SEEDLING AND SOMACLONAL VARIATION AND THEIR CHARACTERIZATION IN VANILLA.

Thesis submitted to

University of Calicut

for the award of the degree of **Doctor of Philosophy** in Botany

By

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UNIVERSITY OF CALICUT

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CERTIFICATE

This is to certify that the thesis entitled '***Seedling and somaclonal variation and their characterisation in Vanilla.***' submitted by Mrs. Minoo Divakaran, for the award of the degree of Doctor of Philosophy in Botany, University of Calicut, contains the results of bonafide research work done by her during 1996 – 2002, at the Indian Institute of Spices Research, Calicut, under my supervision and guidance. No part of this thesis has been submitted to any other university for the award of any other degree or diploma. All sources of help received by her during the course of this investigation have been duly acknowledged.

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DECLARATION

I hereby declare that this thesis '*Seedling and somaclonal variation and their characterization in Vanilla.*' submitted by me for the award of the degree of Doctor of Philosophy in Botany, University of Calicut, contains the results of bonafide research work done by me at the Biotechnology Laboratory, Indian Institute of Spices Research, Calicut under the supervision and guidance of Prof. (Dr.) K. V. Peter. This thesis or part of it has not been submitted to any other university for the award of any other degree or diploma. All sources of help received by me during the course of this study have been duly acknowledged.

Place: Calicut

Date: 7-12-2002

(Minoo Divakaran)

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Dedicated

to… my parents, who made me dare a dream
to… my husband, who made my dream into a reality
to… my teachers who guided through the way
and
to... God, the essence of all.

Every active mind must form opinions without direct evidence, else the evidence too often would never be collected - *RA Fischer*

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Introduction

Fig. 1 Cultivated vanilla, *Vanilla planifolia* Andrews

Vanilla, an important and popular flavouring material and spice, is the fermented and cured fruit of the orchid, *Vanilla planifolia* Andrews (*syn. V. fragrans* Salisb.) (Fig.1). The beans of two other species namely, *V. pompona* Schiede (West Indian vanilla) and *V. tahitensis* J. M. Moore (Tahiti vanilla) are also commercially used as vanilla, but are of inferior quality. The fruits are usually referred to as vanilla beans. Vanilla extract is obtained by macerating the cured beans in alcohol. Vanilla is used extensively to flavour ice creams, chocolates, beverages, cakes, custard, puddings and other confectionery. It is also used in perfumery and to a small extent in medicine as nerve stimulant.

Vanilla planifolia is a tropical climbing orchid, indigenous to wet low land forests of South East Mexico, Gautemala and Central America. It is now cultivated in other parts of the tropics, especially in the Malagasy Republic, Indonesia, Reunion and the Comoro Islands (Purseglove *et al.*, 1981).

The fragrance and flavour of vanilla beans are due to numerous aromatic compounds produced during the curing operation, among which vanillin is most abundant. The widest use of highly purified vanillin is as a chemical intermediate in the

synthesis of numerous pharmaceutical products as vanillin is an antioxidant (Gopinath, 1994). The flavour of vanilla from different parts of the World varies due to climate, soil, extent of pollination, degree of ripeness at harvesting and method of curing. Vanillin can now be produced synthetically and is much cheaper than natural vanillin. However, the flavour of vanilla beans is far superior to that synthetic vanillin due to the presence of other flavour compounds in the natural product and this seems to be the deciding factor for the preference of natural product (Purseglove *et al.*, 1981). Due to the health hazards of synthetic vanillin, the importance of natural vanillin is increasing.

Vanilla has been used as a flavouring agent since 14th century by Azects. In the 16th century the Spaniards introduced vanilla into Spain, and they established factories for the manufacture of chocolate flavoured with vanilla. The introduction of vanilla continued and spread to England, Reunion, Mauritius and Malagasy Republic. Vanilla cultivation started in Java in 1846. The cultivation spread to Seychelles, West Indies, Ceylon, Uganda and Indonesia. Although the plants grew well in the Old World tropics fruits were not produced, until it was discovered that the vanilla blooms could be pollinated by hand, due to the absence of natural pollinators. In its natural habitat in Central America, *V. planifolia* is pollinated by *Melipona* bees and tiny humming birds. Vanilla was introduced to India in 18th century (Spices Board, 2000).

Vanilla is the second most expensive spice traded on the world market. The world wide annual consumption was 1900 tonnes in 1995, with 1400 tonnes imported to USA alone. The total area under vanilla cultivation in the world during 1999 was 37,525 ha with a production of 4,403 MT. At present Indonesia is the largest producer of vanilla, producing about 2,102 MT from about 9,689 ha

followed by Malagasy Republic, which produces about 1,650 MT in about 25,000 ha. The other important vanilla cultivating countries are Mexico with an area of 900 ha and production of 300 MT, Comoro with an area of 700 hectares and production of 150 MT and Reunion Islands with area of 600 ha and production of 30 MT (Spices Board, 2000).

