INDUCTION OF VARIABILITY IN *Vanilla planifolia* Andrews THROUGH INTRA/INTER SPECIFIC HYBRIDISATION AND EMBRYO CULTURE TECHNIQUE

By

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THESIS

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2005

DECLARATION

I hereby declare that the thesis entitled "Induction of variability in *Vanilla planifolia* Andrews through intra/interspecific hybridisation and embryo culture technique" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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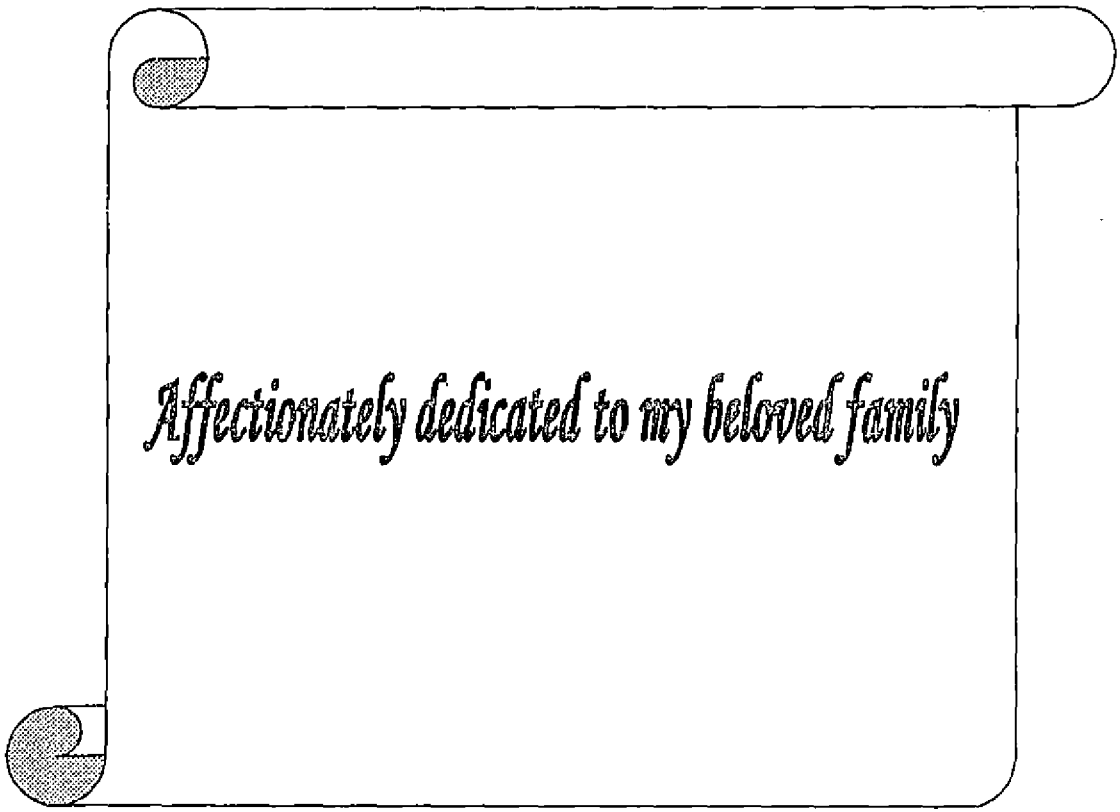
Affectionately dedicated to my beloved family

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LIST OF ABBREVIATIONS

BAP	: Benzyl Amino Purine
cm	: centimeter
CPBMB	: Center for Plant Biotechnology and Molecular Biology
COH	: College of Horticulture
CRD	: Completely Randomised Design
CTAB	: Cetyl Trimethyl Ammonium Bromide
d NTP	: deoxy Nucleotide Tri Phosphate
DAP	: Days after pollination
DNA	: Deoxy ribonucleic acid
EDTA	: Ethyl Diamine Tetra Acetic acid
IAA	: Indole Acetic Acid
MA	: milli Ampere
mg/l	: milligram per litre
MS	: Murashige and Skoog
NAA	: Naphthalene Acetic Acid
OD	: Optical Density
PCR	: Polymerase Chain Reaction
ppm	: parts per million
RAPD	: Random Amplified Polymorphic DNA
RNA	: Ribonucleic acid
RNAase	: Ribonuclease
rpm	: revolutions per minute
SAHN	: Sequential Agglomerative Heirarchial Nested Clustering
SD	: Standard Deviation
TAE	: Tris Aceticacid EDTA

Taq	: Thermus aquaticus
TE	: Tris EDTA
UPGMA	: Unweighted Pair Group Method of Arithmetic Averages
UV	: Ultraviolet
VH	:Vanilla Hybrid
V. p	: *Vanilla planifolia*
V. t	: *Vanilla tahitensis*
WAP	: Weeks after pollination

INTRODUCTION

1. INTRODUCTION

Spices are high value export oriented products extensively used for flavouring food and beverages and also in medicines, cosmetics and perfumery. In the family of spices, vanilla (*Vanilla planifolia* Andrews) is often quoted as "Princess of Spices", owing to its unique flavour and pleasant aroma. Vanilla cultivation in India is mainly concentrated in certain areas in Kerala, Tamil Nadu and Karnataka. During 2004-2005 the export of vanilla from India was 38 Mt and value accounted to 2759 lakh rupees. Prevailing demand for vanilla flavour in the international market makes it one of the most remunerative crop of the country.

Being a climbing orchid, vanilla has to be trailed on suitable standards. It can be grown as an intercrop in coconut and arecanut plantations and as an alternate crop in cardamom and coffee plantations (Rao *et al.*, 1993). It requires a warm humid tropical climate for better growth. Flowering commences in the third year of planting usually. The flowers are to be artificially pollinated, as the natural pollinators are not occurring in India. Vanilla beans mature within seven to nine months of pollination. Timely scientific processing of harvested beans is very essential for premium quality of vanilla. The cured beans are sorted into grades according to their length, aroma quality, moisture content, colour and general appearence.

The best quality 'vanilla beans of commerce' are derived from *Vanilla planifolia* Andrews (*V. fragrans*). The other two vanilla species that yield natural vanillin are *Vanilla tahitensis* Moore and *Vanilla pompona* Schiede. Based on the climate, soil where it is grown and method of curing, there are three distinct quality grades of natural vanilla namely Bourbon vanilla produced in Madagascar, Comoro, Reunion and in India, the Mexican vanilla and the Java vanilla.

The fragrance and flavour of vanilla is due to numerous aromatic compounds, among which vanillin is the most important. Vanillin can now be produced synthetically and is much cheaper than natural vanilla flavour. Nevertheless, the flavour of vanilla beans from *V. fragrans* is far superior to that of synthetic vanillin and the people in developed countries are very much health conscious and prefer products of natural origin. Vanilla's mellow fragrance enhances acceptability of a variety of sweet dishes like puddings, cakes, custards and ice creams and has many non culinary uses like aromatizing perfumes, cigars and liquors.

Diseases of fungal and viral origin pose serious threat to vanilla. Various types of rots and wilts caused by pathogenic fungi have been reported from almost all the vanilla growing countries. Species of *Phytophthora, Fusarium, Sclerotium* and *Colletotrichum* are the commonly occurring pathogens.

Comparatively little work has been done on the improvement of vanilla by breeding. Since vanilla has narrow genetic base, exploration of genetic variability and its exploitation for desirable traits such as yield and tolerance to biotic and abiotic stresses are primarily targeted in the varietal improvement programme. As vanilla belongs to the Orchid family, seeds lack mature endosperm and seed germination is difficult. However, embryo culture techniques can be used to overcome this obstacle and hybridisation can be attempted to facilitate heterosis breeding. Establishment of clonal population of hybrids is easier as micropropagation is standardised in this crop. According to TVC (2004) quality hybrids can be produced by interspecific hybridization of *V. planifolia* and *V. tahitensis*. The Center for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara maintains *V. planifolia* plants derived *in vitro* through seed culture and *V. tahitensis* plants. In the present study, an attempt was undertaken to develop recombinants of *V. planifolia* and *V. tahitensis* through intra/interspecific hybridisation and embryo culture technique. This will broaden the genetic base of

vanilla and can be used to develop vanilla hybrids that are of higher yields, richer vanilla flavor, and disease resistant.

The present study was conducted with the following objectives:

- To induce variability in *Vanilla planifolia* Andrews through intraspecific hybridisation among superior clones of *Vanilla planifolia* and interspecific hybridisation between *Vanilla planifolia* Andrews and *Vanilla tahitensis* Moore and embryo culture technique.
- Morphological and molecular characterisation of selected parents and seedling progenies for assessing genetic variability.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The present study aims at induction of variability in *Vanilla planifolia* Andrews through intra/interspecific hybridisation and embryo culture technique. A comprehensive literature relevant to the study is reviewed in this chapter.

2.1. BOTANY

2.1.1. Systematics

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. The basic chromosome number for the genus vanilla is x =16 and *V. planifolia* is a diploid with 2n =32. One hundred and ten species of vanilla are reported consisting of terrestrial, climbing, epiphytic and saprophytic species (Purseglove *et al.*, 1981). Apart from *Vanilla planifolia* Andrews, two other cultivated species are *Vanilla pompona* Schiede (West Indian vanilla) and *Vanilla tahitensis* J. W. Moore (Tahitian vanilla).

2.1.2. Origin

Vanilla planifolia Andrews (syn *V. fragrans*)(Salisb.)(Ames) is native to the humid tropical rainforests 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. The major vanilla growing countries are Indonesia, Madagascar, Mexico, Tahiti, Comoros and Reunion (Madhusoodanan *et al.*, 2003).

Vanilla growing was started in India with the blessings of the East India Company nearly 250 years back in the Spice garden at Kurtallam in Tamil Nadu. Vanilla was experimentally grown in Assam, West Bengal, Bihar, Tamil Nadu,

Pondichery, Karnataka and Kerala nearly 100 years ago. Fairly systematic investigations on this crop were initiated in 1960 at the RARS, Ambalavayal in Wayanad district and Kallar and Barliar fruit research station in Nilgiri district of Tamil Nadu in 1960 under a scheme of the ICAR, New Delhi (George, 2005).

2.1.3. Cultivated species as spice

The vanilla of international commerce is derived from *Vanilla planifolia* H.C. Andrews (syn *V. fragrans*)(Salib.)(Ames). Other cultivated species of vanilla in the trade include the West Indian vanilla, *V. pompona* Schiede (syn *V. grandiflora* Lindl.) and Tahitian vanilla, *V. tahitensis* J.W. Moore.

2.2. MORPHOLOGY

Purseglove *et al.* (1981) and Straver (1999) provided some important distinctions among the vanilla species:

+ ***V. planifolia*** : The plant is a fleshy, herbaceous perennial vine, climbing by means of long, whitish and aerial adventitious roots, about 2 mm in diameter produced singly opposite the leaves and adhere firmly to the support plant. In cultivation, it is trained to a height that will facilitate hand pollination and harvesting. The long, cylindrical, succulent monopodial stems are simple or branched. The internodes are 5 – 15 cm in length. Leaves are large, flat, fleshy, subsessile, alternate, oblong – elliptic to lanceolate and are 8 – 25 cm long and 2 – 8 cm broad. The tip is acute to acuminate and the base is somewhat rounded. Venation is parallel and veins are distinct. The petiole is thick, short and canalized above. The capsule, known in the trade as a bean, is pendulous, narrowly cylindrical, obscurely three angled, 10–25 cm long and 5–15 mm in diameter. Worldwide *V. planifolia* is considered as superior for the source of vanilla flavour and it is characterised by long pod and vanillin content of 1.3 to 3.8 per cent.

+ *V. tahitensis*: The plant is indigenous to Tahiti and cultivated there and in Hawaii. Correll (1944) reported that tahitian vanilla differs from *V. planifolia* by having more slender stems, narrower leaves which are 12 – 14 cm long 2.5 – 3.0 cm wide and longer perianth segments. The labellum or lip is shorter than the sepals. The pods are 12 – 14 cm long and about 9 – 10 mm wide, tapering towards each end and are indehiscent on maturity. It possesses a characteristic aroma and is used both for flavouring and in perfumery. Tahiti vanilla has been reported to have vanillin contents ranging from 0.9 to 3.3 per cent and is characterised by short pods compared to *V. planifolia*.

2.3. HABIT AND HABITAT

In its original habitat vanilla was seen wild as a climber. Vanilla requires warm and moist conditions of humid tropics 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 upto 1500 m above mean sea level. It grows well on a variety of soils ranging from sandy loam to laterites provided the soil is loose and friable with high organic matter. It flourishes well in partial shade of about 50 per cent. A moderate slope and good drainage aids its easy establishment. The ideal growing condition for vanilla is moderate rainfall evenly distributed throughout ten months of the year, with dry spells during flowering and harvesting period (Madhusoodanan and Radhakrishnan, 2001).

2.4. PRODUCTION AND TRADE

Global cultivation of vanilla during 2004 was 39,191hectares (FAO, 2005) and the annual world production is estimated to be 3000 – 4000 Mt (Peter *et al.*, 2004). In India, it is grown in an area of 2545 hectares covering Kerala, Tamil Nadu and Karnataka producing around 100 Mt (Kuruvilla *et al.*, 2004). During 2004 - 2005 the export quantity of vanilla from India is 38 Mt and value accounts to 2759 lakh rupees. Eventhough our present share in the world trade of vanilla is negligible, there is every possibility that India can emerge as an

important source of vanilla in the years to come. At present, Indonesia and Madagascar are the major centres of vanilla production and export.

2.5. PHENOLOGY OF FLOWERING

Vanilla vines come to flowering in the third year of planting. Flowering generally takes place on the branches that are one and a half years old. Inflorescence initials are generally activated in the leaf axils during December to January as light green protuberances. These buds attained a length of 3.56 cm in the first fortnight. After 30 days length was 4.59 cm. At this stage flower buds commence to emerge out from the base of the inflorescence. Inflorescence is racemose type with a peduncle 2.5cm long. After about 45 days of formation, the flowers were fully formed and blooming commences, the length of inflorescence at this stage was 6.49 cm. Linear growth of inflorescence was observed upto 60 days, by which time it attained a mean length of 7.34 cm (Bhat and Sudharshan, 2004).

2.5.1. Floral biology

Vanilla flowers only once in a year, staggered over an average of two to three months, depending upon the variation in local climate. In a typical hill zone climate (around 2000 mm rainfall distributed over 6 months, maximum temperature of 32°C during summer, relative humidity of more than 90 per cent during rainy and winter mornings in most of the days) flowering starts by February or March preceeded with tip nipping by November followed by two months dry period. But in a relatively drier climate (around 1000 – 1200 mm rainfall distributed over 4 months, maximum temperature of 35°C during summer, varying relative humidity levels) it is being noticed from October itself (Shadakshari *et al.*, 2003).

In Mexico flowering is observed from April – May, in Madagascar and the Comoro islands between November and January (Correll, 1953). In

Indonesia, this occurs between July and August and in India from December to February (Anandaraj *et al.*, 2001).

Vanilla flowers last only for a day. The flower opening occurs between 10.30 p.m. and 1 a.m. and is completed by 6 p.m (Bhat and Sudharshan, 1998).

Time taken for floral initiation to first flower opening varied from 30–45 days in different vines due to the variation in health of vine and number of inflorescence it produced. In a single inflorescence, frequency of flower opening is not regular. It varied from a day to three; rarely a twin flower opened in a day. Total blooming period varies depending on number of flowers produced. For a 15 – 18 flowers it varied from 18 – 31 days (Shadakshari *et al.*, 2003). Bhat and Sudharshan (2004) have reported that blooming period varies from 5 to 40 days depending upon flowers in a particular inflorescence.

2.5.2. Floral morphology

The inflorescence is characteristically raceme with 20 or more flowers. The fruit popularly termed as 'beans' or 'pod' in the vanilla market, botanically a capsule, nearly cylindrical and about 20 cm long (Bailey and Bailey, 1976). Flowers are large, bisexual, zygomorphic with inferior ovary 5 cm in length and 1.4 cm in girth. They are either yellowish, wholly green or white within with oblanceolate sepals and petals. There are three oblong-lanceolate sepals, obtuse to subacute, slightly reflexed at the apex, 4 - 7cm long and 1 - 1.5cm wide. The two upper petals resemble the sepals in shape, but are 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 at its widest point. The lower part of the labellum envelops a central structure called 'column' (gynostemium). The column or gynostemium is 3 – 5 cm long and is attached to the labellum for most of its length. The tip of the column bears a single stamen with two pollinia filled with pollen grains and covered by a cap or hood like structure, and below is the concave sticky stigma, which is separated from the

stamen by the thin flap like rostellum, which prevents natural pollination (Purseglove *et al.*, 1981). The slender stalk like portion is the ovary, which is 4 - 5 cm in length (Kuruvilla *et al.*, 1996).

2.6. POLLEN STUDIES

2.6.1. Pollen morphology

All the normal mature (fertile) pollen grains are similar in morphological features. They are round in shape and possess two distinct coats. The size of the mature pollen varies from 60 – 90 µm and the pollen fertility ranges from 72 – 87 per cent (Kuruvilla *et al.*, 2004).

2.6.2. Pollen viability and stigma receptivity

As a pre-requisite for hybridization work, it is essential to test the germination of pollen *in vitro* and to maintain the viability of pollen. Shadakshari *et al.* (2003) studied pollen viability and stigma receptivity period before and after complete flower opening by hand pollinating the flowers/flower buds with its own pollen. They found that the grains are viable 23 hours prior to anthesis and 16 hours 30 minutes after anthesis. The stigma is receptive 41 hours prior to anthesis and 17 hours after anthesis under hill zone conditions. Pollen viability deteriorates markedly after anthesis, with only 10 – 15 per cent fruit set when 2 – 3 day old pollen grains are used (De Guzman, 2004).

2.6.3. Pollen germination media studies

Ravindran (1979) cultured vanilla pollen grains on a nutritive medium consisting of 10 per cent sucrose, 100 mg l^{-1} boric acid and 100 mg l^{-1} calcium nitrate and the germination count after incubation of 5 – 24 hours was 45 per cent. He also reported abnormalities in pollen grain mitosis and pollen sterility percentage of about 65 per cent in *Vanilla planifolia*.

Bhat and Ratnambal (1997) observed highest percentage of germination (68.8%) of coconut pollen in the medium containing 10 per cent sucrose and 100 mg l^{-1} boric acid. According to O'Kelly (1955) and Vasil (1960) sugars play major roles in pollen germination. Linskens and Kroh (1970) had optioned that sucrose is probably the best and commonly used source of carbohydrate energy for pollen.

Spurr (1957), O'Kelly (1957), Raghavan and Baruah (1959) and Young *et al.* (1966) has reported that boron stimulates pollen germination and tube growth. They also suggested that boron is involved in the synthesis of pectic substances for the growing pollen tube walls.

Calcium Chloride also plays a major role in pollen germination. Cook and Walden (1965) observed that Calcium of any of the three different salts, namely Calcium Chloride, Calcium Nitrate or Calcium Sulphate improved germination four to five fold in maize. Kwack (1967) found that Calcium protects the pollen tubes against the growth inhibitory action of many substances.

2.6.4. Pollen storage

2.6.4.1. Vanilla

The main objective of pollen storage is its subsequent use for pollination to obtain fruit set. Cryopreserved pollen of *Vanilla planifolia* and *Vanilla aphylla* resulted in successful fruit set and seed germination. For cryopreservation, fresh vanilla pollen was collected, dessicated for 10 minutes and treated with 10 per cent DMSO for 10 minutes and cryopreserved in liquid nitrogen. The cryopreserved pollen after thawing retained its viability as seen by *in vitro* germination and pollination (IISR, 2002).

2.6.4.2. Other crops

Patel (1938) maintained the viability of coconut pollen for about 14 days when stored in a dessicator at about 50 per cent relative humidity. Whitehead (1963) reported that freeze dried coconut pollen could be stored for prolonged periods at deep freeze temperatures. By the combined action of low temperature (0°C) and relative humidity 50 – 60 per cent, pollen can remain viable for an year or more (Manthriratne, 1965). Khui *et al.* (1979) obtained best results with regard to viability of date palm pollen when stored at 20 – 40 per cent relative humidity over 2 – 3 weeks. De Lamothe *et al.* (1980) reported that in order to increase the storage of pollen, its moisture content should be reduced to 50 per cent. Ekaratne and Sanathirajah (1983) found that when the humidity was zero per cent, oil palm pollen could be stored for many days. Long term preservation of pollen of many species at very low temperature was reported by Barnabas and Rajki (1981), Eeink (1983), Filippova (1986) and Hanna *et al.* (1986).

Coconut pollen could be stored six days (with 45% germination) and eight days (with 56.8% germination) at 70 and 50 per cent relative humidity, respectively. However, in a dessicator containing fused Calcium Chloride (0% relative humidity), the pollen could be stored even upto 12 days giving germination of 53.4 per cent. The coconut pollen could be successfully stored at sub-zero temperature in a refrigerator for 90 days retaining atleast 50 per cent pollen germination (Bhat and Ratnambal, 1997).

Hermetic cold storage without previous drying (wet-cold storage) was reported by Daniel *et al.* (2002) for the preservation of yam pollen grains. Honma *et al.* (2003) reported long term pollen storage in *Vitis coignetiae* with organic solvents. Deng and Harbaugh (2004) reported a technique for *in vitro* pollen germination and short term pollen storage in caladium.

Sugiyama *et al.* (2002) reported that pollen germination was retained upto 20 days in water melon when stored with silica gel in sealed container and kept at

5°C. He also reported that the use of dehydrated ethyl acetate as organic solvent and storage at -20°C maintained the germinability of the pollen.

2.7. POLLINATION AND FRUITING

The vanilla flower is so constructed that self pollination of the individual flower is impossible, unless hand pollinated due to separation of stamen from the stigma by the rostellum. Although vanilla plants grew well in the Old World tropics, fruits were not produced because of the absence of natural pollinators. In Mexico and Central America where vanilla is indigenous, some of the flowers are pollinated by stingless bees of the genus *Mellipona* and by humming birds. Elsewhere hand pollination is unavoidable for fruit set. Charles Morren discovered the method of hand pollination in 1836. Later Edmond Albius, a former slave in Reunion, identified a practical method of artificial pollination in 1841 that is still used in commercial production (Purseglove *et al.*, 1981). Natural fruit production has been reported in *V. wightiana*, a wild species from Andhra Pradesh (Rao *et al.*, 1994).

The ideal time for pollination is between 6 a.m. and 1 p.m. (Bhat and Sudharshan, 1998). The fruit setting percentage was maximum at 8 a.m. and showed a decreasing trend as pollination gets delayed. Studies have shown that complete transfer of pollen results in maximum fruit growth and with less than 50 per cent pollen, the fruit size reduced considerably (Bhat and Sudharshan, 2000). Effective fruit set in vanilla was observed for 16 hour 30 minutes after complete flower opening (6 a.m. in the morning to night 10.30 p.m.) by hand pollinating the flowers on the day of anthesis with its own pollen (Shadakshari *et al.*, 2003). The fertilized flower develops into mature fruit in about 9 to 11 months period under various conditions.

In Reunion, it takes about six months for the fruits to mature in Mexico, Indonesia and Philippines about nine months (David, 1950; Correll, 1953). The harvesting period varies according to altitude and latitude (Theodose, 1973) and

differs from one country to other. The fruit is a capsule, which is indehiscent in tahitian vanilla (Purseglove *et al.*, 1981) and dehiscent in *V. planifolia* (Madhusoodanan *et al.*, 2003). About 50 – 150 fruits are allowed to develop and mature per vine (Purseglove, 1972). It becomes aromatic on processing. Each bean when ripe contains thousands of minute globose seeds.

Parthenocarpic development of pod can be induced in vanilla with growth regulating chemicals though they are poor both in quantitative and qualitative characters (Gregory *et al.*, 1953).

2.7.1. Pod development studies

Swamy (1947) has noted that ovule formation did not begin in *Vanilla planifolia* until after pollination, and 15 - 20 days were necessary for the archesporial cells to form. The mature pod size was thus attained by the time the pod was 60 days of age.

Duncan and Curtis (1942) have worked out the growth curves for orchid pods and correlated the increments with the internal events of fertilization and ovule formation. According to Withner (1955) the vanilla pods reached a mature size in about six weeks. The data for the size of vanilla pods showed the typical growth curves with two dips at approximately 10 and 35 days after pollination. The three peaks in the curves occurred at 5, 25 and 55 days. On the basis of Duncan and Curtis' (1942) work the initiation of ovule formation would be related to the dip at 10 days and fertilization would be correlated with the second dip at about 35 days. Kuruvilla *et al.* (2004) has reported the elongation of fruit continues for a period of three to four months and attains a maximum length of 21 cm. They observed that rate of elongation is more (13 cm) during the first month after fertilization.

2.7.2. Effect of seed maturity on germination

Mature seeds of tropical vanilla species and hybrids survive atleast three years on synthetic media, germination occurring only where appropriate high temperature and low light levels are present (Knudson, 1950; Lugo, 1955). Withner (1955) has reported that seeds from 45 - 60 day old vanilla pods have young developing embryos that would grow when cultured. The marked hardening of the seed coat took place about 60 days after pollination, and this time seemed to be optimal one for obtaining the best growth in culture. He also observed that vanilla seeds that 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 naturally occurring 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. The rate of germination seemed definitely to decline with the age of the pod and with increasing maturity of the seed coat.

2.7.3. Embryo development studies

Curtis (1947) has reported that vanilla protocorms might be a potential callus producing tissue for *in vitro* culture of monocot tissue. Withner (1955) has noted that black seed coats of vanilla during germination will split in half and small white spherical or irregular protocorms were produced. Philip and Nainar (1988) have reported histogenesis and organogenesis during the processes leading upto seedling formation in cultures of *Vanilla planifolia*. Prior to germination, all cells of the embryo increased in size rupturing the seed coat and initiating the protocorm stage. Bipolar differentiation within the meristem produced the shoot, and after a few leaves had been formed, the first root initiated endogenously from the base of the meristem. The development of roots in vanilla seedlings may require a high level of endogenous auxin at the organogenetic centre of the meristem.

2.8. VANILLIN DERIVATIVES

The unique fragrance and flavour of vanilla is due to vanillin ($C_8H_8O_3$) and the end products are extract, flavouring and tincture as well as their concentrates. Vanillin, the most abundant volatile aromatic constituent of vanilla beans, was first isolated and identified by Gobley in 1858 (Purseglove *et al.*, 1981). Busse (1900) and Pritzer and Jungkunz (1928) have reported the presence of piperonal (heliotropin) in tahitian vanilla. Walbaum (1909) examined tahitian vanilla and identified anisyl alcohol, anisaldehyde and anisic acid as constituents. Broderick (1955) has reported that Java beans (*Vanilla fragrans*) possess a very heavy and woody flavour. Tahiti vanilla is used both for flavouring purposes and in perfumery (Purseglove *et al.*, 1981). The international trade of vanilla is important due to its high price. Of the total vanilla flavour used all over the world, less than 15 per cent is from the natural source (Madhusoodanan and Radhakrishnan, 2001).

Sullivan (1984) examined the vanilla extracts and identified vanillin, coumarin, umbelliferone, ethyl vanillin and piperonal as the aromatic constituents. Larcher (1989) examined the effect of harvesting time on vanillin content of pods of *Vanilla tahitensis* and observed maximum vanillin content when the pods were started to turn brown. The same trend was seen in the contents of the other aromatic constituents except that of anisic acid, which remained nearly constant. Analysis of tahitian beans by high performance liquid chromatography found relatively low amounts of vanillin, vanillic acid and relatively high amounts of para hydroxybenzoic acid and considerable amounts of anisic acid anisyl alcohol (Ehlers *et al.*, 1994). Ramaroson *et al.* (1997) reported large amounts of n-alkanes and n-alkenes in *Vanilla planifolia* and branched alkanes in *Vanilla tahitensis*.

2.8.1. Vanilla curing

The fragrance and flavour of vanilla beans is due to numerous aromatic compounds produced during the curing operation, among which vanillin is the most abundant. So the pods of vanilla need to be cured to develop the characteristic natural flavour associated with the product. Curing can be defined as the sum total changes that occur during the primary processing of a given raw material to a desired finished product, which is ready for market (Jones and Vicente, 1949).

Vanillin, the main flavouring chemical of vanilla, is present only in trace amounts in the green mature beans; upon curing, however, vanillin content increases. The chemical compound from which vanillin is derived occurs in the uncured pods in the form of a glucoside called glucovanillin (Arana, 1943). During the curing process, this glucoside is hydrolysed to form vanillin and glucose through the action of a β glucosidase. The activity of this enzyme changes with the maturity of the vanilla beans, being negligible in the green beans and highest in the split, blossom end yellow beans. One kilogram of processed produce was obtained from 245 to 260 beans. The moisture content of the beans after processing was 24 per cent. The vanillin content observed was 2.41 per cent (Kuruvilla *et al.*, 2004).

2.8.1.1. Curing of Tahiti vanilla

Since *Vanilla tahitensis* is indehiscent, the beans are not killed artificially but are harvested when mature. This is characterized by the tip of the bean turning brown. The curing is carried out by specialist firms, which employ a method bearing similarities to the Mexican procedure (Correll, 1953; Hibon, 1966; Theodose, 1973).

2.9. CROP IMPROVEMENT

Vanilla is a vegetatively propagated climbing orchid. The genetic variability of the crop in the nature is very limited as much of the vanilla in the Old World originated from a single clonal source and due to lack of outbreeding nature. In India it is an introduced crop and it possesses narrow genetic base. Considering the breeding behaviour and mode of propagation of the crop, the breeding methods such as selection, hybridization and mutation breeding are employed (Kuruvilla *et al.*, 2004).

2.9.1. Hybridisation

Hybridisation was initiated in vanilla in recent years with an objective of transferring alien genes responsible for natural bean formation and abiotic stress. Inter and intraspecific vanilla hybrids are relatively easy to make with the modern techniques. *Vanilla fragrans* has been crossed with other species including *Vanilla phaeantha*, which is resistant to Fusarium root rot and produced seedlings (Withner, 1955; Purseglove *et al.*, 1981). Propagation by seeds is confined to breeding work (De Guzman, 2004). Now satisfactory methods exist for growing orchid seeds and the culture of orchid seedlings, it should not be too difficult to raise and test vanilla hybrids, which would then be increased rapidly by meristem culture.

Interspecific hybrids between *Vanilla aphylla* and *Vanilla planifolia* were produced at IISR and among the 50 progenies generated; none expressed the leaf characters of the male parent. All the progenies of aphylla types had only scale leaves and none exhibited the normal leaf character of *Vanilla planifolia* (IISR, 2002). The economic importance of *Vanilla tahitensis* was documented by TVC (2004). According to them quality hybrids can be produced by interspecific hybridization of *Vanilla planifolia* and *Vanilla tahitensis*. Interspecific hybrids were produced between *Vanilla planifolia* and *Vanilla wightiana* using conventional crosses and 50 per cent fruit set was observed (ICRI, 2003).

Electrophoresis with seven polymorphic enzymes supported the finding of Nielsen and Siegismund (1999) about the interspecific differentiation and natural hybridization in localities where *Vanilla claviculata* and *Vanilla barbellata* coexists. Nielsen (2000) identified natural hybridization between *Vanilla claviculata* and *Vanilla barbellata* and is supported by genetic, morphological and pollination experimental data.

2.9.2. Mutation breeding

Mutation breeding is one of the methods available to the plant breeders when the crop is amenable to vegetative propagation. Some mutations have beneficial effects and are useful in crop improvement. Mutations in both qualitative and quantitative traits have been exploited for the development of various crops (Singh, 1998).

