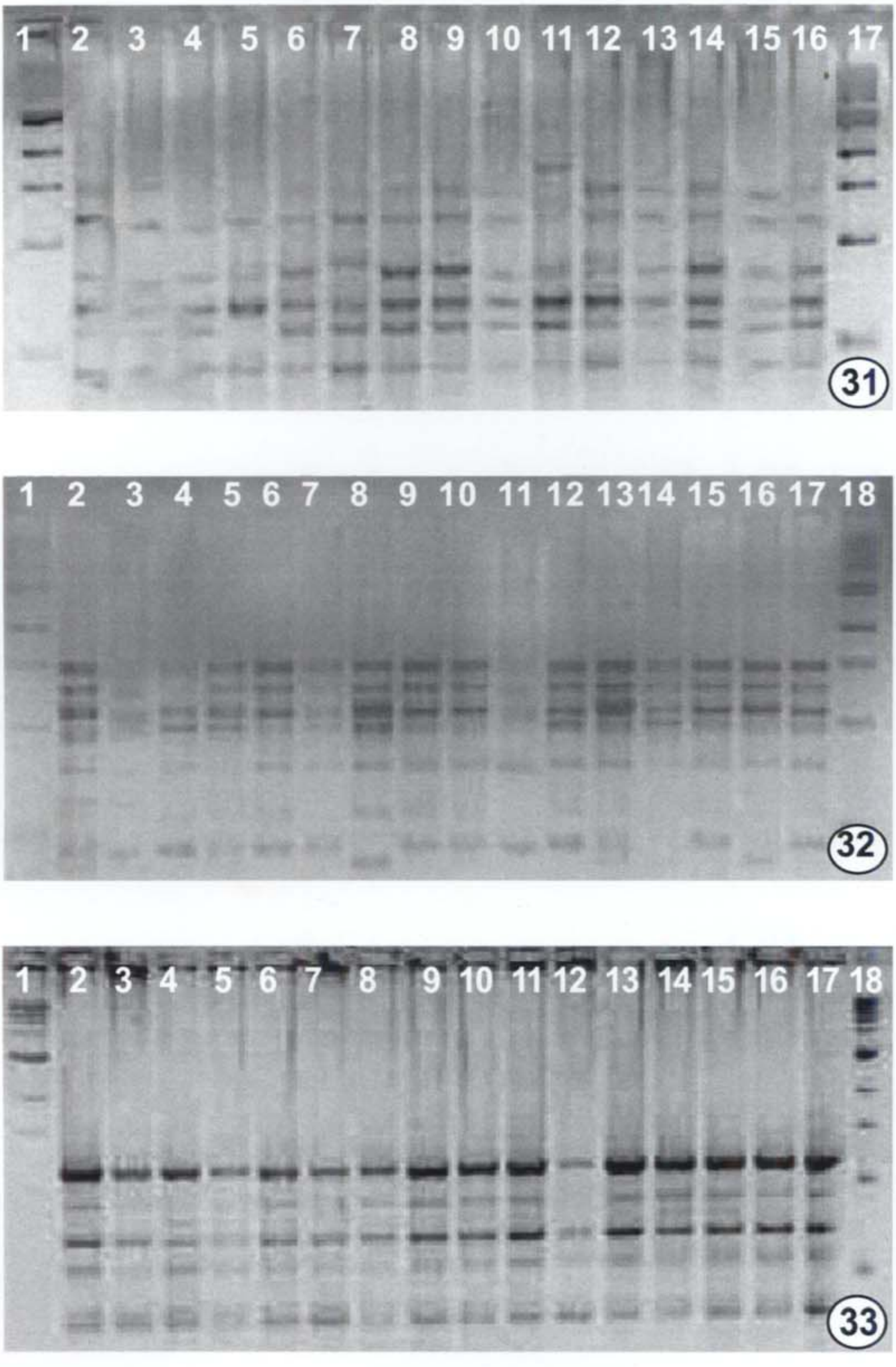

**Fig: 31-33: RAPD profiles of colchicine treated accessions using OPERON primers; 31: OPB 01; 32: OPB 05; and 33: OPE 07;** 1.1Kb ladder, 2.V260, 3. V 56(0.05%)*, 4.V 89(0.2%), 5. V 92(0.2%), 6.V 113(0.2%), 7. V124(0.1%), 8.V 145(0.4%), 9. V161(0.05%), 10. V.260(0.1%), 11. VCP 2(0.4%), 12. VCP 3(0.4%), 13. VCP 4(0.4%), 14. VCP 5(0.4%), 15. V seed 2(0.1%), 16. V seed 4(0.4%), 17. V seed 5(0.2%), 18. 1 Kb ladder; * colchicine percentage in parenthesis.

## In vitro mutation

*Responses of explants after EMS treatment:*

Inhibition of seed germination increased with increasing EMS concentration; the highest percentage of inhibition was observed at 0.1% EMS. A dose dependent reduction in seed growth and seedling development was recorded. Chlorophyll chimeras increased as mutagen concentration increased (Fig 35 d) and depended on the starting material for mutation breeding. Survival percentage decreased considerably with increase in concentration of EMS.

***Morphological characterization***

The primary effect of treatment includes changes in leaf shape and a reduction in growth vigor (Fig 35a, 35e). Fasciation (Fig 35 c) and chlorophyll mutations were common (Fig 35 f). One short internodal mutant with narrow leaf was obtained from the treatment of nodal explant with 0.75 % EMS (Fig 35 b). The seedling progenies treated with 0.75 % EMS showed about three times stem length than the control (Table-37). Thus the maximum visible morphological variations were observed at the concentration of 0.75% of EMS.

The morphological characterization of the treatments one year after planting showed that the characters varied with change in concentration of EMS (Table-37), the type of explant used for the experiment (Table-38) and among the different EMS treated seedling progenies (Table-39). The comparative morphology of seedling progenies and EMS treated seedling progenies is represented in the form of pie charts (Fig 34).

**Table-37: Effect of different concentration of EMS on quantitative morphological characters.***

| Sl.No. | Characters* | | EMS % | | | | | SE | F value |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 0.25 | 0.5 | 0.75 | 1 | | |
| 1 | Stem length (in cm) | Mean | 42 | 22 | 31.6 | 60.6 | 14.1 | **5.74** | **2.11** |
| | | SD | 25.3 | 13 | 13.4 | 57 | 5.2 | | |
| 2 | Stem girth (in cm) | Mean | 1.16 | 1.4 | 1.46 | 1.46 | 1.4 | **0.03** | **6.16** |
| | | SD | 0.13 | 0.09 | 0.05 | 0.16 | 0.11 | | |
| 3 | Internodal length | Mean | 3.36 | 3.2 | 3.68 | 5.16 | 3.27 | **0.25** | **2.45** |
| | | SD | 1.21 | 1.06 | 1.11 | 1.39 | 1.28 | | |
| 4 | Lamina length | Mean | 4.76 | 4.67 | 4.98 | 5.78 | 5.72 | **0.88** | **0.57** |
| | | SD | 1.5 | 1.6 | 1.3 | 2 | 1.8 | | |
| 5 | Lamina width | Mean | 2.4 | 1.87 | 1.74 | 1.76 | 2.12 | **0.1** | **1.4** |
| | | SD | 0.86 | 0.45 | 0.4 | 0.24 | 0.17 | | |
| 6 | Leaf number | Mean | 5.83 | 6.25 | 5.2 | 3.8 | 6 | **0.27** | **3.2** |
| | | SD | 1.6 | 0.88 | 0.83 | 0.83 | 2.2 | | |
| 7 | Root number | Mean | 4 | 3.62 | 3.6 | 2.8 | 3.7 | **0.23** | **0.7** |
| | | SD | 1.9 | 0.74 | 1.14 | 1.3 | 0.5 | | |
| 8 | Root length (in cm) | Mean | 3.5 | 2.4 | 1.8 | 3.3 | 2 | **0.35** | **0.86** |
| | | SD | 3.03 | 1.04 | 1.27 | 2.09 | 1.26 | | |

**SD: Standard Deviation; SE: Standard Error; * Mean of 5 replications**

*** Data taken one year after planting.**

**Table-38: Effect of EMS on quantitative morphological characters in different types of explants.**

| Sl No. | Characters | | Explant type | | | | | SE | F value |
|---|---|---|---|---|---|---|---|---|---|
| | | | Seed | PLB | Callus | Node | Hybrid | | |
| 1 | Internodal length | Mean | 0.78 | 1.35 | 0.33 | 1.97 | 0.35 | **0.25** | **1.99** |
| | | SD | 0.92 | 1.59 | 0.65 | 0.84 | 0.7 | | |
| 2 | Lamina length | Mean | 0.88 | 1.4 | 0.3 | 2 | 0.4 | **0.25** | **1.85** |
| | | SD | 1.08 | 1.59 | 0.6 | 0.8 | 0.7 | | |
| 3 | Lamina width | Mean | 0.43 | 0.5 | 0.1 | 0.68 | 0.15 | **0.09** | **1.27** |
| | | SD | 0.57 | 0.63 | 0.2 | 0.28 | 0.3 | | |

**SD: Standard Deviation; SE: Standard Error**

**Table-39: Effect of EMS on quantitative morphological characters of different seedling progenies.**

| Sl.No. | Characters* | | Accession No. | | | | | SE | F value |
|---|---|---|---|---|---|---|---|---|---|
| | | | V77 | V89 | V113 | V161 | V56 | | |
| 1 | Stem length | Mean | 25 | 42 | 23.3 | 26.7 | 74.3 | **8.93** | **1.18** |
| | | SD | 13.9 | 19 | 13.5 | 9.2 | 76 | | |
| 2 | Stem girth | Mean | 1.53 | 1.4 | 1.3 | 1.5 | 1.5 | **.02** | **7.5** |
| | | SD | 0.05 | 0.1 | 0.08 | 0.05 | .01 | | |
| 3 | Internodal length | Mean | 4.8 | 4.8 | 3.1 | 3.9 | 3.6 | **.36** | **.89** |
| | | SD | 2.4 | 0.34 | 0.66 | 1.7 | 1.4 | | |
| 4 | Lamina length | Mean | 6.17 | 5.87 | 4.48 | 5.23 | 4.46 | **.41** | **.68** |
| | | SD | 3.15 | 1.87 | 0.43 | 0.95 | 1.1 | | |
| 5 | Lamina width | Mean | 1.6 | 1.9 | 1.67 | 1.93 | 1.9 | **.08** | **.65** |
| | | SD | 0.44 | 0.26 | 0.29 | 0.35 | 0.35 | | |
| 6 | Leaf number | Mean | 4.66 | 3.66 | 5.7 | 5.6 | 5.3 | **.38** | **1** |
| | | SD | 1.15 | 1.15 | 0.96 | 2.5 | 1.5 | | |
| 7 | Root number | Mean | 2.3 | 3 | 4.75 | 3.3 | 3 | **.27** | **5.3** |
| | | SD | 0.57 | 1 | 0.5 | 1.15 | .00 | | |
| 8 | Root length | Mean | 1.3 | 2.3 | 1.5 | 3.8 | 3.4 | **.38** | **2.2** |
| | | SD | 0.88 | 1.05 | 0.66 | 2.5 | 1.11 | | |

**SD: Standard Deviation; SE: Standard Error * Data taken one year after planting**

### *Cytological characterization*

A study of the chromosomes in root tip squashes of the controls and the EMS treated mutants selected randomly showed that the mutants did not differ from their parental stocks in chromosome number. Root tip examination failed to detect any structural aberrations in chromosomes.