Vanilla beans differ in chemical, physical and organoleptic properties not only between the species, but also within a species, depending on the geographical source and the physical form or grade. Commercial vanilla is obtained after special processing of the fruits. The primary quality determinant for cured vanilla beans is the aroma/flavour character. Other factors of significance in quality assessment are the general appearance, flexibility, the length and the vanillin content. The relative importance of these various quality attributes is dependent upon the intended end-use of the cured beans. Traditionally, the appearance, flexibility and size characteristics have been of importance since there is a fairly close relationship between these factors and the aroma/flavour quality. Consequently, preferences are expressed by consumers for particular types for certain applications. Malagasy Republic, Comoros and Reunion Islands, who produce 'Bourbon' vanilla, dominate the trade in true vanilla at present. Indonesia, Mexico, and Tonga are next in importance, followed by the West Indies and South America.

Vanilla is always propagated by stem cuttings. The production of flowers begins by 3rd year and maximum reaches in 7 to 8 years. The number of beans per vine varies greatly and is usually about 30 - 150. The time between flowering and harvesting is 6 - 9 months. Yields are variable and a good vanillery is said to yield

about 500 - 800 kilograms of cured beans per hectare per annum during a crop life of about 7 years ((Purseglove *et al.*, 1981).

Vanilla planifolia is a crop little different from the wild progenitors. This can be attributed to a limited breeding and recent domestication. The germplasm base of *V. planifolia* is further threatened by deforestation and over collection. Since the narrow primary gene pool is evidently threatened, the secondary gene pool, that is, the close relatives of *V. planifolia*, becomes more important as a source of desirable traits like self-pollination, higher fruit set and disease resistance. Some of these traits are found in its near relatives (Rao *et al.*, 2000).

Vanilla was introduced into India in the 18th century and commercial cultivation is limited to Kerala, Karnataka and Tamil Nadu. The area under vanilla cultivation in India is estimated to be around 1000 ha and production of cured beans in 1999 – 2000 was 6-8 tonnes (Spices Board, 2000). Much of the planting material of vanilla was originated from limited clonal source and hence practically no variability is available for crop improvement. This leads to monoculture making vanilla susceptible to diseases and pests. The most common diseases are of fungal origin *viz.*, foot rot and wilting caused by *Phytophthora meadii* (Suseela and Thomas, 2000), *Fusarium oxysporum, Calospora vanillae*, *Sclerotium* rot (Thomas and Suseela, 2000), leaf rot, blights and brown spots or anthracnose by *Colletotrichum gloeosporioides* (Gopinath, 1994). Vanilla beans are quite susceptible to infection by *Penicillium* and *Aspergillus* molds and this generally occurs during the conditioning and subsequent storage (Bouriquet, 1954). Vanilla beans are prone to attack by mites, (*Tyrophagus* species), which imparts a disagreeable odour to the beans.

Broadening the genetic base of vanilla by introduction from its native zone or from areas of diversity is difficult due to severe restrictions imposed by the host countries for germplasm exchange. This leaves behind no option but to look for other sources of variability. Vanilla is suspected to be highly heterozygous because of its cross-pollinated nature and variations could be obtained in the segregating seedling progenies. Nair and Ravindran (1994) reported mitotic association in vanilla, which could give rise to further variation in seedling progenies. Somaclonal variation can also form important source of novel genetic variability. Interspecific hybridization will help in bringing together certain useful characters into cultivated vanilla from wild relatives. The resultant variation among the progenies, somaclones and hybrids could be used in crop improvement especially to develop high quality, self-pollinating and disease resistant genotypes.

Another major challenge is to conserve the gene pool of vanilla from the onslaught of habitat destruction, over collection and climate changes. *In vitro* conservation can be a very practical approach to augment the conventional efforts adopted to conserve these plant species.

The main objectives of the present investigation are to study the existing variability in some of the vanilla cultivars and species available in India, to broaden the spectrum of variations in vanilla gene pool using conventional as well as biotechnological approaches, to characterize the extent of variability generated and to standardize protocol for *in vitro* conservation of vanilla germplasm. Attempts were made to standardize micropropagation, callus regeneration and protoplast culture protocols, produce large number of selfed progenies, somaclones and interspecific hybrids. Attempts were also made to characterize some of the species, progenies, somaclones and hybrids using morphological,

cytological, biochemical and molecular markers, wherever possible. A few of them were also screened for reaction against *Phytophthora meadii* and *Fusarium oxysporum,* the two major pathogens affecting vanilla plantations.

This study gives us better understanding of existing variability in Indian vanilla, and newer sources of variability for increasing production and productivity of vanilla through crop improvement.

The various aspects attempted in the present study and the organization of work elements are represented as flow chart in Fig.2.