In vitro mutagenesis in vanilla was induced in seed cultures and protocorms using mutagens and growth regulators like Ethyl Methyl Sulfonate (EMS), Cycocel (CCC), Maleic Hydrazide (MH) and 2,4,5-T in the medium (ICRI, 2002).

2.10. PROPAGATION

2.10.1. Use of cuttings

Vanilla is commercially propagated by stem cuttings. In vegetative propagation, the cuttings should be taken from healthy vigorous plants and may be cut from any part of the vine. Cuttings of 2 - 3.5 m long are preferred since they will flower in one or two years, as opposed to 30 cm cuttings which will bear flowers and fruits in three to four years (Correll, 1953; Purseglove *et al.*, 1981). When the vines attain sufficient length later, they are coiled or trailed on supports in the plantations to promote flower formation (George, 1981).

Sasikumar *et al.*, (1992) has reported that the cuttings of 1 m long are generally preferred for planting.

2.10.2. Use of *in vitro* techniques

2.10.2.1. Micropropagation

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 development of the mother plant (Ayyappan, 1990). Vanilla, being a monopodial orchid, yields only a small number of planting material. Moreover the market demand for the propagules is hardly met with such cuttings. As the growers are looking for alternate sources, micropropagated plantlets serve the purpose and are popularly used (Geetha and Shetty, 2000).

Vanilla is successfully propagated using *in vitro* techniques. Shoot proliferation with aerial root formation can be induced using nodal stem segments (Kononowicz and Janick, 1984). Plantlet or protocorm formation using stem discs with a node has been reported to be dependent upon the composition of the medium, size of the explant or both. Production of multiple plantlets is also possible using aerial root tips. This occurs in the absence of a callus interphase, reducing the possibility of induced epigenetic changes in the derived plant. The aerial root tips cultured on liquid MS medium supplemented with 2 mg l^{-1} of IAA and 0.2 mg l^{-1} of kinetin, swelled considerably within two months (Philip and Nainar, 1986). This technique involving transformation of aerial root meristems to shoot meristems was recommended by them for the production of planting materials free from *Fusarium batatis* var. vanillae. In another account by Philip and Padikkala (1989), the role of IAA in the conversion of root meristems to shoot meristems was discussed. 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*.

There are several accounts of micropropagation through enhanced axillary branching in vanilla. Medium based on Knudson (1950) /, Murashige and Skoog (1962) supplemented with various concentrations of BAP, IAA and NAA is generally employed for initiation of culture. Protocols on micropropagation of vanilla for shoot proliferation have been reported through the culture of callus masses (Gu *et al.*, 1987; Davidonis and Knorr, 1991) and using nodal explants (Rao *et al.*, 1993; Ganesh *et al.*, 1996; George and Ravishankar, 1997).

Ganesh *et al.* (1996) reported that BAP alone was found to induce rosetted buds with condensed internodes. Newly formed buds can be transferred to multiplication media within 28 – 35 days of culture initiation. Rao *et al.* (1999) have also reported *in vitro* propagation system for vanilla through nodal segments and root tips. Culture establishment and multiple shoot production from nodal segments was achieved in the MS medium with BAP 1 mg l^{-1} and IAA 0.1 mg l^{-1}. Elongation and rooting was reported in KC medium with kinetin and NAA each at 0.1 mg l^{-1}. Root tips from *in vitro* shoots were excised and inoculated directly on the medium with tip upwards. Vertically oriented root tips exhibited direct transformation to multiple shoot buds within 40 days in culture on MS + BAP 2 mg l^{-1} + IAA 0.1 mg l^{-1}. Geetha and Shetty (2000) have also reported a simple and efficient micropropagation protocol for *Vanilla planifolia* using shoot tip and nodal segments. They observed that the axillary proliferation was initiated only when giving a longitudinal bisection injury to the shoot tip.

George and Ravishankar (1997) observed that vigorous multiplication can be triggered if shoot cultures are maintained in liquid medium for 14 days before transferring to semi-solid medium. High rate of multiplication of *Vanilla planifolia* clonal propagules was obtained from axillary bud explants using semi-solid medium (MS supplemented with BAP 2 mg l^{-1} and NAA 1 mg l^{-1}) succecssively. Mass propagation for commercial cultivation requires a simple,

economical, rapidly multiplying and highly reproductive protocol (Geetha and Shetty, 2000). Mutiple shoots were developed from axillary bud explants using MS medium supplemented with zeatin, BAP + zeatin, thidiazuron (TDZ) and thidiazuron + coconut milk (Giridhar and Ravishankar, 2004).

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

Generally silver nitrate inhibits ethylene action through the Ag^{2+} ions, by reducing the receptor capacity to bind ethylene. The adverse effect of accumulated ethylene produced by *in vitro* grown tissues on shoot proliferation was well documented by Hussey and Stacey (1981). The use of ethylene inhibitors like $AgNO_3$ was found to be beneficial in overcoming this problem (Chi and Pua, 1989). Agrawal *et al.* (1992) devised a method of clonal propagation for *Vanilla walkeriae,* which is restricted to the tropical forests of Tamil Nadu and Kerala. Ganesh *et al.* (1996) investigated the effects of culture media type and BAP, GA_3 or $AgNO_3$ on *in vitro* shoot proliferation of *Vanilla planifolia*; good shoot proliferation was observed only in the presence of BAP. 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 *Vanilla planifolia* when used in micromolar concentrations (Giridhar *et al.*, 2001).

In trials on the micropropagation of *Vanilla tahitensis* and *Vanilla planifolia,* it was found that explants obtained from the middle part of the donor plants exhibited the best growth in terms of length and number of nodes, although explants from the base and tip produced more shoots (Pett *et al.*, 1999). Mathew *et al.* (2000) standardized a viable protocol for mass propagation of *Vanilla tahitensis*. Among the various growth regulators tested, MS with BAP at 1 mg l^{-1}

and NAA 0.1 mg l^{-1} proved most suitable for efficient multiplication. A multiplication ratio of 1: 4.7 was observed over a culture period of 60 – 70 days.

2.10.2.2. Embryo culture

In crop improvement programme, post fertilization barriers can be overcome by embryo rescue technology (Bhojwani and Razdan, 1983). When embryos cannot be easily excised, whole ovule can be cultured and similarly when ovule is too small to be removed, whole ovary can be cultured. Thus embryo rescue technique can be done by ovule culture, embryo culture and ovary culture. This technique is utilized for the production of certain rare hybrids, which are otherwise impossible in nature due to embryo abortion.

Embryo culture is an aspect of embryo rescue technology and is the sterile isolation and growth of an immature or mature embryo *in vitro*, with the goal of obtaining a viable plant. Culturing immature embryos that normally do not survive to maturity *in situ* has produced many interspecific and intergeneric hybrids.

As in any other orchid, the seeds of vanilla are unique being extremely small in size, having an undifferentiated embryo with limited food reserves and lacking endosperm and do not germinate unless in association with a mycorrhizal fungus (Singh, 1981; Smereciu and Currah, 1989; Singh, 1995).

In 1902, M. Dupont made experiments at the Government Botanic Station of the Seychelles to raise vanilla from seed (Ridley, 1912). Aseptic germination technique ensures nearly 100 per cent germination of seeds for many orchid seeds (Bouriquet and Boiteau, 1937; Knudson, 1922; 1947) including vanilla and has been used largely for breeding varieties resistant to Fusarium root rot (Knudson, 1950; Lugo, 1955; Withner, 1955). Paul (1995) reported that seed propagation in vanilla is cumbersome and a few heterogenous seedlings that can be raised are very slow growing requiring a longer period to bear flowers. *In vitro* seed

germination can be used in hybridisation programme for rescuing embryos of artificial hybrid of vanilla; thereby creating improved variant lines (Rao *et al.*, 1999; Madhusoodanan *et al.*, 2003). Seed culture is an important technique in propagation of orchids (Chawla, 2002).

The first attempt to grow the embryos of angiosperms was made by Hannig in 1904, which obtained viable plants from *in vitro* isolated embryos of two crucifers Cochleria and Raphanus (Hannig, 1904). Dietrich (1924) grew embryos of different plant species and established that mature embryos grew normally but those excised from immature seeds failed to achieve the organisation of a mature embryo. Laibach (1929) demonstrated the practical application of this technique by isolating and growing the embryos of interspecific cross *Linum perenne* and *L. austriacum* that aborted *in vivo*.

Embryo culture has been used as an effective tool for developing interspecific hybrids involving various *Trifolium* species (Phillips *et al.*, 1982; Pandey *et al.*, 1987; Przywara *et al.*, 1989; Ferguson *et al.*, 1990; Abberton *et al.*, 2002; Kaushal *et al.*, 2004). Hybrid plants raised by embryo culture have recombined desirable genes such as earliness, disease and pest resistance (Chawla, 2002). Successful interspecific hybrids in cotton (Rajashekhar *et al.*, 2003), barley, tomato, rice, legume and intergeneric hybrids are produced through embryo culture.

2.10.2.2.1. Embryo age

2.10.2.2.1.1. Orchids

Withner (1955) observed that the best embryo germination was obtained from the two month old pod cultures of vanilla. Lo *et al.* (2004) has observed that maximum number of seeds germinated from immature capsules of *Dendrobium tosaense*, a medicinally important orchid.

2.10.2.2.1.2. Other crops

It has been reported that ovules, three day after pollination were found suitable for *in vitro - in ovulo* embryo culture in cotton (Joshi and Johri, 1972). Stewart (1981) obtained better results by culturing entire ovule containing embryo in cotton. Finer (1987), Wilcox *et al.* (1988) and Witrzens *et al.* (1988) described the regeneration of plants from immature embryos of sunflower hybrids. Culture media and embryo age directly influenced germination percentage (Power, 1987; Witrzens *et al.*, 1988; Espinasse *et al.*, 1989; Chraibi *et al.*, 1992).

Pandey *et al.* (1987) and Kaushal *et al.* (2004) reported that embryos between the heart shaped and torpedo stages were the best for embryo rescue in *Trifolium* species. For many recalcitrant species, such as cereals, cotton and pines, immature zygotic embryos are the most suitable explants with which to establish regenerating cultures because of their juvenile nature (Witrzens *et al.*, 1988; Bhojwani and Razdan, 1983). Chang *et al.* (2003) has reported high frequency plant regeneration from immature embryos of *Hordeum vulgare* L. cv Morex. They found that smaller embryos (0.5 – 1.5 mm) showed a much higher ability to produce embryogenic callus capable of regenerating green plants. Chaturvedi *et al.* (2004) produced highly regenerative cultures from immature zygotic embryo cultures of neem. They observed that early dicotyledenous stage embryos were the most responsive followed by torpedo stage embryos.

2.10.2.2.2. Surface sterilization

Knudson (1950) recommends a treatment with 95 per cent alcohol to get rid of fatty material, followed by several washings in distilled water. Philip and Nainar (1988) has reported a surface sterilisation step for the aseptic culture of vanilla seeds by dipping the pods in alcohol followed by flaming and then taking out the seeds by splitting apart the pods. Mary *et al.* (1999) has reported a surface sterilisation technique for green pod culture in vanilla.

Lo *et al.* (2004) surface sterilized the pods of *Dendrobium tosaense*, a medicinally important orchid for pod culture by dipping in 70 per cent ethanol for 30 seconds followed by treating in one per cent sodium hypochlorite with two drops of Tween 20® per 100 ml for 10 minutes. Kitsaki *et al.* (2004) also has reported a surface disinfection method for mature and immature pods of *Ophrys* species (Orchidaceae).

2.10.2.2.3. Culture requirements

The most important aspect of embryo culture work is the selection of medium necessary to sustain continued growth of the embryo. Hanning (1904) reported that mineral salt-sucrose solution was sufficient to culture mature embryos (2mm long) of crucifers. Monnier (1978) observed that though MS medium supported maximum growth of embryos of *Capsella bursa-pastoris*, the survival frequency of the embryos were very low, whereas in the Knop's medium which was least toxic, the growth of the embryo was very poor. So he developed Monnier's medium, which was a modification of MS medium, containing high concentrations of K^+ and Ca^{2+} and a reduced level of NH_4^+ ions supported both growth and survival of embryos.

Many factors influence the germination and growth of orchid seeds. The mineral requirement of orchids is generally not high and a salt poor medium of Knudson (1946) and Vacin and Went (1949) are good. Knudson (1950) found that germination of seeds of *Vanilla fragrans* is possible if the cultures are maintained in a dark incubator at 32°C and that exposure of the seeds to greenhouse conditions before incubation resulted in a higher percentage of germination. Orchid seeds induced to germinate symbiotically or asymbiotically relies on an exogenous supply of nutrient for their further growth (Manning and Van Staden, 1987). Rao *et al.* (1999) reported that culturing in dark for a period of 3 - 4 weeks was essential for seed germination. Germination recorded was 70 - 80 per cent. Half strength MS basal medium devoid of plant growth regulators

found to be the best for asymbiotic germination of seeds of *Dendrobium tosaense*, a medicinally important orchid.

Brar and Sandhu (1984), Gill and Bajaj (1984) and Bajaj and Gill (1985) observed the possibility of growing embryo axes on MS medium with minor modifications in application of growth regulator in cotton. Murashige and Skoog basal medium was found to be the best for embryo growth and regeneration in many *Trifolium* interspecific crosses (Ferguson *et al.*, 1990; Roy *et al.*, 2004). Solid SH medium was used for embryo culture in black pepper as reported by Nair and Gupta (2003). Kaushal *et al.* (2004) has reported that MS basal salts containing only kinetin (0.5 mg l^{-1}) and moderate sucrose (3%) was the best for embryo germination in *Trifolium* species.

2.10.2.2.4. Growth regulators

The nutrient requirements may change rapidly during the autotrophic stages of germination and the relatively high sugar and mineral content of standard media may be toxic to such seedlings. An auxin or cytokinin, or both are required for the growth of embryo and for callusing embryos. When embryo ceases to grow, it must be transferred to a second medium with normal sucrose concentration, low level of auxin and moderate level of cytokinin, which allows for renewed embryo growth with direct shoot germination (Chawla, 2002).

2.10.2.2.4.1. Orchids

Withner (1951) observed that Indole Butyric Acid (IBA) have a beneficial effect upon the growth and development of orchid seedlings. Withner (1955) has reported that Indole Butyric Acid (IBA) in low amounts seemed to have a definite stimulating effect on the vanilla seedlings. Excess of carbohydrate in the medium produced fasciation in the development of protocorms and seedlings of vanilla. He also observed the beneficial effect of arginine (also lysine) on vanilla

seedlings. Divakaran *et al.* (1996) recommended 1.0 mg l^{-1} BAP for production of multiple shoots from vanilla seedlings.

Mary *et al.* (1999) has reported a procedure for embryo germination from mature green pods. The medium of half MS with NAA and BAP was found to give high and early germination. The micropropagated progenies were field planted and showed variability for morphological characters and vigour. Shoot proliferation was high in medium containing half MS supplemented with BAP and IAA 1.0 mg l^{-1}. For further elongation of the shoots these were subcultured to media containing a lower level of BAP. Good rooting was achieved in the media containing 0.5 mg l^{-1} NAA and 0.2 mg l^{-1} BAP.

Rao *et al.* (1999) reported a complete protocol for aseptic nonsymbiotic seed germination in vanilla on KC medium with BAP (0.5 - 1.0 mg l^{-1}). The germinated seedlings elongated and developed into plantlet on KC medium containing NAA 0.1 mg l^{-1} + Kinetin 0.1 mg l^{-1}. For mass multiplication of the seedling, KC medium with BAP 1.0 mg l^{-1} and IAA mg l^{-1} was the best.

2.10.2.2.4.2. Other crops

Chang *et al.* (2003) has reported that both 2,4-D and dicamba induced vigorously growing, friable embryogenic callus in barley. Tiwari and Tripathi (2004) have reported interspecific hybridisation in *Helianthus* through embryo rescue. The best results were obtained on B_5 medium (Gamborg *et al.*, 1968) fortified with 90.0 g l^{-1} sucrose + 0.5 mg l^{-1} NAA + 100 mg l^{-1} myo-inositol + 600 mg l^{-1} L-alanine + 120 mg l^{-1} L-serine + 40 mg l^{-1} tryptophan + 6 mg l^{-1} cysteine. Sreelatha *et al.* (2005) succeeded in getting positive response of Kannanthali (*Exacum bicolor*) seeds to *in vitro* seed culture. The seeds of this plant are not reported to germinate in natural condition. By culturing the seeds in the medium half MS + 2.0 mg l^{-1} IBA they could get the best response.

2.10.2.2.5. Carbohydrate of culture medium

Sucrose is the commonly used carbohydrate for embryo culture (Beek *et al.*, 1944). Sucrose is also essential in the medium to maintain suitable osmolarity, which is extremely important for immature embryos. Eight to twelve per cent of sucrose was required for the culture of pro-embryos (Norstog, 1961 and Monnier, 1978). Pancholi *et al.* (1995) reported that immature embryos excised from *Musa velutine* seeds when cultured on half strength MS medium with two per cent sucrose formed plantlets. Mature embryos can be grown in a basal salt medium with a carbon energy source such as sucrose. But young embryos in addition require different vitamins, aminoacids and growth regulators and in some cases natural endosperm extracts. Young embryos should be transferred to medium with high sucrose concentration (8-12%) and a combination of hormones, which supports the growth of heart stage embryos (Chawla, 2002).

2.10.2.2.6. Amino acids, vitamins and natural plant extracts

Hanning (1904) reported that asparagines were very effective in embryo growth of crucifers. Beek *et al.* (1944) observed that normal seedlings from Datura embryos as small as 150 - 200 µm long and as young as 10 DAP could be developed by incorporating coconut water in the culture medium containing mineral salts, vitamins and amino acids. The addition of amino acids, singly or in combination to the culture medium may stimulate embryo growth. Reduced organic nitrogen such as asparagine, glutamine or casein hydrolysate are always beneficial to embryo culture. Malic acid is also beneficial for embryo culture (Chawla, 2002).

Beneficial effects of organic additives such as coconut water and/or banana homogenate and/or potato homogenate added to medium on seedling growth have been reported in many orchid species (Kim *et al.*, 1970; Lu and Lee, 1990). Lo *et al.* (2004) has reported that germinated seedlings after transfer to

MS medium with 1.5 per cent sucrose and 8 per cent banana homogenate or potato juice or coconut water developed into healthy plantlets. Kitsaki *et al.* (2004) has observed *in vitro* germination of seeds from *Ophrys* species (Orchidaceae) in Malmgren's medium as a basal sowing and growing medium supplemented with pineapple juice or coconut milk.

2.11. CHARACTERISATION OF VARIATION

2.11.1. Morphological characterization

Shankaran *et al.* (1994) has developed a model for determination of vine length based on biometrical observations. Hundred plants of *Vanilla planifolia* Andrews were randomly selected and observations were made on vegetative characteristics. Number of nodes, leaves and internodal length have been reported to be significantly and positively correlated with vine length. A multiple regression equation was derived which had 82.5 per cent precision. Divakaran *et al.* (1996) studied various morphological parameters like leaf size, internodal length, growth rate and isozyme pattern in vanilla seedlings raised through ovule culture. Variability has been observed in leaf morphology and phyllotaxy of vanilla seedlings raised through *in vitro* seed culture (Mary *et al.*, 1999). Parthiban *et al.* (2003) has evaluated vanilla ecotypes in lower Pulney hills region of Western Ghats. They observed significant differences on number of fruits, average fruit weight, bean length and yield.

Krishnakumar *et al.* (1997) has worked out a method to estimate the leaf area in vanilla. A minimum descriptor for vanilla has been published by Kuruvilla *et al.* (2000). Observations on morphological characters of vanilla like vine length, number of leaves, internodes, leaf area and yield have been taken and analysed (ICRI, 2002). Morphological characterization of variability in *in vitro* derived seedlings of vanilla was done by Hena (2005) and Kuriakose *et al.* (2005). They assessed the variability by studying the morphological characters like leaf length, leaf width, leaf area, number of leaves, total growth, leaf shape,

phyllotaxy, root origin and number of roots. Clones derived from regenerants of the same seed, seedlings raised from the same pod and those from different pods were evaluated. The intraclonal variation recorded for tissue culture derived vanilla was insignificant, while it was significant in clones derived from different seeds in the same pod and also from different pods.

2.11.2. 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 or 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) and Variable Number of Tandom Repeats (VNTR) or Simple Sequence Repeats (SSR). Among these, the Polymerase Chain Reaction (PCR) based RAPD method has the advantages such as ease of use, low cost, 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.

2.11.2.1. Random Amplified Polymorphic DNA

Williams and coworkers (1990) and Welsh and Mc Clelland (1990) described the use of single short oligonucleotide primer of arbitrary sequences to the amplification of randomly distributed segments of genomic DNA. The RAPD (Williams *et al.*, 1990) technique is based on the amplification of the DNA segments between pairs of small inverted DNA sequences scattered throughout a genome. The RAPD polymorphism results from mutation or rearrangements at or between oligonucleotide primer binding sites in a genome. This polymorphism can be analyzed on either agarose or polyacrylamide gels. RAPD's are visualized

as dominant markers. To assure reproducibility of the assay however special attention must be paid to the optimization of RAPD experimental conditions.

One pre-requisite of amplification *via* PCR is contamination free DNA. The high quality DNA obtained is suitable for amplification through the PCR for producing RAPD markers. Lim *et al.* (1997) has reported a simple and reliable protocol for extracting DNA from orchid species and hybrids.

Divakaran *et al.* (1996) has reported genetic variability in vanilla seedlings raised through ovule culture. Studies conducted at Indian Institute of Spices Research, Calicut indicated that the progenies of vanilla are more similar to their parent *Vanilla planifolia* and to each other. There was reasonable degree of variability within the selfed progenies of vanilla. The polymorphism observed using RAPD indicated that *Vanilla planifolia* and *Vanilla aphylla* are distinct. The profiles coupled with morphological characters indicated that VH1, VH4 and VH5 are true interspecific hybrids between *Vanilla planifolia* and *Vanilla aphylla* as they are approximately equidistant from the parents (IISR, 2000).

Hena (2005) and Kuriakose *et al.* (2005) has reported molecular characterization of *in vitro* derived seedlings of vanilla. The dendrogram constructed from the pooled data of the RAPD scores with ten different primers expressed great variability in the genetic makeup of vanilla plants evaluated. The variation recorded ranged from 12 to 55 per cent between the major clusters (Hena, 2005).

Babu (1997) optimized the protocol for RAPD analysis in black pepper. The primers identified for varietal screening and the RAPD profile developed for the five important varieties could be utilized for fingerprinting of other varieties. Genetic fingerprinting of coffee hybrids (Ligenioides Hibridode- Timor) has been reported by Ram *et al.* (2002). They observed a large genetic similarity and uniqueness between HDT and Ligenioides and their F1 hybrids by RAPD analysis. The RAPD is referred as an appropriate tool for certification of genetic

stability and clonal fidelity of *in vitro* propagated chestnut hybrids (Gupta and Rao, 2002; Carvalho *et al.*, 2004).

The RAPD assay has been used for analysis of genetic diversity in crop plants like brassica species (Demeke *et al.*, 1992), rice (Fukuoka *et al.*, 1992; Dey *et al.*, 2005) and lilies (Lee *et al.*, 1996). Ravishankhar *et al.* (2000) assessed the genetic relatedness among mango cultivars of India using RAPD markers. Attanayaka *et al.* (2000) used RAPD technique in the genetic differentiation of RRIC 100 series and two germplasm clones of rubber. Shashidhara *et al.* (2003) estimated the genetic diversity using RAPD markers in 51 genotypes of *Santalum album* procured from different geographical regions of India and three exotic lines from Australia. The cluster analysis indicated that sandalwood germplasm within India constitutes a broad genetic base with values of genetic similarity ranging from 15 to 91 per cent. Gomes *et al.* (2004) analysed the genetic relatedness in seven *Gossypium* species using RAPD markers. Suma and Balasundaran (2004) have carried out biochemical and molecular analyses in somatic embryos of *Santalum album.* Remarkable degree of heterogeneity was observed in RAPD profiles at different stages can be attributed to culture induced variations at the DNA level.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The experiments in the present study were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture (COH), Kerala Agricultural University, Vellanikkara during the period from 2003 to 2005. The materials used and methodologies adopted are discussed in this chapter.

3.1 MATERIALS USED

3.1.1 Plant material

The different species of vanilla and the clones generated through embryo culture of self pollinated pods maintained in the DBT funded network project for improvement of vanilla at CPBMB, COH was utilised for the study. Details of plants used for inter/intraspecific hybridization are given in Table 1.

Table: 1 List of plants used for hybridization

Sl no:	Genotype	Clone/acession used as parents	Identity code
1	*V. planifolia clone*	*a 82*	V_1
2	*V. planifolia clone*	*a 94*	V_2
3	*V. planifolia clone*	*a 425*	V_3
4	*V. planifolia clone*	*a 428*	V_4
5	*V. planifolia* germplasm accession	*vv 97/84*	V_5
6	*V. tahitensis*	*V. t*	V_6

3.2. METHODS

3.2.1. Plant morphology of vanilla plants selected as parents

Parents belonging to *Vanilla planifolia* viz., *a 82, a 94, a 425, a 428, vv 97/84* and *V. tahitensis* were morphologically analysed for the following characters. In each genotype five plants were observed.

3.2.1.1. *Leaf characters*:

1) Leaf shape	: Oblong/elliptic/lanceolate/ovate
2) Leaf length	:Tip of the leaf to the base of the leaf lamina (cm)
3) Leaf width	:Widest portion on the leaf lamina (cm) (5^{th} leaf from tip)
4) Leaf area	: -62.246 + 3.376 L + 13.294 W where L and W are the length and width of a leaf respectively (Krishnakumar *et al.*, 1997).
5) Leaf phyllotaxy	: alternate/adjacent

3.2.1.2. Stem characters

1) Stem type	: Simple/Branched
2) Vine colour	: Green/pale green/dark green
3) Internodal length	: 5^{th} internode from the tip (cm)

3.2.1.3. Aerial root characters:

1) Root number	: Number of roots emerging from a node
2) Root origin	: Position from where the roots emerged

3.2.1.4. Statistical analysis

The data recorded for morphological characters were analysed statistically. The T test was done to assess variation in morphological characters

between two species of vanilla. The interclonal variation among clones of *V. planifolia* was evaluated by analysing the data using CRD.

3.2.2. Floral biology and morphology

The *V. planifolia* clones *viz., a 82, a 94, a 425, a 428, vv 97/84* and *V. tahitensis* plants selected as parents were subjected to floral biology and morphology studies. Totally 18 plants were observed i.e., three plants from each parent accession. Observations on different characters were recorded for each parent plant. The characters used for floral morphological and biological analysis were as follows:

3.2.2.1. Floral biology

1) Flowering season in parent plants
2) Percentage of plants flowered per clone
3) Number of days from inflorescence emergence to first flower opening
4) Days of first flower opening to the opening of the last flower (Blooming period of plants)

3.2.2.2. Inflorescence characteristics

1) Number of inflorescence per plant
2) Number of flowers per inflorescence
3) Length of inflorescence (from base upto the longest flower)

3.2.2.3. Floral morphology

1) Length of flower
2) Length of ovary
3) Girth of ovary
4) Shape of sepals

5) Length of sepals
6) Width of sepals
7) Length of labellum
8) Width of labellum
9) Length of gynostemium

3.2.3. Pollen studies

3.2.3.1. Pollen fertility and viability studies

The pollen fertility and viability of *Vanilla planifolia* and *Vanilla tahitensis* were recorded through stain test and germination studies.

3.2.3.1.1. Estimation of pollen fertility

Pollen grains scooped out from flower buds at the time of anthesis were stained using 1 per cent acetocarmine (Table 2) and viewed at 40 X and 100 X magnifications. All the pollen grains that were well stained were counted as fertile and unstained as sterile. The observations were recorded from ten microscopic fields (40 X). Photomicrographs of the stained pollen grains were also taken. The fertility percentage was calculated using the formula:

$$\frac{\text{No.: of well stained pollen grains in a field}}{\text{Total no: of pollen grains in a field}} \times 100$$

Table: 2 Composition of acetocarmine stain

Sl no:	Components	Quantity
1	Acetic acid	45 ml
2	Carmine	1 gm
3	Distilled water	55 ml

3.2.3.1.1.1. Preparation of 1 per cent acetocarmine

Acetic acid (45 ml) and distilled water (55 ml) were boiled gently in a conical flask. A funnel was put while boiling to prevent the acid from coming out. Then added 1 gm carmine to boiling solution and continued boiling for 10 to15 minutes. It was then cooled and filtered to get a clear solution.

3.2.3.1.2. Pollen viability studies

The pollen viability was tested in the following three pollen germination media combinations:

1) Medium containing sucrose, calcium nitrate and boric acid (Ravindran, 1979) (Table 3)
2) Brewbaker and Kwack's medium (Brewbaker and Kwack, 1963) (Table 4)
3) Modified ME_3 medium (Leduce *et al.*, 1990) (Table 5)

The pollen viability was tested with the pollen grains collected from flower buds at the time of anthesis and was incubated in a moisture chamber along with a drop of germination medium. The observations were recorded after 24 hours incubation. The number of germinated pollen grains and total number of pollen grains were counted from 10 microscopic fields viewed at 40X and 100X magnification and the mean germination percentages were worked out. Photomicrographs of the germinated pollen grains were also taken. The germination percentage was calculated using the formula:

$$\frac{\text{No. of germinated pollen grains in a field}}{\text{Total no: of pollen grains in a field}} \times 100$$

Table: 3 Composition of pollen germination medium (Ravindran, 1979)

Sl no:	Components	Concentration
1	Sucrose	10 per cent
2	Boric acid	100 mg l^{-1}
3	$Ca(NO)_3$	100 mg l^{-1}

Table: 4 Composition of Brewbaker and Kwack's medium (Brewbaker and Kwack, 1963)

Sl no:	Components	Concentration (mg l^{-1})
1	Sucrose	100000.00
2	$Ca(NO_3)_2.4H_2O$	300.00
3	$MgSO_4.7H_2O$	200.00
4	KNO_3	100.00

Table: 5 Composition of modified ME_3 medium (Leduce *et al.*, 1990)

Components	Concentration (mg l^{-1})
Macronutrients	
$MgSO_4.7H_2O$	370.00
KNO_3	950.00
KH_2PO_4	85.00
$CaCl_2$	880.00
NH_4NO_3	412.50
KCl	175.00
Na_2EDTA	7.45
$FeSO_4.7H_2O$	5.55
Micronutrients	
H_3BO_3	50.00
$MnSO_4.H_2O$	16.80
$ZnSO_4.7H_2O$	10.50
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Thiamine	1.00
Pyridoxine	1.00
PEG 4000	120000.00
pH	5.8

3.2.3.1.2.1. Preparation of pollen germination medium

The components for the three pollen germination media were weighed, dissolved in distilled water and volume was made upto 100ml. Sterilization of the medium was done by subjecting them to temperature of 121^{0}C

at a pressure of 15 psi for 20 minutes. After sterilization, the medium was allowed to cool to room temperature and stored in cool, dry place.

3.2.3.1.3. Statistical analysis

The data recorded for pollen fertility and viability was analysed using T test to assess the significant variation between two species of vanilla.

3.2.3.2. Pollen storage

Since the flowering of *V. tahitensis* and *V. planifolia* was not in synchronisation, pollen grains of *V. tahitensis* were stored in cryovial containing calcium chloride at 4°C. Pollen grains for this purpose were collected on the day of anthesis between 7 and 8 a.m.