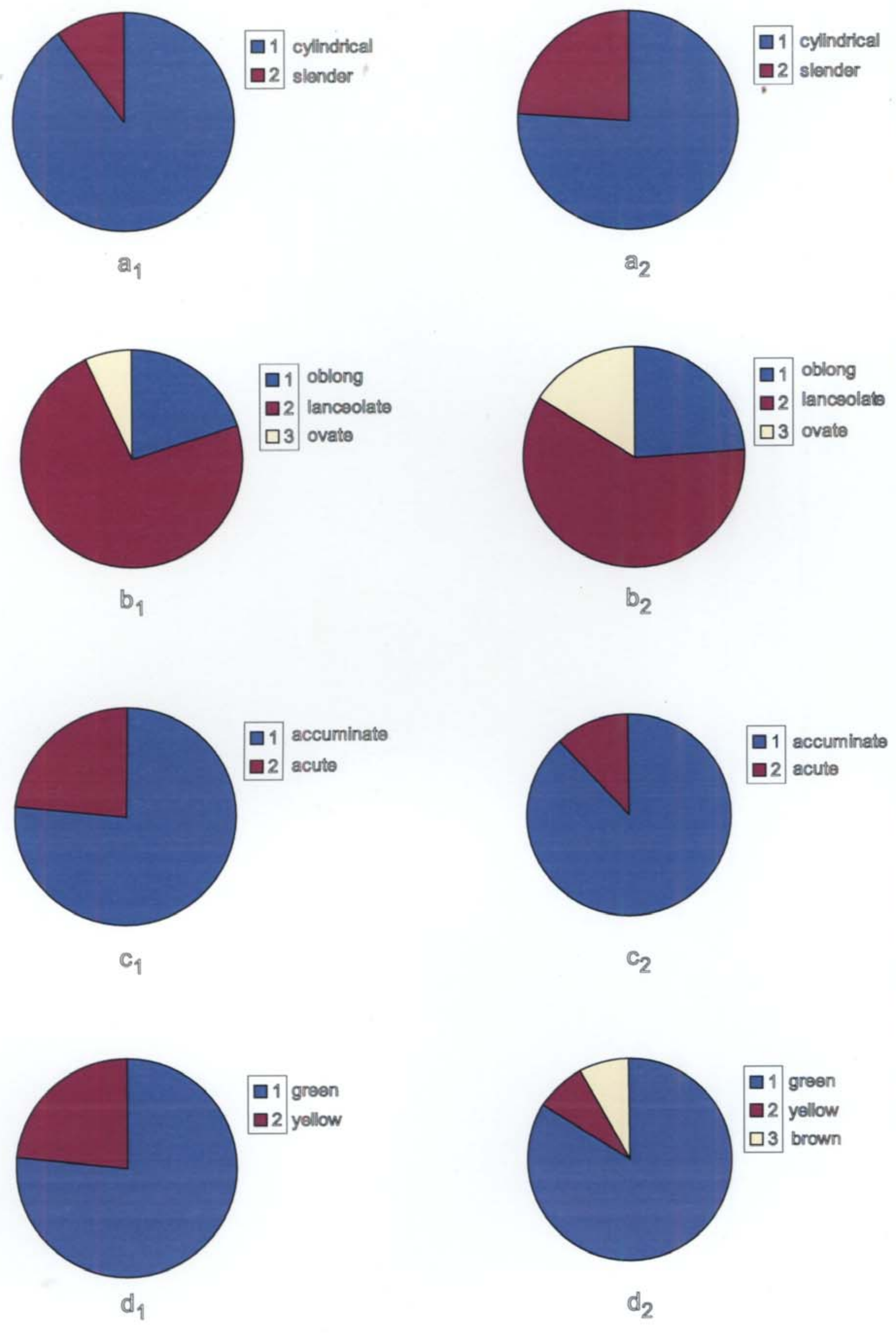

Fig-34: Pie charts showing comparative morphology of seedling progenies ($_1$) and EMS treated seedling progenies ($_2$) with regard to (a): stem shape, (b): leaf shape, (c): leaf tip nature, (d): aerial root colour.

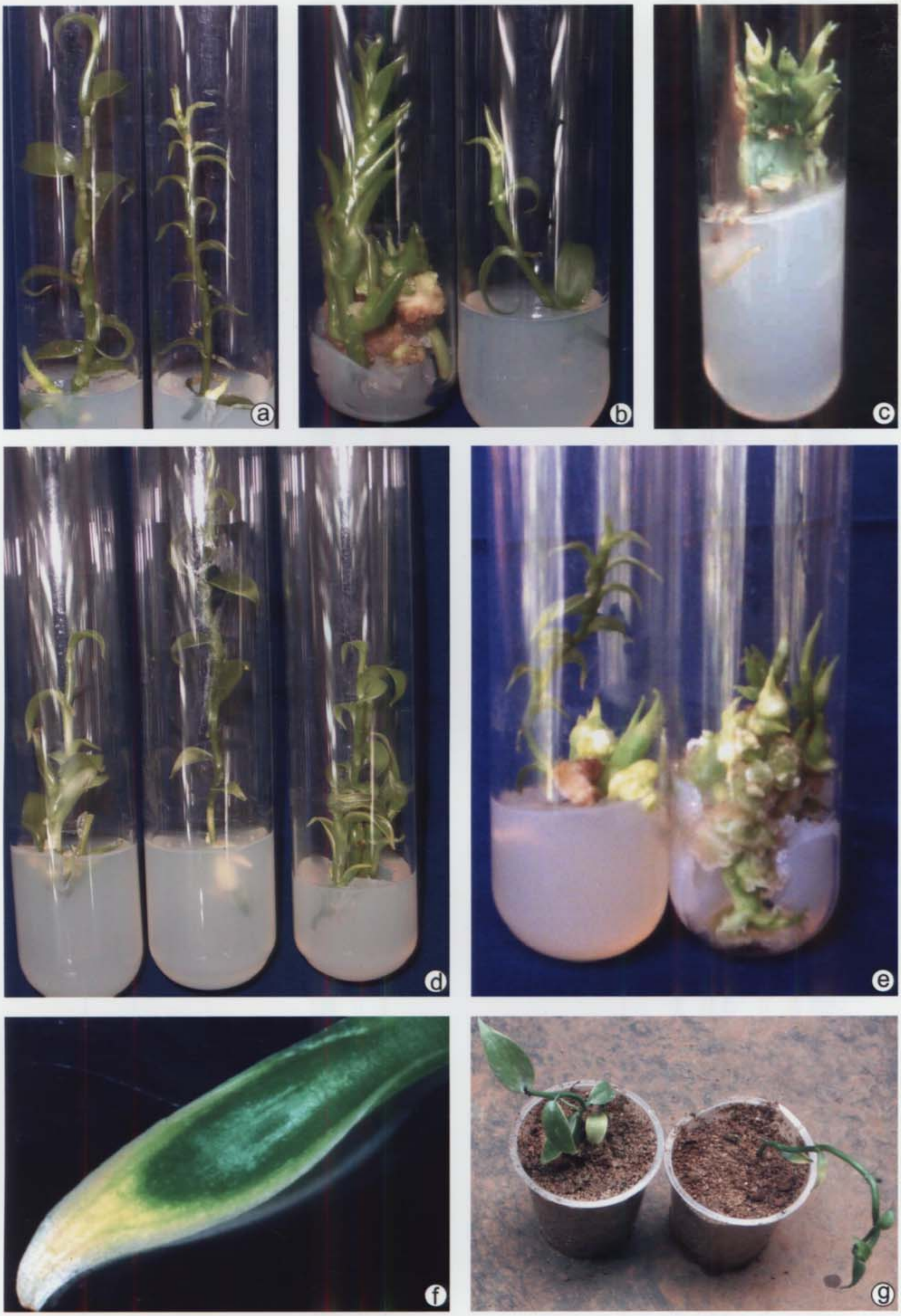

**Fig 35: a-f: *In vitro* responses in EMS treated cultures**; a: an EMS induced narrow leaf type (V145, 0.75% EMS) and its control; **b:** condensed internodal type (V113, 0.75% EMS) and its control. **c:** fasciation; **d:** chlorophyll mutants; **e:** characteristic multiplication of an EMS treated plant; **f:** a leaf with chlorophyll chimeras ; **g:** hardening stage of EMS treated plants.

### *Molecular characterization*

*Genotypes Used:*

Fifteen lines from EMS treated plants of *V. planifolia* were used in the present study. The seed derived progeny V260 was used as control. V56 (0.75% EMS), V77 (0.75% EMS), V89 (0.75% EMS), V92 (0.25% EMS), V113 (0.25% EMS), V113 (0.25% EMS), V113 (0.5% EMS), V113 (0.75% EMS), V124 (1% EMS), V153 (0.25% EMS), V153 (0.75% EMS), V161 (0.75% EMS), V260 (1% EMS), V seed 1 (0.25% EMS) and V seed 3 (0.5% EMS) were the treatments used.

*Screening for primers:*

Out of the 24 primers tested, specific amplifications could be obtained from most of the primers but only seven primers were used for detecting polymorphisms.

*Test for polymorphism:*

Out of the twenty four primers used for screening, seven were selected and used in seven separate reactions to detect polymorphism among the fifteen different EMS treated progenies of vanilla, with V 260 as control. Out of these only three primers, *viz,* OPB 01, OPB 05 and OPE 07 could produce polymorphisms.

Primer OPB 01 (Fig 36) gave specific amplification products in all the genotypes. In the seedling progeny V113 treated with 4 different concentrations (0.25%, 0.5%, 0.75% and 1%) of EMS, this primer produced significant polymorphism. Among the seeds directly treated with EMS, the seed treated with 0.5% EMS showed significant polymorphism with 72.2% similarity and the seed treated with 0.25% EMS showed approximately 94.4% similarity with the control (Table 40).

Primer OPB 05 (Fig 37) could produce polymorphisms in all genotypes except V 92 (0.25% EMS) and V113 (0.25%, 0.5%, and 1% EMS).

Primer OPE 07 gave good amplification in all the genotypes studied (Fig 38). This primer could detect polymorphism between three genotypes with presence or absence of bands at 1.8 Kb region. They are V 56 (0.75 % EMS), V 260 (1% EMS) and V seed 3 (0.5 % EMS). This also indicates that these genotypes are genetically different.

The polymorphic bands were scored and the data is given in Table-40.

**Table-40: RAPD markers showing polymorphism in EMS treated progenies**

| S.N | Primer | A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | A11 | A12 | A13 | A14 | A15 | A16 | Kb |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | OPB 01-1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.3 |
| 2 | OPB 01-2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0.7 |
| 3 | OPB 01-3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.75 |
| 4 | OPB 01-4 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0.95 |
| 5 | OPB 01-5 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1.2 |
| 6 | OPB 01-6 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 2 |
| 7 | OPB 05-1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0.3 |
| 8 | OPB 05-2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0.4 |
| 9 | OPB 05-3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.85 |
| 10 | OPB 05-4 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1.1 |
| 11 | OPB 05-5 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1.2 |
| 12 | OPB 05-6 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 2 |
| 13 | OPE 07-1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.5 |
| 14 | OPE 07-2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.6 |
| 15 | OPE 07-3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0.8 |
| 16 | OPE 07-4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| 17 | OPE 07-5 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1.8 |
| 18 | OPE 07-6 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |

**A1:** V260, **A2:** V56 (0.75% EMS), **A3:** V77 (0.75% EMS), **A4:** V89 (0.75% EMS), **A5:** V92 (0.25% EMS), **A6:** V113 (0.25% EMS), **A7:** V113 (0.25% EMS), **A8:** V113 (0.5% EMS), **A9:** V113 (0.75% EMS), **A10:** V124 (1% EMS), **A11:** V153 (0.25% EMS), **A12:** V153 (0.75% EMS), **A13:** V161 (0.75% EMS), **A14:** V260 (1% EMS), **A15:** V seed 1 (0.25% EMS) and **A16:** V seed 3 (0.5% EMS).

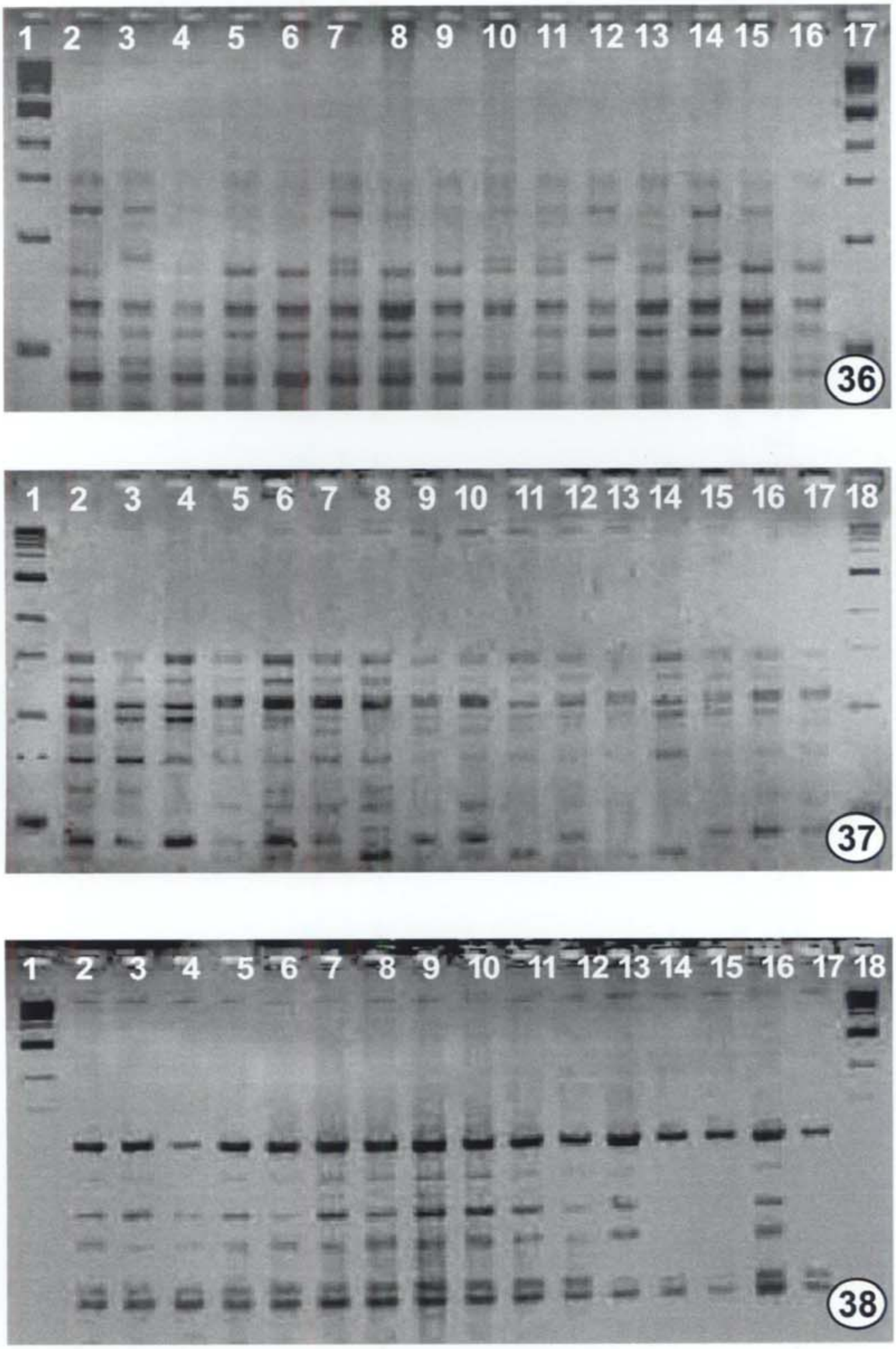

**Fig: 36-38: RAPD profiles of EMS treated accessions using OPERON primers; 36: OPB 01; 37: OPB 05; and 38: OPE 07;** 1.1Kb ladder, 2.V260, 3. V 56(0.75%)*, 4.V 77(0.75%), 5. V 89(0.75%), 6.V 92(0.25%), 7. V113(0.25%), 8.V 113(0.5%), 9. V113(0.75%), 10. V 113(1%), 11. V 124(1%), 12. V153 (0.25%), 13. V153(0.75%), 14. V 161(0.75%), 15. V 260(1%), 16. V seed 1(0.25%), 17. V seed 3(0.5%), 18. 1 Kb ladder; * EMS percentage in parenthesis.