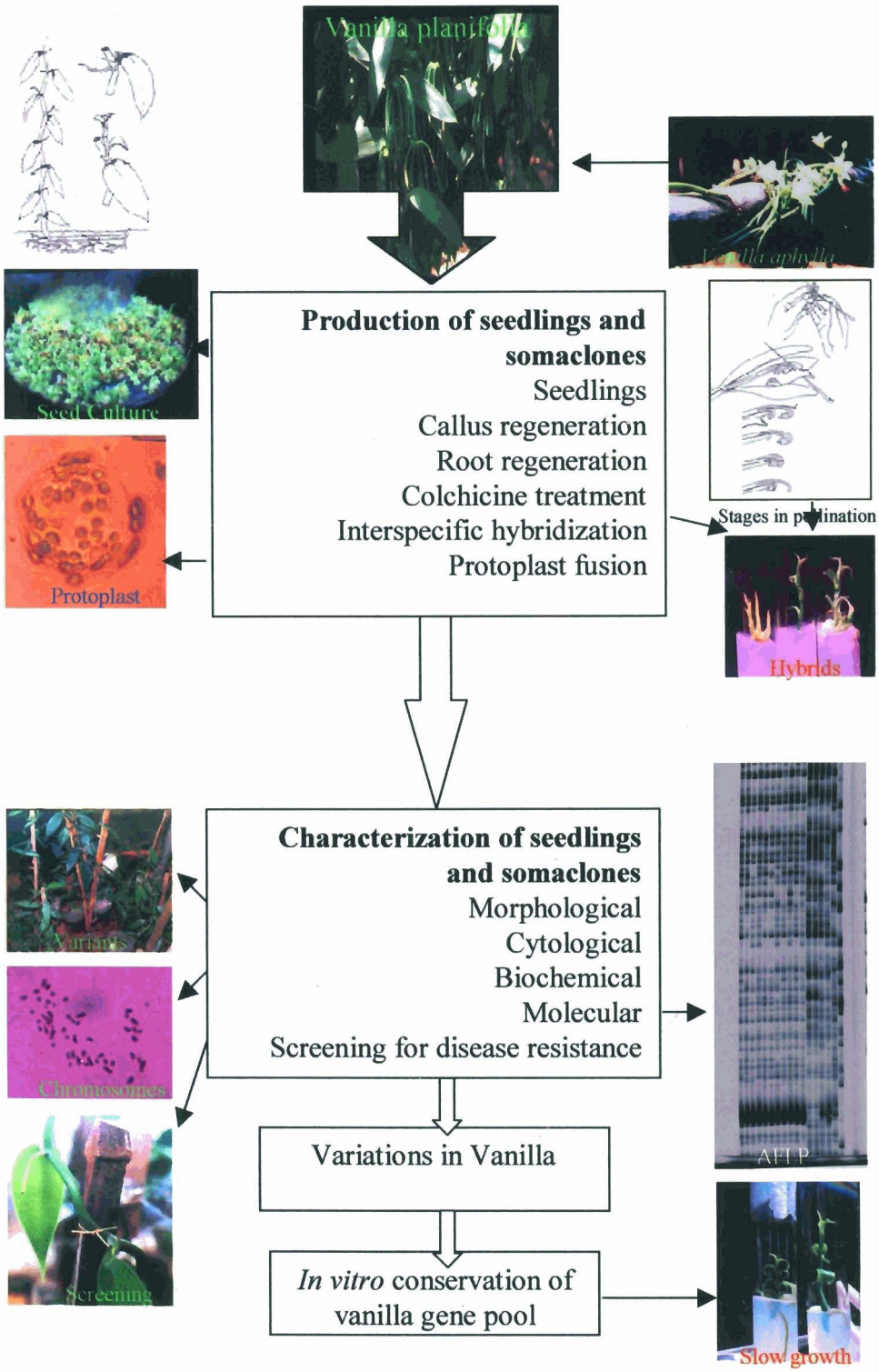


Fig. 2 Flow chart showing various aspects attempted in the present study

Review of Literature

Minoo Divakaran “Seedling and somaclonal variation and their characterization in Vanilla ” Thesis. Indian Institute of Spices Research Calicut, University of Calicut, 2002

Review of Literature

VANILLA

Vanilla, an important and popular flavouring material and spice, is the fully grown fruit of tropical climbing orchid, *Vanilla planifolia* Andrews, (syn. *V. fragrans* Salisb.), harvested before it is ripe, and fermented and cured. Vanilla belongs to the family Orchidaceae, which is the largest family of flowering plants. The genus *Vanilla* comprises of about 110 species, distributed in tropical parts of the world (Purseglove *et al.*, 1981).

Commercial vanilla is obtained from three different species of *Vanilla*, namely, *V. planifolia* Andrews (Mexican vanilla), *V. pompona* Schiede (West Indian vanilla) and *V. tahitensis* J. M. Moore (Tahiti vanilla). The most important one is *V. planifolia*, from which almost all vanilla fruits, come from (Ferrão, 1993). The other two species are cultivated rarely, yield an inferior quality of vanilla. Another species, *V. barbellata*, is used in indigenous medicine. *Vanilla phaeantha* Rchb.f, is reported to be resistant to *Fusarium* root rot (Purseglove *et al.*, 1981).

The name vanilla is derived from the Spanish 'vainilla' which means a small pod, due to the great similarity between this fruit and a true pod and its species name, 'planifolia', refers to the broad, flat leaf of the plant (Ferrao,1992; Mabberley, 1993). Vanilla stems are thick and fleshy green, the leaves are alternate, long elliptical, sessile and bright green. The flowers in clusters appear in the leaf axils. The plant blooms three years after cuttings are planted and the green fruits take 5-7 months to mature and many have seeds upto 90,000. The fruit is scentless when harvested, it has a length between 10-25 cm and a weight of 5g to 30g (Ferrao, 1993). The fully grown fruits or pods, usually referred to as beans, yield vanillin, the popular flavouring material and spice. The substance chiefly

responsible for the unique fragrance and flavour of the vanilla bean is vanillin (Purseglove *et al.*, 1981).