3.2.3. Inter/intraspecific hybridization

Interspecific hybridization between *V. planifolia* and *V. tahitensis* and intraspecific hybridization between superior clones of *V. planifolia* was done in the following combinations:

1) *V. planifolia* (*a 425*) x *V. tahitensis*
2) *V. tahitensis* x *V. planifolia* (*a 428*)
3) *a 82* x *a 94*
4) *a 94* x *a 82*
5) *a 425* x *a 94*
6) *vv 97/84* x *a 94*

3.2.4.1. Pollination and pod development

In vivo pollination was done as per routinely practiced pollination technique of *V. planifolia* for commercial cultivation. The pollinia from the male parent were deposited on the stigmatic surface of the female parent using a tooth

prick and the flower was labelled. The flowers were hand pollinated between 8 and 9 a.m. on the day of flower opening. The date of pollination and length of ovary were recorded. The pod set percentage was scored 40 days after pollination. The pod length was taken at monthly intervals.

3.2.5. *Invitro* embryo culture

3.2.5.1. Containers for embryo culture

Flasks and bottles of 500ml capacity were used for seed culture initiation and glass test tubes (150 x 25 mm) (Borosil India, Ltd) for multiplication. They were soaked in hot water of 100°C for half an hour. On cooling, they were washed with detergent solution, rinsed with potassium dichromate solution, then washed free of detergent and acid using tap water and finally rinsed with distilled water. Washed glasswares were dried in hot air oven at 60^0C and were stored away from dust and contaminants.

3.2.5.2. Media preparation

Half strength MS (Murashige and Skoog, 1962) supplemented with various concentrations of auxins and cytokinins were used for the different aspects of embryo culture. Separate stocks were prepared by dissolving required quantities of macronutrients, micronutrients, vitamins and aminoacids (Sisco Research Laboratories, Hi–Media, Bombay, Sigma, USA and E Merck, Germany) in distilled water and were stored under refrigerated conditions. Composition of MS media and strength is given in Table 6. Separate stocks of growth regulators (Sigma, USA) used viz., Indole Acetic Acid (IAA), Naphthalene Acetic Acid (NAA), Benzyl Amino Purine (BAP) were prepared by dissolving in NaOH, volume was made up with distilled water and stored under refrigerated conditions.

Table: 6 Composition of Murashige and Skoog basal medium

Stock	Composition	Concentration (mg/l)	Stock strength	Quantity/L medium
I.	**Macronutrients**		50X	20 ml
	NH_4NO_3	1650.00		
	KNO_3	1900.00		
	$CaCl_2.2H_2O$	440.00		
	KH_2PO_4	170.00		
	$MgSO_4.7H_2O$	370.00		
II.	**Micronutrients**		50X	20 ml
	H_3BO_3	6.20		
	$MnSO_4.4H_2O$	22.30		
	KI	0.83		
	$ZnSO_4.7H_2O$	8.60		
	$Na_2MoO_4.2H_20$	0.25		
	$CuSO_4.5H_2O$	0.025		
	$CoCl_2.6H_2O$	0.025		
III.	**Fe stock**		100X	10 ml
	Na_2EDTA	37.30		
	$FeSO_4.7H_2O$	27.80		
IV.	**Vitamins**		100X	10 ml
	Thiamine HCl	0.10		
	Nicotinic acid	0.50		
	Pyridoxine HCl	0.50		
V.	**Amino acid**		100X	10 ml
	Glycine	2.00		

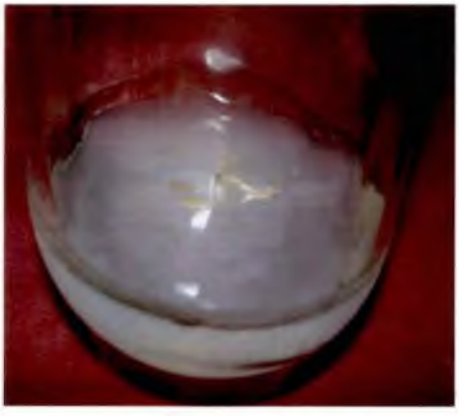
The MS medium was prepared according to the standard procedure given by Gamborg and Shyluck (1981). Specific quantities of stock solutions of chemicals were pipetted out into 1000 ml standard flask. To this, the required quantity of sucrose, inositol and growth regulators were added and the volume was made up with double glass distilled water. Then the pH of the solution was checked and adjusted to 5.8 using one per cent NaOH/HCl. To get the solid medium, agar (0.7%) was added and final volume of the medium was made upto 1000 ml. Agar was then dissolved in the medium by heating. Then the medium was poured hot into clean and dry culture vessels. They were plugged with non-absorbent cotton and sterilized at a pressure of 1.1 kg cm^{-2} at 121°C for 20 to 30 minutes. After sterilization, the media were transferred to an air-conditioned culture room.

3.2.5.3. ***Embryo culture***

The embryo culture was done as per the technique reported by Mary *et al.* (1999). The pods were harvested at different stages starting from 6 weeks after pollination from different crosses as mentioned in section 3.2.3. and embryo culture was done (Plate 1). The length and girth of the pods were measured at the time of harvest and the embryos from each pod were cultured separately in bottles.

The pods collected were first washed in teepol and wiped with 70 per cent alcohol. Then they were treated with 0.1 per cent mercuric chloride for 12 minutes followed by rinsing four times with sterile distilled water to remove traces of sterilant and dried on sterile filter paper. In the laminar air flow (Kirloskar, India), the pods were then dipped in 100 per cent alcohol and flame sterilized. Inoculations were done as per the standard procedures to maintain the asepsis.

The pods were split open and seeds scooped out into the seed germination medium (half strength MS + 3 per cent sucrose + 1.0 mg l^{-1} NAA +



6 weeks old

14 weeks old

18 weeks old

28 weeks old

Plate 1. Embryo culture from pods of different age

1.0 mg l^{-1} BAP) reported by Mary *et al.* (1999). The cultures were incubated at 25 $\pm$ 2^0C and were given a photoperiod of 16 hours with a light intensity of 1000 lux, provided by 'Philips' cool white fluorescent tubes. Following observations were recorded during seed germination:

1) Time taken for embryo germination
2) Intensity of germination
3) Number of days taken for shoot differentiation

3.2.5.3.1. Induction of multiple shoots

Multiple shoots were induced in the germinated seedling by transferring to the multiple shoot inducing medium (half strength MS + 3 per cent sucrose + 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BAP) reported by Mary *et al.* (1999). They were subcultured in the same medium for a period of 8 to 9 weeks at an interval of 4 weeks so as to establish a clonal population of 5 to 6 plantlets per seedling. The following observations were recorded during the multiplication stage:

1) Number of shoots/buds per culture for each genotype
2) Length of shoots (cm) for each genotype

3.2.5.3.2. Elongation and rooting

The proliferated shoots from each seedling were separated and transferred to elongation and rooting media (half strength MS + 3 per cent sucrose + 1.0 mg l^{-1} IAA + 0.2 mg l^{-1} BAP) reported by Mary *et al.* (1999). The following observations were recorded for each genotype during the elongation and rooting stage:

1) Length of shoots (cm)
2) Number of leaves or nodes produced
3) Length of roots (cm)
4) Number of roots produced

3.2.5.3.3. Hardening and planting out

The rooted seedlings were washed under running water to remove traces of culture media and were transferred to polythene bags (15 x 10 cm) containing potting mixture (1 soil: 1 sand: 1 organic manure). The seedlings were given a fungicidal dip (Indofil M 45, 0.3 %) for three minutes before planting. After planting, a drench was also given with the same fungicide solution. The seedlings were hardened in a net house of 75 – 80 per cent shade provided with mist. High humidity was maintained by misting for five minutes twice a day for first two weeks and then thrice in a week. Regular fungicidal sprayings were given using the contact fungicide Indofil-M 45 (0.3 %) at weekly interval. One- fourth strength MS was given once in two weeks. The plant out success was recorded for each genotype.

3.2.6. Estimation of vanillin content of *V. tahitensis* beans

The curing of tahiti vanilla was done as reported by Purseglove *et al.* (1981) to estimate the vanillin content of beans grown in Kerala conditions. Beans were harvested when mature i.e., when the tip of the bean turned brown. The weight of the harvested beans was 568 gm. Harvested beans were first washed in teepol followed by rinsing with sterile distilled water to remove dust and other contaminants and then allowed to air dry on sterile filter paper. Since *Vanilla tahitensis* is indehiscent, the beans were not killed artificially but were wrapped in newspaper to turn brown and kept for one week. The beans that turned brown were spread in blanket and kept in oven for six hours for sweating followed by wrapping in the blanket to maintain the temperature for enzymatic action and kept in thermo cool box. The process of oven wilting was continued for three weeks. Then the beans were kept for slow drying in a moisture chamber to maintain relative humidity of 75 to 85 per cent for four weeks. By this time the weight of the beans became 155 gm. Beans were kept for conditioning for a period of two months wrapped in butter paper. Vanillin content of three samples 30 gm each of cured

beans of tahiti vanilla was analysed as per AOAC method (Horwitz, 1980) at Spices Board, Kochi.

3.2.7. Variability in hybrid progenies

3.2.7.1. Morphological characterisation

Variability in morphological characters of parents and progenies of different crosses derived by *in vitro* embryo culture were recorded as per the descriptor of vanilla (Kuruvilla *et al.*, 2000). Hybrid seedlings derived from the same cross and from different crosses were evaluated. The characters studied included both qualitative and quantitative ones as shown below:

3.2.7.1.1. Stem characters:

1) Stem type : Simple/Branched
2) Vine colour : Green/pale green/dark green
3) Internodal length : third internode from the tip (cm)
4) Plant height : in (cm)
5) Plant growth : More vigorous/vigorous/less vigorous

3.2.7.1.2. Leaf characters:

1) Leaf shape : Oblong/elliptic/lanceolate/ovate
2) Leaf length : Tip of the leaf to the base of the leaf lamina (cm)
3) Leaf width : Widest portion on the leaf lamina (cm) (3^{rd} leaf from tip)
4) Leaf phyllotaxy : alternate/adjacent
5) Number of leaves

3.2.7.1.3. Aerial root characters:

1) Root number : Number of roots emerging from nodes
2) Root origin : Position from where the roots emerged

3.2.7.2. Statistical analysis

Morphological observations were recorded for a period of 3 months at monthly intervals for hybrid seedlings. Mean and standard deviation (SD) were calculated for the observations recorded.

3.2.8. Molecular characterization by RAPD

Molecular characterisation of parents and progenies was done by RAPD technique. Seventeen progenies from the cross between superior clones of *V. planifolia* (*a 82* x *a 94*, *a 94* x *a 82*, *a 425* x *a 94*, *vv 97/84* x *a 94*) and seven progenies from the cross between *V. tahitensis and V. planifolia* were used for molecular characterisation. The list of parents and progenies is given in Table 7.

3.2.8.1. DNA isolation

A modified CTAB extraction procedure reported by Doyle and Doyle (1987) was followed for the extraction of genomic DNA in vanilla.

Tender vanilla leaves were taken from the selected plants using sterile blade. The leaf samples were washed in tap water, wiped with alcohol, weighed and immediately used for extraction.

Reagents

1. 4X Extraction buffer

Tris HCl	: 4.8g
EDTA disodium salt	: 0.74g
Sorbitol	: 2.56g
Sterile milli Q water to	: 100 ml

Table: 7 List of parents and progenies used for molecular characterization

Sl no:	Source	Genotype	Identity code
1	*V. planifolia* clone	*a 82*	V_1
2	*V .planifolia* clone	*a 94*	V_2
3	*V. planifolia* clone	*a 425*	V_3
4	*V..planifolia* clone	*a 428*	V_4
5	*V..planifolia* germplasm acession	*vv 97/84*	V_5
6	*V. tahitensis* clone	*V.tahitensis*	V_6
7	*V.t* x *V.p*	383 a	VHI
8	*V.t* x *V.p*	385 b	VH2
9	*V.t* x *V.p*	387 a_2	VH3
10	*V.t* x *V.p*	387 e_4	VH4
11	*V t* x *V:p*	387 a_4	VH5
12	*V.t* x *V.p*	382 a	VH6
13	*V.t* x *V.p*	384 f	VH7
14	*a 82 x a 94*	$18a_3$	VH8
15	*a 82 x a 94*	24e	VH9
16	*a 82 x a 94*	$9d_4$	VH10
17	*a 82 x a 94*	$9d_5$	VH11
18	*a 425 x a 94*	207 a	VH12
19	*a 425 x a 94*	207 b	VH13
20	*a 425 x a 94*	208 c	VH14
21	*a 425 x a 94*	208 j_1	VH15
22	*a 94 x a 82*	574 b	VH16
23	*a 94 x a 82*	574 c	VH17
24	*a 94 x a 82*	575 a	VH18
25	*a 94 x a 82*	579 b	VH19
26	*a 94 x a 82*	579 c	VH20
27	*vv 97/84 x a 94*	17 c	VH21
28	*vv 97/84 x a 94*	17 a	VH22
29	*vv 97/84 x a 94*	24 c	VH23
30	*vv 97/84 x a 94*	24 d	VH24

Tris HCl, EDTA and sorbitol were dissolved in about 80 ml sterile milli Q water and the pH was adjusted to 7.5 using concentrated HCl. The volume was then made up to 100 ml.

2. Lysis buffer

1M Tris HCl, pH 8	: 20.0 ml
0.25M EDTA	: 20.0 ml
5M NaCl	: 40.0 ml
Sterile milli Q water	: 20.0 ml
Cetyl Trimethyl Ammonium Bromide (CTAB)	: 2.0g

3. TE buffer

10mM Tris HCl, pH 7.6	: 0.3028g
1mM EDTA	: 0.0930g
Distilled water	: 200ml

4. Sarcosine (5%)

Sarcosine	: 5.0g
Distilled water	: 100ml

5. Chilled isopropanol
6. Chloroform: isoamyl alcohol mixture (24:1 v/v)
7. β mercaptoethanol
8. Ethanol (100% and 70%)

The reagents 1 to 4 were prepared and autoclaved before use.

3.2.8.1.1. Procedure

Leaf sample (0.5g) collected was ground in sterile mortar, with 3ml of 2X extraction buffer and 10 µl β mercaptoethanol. A pinch of sodium meta bisulphate was added to it just before grinding. The homogenate was then poured into a centrifuge tube (50ml) containing 7.5ml pre-warmed lysis buffer and 1.25 ml

(5%) sarcosine. The tube was kept for 15 minutes at 65^{0}C and was occasionally mixed by inversion. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged at 10,000 rpm for 15 minutes at 4^{0}C. The upper aqueous phase was pipetted out and saved in a centrifuge tube. To the tube containing the aqueous phase, 0.6 volume pre chilled isopropanol was added. The tube was kept at –20^{0}C for half an hour for precipitation. The contents were mixed gently by inversion until the DNA was precipitated. The DNA was pelleted by centrifuging at 10,000 rpm for 15 minutes at 4^{0}C. The isopropanol was poured off, drained well and the pelleted DNA was washed with 70 per cent alcohol and centrifuged at 10,000 rpm for 10 minutes. The pellet was air dried and resuspended in 200 µl TE buffer.

3.2.8.2. Purification of DNA

The DNA isolated will also contain RNA and residual protein. To exclude the RNA and residual proteins, the sample was treated with RNAase and Proteinase K.

3.2.8.2.1. Preparation of RNAase and Proteinase K

The ribonuclease A from Genei, Bangalore was used to prepare RNAase. Ribonuclease was dissolved in 0.10 M sodium acetate (pH 5.2) at the rate of 10 mg ml^{-1}. Solution was heated at 100°C for 15 minutes and allowed to cool to room temperature. The pH was adjusted to 7.4 by adding 100 µl 1M Tris base and stored at -20°C.

The Proteinase K from Genei, Bangalore at 20 mg ml^{-1} concentration was prepared in distilled water and stored at -20°C.

3.2.8.2.2. Incubation of DNA with RNAase and Proteinase K

The extracted DNA suspended in TE buffer (100 µl) was treated with 20 µg/100ml of RNAase solution and incubated at 37°C for 1hour. The extracted DNA

after RNAase digestion was treated with 50 µg ml^{-1} Proteinase K solution and incubated at 45°C for 1 hour.

After incubation, the sample was then made upto 500 µl with sterile distilled water. Equal volumes (250 µl) of phenol : chloroform-isoamyl alcohol (24:1) mixture was added. It was then centrifuged at 10,000 rpm for 10 minutes at 4°C. The aqueous phase was saved using a micropipette and transferred to a sterile eppendorff tube. To it equal volume of chloroform-isoamyl alcohol (24:1) mixture was added and centrifuged at 10,000 rpm for 10 minutes at 4°C. The aqueous phase obtained was again saved and centrifuged at 10,000 rpm for 10 minutes at 4°C with equal volume of chloroform-isoamyl alcohol (24:1) mixture. The final aqueous phase was saved in a sterile eppendorff tube and 0.6 volume of chilled isopropanol was added, mixed gently and kept at -20°C for half hour until the DNA was precipitated. It was then centrifuged at 10,000 rpm for 10 minutes at 4°C. The isopropanol was poured off and the DNA pellet was washed first with 70 per cent alcohol and then with absolute alcohol. The DNA was then allowed to air dry, dissolved in 25-50 µl of TE buffer and stored at -20°C for further use.

3.2.8.3. Estimation of quality of DNA

The quality of isolated DNA was evaluated through electrophoresis.

3.2.8.3.1. Electrophoresis of DNA samples

Reagents

1. Agarose
2. 50X TAE buffer

Tris base	:242g
0.5M EDTA, pH 8	:100ml
Glacial acetic acid	: 57.1ml

The contents were mixed well, autoclaved and stored at room temperature.

3. 6X Gel loading dye

Bromophenol blue : 0.25%

Xylene cyanol FF : 0.25%

Glycerol in water : 30%

The components were mixed well, autoclaved and stored at 4^0C

4. Ethidium bromide

3.2.8.3.1.1. Procedure

Gel buffer 1X TAE was prepared from the 50X TAE stock solution. Gel buffer 1X TAE was taken in a conical flask (100 ml for large gel and 30 ml for small gel). Agarose (1.0% for DNA samples and 1.4% for RAPD samples) was weighed, added to the flask, stirred and boiled till the agarose dissolved completely. It was then allowed to cool to 40^0C and ethidium bromide (2 µl) was added into the flask, mixed well. The open ends of the gel-casting tray was sealed with cello tape and placed on a leveled horizontal surface and the comb was placed properly on the tray. The dissolved agarose was poured gently into the tray. The gel was allowed to solidify for 30 minutes and then the comb was removed carefully. The gel was then placed in electrophoresis unit (Hoefer Pharmacia) with the well side in the cathode position. The TAE buffer (1X) was poured into the buffer tank till the buffer level is 2 to 3 millimeter above the gel. The DNA samples (5 µl and 20 µl in the case of RAPD products) were mixed with gel loading dye and then loaded carefully into the wells using micropipette. Standard DNA molecular weight marker (λ DNA + *Eco*RI + *Hin*dIII) was loaded in one well. The cathode and anode of the electrophoresis unit were then connected to the power supply and the gel was run at constant voltage (45 mA). The power supply was turned off when the loading dye moved three fourth of the gel.

3.2.8.3.1.2. Gel documentation

The gel was taken from electrophoresis unit and viewed in the UV transilluminator (HeroLab, Germany) under UV light of 320nm. The image of the

gel was monitored and stored in a gel documentation system Alpha Imager-2000 (Alpha InfoTech, USA).

3.2.8.4. Estimation of quantity of DNA

The quantity of DNA from parents and progenies of vanilla was measured using UV visible spectrophotometer (Spectronic R Genesys 5 from Spectronic Instruments Inc., USA). The isolated DNA was diluted by adding 3 µl of DNA to 1.5 ml sterile water (dilution 1:500) and absorbance was read at 260 nm and 280 nm against distilled water blank. The purity of DNA was assessed from the ratio of OD value at 260 to OD value at 280. A ratio of 1.8 to 2.0 indicates pure DNA. The quantity of DNA in the pure sample was calculated using the formula:

OD 260 = 1 which is equivalent to 50 µg double stranded DNA/ml

Therefore, the quantity of DNA in µg/ml = OD260 x 50 x 500.

3.2.8.5. Random Amplified Polymorphic DNA analysis

To detect the genetic base of morphological variability, parents of different crosses and their progenies as listed in Table 7 were subjected to RAPD analysis. The procedure of Demeke *et al.* (1992) was modified and was used for the amplification of genomic DNA.

3.2.8.5.1. Screening of random primers for RAPD assay

The decamer primers obtained from 'Operon Technologies', USA, were used for the study. Thirty decamer primers in the series OPF, OPP and OPE were screened with genomic DNA of clone ***a 82*** as the template. Those primers, which gave good amplification with more than eight bands were selected for further studies. Details of primers used for screening is given in Table 8. The six primers selected were OPF3, OPP6, OPE 14, OPE 15, OPE 18 and OPE 19. Genomic

DNA (20-50 ng) was subjected to amplification using these selected random primers.

The amplification products were resolved on 1.4 per cent agarose gel with TAE buffer system, stained with ethidium bromide, visualized under UV transilluminator and documented using alpha imager.

Amplification reaction was carried out in a Master cycler (PTC 200 model - MJ Research, USA) with the following program. The PCR was programmed for 40 cycles to get proper amplification. One PCR reaction cycle was completed as shown below:

- Primary denaturation at 94^0C for 3 minutes
- DNA denaturation at 92^0C for 1 minute
- Annealing of the primer to the template DNA at 37^0C for 1 minute
- Primer extension at 72^0C for 2 minutes

Composition of reaction mixture for RAPD assay:

10x Assaybuffer for Taq DNA polymerase (15mM $MgCl_2$)	2.5 µl
$MgCl_2$	1.0 µl
d NTP mix (100µM each of dATP, dCTP, dGTP, dTTP)	1.5 µl
Decamer primer (5pM)	1.5 µl
Taq DNA polymerase (1U)	1.0 µl
Genomic DNA (40-50ng)	4.0 µl
Milli Q water	13.5 µl
Total volume	**25.0 µl**

A master mix without the template DNA was prepared using the reaction mixture for the required number of reactions. From this master mix, 21.0 µl was pipetted into each PCR tube (Merck India and Tarsons India Ltd.). Template DNA (4.0 µl) were added. Heated lid of the instrument was used to prevent vapourisation of the reaction mix. The PCR tubes were kept in the hermal cycler and the program was run for 40 cycles and was completed in 4 hours.

Table:8 List of operon decamer primers used for screening

Sl no:	Primer code	Primer sequence
1	OPP-1	GTAGCACTCC
2	OPP-2	TCGGCACGCA
3	OPP-3	CTGATACGCC
4	OPP-4	GTGTCTCAGG
5	OPP-5	CCCCGGTAAC
6	OPP-6	GTGGGCTGAC
7	OPP-7	GTCCATGCCA
8	OPP-8	ACATCGCCCA
9	OPP-9	GTGGTCCGCA
10	OPP-10	TCCCGCCTAC
11	OPF-1	ACGGATCCTG
12	OPF-2	GAGGATCCCT
13	OPF-3	CCTGATCACC
14	OPF-4	GGTGATCAGG
15	OPF-5	CCGAATTCCC
16	OPF-6	GGGAATTCGG
17	OPF-7	CCGATATCCC
18	OPF-8	GGGATATCGG
19	OPF-9	CCAAGCTTCC
20	OPF-10	GGAAGCTTGG
21	OPE-11	GAGTCTCAGG
22	OPE-12	TTATCGCCCC
23	OPE-13	CCCGATTCGG
24	OPE-14	TGCGGCTGAG
25	OPE-15	ACGCACAACC
26	OPE-16	GGTGACTGTG
27	OPE-17	CTACTGCCGT
28	OPE-18	GGACTGCAGA
29	OPE-19	ACGGCGTATG
30	OPE-20	AACGGTGACC

3.2.8.5.2. Data analysis

The amplification products for the selected primers were scored across the lanes as 1 or 0 to denote the presence or absence of bands of identical molecular weights. The total number of amplification products, monomorphic and polymorphic bands were scored for hybrids and progenies. Statistical analysis with UPGMA analysis was done and dendrogram was generated using NTSYS software. Similarity indices were computed as JACCARD's coefficient through 'Simqual ' routine and clustering was done using Sequential Agglomerative Heirarchial Nested Clustering (SAHN) routine of the NTSYS package. Dendrograms were constructed for different crosses based on the clustering using Unweighted Pair Group Method of Arithmetic Averages (UPGMA).

RESULTS

4. RESULTS

The intraspecific hybridisation among superior clones of *Vanilla planifolia* and interspecific hybridisation between *Vanilla planifolia* and *Vanilla tahitensis* were conducted. The hybrid seedlings were raised through embryo culture technique and variability in the seedling progenies was assessed by morphological and molecular characterisation. The results obtained from this study are presented in this chapter.

4.1 VARIABILITY ANALYSIS IN PARENTS FOR PLANT MORPHOLOGY

Various foliar and cauline characters of selected plants as parents were studied for assessing the morphological variability in *Vanilla planifolia* and *Vanilla tahitensis* plants.

4.1.1. Foliar characteristics

Various foliar characteristics studied were leaf length, leaf breadth, leaf area, leaf shape and leaf phyllotaxy. Results are presented in Tables 9 & 10.

4.1.1.1 Leaf length

The observations showed that *V. tahitensis* plants have a mean leaf length of 20.84 cm while *V. planifolia* clones recorded only 17.14 cm. Among the *V. planifolia* clones mean leaf length ranged from 14.74 cm in the genotype *a 428* to 18.14 cm in *vv 97/84*. The statistical analysis showed that there was no significant variation in leaf length between two species of vanilla and among clones of *V. planifolia* (Plate 2A).

4.1.1.2. Leaf breadth

The observations showed that *V. planifolia* clones have a mean leaf breadth of 5.02 cm while *V. tahitensis* plants recorded only 3.6 cm. Among the *V. planifolia* clones mean leaf breadth ranged from 4.8 cm in *a 428* to 5.4 cm in *a 82*. The statistical analysis showed that there was no significant variation in leaf breadth between two species of vanilla and among clones of *V. planifolia* (Plate 2A).

4.1.1.3. Leaf area

The observations showed that *V. planifolia* clones have a mean leaf area of 62.08 cm^2 while *V. tahitensis* plants recorded only 55.96 cm^2. Among the *V. planifolia* clones mean leaf area ranged from 50.82 cm^2 in *a 428* to 69.2 cm^2 in *a 82*. The statistical analysis showed that there was no significant variation in leaf area between two species of vanilla and among clones of *V. planifolia*.

4.1.1.4. Leaf shape

Oblong – elliptic leaf shape was observed for all clones of *V. planifolia* while it was narrow lanceolate for *V. tahitensis* plants (Table 10 & Plate 2A).

4.1.1.5. Leaf phyllotaxy

The leaf phyllotaxy was alternate spiral for both *V. planifolia* and *V. tahitensis* plants (Table 10).

4.1.2. Cauline characteristics

Various stem characters i.e. stem type, vine colour and internodal length were studied in the selected parent plants and are presented in Table 11.

Table: 9 Leaf length, breadth and leaf area of *Vanilla planifolia* and *Vanilla tahitensis* parent plants

Vanilla planifolia					*Vanilla tahitensis*			
Clone no:	Accession no:	Leaf length (cm)	Leaf breadth (cm)	Leaf area	Accession no	Leaf length (cm)	Leaf breadth (cm)	Leaf area
a 82	1	18.9	6.1	82.66	1	20.6	3.6	55.16
	2	16.6	6.1	74.86	2	21.6	3.9	62.5
	3	18.7	4.9	66.03	3	18.7	3.2	43.43
	4	17.4	4.6	57.65	4	22.1	3.4	57.56
	5	16.7	5.3	64.60	5	21.2	3.9	61.17
	Mean	**17.7**	**5.4**	**69.2**	**Mean**	**20.84**	**3.6**	**55.96**
a 94	1	18.8	5.4	73.0				
	2	17.0	4.7	57.64				
	3	17.1	4.8	59.30				
	4	18.3	5.1	67.35				
	5	17.3	5.0	62.62				
	Mean	**17.7**	**5.0**	**64.0**				
a 428	1	17.4	5.2	65.62				
	2	17.0	4.3	52.31				
	3	17.5	6.5	83.27				
	4	10.5	3.9	25.1				
	5	11.3	3.9	27.81				
	Mean	**14.74**	**4.8**	**50.82**				
a 425	1	16.3	4.4	51.28				
	2	19.4	4.7	65.74				
	3	18.0	5.1	66.40				
	4	17.5	5.0	63.3				
	5	16.0	4.7	54.24				
	Mean	**17.4**	**4.8**	**60.19**				
vv 97/84	1	18.9	5.7	77.34				
	2	20.0	5.5	78.40				
	3	18.1	5.1	66.67				
	4	16.8	4.6	55.63				
	5	16.4	4.5	52.95				
	Mean	**18.14**	**5.1**	**66.20**				
	Over all mean	**17.14**	**5.02**	**62.08**				
	CD	-	-	-				
		NS	NS	NS				

Table: 10 Leaf shape and phyllotaxy of *Vanilla planifolia* and *Vanilla tahitensis* parent plants

Vanilla planifolia				*Vanilla tahitensis*		
Clone no:	Access-ion no:	Leaf shape	Leaf phyllotaxy	Acess-ion no.	Leaf shape	Leaf phyllotaxy
a 82	1	Oblong – elliptic	Alternate	1	Narrow lanceolate	Alternate
	2	Oblong – elliptic	Alternate	2	Narrow lanceolate	Alternate
	3	Oblong – elliptic	Alternate	3	Narrow lanceolate	Alternate
	4	Oblong – elliptic	Alternate	4	Narrow lanceolate	Alternate
	5	Oblong - elliptic	Alternate	5	Narrow lanceolate	Alternate
a 94	1	Oblong – elliptic	Alternate			
	2	Oblong – elliptic	Alternate			
	3	Oblong – elliptic	Alternate			
	4	Oblong – elliptic	Alternate			
	5	Oblong - elliptic	Alternate			
a 428	1	Oblong – elliptic	Alternate			
	2	Oblong – elliptic	Alternate			
	3	Oblong – elliptic	Alternate			
	4	Oblong – elliptic	Alternate			
	5	Oblong - elliptic	Alternate			
a 425	1	Oblong – elliptic	Alternate			
	2	Oblong – elliptic	Alternate			
	3	Oblong – elliptic	Alternate			
	4	Oblong – elliptic	Alternate			
	5	Oblong - elliptic	Alternate			
vv 97/84	1	Oblong – elliptic	Alternate			
	2	Oblong – elliptic	Alternate			
	3	Oblong – elliptic	Alternate			
	4	Oblong – elliptic	Alternate			
	5	Oblong - elliptic	Alternate			

4.1.2.1. Stem type

Simple stem was observed for all the plants belonging to *V. planifolia* and *V. tahitensis* (Plate 2B).

4.1.2.2. Vine colour

Vine colour was green for all *V. planifolia* clones while it was dark green for *V. tahitensis* (Table 11).

4.1.2.3. Internodal length

The observations showed that *V. tahitensis* plants have a mean internodal length of 11.08 cm while *V. planifolia* clones recorded only 8.34 cm. Among the *V. planifolia* clones mean internodal length ranged from 6.84 cm in the genotype *a 428* to 10.36 cm in *vv 97/84*. The statistical analysis showed that there was significant variation in internodal length at 5 per cent level among clones of *V. planifolia*; whereas no significant variation between the two species of vanilla (Table 11).

4.1.3. Aerial root characteristics

4.1.3.1. Root origin and number of roots from a node

The aerial roots were produced singly from a node opposite to the leaves in all parent plants studied (Table 12 and Plate 2C).