The RAPD data was analyzed using Paired Affinity Indices and percentage similarity was calculated (Table-41). A dendrogram was constructed based on the paired values to show the relation ship between the different EMS treated progenies (Fig 39).

**Table-41: Paired Affinity Indices (PAI; % similarity) of the EMS treated progenies**

| | A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | A11 | A12 | A13 | A14 | A15 | A16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | 100 | | | | | | | | | | | | | | | |
| A2 | 83 | 100 | | | | | | | | | | | | | | |
| A3 | 77 | 83 | 100 | | | | | | | | | | | | | |
| A4 | 83 | 77 | 83 | 100 | | | | | | | | | | | | |
| A5 | 94 | 77 | 83 | 88 | 100 | | | | | | | | | | | |
| A6 | 94 | 88 | 72 | 77 | 88 | 100 | | | | | | | | | | |
| A7 | 100 | 83 | 77 | 83 | 94 | 94 | 100 | | | | | | | | | |
| A8 | 88 | 83 | 88 | 94 | 94 | 83 | 88 | 100 | | | | | | | | |
| A9 | 88 | 83 | 77 | 83 | 94 | 94 | 88 | 88 | 100 | | | | | | | |
| A10 | 72 | 66 | 61 | 77 | 77 | 77 | 72 | 72 | 83 | 100 | | | | | | |
| A11 | 88 | 83 | 66 | 83 | 83 | 94 | 88 | 77 | 88 | 83 | 100 | | | | | |
| A12 | 77 | 72 | 66 | 72 | 72 | 72 | 77 | 66 | 66 | 72 | 77 | 100 | | | | |
| A13 | 72 | 66 | 61 | 66 | 77 | 77 | 72 | 72 | 83 | 77 | 72 | 61 | 100 | | | |
| A14 | 72 | 77 | 72 | 66 | 66 | 77 | 72 | 72 | 72 | 55 | 72 | 50 | 77 | 100 | | |
| A15 | 94 | 88 | 83 | 88 | 88 | 88 | 94 | 94 | 83 | 66 | 83 | 72 | 66 | 77 | 100 | |
| A16 | 72 | 66 | 83 | 88 | 77 | 66 | 72 | 83 | 72 | 66 | 72 | 72 | 55 | 66 | 77 | 100 |

**A1:** V260, **A2:** V56 (0.75% EMS), **A3:** V77 (0.75% EMS), **A4:** V89 (0.75% EMS), **A5:** V92 (0.25% EMS), **A6:** V113 (0.25% EMS), **A7:** V113 (0.25% EMS), **A8:** V113 (0.5% EMS), **A9:** V113 (0.75% EMS), **A10:** V124 (1% EMS), **A11:** V153 (0.25% EMS), **A12:** V153 (0.75% EMS), **A13:** V161 (0.75% EMS), **A14:** V260 (1% EMS), **A15:** V seed 1 (0.25% EMS) and **A16:** V seed 3 (0.5% EMS)

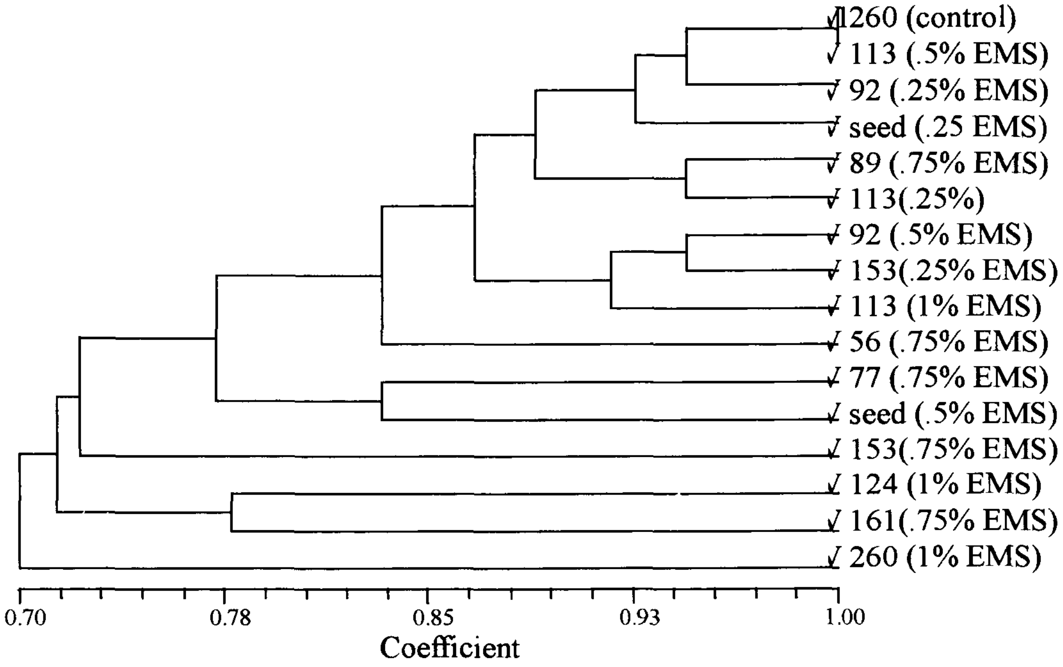

**Fig 39: Dendrogram of EMS treated progenies as expressed by RAPD polymorphisms.**

# Studies on Genetic Variability in Open Pollinated Progenies of Vanilla.

Thesis submitted to
**University of Calicut**
for the award of **Doctor of Philosophy** in Botany

By
**Vimala Jose**

**University of Calicut**
**Kerala, India**
**2005**

# Discussion

*The Malayan species also found in South India*

## VANILLA

*Vanilla* Mill. is Pan tropical, comprises more than 100 species, and thus is noteworthy among genera of the tribe *Vanilleae*. Other genera of *Vanilleae* have 20 or fewer species and are comparatively restricted in distribution (Dressler, 1993; Mabberley, 1997).

Hooker (1885) and Fischer (1928) reported two species from South India – *V.walkeriae* Wt. and *V. wightiana* Lindl. Karthikeyan *et al* (1989) in the Flora of India, reported five species from India -*V. andamanica* Rolfe, *V.parishii* Reichb., *V. pilifera* Holtt., *V.walkeriae* Wt. and *V. wightiana* Lindl. Amongst the five species reported from India, *V.pilifera* Holt. is endemic to Assam while, *V.andamanica* Rolfe.is reported only from Andaman Islands, *V.aphylla* Blume., previously known from Thailand, Laos, Vietnam, Malaya and Java is found occurring in Kerala and Tamilnadu, *V.walkeriae* Wight. from Travancore and Trivandrum and *V.wightiana* Lindl. from the states of Andhra Pradesh, Karnataka and Kerala (Kumar and Manilal, 1993).

Backer and Brink (1968) in the Flora of Java reported three species from Andaman Islands – *V.aphylla, V.albida* and *V.mexicana. V.andamanica* is known only from the Andaman group of islands. Seidenfaden (1978) believes this to be the same as *V. albida* Blume. Dassanayake and Fosberg (1972) in the Flora of Ceylon described only two species endemic to Sreelanka – *V.walkeriae* and *V.moonii.*

*Vanilla* is the only orchid genus that enters into commercial trade, other than those orchids prized for their ornamental value. The famed vanilla flavoring is the product primarily of *V. planifolia* Andrews, and to a lesser extent *V. pompona* Schiede and *V.tahitensis* J. W. Moore (Purseglove, 1972). These and other Vanilla species have been treated in various monographic works, particularly in *le Vanillier et Vanilla Dans le Monde* by Boriquet (1954).

Vanilla is suspected to be highly heterozygous with extensive genetic variations because of its cross-pollinated nature. As much of the cultivated vanilla in the Old World, where introduced from New World; is propagated vegetatively and is of limited clonal origin, there is little variation. Continuous clonal propagation of the existing introduced gene pool leads to narrow genetic base. This is one of the most important limiting factors in varietal improvement of vanilla.

The present investigation was conducted with the broad objective of improvement of vanilla by generating variability in the available gene pool. In this study, seedling progenies, interspecific hybrids, colchicine treated and EMS treated progenies were analyzed for variation as expressed by morphological, cytological, anatomical and RAPD markers.

## IN VITRO CULTURE

The young protocorms of *V. planifolia* is generally elongated and its tip often curved; it is much less thick than the PLBs (Veyret, 1974) and fails to develop chlorophyll. Protocorms of *Vanilla aphylla* consistently produces green protocorms while that of *V.planifolia* are chlorophyll free. The fact that PLBs and protocorms of the cultivated Vanilla are slow to develop chlorophyll in experimental conditions suggests that they would be obligatory mycorrhizal in natural conditions.

Among the vanillas (Bouriquet, 1947; Knudson, 1950), the formation of the roots in the protocorms is much later. The seedling development of all the species is unique in that the first growth is positively geotropic with the developing stem growing downward into the medium rather than upward. Later negatively geotropic leafy stems are formed. The first roots arise at the level at which stem differentiation began.

### Effect of age of the embryo on germination

This experiment was planed to find out the optimum stage at which the *Vanilla planifolia* embryo could be rescued and grown *in vitro* to get maximum germination so that this knowledge could be further used in the hybridization experiments where there is more chance of embryo abortion.

The mature pod size was attained by the time the pod was 6 weeks of age and maximum percentage of germination was obtained 56 days after pollination. The rate of germination seemed definitely to decline after about 27 weeks until no germination after 30 weeks.

From the present study, it was observed that, at the time of pollination the length and girth of the ovary was 5 cm and 1.4 cm respectively which later developed into 15.3 cm and 3.9 cm respectively. The failure to transfer complete pollen mass to the stigma during hand pollination could be the main reason for the differences observed in pod size at the time of harvest in addition to genotype difference if any. This is in agreement with the earlier finding of Bhatt and Sudharshan (2000) about the effect of pollen load on the growth and development of vanilla fruits.

Pollination has a stimulating effect upon the ovary prior to fertilization (Madhusoodanan *et al.*, 2003). Swamy (1947) has noted that ovule formation did not begin in *V. planifolia* until after pollination, and 15 - 20 days were necessary for the archesporial cells to form. Pods containing these stages before fertilization, or even before the ovules were formed, would hardly produce growth if cultured. The best results were obtained when 8 weeks old fruits were cultured.

Embryo age has statistically a significant influence on direct regeneration. The highest percentage was observed with 8 weeks old and older embryos. The lower germination percentage observed with 22 day old embryos might be explained by developmental differences: being at the globular phase, they probably require more complex media for optimal development (Raghavam, 1966). Pod size does not directly influence *in vitro* germination responses; rather it is a reflection of percentage of pollen load (Bhatt and Sudharshan, 2000). The under sized pod developed probably by parthenocarpy or insufficient pollen load failed to germinate in culture even when the harvesting time was increased. Thus, the pollinator should ensure that sufficient pollen mass is deposited on the stigma and all under sized beans should be removed two weeks after pollination to help other pods to enlarge.

Harvesting of the pods for *in vitro* germination at an early period that is in the first two weeks of pollination resulted in failure of germination. This is because there is considerable delay before fertilization of the ovules takes place after pollination, and after that the limited development of the Vanilla seed takes place.