Origin and History

The use of vanilla by the *Aztecs* was recorded by the Spanish conquistadors. Correll (1953) states the "Bernal Diaz, a Spanish officer under Hernando Cortes, was perhaps the first white man to take note of this spice when he observed Montezuma, the intrepid Aztec emperor, drink "chocolatl", a beverage prepared from pulverized seeds of the cacao tree, flavoured with ground vanilla beans which the Aztecs call 'tlilxochitl', meaning 'black pod'. Vanilla beans were considered to be among the rarer tributes paid to the Aztec emperor by his subject tribes. Legend has it that Cortes in 1520 was given chocolate flavoured with vanilla by Montezuma, served in golden goblets. Local aristocracy used it to flavour chocolate, a custom still practised today (Brosse *et al.,* 1989). Bernardino de Sehagun, a Franciscan friar, who arrived in Mexico in 1529, wrote about vanilla, saying the Aztecs used it in cocoa, sweetened with honey, and sold the spice in their markets, but his work, originally written in the Aztec language, was not published until 1829-1830. The Spaniards early imported vanilla beans into Spain, where factories were established in the second half of the sixteenth century for the manufacture of chocolates flavoured with vanilla.

Fransisco Hernandez, who was sent to Mexico by Philip II of Spain, gave an illustrated account of vanilla in his Rerum Medicarum Novae Hispaniae Thesaurus, which was first published in Rome in 1651. In it, he translated "tlilxochitl" as "black flowers', a fallacy which Correll (1953) say remained in the literature for many years, although the flowers are greenish yellow in colour.

Hugh Morgan, apothecary to Queen Elizabeth I of England, suggested vanilla as flavouring in its own right. He gave some cured beans to the Flemish botanist, Carols Celsius, in 1602 and the latter describes them in his Exotic rum Libra Deem of 1605. William Dampier observed vanilla growing in 1626 in the Bay of Capuche in southern Mexico and in 1681 at Boca-Toro in Costa Rica. Formerly, vanilla was used in medicine, as a nerve stimulant, and along with other spices had a reputation as an aphrodisiac. It was also used for scenting tobacco.

The plant appears to have been taken to England prior to 1733 and was then lost (Purseglove, 1972). It was re-introduced by the Marquis of Blandford at the beginning of the nineteenth century and flowered in Charles Greville's collection at Paddington in 1807, Greville supplied cuttings to the botanic gardens in Paris and Antwerp. Two plants were sent from Antwerp to Buitenzorg (Bogor), Java, in 1819, only one of which survived the journey. It flowered in 1825, but did not fruit. Plants were taken to Reunion and from there to Mauritius in 1827. Vanilla was taken to the Malagasy Republic about 1840. The Totonacas people of this region still grow vines with almost religious devotion because to them it was the gift of the Gods. It is not uncommon to have a few vines growing around their houses, which they water every day as if they were their most valuable possession. Also they make with the beans all kinds of crafts such as frogs, baskets, little houses, etc. which people hang in the rearview mirror of their cars as an air purifier with a very artistic twist. In the linen closets of Veracruz, it is very common to find a few beans among the bedding sheets.

Crop introduction

The Dutch introduced vanilla into Java (Indonesia), a former European colony in East Indies, at the beginning of the nineteenth century and the French

did the same in the Reunion Island, Mauritius and Malagasy Republic, all located in the Southwest Indian Ocean. Vanilla cultivation on a systematic basis was introduced into Java in 1846 by Teysmann, Director of the Buitenzorg (now Bogor) Botanic Gardens. It was the discovery of a satisfactory method of hand pollination and the failure of the sugarcane crop in 1849-56 that gave impetus to cultivation of the crop in Reunion Islands. Vanilla cuttings are said to have been first introduced into the Seychelles in 1866. Vanilla was introduced from Manila to Tahiti by Hamelin in 1848, where an important industry developed. Cultivation of the crop began in the Comoros Islands in 1893 and soon spread throughout the islands. Vanilla was cultivated as early as 1839 in Martinique in the West Indies and probably about the same time in Guadeloupe. The plant was introduced into Uganda from Ceylon in 1912 and now has a small production. Former French island possessions are now the main producers of vanilla, with the Malagasy Republic as the major producer followed by Indonesia. Mexico still exports this spice. Vanilla has been widely introduced throughout the tropics where climatic conditions are suitable, but has achieved little importance, except in the countries mentioned above.

Although the plant grows well in the Old World Tropics, fruits were not produced because of the absence of natural pollinators. In its native home in Central America, vanilla is pollinated by bees of the genus *Melapona* and humming birds. When it was taken to other parts of the world, it did not produce beans until it was discovered that the small orchid blooms have to be pollinated by hand. This is due to the separation of the stamen from the stigma by the rostellum. It was not until Professor Charles Morren of Liege discovered the artificial means of pollination for the production of capsules in 1836 and Edmond Albius, a former

slave in Reunion, developed a practical method of artificial pollination in 1841, and which is still used, that commercial production was possible in the eastern hemisphere away from the center of origin. In 1846, Teysmann Java discovered a satisfactory method of hand pollination (Purseglove *et al.,* 1981; Ferrão, 1993).

Gregory *et al.,* (1967), have shown that the growth regulating chemicals 2,4-D (2,4-Dichloro phenoxy acetic acid), Dicamba (2-methoxy 3,6-dichloro benzoic acid) and IAA-IBA (Indole 3-acetic acid –Indole 3-butyric acid) will induce the development of parthenocarpic fruits and give a high percentage of fruit set. Thus, it may be possible to obtain vanilla beans without the need for hand pollination.