4.3. FLORAL BIOLOGY AND MORPHOLOGY STUDIES

The data was recorded on floral biological (Table 13) and morphological characters of *V. planifolia* and *V. tahitensis* plants.

Table: 11 Cauline characteristics of *Vanilla planifolia* and *Vanilla tahitensis* parent plants

Vanilla planifolia					*Vanilla tahitensis*			
Clone no:	Accession no:	Stem type	Vine colour	Internodal length (cm)	Acession no.	Stem type	Vine colour	Internodal length (cm)
a 82	1	Simple	Green	8.7	1	Simple	Dark green	11.2
	2	Simple	Green	7.0	2	Simple	Dark green	10.7
	3	Simple	Green	8.0	3	Simple	Dark green	10.4
	4	Simple	Green	7.4	4	Simple	Dark green	11.7
	5	Simple	Green	7.0	5	Simple	Dark green	11.4
	Mean			**7.62**	**Mean**			**11.08**
a 94	1	Simple	Green	7.7				
	2	Simple	Green	7.1				
	3	Simple	Green	7.2				
	4	Simple	Green	7.9				
	5	Simple	Green	8.0				
	Mean			**7.58**				
a 428	1	Simple	Green	8.0				
	2	Simple	Green	10.4				
	3	Simple	Green	4.7				
	4	Simple	Green	6.1				
	5	Simple	Green	5.0				
	Mean			**6.84**				
a 425	1	Simple	Green	8.1				
	2	Simple	Green	11.0				
	3	Simple	Green	8.0				
	4	Simple	Green	10.6				
	5	Simple	Green	8.9				
	Mean			**9.32**				
vv 97/84	1	Simple	Green	7.2				
	2	Simple	Green	12.6				
	3	Simple	Green	10.0				
	4	Simple	Green	12.6				
	5	Simple	Green	9.4				
	Mean			**10.36**				
	Overall mean			**8.34**				
	CD			**2.53**				
				S*				

* significant at 5% level

Table: 12 Aerial root characteristics of *Vanilla planifolia* and *Vanilla tahitensis* parent plants

Vanilla planifolia				*Vanilla tahitensis*		
Clone no:	Accession no:	Root origin	No: of roots from node	Accession no:	Root origin	No: of roots from node
a 82	1	Opposite	1	1	Opposite	1
	2	Opposite	1	2	Opposite	1
	3	Opposite	1	3	Opposite	1
	4	Opposite	1	4	Opposite	1
	5	Opposite	1	5	Opposite	1
a 94	1	Opposite	1			
	2	Opposite	1			
	3	Opposite	1			
	4	Opposite	1			
	5	Opposite	1			
a 428	1	Opposite	1			
	2	Opposite	1			
	3	Opposite	1			
	4	Opposite	1			
	5	Opposite	1			
a 425	1	Opposite	1			
	2	Opposite	1			
	3	Opposite	1			
	4	Opposite	1			
	5	Opposite	1			
vv 97/84	1	Opposite	1			
	2	Opposite	1			
	3	Opposite	1			
	4	Opposite	1			
	5	Opposite	1			

Plate 2A: Variation in leaf size, shape and colour of *V.planifolia* clones (a 82, a428) and *V.tahitensis* plants

Plate 2B: Simple stem type in *V.planifolia* and *V.tahitensis* plants

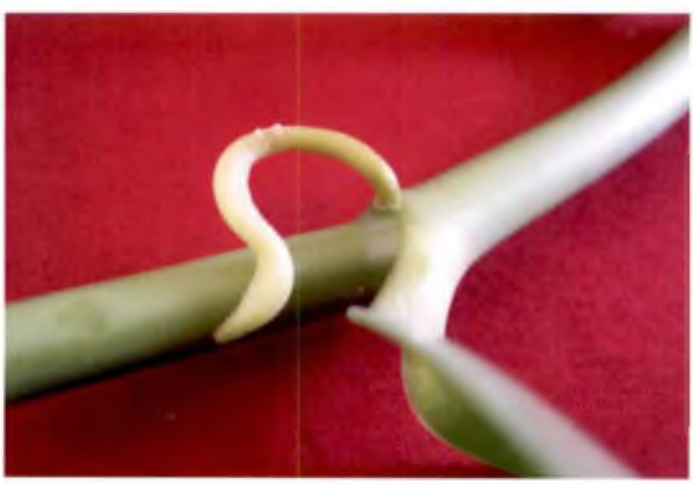


Plate 2C: Root origin and number of roots originated from a single node in vanilla

4.3.1. Floral biology

4.3.1.1. Flowering season in parent plants

The flowering of *V. tahitensis* plants was observed during September to October and December to January. All the clones of *V. planifolia* except *a 428* flowered during January to April. The clone *a 428* flowered twice in an year i.e., October to November and February to March.

4.3.1.2. Number of days from infloroscence emergence to first flower opening

The mean developmental period from floral initiation to first flower opening in *V. planifolia* clones was 15.6 days and ranged from 15.3 to 16.1 days among clones. The mean developmental period in *V. tahitensis* plants was 13.8 days.

4.3.1.3. Blooming period

Mean blooming period of a plant in *V. planifolia* was 30.95 days while it was 26.2 days in *V.tahitensis*. Among the *V. planifolia* clones it ranged from 27.7 days in *a 94* to 38.1 days in *a 425*.

4.3.1.4. Percentage of parent plants flowered

Maximum flowering was observed in plants of clone *a 82* of *V. planifolia* and *V. tahitensis* (80 %). In other *V. planifolia* clones it was 60 per cent.

4.3.2. Infloroscence characteristics

4.3.2.1 Number of infloroscence per plant

The observations showed that mean number of infloroscence per plant in *V. planifolia* clones was 2.58 and ranged from 1.0 to 4.36 in *a 428* and *a 82*

Table: 13 Floral biology of *Vanilla planifolia* and *Vanilla tahitensis* parent plants

Vanilla planifolia						*Vanilla tahitensis*				
Clone no:	Accession no:	Flowering season	Plants flowered in a clone (%)	No. of days from inflorescence emergence to flower opening	Blooming period of plants	Accession no.	Flowering season	Plants flowered in a clone (%)	No. of days from inflorescence emergence to flower opening	Blooming period of plants
a 82	1	Mar – April	80%	16.0	24.0	1	Sep – Oct, Dec - Jan	80%	13.0	29.3
	2			15.0	31.5	2			15.0	21.7
	3			15.0	30.7	3			13.3	27.7
	Mean			**15.3**	**28.7**	**Mean**			**13.8**	**26.2**
	SD			**0.58**	**4.12**	**SD**			**1.08**	**4.02**
a 94	1	Feb – Mar	60%	14.3	33.7					
	2			15.5	25.5					
	3			16.0	24.0					
	Mean			**15.3**	**27.7**					
	SD			**0.87**	**5.2**					
a 428	1	Jan - Mar	60%	16.3	39.3					
	2			18.0	36.0					
	3			14.0	39.0					
	Mean			**16.1**	**38.1**					
	SD			**2.01**	**1.82**					
a 425	1	Oct – Nov, Feb – Mar	60%	19.0	31.0					
	2			14.0	29.0					
	3			14.0	28.0					
	Mean			**15.7**	**29.3**					
	SD			**2.89**	**1.53**					
	Overall mean			**15.6**	**30.95**				**13.8**	**26.2**

respectively. The mean number of infloroscence per plant in *V. tahitensis* was 2.65. (Table 14).

4.3.2.2. Number of flowers per infloroscence

The observations showed that mean number of flowers per infloroscence in *V. planifolia* clones was 15.97 and ranged from 14.28 to 19.56 in *a 82* and *a 425* respectively. The mean number of flowers per infloroscence in *V. tahitensis* plants was 15.1 (Table 14).

4.3.2.3. Length of infloroscence

The observations showed that *V. tahitensis* plants have a mean infloroscence length of 15.46 cm while *V. planifolia* clones recorded only13.77 cm. Among the *V. planifolia* clones mean infloroscence length ranged from 13.8 cm to 15.2 cm in *a 82* and *a 428* respectively (Table 14).

4.3.2.4. Floral morphology

The flowers of *Vanilla planifolia* were 9 - 11 cm long and that of *Vanilla tahitensis* were about 8-9 cm long. The flower colour of *V. planifolia* was greenish cream with less floral diameter while that of *V. tahitensis* was pale greenish cream with more floral diameter (Plate 3). Among the clones of *V. planifolia*, clone *a 428* exhibited more greenish tinged flowers and deeply embedded pollinium with less pollen mass. Sepal shape was similar in both species. There were three oblong – lanceolate sepals, slightly reflexed at the apex. The sepals were 5 - 6 cm long and 1.0 - 1.3 cm wide in *V. tahitensis*. The length and width of the sepals in *V. planifolia* was 5.5 - 6.5 cm and 1 - 1.5 cm. In both species of vanilla the length of upper petals was almost equal to the sepals; whereas width of the petals was slightly less than the sepals. The width of upper petals ranged from 0.8 - 1.0 cm. The labellum or lip was shorter than the other perianth lobes.

Table: 14 Inflorescence characteristics of *Vanilla planifolia* and *Vanilla tahitensis* parent plants

Vanilla planifolia					*Vanilla tahitensis*			
Clone no:	Accession no:	No: of inflorescence/ plant	No: of flowers/ inflorescence	Length of* inflorescence (cm)	Accession no.	No: of inflorescence/plant	No: of flowers/ inflorescence	Length of* inflorescence (cm)
a 82	1	1	9.0	12.7	1	4	18.7	17.0
	2	2	14.5	14.7	2	3	10.3	13.9
	3	9	19.3	13.9	3	8	16.3	15.5
	Mean	**4**	**14.28**	**13.8**	**Mean**	**5**	**15.1**	**15.46**
	SD	**4.36**	**5.2**	**1.01**	**SD**	**2.65**	**4.31**	**1.57**
a 94	1	6	16.7	14.1				
	2	2	13.5	13.9				
	3	1	14.0	14.0				
	Mean	**3**	**14.72**	**14.0**				
	SD	**2.65**	**1.7**	**0.1**				
a 428	1	5	22.7	15.9				
	2	1	14.0	12.5				
	3	1	22.0	13.9				
	Mean	**2.3**	**19.56**	**14.1**				
	SD	**2.3**	**4.82**	**1.71**				
a 425	1	1	19.0	16.0				
	2	1	15.0	15.3				
	3	1	12.0	14.4				
	Mean	**1**	**15.3**	**15.2**				
	SD	**0**	**3.51**	**0.8**				
	Overall mean	**2.58**	**15.97**	**13.77**	**5.0**		**15.1**	**15.46**

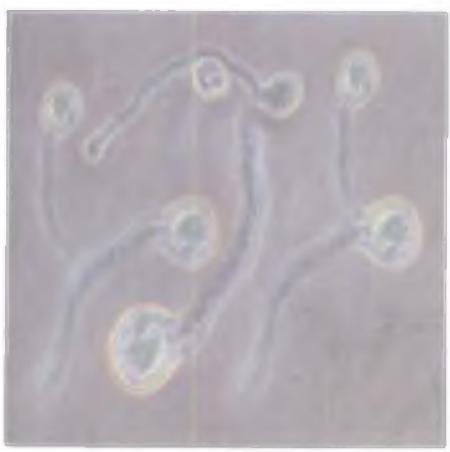
*from base to longest flower

** *Vanilla planifolia* clones

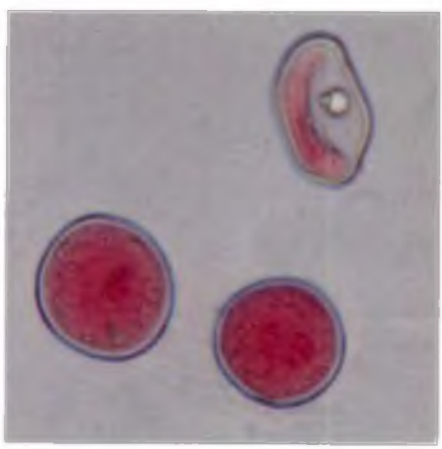
Vanilla planifolia

Vanilla tahitensis

Plate 3. Flowers of *Vanilla planifolia* and *Vanilla tahitensis*



Pollen germination
(100X magnification)



Pollen fertility
(Acetocarmine stain)
(100X magnification)

The length of the ovary of *Vanilla planifolia* ranged between 4 - 6 cm and that of *V. tahitensis* ranged from 3 - 4 cm. The girth of ovary of *V. planifolia* ranged between 1.3 – 1.6 cm and that of *V. tahitensis* ranged between 1.2 – 1.4 cm. The length of gynostemium or column was 3.4 - 3.7 cm in *V. tahitensis* and 3.6 - 4.0 cm in *V. planifolia*. In both species the tip of the column bears a single stamen with two pollen masses (pollinia) covered by a cap or hood like structure called rostellum, which prevents natural pollination. Hence artificial hand pollination has to be done for pod set.

4.4. POLLEN STUDIES

4.4.1. Pollen fertility and viability

Mean pollen fertility as per acetocarmine stain test for *V. planifolia* was 61 per cent while it was 74.69 per cent for *V. tahitensis* flowers. The statistical analysis (T test) showed that there was no significant variation in pollen fertility and viability percentages between *V. planifolia* and *V. tahitensis* (Table 15). The stained (fertile) pollen grains and germinated pollen grains of vanilla are shown in Plate 4.

Pollen viability of *V. planifolia* and *V. tahitensis* was checked in three media combinations. Pollen germination was obtained only in the media reported by Ravindran (1979), which consisted of sucrose, calciun nitrate and boric acid. The mean pollen viability percentages of *V. planifolia* and *V.tahitensis* were 39 per cent and 45.3 per cent respectively in this medium. There was no pollen germination in the modified ME_3 and Brewbaker and Kwack's media.

4.4.2. Pollen storage

Since the flowering of *V. planifolia* and *V. tahitensis* was not in synchronisation, pollen of *V.tahitensis* was stored for interspecific hybridization. The pollen storage was done in cryovials containing $CaCl_2$ and kept at 4°C. The

Table: 15 Pollen fertility and viability of *Vanilla planifolia* and *Vanilla tahitensis* flowers

Genotype	Mean pollen fertility (%)	Mean pollen viability (%)
V. planifolia	61.0	39.0
V. tahitensis	75.6	45.3

T test was done and found non significant

storage of pollen in $CaCl_2$ retained it's viability upto 7 to 8 weeks. The pod set percentage of *V. planifolia* x *V. tahitensis* hybrid pods developed after pollinating with stored *V. tahitensis* pollen grains is given in Table 16. Pod set percentage was taken as an index of pollen viability after storage. The vanilla pollen became dried on long term storage of three months in $CaCl_2$ at 4°C and pod set was not observed on using this pollen for pollination.

4.5.INTER/INTRASPECIFIC HYBRIDIZATION

4.5.1. Pollination and pod set

Artificial hand pollination was done between 7 and 8 a.m. for pod set in both interspecific and intraspecific crosses. Successful pod set after pollination was indicated by the retention of floral appendages 4 to 5 days after pollination. The intraspecific (*V. p* x *V. p*) and interspecific (*V. t* x *V. p*) hybrid pods are shown in Plate 5. The pod set percentage for crosses of different parental combinations were recorded 40 days after pollination and it varied from 9.1 to100 per cent (Table 16). In the interspecific cross *V. planifolia* x *V. tahitensis* pod set was 70 per cent. However, only four hybrid pods could be recovered for embryo culture because of premature dropping of pods at four months after pollination. Cent per cent pod set was observed for the hybrid crosses *a 82* x *vv 97/84* and *a 428* x *a 82*. Minimum pod set percentage of 9.1 was recorded for the cross of *a 94* x *a 425*. The pod set percentage of other crosses varied from 14.3 to 96.9 per cent.

4.5.2. Length increment of hybrid beans

The mean length of the ovary of *V. planifolia* on the day of pollination was 5.18 cm and it increased to 6.88 cm within 4 DAP with pollen grains of *V. tahitensis*. It attained a length of 9.28 cm within a period of 1 month. After 1 month, length increment was meagre and it attained a maximum of 9.87 cm at 120 DAP. The length was reduced to 9.8 cm at 150 DAP. In the intraspecific cross, the mean length of the ovary of *V. planifolia* on the day of pollination was

V. tahitensis

x

V. planifolia

Interspecific hybrid pods
(V.t x V.p)

V. planifolia

x

V. planifolia

Intraspecific hybrid pods
(V.p x V.p)

Plate 5.Interspecific *(V.t x V.p)* and intraspecific *(V.p x V.p)* hybrid pods

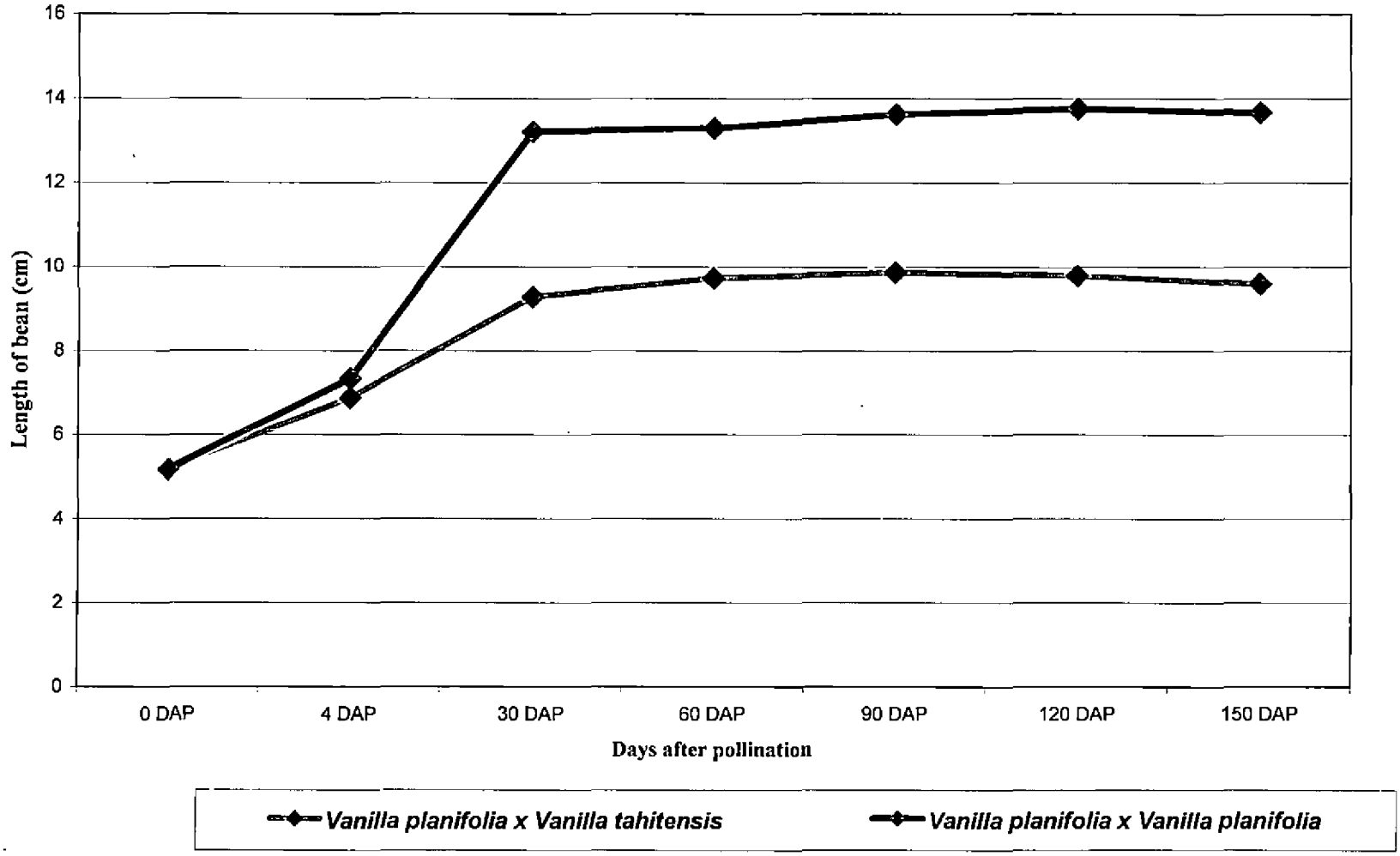
Table: 16 Pod set percentage in different parental combinations

Sl no:	Genotype	Total crossed	Pod set	Pod set (%)
1	*V. planifolia* × *V. tahitensis* (*a 425*)	20	14	70
2	*a 82*× *a 94*	24	23	95.8
3	*a 94* × *a 82*	32	31	96.9
4	*a 428* × *a 94*	17	16	94.1
5	*a 425* × *a 94*	18	16	88.9
6	*a 94* × *a 425*	11	1	9.1
7	*a 428* × *a 82*	10	10	100
8	*a 82* × *vv 97/84*	15	15	100
9	*a 82* × *a 428*	15	9	60
10	*a 94* × *a 428*	7	1	14.3
11	*a 82* × *a 425*	3	2	66.7

Table 17. Pod development as length increment in hybrid beans at various time intervals

Genotype	Mean length of bean (cm)						
	0 DAP	4 DAP	30 DAP	60 DAP	90 DAP	120 DAP	150 DAP
V. planifolia x *V. tahitensis*	5.18	6.88	9.28	9.73	9.79	9.87	9.8
V. planifolia x *V. planifolia*	5.18	7.34	13.21	13.3	13.63	13.76	13.67

Fig 1. Pod development as length increment in hybrid beans at various time intervals



5.18 cm and it increased to 7.34 cm within 4 DAP with pollen grains of *V. planifolia*. It attained a length of 13.21 cm within a period of 1 month. After 1 month, length increment was meagre and it attained a maximum of 13.76 cm at 120 DAP. The length was reduced to 13.67 cm at 150 DAP. The pod development pattern was similar in both inter and intraspecific crosses (Table 17 and Fig 1).

4.5.3. Embryo culture studies

4.5.3.1. Embryo germination

The seeds of hybrid pods were germinated in the media half strength MS + 3 per cent sucrose + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BAP. The cultures were incubated at 25 $\pm$ 2^{0}C under a photoperiod of 16 hours with a light intensity of 1000 lux. Embryo germination from interspecific and intraspecific hybrid pods are shown in Plate 6. Green and creamy white protocorms were produced initially on seed germination (Plate 7). The time taken for embryo germination varied significantly with the age of pods (Table 18 & Fig 2). Seeds from pods (*a 82* x *a 94*) of 6 weeks maturity germinated within 34 days whereas seeds from 28 - 36 week old pods failed to germinate. In the interspecific hybrid pods (*V. t* x *V. p*) of 18 weeks maturity, seeds germinated in 42 days. Seeds from hybrid pods (*V. p* x *V. t*) of 14 and 20 weeks maturity germinated in 35 and 51 days respectively. The protocorms produced later developed shoots (Plate 8). The time recorded from embryo germination to shoot differentiation was 52 - 55 days.

Although many seeds started to germinate and formed small protocorms, some turned brown and died. The intensity of seed germination varied with genotype and age of the pods (Table 18 & Fig 2). Seeds from pods (*a 82* x *a 94*) of 6 weeks maturity showed high (+++) intensity of germination whereas seeds from 28 - 36 week old pods showed no germination. The interspecific hybrid pods, *V.t* x *V. p* of 18 weeks maturity showed moderate (++) intensity of germination whereas *V. p* x *V. t* pods of 14 and 20 weeks maturity yielded only

Embryo germination from a 82 x a 94 *(V.p x V.p)* hybrid pod

Embryo germination from
V.t x V.p hybrid pod

Embryo germination from
V.p x V.t hybrid pod

Plate 6. Embryo germination from hybrid pods of vanilla

Table: 18 Effect of genotype and maturity of bean on *in vitro* germination of hybrid embryo

Genotype	Age of pod	Time taken for germination	Intensity of germination**
a 82 x *a 94*	6 WAP*	34 days	+++
V. planifolia x *V. tahitensis*	14 WAP	35 days	+
a 425 x *a 94*	16 WAP	49 days	++
V. tahitensis x *V. planifolia*	18 WAP	42 days	++
vv 97/84 x *a 94*	20 WAP	49 days	++
V. planifolia x *V. tahitensis*	20 WAP	51 days	+
a 94 x *a 82*	20 WAP	49 days	+
a 425 x *a 94*	22 WAP	49 days	+
vv 97/84 x *a 94*	24 WAP	47 days	++
vv 97/84 x *a 94*	28 WAP	-	-
V. tahitensis	28 WAP	-	-
V. tahitensis	36 WAP	-	-

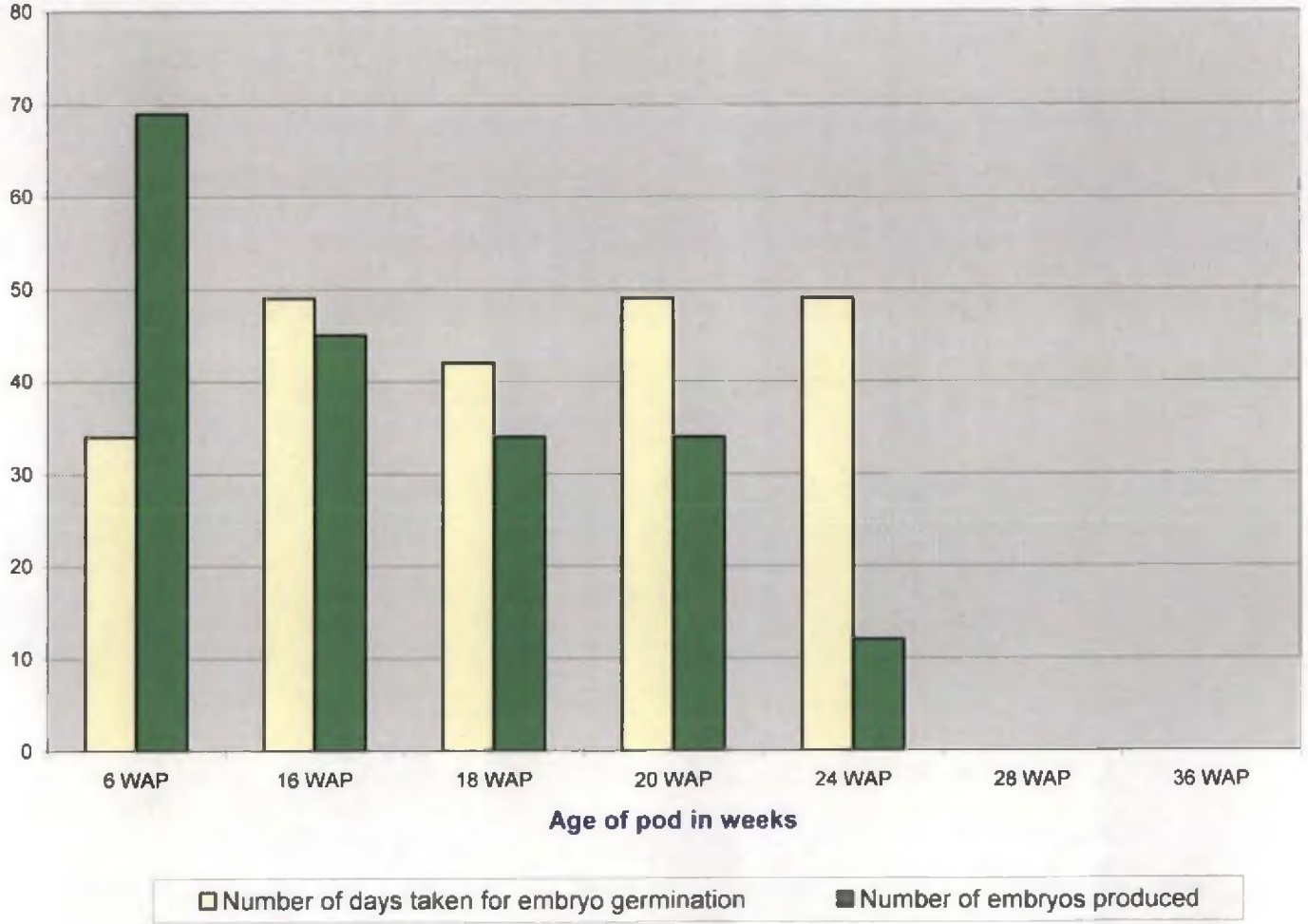
Basal medium: half strength MS + 1.0 mg l^{-1} NAA+ 1.0 mg l^{-1} BAP

* WAP: Weeks after pollination

** + : 0-30 embryos, ++ : 30-60 embryos, +++ : Above 60 embryos

(Scoring 4 months after inoculation)

Fig 2. Effect of maturity of pod on time taken for germination and intensity of germination



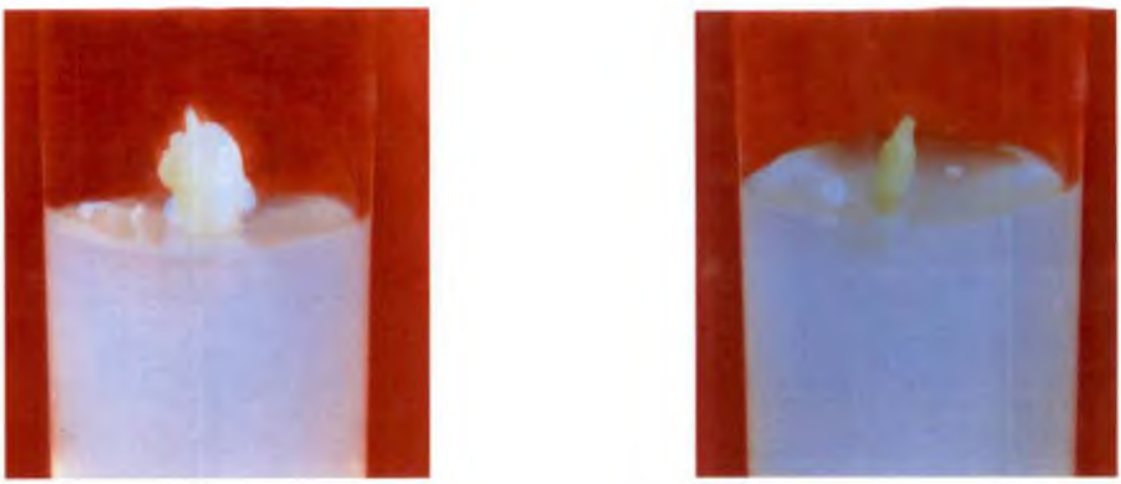


Plate 7. Protocorm formation from embryo in vanilla

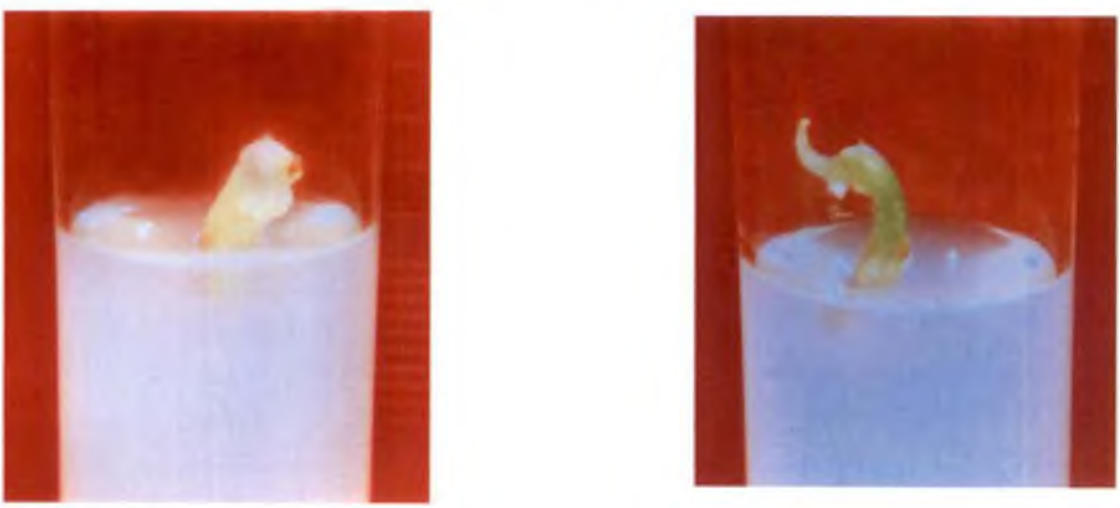


Plate 8. Shoot differentiation from protocorms in vanilla

very few seeds and these seeds showed less (+) intensity of germination. Seeds from *vv 97/84* x *a 94* hybrid pods of 20 and 24 weeks maturity showed moderate (++) germination whereas seeds from *a 425* x *a 94* pods of 16 and 22 weeks maturity showed moderate (++) and less (+) intensity of germination respectively.