The results indicate that when embryos under the age of 22 days were cultured, they are not able to germinate and may be due to the minimum time required for the orchid pollen tube to reach the ovules, which develop only 26 days after pollination. The minimum period required for pod harvesting for *in vitro* culture is about 26 days. Vanilla seeds which were collected from pods about nine months old; *i.e*, in its full maturity failed to germinate and may be due to the fully developed sclerotic seed coat, which acts as mechanical barrier to germination (Garay, 1960; Beer, 1863); hence need much more time for germination than the time studied in the present experiment. Withner (1955) based on seed coat anatomy concluded that marked hardening of the seed coat took place about 60 days after pollination, and this time seemed to be an optimal one for obtaining the best growth in culture. This germination inhibition may also be due to the vanillate compounds in the placental tissues and glandular hairs. As the seeds and pods mature, these materials form in increasing amounts, in sufficient quantity to reduce germination by chemical means (Withner, 1955).

To avoid chemical or mechanical disadvantages for vanilla seed germination and the effects of aging, ovule culturing should be initiated at an earlier stage. It was inferred from the experiment that the simple retention of the pod in the plant for long would not increase *in vitro* germination percentage, even though it may increase the quality attributes of vanilla bean for marketing.

The growth curve of the vanilla pod can be used to correlate it with pod size. Growth curves to show pod size illustrate the increments after 2 days of pollination with little or no size increase after 42 days. The most preferable time of seed culture is 8 weeks after pollination (Table 12).

Results from this experiment were used to speed up the breeding procedures, as this would shorten the reproductive cycle of vanilla seeds *in vitro*. In the case of incompatible crosses (*e.g.*, interspecific hybridization), embryo rescue at an earlier period would prevent embryo abortion.

### Effect of different media combinations on germination

In the second set of experiment, vanilla pods of different ages were used for ovule culture in order to determine the feasibility of ovule cultures in the different media combinations and to select out an optimal media combination for the further studies.

It was found that the combination MS + BA 1mg/L + IBA 0.5mg/L + Tryptone 2g/L resulted in a relatively higher embryo germinating percentages (>80%) when compared to the control treatment (MS media without hormones). There was considerable variation in growth pattern of the seedlings in the media with various organic additives.

## INDUCTION OF VARIATION

Seed culture, interspecific hybridization, polyploidy induction and mutation induction were the techniques used to induce variations.

### Development of segregating seedling progenies

Embryo culture is used to break dormancy in the seeds, there by shortening the breeding cycle by months or even years. It can also be used when important seed lots have lost viability during storage and have poor germination (Biggs *et al.*, 1986).

Seeds were cultured in MS media supplemented with BA 1mg/L, IBA 0.5mg/L and tryptone 2g/L, sub culturing was carried out in hormone free MS media and hardened under nursery conditions in river sand taken in plastic cups.

#### *Morphological characterization*

From this study, it was observed that the seedling progenies differed among themselves in their external morphological characters. The differences included variations in leaf shape, leaf size, internodal length, root number, root colour etc. Leaf shape variation included oblong, lanceolate or ovate shapes. The leaf length ranged from

2.1 cm to 8.1 cm, the internodal length ranged from 1.7 cm to 6.1 cm, the root number ranged from 2 to 6 cm and the root length ranged from 0.8 cm to 4.8 cm.

### *Cytological characterization*

Despite the disadvantages of small chromosome size and relatively high chromosome number, vanilla has two advantages, which are not present in most other plant groups. First, the aerial roots which are both clean and readily accessible for collection. Second, because of the nature of pollen development in a pollinium, the nuclear division known as pollen mitosis (which occurs after meiosis but before pollen is fully mature) is well synchronized and provides excellent opportunities for chromosome counting (Keith Jones, 1974).

If we try to trace the reason for the high rate of morphological variation among the selfed progenies, we could not in any way underestimate the role played by pollen grains with different genetic make up, due to pre- meiotic and post – meiotic associations and other abnormalities. The extent of variations among individuals of a given species being so greater, that between different species and among interspecific hybrids would be still higher.

The fact that some of the chromosome pairing during meiosis resulted in multivalent and univalent formation, throws light on the possible origin of *V. planifolia* as a natural hybrid or more accurately as a segmental allopolyploid. This can also explain the high degree of sterility observed in the pollen (Ravindran, 1979), because of the low compatibility between the non-homologous chromosomes of the parent. The high rate of heterozygosity among the selfed progenies may be caused by chromosome pairing among the genomes of the natural allopolyploid. That is, pairing often occurs with one chromosome in one part and another in another part, so that multivalents of three or four chromosomes are formed. When the chromosomes separate again, occasionally the separation is three to one daughter cell and one to the other. These variations in distribution of the chromosomes from multivalents often result in high frequency of aneuploidy in the gametes and subsequently in the offspring. Thus, as a consequence of the meiotic behavior of chromosomes in a segmental allopolyploid like vanilla (Nair and Ravindan 1994), they will on selfing give a high proportion of aneuploids among the progeny.

It is likely that, the natural polyploidy in vanilla with its high heterozygosity would be at an advantage over the diploid, the diploid of a normally cross-fertilizing species might show inbreeding depression relatively quickly. Another explanation is that a large range of variation is expected to result from segregation in segmental allopolyploids, and it would be unexpected at the extremity of the range of a diploid (Stebbins, 1950). This seed derived variability will surely facilitate an extension of the geographical range of vanilla and entry into new habitats.

Chromocenter association is affected by the degree of partitioning of the genome, since the extent of this phenomena correlates positively with the number of chromosomes in the complement, the level of ploidy and negatively with the mean DNA content (Ceccarelli *et.al.*, 1998). The present observation of chromosome association in the pre-meiotic cells confirms the finding of Nair and Ravindan (1994) that homologous chromosomes are associated even in somatic tissue. From the present study, it appears that chromocenter association in vanilla starts even before the cell enters to the meiotic divisional stage. The reduction in the number of chromocenters in the later stages proves that they are closely paired during premeiotic interphase. It is remarkable that premeiotic pairing of homologous does not always occur at the same time. In some of the cells, two chromocenters were associated so closely that they seemed to be fused. Due to the size differences between the pairs of chromocenters it was possible to state that homologous chromosomes were associated. The present observation of chromosome association in the pre – meiotic cells is reported for the first time in *V.planifolia*.

In *Xyris* (Benko and Wanderley, 2002), the heterochromatin parts of the homologous chromosomes are associated early during interphase and prophase.

Synapses of homologous chromosomes and subsequent pairing at meiosis are genotypically controlled (Rees, 1961). In the hexaploid oat, Thomas and Mytton (1970) described a factor in the short arm of chromosome IV control pairing between homologous chromosomes. Thus it can be stated that chromosome pairing in somatic cell and premeiotic cell does not imply a difference in the actual event, but refers to a difference in the ultimate fate of the paired chromosomes, *i.e.*, whether they will be involved only in mitosis or eventually in meiosis.

Association of chromosomes in the root tip cells is significantly less intimate than that of homologous association in premeiotic cells. Differences between the mitotic and meiotic association could be an artifact resulting from different treatments of the two sets of material, while meiotic cells received no pretreatment, root tip cells were given cold treatment. Effect of low temperature on spindle suppression can be similar to that of colchicine (Barber and Callan, 1943; Feldman *et. al.*, 1972) there is a possibility that association in root tip cells was partially suppressed.

According to Gaulden (1987), sticky chromosome may be caused by defective functioning of one or two types of specific non-histone proteins involved in chromosome organization that might be necessary for chromatid separation and segregation. The altered functioning of these proteins leading to stickiness may be caused by mutations in the genes that code for the protein (hereditary stickiness) or by the direct action of mutagens in the proteins (induced stickiness). Environmental factors and certain chemical agents are known to increase the frequency.

It has been suggested that blocks of repetitive DNA in constitutive heterochromatin could play role in homologous chromosome pairing by providing large sites for DNA-DNA hybridization (Yunis and Yasminch, 1972; Stack and Clarke, 1973). Late in the premeiotic interphase, the prochromosomes are difficult to distinguish at all. This last period may correspond to the period of replication of DNA (Stack and Brown, 1969).

***Molecular characterization***

Of the six primers screened to detect RAPD polymorphisms among the seedling progenies, four primers (OPB 01, OPB 05, OPE 07 and OPE 18) showed faintly developed DNA fragments showing polymorphism, while two primers (OPE 12 and OPE 19) showed little or no polymorphism. This indicates that there is certain amount of minor differences among the seed derived plants but most of the primers failed to detect polymorphism between these genotypes.

As explained earlier, there must be high amount of variability among the seed derived progenies. However, this could not be detected with present study by RAPD polymorphisms because of poor gels and probably the presence of monomorphic primers.

## Interspecific hybridization

Results showed that hybridization influenced cytological, anatomical and molecular characteristics of the progenies in addition to morphological characters.

### *Morphological characterization*

In the cross between *V.planifolia (♀)* and *V.aphylla (♂)*, the progenies segregated into *V. planifolia* type (broad leaves) and *V. aphylla* type (narrow leaves), the '*aphllya* type' showing chlorosis. In the cross between *V. aphylla (♀)* and *V. planifolia (♂)*, all the progenies showed 'scale leaf morphology', showing chlorosis. The albino plants could not be established when planted out.

In the cross between *V. aphylla* (♀) and *V. planifolia* (♂), maternal inheritance was observed. The maternal inheritance of the above cross may indicate the parthenocarpic fruit development due to pseudofertilisation. Hybridization between *V.tahetensis* (♀) and *V.aphylla* (♂) yielded very few seeds and the seeds obtained did not germinate. In the cross between *V. aphylla* (♀) and *V. tahetensis* (♂), the progenies segregated into broad-leaved type and narrow leaved type.

### *Cytological characterization*

The purpose of this work is to provide preliminary chromosomal data towards the species of vanilla used as parents in the hybridization experiment; and to study the effects of hybridization on the chromosome counts of the progenies. The difficulty with cytological study in this genus is the small chromosome size, the chromosome association and the relatively high chromosome number. The possibility of the improvement and cultivation of vanilla can only be worked out, provided a complete spectrum of genetic variance is fully understood from a cytological standpoint.

In a hybrid, the chromosomes of both parents are brought together, each contributing half of its own total chromosome complement. If the parents differ in their chromosome number, then, of course, it will be possible to detect the hybrids, which will have an intermediate chromosome number. This is the first and the simplest use to which the chromosomes can be put. The chromosome number can be used for unequivocally identifying hybrids when the parents possess different chromosomes (Takatsu *et.al;* 2001).

The chromosome number of interspecific hybrids resulting from a mating between *V.planifolia* (2n=32) and *V. aphylla* (2n=64) would be expected to be 2n=4x=48. This prediction was confirmed by chromosome counts employing root tip meristematic cells recovered from allopolyploids VH7, VH6 and VH19. The progenies with the diploid chromosome number of 2n > 60 in the first cross (VH4 and VH5) most likely rose from the union between unreduced gamete from *V.planifolia* (n=2x=32) and reduced gamete from *V.aphylla* (n=2x=32). The hybrids $VH_2$-1and $VH_2$-2 had a diploid number around 30, and may be due to parthenogenetic development of unreduced gamete due to pseudo fertilization.

Parthenogenesis has been discovered in a certain number of orchids, particularly those in which fertilization is late in occurring (Hagerup, 1945, 1947; Maheswery and Narayanaswamy, 1950). Miduno (1940) reported a haploid parthenogenic origin of an F1 hybrid in a cross between the two Orchid species *Bletilla striata* and *Eleorchis japonica*. Somatic apospory (Swamy, 1948; Wirth and Withner, 1959) and apomixis (Afzelius, 1959) occur in *Orchidaceae* when pollinated with foreign pollen. The anomalies in the development of the gametes, male or female, and degeneration of the pollen *insitu*

(Seshagiriah, 1934) may also result in parthenogenesis. Nucellar origins of the hybrid *Cynorkis lilarcina* x *C. ridleyi* (Veyret, 1972) and integumentery embryony in *Spiranthes* (Swamy, 1948) are some of the reported cases of diploid hybrids in *Orchidaceae*. Ravindran (1976) reported meiotic abnormalities of sterile pollen of *V.planifolia*. Somatic association of chromosomes also exists in *V.planifolia* (Nair and Ravindran, 1994). The cumulative effect of all these factors may be the reason for the deviation from the expected hybrid chromosome number of 2n=48 in some of the hybrids obtained.

Genetic variation in vanilla is relatively limited. Interspecific hybridization is meant to unite the divergent genomes of *V. planifolia* and *V. aphylla*, to produce fertile progeny. Transfer of economically important characters (*e.g.*, disease resistance, adaptability to low humidity) from the wild species could ultimately broaden the narrow germplasm of vanilla.

### ***Anatomical characterization***

*i. species:*

Orchid taxonomy is based mainly on floral morphology and external vegetative characters. It is rather impossible to distinguish one orchid taxon from another in vegetative phase; therefore, anatomical features can be utilized to distinguish species in vegetative condition (Metcalfe, 1961). Earlier anatomical studies on this genus are highly confusing; we could clarify it with some species-specific characters and some species interrelationships based on which a classification of the available species of this genus was attempted. The results obtained from the present investigation revealed a number of interesting features that may be useful in identifying these plants before flowering.