The vines grow around trees and when the flowers fall, the bean stops growing, thus it is very important to keep the flower from falling. Hence in Mexico, it was grown under the jungle canopy to protect it from high winds and hurricanes common to the tropics. It is important not to over pollinate the vine because this will lead to drying and death of the vine.

Botany

The genus *Vanilla* has about 110 species, distributed throughout the tropics (Bouriquet, 1954). It belongs to Orchidaceae which is the largest family of flowering plants with about 700 genera and 20,000 species. Etymologically, the word vanilla came from the Spanish *vainilla,* which means a small pod, due to the great similarity between this fruit and a true pod (Purseglove *et al.,*1981; Ferrão, 1992; Mabberley, 1993). The stems are thick and fleshy green; the leaves are alternate, long elliptical, sessile and bright green. The flowers, in clusters, appear in the leaf axils. They live only 8 hours and die if fertilization fails to occur. The plant blooms three years after the cuttings are planted and the yellow greenish

fruits many have up to 90,000 seeds, taking five to seven months to mature. The fruit is scentless when harvested, it has a length between 10 to 25 cm and a weight of 5 to 30g (Ferrão, 1993).

Cytology

Earlier studies have shown that the basic chromosome number for the genus is 16 (x=16) and V*anilla planifolia* is a diploid with 2n=32 (Heusser, 1938). The chromosome number of *V. pompona* and *V. tahitensis* is 2n=32 (Purseglove *et al.*, 1981). However, Nair and Ravindran (1994) reported that somatic chromosome number of vanilla ranged from 2n = 20 - 32, with 28 being the most frequent number. Ravindran (1979) reported abnormalities in pollen grain mitosis combined with high pollen sterility. He also reported chromosome associations during pollen mitosis. The above studies indicate the possible occurrence of cytotypes in the seedling progenies of vanilla.

Habit

Vanilla planifolia is a fleshy, herbaceous perennial vine, climbing by means of adventitious roots on trees or other supports to a height of 10-15 meters. In cultivation it is trained to a height which will facilitate hand pollination and harvesting. It is also grown on temporary supports (Fig.3). The plant flowers only once in a year over a period of about two months. In Mexico, it flowers around April-May while in Malagasy Republic, Reunion, Comoro Islands and India, it flowers between November and January. The pendulous capsule, commonly called as bean is 10-25 cm long and 5-15 mm in diameter. It is aromatic on drying myriads of very minute globose seeds about 0.3mm in diameter which are liberated by splitting open the beans longitudinally. In commercial production the

capsules are harvested before they are ripe. Cured fruit pods enter international trade either in the powdered form or as solvent extracts.

Pollination

The flower of vanilla is so constructed that self pollination is impossible due to separation of the stamens from the stigma by a membranous rostellum. Hand pollination is necessary for fruit set and development. In Mexico and Central America where vanilla is indigenous, some of the flowers are pollinated by bees of the genus *Melapona.* Nectar is secreted by the base of the lip and the flowers are sweet scented. Humming birds also act as pollinating agents. Even in Mexico, only small percentage of fruits set naturally and hand pollination is required. Flowers open from the base of the raceme upwards and usually 1 or 2 open on the inflorescence at a time. The flowers open early in the morning, remain receptive for 8 hours and wither the following day. Fruit set is highest when pollination is done early on a bright morning. If, fertilization is successful, the flowers remain on the rachis. The flowers drop off in 2 or 3 days, if the fertilization is unsuccessful. Thus it is possible to judge the fruit set and to discontinue pollination when the desired number has been obtained.

Cultivars

Except in the countries of origin, vanilla is likely to be of clonal origin and very little variation can be expected. As per the present information, no difference in cultivated types of *V. planifolia* is recognized. The vanilla plantations of Reunion, Mauritius, Seychelles and the Malagasy Republic have all derived from a single cutting (Lionnet, 1958).

Major types of vanilla

Vanilla beans differ in chemical, physical and organoleptic properties not only according to the species, but also within a species, depending on the geographical source and the physical form or grade. Consequently, preferences are expressed by consumers for particular types for certain applications. The trade in true vanilla (from *V. planifolia*) in the twentieth century has been dominated by Malagasy Republic, the Comoros Islands, and Reunion; producers of "Bourbon" vanilla. Indonesia, Mexico, and Tonga have been next in importance, followed by the West Indies and South America. Other minor and intermittent suppliers are Rodriguez, Mauritius and Seychelles, Uganda, and Fiji. During the last ten years, Indonesia, and also Tonga in smaller proportion, has improved the quality and the quantity of their production. The different commercial types of vanilla are given below.

Mexican vanilla

Mexico has traditionally been regarded as the supplier of vanilla possessing the finest aroma and flavour. Consumers accustomed to artificial vanilla, however, often dislike the aroma of Mexican vanilla.

Bourbon vanilla

This has a deeper "body" flavour than Mexican vanilla but lower aroma. Bourbon vanilla is frequently "frosted" and is marketed in grades of whole and split beans and a category known as "vrac", comparable to Mexican cuts.