4.5.4. Multiple shoot production in hybrid progenies

The multiple shoots were produced from each seedling in the media ½ MS + 3 per cent sucrose + 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BAP (Mary *et al.*, 1999) and is shown in Plate 9. Observations were made on number of shoots or buds per culture and length of shoots in cm for different genotypes after a culture period of 8 - 9 weeks.

The data regarding multiple shoot production is given in Table 19. Variation in the number of shoots/buds produced per progeny was observed for different genotypes. Multiple shoots produced varied from 3.8 to 5.8 in the progenies of *a 94* x *a 82* and *a 82* x *a 94* crosses. In the interspecific cross it was 4.9 per progeny.

The mean shoot length varied from 1.55 to 1.72 cm in progenies of genotypes *a 425* x *a 94* and *a 82* x *a 94* crosses. In the interspecific hybrids it was 1.6 cm.

4.5.5. Elongation and rooting of progenies

Observations were recorded on length of shoots, number of leaves, length of roots and number of roots (Table 20). The elongation and rooting of the proliferated shoots was obtained in the media half srength MS + 3 per cent sucrose + 1.0 mg l^{-1} IAA + 0.2 mg l^{-1} BAP within a culture period of 7 - 8 weeks under light intensity of 1000 lux for a photoperiod of 16 hours (Plate 10).

Table:19 Multiplication rate of hybrid progenies

Sl no:	Genotype	Mean no: of shoots/buds per progeny ± SD	Mean shoot length of progeny (cm) ± SD
1	*V.t* x *V.p*	4.9 ± 1.77	1.60 ± 0.82
2	a 82 x a 94	5.8 ± 2.35	1.72 ± 0.44
3	a 94 x a 82	3.8 ± 1.30	1.67 ± 0.74
4	a 425 x a 94	5.4 ± 2.74	1.55 ± 0.52
5	vv 97/84 x a 94	5.2 ± 2.4	1.68 ±0.49

Basal medium- Half strengh MS + 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} IAA, culture period 8–9 weeks

Table:20 Elongation and rooting rate of hybrid progenies

Sl no:	Genotype	Mean length of shoots (cm) ± SD	Mean no: of leaves or nodes ± SD	Mean length of roots (cm) ± SD	Mean no: of roots ± SD
1	*V.t* x *V.p*	4.27 ± 1.0	4.73 ± 0.91	2.75 ± 1.91	3.91 ± 1.22
2	a 82 x a 94	4.85 ± 1.85	5.41 ± 1.77	3.67 ± 2.47	3.29 ± 1.46
3	a 94 x a 82	5.06 ± 1.35	5.33 ± 1.12	3.40 ± 2.39	2.78 ± 0.97
4	a 425 x a 94	5.25 ± 1.63	4.73 ± 1.28	3.23 ± 1.8	2.66 ± 1.11
5	vv 97/84 x a 94	5.55 ± 1.23	6.00 ± 1.34	3.61 ± 2.48	1.91 ± 0.83

Basal medium- Half strength MS + 0.2 mg l^{-1} BAP + 1.0 mg l^{-1} IAA, culture period 7– 8 weeks

Plate 9. Hybrid seedlings in the multiple shoot inducing medium

Plate 10. Hybrid seedlings in the elongation and rooting medium

The mean shoot length varied from 4.27 to 5.55 cm in progenies of *V. t* x *V. p* and *vv 97/84* x *a 94* crosses. In the interspecific hybrids minimum shoot length was observed.

The mean number of leaves varied from 4.73 in progenies of *V. t* x *V. p* and *a 425* x *a 94* to 6.0 in progenies of *vv 97/84 x a 94* crosses.

The mean root length varied from 2.75 to 3.67 cm in progenies of *V. t* x *V. p* and *a 82* x *a 94* crosses. In the interspecific hybrids minimum root length was observed.

The mean number of roots varied from 1.91 to 3.91 in progenies of *vv 97/84* x *a 94* and *V. t* x *V. p* crosses. In the interspecific hybrids maximum number of roots was observed.

4.5.6. Plant out success

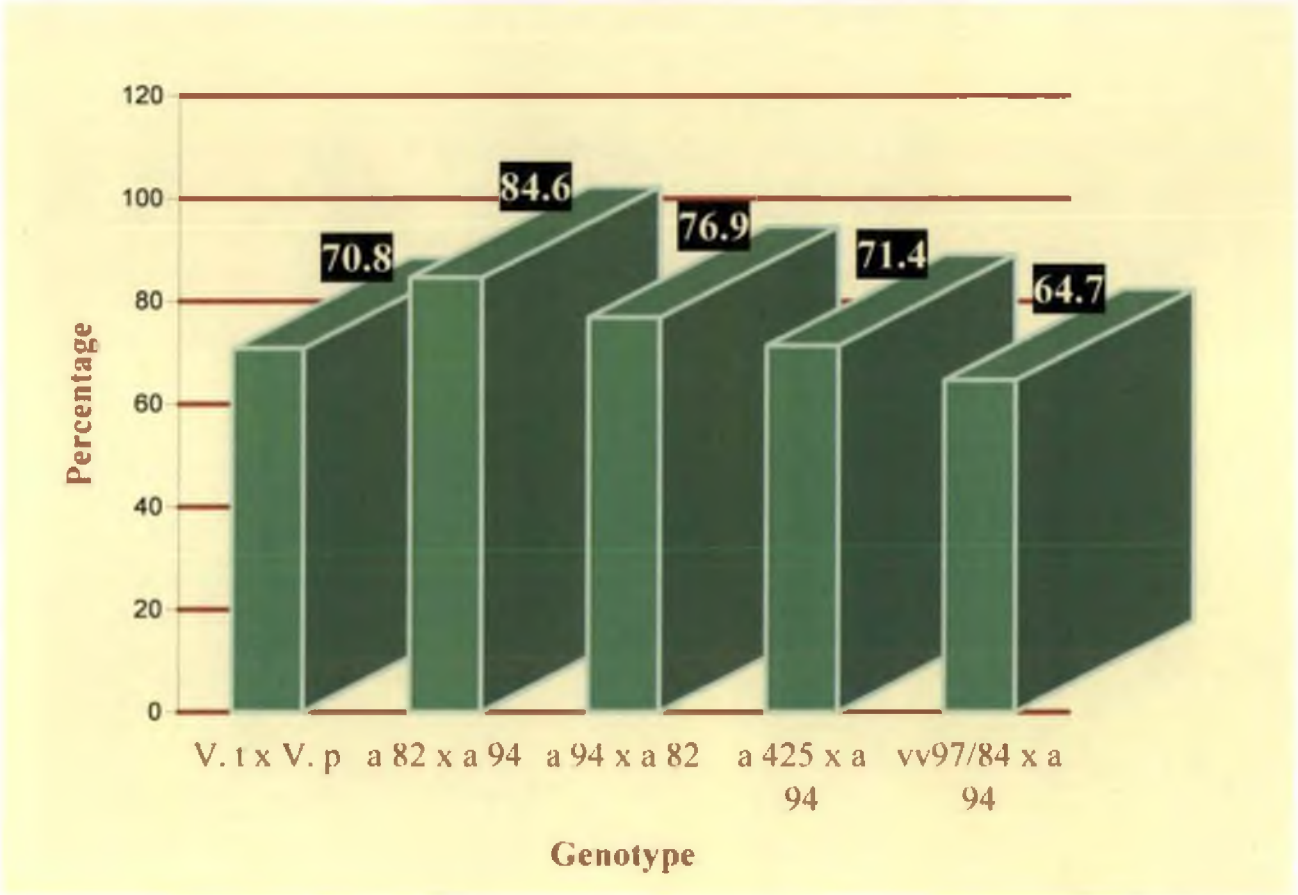
The seedlings having 3 to 5 leaves and 1 to 3 roots were planted out in the net house. The planting out of hybrid seedlings was done in polybags (15 x 10 cm) containing potting mixture (1 soil:1 sand:1 organic manure). Fungicidal dip and drenching gave moderate protection to the seedlings. Plants got established in the net house with 75 to 80 per cent shade provided with mist. Aftercare details of the planted out seedlings are given in section *3.2.5.3.4.*

The percentage of plant out success for different parental combinations varied from 64.7 to 84.6 (Table 21 & Fig 3). In the interspecific cross (*V.t* x *V. p*), plant out success recorded was 70.8 per cent. Among the intraspecific crosses, *a 82* x *a 94* genotype recorded maximum (84.6%) and *vv 97/84* x *a 94* recorded minimum (64.7%) plant out success.

Table: 21 Plant out success under controlled conditions

Sl no:	Genotype	Total no: of hardened plants	No: of plants survived	Percentage of plant out success
1	*V.t* x *V.p*	24	17	70.8
2	*a 82* x *a 94*	26	22	84.6
3	*a 94* x *a 82*	13	10	76.9
4	*a 425* x *a 94*	21	15	84.2
5	*vv97/84* x *a 94*	17	11	64.7

Fig 3. Plant out success



4.5.7.Curing of *V. tahitensis*

The curing of tahiti vanilla was done to estimate the vanillin content. The proportion of cured beans to fresh beans after slow drying was 1:3.5. The cured beans were dark brown in colour with aroma. Vanillin content estimated as per AOAC method ranged between 0.43 - 0.52 per cent.

4.6. VARIABILITY ANALYSIS IN HYBRID PROGENIES

4.6.1. Variability in foliar morphology

Hybrid seedlings obtained from same and different parental combinations were evaluated for different foliar characters. Leaf characters studied were total number of leaves, leaf length, leaf breadth, leaf shape and leaf colour and is shown in Table 22.

4.6.1.1. Number of leaves

Hybrid seedlings from same and different parental combinations varied in mean number of leaves produced. It ranged from 5.0 to 9.3 between different parental combinations. The mean number of leaves for the hybrid seedlings derived from *V. t* x *V. p* cross varied from 5 in VH 386 and VH 381 to 8 in VH 383 hybrids. In the intraspecific cross *a 82* x *a 94* it varied from 6.33 to 8.0 in VH 18 and VH 30 hybrids. The mean leaf length in the cross *a 94* x *a 82* varied from 5.0 in VH 574 and VH 577 to 5.5 in VH 571 hybrids. In the cross *a 425* x *a 94*, it varied from 5.0 to 5.5 in VH 207 and VH 197 hybrids. The mean number of leaves in the cross *vv 97/84* x *a 94* varied from 7.0 to 9.3 in VH 26 and VH 25 hybrids.

4.6.1.2. Leaf length

Hybrid seedlings from same and different parental combinations varied in mean leaf length. It ranged from 1.8 to 4.5 cm in VH 381 (*V. t* x *V. p*) and VH 207 (*a 425* x *a 94*) hybrids between different parental combinations. The mean

Plate 11. Cured beans of *Vanilla tahitensis*

leaf length for the hybrid seedlings derived from *V. t* x *V. p* cross varied from 1.8 to 4.4 cm in VH 381 and VH 384 hybrids. In the intraspecific cross *a 82* x *a 94* it varied from 2.64 to 3.41 cm in VH 18 and VH 9 hybrids. The mean leaf length in the cross *a 94* x *a 82* varied from 2.5 to 3.6 cm in VH 571 and VH 574 hybrids. In the cross *a 425* x *a 94*, it varied from 2.7 to 4.5 cm in VH 208 and VH 207 hybrids. The mean leaf length in the cross *vv 97/84* x *a 94* varied from 3.65 to 4.3 cm in VH 17 and VH 26 hybrids.

4.6.1.3. Leaf width

Hybrid seedlings from same and different parental combinations varied in mean leaf width. It ranged from 0.6 to 1.55 cm in VH 381 (*V. t* x *V. p*) and VH 17 (*vv 97/84* x *a 94*) hybrids between different parental combinations. The mean leaf width for the hybrid seedlings derived from *V. t* x *V. p* cross varied from 0.6 to 1.15 cm in VH 381 and VH 383 hybrids. In the intraspecific cross *a 82* x *a 94* it varied from 1.22 to 1.38 cm in VH 18 and VH 9 hybrids. The mean leaf length in the cross *a 94* x *a 82* varied from 0.85 to 1.2 cm in VH 571 and VH 577 hybrids. In the cross *a 425* x *a 94*, it varied from 0.85 to 0.97 cm in VH 207 and VH 208 hybrids. The mean leaf length in the cross *vv 97/84* x *a 94* varied from 0.87 to 1.55 cm in VH 25 and VH 17 hybrids.

4.6.1.4. Leaf shape

The leaf shapes varied remarkably in hybrid seedlings from the same and different parental combinations. It varied from oblong - elliptic to linear lanceolate between different parental combinations. The leaf shapes for the hybrid seedlings derived from *V. t* x *V. p* were broadly ovate in VH 381, oblong-lanceolate in VH 383 and VH 387, lanceolate in VH 384, VH 386 and VH 387 and linear-lanceolate in VH 386 hybrids. In the intraspecific cross *a 82* x *a 94* and *a 425* x *a 94* oblong-elliptic leaf shape was observed in all hybrids. The leaf shapes in the cross *a 94* x *a 82* were lanceolate in VH 574 and oblong - elliptic in VH 571 and VH 577 hybrids. In the cross *vv 97/84* x *a 94*, leaf shapes observed

Table: 22 Variability in foliar morphology of hybrid progenies three months after plant out

Genotype	Hybrid no.	Mean no: of leaves	Mean leaf length (cm)	Mean leaf width (cm)	Leaf shape	Leaf colour
V.t x *V.p*	VH 381	5.0	1.8	0.6	Broadly ovate	Green
	VH 383	8.0	3.45	1.15	Oblong -Lanceolate	Green
	VH 384	6.3	4.4	1.2	Lanceolate	Dark Green
	VH 386	5.0	3.23	0.83	Linear-Lanceolate, Lanceolate	Green
	VH 387	5.2	3.2	0.92	Oblong -Lanceolate Lanceolate	Green
a 82 x *a 94*	VH 9	7.89	3.41	1.38	Oblong-eliiptic	Green
	VH 18	6.33	2.64	1.22	Oblong-elliptic	Green
	VH 30	8.0	3.1	1.25	Oblong-elliptic	Green
a 94 x *a 82*	VH 571	5.5	2.5	0.85	Oblong –elliptic	Green
	VH 574	5.0	3.6	1.1	Lanceolate	Green
	VH 577	5.0	3.1	1.2	Oblong –elliptic	Green
a 425 x *a 94*	VH 197	5.5	3.2	0.95	Oblong – elliptic	Green
	VH 207	5.0	4.5	0.85	Oblong – elliptic	Green
	VH 208	5.1	2.7	0.97	Oblong – elliptic	Green
vv 97/84 x *a 94*	VH 17	8.0	3.65	1.55	Oblong – elliptic	Green
	VH 25	9.3	3.87	0.87	Oblong -Lanceolate	Green
	VH 26	7.0	4.3	1.3	Oblong -Lanceolate	Green

were oblong - elliptic in VH 17 and oblong - lanceolate in VH 25 and VH 26 hybrids.

4.6.1.5. Leaf colour

There was not much variation in leaf colour for hybrid progenies of same and different parental combinations. All hybrid seedlings showed green leaf colour except VH 384 hybrid of *V. t* x *V. p* cross which showed dark green leaves.

4.6.2. Variability in cauline morphology

Hybrid seedlings obtained from same and different parental combinations were evaluated for different stem characters as shown in Table 23. Stem characters studied were plant height, internodal length, stem type and number of aerial roots from single node.

4.6.2.1. Plant height

Hybrid seedlings from same and different parental combinations varied in plant height. It ranged from 5.0 to 13.9 cm in VH 574 (*a 94* x *a 82*) and VH 383 (*V. t* x *V. p*) hybrids between different parental combinations. The mean plant height for the hybrid seedlings derived from *V. t* x *V. p* cross varied from 5.1 to 13.9 cm in VH 381 and VH 383 hybrids. In the intraspecific cross *a 82* x *a 94* it varied from 9.12 to 11.69 cm in VH 18 and VH 9 hybrids. The mean plant height in the cross *a 94* x *a 82* varied from 5.0 to 7.7 cm in VH 574 and VH 577 hybrids. In the cross *a 425* x *a 94*, it varied from 5.7 to 9.1 cm in VH 207 and VH 197 hybrids. The mean leaf length in the cross *vv 97/84* x *a 94* varied from 9.3 to 11.4 cm in VH 26 and VH 25 hybrids.

4.6.2.2. Internodal length

Hybrid seedlings from same and different parental combinations varied in internodal length. It ranged from 0.8 to 2.9 cm in VH 381 (*V. t* x *V. p*) and VH

197 (*a 425 x a 94*) hybrids between different parental combinations. The mean internodal length for the hybrid seedlings derived from *V. t* x *V. p* cross varied from 0.8 to 1.95 cm in VH 381 and VH 383 hybrids. In the intraspecific cross *a 82* x *a 94*, it varied from 1.55 to 2.1 cm in VH 30 and VH 9 hybrids. The mean internodal length in the cross *a 94* x *a 82* varied from 1.1 to 1.4 cm in VH 577 and VH 574 hybrids. In the cross *a 425* x *a 94*, it varied from 1.2 to 2.9 cm in VH 207 and VH 197 hybrids. The mean internodal length in the cross *vv 97/84* x *a 94* varied from 1.0 to 1.73 cm in VH 26 and VH 25 hybrids.

4.6.2.3. Stem type

Simple and branched stem type was observed in hybrid seedlings of same and different parental combinations. The stem type for the hybrid seedlings derived from *V. t* x *V. p* cross was simple in VH 381, 383, 384 and 383 hybrids whereas simple and branched in VH 387 hybrid. In the intraspecific cross *a 82* x *a 94* simple and branched stem was observed in VH 18 hybrid. The stem type in the crosses *a 94* x *a 82* and *a 425* x *a 94* was simple for all hybrids. Simple and branched stem type was observed in the cross *vv 97/84* x *a 94* for VH 17 hybrid.

4.6.2.4. Number of aerial roots per node

Striking difference was noted in the number of aerial roots produced from single node in hybrid seedlings from same and different parental combinations. Some hybrids produced two aerial roots from a node as against the normal single aerial root. This character was observed in VH 383 (*V. t* x *V. p*), VH 9, VH 18 (*a 82* x *a 94*) and VH 208 (*a 425* x *a 94*). But the clonal progenies of the aforesaid hybrids showed single as well as double aerial roots.

4.6.2.5. Plant growth as vigorous/less vigorous

Hybrid seedlings from same and different parental combinations varied in plant growth. It varied from less vigorous to more vigorous between different parental combinations. In the parental combination of *V. t* x *V. p*, the hybrids VH

Table:23 Variability in cauline morphology of hybrid progenies three months after plant out

Genotype	Hybrid no.	Plant ht (cm)	Internodal length (cm)	*Plant growth	Stem type	No: of aerial roots/node
V.t x *V.p*	VH 381	5.1	0.8	+	Simple	1
	VH 383	13.9	1.95	++	Simple	1 and 2
	VH 384	11.1	1.93	++	Simple	1
	VH 386	5.6	1.23	+	Simple	1
	VH 387	5.3	0.84	+	Simple, branched	1
a 82 x *a 94*	VH 9	11.69	2.1	+++	Simple	1 and 2
	VH 18	9.12	1.85	++	Simple, branched	1 and 2
	VH 30	9.85	1.55	++	Simple	1
a 94 x *a 82*	VH 571	5.9	1.2	+	Simple	1
	VH 574	5.0	1.4	+	Simple	1
	VH 577	7.7	1.1	+	Simple	1
a 425 x *a 94*	VH 197	9.1	2.9	++	Simple	1
	VH 207	5.7	1.2	+	Simple	1
	VH 208	7.9	1.83	+	Simple	1 and 2
vv 97/84 x *a 94*	VH 17	10.8	1.7	++	Simple, branched	1
	VH 25	11.4	1.73	+++	Simple	1
	VH 26	9.3	1.0	++	Simple	1

*Scoring- More vigorous (+++), Vigorous (++), less vigorous (+)

383 and VH 384 were vigorous compared to other hybrids VH 381, VH 386 and VH 387 that were less vigorous. All hybrids derived in *a 82* x *a 94* and *vv 97/84* x *a 94* crosses were vigorous; whereas all hybrids derived in *a 94* x *a 82* cross were less vigorous. In the cross *a 425* x *a 94*, the hybrid VH 197 was vigorous compared to VH 207 and VH 208 hybrids that were less vigorous (Plate 12).

4.7. MEAN VARIABILITY IN FOLIAR MORPHOLOGY OF HYBRID PROGENIES IN DIFFERENT PARENTAL COMBINATIONS

The mean number of leaves in seedlings of different parental combinations ranged from 5.1 to 8.4 in *a 425* x *a 94* and *vv 97/84* x *a 94* crosses. In the interspecific cross *V. t* x *V. p*, it was 5.8 (Table 24).

The mean leaf length in seedlings of different parental combinations ranged from 2.96 to 3.81 cm in *a 94* x *a 82* and *a 82* x *a 94* crosses. In the interspecific cross *V. t* x *V. p*, it was 3.47 cm (Table 24).

The mean leaf width in seedlings of different parental combinations ranged from 0.95 to 1.39 cm in *a 425* x *a 94* in *a 82* x *a 94* crosses. In the interspecific cross *V. t* x *V. p*, it was 0.99 cm (Table 24).

Leaf shape variations in hybrid seedlings of various parental combinations are given in Table 24. In the interspecific cross of *V. t* x *V. p* the variations in leaf shape were remarkable and four leaf shapes broadly ovate, oblong-lanceolate, lanceolate and linear-lanceolate were observed in the progenies as against narrow lanceolate in *V. t* parent and oblong elliptic in *V. p* parent (Plate 13). The progenies of the intraspecific crosses also showed variation with respect to leaf shape. In the progenies of *vv 97/84* x *a 94* two leaf shapes oblong-elliptic and oblong-lanceolate were observed as against oblong elliptic leaf shape in parents (Table 24). In the cross of *a 94 x a 82*, the progenies showed two leaf shapes oblong - elliptic and lanceolate as against oblong elliptic leaf shape in parents

Plate 12. Variability in growthof hybrid progenies in vanilla

Table:24 Mean variability in foliar morphology of hybrid seedlings of different parental combinations

Genotype	Mean no. of leaves ± SD	Mean leaf length(cm) ± SD	Mean leaf breadth(cm) ± SD	Leaf shape	Leaf colour
V. t x *V. p*	5.8 ± 1.37	3.47 ± 0.95	0.99 ± 0.24	broadly ovate, oblong-lanceolate, lanceolate & linear-lanceolate	Green & dark green
a 82 x *a 94*	7.4 ± 1.97	3.81 ± 1.09	1.39 ± 0.41	oblong–elliptic	Green
a 94 x *a 82*	5.2 ± 0.45	2.96 ± 0.47	0.96 ± 0.18	oblong-elliptic & lanceolate	Green
a 425 x *a 94*	5.1 ± 0.86	3.0 ± 1.1	0.95 ± 0.37	oblong-elliptic	Green
w 97/84 x *a 94*	8.4 ± 1.42	3.78 ± 0.76	1.14 ± 0.34	oblong-elliptic & oblong-lanceolate	Green

Plate 13. Variation in leaf shape of hybrid progenies

1. Broadly Ovate 2. Oblong - lanceolate 3. Lanceolate 4. Linear lanceolate

Plate 14. Variation in leaf size
and leaf colour of hybrid progenies

(Table 24). The progenies of *a 82* x *a 94* and *a 425* x *a 94* produced only oblong - elliptic shaped leaves.

All hybrid progenies of all intraspecific crosses produced green coloured leaves; whereas the interspecific cross *V. t* x *V. p* showed green and dark green leaf colour (Table 24 & Plate 14).

The parental combination *vv 97/84* x *a 94* produced hybrids with maximum mean number of leaves (8.4) followed by the cross *a 82 x a 94* (7.4). The parental combination *a 82* x *a 94* produced hybrids with maximum mean leaf length (3.81 cm) and leaf breadth (1.39 cm) followed by the cross *vv 97/84* x *a 94* with leaf length (3.78 cm) and leaf breadth (1.14 cm).

4.8. MEAN VARIABILITY IN CAULINE MORPHOLOGY OF HYBRID PROGENIES IN DIFFERENT PARENTAL COMBINATIONS

The mean plant height in seedlings of different parental combinations ranged from 5.7 to 10.57 cm in *a 94* x *a 82* and *a 82* x *a 94* cross. In the interspecific cross, *V. t* x *V. p* it was 7.52 cm (Table 25).

The mean internodal length in seedlings of different parental combinations ranged from 1.12 to 1.94 cm in *a 94* x *a 82* and *a 82* x *a 94* cross. In the interspecific cross *V. t* x *V. p*, it was 1.35 cm (Table 25 & Plate 15).

Simple and branched stem type was observed in the interspecific cross *V. t* x *V. p* and intraspecific crosses *a 82* x *a 94* and *vv 97/84* x *a 94*. Simple stem type was observed in *a 425* x *a 94* and *a 94* x *a 82* crosses (Plate 16A & B).

Single and double aerial roots were produced from single node in the interspecific cross *V. t* x *V. p* and intraspecific crosses *a 82* x *a 94* and *a 425* x *a 94* (Plate 17). The single root origin was observed in the progenies of the crosses *vv 97/84* x *a 94* and *a 94* x *a 82*.

Table: 25 Mean variability in cauline morphology of hybrid seedlings of different parental combinations

Genotype	Mean plant height(cm) ± SD	Mean internodal length(cm) ± SD	Stem type	No. of aerial roots from single node ± SD
V. t x *V. p*	7.52 ± 4.3	1.35 ± 0.68	Simple, branched	1 and 2
a 82 x *a 94*	10.57 ± 5.36	1.94 ± 0.53	Simple, branched	1 and 2
a 94 x *a 82*	5.7 ± 1.37	1.12 ± 0.22	Simple	1
a 425 x *a 94*	7.7 ± 3.7	1.89 ± 0.9	Simple	1
vv 97/84 x *a 94*	10.1 ± 3.1	1.56 ± 0.42	Simple, branched	1 and 2

Plate 15. Variation in internodal length of hybrid progenies

Plate 16A. Branched stem type in hybrid progenies

Plate 16B. Branched stem type in hybrid progenies

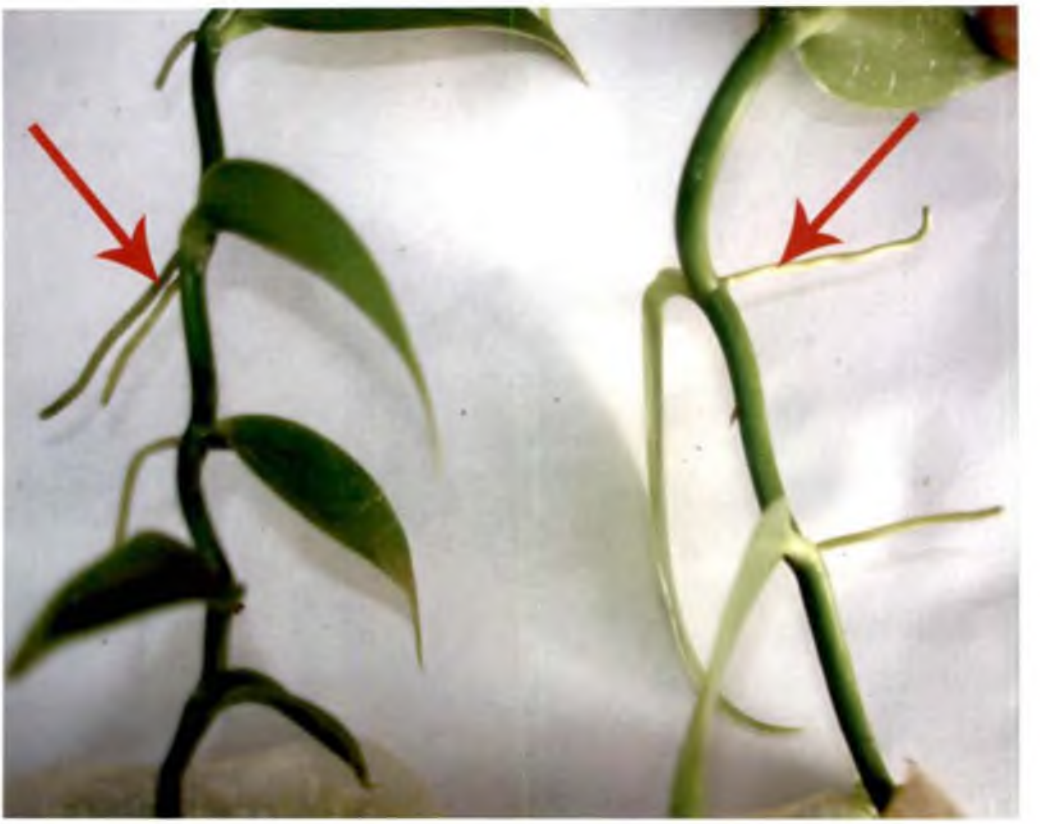


Plate 17. Single and double aerial roots
produced from single node
of hybrid progenies

The parental combination *a 82* x *a 94* produced hybrids with maximum mean plant height (10.57 cm) followed by the cross *vv 97/84* x *a 94* (10.1 cm).

4.9. MOLECULAR CHARACTERIZATION

Molecular characterisation of parents and progenies was done by RAPD technique.

4.9.1. DNA isolation

The genomic DNA was successfully isolated from tender and fresh leaves of vanilla plants using modified CTAB method of Doyle and Doyle, (1987). There was considerable amount of RNA contamination in the isolated samples.

4.9.1.1. Purification of DNA

The RNAase treatment substantially reduced the RNA contamination. The electrophoresis of the DNA samples after RNase treatment is shown in Plate 18.

Table 26 shows the quantity and quality of DNA extracted from parents and progenies. The quality of DNA was checked by electrophoresis and the presence of an intact discrete band indicated non-degraded RNA free DNA and these were used for RAPD assay.

4.9.2. RAPD assay

4.9.2.1. Genotypes used

Seventeen progenies from the cross between superior clones of *V. planifolia* (*a 82* x *a 94*, *a 94* x *a 82*, *a 425* x *a 94*, *vv 97/84* x *a 94*) and seven progenies from the cross between *V. tahitensis and V. planifolia* were used for molecular characterisation (Table 7).