Leaf anatomy:

Anomocytic stoma was shown by Singh and Singh (1974) to be characteristic of the orchidaceae. *V. tahetensis* have slightly sunken stomata often with a narrow pore formed by the overgrowing of the cuticle. The anticlinal walls of the subsidiary cells in the mature leaf of *V.piliferae* are dissolved. Hence the stomata appear to be of floating type as reported in some ferns (Cotthem, 1970). The occurrence of floating stomata only in *V.piliferae* actually, separated it from other species that possessed normal stomata. Singh and Singh (1974) have reported this condition earlier in the family Orchidaceae.

Edeogo and Osawe (1996) recognized the significance of stomatal index values in distinguishing between the leaves of closely related *Senna* species. Considerable differences in stomatal characters were shown by all the seven species of vanilla.

The major feature that seems to differentiate *V.andamanica* from others is the presence of a series of transverse bundles running through the mesophyll. These bundles are composed of sclerenchyma fibers and xylem and phloem elements. They strengthen the leaf and seemingly provide for greater protection for the inner mesophyll from the outside environment. The increase in the quantity of these corresponds to the degree of toughness characteristic of the leaf.

Root anatomy:

It is evident that the diameters of metaxylem cells in terrestrial roots are appreciably wider than comparable cells in aerial roots of the same species. These differences are readily explained in that wide xylem conducting cells in terrestrial roots are clearly modified for rapid water transport; where as those in aerial roots are not so structured.

The epiphytic roots are made impermeable to a considerable degree by the velamen (Daces and Knudson, 1957), so that most water absorption is presumably through the terrestrial roots. Here the terrestrial root is structurally modified so that velamen is absent or modified and absorption can occur freely (Freire, 1939). Thus the velamen roots serves mainly for clinging to the substratum and may be of late in development in their epiphytic way of evolution.

The dimension of metaxylem cells in terrestrial *vs*. aerial roots is indicative of the possibility that aerial roots are non-absorptive of water. Capesius and Barthlott (1975), using radioactive tracers have stated that aerial roots of *V.planifolia* cannot absorb water through their velamina. They report that root tips are clothed with hairs and secrete cementing mucilage on the surface of the root facing the supporting surface, i.e. the ventral surface.

Rosso (1966) suppose that the velamen is coincidental with the epiphytic habit. The possible evidence suggests that velamen root help in anchoring rather than absorption and this again depends on the location of the roots; once they touch the soil they develop into terrestrial roots.

Stem anatomy:

The internal anatomy of the stem of *V.planifolia* and *V. tahitensis* is unique and deserves special mention because of the sclerenchymatous band. Heckel (1899), Hafliger (1901) and Roux (1954) considered this layer as a sclerenchymatous endodermis. Stern and Judd (1999) based on the ecological study of Roux (1954) and Priestley (1926) suggests the role of light exposure in controlling the differentiation of the endodermis.

The shaded habitats occupied by leafy vanillas enhance the formation of endodermis; and the heliophilous nature of leafless species inhibits the differentiation of the endodermis.

The distribution pattern of sclerenchyma can be of great value, but only in plants where this tissue is well developed. It is of particular value in monocotyledons. The distribution pattern of sclerenchyma in the stem of *Vanilla* enables that are other wise difficult to identify species to be distinguished from one another. Stern and Judd (1999) reported that the presence of a sclerenchymatous cauline ring was a useful taxonomic indicator separating the leafy and leafless species. They asserted that in all the members studied of the genus vanilla, the leafy ones had a cauline sclerenchymatous ring while the leafless species lacks this ring. They argued that the sclerenchyma bands act to support leafy stems that might be stressed owing to their foliar burden. Accordingly, in their cladistic analysis they divided the whole genus into two groups based upon the presence and absence of cauline sclerenchymatous ring, which corresponds to leafy nature and leafless nature respectively. This overlooks the role of aerial clasping roots that attach the stem to the substrate and the absence of sclerenchyma bands in some leafy species like *V.pilifera* and *V.andamanica* as observed in the present study. In the present study, hand cut sections from stems of all leafy species were examined. Sections were treated with saffrenin to test for the presence of lignin. In *V.andamanica* and *V.pilifera* the walls of cells in the cortex showed no positive reaction. In our present study, results contrary to the earlier report of Stern and Judd (1999) was found when the leafy species *V.pilifera* and *V.andamanica* were studied.

The water storage cells in the cauline ground tissue are characteristic of those vanilla species lacking the sclerenchymatous ring in their cortex. The larger cell size of the water storage is associated with the pleated cell wall, secondary thickenings in bands on the walls. These thickenings apparently strengthen the cells and hence lend support against collapse due to dehydration. Thus, it seems that the mechanical support offered to the stem by the sclerenchyma ring in some vanillas is compensated in those, which lack the sclerenchymatous ring, by the presence of the water storage cells with its secondary thickenings.

The bundles are scattered in the ground tissue. No definite pattern of scattering arrangement can be detected, though all bundles are oriented with phloem facing outside. The anatomy of individual bundles conforms to the general plan, although small bundles are towards the periphery. In mature bundles, the phloem and xylem are completely surrounded by sheath of thick walled sclerenchyma fibers while in immature ones they are only partial. Parenchyma cells are small, isodiametric and fairly thick walled with simple pits. In *V.planifolia*, the central two adjacent bundles seem to orient side by side.

The xylem elements of vanilla are larger in diameter than is usual for orchids. Withner *et.al.* (1974) while studying the anatomy of orchids considered vanilla as a class by itself because of its vine habit and uniqueness in internal anatomy. Unlike those of other orchids, the conducting cells are vessel elements, with gently slopping end walls and scalariform perforation plates, instead of the usual tracheids. The walls of the elements show scalariform pitting; smaller protoxylem elements have spiral thickenings. The evolutionary development of the vining habit of vanilla perhaps depended upon the greater efficiency for water conduction by vessel elements as compared to the basic trachids. This indicates a more advanced evolutionary state for the stems of vanilla, since it is generally accepted that vessel elements developed as a specialization for the primitive tracheid-conducting cell.

From the analysis of the present data, the following comments may be of interest; The genus vanilla is relatively heterogeneous anatomically than is commonly realized. This lack of understanding has arisen because many botanists have concentrated mainly on morphological characters alone. Anatomical evidences are important because it may enable us to identify fragmentary material where the morphological characters are of no help.

The "leafy" species *V.andamanica* is more related to the "leafless" species *V.aphylla*. One example is provided by the organization of stem cortex in these two groups, the anatomical similarity may be due to the Old World origin of these two species.

The two New World species of vanilla (*V.planifolia and V.tahetensis*) share many common anatomical characters and the rest of the species, which belongs to the Old World, have their own common anatomical characters. The anatomical characters were found to be species specific, based on which a key was prepared. The anatomical key to the species is the first of its kind in *Vanilla.*

The aim of the present study is to use comparative anatomy as an aid to the present taxonomic grouping of the species and to assess whether the data support the continued placement of them in their respective grouping. The differences in anatomy between the New World and Old World species of vanilla may be the result of divergence due to geographical isolation. From this study, it is able to come up with very clear-cut anatomical characters, which could be dependent upon in assessing the specific delineation of these species. In such a genus, one cannot and should not separate anatomical from morphological characters.

*ii. Interspecific hybrids:*

The information from the anatomical study of the species was put to solve the problem of hybridity of the progenies, which showed intermediate character. Comparative juvenile anatomy of vanilla hybrids was undertaken to investigate the anatomical characters of the hybrids and to compare it with that of the parents to confirm hybridity.

Interspecific hybrids were studied anatomically and found to be intermediate between the maternal and paternal parents in some of the characters; or showed one or more maternal / paternal characters. Anatomical features of vanilla are of great taxonomic value. They help to distinguish at the specific level, and also aids in the study of interspecific hybrids (Louro *et.al.*, 1999). From this study, it can be concluded that the failure of establishment of the hybrids (VH4 and VH5) can be attributed to the lower photosynthetic capacity due to low frequency of chloroplasts in the stem (Neo and Bonimi, 1996). Other anatomical factors contributing to high *ex vitro* seedling mortality are the very thin cuticle layer; the discontinuous root pith; and the malformed root stele (Padmaja *et.al.*, 1990).

Thus in short, three clades are evident from the dendrogram- these clades are: the *V.planifolia* clade in which the hybrids VH1, VH6, VH7, VH9, VH15 and VH19 are included; the *V.tahetensis* clade in which the hybrids $VH_2$-1 and $VH_2$-2 are included and the *V.aphylla* clade with the hybrids VH4, VH5, VH60, VH62, VH63, VH71, VH97, VH99, $VH_2$-3 and $VH_2$-4 are included.

The first clade is easily diagnosed by the presence of cauline sclerenchyma band, assimilatory cells and polygonal root pith cells and lack of foliar bundle sheath corona. The second clade in general had cauline sclerenchyma band, assimilatory cells, foliar bundle sheath corona and circular root pith cells. The aphylla clade with its hybrids is characterized by the absence of cauline sclerenchyma band, presence of water storage cells and stomata on both surfaces.

Anatomical characteristics have undoubtedly aided in the study of hybrids as much as that of the parent species, for these features are of great help in pin pointing parentage (Stace, 1970). They help to distinguish at the specific level, and aids in the study of interspecific hybrids (Louro *et.al.*, 1999).

From this study, it is able to come up with very clear-cut anatomical differences among the hybrids, which could be used to confirm hybridity of the plantlets developed. Anatomical characters could be used in breeding programs in the selection of vanilla hybrids for the purpose of establishment. Introducing anatomical parameters in primary screening would in turn reduce the number of expensive transplantation experiments (Sliwinska *et.al.*, 1995).

### *Molecular characterization*

The parentage identification of the hybrids derived from the two different interspecific crosses was analyzed by RAPD technique. The results verify that the hybrids are descendents of the respective crosses, viz, *V. planifolia (♀)* X *V. aphylla (♂)* and *V. planifolia (♀)* X *V. aphylla (♂).*

*Interspecific hybrids of V. planifolia (♀)* X *V. aphylla* (♂):

The present studies indicated that *V. planifolia* and *V. aphylla* are widely distant with their similarity index around 36.4%. Six primers (OPB 01, OPB 05, OPE 07, OPE 12, OPE 18 and OPE 19) provided discrete DNA fragments specific for the male parent. The interspecific hybrids VH4 and VH5 in general showed equidistance from *V. planifolia* and *V. aphylla*, while the hybrids VH7, VH9, VH10, VH13, VH14, VH15 and VH19 are more nearer to *V. planifolia* mother plant. The RAPD profiles support the morphological data with regard to the leaf nature.

*Interspecific hybrids of V. planifolia (♀) X V. aphylla (♂):*

Among the six different interspecific hybrids of this group, $VH_2$ -1 and $VH_2$ -2 resembled *V. tahetensis* male parent with 68.2% similarity while $VH_2$ -7 was least similar with 36.4% similarity. Morphologically $VH_2$ -1 and $VH_2$ -2 are 'tahetensis types' and the others are 'aphylla types'. Thus, the RAPD profiles coupled with morphological characters indicate the hybridity of the six genotypes.

In conclusion, the results indicated that there are genetic variations among the hybrids of vanilla as supported by RAPD profile difference in addition to morphological and cytological difference.

**In vitro polyploidy**

Polyploidy is recognized as the most important feature of evolution of plants. Induction of polyploidy has more importance in vegetatively propagated plants, as low seed set in autoploids can be overcome by its vegetative propagation (Hermsen, 1984).

Polyploidisation of interspecific hybrids proved to be the most practical in breeding work. This approach could solve sterility problems of interspecific hybrids. Inducing tetraploidy through colchicine treatment of the F1 hybrid can restore pollen fertility. With this intention, interspecific hybrids were used for polyploidy induction in the present study.

***Preparation of explants for polyploidy induction:***

Embryos are ideal materials for mutational and polyploidy studies due to its juvenility. Therefore, seed explants are very much used in preliminary mutational experiments. The seed treatment is not practical when clones with specific genotypes are to be doubled, because the seeds are not true to the genotype of the parent. Therefore, the treatment of axillary buds is the suitable method of doubling clones of specific genotypes. The production of single clones of embryos is another method to tackle this problem. This could be achieved if we use somatic embryos for mutational studies. Thus, a protocol for obtaining a ready source of somatic embryos (PLBs) was developed. Thin cell layer culture of PLBs can be used as a commercial application of tissue culture technology for mass micro-propagation as well as mutational study.

The pattern of PLB formation was common to all the media additives. PLBs presented two basic patterns of development; one of them represented by direct plant formation and the other by a secondary PLB regeneration giving rise as a consequence, to clusters of new PLBs. Basically PLB formation in root tips and shoot tips of vanilla involves a direct conversion of the apices, while in most other orchid species studied a callus stage has proved to be a 'sine qua non' condition for *in vitro* regeneration (Stewart and Button, 1978; Kerbauy, 1984 b).