Indonesian vanilla

The main area of production is initially Bali and South Java. But during the last five years, vanilla culture has spread all over Sulawesi, Sumatra (North and South), Lomboc, Flores and Timor Timor. Generally speaking, Indonesian vanilla is known as a mixed quality with little attention paid to grading. However,

quality was improved year after year and today it is possible to find Indonesian vanilla comparable to "bourbon" vanilla. Indonesian vanilla possesses a deep full-bodied flavour well appreciated in America.

South American and West Indian vanilla

The small volume of true vanilla entering trade from this region is more similar in properties to Bourbon vanilla than Mexican vanilla. It is poor in quality. *Vanilla pompona* is the main source of West Indian vanilla.

Tahiti vanilla

The vanilla produced in French Polynesia is obtained from *V. tahitensis* and possesses a characteristic aroma of commarin and usually has a lower vanillin content than true vanilla. It is generally less favoured for flavouring due to its relatively high volatile-oil content which can result in cloudy extracts. Tahiti vanilla is exported in five main grades which rarely frost.

Vanillons (Guadeloupe vanilla or Antilles vanilla)

This is produced from *V. pompona* in certain of the former French West Indian islands, principally in Guadeloupe. Vanillons has a low vanillin content and possesses a characteristic floral aroma, bearing similarities to Tahiti vanilla. It is mainly employed in perfumery as its flavour is considered to be poor and it tends to provide gummy aqueous alcohol extracts. Earlier vanillons were exported in three grades, but today this quality is not traded anymore (Purseglove *et al.*, 1981).

Ecology

Climate

Vanilla usually grows climbing on trees in wet tropical lowland forests from sea-level to 600 meters. It thrives best in hot, moist, insular climate, with

frequent, but not excessive rain. The optimum temperature is 21-32 ^{0}C, with an average around 27^{0}C and with an evenly distributed rainfall of 2000-2500 mm per annum, but with two dry months to check vegetative growth and make the vines to flower. Regions with a prolonged dry season are not suitable for vanilla cultivation.

Soil

Vanilla, in general prefers sloping land with soil of high organic matter and grows best under 40-50% of normal sunlight intensity. A shaded area, rainfall between 1000-3000mm and an average temperature of 23^{0}C to 29^{0}C year round are the preferred conditions (Havkin-Frenkel and Dorn, 1997). Soil parameters such as texture and pH appear to be more important than nutrients. The best soil appears to be limestone with a pH of 6.0-7.0 and a deep layer of mulch that provides nutrients over and around the roots (Purseglove *et al.*, 1981). A climbing orchid, vanilla needs support for growth, convenient access for pollination and harvesting. Fast growing trees with small branches providing shade but not competing for water, is preferred (Ranadive, 1994). The most suitable soil is friable soil with adequate drainage, and a thick surface layer of humus or mulch in which the roots can spread.

Cultivation

Propagation

Commercial vanilla is always propagated by stem cuttings. Cuttings should be taken from healthy vigorous plants and may be cut from any part of the vine. The length of the cutting is usually determined by the amount of planting material available. Short cuttings, 20 cm in length, will take 3 to 4 years to flower and fruit. Cuttings, 90-100 cm in length, are usually preferable as they tend to

flower early. In some regions, cuttings 2-3.5 m in length are used. When available, with their free ends hanging over supports; these will flower and fruit in 1 to 2 years. In short cuttings, at least two nodes should be left above ground. The portions above ground should be tied to the support until the aerial roots have obtained a firm grasp. Cuttings are usually planted in situ, but they can be started in nursery beds when necessary. Because of their succulent nature, cuttings can be stored or transported for a period of up to two weeks if required.

For breeding purposes, vanilla can be grown from the seeds. The seeds should be disinfected, washed in sterile distilled water and cultured on nutrient medium (Knudson 1950) . Germination of vanilla seeds is better if the cultures were maintained in dark incubator at 32^0C. Seeds of interspecific crosses between *V. planifolia* and *V. pompona* required a higher temperature of 34^0C for germination. Hybridization and production of plants from seeds have been carried out in Puerto Rico and Malagasy Republic.

Supports

The vines of vanilla require some form of support to climb, and also light shade; too dense shade and full sunlight are both deleterious. The ideal support tree should be quick-growing, providing light, checkered shade, have sufficient low branches providing easy access to the vanilla; be strong enough to support the vines in strong winds; and be easily pruned when necessary. It is an advantage to plant large cuttings since this may flower early. The two trees most commonly used in the Malagasy Republic are the physic nut, *Jatrpopha curcas* L, which can be propagated from cuttings or grows rapidly from seeds, and *Casuarina equiserifolia* L. In the early stage, lateral shade may be provided by bananas or maize. Windbreaks should be provided wherever necessary. If vanilla is grown up

posts or trellises, it will also be necessary to supply some form of partial shade (Fig.3). In Indonesia, it is grown on *Glyrcidia.*

Planting and after-care

The cuttings are usually planted about 3 meters apart at the base of the supporting trees or poles. A spacing of 1.2 - 1.5 meters in rows 2.5 – 3.0 meters between rows is also sometimes recommended. In the early vanilleries, the plants were often planted so close together that they became entangled. This usually gave very high initial yields, but presented grave problems of access and disease control . It is necessary to train the vines so that they may grow at a convenient height for pollination and harvesting. The vines are twisted round the lower branches of the supporting tree or over the lattice of the trellis so that they may hang down. Care is required so as not to tear or bruise the leaves, branches or roots.