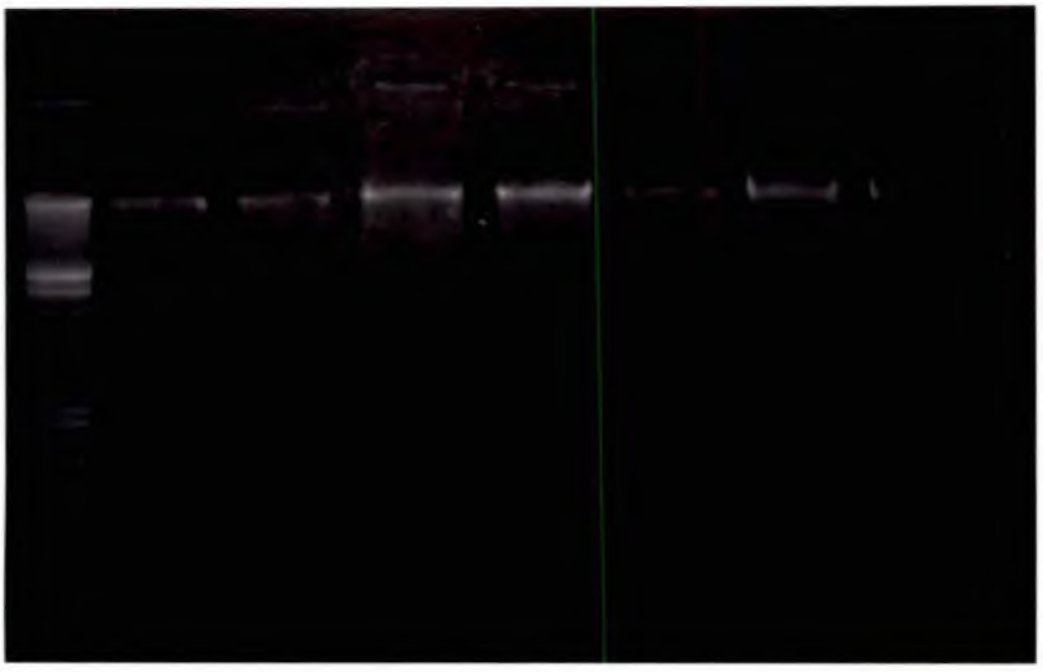


Plate 18: Quality of genomic DNA isolated from vanilla leaves

Table: 26 Quality and quantity of DNA extracted from parents and progenies

Sl no:	Plant code	Absorbance		260/280 ratio	Quantity (µg/ml)	Quality
		at 260 nm	at 280 nm			
1	V_1	0.032	0.017	1.88	800	Good
2	V_2	0.020	0.011	1.82	500	Good
3	V_3	0.017	0.009	1.89	425	Good
4	V_4	0.027	0.016	1.70	675	Average
5	V_5	0.023	0.013	1.77	575	Good
6	V_6	0.011	0.006	1.83	275	Good
7	VHI	0.020	0.011	1.82	500	Good
8	VH2	0.009	0.005	1.80	225	Good
9	VH3	0.017	0.009	1.89	425	Good
10	VH4	0.018	0.010	1.80	450	Good
11	VH5	0.015	0.008	1.88	300	Good
12	VH6	0.013	0.007	1.86	325	Good
13	VH7	0.015	0.008	1.88	375	Good
14	VH8	0.023	0.012	1.94	575	Average
15	VH9	0.013	0.007	1.86	325	Good
16	VH10	0.017	0.009	1.89	425	Good
17	VH11	0.016	0.009	1.78	400	Good
18	VH12	0.013	0.007	1.86	325	Good
19	VH13	0.022	0.012	1.83	550	Good
20	VH14	0.019	0.010	1.90	475	Good
21	VH15	0.026	0.014	1.86	650	Good
22	VH16	0.020	0.011	1.82	500	Good
23	VH17	0.019	0.010	1.90	475	Good
24	VH18	0.013	0.007	1.86	325	Good
25	VH19	0.020	0.011	1.82	500	Good
26	VH20	0.015	0.008	1.83	375	Good
27	VH21	0.033	0.018	1.83	825	Good
28	VH22	0.017	0.009	1.89	425	Good
29	VH23	0.022	0.012	1.92	575	Average
30	VH24	0.009	0.005	1.80	225	Good

4.9.2.2. Screening of random primers

. The amplification pattern produced for thirty primers in OPF, OPP and OPE series is presented in Table 27. The reaction mixture was found optimum for perfect amplification. The thermal cycle followed was ideal and gave good amplification. Six random primers that gave very good amplification were selected to detect polymorphism among the twenty four progenies.

4.9.2.2.1. OPF series

The amplification pattern produced for ten primers in OPF series is shown in Plate 19. The number of bands ranged between 0 and 12. Out of the ten primers screened OPF 3, OPF 1 and OPF 10 gave good amplification. The primer OPF 3 was selected for further studies.

4.9.2.2.2 OPP series

The number of bands produced ranged from 3 to 12. Out of the ten primers screened OPP 6, OPP 3, OPP 8 and OPP 2 gave good amplification with 12, 9, 8 and 7 number of bands respectively. The primer OPP 6 was selected for further analysis.

4.9.2.2.3. OPE series

The amplification pattern produced for ten primers in OPF series is shown in Plate 20. The number of bands produced by OPE series ranged between 5 and 12. Number of amplification products was also more for these series of primers. Out of these OPE 14, 15, 18 and 19 gave good amplification with 11, 10, 10 and 9 number of bands respectively. Four primers OPE 14, 15, 18 and 19 were selected for further analysis.

Table: 27 Amplification pattern of vanilla genomic DNA with different decamer primers under OPP, OPF and OPE series

Primer code	Primer sequence	No: of bands	Quality of amplification
OPP-1	GTAGCACTCC	2	Poor
OPP-2	TCGGCACGCA	7	Good
OPP-3	CTGATACGCC	9	Good
OPP-4	GTGTCTCAGG	6	Average
OPP-5	CCCCGGTAAC	5	Average
OPP-6	GTGGGCTGAC	12	Very good
OPP-7	GTCCATGCCA	4	Average
OPP-8	ACATCGCCCA	8	Good
OPP-9	GTGGTCCGCA	6	Average
OPP-10	TCCCGCCTAC	3	Poor
OPF-1	ACGGATCCTG	8	Good
OPF-2	GAGGATCCCT	1	Poor
OPF-3	CCTGATCACC	12	Very good
OPF-4	GGTGATCAGG	2	Poor
OPF-5	CCGAATTCCC	6	Average
OPF-6	GGGAATTCGG	1	Poor
OPF-7	CCGATATCCC	5	Average
OPF-8	GGGATATCGG	1	Poor
OPF-9	CCAAGCTTCC	1	Poor
OPF-10	GGAAGCTTGG	10	Very good
OPE-11	GAGTCTCAGG	7	Good
OPE-12	TTATCGCCCC	12	Very good
OPE-13	CCCGATTCGG	1	Poor
OPE-14	TGCGGCTGAG	11	Very good
OPE-15	ACGCACAACC	10	Very good
OPE-16	GGTGACTGTG	6	Average
OPE-17	CTACTGCCGT	5	Average
OPE-18	GGACTGCAGA	10	Very good
OPE-19	ACGGCGTATG	9	Good
OPE-20	AACGGTGACC	8	Good

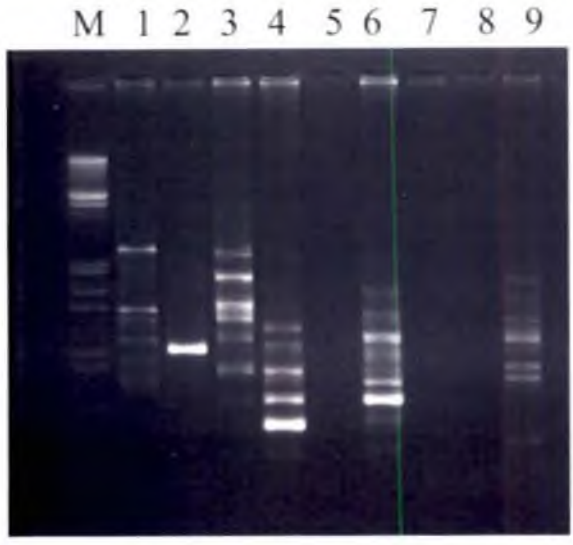


Plate 19: Amplification pattern of vanilla genomic DNA using decamer primers in OPF series

Lanes 1- 9 : Molecular wt. marker
(*Lambda* DNA / *Eco*RI / *Hind*III), OPF 1, OPF 2, OPF 3, OPF 5, OPF 6, OPF 7, OPF8, OPF 9, OPF 10

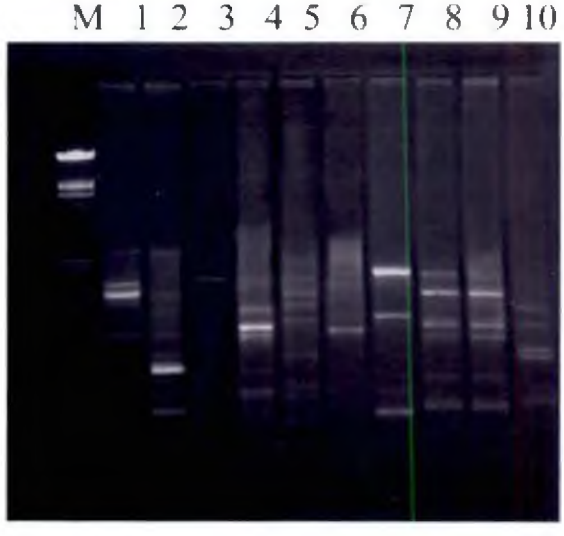


Plate 20:Amplification pattern of vanilla genomic DNA using different decamer primers in OPE series

Lanes 1- 10 : Molecular wt.marker
(*Lambda* DNA + *Eco*RI + *Hind*III), OPE11, OPE 12, OPE 13, OPE 14, OPE 15, OPE 16, OPE 17, OPE 18, OPE 19, OPE 20

4.9.2.3. Screening of parents and hybrid progenies with selected primers

The genomic DNA extracted from the samples was amplified using the selected primers. Tables 28 - 33 shows the results of screening of hybrid progenies and their parents using primers selected from the initial screening.

4.9.2.3.1. Amplicons in V. planifolia and V. tahitensis plants selected as parents using selected primers

Table 28 shows the amplicons obtained in the parents using selected primers. Amplification with the primers OPF 3 and OPE 19 resulted in five amplification products common to all the parents. The primer OPE 14 produced six polymorphic bands in parents V_1 and V_5. The primer OPE 15 produced no polymorphic bands in parents V_2 and V_6 and the primer OPE 18 produced no polymorphic bands in V_6 parent.

4.9.2.3.2. Amplicons in parents and progenies of cross V. t x V. p (V_6 x V_4) *using selected primers*

Amplification with the selected six primers resulted in polymorphic and monomorphic bands in parents and progenies (Table 29). Three monomorphic bands were produced in parents and progenies with primers OPF 3, OPE 15 and OPE 18. The primer OPE 14 produced no monomorphic bands. The number of polymorphic bands produced by the primer OPP 6 varied from two to eight. The maximum number of polymorphic bands was produced in V_6 parent by OPP 6 primer.

4.9.2.3.3. Amplicons in parents and progenies of cross a 82 x a 94 (V_1 x V_2) *using selected primers*

The primers OPE 14 and OPE 19 produced five monomorphic bands in V_1, V_2 parents and their progenies. The primer OPE 15 produced eight polymorphic bands in VH9 hybrid. Table 30 shows the amplicons produced by

selected primers in parents and their progenies of *a 82* x *a 94* cross. The primers OPE 18 and OPE 19 produced no polymorphic band in VH10 and VH11 hybrids.

4.9.2.3.4. Amplicons in parents and progenies of cross vv 97/84 x a 94 (V_5 x V_2) using selected primers

Seven amplification products common to parents and their progenies were produced by the primer OPE 19. The maximum number of polymorphic bands were produced with the primer OPE 14. The primer OPE 18 produc 111 polymorphic band in the progeny VH21. Table 31 shows the number of amplicons produced in parents and their progenies of the cross *vv 97/84* x *a 94* with the selected primers.

4.9.2.3.5. Amplicons in parents and progenies of cross a 94 x a 82 (V_2 x V_1) using selected primers

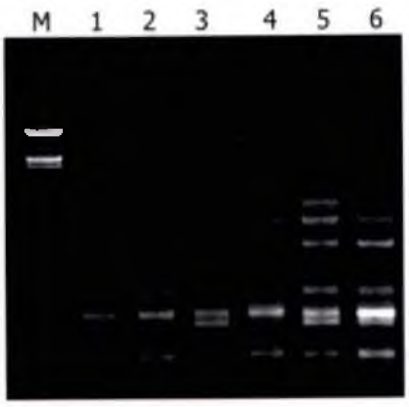
Amplification with the primer OPE 15 resulted in seven amplification products common to parents and their progenies (Table 32). The primers OPP 6 and OPE 18 produced maximum number of polymorphic bands in parents and progenies. The primer OPE 19 produced no polymorphic band VH18 progeny.

4.9.2.3.6. Amplicons in parents and progenies of cross a 425 x a 94 (V_3 x V_2) using selected primers

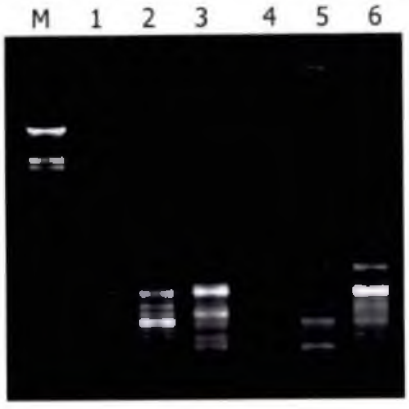
The primer OPE 14 produced six monomorphic bands in parents and progenies. The primer OPF 3 produced maximum number of polymorphic bands. No polymorphic bands were produced in V_3, V_2, VH12 and VH13 genotypes by the primer OPE 14. The data is given in Table 33.

4.9.2.4. Cluster analysis

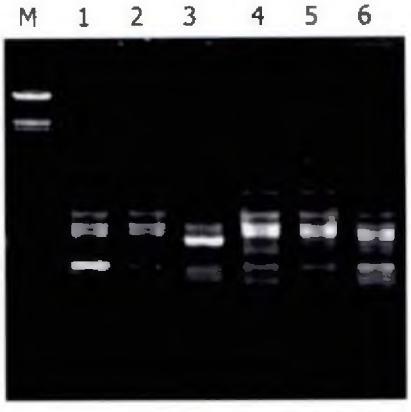
The dendrogram constructed from the pooled data of the RAPD scores



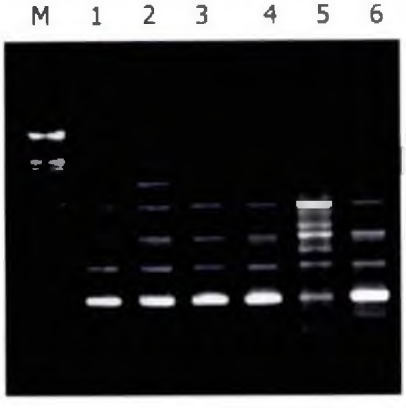
OPF 3



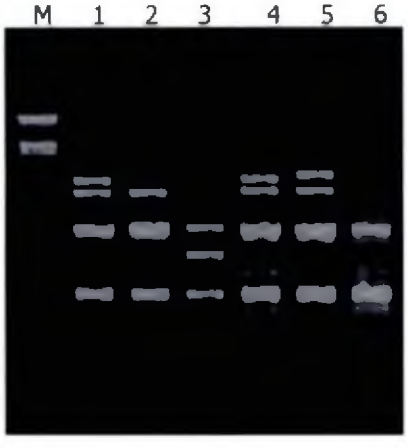
OPP 6



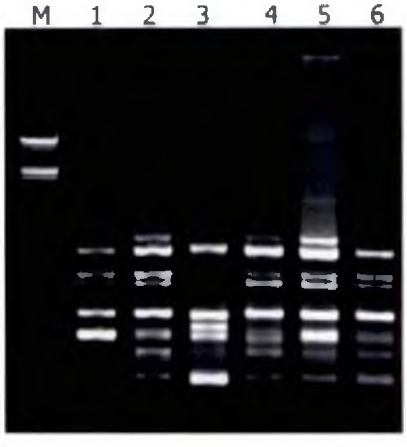
OPE 14



OPE 15



OPE 18



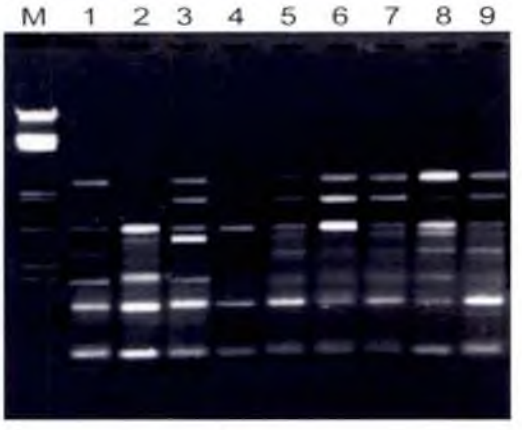
OPE 19

Plate 21. RAPD profile for parents with selected primers

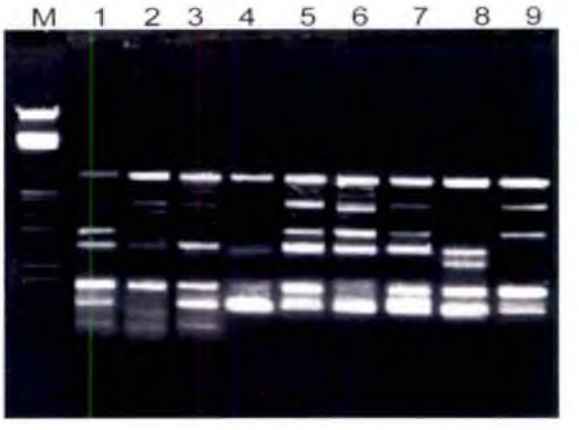
Lanes 1-7: Molecular wt.marker(*Lambda* DNA/ *Eco*RI/*Hind* III), a 425, a 428, *V. t*, vv97/84, a 82, a 94

Table: 28 Amplicons obtained in *V. planifolia* and *V. tahitensis* parent plants using selected primers

Primer	Identity code	No: of polymorphic bands	No: of monomorphic bands
OPF 3	V1	3	5
	V2	3	5
	V3	3	5
	V4	4	5
	V5	2	5
	V6	2	5
OPP 6	V1	3	2
	V2	5	2
	V3	1	2
	V4	5	2
	V5	2	2
	V6	5	2
OPE 14	V1	6	3
	V2	3	3
	V3	5	3
	V4	4	3
	V5	6	3
	V6	4	3
OPE 15	V1	2	4
	V2	0	4
	V3	1	4
	V4	2	4
	V5	2	4
	V6	0	4
OPE 18	V1	3	3
	V2	3	3
	V3	3	3
	V4	2	3
	V5	5	3
	V6	0	3
OPE 19	V1	3	5
	V2	3	5
	V3	4	5
	V4	4	5
	V5	4	5
	V6	3	5



OPF 3



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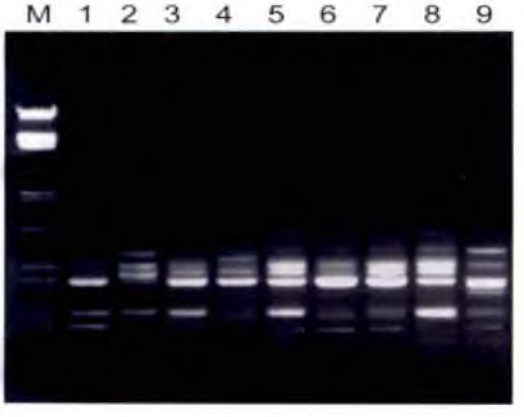
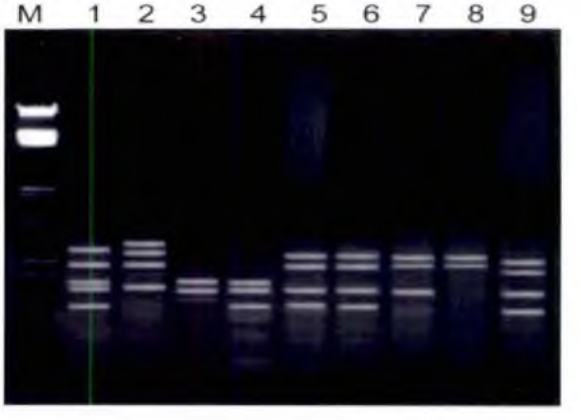
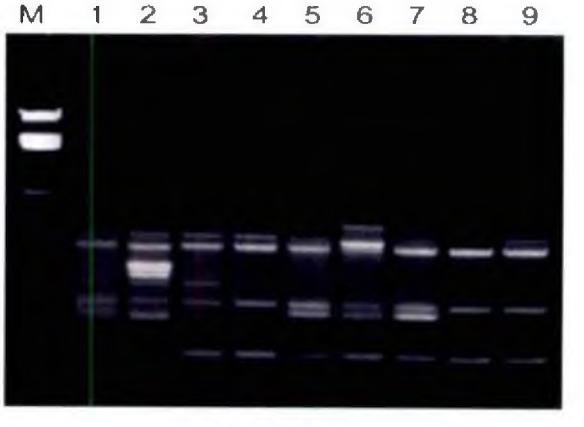


OPE 14



OPE 15

OPE 18



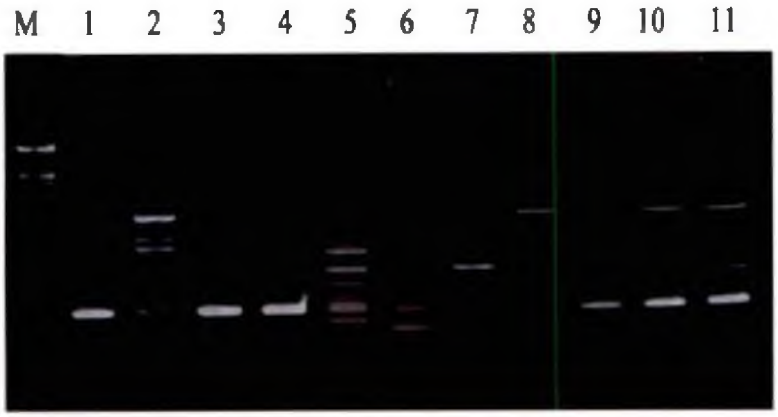
OPE 19

Plate 22: RAPD profile for parents and progenies of cross *V. t x V. p* (a 428) with selected primers

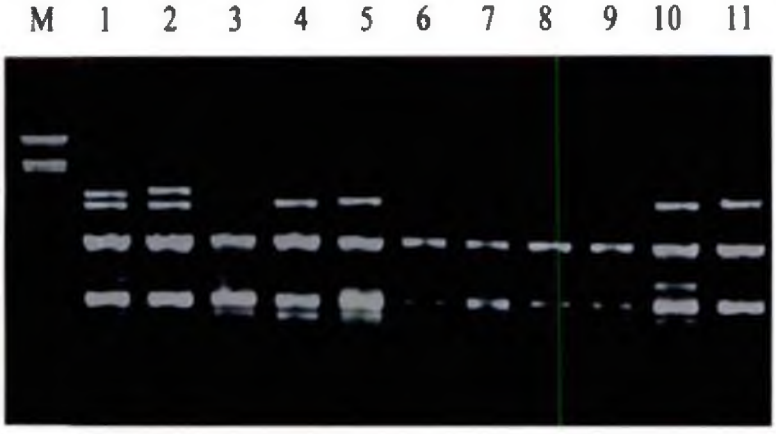
Lanes 1 - 10: Molecular wt.marker(*Lambda* DNA/ *Eco*RI/*Hin*dIII), *V. t* , a 428, VH1, VH2, VH3, VH4, VH5, VH6, VH7

Table: 29 Amplicons obtained in parents and progenies of *V. tahitensis* x *V. planifolia* cross using selected primers

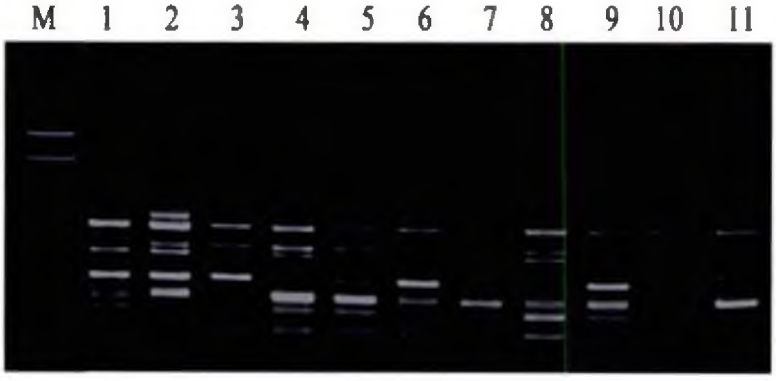
Primer	Identity code	No: of polymorphic bands	No: of monomorphic bands
OPF 3	V6	3	3
	V4	2	3
	VH1	4	3
	VH2	0	3
	VH3	5	3
	VH4	4	3
	VH5	5	3
	VH6	5	3
	VH7	4	3
OPP 6	V6	8	1
	V4	4	1
	VH1	6	1
	VH2	2	1
	VH3	5	1
	VH4	5	1
	VH5	6	1
	VH6	5	1
	VH7	5	1
OPE 14	V6	4	0
	V4	5	0
	VH1	4	0
	VH2	4	0
	VH3	6	0
	VH4	6	0
	VH5	7	0
	VH6	6	0
	VH7	6	0
OPE 15	V6	2	3
	V4	1	3
	VH1	0	3
	VH2	2	3
	VH3	1	3
	VH4	1	3
	VH5	0	3
	VH6	0	3
	VH7	1	3
OPE 18	V6	1	3
	V4	2	3
	VH1	1	3
	VH2	1	3
	VH3	2	3
	VH4	2	3
	VH5	2	3
	VH6	2	3
	VH7	2	3



OPE 15



OPE 18



OPE 19

Plate 23. RAPD profile for a 82, a94, vv97/84 parents and hybrid progenies with selected primers

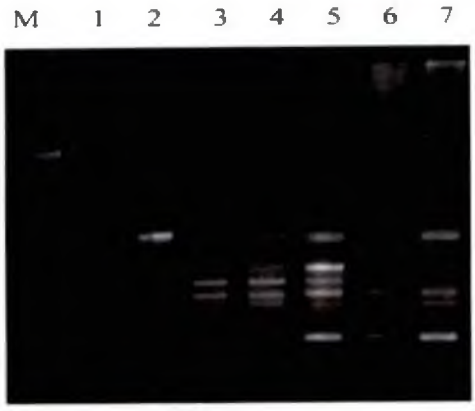
Lanes 1-12: Molecular wt.marker(*Lambda* DNA/ *Eco*RI/*Hind* III), vv97/84, a 82, a94,VH8,VH9,VH10,VH11, VH21,VH22,VH23,VH24

Table: 30 Amplicons obtained in parents and progenies of a 82 x a 94 cross using selected primers

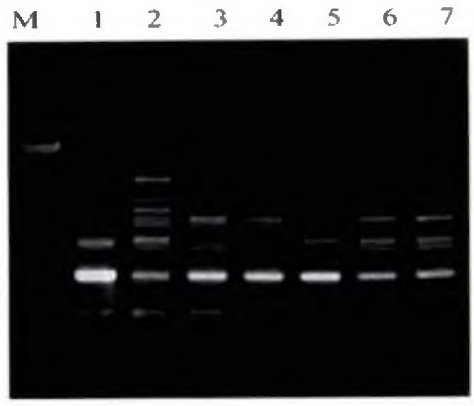
Primer	Identity code	No: of polymorphic bands	No: of monomorphic bands
OPF 3	V1	5	4
	V2	7	4
	VH8	4	4
	VH9	1	4
	VH10	7	4
	VH11	7	4
OPP 6	V1	2	3
	V2	5	3
	VH8	5	3
	VH9	2	3
	VH10	7	3
	VH11	4	3
OPE 14	V1	3	5
	V2	1	5
	VH8	4	5
	VH9	4	5
	VH10	4	5
	VH11	5	5
OPE 15	V1	5	2
	V2	3	2
	VH8	2	2
	VH9	8	2
	VH10	7	2
	VH11	6	2
OPE 18	V1	4	2
	V2	3	2
	VH8	4	2
	VH9	4	2
	VH10	0	2
	VH11	1	2
OPE 19	V1	5	5
	V2	3	5
	VH8	4	5
	VH9	3	5
	VH10	3	5
	VH11	0	5

Table: 31 Amplicons obtained in parents and progenies of vv 97/84 x a 94 cross using selected primers

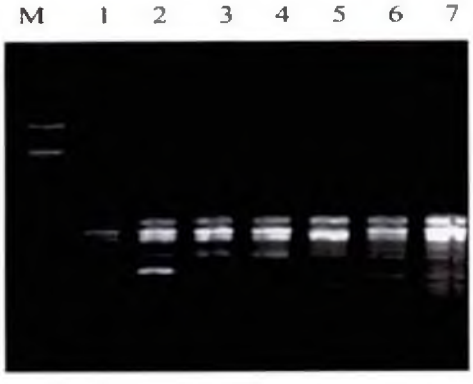
Primer	Identity code	No: of polymorphic bands	No: of monomorphic bands
OPF 3	V5	1	6
	V2	5	6
	VH21	2	6
	VH22	3	6
	VH23	2	6
	VH24	3	6
OPP 6	V5	1	3
	V2	5	3
	VH21	2	3
	VH22	3	3
	VH23	3	3
	VH24	4	3
OPE 14	V5	7	2
	V2	4	2
	VH21	6	2
	VH22	3	2
	VH23	4	2
	VH24	5	2
OPE 15	V5	3	3
	V2	2	3
	VH21	3	3
	VH22	2	3
	VH23	5	3
	VH24	5	3
OPE 18	V5	5	2
	V2	3	2
	VH21	0	2
	VH22	2	2
	VH23	4	2
	VH24	3	2
OPE 19	V5	3	7
	V2	1	7
	VH21	2	7
	VH22	1	7
	VH23	1	7
	VH24	2	7



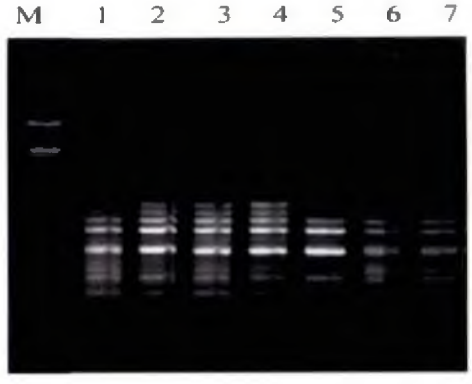
OPF 3



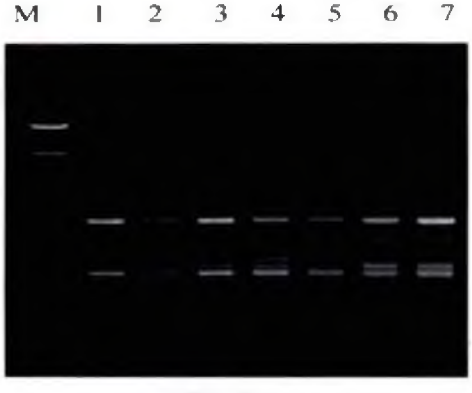
OPP 6



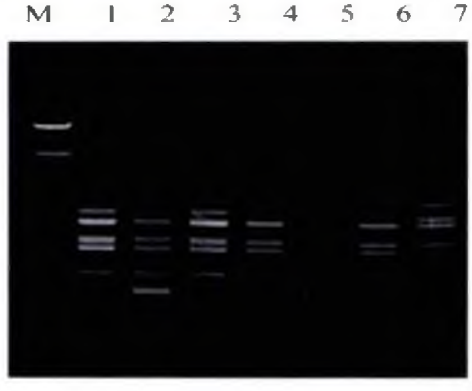
OPE 14



OPE 15



OPE 18



OPE 19

Plate 24. RAPD profile for parents and progenies of a 94 x a 82 cross with selected primers