***Responses of explants after colchicine treatment:***

Present investigation indicated that axillary bud treatment was not so effective in inducing autotetraploidy. In axillary bud treatments, ninety explants of both the hybrids and the seedling progenies were treated with different concentrations of colchicine for different durations. Some of the treated plants died before attaining maturity. Some of the seedlings showed marked morphological deformity such as misshaped and contorted leaves, swelling below the apical bud and stunted growth during early stages. Polyploidy was induced only in a very few cases. Callus treatment failed to produce polyploid plants. Surviving explants were maximum in the case of nodal explants. Some of the plantlets showed gigantic characteristics typical of doubled diploids. However, chromosome counts of this morphological suspect genotype did not confirm polyploidy. Maximum number of autotetraploids was produced when germinating seeds were used for treatment. Unfortunately, the seeds of the hybrids could not be used for colchicine treatment. From this study, it was found that the ideal condition for colchicine induction was to treat germinating seeds with 0.4% of colchicine for 15 days by media supplementation method.

Polyploidy induces the following morphological characters in Vanilla - the immediate effects noticed in the suspected polyploids of all the treatments were reduction in plant height, thicker as well as dark green leaves and swelling of root/ shoot bud tips. Considerable changes in the shape and size of leaves in the tetraploid plants of all the treatments were observed at maturity. Leaves of the tetraploids became narrower, but thicker and greener than that of the respective diploids. Eight polyploid plants were established from the experiment, and as expected, there appeared no obvious differences among these in general morphology or cytology.

Induced polyploidy is known to enhance the content and yield of secondary metabolites in various plant species like *Papaver somniferum* (Andreev, 1963), *Mentha* *arvensis* (Janaki and Sobti, 1962), *Catharanthus roseus* (Kulkarni *et al.*, 1984), *Rauvolfia serpentina* (Janaki, 1962) and others. However, no attempt has been made so far towards production of polyploids in *Vanilla planifolia*.

Polyploidy often leads to adaptive tolerances in orchidaceae and have been strongly selected for in those geographical regions, which may place greater environmental pressure in diploid plants (Barber, 1970). The production of auto polyploidy by colchicine treatment was reported for the first time in *V.planifolia*.

### ***Morphological characterization***

Plant height may be used as an index for the early identification of the chromosome doubled plants in vanilla. Autotetraploids were relatively slow growing in early stages but at maturity, the growth was normal. Significant decrease in length and width of leaf was noted in the autopolyploids.

On an average, with increase in concentration, the width of the leaf tends to diminish. This change was observed only when germinating seeds were used as explants. Polyploid leaves were thicker with more narrow and entire leaf blade than diploids. Significant decrease in length, width and area of leaf was noted in these plants than in their diploid controls. One of the colchicine treated plants (plant derived from nodal explant of V145 treated with 0.1 % colchicine) is distinguishable from the control and other treatments by its remarkable stout nature and other characteristics, although, it was diploid in cytological studies. This specific plant was designated as a mutant. Compared to the control this mutant exhibited a vigorous growth rate and plant height from the early stage.

### ***Anatomical characterization***

Stomata of regenerated plants were smallest in the diploid genotype, followed by tetraploid. Increased guard cell size was found to be a consistent indicator of a doubling of the chromosome number (Watrous and Wimber, 1988). Stomatal size decreased with increase in stomatal density. High positive correlations were found between area and stomatal size. Stomatal size was negatively correlated with stomatal density. It was concluded that stomatal size and density on the abaxial leaf surface could be used to identify tetraploid vanilla early on its development.

Chloroplast number in guard cells is found to be positively correlated with ploidy, and provided a reliable method to distinguish diploid, triploid and tetraploid and hexaploid plants from each other regardless of their taxonomic backgrounds (Singsit and Ozias-Akins, 1992). This was done by counting the chloroplasts in guard cells of 10 stomata of young leaves after staining with potassium iodide. The chloroplast count technique is a rapid and reliable method of determining ploidy level in the plant species (Singsit and Ozias, 1992).

Correlating these traits with chromosome counts using root tips assessed the reliability of ploidy determination using stomatal measurements. In general, size and density of the stomata, which are negatively correlated, varied according to ploidy level. Diploids had, on average, 29 stomata per sq. mm. while tetraploids had an average 15 stomata per sq. mm. Both traits have linear relation ships with changes in ploidy level. Stomatal size and ploidy were positively correlated while stomatal density and ploidy were negatively associated.

It was found that the induction of polyploidy in vanilla *via* colchicine treatment is dose dependent and explant dependent and macro and micro phenotypic features could be used in ascertaining ploidy level. The ploidy level variations can be easily confirmed by root tip chromosome counts. Induction of polyploidy in *Vanilla planifolia* by using seeds was simple and reliable and may be effective in developing new germplasm of *Vanilla planifolia.*

The induced polyploids were characterized by reduction in size to their diploid counterparts. It is often stated that 'gigantism' is one of the chief attributes of polyploidy. However, in this case results have been obtained contrary to the general expectations. Instead of "gigas" characteristics typical of doubled diploids, the chromosome doubling in *Vanilla planifolia* often showed a tendency towards morphological diminishing especially in leaf area. Bhattacharjee (1956) working on autotetraplod pigeon pea observed that the autotetraplods are often shorter than diploids in some of the morphological parameters. Such an effect often results from fewer cell division accompanied by increased cell size and larger stomata. These facts also suggest that duplication of genes responsible for quantitative characters does not always enhance but some times cause reduction (Gupta and Sinha, 1978).

This underlines the general concept that *Vanilla planifolia* is a natural allopolyploid (Nair and Ravindran, 1994) and further doubling of chromosome number after reaching the optimal level of ploidy often leads to reduction in size.

***Molecular Characterization***

The RAPD primers used in the present study could not detect significant polymorphisms among colchicine treated ones. The occurrence of monomorphic bands in may be due to failure of amplification of the genotypes. Use of more primers may resolve the problem.

In general, similarities were noticed between colchicine treated ones and this might be due to the fact that colchicine simply duplicates the DNA with no significant genomic alterations and duplications could not be detected by the RAPD technique.

**In vitro mutation**

Vanilla is the second most costly spice crop in the world. Due to the low priority attached to this crop in the past, no systematic breeding work was under taken to develop improved varieties resulting in a narrow genetic base of these crops. Hence, EMS was used to induce genetic variability in traits of economic importance thus diversifying the genetic base of vanilla. The use of mutagenic agents to induce variability has been a practical tool especially when natural variability is not available (Tulmann, 1990).

***Morphological characterization***

Attempts were made to standardize a micro technique for plant regeneration from mutated tissue of seeds, stem node, callus and PLB. One of the main objectives in vanilla research is to obtain short internodal varieties in order to reduce production costs, increase planting density and greater number of inflorescences resulting in higher yield. From the study, few short internodal variants were identified and could be used as promising planting material. The short internodal mutant (V89 treated with 0.75% EMS) with narrow leaf obtained in this experiment is of potential use in breeding sun scorching resistant cultivars. Some of the EMS induced variants (V145 treated with 0.75% EMS) showed a reduction in leaf area and they may be ideally suited in agro climatic areas of low humidity and reduced water availability. Some of the EMS induced variants (V113 treated with 0.75% EMS) were characterized by thicker stems and shorter internodes, hence the rosetted appearance. Fasciation and branching were common. Color breaks or sectors with less chlorophyll, frequently appeared in both stem and leaves.

Although the large fleshy leaf can store considerable water, it has the greater disadvantages of overheating and of therefore being very inefficient in photosynthesis. The small narrow leaf on the other hand, may cool rapidly (Sanford, 1974). The broad leaf types are more adaptable to exposed, sunny habitats and narrow-leaf with moist shaded habitats.

### ***Molecular characterization***

The present study indicated that concentration of EMS, the type of explant used for treatment along with the genotype of the treatment influenced the variation as expressed by RAPD polymorphism.

The EMS concentration of 0.75% was most effective in inducing variation with only 72.2 % similarity with the control, while EMS concentration of 0.25% failed to produce significant polymorphisms as is evident from the paired affinity indices value of 94.4 % similarity with the control. Among the different types of explants used, the protocorms (V153) and seeds (V seed) were equally efficient in producing variations as is evident from the similarity indices. However, here also the concentration of EMS influenced the degree of polymorphism.

From this study, it was found that variation among EMS treated plants was more than that found in seedling progenies. The appearance of reduced leaf size, a primitive character, in the colchiploids and EMS variants is an indication that disease resistance associated with the leafless vanillas could be transferred by this approach also in addition to hybridization approach. The RAPD primers used in the present study could not detect significant polymorphisms among seed derived progenies and colchicine treated ones. The occurrence of monomorphic bands in seedling progenies may be due to failure of amplification of the genotypes. Use of more primers may resolve the problem.

Thus, in conclusion, the results indicated that variability of the cultivated vanilla could be obtained from segregating progenies of *V.planifolia*; this variability can be further enhanced by *in vitro* polyploidy, *in vitro* mutation and interspecific hybridization.

The modal vanilla of the future will be a disease resistant one, which is adapted to a wide range of agro climatic conditions, with a shorter vegetative phase and greater inflorescence density, with bigger pods and higher vanillin content and preferably, flowers with out rostellum so that the laborious hand pollination could be avoided. It needs a lot more to do in vanilla breeding especially *ex vitro* screening trials to achieve the ultimate goal.

## Evolutionary trends in vanilla

The *Orchidaceae* excel in an overwhelming polymorphy (Vermeulen, 1966). Determination of the evolutionary rate is extremely complicated. Besides the inherent characters in the given group, environmental factors play a large part. Not all species of a particular genus evolve at the same speed. The rate of evolution depends upon the evolutionary potential, genetic plasticity and the types of selection pressure operating upon them (Sivarajan, 1991).

The issue of distinguishing between anatomical characters that are related to habitat and environment and those that point to taxonomic affinity is constant and complicated. In an attempt to produce a more phylogenetic classification comparative anatomy will provide a new scope. The different types of tissues in related groups of plants may be the result of divergent evolution. Population of the same species and even different organs of the same taxon are known to have evolved at different speeds (Stebbins, 1984a). This makes determination of the polarity of characters extremely difficult. Earlier studies on RAPD profiling indicated that the species form three distinctly different groups – *V.planifolia* and *V. tahetensis* in one group; *V.aphylla* and *V.pilifera* in another distinct group and *V. andamanica* is widely separated from both these groups (Minoo, 2002).

The center of maximum diversity is considered as the center of origin of any particular taxa (Davis and Haywood, 1963). Accordingly, the probable centers of origin of the different species of Vanilla are given in Table 42 and Figure 40.

**Table 42-: List of species used in the present study:**

| Sl# | **Species studied** | **Centre of distribution** | **Morphological type** | **Reference** |
|---|---|---|---|---|
| 1 | *V. planifolia* Andr. | Mexico, C America | Group I * | Purseglove, 1974 |
| 2 | *V. aphylla* Blume. | Malaya, Kerala, Tamil Nadu | Group II ** | Backer & Bakhuizen, 1968 |
| 3 | *V. andamanica* Rolfe. | Andaman Islands | Group I | Rolfe, 1918 |
| 4 | *V. pilifera* Holt. | Malaya, Tailand and Assam | Group I | Borthakur & Hajra, 1976 |
| 5 | *V.tahitensis* Moore. | Tahiti and Hawaiian Islands | Group I | Purseglove, 1974 |
| 6 | *V. whighti**ana* Lindl. | Andhra Pradesh, Karnataka | Group II | Hooker, 1890 |
| 7 | *V. walkierie* Wight. | South India to Sreelanka | Group II | Hooker, 1890 |

***Group 1**- those with persistent foliage leaves, **** Group II** - those with scale leaves.

The epiphytic habitat of *vanilla* appears to be equivalent to the distributed habitat in that there are few competitors. Moreover, epiphytic orchids have been called pioneers (Pettendrigh, 1948). Van Oye's early work (1921, 1924) in Java clearly demonstrated the reality of epiphytic succession with ferns and orchids following algae, lichens and bryophytes. Olwer (1930) found much the same thing in New Zealand: the first settlers are usually small lichens and mosses; ferns appear next, together with orchids.

Thorne (1973) stated that continental drift was too early to help much in explaining present day distributions of orchids. Long distance dispersal of orchid seeds across ocean gaps is doubtful, however, both because of distances and time involved and, more especially, because of the toxic salinity of sea water (Wright, 1946).

The evolution and proliferation of orchid species have been accompanied by responses to environments where rainfall and temperatures are seasonal and sun and heat are considerably more direct than in shady moist forests. These conditions necessitate ample provisions for water storage and retention as a prerequisite for survival (Withner *et al.*, 1974).

