Vimala Jose “Studies on genetic variability in open pollinated progenies of vanila” Thesis. Indian Institute of Spices Research, University of Calicut, 2005
Review of Literature
Origin

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

Morphology

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

In *V. planifolia* the inflorescence is axillary, simple, only rarely branched, 5-8 cm long, up to 20-30 flowers, opening from base upwards, generally with only 3 open at a time, each lasting one day. The rachis is stout, often curved, and 4 -10 mm diameter. The bracts are rigid, concave, persistent, 5 -15 mm long.
Flowers are large, waxy, fragrant, pale greenish yellow 10cm diameter; pedicel very short. The interior cylindrical, tricarpellary ovary often curved, 4 -7cm long and 3 -5 mm diameter. Three oblong lanceolate sepals, obtuse to sub acute, slightly reflexed at the apex, 4-7 cm long. The two upper petals resembles sepals in shape, but slightly smaller. The lower petal is modified as a trumpet - shaped labellum or lip, which is shorter than the other perianth lobes and is 4-5 cm long and 1.5-3 cm broad. It is attached to the column which it envelops. The tip of the lip is obscurely 3-lobe and is irregularly toothed on its revolute margin. Stamen containing the pollen masses or pollinia covered by a cap, and below is the concave sticky stigma, which is separated from the stamen by the thin flap like rostellum (Purseglove, 1981).

Habit and habitat.

The Orchidaceae comprises a group of fast evolving angiosperm species (Dressler and Dodson, 1960) with a high incidence of gene exchange within and between genera through interspecific hybridization. The wide range of morphological variation found between orchid species is attributed to gene mutation; random genetic drift (Sanford, 1974) and adaptive changes in response to intense natural selection. The Orchidaceae with about 20,000 species classified into 750 genera, is one of the larger families among flowering plants, occurring widely in the tropical and temperate regions of the world. The Indo-Malaysian belt ranging from the Himalayas to New Guinea and Columbia in the South America is a major center of Orchid distribution. Based on phytogeographical evidences India was suggested as the primary or secondary centre of origin for orchids (Jain, 1986).
The *Orchidaceae* is an extremely variable group, as has been made obvious by emphasis of the tremendously high species number (Sanford, 1974). Variants resulting from plastic response of identical genotypes will not be of taxonomic significance. Genotypic variants arise by gene and chromosomal mutation and by recombination of genes, through cross over, and recombination of chromosomes through hybridization and introgression (Sanford, 1974).

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

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

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

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

History

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

**Pollination**

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

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

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

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

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

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

**Distribution**

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

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

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

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

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

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

- **Stem leafless:**

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


  Distribution: South India.

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


  Distribution: Deccan peninsula.

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

Distribution: South India.

- Stem leafy

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


Distribution: Andaman and Nicobar Islands.

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


Distribution: N-E India.

**Systematic studies**

Vanilla belongs to the family *Orchidaceae*, an advanced group of monocotyledons. The family is the largest one of the flowering plants with about 700 genera and 20,000 species. One hundred and ten species of vanilla are reported (Purseglove et al., 1981) consisting of terrestrial, climbing, epiphytic and saprophytic species. Apart from *V. planifolia* Andrews, two other cultivated species are *V. pompona*
Schiede (West Indian vanilla) and *V. tahitensis* J. W. Moore (Tahitian vanilla). The basic chromosome number for the genus vanilla is \( x = 16 \) and *V. planifolia* is a diploid with \( 2n = 32 \). In countries where vanilla has been introduced, variability is likely to be highly limited. The material is propagated vegetatively, hence of clonal origin.

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

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

**Production and trade**

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

**World Scenario**

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

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

**Shift in Production base**

Madagascar used to meet 70 per cent of the world supply during 1970’s. Now the position has changed and Madagascar stands next to Indonesia in its production. Indonesia was an insignificant producer with about 24 Mt production during late 1940’s. Later it increased the production and become the world’s second largest producer during
1980’s. Production went up in subsequent years and it stands number one in production figures, now it is having a production of 1800 Mt during 2002 (FAO, Rome, 2003). These two countries together contribute about 76 per cent of the world production. The center of maximum world production of vanilla is getting shifted in course of time, from the native Mexico it got shifted to Sub-Saharan Africa during 1950’s and from they’re to countries in pacific basin during late nineties. Unstable production and a very small number of countries that enjoy large market power characterize the supply side.