Lanes 1 - 8: Molecular wt.marker(*Lambda* DNA/ *Eco*RI/*Hind* III), a 94, a 82, VH 16, VH 17, VH 18, VH 19, VH 20

Table: 32 Amplicons obtained in parents and progenies of a 94 x a 82 cross using selected primers

Primer	Identity code	No: of polymorphic bands	No: of monomorphic bands
OPF 3	V2	4	2
	V1	3	2
	VH16	4	2
	VH17	4	2
	VH18	4	2
	VH19	4	2
	VH20	4	2
OPP 6	V2	2	2
	V1	6	2
	VH16	4	2
	VH17	5	2
	VH18	5	2
	VH19	4	2
	VH20	4	2
OPE 14	V2	0	4
	V1	3	4
	VH16	3	4
	VH17	3	4
	VH18	1	4
	VH19	1	4
	VH20	3	4
OPE 15	V2	2	7
	V1	1	7
	VH16	2	7
	VH17	2	7
	VH18	0	7
	VH19	2	7
	VH20	2	7
OPE 18	V2	6	2
	V1	1	2
	VH16	5	2
	VH17	4	2
	VH18	2	2
	VH19	5	2
	VH20	5	2
OPE 19	V2	4	4
	V1	4	4
	VH16	3	4
	VH17	3	4
	VH18	0	4
	VH19	2	4
	VH20	5	4

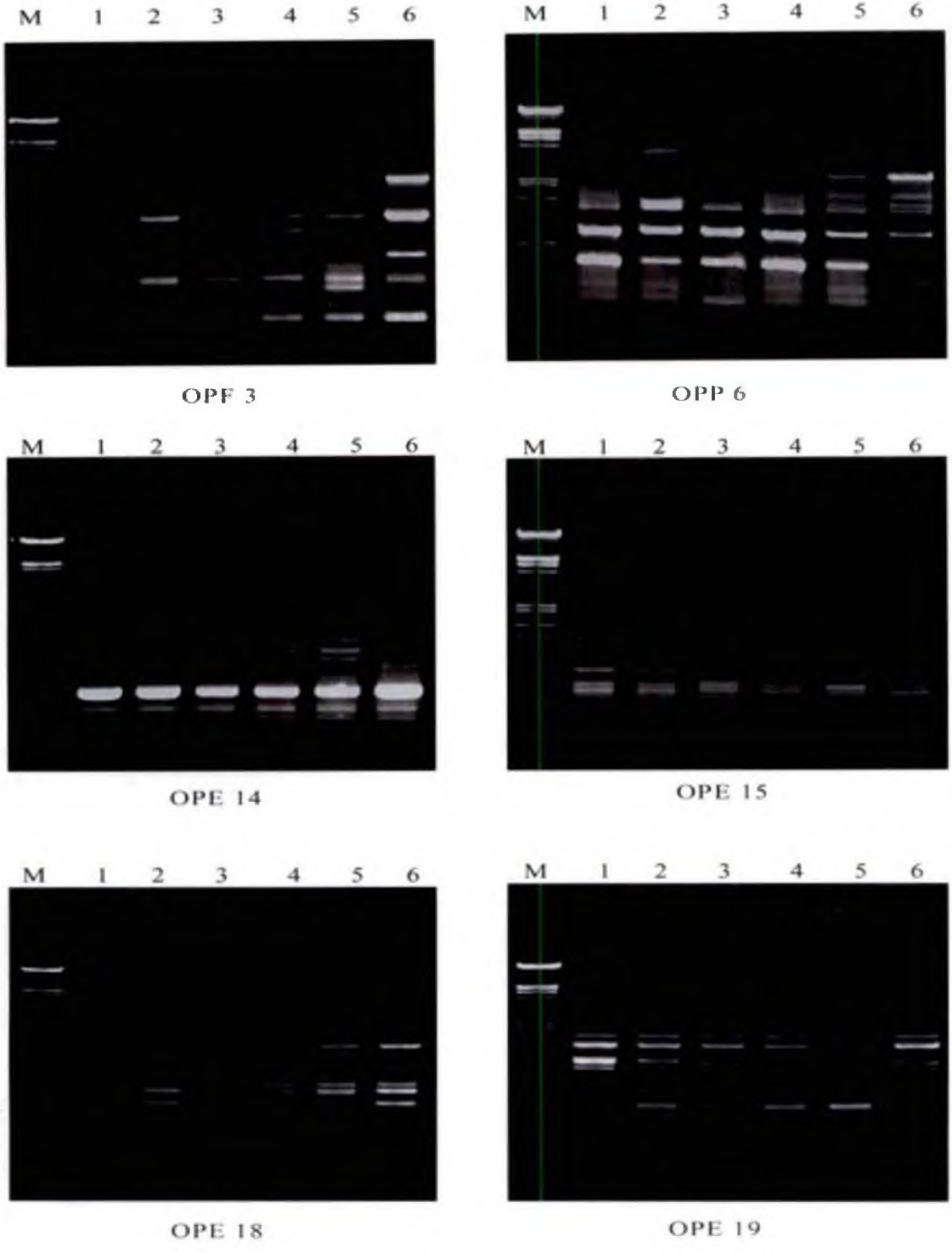


Plate 25. RAPD profile for parents and progenies of a 425 x a 94 cross with selected primers

Lanes 1- 7: Molecular wt.marker(*Lambda* DNA/ *Eco*RI/*Hind* III), a 425, a 94, VH 12, VH 13, VH 14, VH 15

Table: 33 Amplicons obtained in parents and progenies of a 425 x a 94 cross using selected primers

Primer	Identity code	No: of polymorphic bands	No: of monomorphic bands
OPF 3	V3	5	3
	V2	6	3
	VH12	3	3
	VH13	3	3
	VH14	5	3
	VH15	4	3
OPP 6	V3	5	2
	V2	4	2
	VH12	2	2
	VH13	5	2
	VH14	5	2
	VH15	4	2
OPE 14	V3	0	6
	V2	0	6
	VH12	0	6
	VH13	0	6
	VH14	1	6
	VH15	1	6
OPE 15	V3	3	3
	V2	3	3
	VH12	3	3
	VH13	3	3
	VH14	1	3
	VH15	2	3
OPE 18	V3	3	3
	V2	2	3
	VH12	2	3
	VH13	2	3
	VH14	2	3
	VH15	2	3
OPE 19	V3	2	5
	V2	3	5
	VH12	3	5
	VH13	3	5
	VH14	3	5
	VH15	2	5

with six different primers expressed variability in the genetic make up of the parents and hybrid progenies of vanilla.

4.9.2.4.1. Cluster analysis of parents

The variation recorded for parents ranged between 11 per cent and 47 per cent forming three clusters (Fig 4). The first cluster included *a 425*, *a 428*, *vv 97/84* and *a 82* parents with a total variability of about 17 per cent. The parents *a 82* and *vv 97/84* showed 89 per cent similarity in RAPD pattern. The other pair of parents (*a 425* and *a 428*) also showed about 86 per cent similarity. The parent *V. t* was very distinct and showed maximum variability of 47 per cent followed by *a 94* parent with 27 per cent variability.

4.9.2.4.2. Cluster analysis of parents and progenies of V. tahitensis x V. planifolia

Hierarchical cluster analysis of RAPD data with six primers indicated that the two parents showed great variability. The total variation for parents and progenies ranged between 8 and 40 per cent (Fig 5). The hybrid progeny VH7 was equidistant from both the parents. The two progenies VH3 and VH5 showed 92 per cent similarity. Another progeny VH4 showed 85 per cent similarity with VH3 and VH5 progenies. The VH2 progeny showed 40 per cent variability and is more similar to *a 428* (♂) parent whereas all other progenies were similar to *V. t* (♀) parent.

4.9.2.4.3. Cluster analysis of parents and progenies of a 82 x a 94

The total variation for parents and progenies ranged between 16 and 36 per cent (Fig 6). Hierarchical cluster analysis of RAPD data revealed that *a 94* parent and VH 8 progeny resembled each other very closely in their genetic architecture with 82 per cent similarity. The two progenies VH10 and VH11

showed 84 per cent similarity in RAPD pattern. The progeny VH9 showed 33 per cent variability and was more similar to *a 82* parent.

4.9.2.4.4. Cluster analysis of parents and progenies of a 94 x a 82

The variation recorded ranged between 4 and 31 per cent (Fig 7). The two progenies VH16 and VH17 showed 96 per cent similarity in RAPD pattern. All the progenies were more similar to their parent *a 82* and to each other.

4.9.2.4.5. Cluster analysis of parents and progenies of a 425 x a 94

The variation recorded ranged between 10 and 34 per cent (Fig 8). The progeny VH15 was very distinct and showed maximum variability of 34 per cent. The progenies VH12 and VH13 showed 90 per cent similarity. The progenies VH14, VH12 and VH13 were more similar to *a 425* parent and to each other.

4.9.2.4.6. Cluster analysis of parents and progenies of vv 97/84 x a 94

Hierarchical cluster analysis of RAPD data revealed that all progenies were more similar to *a 94* parent. The two progenies VH23 and VH24 showed 88 per cent similarity. The progeny VH22 and *a 94* parent showed 77 per cent similarity. All the progenies were more similar to *a 94* parent (Fig 9).

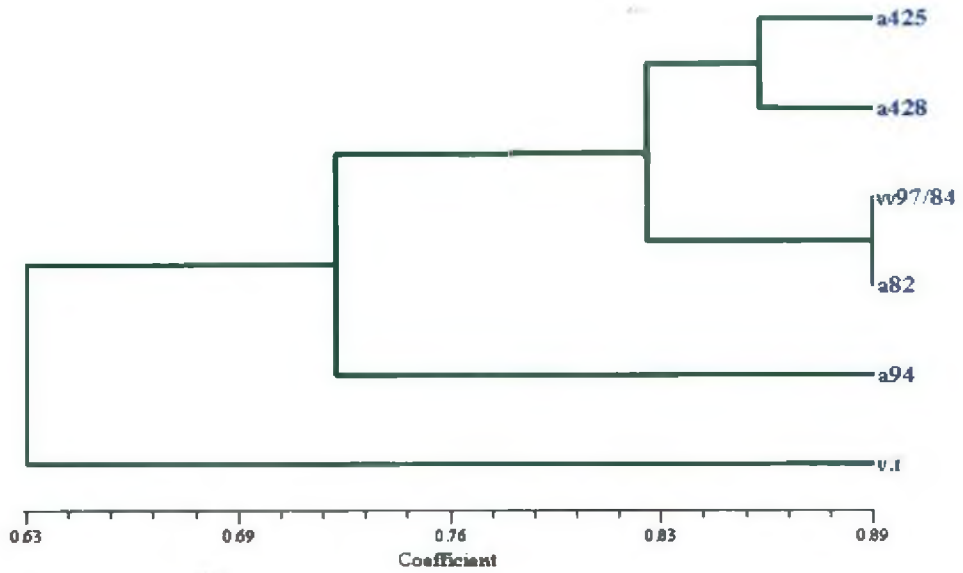


Fig. 4 Dendrogram showing clusters of *V. planifolia* and *V. tahitensis* plants selected as parents after RAPD analysis

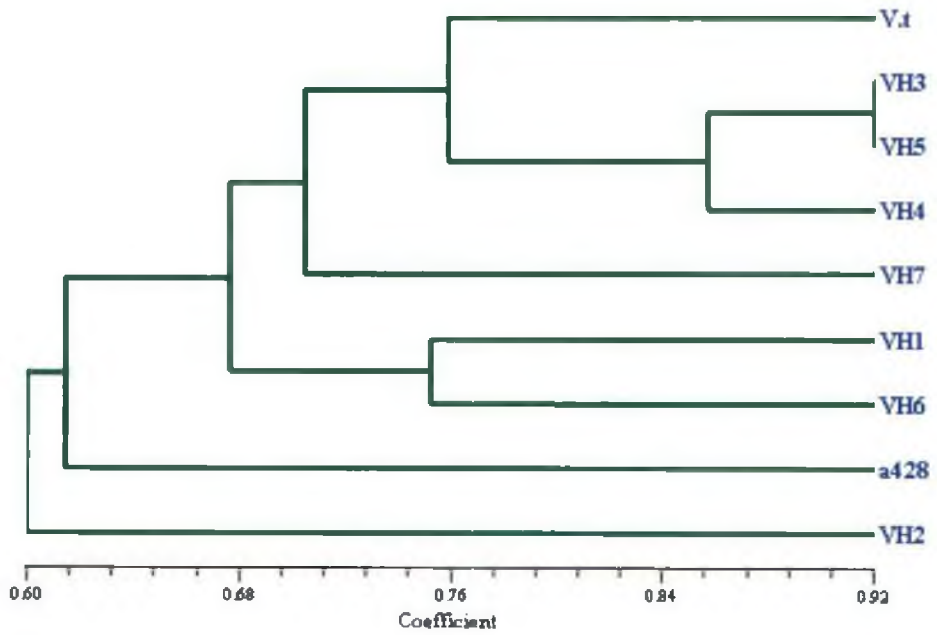


Fig. 5 Dendrogram showing clusters of parents and progenies of *V. tahitensis (V. t)* x *V. planifolia (a 428)* cross after RAPD analysis

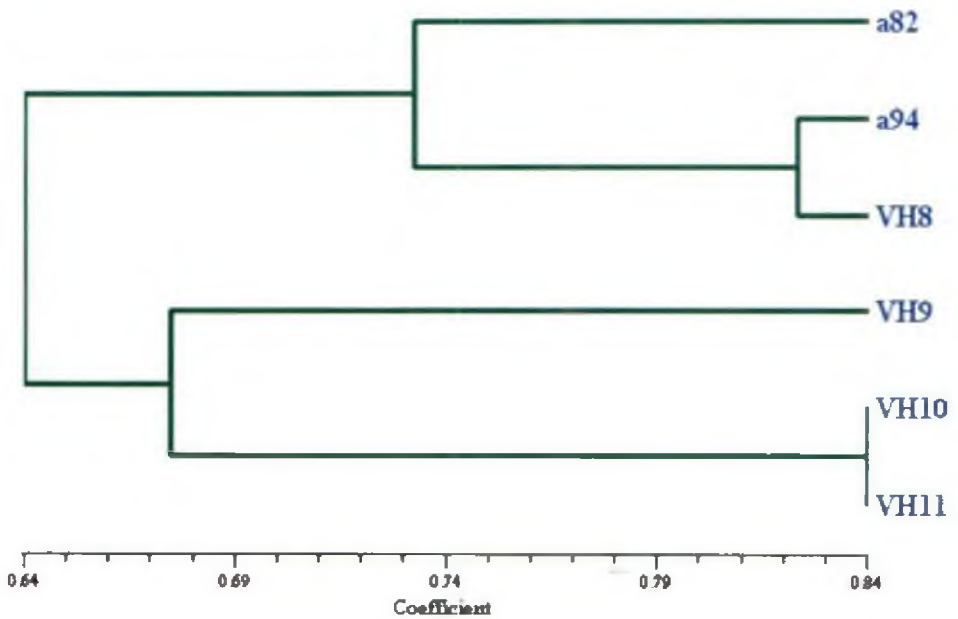


Fig. 6 Dendrogram showing clusters of parents and progenies of *a 82* x *a 94* cross after RAPD analysis

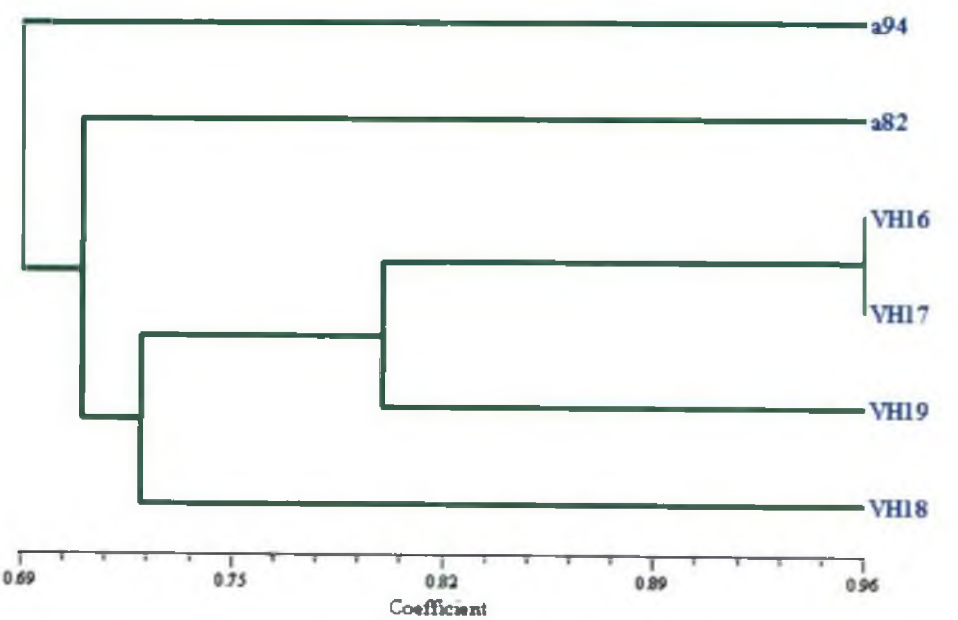


Fig. 7 Dendrogram showing clusters of parents and progenies of *a 94* x *a 82* cross after RAPD analysis

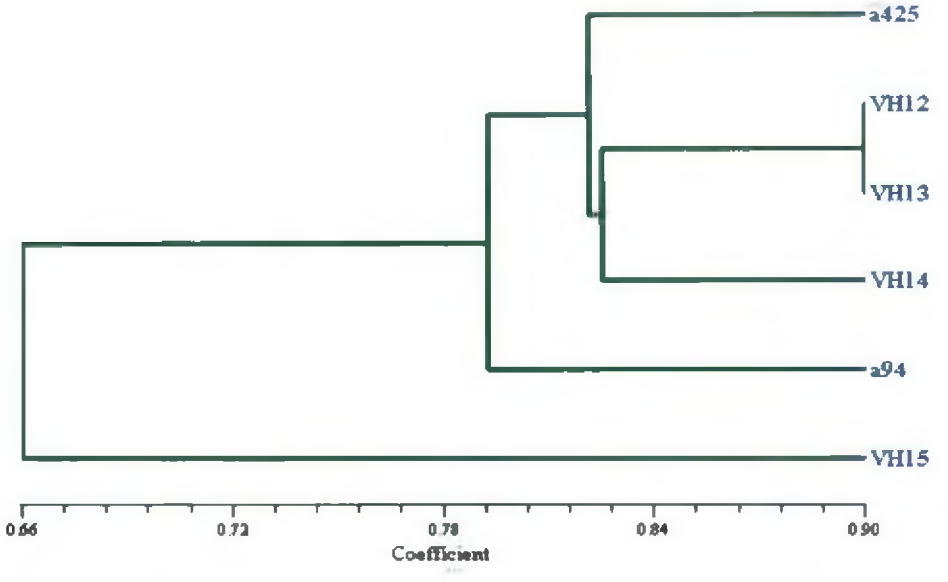


Fig. 8 Dendrogram showing clusters of parents and progenies of *a 425* x *a 94* cross after RAPD analysis

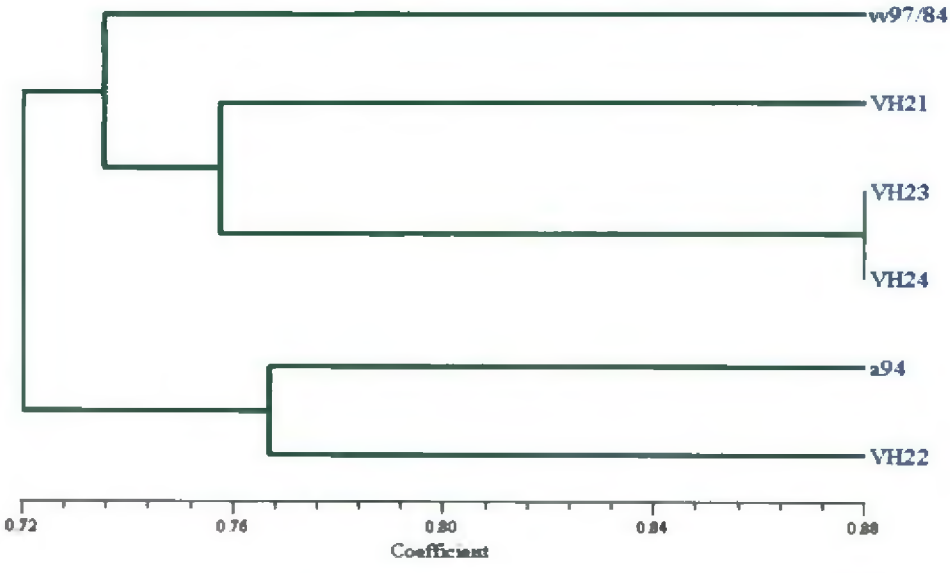


Fig. 9 Dendrogram showing clusters of parents and progenies of *vv 97/84* x *a 94* cross after RAPD analysis

DISCUSSION

5. DISCUSSION

Vanilla is the second costliest spice in the world next to saffron. The best quality 'vanilla beans of commerce' are derived from *Vanilla planifolia* Andrews (*V. fragrans*). The other two vanilla species that yield natural vanillin are *Vanilla tahitensis* Moore, the Tahitian vanilla, which is cultivated in Tahiti islands and *Vanilla pompona* Schiede, cultivated in some of the South Pacific islands. (Purseglove, 1978).

The genetic base of vanilla is narrow as it is propagated vegetatively. Continuous clonal propagation of the limited gene pool leads to evolutionary stagnation. Since vanilla is a seed setting crop there is scope for varietal improvement through hybridization. Hybridisation was initiated in vanilla in recent years for getting variants with higher yields, richer vanilla flavor, disease resistance and natural self pollination. But natural seed germination in vanilla is difficult due to lack of endosperm. Embryo culture can be used to overcome this obstacle and the production of inter/intraspecific vanilla hybrids is feasible. The present study was conducted with the objective of improving the gene pool of vanilla by generating variability through intra/interspecific hybridization and embryo culture technique. Interspecific hybridisation was done between *V. planifolia* and *V. tahitensis* and intraspecific hybridisation was done among superior clones of *V. planifolia* derived from *in vitro* seed culture of self pollinated pods.

Major aspects of the study were the following:

1) Variability analysis in parents for plant morphology
2) Floral biology and morphology of parents
3) Pollen studies
4) Inter/intraspecific hybridization
5) Embryo culture studies
6) Variability analysis in hybrid progenies for morphological characters

7) Variability analysis in parents and hybrid progenies by RAPD

5.1. VARIABILITY ANALYSIS IN PARENTS FOR PLANT MORPHOLOGY

Various foliar characteristics studied were leaf length, leaf breadth, leaf area, leaf shape and leaf phyllotaxy (Tables 9 & 10). The stem characters studied were stem type, vine colour and internodal length (Table 11).

The mean leaf length was 20.84 cm in *V. tahitensis* plants and 17.14 cm in *V. planifolia* clones. Among the *V. planifolia* clones it ranged from 14.74 to 18.14 cm in *a 428* and *vv 97/84*. The mean leaf breadth was 3.6 cm in *V. tahitensis* plants and 5.02 cm in *V. planifolia* clones. Among the *V. planifolia* clones, it ranged from 4.8 to 5.4 cm in *a 428* and *a 82*. The mean leaf area was 55.96 cm^2 in *V. tahitensis* plants and 62.08 cm^2 in *V. planifolia* clones. Among the *V. planifolia* clones, it ranged from 50.82 to 69.2 cm^2 in *a 428* and *a 82*. Oblong – elliptic leaf shape was observed for all clones of *V. planifolia* while it was narrow lanceolate for *V. tahitensis* plants. The leaf phyllotaxy was alternate spiral for both *V. planifolia* and *V. tahitensis* plants.

Simple stem was observed for all the plants belonging to *V. planifolia* and *V. tahitensis*. Vine colour was green for all *V. planifolia* clones while it was dark green for *V. tahitensis*. The mean internodal length was 8.34 cm in *V. planifolia* clones and 11.08 cm in *V. tahitensis* plants. Among the *V. planifolia* clones, it ranged from 6.84 cm in *a 428* to 10.36 cm in *vv 97/84*. The aerial roots were produced singly from a node opposite to the leaves in all parent plants studied.

The *V. planifolia* plants differed from *V. tahitensis* plants in leaf length, leaf breadth, leaf area, leaf shape and leaf colour. But these variations were not statistically significant. Among *V. planifolia* clones *a 82* (69.2 cm^2) has the maximum leaf area followed by *vv 97/84* (66.2 cm^2) and *a 94* (64.0 cm^2). There was no variation for leaf shape and leaf colour among *V. planifolia* clones.

Significant variation was observed in internodal length at 5 per cent level among clones of *V. planifolia.*

Hena (2005) and Kuriakose *et al.* (2005) have assessed morphological variability in clones derived through *in vitro* culture comprising clones *a 82, a 94* and *a 425.* According to Hena, intraclonal variation for leaf characters was insignificant; while it was significant for clones derived from different seeds in the same pod and also from different pods. In their studies *a 82* and *a 94* were found as superior clones. In the present study also they were found superior based on leaf area.

Worldwide *V. planifolia* is considered as superior for the source of vanilla flavour and it is characterised by long pod and vanillin content of 1.3 to 3.8 per cent. Tahiti vanilla has vanillin contents ranging from 0.9 to 3.3 per cent and is characterised by short pods compared to *V. planifolia.* In cultivation, diseases like stem rot, root rot, bean rot, shoot tip rot, bean yellowing and anthracnose threaten vanilla crop. The tolerance/susceptibility of *V. tahitensis* to these diseases is not studied so far. The possibility of getting disesase resistant variants in the interspecific cross can be explored. The economic importance of *Vanilla tahitensis* was documented by TVC (2004). According to them quality hybrids can be produced by interspecific hybridization of *V. planifolia* and *V. tahitensis.*

5.2. FLORAL BIOLOGY AND MORPHOLOGY STUDIES

5.2.1. Floral biology

The flowering of *V. tahitensis* plants was observed during September to October and December to January; whereas all the clones of *V. planifolia* except *a 428* flowered during January to April. The clone *a 428* flowered twice in an year i.e., October to November and February to March. The mean developmental period from floral initiation to first flower opening varied from 15.6 days in *V. planifolia* clones to 13.8 days in *V. tahitensis* plants. Mean blooming period of a

plant in *V. planifolia* was 30.95 days while it was 26.2 days in *V. tahitensis*. Maximum flowering (80%) was observed in plants of clone *a 82* of *V. planifolia* and *V. tahitensis* plants.

In *V. tahitensis* plants two flowering season was observed as stated above. But for all *V. planifolia* clones except *a 428*, single season was observed. Same plant was not flowered in both seasons.

Anandaraj *et al.* (2001) has reported vanilla flowering from December to February in India. Climate plays a dominant role in floral initiation. In a typical hill zone climate flowering starts by February or March; but in a relatively drier climate it occurs from October onwards (Shadakshari *et al.*, 2003). In the present study, main flowering season for *V. tahitensis* was September to October while it was January to April for *V. planifolia*. Time taken for floral initiation to first flower opening varied from 30 – 45 days in different vines. Total blooming period varies depending upon number of flowers produced per inflorescence. For an inflorescence of 15 – 18 flowers it varied from 18 – 31 days (Shadakshari *et al.*, 2003). Bhat and Sudharshan (2004) have reported that blooming period varies from 5 to 40 days depending upon flowers in a particular inflorescence. In the present study, *V. planifolia* clones and *V. tahitensis* plants recorded a mean floral developmental period of 15.6 days and 13.8 days and mean blooming period of 30.95 days and 26.2 days.

In the present study, the flowering season of *Vanilla planifolia* and *Vanilla tahitensis* was not in synchronization. So in interspecific hybridization programme pollen storage may be necessary. *Vanilla tahitensis* was early to flower and showed two flowering season. The advantage of this aspect in crop husbandry of vanilla has to be explored.

5.2.2. Inflorescence characteristics

The mean number of infloroscence per plant was 2.58 in *V. planifolia* clones and 2.65 in *V. tahitensis*. Among the *V. planifolia* clones, it ranged from 1.0 to 4.36 in *a 428* and *a 82*. The mean number of flowers per infloroscence was 15.97 in *V planifolia* clones and 15.1 in *V. tahitensis*. Among the *V. planifolia* clones it ranged from 14.28 to 19.56 in *a 82* and *a 428*. The mean inflorescence length was 15.46 cm in *V. tahitensis* plants and 13.77 cm in *V. planifolia* clones. Among the *V. planifolia* clones it ranged from 13.8 to 15.2 cm in *a 82* and *a 425*.

There was not much difference in *V. planifolia* and *V. tahitensis* with respect to number of inflorescence per plant and number of flowers per inflorescence. But the length of inflorescence was more in *V. tahitensis*. Bhat and Sudharshan (2004) studied inflorescence characters in vanilla and is in tune with present study.

5.2.3. Floral morphology

The floral morphology of *V. planifolia* and *V. tahitensis* was almost similar except for flower colour, length and girth of the ovary. The flower colour of *V. planifolia* was greenish cream with less floral diameter while that of *V. tahitensis* was pale greenish cream with more floral diameter (Plate 3). Among the clones of *V. planifolia*, clone *a 428* exhibited more greenish tinged flowers and deeply embedded pollinium with less pollen mass. The length of the ovary of *Vanilla planifolia* ranged between 4 - 6 cm and that of *V. tahitensis* ranged between 3 - 4 cm. The girth of ovary of *V. planifolia* ranged between 1.3 – 1.6 cm and that of *V. tahitensis* ranged between 1.2 – 1.4 cm. In both species the tip of the column bears a single stamen with two pollen masses (pollinia) covered by a cap or hood like structure called rostellum, which prevents natural pollination. Hence artificial hand pollination has to be done in both species for pod set.

The floral morphology details observed in the present study were similar with the description given by Purseglove *et al.* (1981) and Kuruvilla *et al.* (1996).

5.3. POLLEN STUDIES

5.3.1. Pollen fertility

The mean pollen fertility per cent as per acetocarmine stain test for *V. planifolia* was 61 per cent while it was 74.69 per cent for *V. tahitensis* flowers. But these variations between species were not statistically significant. According to Kuruvilla *et al.* (2004) the pollen fertility per cent of *V. planifolia* ranged from 72 – 87 per cent.

5.3.2. Pollen viability

Pollen viability of *V. planifolia* and *V. tahitensis* was checked in three media combinations. Pollen germination was obtained only in the media reported by Ravindran (1979), which consisted of sucrose, calcium nitrate and boric acid. The mean pollen viability per cent of *V. planifolia* and *V. tahitensis* were 39 per cent and 45.3 per cent in this medium. There was no pollen germination in the modified ME_3 and Brewbaker and Kwack's media.

The results of pollen viability studies were in line with the reports of Ravindran (1979). He cultured *V. planifolia* pollen grains on a nutritive medium consisting of 10 per cent sucrose, 100 mg l^{-1} boric acid and 100 mg l^{-1} calcium nitrate and the germination count after incubation of 5 – 24 hours was 45 per cent. According to O'Kelly (1955) and Vasil (1960) sugars play major roles in pollen germination. Spurr (1957), O'Kelly (1957), Raghavan and Baruah (1959) and Young *et al.* (1966) has reported that boron stimulates pollen germination and tube growth. They also suggested that boron is involved in the synthesis of pectic substances for the growing pollen tube walls.