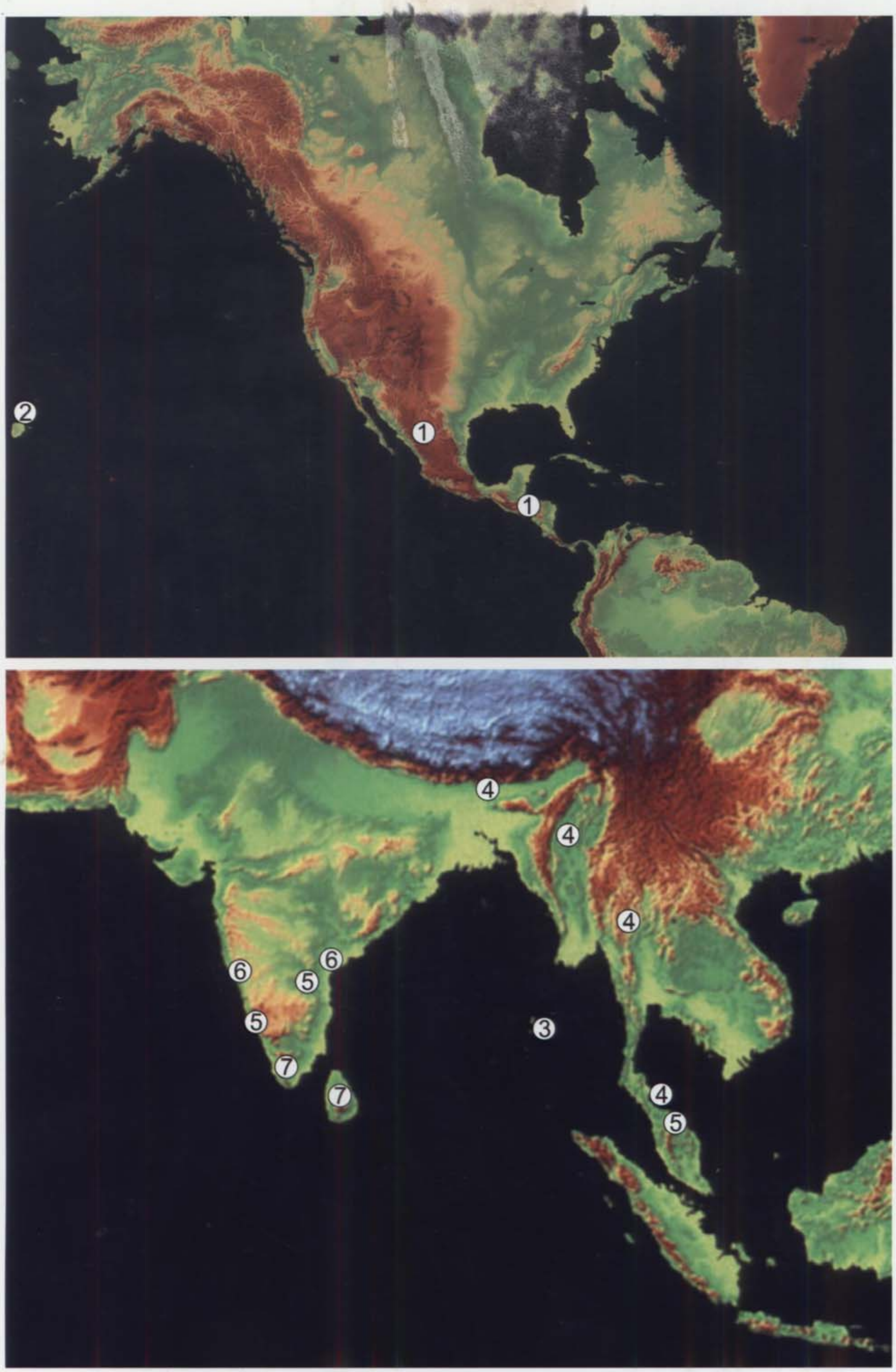

**Fig 40: Geographical distribution of the species of vanilla as per Table 42:** distribution range of **1:** *V.planifolia*; **2:** *V.tahetensis*; **3:** *V.andamanica*; **4:** *V.pilifera*; **5:** *V.aphylla*; **6:** *V.wightiana* and **7:** *V.walkieriae*.

The phytogeography also supports this idea. In the Old World, the origin of cultivated plants is associated with a zone between the latitudes of 20° and 40° N. This mountain area is delimited by the deserts of Central Asia and by the Sahara and, as far as the diversity of the climates and soils is concerned, it represents optimal conditions for the manifestation of the type forming process (Vavilov, 1992).

The Old World species of *Vanilla*, both the scale leaf type and the foliage leaf type provide splendid modifications for water storage, but they depend on shaded or cool environment. The hard leathery foliage leaves of New World species of *Vanilla* provide the most effective water retention. The thick epidermal cells and more important, the ring of cauline sclerenchyma fibers provide excellent protection against dehydration, as well as affording support.

Mexico and Central America are the basic centers of origin of cultivated plants in the New World (Vavilov, 1992). There can be no doubt that the agricultural crops of Mexico and Central America arose independently and without any influence from the Old World (Merrill, 1933, 1934, 1938).

The agriculture of Central America and Southern Mexico is primitive. Therefore, there is no sharply reflected differential localization of it associated with river basins or water reservoirs, such as is found in areas with irrigated agriculture of the Old World. All the Mexican agriculture was essentially non-irrigated in the past. Irrigation was developed in Northern Mexico only during the 19th century (Vavilov, 1992). The non-irrigated ancestry of the New World species is reflected in their xerophytic adaptations.

In this genus, there is a mixture of mesophytic and xerophytic features such as epidermis with thick cuticle, anomotetracytic stomata, water storage cells, crystals etc. It can be concluded that the anatomical features of these lend support to the phylogenetic trends within and between them. At any rate, the fact that the difference between the two groups – the Old World species (mesic) and the New World species (xeric) are so consistent clearly suggests that the two groups have developed independently.

*Vanilla andamanica* being endemic to the islands of Andamans makes it to consider it as a special distribution area apart from New World and Old World. The Andaman and Nicobar islands (Lat.6 ° to 14 ° N and Long. 90 ° to 94 ° E) situated in the Bay of Bengal are well known for their plant diversity and floristic richness. The tropical forests, which clothe these islands, are the home for many flowering plants and there are about 215 endemic species in these islands. Andamans is the closest large landmass. This island has 971 orchid species distributed over 139 genera (Backer, 1968).

The problem of orchid floras of islands is based on two geographical factors: the relative positions and their sizes (Tobler et al., 1970). Preston (1962) has given statistical reasons for caution in considering island flora depauperate. He maintains that islands are 'isolates' and equal areas on the main land are 'samples' and so direct comparison is invalid. Although this is true, it is still instructive to compare speciation in 'isolates' and in 'samples' and his statistical caution rather misses the point.

*Vanilla tahetensis* is endemic to Tahiti and Hawaiian Islands (Purseglove, 1972). The Hawaiian Islands are separated from other landmasses by very great sea distances. Geographically this is the most isolated of all the floristic regions, a fact that undoubtedly accounts largely for its most prominent botanical feature, the huge proportion of its flora, which is endemic (Good, 1974). However, according to Stone, (1967) these islands have only 2 % endemism in the monocots. Orchid species of Hawaiian Islands are only 3 in 3 genera (Dillon, 1953).

From the study of distribution of *V. tahetensis* across Tahiti and Hawaiian Islands, we can conclude that some long-range dispersal of orchids over water may have occurred and may still occur, although to a reduced extent (Sanford, 1974). *V.tahetensis*, the Hawaiian species of vanilla shows well-marked adaptation due to their evolution in the coastal habitat.

The anatomical study revealed that species of a genus endemic to a particular geographical area might not have much anatomical differences. *V.aphylla* and *V. andamanica* are endemic to South Asiatic tropic and they resemble each other anatomically. In spite of the superficial similarity in morphology *V.andamanica* is not so closely related to *V.planifoli*a. They have some anatomical characters in common due perhaps to convergence; but there are notable anatomical differences. One difference is the absence of sclerenchymatous ring in the stem cortex of *V. andamanica*, which is present in *V.planifolia*. This was not only an important anatomical character to distinguish the species based on phytogeography, but also has potential value in the study of hybridization and breeding between them. Diversification into new species in the Old World is largely dependent upon diversity of available habitats, which is normally greater in larger areas (Sanford, 1974).

Evolutionary forces acting on geographically isolated demes results in primary speciation (Stebbins, 1974). This evolutionary trend is evident even in the evolution of pollinators along with their crop. Orchid species have evolved through the development of pollinator specificity as opposed to the incompatibility mechanisms that are usual in other plants (van Steenis, 1969). It is also interesting that the bees of Mexico belong to the genus *Melipona*, in contrast to the domesticated bees of the Old World, which belong to the genus *Apis*. (Norden-skiold, 1929; Sapper, 1935). *Mellipona* bees naturally pollinate *Vanilla planifolia* in their native home (Purseglove,197 ) and thus there is better chance of evolution.

In nature, the Old World species of vanilla rarely set fruits and is mostly propagated vegetatively (except for *V.wightiana* and *V.andamanica* where natural fruit set is reported) due to the absence of natural pollinators. As the Old World species of vanilla rarely undergo sexual reproduction, a great amound of divergence of these group from the New World species may have resulted from the cumulative effect of spacial and reproductive isolation.

The anatomical differences between the New World species and Old World Species may be the result of geographical isolation, which subjects them to different selection pressures and initiates their divergence. *V.planifolia* of Central America and *V. aphylla* of Indian subcontinent provides very good examples of how special isolation has resulted in divergence, which is also reflected in their anatomy.

Stomatal structure is another character, which may indicate phylogenetic trends. All the species of vanilla has conspicuous stomata of the anomocytic type, in which no subsidiary cells accompanying the guard cells. According to Stebbins and Khush (1961) this type of stoma is more advanced and derived from one with two or more subsidiary cells and may have originated under the influence of xerophytic conditions.

Ontogeny recapitulates phylogeny so often that this criterion could be used for phylogenetic reconstruction and is determining character polarity (Hennipman, 1987).

Evidence from ontogeny is of great value in the assessment of homology, as an extra source of information from which one can judge historical relationships, and as an aid for polarizing character transformations (Kluge, 1985). Dressler and Dodson (1960) point out that both the leafy and leafless species of vanilla have similar ontogeny. All the species shows convolute leaf formation during development and only the foliage leaf types shows conduplicate appearance when mature. If ontogeny reflects evolution, it could be concluded that the character state of convolute leaf formation implying leaflessness is likely to be more primitive in the group.

The mitotic chromosome association and the formation of bivalent, trivalent and quadrivalent- like structures during meiosis in vanilla raise the question whether it is of any phylogenetic significance and can throw new light on the basic chromosome number of this genus. If the chromosome association observed in this taxa is the result of pairing and chiasma formation between non-homologous chromosomes, it should be assumed that there are duplicated segments in the genome. Cultivated vanilla from the so-called diploid cultivar of *Vanilla planifolia* has been propagated vegetatively since its introduction into India. In such situation, it is likely that many structural changes may have been produced and preserved to a great extent in the plant.

The gametophyte is of special significance in plant evolution and taxonomy, for it is the forrunner of the sporophyte. Almost all palynological discussions on plant relationships and phylogeny are based on the form, number and position of the apertures (Nair, 1971). A palynological examination of the two species representing leafy and leafless vanilla revealed that the pollen grains of its members fall into two groups, colpate in leafless forms and porate in leafy forms (Fig. ), and that in combination with other characters this can effectively utilized in analyzing the phylogenetic trends. Colpate type is the primitive type of aperture associated with the Magnolian stocks of Angiosperms (Sivarajan, 1992) and the occurrence of this type of pollen in *V.aphylla* further supports the idea that leaf less types are the forerunners of leafy types.

The dependence of leafy vanilla on mycorrhiza for seed germination and seedling establishment is a major factor in habitat determination. Arditti and Ernst (1972) considered such orchids as parasites upon saprophytic fungi in the initial part of their life cycle. This made life on the tree surface possible where by cellulose and other organic materials could be broken down and utilized. In this course of evolution they also developed xerophytic modifications for water conservation. Evolution among orchids proceeded in this fashion - from autotropic to saprophytic, from terrestrial to epiphytic, from mesophytic to xerophytic and from self pollinated to insect pollinated (Sanford, 1974).

Thus evidences from the various fields like embryology, palynology, anatomy, morphology and phytogeography support the possible evolution of leafy vanillas from leafless progenitors. In the course of evolution, more specialized adaptations evolved in the vanillas - the green protocorms of leafless forms loosing chlorophyll in the leafy forms and completely depending on michorrhizal nutrition; the development of pollinator specificity in the leafy forms because of the well differentiated rostellum etc. (Hirmer, 1920; Gellert, 1923). However, the remenents of this evolutionary leap from leafless to leafy is conserved in intermediate forms like *V. andamanica* and *V. pilifera*.

The question of origin of the leafy vanillas with a much lower chromosome number from the leafless ones with a higher chromosome number is another aspect which has evolutionary implications. It has been widely held that diploids are more primitive forms from which polyploids arose and that this change is irreversible. The findings of Raudolph and Fischer (1939) that tetraploid strains of maize could give rise to diploid ones and the hypothesis of Ornduff (1970) about the evolution of plants with low chromosome number from those having higher numbers are evidences that the other way is also possible.