Flowering

The top 7.5 - 10 cm of the vine is usually pinched 6 - 8 months before the flowering season to encourage the production of inflorescences in the axils of the leaves on the hanging branches. Vanilla usually starts flowering in its third year after planting, but depends on the size of the original cuttings planted. The maximum production of flowers is reached in 7 to 8 years. Given proper care this may continue for years, but in some vanilleries the production period is shorter.

Fig.3 Cultivation of *Vanilla planifolia*

a. Fruit set in vanilla, b, c. *V.planifolia* – a bearing vine, d. Vanilla cultivation on live standards, e. Vanilla cultivation under shade.

As the flower opens, the requisite number is hand-pollinated. Only the flowers on the lower side of the raceme are pollinated so that the fruits may hang perpendicularly to produce straight beans; those on the upper side would produce crooked beans of inferior quality. Usually only one flower opens in each inflorescence in a day and is receptive for about 8 hours. Consequently, most of the pollination must be done in the mornings and is continued for 1 or 2 months until the required number of fruits have set. The number of inflorescences and flower per vine, and the number which are pollinated and allowed to produce mature beans, 8 - 10 flowers on 10 - 20 inflorescences are pollinated, of which 4 - 8 capsules are allowed to grow to maturity on each size. If pollination has been a failure, the flowers drop off the next day. When the desired number of fruits has set, the remaining buds are removed, which may be done by clipping off the tip of the inflorescence. Damaged and malformed capsules are removed during growth. The final number of beans per vine varies greatly and is usually about 30 – 150 (Ferrão, 1993).

Harvesting

The time between flowering and harvesting is 6 - 9 months. The pods are harvested when they are fully grown and as they begin to ripen, as shown by the tips becoming yellow. It is essential to pick the pods at the right stage as immature pods produce an inferior product and if picked too late they will split during curing. The commercial fruit has a black to brown colour and a soft and flexible touch, due to several oxidations that take place inside it. Usually, 6 kg of green fruits produce 1 kg of commercial vanilla. The fruit must not have a water content above 25% and should always be kept in a very tight box to avoid the loss of aroma (Boisvert et Hubert, 1998).

Diseases and Pests

Vanilla is affected a large number of pests and diseases. Diseases are more serious than pests and sometimes cause heavy losses. The important diseases are root rot caused by *Fusarium batatatis* var.*vanillae*, wilt and stem rot caused by *Fusarium oxysporum* var. *vanillae;* foot rot and wilting caused by *Phytophthora meadii* (Suseela and Thomas, 2000); *Sclerotium* rot (Thomas and Suseela, 2000); anthracnose caused by *Calospora vanillae*, leaf spot and shoot tip rot caused by *Colletotrichum vanillae;* leaf rot, blights and brown spots or anthracnose caused by *Colletotrichum gloeosporioides* (Gopinath, 1994).

A few minor pests like lamellicorn beetle (*Hoplia retusa*) and Ashygrrey weevil (*Cratopus retusa*) sometimes infests vanilla plantations, makes holes in flowers and often destroy the column. A few caterpillars, earwigs, snails and slugs damage tender parts of the plant such as, shoot, flower buds and immature beans (Spices Board, 2000).

Vanilla beans are quite susceptible to infection by *Penicillium* and *Aspergillus* molds and this generally occurs during the conditioning and subsequent storage (Bouriquet, 1954). Vanilla beans are prone to attack by mites of the *Tyrophagus* species, which imparts a disagreeable odour to the beans.

Area and production

Vanilla is the second most expensive spice traded on the world market, second only to saffron (Ferrao,1992). The world wide annual consumption was 1900 tons in 1995, with 1400 tons imported to USA alone. The total area of vanilla cultivation in the world during 1999 was 37,525 ha and production 4,403MT. Though Malagasy Republic holds the prominent position having a cultivated area of 25,000 ha, of late, Indonesia has started to produce more with a

production of 2,102 MT from 9,689 ha. The area and production of vanilla in different countries are given in Table 1 (Spices Board, 2000).

The primary growing regions in the Indian Ocean are Malagasy Republic, Comoros and Reunion. Beans from this area are called Bourbon and represent half of the world's vanilla production. Tahitian vanilla beans, which represent another variety, are grown mainly on the island of French Polynesia.

Table 1 : Area and production of vanilla during 1999

Country	Area (ha)	Production (MT)
Comoro	700	150
Cook Islands	1	2
French Polynesia	200	34
Gaudeloupe	40	40
Indonesia	9689	2102
Malagasy Republic	25000	1650
Mexico	900	300
Reunion	600	30
Tonga	350	50
Turkey	-	35
Zimbabwe	45	10
Total	37,525	4,403

Spices Board, 2000

In the nineteenth century, Mexico was the largest vanilla producer, but since the First World War some islands in the Indian Ocean have become the world's largest centre of production. Today the largest exporters are Malagasy Republic, Comores Islands, Reunion Island, China and Indonesia. The most important consumers are USA, Canada, EEC countries, Japan and Australia (Ferrão, 1993).