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

<table>
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<th>Country</th>
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<th>2002</th>
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Analysis of time series data on country wise production indicates that no country could maintain its hold on vanilla production for as long period. Leadership in production went from Mexico to Madagascar and there to Indonesia in 1990's. Now there is a decreasing trend in Indonesia’s production for the last few years, mainly due to unforeseen agro-climatological factors (Madan *et al.*, 2004).

**Supply, Demand gap analysis**

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

**Vanillin derivatives**

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

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

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

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

IN VITRO TECHNOLOGY

Embryo Culture

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

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

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

Protocorm like bodies (PLBs), which appear on the vegetative bud in culture, are adventive embryos analogous to the gametic embryos. There were morphological and physiological similarities between the protocorm like bodies and the protocorms of orchids (Huang *et al*, 1990). Orchid PLBs, which originates from vegetative tissues, were considered to be true somatic embryos by Moral (1974). Vegetative bud of an adult plant rejuvenates to from PLB, which has close similarities with the protocorms produced by seed germination. Ironically, the earliest commercialization of micropropagation involved the mass propagation of orchids, which involved the large-scale production of protocorms
According to Peterson (1975), bud regeneration in the region of root apical meristem could be considered a relatively rare process in higher plants.

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

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

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

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

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

**Micro propagation**

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

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

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

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

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

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

Silver nitrate, an inhibitor of ethylene activity, has been reported to be responsible for inducing positive response not only on shoot initiation and growth but also increased root number length of V. planifolia when used in micro molar concentrations (Giridhar et al, 2001). Generally, silver nitrate inhibits ethylene action through the Ag2+ ions, by reducing the receptor capacity to bind ethylene (Yang, 1985). The investigation by Giridhar et al (2001) is of importance in demonstrating efficient multiplication of shoots along with root growth under the influence of silver nitrate during in vitro propagation of V. planifolia. Ganesh et al. (1996) investigated the effects
of culture media type and BAP, GA or AgNO₃ on shoot proliferation in vitro of *V. planifolia*; good shoot proliferation was observed only in the presence of BAP.

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

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

**INDUCTION OF VARIATION**

**Seed culture**

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

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

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

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

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

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

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

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

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

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

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

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

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

Embryo development.

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

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

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

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

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

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

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

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

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

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

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

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

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

**Hybridization and embryo culture**

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

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

**In vitro polyploidy**

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

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

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

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

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

*Callus as explants for colchicine treatment:*

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

Seed as explants for colchicine treatment:

Chromosome doubling via colchicine treatment of seed explants was attempted in Sesamum indicum (Zhang et al., 2001), grass pea (Dibyendu et al., 2001), and ryegrass (Pasakinskiene, 2000). Mehra et al. (1999) studied the effect of EMS and colchicine alone and in combination on the seeds of chilli and found that combined treatments yielded higher numbers of morphological and quantitative mutations than individual treatments. Pasakinskiene (2000) could attain a polyploid production rate of 65.8% by using a high concentration of sucrose (100g/l) and vacuum (0.14 kg/cm²) to infiltrate colchicine solution in germinating embryos of ryegrass. Levites et al. (2000) suggested that colchicine treatment of the apozygotic seeds increases the proportion of the cells with high level of chromosome endo reduplication resulting in embryogenesis through sporophytic agamospermy. Increased guard-cell size is a consistent indicator of a doubling of the chromosome number (Watrous and Wimber, 1988).

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

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

**In vitro mutagenesis**

The extreme dispersibility of orchids has placed them in such a variety of habitats that mutations not only would be selected for through great variety of environmental pressures but also often could be isolated easily from the parent forms and so preserved. This would superficially give the appearance of a higher mutation rate (Sanford, 1974).
Mutation breeding is one of the methods available to the plant breeders when the crop is amenable to vegetative propagation. The generally high degree of heterozygosity, which causes a complex inheritance of genetic factors as well as a frequent polyploidy, both serious handicaps in the conventional methods of breeding, are advantageous in mutation breeding, as large variations can often be observed in the mutated populations. The most promising aspect of mutation induction in the vegetatively propagated plants, compared to the cross breeding methods is the ability to change only a very few characters of an otherwise good cultivar without altering significantly the remaining and often unique genotype (Broertjes, 1977).