Stebbins (1950) envisaged a diminution in the chromosome base number during the progress of evolution and the results of studies in *Crepis*, *Haplopappus* and many other genera have agreed with his contention. Jones (1970) is of openion that reduction in number is much more frequent than increase and he cited that 'polyploidy can raise total chromosome number to high levels but then further progression is frequently by reduction'. In Vanilla it is more appropriate to think that there occurred a drop in chromosome number during the process of evolution as presumed by Darlington (1956) by the phenomenon of 'polyploid drop' and the primitive leafless forms have become modified to leafy forms.

Like all approaches to taxonomy, phylogenetic approach has its limitations. Variation is a prerequisite for biological diversity. Only if more data from other fields is accumulated, and their taxonomic significance is analyzed, it is to be expected that this approach will be fully understood.

# Studies on Genetic Variability in Open Pollinated Progenies of Vanilla.

Thesis submitted to
**University of Calicut**
for the award of **Doctor of Philosophy** in Botany

By
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**2005**

# Summary and Conclusion

*The endangered South Indian Vanilla*

## VANILLA

Vanilla *(Vanilla planifolia)* is one of the most expensive spice traded in the global market after Saffron (*Crocus sativus L*). Vanilla pods are the cured fruits obtained from three different species, namely, *V. Planifolia* Andrews (Mexican vanilla), *V. tahitensis* Moore (Tahiti vanilla) and *V. pompona* Scheiede (West Indian vanilla). Five species of vanilla are reported from India - *V.pilifera* Holt., *V.andamanica* Rolfe., *V.aphylla* Blume., *V.walkeriae* Wight. and *V.wightiana* Lindl. (Kumar and Manilal, 1993).

Genetic variation in the cultivated germplasm of *Vanilla planifolia* (2n=32) is relatively limited because of the continuous clonal breeding of the existing introduced gene pool. This is one of the most important limiting factors in varietal improvement of vanilla.

The present work was taken up with the following main objectives: they are, induction of variations in segregating progenies of vanilla and enhancing the spectrum of variability by induction of polyploidy and mutations. Interspecific hybridization was also attempted. The *in vitro* technology for culture and propagation of vanilla was improved for efficient and early development of plants and regeneration of the plantlets. The extent of variability of the resultant plantlets was estimated by morphological, cytological, anatomical and molecular characterization. A study on vegetative anatomy was conducted in 7 species of vanilla for the possible preparation of anatomical key and identification of the species. A brief discussion on the evolutionary trends of this genus was also attempted.

## IN VITRO CULTURE

*In vitro* culture of the seeds were tried to produce sufficient seeds and seedlings for further studies.

### Effect of age of the embryo on germination

This experiment was planed to find out the optimum stage at which the *Vanilla planifolia* embryo could be rescued and grown *in vitro* to get maximum germination so that this knowledge could be further used in the hybridization experiments where there is more chance of embryo abortion.

The mature pod size was attained by the time the pod was 6 weeks of age and maximum percentage of germination was obtained 56 days after pollination. The rate of germination seemed definitely to decline after about 27 weeks until no germination after 30 weeks.

The results indicated that when embryos under the age of 22 days were cultured, they are not able to germinate and may be due to the minimum time required for the orchid pollen tube to reach the ovules. It was inferred from the experiment that the simple retention of the pod in the plant for long would not increase *in vitro* germination percentage, even though it may increase the quality attributes of vanilla bean for marketing. To avoid chemical or mechanical disadvantages for vanilla seed germination and the effects of aging, ovule culturing should be initiated at an earlier stage. Results from this experiment were used to speed up the breeding procedures, as this would shorten the reproductive cycle and reduce the time from one generation to another.

### Effect of different media combinations on seed germination

In the second set of experiment, vanilla pods of different ages were used for ovule culture in order to determine the feasibility of ovule cultures in the different media combinations and to select out an optimal media combination for the further studies.

It was found that the combination MS + BA 1mg/L + IBA 0.5mg/L + Tryptone 2g/L resulted in a relatively higher embryo germinating percentages (>80%) when compared to the control treatment (MS media without hormones). There was considerable variation in growth pattern of the seedlings in the media with various organic additives.

## INDUCTION OF VARIATION

Seed culture, interspecific hybridization, polyploidy induction and mutation induction were the techniques used to induce variations.

### Development of segregating seedling progenies

Seeds were cultured in MS media supplemented with BA 1mg/L, IBA 0.5mg/L and tryptone 2g/L, sub culturing was carried out in hormone free MS media and hardened under nursery conditions in river sand taken in plastic cups.

#### *Morphological characterization:*

From this study, it was observed that the seedling progenies differed among themselves in their external morphological characters. The differences included variations in leaf shape, leaf size, internodal length, root number, root colour etc.

#### *Cytological characterization:*

*Meiotic studies*: Heterochromatin is distinctly visible as chromocenters in the inter phase nuclei of *V.planifolia*. Early leptotene stages showed even and thin chromatin interrupted by heterochromatic region, which seem to lie in pairs. Interphase nuclei preceding leptotene showed darkly stained, compact and thick chromocenters whose number equals the diploid number of chromosomes. Early metaphase stage was characterized by the presence of bivalents and multivalents laying together, the number of pairs often less than 16. The present observation of chromosome association in the pre-meiotic cells confirms the finding of Nair and Ravindan (1994) that homologous chromosomes are associated even in somatic tissue.

*Mitotic studies*: The cytological examination of the progenies produced by selfing showed the expected number of 2n=32 in most of the *V planifolia* progenies. Apart from the diploid chromosome number some aneuploids with chromosome number less than 2n =32 were also observed. Cells showing mitotic associations were common in most of the preparations.

If we try to trace the reason for the high rate of morphological variation among the selfed progenies, we could not in any way underestimate the role played by pollen grains with different genetic make up, due to pre- meiotic and post – meiotic associations and other abnormalities. The present observation of chromosome association in the pre – meiotic cells is reported for the first time in *V.planifolia*.

If the chromosome association observed in this taxa is the result of pairing and chiasma formation between non-homologous chromosomes, it should be assumed that there are duplicated segments in the genome. The fact that some of the chromosome pairing during meiosis resulted in multivalent and univalent formation, throws light on the possible origin of *V. planifolia* as a natural hybrid or more accurately as a segmental allopolyploid. This can also explain the high degree of sterility observed in the pollen

(Ravindran, 1979), because of the low compatibility between the non-homologous chromosomes of the parent.

### *Molecular characterization:*

Of the six primers screened to detect RAPD polymorphisms among the seedling progenies, four primers showed faintly developed DNA fragments showing polymorphism, while two primers showed little or no polymorphism. This indicates that there is certain amount of minor differences among the seed derived plants but most of the primers failed to detect polymorphism between these genotypes.

It was concluded that the seed derived variability would surely facilitate extension of the geographical range of vanilla and entry into new habitats.

## Interspecific hybridization

Interspecific hybridization between cultivated vanilla (*V. planifolia, V. tahetensis)* and wild vanilla (*V. aphylla*) was done to produce fertile progeny which may carry economically important genes. Results showed that hybridization influenced cytological, anatomical and molecular characteristics of the progenies in addition to morphological characters.

### *Morphological characterization:*

Most of the hybrid plants showed a phenotypic similarity to the maternal parent. The hybrids of the cross *Vanilla aphylla (♀)* x *Vanilla planifolia (♂)*, come under the scale leaf morphotype. In the other two crosses, the foliage leaf type and scale leaf type segregated in the progenies.

### *Cytological characterization:*

The chromosome number of interspecific hybrids resulting from a mating between *V.planifolia* (2n=32) and *V. aphylla* (2n=64) would be expected to be 2n=4x=48. This prediction was confirmed by chromosome counts employing root tip meristematic cells recovered from some of the allopolyploids. The progenies with the diploid chromosome number of 2n > 60 in the first cross most likely rose from the union between unreduced gamete from *V.planifolia* (n=2x=32) and reduced gamete from *V.aphylla* (n=2x=32). The hybrids with a diploid number around 30 may be due to parthenogenetic development of unreduced gamete.

***Anatomical characterization:***

Orchid taxonomy is based mainly on floral morphology and external vegetative characters. It is rather impossible to distinguish one orchid taxon from another in vegetative phase; therefore, anatomical features can be utilized to distinguish species in vegetative condition (Metcalfe, 1961).

i. Species:

The aim of the present study is to use comparative anatomy as an aid to the present taxonomic grouping of the species and to assess whether the data support the continued placement of them in their respective grouping. Earlier anatomical studies on this genus are highly confusing; we could clarify it with some species-specific characters and some species interrelationships based on which a classification of the available species of this genus was attempted. The results obtained from the present investigation revealed a number of interesting features that may be useful in identifying these plants before flowering.

The species could be demarcated into two distinct classes - the New World Species and Old World species. The two New World species of vanilla (*V.planifolia and V.tahetensis*) share many common anatomical characters and the rest of the species, which belongs to the Old World, have their own common anatomical characters. The "leafy" species *V.andamanica* is more related to the "leafless" species *V.aphylla*. The anatomical similarity may be due to the Old World origin of these two species.

The anatomical characters were found to be species specific, based on which a key was prepared. The anatomical key to the species is the first of its kind in *Vanilla.*

ii. Interspecific hybrids:

Comparative juvenile anatomy of vanilla hybrids was undertaken to investigate the anatomical characters of the hybrids and to compare it with that of the parents to confirm hybridity.

Twenty features of vegetative anatomy of the three species and eighteen hybrids were scored and used as the basis for analyzing the segregation pattern among the hybrids. Based on the scored values, a dendrogram was constructed which was used to study the interrelationship between the hybrids and their parents.

***Molecular characterization:***

The parentage identification of the hybrids derived from the two different interspecific crosses was analyzed by RAPD technique. The results proves that the hybrids are descendents of the respective crosses, *viz, V. planifolia* (♀) X *V. aphylla (♂)* and *V. planifolia* (♀) X *V. aphylla (♂).*

## In vitro polyploidy

This research was carried out in order to determine the best method of autotetraploid induction in an attempt to exploit the characters associated with chromosome duplication in this crop. Five groups of explants were used for this experiment: they are - germinating seeds from selfed *V. planifolia,* somatic embryos (PLBs), callus, axillary bud explants from seed derived progenies of *V. planifolia* and axillary bud explants from *V. planifolia* (♀) X *V. aphylla* (♂) interspecific hybrids. A protocol for obtaining a ready source of PLBs and callus was developed.

The production of auto polyploidy by colchicine treatment was reported for the first time in *V.planifolia.* Survival rate was lowest using colchicine concentration of 0.4%; however, at this concentration the chromosome-doubling rate was highest. From this study, it was found that the ideal condition for colchicine induction was to treat germinating seeds with 0.4% of colchicine for 15 days by media supplementation method.

### *Morphological characterization:*

Tetraploid plants were characterized by slow growth, greener stem, and narrower and thicker leaves and could be first selected based on these morphological characters. Stomatal size and stomatal density on the abaxial leaf surface could be used to identify tetraploid vanilla early on its development. Instead of 'gigas' characteristics typical of doubled diploids, the chromosome doubling in *Vanilla planifolia* often showed a tendency towards morphological diminishing especially in leaf area.

### *Cytological characterization:*

A modified root tip squash technique using lactopropionic orcein was used for positive confirmation of ploidy change by chromosome counts. Tetraploid plants were produced at the frequency of 30% for the concentration of 0.4% when seeds were used for treatment. PLB treatment was also effective in inducing polyploidy. The doubling seldom occurred when the concentration of colchicine was lower than 0.2%.

### *Molecular characterization:*

The results of different primers showed that the banding pattern were not homogenous in all the genotypes studied. Thus, the primers identified minor polymorphism between the colchicine treated progenies. In general, similarities were noticed between colchicine treated ones and this might be because colchicine simply duplicates the DNA with no significant genomic alterations and duplications could not be detected by the RAPD technique.

## In vitro mutation

Induced mutation breeding was initiated to augment the spectrum of variation and select desirable mutants. Attempts were made to standardize a micro technique for plant regeneration from mutated tissue of seeds, stem node, callus and PLB.

Inhibition of seed germination increased with increasing EMS concentration; the highest percentage of inhibition was observed at 0.1% EMS. A dose dependent reduction in seed growth and seedling development was recorded. Survival percentage decreased considerably with increase in concentration of EMS.

### *Morphological characterization:*

The primary effect of treatment includes changes in leaf shape and a reduction in growth vigor. Some of the EMS induced variants showed a reduction in leaf area and they may be ideally suited in agro climatic areas of low humidity and reduced water availability. The morphological characterization of the treatments one year after planting showed that the characters varied with change in concentration of EMS, the type of explant used for the experiment and among the different EMS treated seedling progenies..

***Molecular characterization:***

The present study indicated that concentration of EMS, the type of explant used for treatment along with the genotype of the treatment influenced the variation as expressed by RAPD polymorphism. The EMS concentration of 0.75% was most effective in inducing variation. Among the different types of explants used, the protocorms and seeds were equally efficient in producing variations.

In conclusion, the results indicated that the extent of variability of the cultivated vanilla could be enhanced by seed heterozygosity, colchicine effect, mutation effect and hybridization effect.

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