Shadakshari *et al.* (2003) studied pollen viability and stigma receptivity and found that the grains are viable 23 hours prior to anthesis and 16 hours 30 minutes after anthesis. The stigma is receptive 41 hours prior to anthesis and 17 hours after anthesis under hill zone conditions. Pollen viability deteriorates markedly after anthesis; with only 10 --15 per cent fruit set when 2 – 3 day old pollens are used (De Guzman, 2004).

Since the pollen fertility and viability of *Vanilla planifolia* and *Vanilla tahitensis* are high, there is chance of getting good pod set in interspecic and intraspecific hybridization programme.

5.3.3. Pollen storage

Since the flowering of *V. planifolia* and *V. tahitensis* was not in synchronisation, pollen of *V. tahitensis* was stored for interspecific hybridization. The pollen storage was done in cryovials containing $CaCl_2$ and kept at 4°C. The storage of pollen in $CaCl_2$ retained its viability upto 7 - 8 weeks. The pod set per cent of *V. planifolia* x *V. tahitensis* hybrid pods developed after pollinating with stored *V. tahitensis* pollen grains was taken as index of pollen viability after storage. The vanilla pollen became dried on long term storage of 3 months in $CaCl_2$ at 4°C and pod set was not observed on using this pollen for pollination.

Storage of coconut pollen in fused Calcium Chloride has been reported earlier and could be stored upto 12 days (with 53.4 % germination) in a dessicator (Bhat and Ratnambal, 1997). Cryopreserved pollen of *Vanilla planifolia* and *Vanilla aphylla* in liquid nitrogen resulted in successful fruit set and seed germination (IISR, 2002).

Various workers in different crop species reported similar results of pollen storage at low temperature. Whitehead (1963) reported that freeze dried coconut

pollen could be stored for prolonged periods at deep freeze temperatures. Long term preservation of pollen of many species at very low temperature was reported by Barnabas and Rajki (1981), Eeink (1983), Filippova (1986) and Hanna *et al.* (1986).

Since the pollen storage in $CaCl_2$ at 4°C retained viability upto 7 – 8 weeks, interspecific hybrisation could be done using pollen stored in this method.

5.4. INTER/INTRASPECIFIC HYBRIDIZATION

5.4.1. Pollination and pod development

The vanilla flower is so constructed that self pollination of the individual flower is impossible, unless hand pollinated due to separation of stamen from the stigma by the rostellum. Artificial hand pollination was done between 8 and 9 a.m. in both interspecific and intraspecific crosses and pod set was obtained in both cases. In the interspecific cross *V. planifolia* x *V. tahitensis* pod set was 70 per cent. However, only four hybrid pods could be recovered for embryo culture because of premature dropping of pods at four months after pollination. In the intraspecific crosses pod set varied from 9.1 to100 per cent (Table 16). Cent per cent pod set was observed for the hybrid crosses *a 82* x *vv 97/84* and *a 428* x *a 82* and it was minimum for the cross *a 94* x *a 425*.

The practical method of artificial pollination identified by Edmond Albius in 1841 was used for pollination (Purseglove *et al.*, 1981) and pod set was obtained in both interspecific and intraspecific crosses. Pollination between 8 and 9 a.m. has resulted cent per cent pod setting in certain crosses. Bhat and Sudharshan (1998) have reported that the ideal time for pollination is between 6 a.m. and 1 p.m. The fruit setting per cent was maximum at 8 a.m. and showed a decreasing trend as pollination gets delayed. Effective fruit set in vanilla was observed for 16 hour 30 minutes after complete flower opening (6 a.m. in the

morning to night 10.30 p.m.) by hand pollinating the flowers on the day of anthesis with its own pollen (Shadakshari *et al.*, 2003).

The pod set per cent of different genotypes or hybrid crosses showed remarkable variation. From the study it could be concluded that hybridisation between *V. planifolia* x *V. tahitensis* is feasible. But 4 months after pod set, 67 per cent of pods were shed and this may be due to post fertilisation barriers but need further investigation. According to Duncan and Curtis (1942) fertilization in vanilla pods occurred at about 35 days after pollination. Minimum pod set per cent of 9.1 recorded for the genotype *a 94* x *a 425* might be due to some pre fertilization barriers and need further investigation. Minimum pod set per cent for crosses involving *a 428* clone as male parent could be explained due to the presence of deeply embedded pollinia with less pollen mass; since the crosses involving *a 428* as female parent recorded high pod set per cent.

5.4.2. Pod development as length increment in hybrid beans

The mean length of the ovary of *V. planifolia* on the day of pollination was 5.18 cm and increased to 6.88 cm within 4 DAP by crossing with *V. tahitensis*. It attained a length of 9.28 cm within a period of 1 month and afterwards length increment was meagre and attained a maximum of 9.87 cm at 120 DAP. The length was reduced to 9.8 cm at 150 DAP. The same developmental pattern was observed for pods of intraspecific crosses also (Fig 1 & Table 17).

The pod development pattern was similar in both inter and intraspecific crosses. The pod length in interspecific cross was less compared to intraspecific cross. According to Purseglove *et al.* (1981) pod length of *V. tahitensis* was less compared to *V. planifolia*. This might be the reason for less pod length in interspecific cross compared to intraspecific cross.

The study revealed that pod set could be ascertained 4 DAP by observing the length increment. It also showed that within 1 MAP the pod attains 95 per cent of its total length. Length increment after one month was meagre and one per cent reduction was also noticed at 5 MAP. Shadakshari *et al.* (2003) and Kuruvilla *et al.* (2004) has reported same pattern of growth of vanilla pod as observed in the present study. They observed that growth of bean in terms of length and diameter was very rapid upto five weeks after pollination.

5.5. EMBRYO CULTURE STUDIES

The seeds of hybrid pods germinated by producing green and creamish white protocorms in the media half strength MS + 3 per cent sucrose + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BAP under the culture conditions of 25 $\pm$ 2^0C temperature and 16 hours photoperiod of 1000 lux. Seeds from pods (*a 82* x *a 94*) of 6 weeks maturity germinated within 34 days and showed high (+++) intensity of germination while seeds from 28 - 36 weeks old pods did not germinate at all (Table 18). The multiple shoots were produced from each seedling in the media half strength MS + 3 per cent sucrose + 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BAP. The elongation and rooting of the proliferated shoots was obtained in the media MS + 3 per cent sucrose + 1.0 mg l^{-1} IAA + 0.2 mg l^{-1} BAP within a culture period of 7 - 8 weeks under light intensity of 1000 lux for a photoperiod of 16 hours. The seedlings having 3 - 5 leaves and 1 - 3 roots were planted out in the net house with 75 – 80 per cent shade provided with mist. Ganesh *et al.* (1996) reported hardening of vanilla plantlets derived from node cultures by growing within polytunnels in a green house. The percentage of plant out success for different parental combinations varied from 64.7 to 84.6 and in the interspecific cross (*V. t* x *V. p*), it was 70.8 per cent (Table 21).

The unique nature of orchids with undifferentiated embryos and suppressed endosperm formation necessitates symbiotic or *in vitro* germination. The protocol for embryo culture reported by Mary *et al.* (1999) was viable in the present study. As observed in the present study, Withner (1955) and Philip and

Nainar (1988) have reported the production of small white spherical or irregular protocorms during germination of vanilla seeds.

From the present study it could be concluded that age and genotype of the pods greatly influenced the time taken for embryo germination and intensity of germination. In the present study, seed germination was obtained even from 6 weeks mature pods. It was inferred that retention of the pod in the plant for long would not increase *in vitro* germination per cent, eventhough it may increase the quality attributes of vanilla bean for marketing. To avoid chemical or mechanical disadvantages associated with aging, ovule culturing for embryo germination should be initiated at an earlier stage. In the case of incompatible crosses (eg. inter/intraspecific hybridization), embryo rescue at an earlier period could be attempted to prevent embryo abortion. Withner (1955) obtained best growth from two month old pod cultures of vanilla. According to him 45 - 60 day pods with young developing embryos would grow when cultured. Mary *et al.* (1999) has reported that green pods of 5 - 7 months maturity were found to be ideal for *in vitro* seed germination. Pollination has a stimulating effect upon the ovary prior to fertilisation (Madhusoodanan *et al.*, 2003). Lo *et al.* (2004) has observed that maximum number of seeds germinated from immature capsules of *Dendrobium tosaense*, a medicinally important orchid.

Similar works regarding effect of embryo age on embryo germination has been reported by various workers in other crop species. Joshi and Johri (1972) has reported that ovules, three day after pollination were found suitable for *in vitro - in ovulo* embryo culture in cotton. Finer (1987), Wilcox *et al.* (1988) and Witrzens *et al.* (1988) described the regeneration of plants from immature embryos of sunflower hybrids. Pandey *et al.* (1987) and Kaushal *et al.* (2004) reported that embryos between the heart shaped and torpedo stages were the best for embryo rescue in *Trifolium* species.

In the present study, vanilla seeds collected from pods of 28 and 36 weeks old failed to germinate and it might be due to fully developed sclerotic coat,

which acts as mechanical barrier to germination. Withner (1955) reported that vanilla seeds from nine months old pods i.e., in its full maturity failed to germinate due to the naturally occurring vanillate compounds in the placental tissues and glandular hairs.

The study revealed that intensity of seed germination varied with genotype and age of pods. Seeds from pods (*a 82* x *a 94*) of 6 weeks maturity showed high (+++) intensity of germination whereas seeds from *V. p* x *V. t* pods of 14 and 20 weeks maturity showed less (+) intensity of germination. Withner (1955) has reported that seeds from any particular pod were not homogenous in its growth characteristics due to different potentialities of pollen grains and fewer pollen tubes reached the base of the pod and it took a tube longer time to reach an ovule in the base.

From the present study, it could be concluded that hybrid seed germination in vanilla could be obtained from young pods of six weeks maturity.

5.6. CURING OF *V. tahitensis*

Mature pods of *V. tahitensis* were harvested and processed as reported by Purseglove *et al.* (1981) without arresting the biological processes in hot water. The proportion of cured beans to fresh beans was 1:3.5 after slow drying. Vanillin content estimated as per AOAC method ranged between 0.43 - 0.52 per cent. Result shows that the vanillin content of *V. tahitensis* beans under tropical climate of Kerala plains was less compared to *V. planifolia*. But this aspect needs further investigation for conclusive results especially with respect to processing. According to Purseglove *et al.* (1981) tahiti vanilla has vanillin contents ranging from 0.9 to 3.3 per cent.

5.7. VARIABILITY IN MORPHOLOGICAL CHARACTERS OF HYBRID PROGENIES

The hybrid progenies obtained from same and different parental combinations showed variability in foliar and cauline characters. Leaf characters studied were total number of leaves, leaf length, leaf breadth, leaf shape and leaf colour (Tables 22 & 24). Stem characters studied were plant height, internodal length, stem type, plant growth and number of aerial roots from a single node (Tables 23 & 25).

In the present study, variation was observed in the hybrid progenies from same and different parental combinations for mean leaf length, leaf breadth, leaf shape and leaf colour. Leaf shape variations in hybrid seedlings of various parental combinations were remarkable. In the interspecific cross of *V. t* x *V. p*, four leaf shapes broadly ovate, oblong-lanceolate, lanceolate and linear-lanceolate were observed in the progenies against narrow lanceolate in *V. t* parent and oblong elliptic in *V. p* parent. In the progenies of *vv 97/84* x *a 94* two leaf shapes oblong-elliptic and oblong-lanceolate were observed against oblong elliptic leaf shape in parents. In the cross of *a 94* x *a 82*, the progenies showed two leaf shapes oblong - elliptic and lanceolate against oblong - elliptic leaf shape in parents. The progenies of *a 82* x *a 94* and *a 425* x *a 94* produced only oblong - elliptic shaped leaves.

Another interesting variation observed in the hybrid progenies was simple and branched stem type. In the interspecific cross *V. t* x *V. p* and intraspecific crosses *a 82* x *a 94* and *vv 97/84* x *a 94* simple and branched stem type (Plates 16A & B) was observed against simple stem type in their parents. Single and double aerial roots were produced from single node in the interspecific cross *V. t* x *V. p* and intraspecific crosses *a 82* x *a 94* and *a 425* x *a 94* against normal single root origin in their parents (Plate 17). In the parental combination of *V. t* x *V. p*, the hybrids VH 383 and VH 384 were vigorous compared to other hybrids. All

hybrid progenies derived from intraspecific crosses of *a 82* x *a 94* and *vv 97/84* x *a 94* were found to be more vigorous with respect to plant growth.

As observed in the present study, variation in morphology of seedling progenies derived through embryo culture was reported by various workers in vanilla. Divakaran *et al.* (1996) studied parameters like proliferation rate, leaf size, shape, colour, internodal length and growth rate as an index for morphological variability in vanilla seedlings derived from ovule culture. Variability has been observed in the vanilla seedlings raised through *in vitro* seed culture especially in the leaf morphology and phyllotaxy (Mary *et al.*, 1999). Interspecific hybrids between *Vanilla aphylla* and *Vanilla planifolia* were produced at IISR and among the 50 progenies generated, none expressed the leaf characters of the male parent. All the progenies of aphylla types had only scale leaves and none exhibited the normal leaf character of *Vanilla planifolia* (IISR, 2002). Morphological characterization of variability in *in vitro* derived seedlings of vanilla was reported by Hena (2005) and Kuriakose *et al.* (2005). They assessed the variability by studying the morphological characters like leaf length, leaf width, leaf area, number of leaves, total growth, leaf shape, leaf phyllotaxy, root origin and number of roots.

5.8. VARIABILITY ANALYSIS IN PARENTS AND HYBRID PROGENIES BY RAPD

The DNA was isolated by the method reported by Doyle and Doyle (1987) with slight modifications. The RNAase treatment was effective to remove RNA from the DNA sample and the quality and purity of sample improved. This is in line with the reports of Babu (1997) and Hena (2005).

The RAPD analysis was carried out for seventeen progenies from the intraspecific crosses of *V. planifolia* (*a 82* x *a 94*, *a 94* x *a 82*, *a 425* x *a 94*, *vv 97/84* x *a 94*) and seven progenies from the cross *V. tahitensis* x *V. planifolia*.

The experiment was carried out using six decamer primers (OPF 3, OPP 6, OPE 14, OPE 15, OPE 18 and OPE 19) that gave very good amplification out of thirty decamer primers.

The variation recorded for *V. planifolia* clones and *V. tahitensis* plants selected as parents ranged between 11 and 47 per cent. The extent of variability was in conjunction with the morphological variability exhibited by the parents. The clones *a 82* and *vv 97/84* characterised by long and widthy leaves were 89 per cent similar. The *V. tahitensis* plants, which exhibited narrow lanceolate leaf shape and dark green colour stood separate in clustering with maximum variability.

Hena (2005) has reported great variability in the genetic make up of *in vitro* derived seedlings of vanilla by RAPD assay. In the RAPD analysis of vanilla done by Kuriakose *et al.* (2005) *V. tahitensis* plants stood distinct in the cluster.

In the interspecific cross (*V. t* x *V. p*), the total variation for parents and progenies ranged between 8 and 40 per cent (Fig 5). Five hybrid progenies (VH 1,VH 3, VH 4, VH 5 and VH 7) showed more similarity with *V. t* (♀) parent while VH 2 hybrid was more similar to *V. p* (♂) parent whereas all other progenies were similar to *V. t* (♀) parent. In the intraspecific cross *a 82* x *a 94*, the total variation ranged between 16 and 36 per cent (Fig 6). The progeny VH 8 showed similarity with *a 94* parent whereas three progenies (VH 9, VH 10 and VH 11) were more similar to *a 82* parent. In the cross *a 94* x *a 82*, the variation recorded ranged between 4 and 31 per cent (Fig 7). All the progenies were more similar to *a 82* parent and to each other. The variation for the progenies and parents of *a 425* x *a 94* cross ranged between 10 and 34 per cent (Fig 8). The progeny VH 15 was very distinct and showed similarity to *a 94* parent than *a 425* parent. The progenies VH 12 and VH 13 showed 90 per cent similarity since they were derived from the same seedling. The progenies VH 12, VH 13 and VH 14 were more similar to *a 425* parent and to each other. In the cross *vv 97/84* x *a 94*

the variation recorded ranged between 12 and 28 per cent (Fig 9). All the progenies were more similar to *a 94* parent.

The results of RAPD analysis from the pooled data of the RAPD scores with six different primers verified the variability in the genetic make up of the parents and hybrid progenies of vanilla and the hybrids derived are the descendents of their respective inter/intraspecific crosses. These results are in confirmation with the findings of various workers. Ram *et al.* (2002) has reported the genetic fingerprinting of coffee hybrids (Ligenioides Hibridode- Timor) and observed a large genetic similarity and uniqueness between parents and their F1 hybrids by RAPD analysis. Divakaran *et al.* (1996) has reported that genetic variability in vanilla seedlings raised through ovule culture. The RAPD profiles coupled with morphological characters indicated that VH 1, VH 4 and VH 5 are true interspecific hybrids between *Vanilla planifolia* and *Vanilla aphylla* as they are approximately equidistant from the parents (IISR, 2000).

Another interesting observation in the present study is the appearance of some RAPD fragments specifically in the progenies, which can be explained on the basis of formation of new primer recognition sites through recombination during hybridization. In all the crosses, clones derived from the same seedling showed cent per cent similarity among them. This is in close confirmation with the reports of Gupta and Rao (2002) and Carvalho *et al.* (2004) that the RAPD is referred as an appropriate tool for certification of genetic stability and clonal fidelity of *in vitro* propagated chestnut hybrids.

In conclusion, the results from present study indicated that variability could be induced in vanilla through intra/interspecific hybridisation and embryo culture. Since the hybrid seedlings produced are only 4 - 5 months old, stable morphological variation could be analysed only at a later stage. This needs *ex vitro* screening.

5.9 FUTURE LINE OF WORK

1) More hybrids has to be produced to get desirable variants
2) Hybrids should be maintained and evaluated for flowering, yield, quality of beans, disease and pest resistance

SUMMARY

6. SUMMARY

The present investigation "Induction of variability in *Vanilla planifolia* Andrews through intra/interspecific hybridisation and embryo culture technique"was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara with the objective of inducing variability in *Vanilla planifolia* through intraspecific hybridisation (among superior clones of *V. planifolia*) and interspecific hybridisation (between *V. planifolia* and *V. tahitensis*) and embryo culture technique. The salient findings/achievements of the present study are stated below.

1. The study was conducted with six clones of *V. planifolia* (*a 82, a 94, a 425, a 428, vv 97/84*) and *V. tahitensis* plants. The existing variability in the selected genotypes for morphological characters was assessed. Variation was observed in foliar and cauline characters. Remarkable variation was observed between *V. planifolia* and *V. tahitensis* plants in leaf size, leaf shape and leaf colour. Statistically significant variation was observed for internodal length among *V. planifolia* clones. Clones *a 82* and *vv 97/84* of *V. planifolia* were found superior plants with respect to leaf area.

2. The floral biology and morphology of selected *V. planifolia* clones and *V. tahitensis* plants were studied.

3. The *V. tahitensis* plants showed two flowering seasons i.e., September-October and December - January. All the *V. planifolia* clones flowered during January - April except *a 428* clone which flowered during October - November also. The synchronised flowering of *V. tahitensis* and *a 428* clone was exploited in this hybridisation programme to get *V. tahitensis* x *V. planifolia* interspecific hybrid pods. The other floral characters namely developmental period from inflorescence emergence to first flower opening, blooming period, percentage of plants flowered, number of

inflorescence per plant, number of flowers per inflorescence and length of inflorescence varied between *V. planifolia* and *V. tahitensis* and among *V. planifolia* clones.

4. The floral morphology of *V. planifolia* and *V. tahitensis* were similar except flower colour, length and girth of the ovary. Among *V. planifolia* clones, *a 428* showed variation in flower colour (more greenish tinged flowers) and pollen production (less pollen mass).

5. The pollen fertility by acetocarmine stain and pollen viability in artificial sterilised media was tested. The pollen grains germinated in the medium of Ravindran (1979). It did not germinate in modified ME_3 and Brewbaker and Kwack's medium.

6. Mean pollen fertility for *V. planifolia* was 61 per cent while it was 74.69 per cent for *V.tahitensis* flowers. The mean pollen viability percentages of *V. planifolia* and *V.tahitensis* were 39 per cent and 45.3 per cent.

7. Since the flowering of *V. planifolia* and *V. tahitensis* was not in synchronisation, pollen grains of *V. tahitensis* was stored for doing interspecific hybridisation between *V. planifolia* and *V. tahitensis*. The storage of pollen in $CaCl_2$ at 4°C retained viability upto 7-8 weeks.

8. Hybridisation was done by hand pollination between 7 and 8 a.m. and pod set was obtained in inter and intraspecific crosses. Successful pod set after pollination was indicated by the retention of floral appendages 4 to 5 days after pollination.

9. The pod set percentage varied from 9.1 - 100 per cent in *V. planifolia* clones depending upon the genotype involved in crosses. The interspecific cross of *V. planifolia* and *V. tahitensis* recorded 70 per cent pod set with the pollen grains stored in $CaCl_2$.

10. The pod development as length increment showed similar growth pattern for both inter and intraspecific crosses. The pods attained 95 per cent of its total length within 1 MAP. Length increment after one month was meagre and one per cent reduction was also noticed at 5 MAP.

11. Seeds of hybrid pods germinated in the media half strength MS + 3 per cent sucrose+ 1.0 mg l^{-1} NAA+ 1.0 mg l^{-1} BAP (Mary *et al.*, 1999) under culture conditions of 25 $\pm$ 2^{0}C temperature and 16 hours photoperiod of 1000 lux. The time taken for embryo germination varied significantly with the age of pods. Seeds from pods of 6 weeks maturity germinated early compared to 28 - 36 weeks old pods, which failed to germinate.

12. Seeds germinated producing green and creamy white protocorms, which differentiated into shoots within 52 - 55 days after germination.

13. The intensity of embryo germination varied with genotype and age of the pods. The seeds of six weeks maturity from pods of cross *a 82* x *a 94* showed high intensity (+++) of germination and seeds of 20 and 24 weeks maturity from cross *vv 97/84* x *a 94* showed moderate intensity (++) of germination.

14. Multiple shoots were produced from each hybrid seedling in the media half strength MS+ 3 per cent sucrose+ 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BAP within a culture period of 8 - 9 weeks.

15. Elongation and rooting of the proliferated shoots was obtained in the media half strength MS+ 3 per cent sucrose + 1.0 mg l^{-1} IAA + 0.2 mg l^{-1} BAP (Mary *et al.*, 1999) within a culture period of 7 - 8 weeks.

16. The hybrid seedlings with 3 - 5 leaves and 1 - 3 roots were planted out in the net house with 75 - 80 per cent shade provided with mist. Plant out was done in standard potting mixture.

17. The percentage of plant out success for different parental combinations varied from 64.7 - 84.6. Plant out success was maximum for *a 82* x *a 94* genotype (84.6%) and interspecific cross (*V. t* x *V. p*) recorded 70.8 per cent plant out success.

18. Results from the embryo culture study could be used to speed up the breeding procedures, as this would shorten the reproductive cycle and reduce the time from one generation to another.

19. Morphological characterisation of hybrid progenies from different crosses was done. The progenies varied in leaf shape, size, number of leaves, leaf colour, plant height, internodal length, plant growth, stem type and number of aerial roots per node.

20. In the interspecific cross of *V. t* x *V. p*, the variations in leaf shape were remarkable and four leaf shapes broadly ovate, oblong lanceolate, lanceolate and linear lanceolate were observed in the progenies against narrow lanceolate in *V. t* parent and oblong - elliptic in *V. p* parent.

21. Progenies of intraspecific crosses also showed variation for leaf shape. In the progenies of *vv 97/84* x *a 94* cross oblong-elliptic and oblong-lanceolate leaves were observed against oblong-elliptic leaf shape in parents In the cross *a 94* x *a 82* oblong-elliptic and lanceolate leaf shape was observed against oblong-elliptic shape in parents.

22. All the hybrid progenies derived from *a 82* x *a 94* and *vv 97/84* x *a 94* crosses were vigorous in plant growth compared to progenies from other crosses.

23. Simple and branched stem type was observed in the progenies derived from interspecific cross (*V. t* x *V. p*) and intraspecific crosses (*a 82* x *a 94* and *vv 97/84* x a94).

24. Variants with single and double aerial roots arising from single node were observed in progenies of interspecific cross (*V. t* x *V. p*) and intraspecific crosses (*a 82* x *a 94* and *a 425* x *a 94*).

25. The genomic DNA isolated from tender and fresh leaves of vanilla plants using CTAB method of Doyle and Doyle (1987) with slight modifications gave good quantity DNA.

26. The purification of isolated DNA with RNAase and proteinase resulted in good quality DNA and was used for RAPD analysis.

27. The screening of parents and progenies from different hybrid crosses done with six primers viz., OPF 3, OPP 6, OPE 14, OPE 15, OPE 18 and OPE 19 resulted in formation of monomorphic and polymorphic bands.

28. Molecular chracterisation by RAPD assay showed variation in the banding pattern of parents and their progenies.

29. The RAPD banding pattern showed that the progenies of a hybrid cross do not uniformly inherit the unique RAPD fragments of both parents but inherit RAPD bands from either of the parents.

30. Appearance of new RAPD fragments that were unique to hybrid progenies were observed which could be explained on the basis of formation of new primer recognition sites through intragenic recombination.

31. The heirarchical cluster analysis of RAPD data indicated variation between parents and their progenies.

32. The variation in the parents and progenies of interspecific cross (*V. t* x *V. p*) ranged from 8 - 40 per cent. The intraspecific crosses *a 82* x *a 94*, *a 94*

x *a 82*, *a 425* x *a 94* and *vv 97/84* x *a 94* showed variation range of 16 - 36 per cent, 4 - 31 per cent, 10 - 34 per cent and 12 - 28 per cent respectively.

Thus in conclusion, morphological characters coupled with molecular characterisation using RAPD confirmed variability in parents and progenies of intra/interspecific crosses.

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* originals not seen

APPENDICES

APPENDIX I

Specific chemicals used	Firm
Cetyl trimethyl ammonium bromide (CTAB)	E Merck, Germany
β mercaptoethanol	E Merck, Germany
Ethidium bromide	Sigma, USA
Agarose	Genei, Bangalore
Taq DNA polymerase	Genei, Bangalore
PCR reagents (10X assay buffer, dNTPs)	Genei, Bangalore
Decamer primers	Operon, USA
Bromophenol blue	Sigma, USA
Tris base	Sigma, USA
EDTA	Sigma, USA
DNA molecular wt. Marker	Genei, Bangalore
Naphthalene acetic acid	Sigma, USA
Benzyl amino purine	Sigma, USA
Indole acetic acid	Sigma, USA
Vitamins (MS media)	Sigma, USA
Agar agar	Sigma, USA Sisco Research Laboratories (SRL)
Sucrose	Sigma, USA Sisco Research Laboratories (SRL)

APPENDIX II

Equipments for the study	Firm
Spectrophotometer	Spectronic Instruments Inc.,USA
Refrigerated high speed centrifuge	Kubota, Japan
Water purification system	Millipore, Germany Aquaguard,Eurekha Forbes
Deep freezer	Sanyo, Japan
Electronic balance	Sarturius
Laminar airflow	Kirloskar, India
Cytocentrifuge	Spinwin
Electrophoresis system	Hoefer, USA Biotech, Madras Genei, Bangalore
Thermal cycler	MJ Research, USA Peltier PTC 200
Transilluminator	Herolab, Germany
Documentation system	Alpha Infotech, USA
Ice flaking machine	Icematics

INDUCTION OF VARIABILITY IN *Vanilla planifolia* Andrews THROUGH INTRA/INTER SPECIFIC HYBRIDISATION AND EMBRYO CULTURE TECHNIQUE

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The present investigation entitled "Induction of variability in *Vanilla planifolia* Andrews through intra/interspecific hybridisation and embryo culture technique" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Vellanikkara during the period from 2003-2005.

Genetic variation in the germplasm of *Vanilla planifolia* is relatively limited because of the continuous clonal breeding of the existing gene pool. The present work was taken up with the main objectives of enhancing the spectrum of variability in vanilla through intra/interspecific hybridisation and thereby to get desirable recombinants of higher yields, richer vanilla flavour and disease resistant.

The superior clones of *V. planifolia* (*a 82, a 94, a 425, a 428, vv97/84*) and *V. tahitensis* plants maintained at CPBMB were taken as parent plants in the hybridisation programme. Variation in the foliar and stem characters of parent plants was observed which could be manifested in the progeny. Floral morphology and biology of *V. planifolia* and *V. tahitensis* plants were studied and variation was observed in flowering season of both species. Pollen fertility was assessed in acetocarmine (1%) stain and was 61 per cent for *V. planifolia* and 74.69 per cent for *V. tahitensis* flowers. The viability of pollen was assessed in pollen germination reported by Ravindran (1979) and was 39 and 45.3 per cent for *V. planifolia* and *V. tahitensis* pollen grains. There was no significant variation in the pollen fertility and pollen viability percentages of both species. *In vivo* crossing was done as per the pollination technique of *V. planifolia* for inter and intraspecific crosses. The pod set percentage varied significantly with genotype. The pod development pattern was similar in both inter/intraspecific hybrid pods. Embryo culture was done as per the protocol reported by Mary *et al.* (1999) to get hybrid progenies. Seeds from pods of different ages were cultured. The time taken for embryo germination and intensity of embryo germination

varied significantly with the maturity and genotype of pods. Seeds from hybrid pods (*a 82* x *a 94)* of 6 weeks maturity germinated early compared to seeds from half mature (14, 18, 20, 22 and 24 weeks) and mature (28 and 36 weeks) pods. The germinated seedlings were multiplied and planted out with the already available protocol.

Variability in the seedling progenies was assessed at monthly intervals for morphological characters. The hybrids obtained from intraspecific crosses *a 82* x *a 94* and *vv 97/84* x *a 94* were found vigorous with respect to plant growth. The hybrid progenies from interspecific cross *V. t* x *V. p* showed remarkable variation in leaf shapes and four leaf shapes broadly ovate, oblong lanceolate, lanceolate and linear lanceolate were observed in the progenies against narrow lanceolate in *V. t* parent and oblong-elliptic in *V. p* parent. Simple and branched stem type were observed in the progenies of interspecific cross (*V. t* x *V. p*) and intraspecific crosses (*a 82* x *a 94* and *vv 97/84* x *a 94*) against normal simple stem in the parents. Single and double aerial roots were produced from single node in the progenies of interspecific cross (*V. t* x *V. p*) and intraspecific crosses (*a 82* x *a 94* and *a 425* x *a 94*) against single root origin in the parent plants. Variation was also observed for number of leaves, plant height, leaf size, leaf colour and internodal length.

Molecular characterisation of selected parents and hybrid progenies was done using RAPD technique. Heirarchical cluster analysis of RAPD data revealed variability between parents and hybrid progenies of intra/interspecific crosses. The variation in the parents and progenies of interspecific cross (*V. t* x *V. p*) ranged from 8 - 40 per cent. The intraspecific crosses *a 82* x *a 94*, *a 94* x *a 82*, *a 425* x *a 94* and *vv 97/84* x *a 94* showed variation range of 16 - 36 per cent, 4 - 31 per cent, 10 - 34 per cent and 12 - 28 per cent respectively.

The results obtained from morphological and molecular characterisation of parents and progenies confirmed that variability was induced in vanilla through intra/interspecific hybridisation and embryo culture technique.