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

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

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

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

Various physical mutagens, such as X-rays, gamma rays, beta rays, neutrons, lasers, electron beams and ion beams, have been used in mutation breeding either individually or in combination with chemical mutagens, such as EMS, DES, NaN3 etc. Not only the genotype of the initial plant material but also the treatment techniques play important roles in mutation induction. On the other hand, the role the genetic background of the initial materials plays in mutation induction has been studied in depth (Wen and Qu, 1996).

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

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

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

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

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

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

CHARACTERIZATION OF VARIATION

Anatomical characterization

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

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

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

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

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

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

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

Table 2: Chromosome list of the different species of Vanilla

<table>
<thead>
<tr>
<th>Name of the species</th>
<th>Meristem chromosome no:</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V. aphylla )</td>
<td>64</td>
<td>Vatsala, 1964</td>
</tr>
<tr>
<td>( V. aromatica )</td>
<td>32</td>
<td>Eftimiu-Heim, 1950</td>
</tr>
<tr>
<td>( V. barbellata )</td>
<td>32</td>
<td>Martin, 1963</td>
</tr>
<tr>
<td>( V. dilloniana )</td>
<td>32</td>
<td>Martin, 1963</td>
</tr>
<tr>
<td>( V. fragrans )</td>
<td>30-32</td>
<td>Eftimiu-Heim, 1950</td>
</tr>
<tr>
<td>( V. haapape )</td>
<td>64</td>
<td>Tonnier, 1951</td>
</tr>
<tr>
<td>( V. hartii )</td>
<td>32</td>
<td>Eftimiu-Heim, 1950</td>
</tr>
<tr>
<td>( V. imperialis )</td>
<td>32</td>
<td>Eftimiu-Heim, 1950</td>
</tr>
<tr>
<td>( V. moonii )</td>
<td>32</td>
<td>Eftimiu-Heim, 1950</td>
</tr>
<tr>
<td>( V. papeno )</td>
<td>32</td>
<td>Eftimiu-Heim, 1950</td>
</tr>
<tr>
<td>( V. phaeantha )</td>
<td>32</td>
<td>Martin, 1963</td>
</tr>
<tr>
<td>( V. planifolia )</td>
<td>28-32</td>
<td>Eftimiu-Heim, 1950; Hoffmann, 1929</td>
</tr>
<tr>
<td>( V. pompona )</td>
<td>32</td>
<td>Eftimiu-Heim, 1950; Martin, 1963</td>
</tr>
<tr>
<td>( V. roscheri )</td>
<td>36</td>
<td>Krupko et al, 1954</td>
</tr>
<tr>
<td>( V. thaitii )</td>
<td>32</td>
<td>Eftimiu-Heim, 1950</td>
</tr>
</tbody>
</table>

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

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

**Somatic association**

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

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

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

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

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

**Molecular basis of RAPD polymorphisms**

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

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

**RAPDs —A breif history**

Two groups developed RAPD assay like the classical Hardy-Weinberg model of population genetics, independently and simultaneously during the year 1989-1990. Williams *et al.* (1990) described the use of single short oligonucleotide primers of arbitrary sequence for the amplification of rapidly distributed segments of genomic DNA.
The RAPD and AP-PCR techniques are based on the amplification of the DNA segments between pairs of small inverted DNA sequences scattered throughout the genome and provide an innovative technology for DNA mapping fingerprinting and related research (Waugh and Powell, 1992). This advance has resulted in a DNA marker technology that of the DNA can be readily employed because of the wide availability of synthetic oligonucleotides. RAPD polymorphisms result from mutations or rearrangements at or between oligonucleotide primer binding sites in a genome. These polymorphisms can be analyzed on either agarose or acrylamide gels, and manifest themselves in the presence or absence of an amplification products. RAPDs are visualized as dominant markers. They require extremely small amounts of genomic DNA, which can be of low quality but also eliminate the need of DNA blotting and the use of radioactivity.

**Various applications of RAPD polymorphism**


In 1990, Williams et al, found that DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Sosinski and Douches (1996) reported using polymerase chain reaction based DNA amplification to fingerprint North American potato cultivars.
Genetic relationships and origins of six grape cultivars of *Galicia* using RAPD markers were done by Vidal and colleagues in 1999. Use of RAPDs for identification of rice accessions was reported by Shuichi *et al.* (1992). Molecular characterization of *Piper nigrum* L using RAPD markers was succeeded by Pradeep Kumar and colleagues (2001).

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

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

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

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

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

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

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

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

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

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

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