Crop variability

Vanilla planifolia is a crop little different from the wild progenitors. This can be attributed to a limited breeding and a recent domestication. In Mexico there are two areas in which *V. planifolia* is grown in some extent, northern Veracruz, and northern Oaxaca. Plantations of northern Veracruz are ancient and the source of the majority of vanilla produced in Mexico, no wild populations are known in

this area. Plantations in Oaxaca were established by the end of the 1980s from wild specimens from the region. Cultivars in *V. planifolia* (like the widely cultivated Mansa) may be an spontaneous wild specimen brought to cultivation by Totonaco Indians. Only two other cultivars are recognized by growers in the traditional plantations of northern Veracruz, one of them self-incompatible. Because of the population pressures in many tropical and semi-tropical countries there is extensive logging and clearing of land for agriculture, resulting in serious loss of habitat and the germplasm of *Vanilla planifolia* being threatened by deforestation and overcollection. Most genetic diversity, that found in the plantations of Oaxaca, is threatened by the bad management of plantations. This suggests that the primary gene pool of this crop maybe severely threatened by demographic causes in wild populations and in plantations by genetic erosion. Electrophoretic data of specimens from the vanilla plantations of northern Veracruz, Oaxaca, and elsewhere were analyzed to study the extent of variability. The allozyme data showed little genetic variation in general. However, plants from the two main areas could be differentiated. There is little variation with homozygotes in Veraruz populations while the populations in Oaxaca showed larger genotypic diversity and heterozygosity. Nucleotide sequence variation within introns of a couple of specific protein-coding genes, namely the Calmodulin and the Glyceraldehyde 3-phosphate dehydrogenase were detected in order to propose genealogies of *V. planifolia*. Enough variation, required to use these genomic regions as molecular markers for the recognition of the different Oaxacan specimens, were found. A phylogenetic framework of the genus *Vanilla* permits us to recognize which species could be the most appropriate sources of the desired traits. It has been indicated that traits like self-pollination. root-rot

resistance, ability to maintain higher fruit sets, and less dependence of flower induction to photoperiod, could be desirable characters in vanilla as a crop. All these traits are found in its near relatives (www.canadianorchidcongress.ca)

Yields are very variable. A good vanillery is said to yield about 500 - 800 kg of cured beans per hectare per annum during a crop life of about 7 years. A 7 to 9 inch vanilla bean will weight approximately 5 grams and will yield about 1 gram or 1/2 teaspoon of vanillin seeds. The vanilla seeds contribute to the essence and flavour of the vanilla, so it is important that the skin should be as thin as possible. A bean with numerous seeds and less moisture will give a plumpy bean with thin skin. Sometimes pure vanillin crystals, called 'sugar crystals', come out of the dried fruits.

Processing

Curing of vanilla beans

Commercial vanilla is obtained after special processing of the fruits. First they are put in hot water and then dried to allow fermentation to occur. In Malagasy Republic, the world's largest producer of bourbon vanilla, the fruits are sundried and wrapped in woolen blankets at night to remain warm. This is a long process that takes months and increases the cost of the product (Ferrão, 1992).

Quality requirements

The primary quality determinant for cured vanilla beans is the aroma/flavour character. Other factors of significance in quality assessment are the general appearance, flexibility, length and the vanillin content. The relative importance of these quality attributes is dependent upon the intended end-use of the cured beans. There is a fairly close relationship between appearance, flexibility and size characteristics and the aroma/flavour quality. Top quality

beans are long, fleshy, supple, very dark brown to black in colour, strongly aromatic and free from scars and blemishes. Low quality beans are usually hard, dry, thin, brown or reddish-brown in colour and possess a poor aroma. At one time, the presence of a surface coating of naturally exuded vanillin crystals (frosting) was regarded as an indicator of good quality. However, the Mexican vanilla, which has the best reputation for quality, rarely "frosts". A high vanillin content is desirable but this value is not directly commensurate with the overall aroma/flavour quality of the bean. Much of the vanilla entering Western markets is used for the preparation of vanilla extract, and for this purpose the appearance of the beans is not of prime importance (Ferrao, 1992).

Traditional curing methods

A number of traditional curing methods have been evolved for the curing of vanilla, but they are all characterized by four phases.

Killing or wilting

Killing stops further development in the fresh bean and initiates the onset of enzymatic reactions responsible for the production of the aroma and flavour. Killing is indicated by the development of a brown colouration in the bean.

Sweating

This involves raising the temperature of the killed beans to promote the desired enzymatic reactions and to provide a first, fairly rapid drying to prevent harmful fermentation. During this operation, the beans acquire a deeper brown colouration and become quite supple, and the development of an aroma becomes perceptible.

Drying

The third stage entails slow drying at ambient temperature, usually in the shade, until the beans have reached about one third of their original weight.

Conditioning

In the final stage, known as "conditioning", the beans are stored in closed boxes for a period of three months or longer to permit the full development of the desired aroma and flavour.

Various traditional procedures for curing vanilla beans are known all over the world (Mexican method, Tahitian method, artificial method). But the most important one used in Malagasy Republic, Comoros, Reunion and to a certain extent in Indonesia is "the Bourbon" method.

In this method, the beans are sorted according to the degree of maturity, size, and into split and unsplit types. Batches of beans, weighing 25 - 30 kg, are loaded into openwork cylindrical baskets, which are then plunged into containers full of hot water heated to 60 - 63^{0}C. Batches of beans which will eventually make up the top three qualities are immersed for 2 - 3 minutes, while smaller and split beans are treated for less than 2 minutes. The warm beans are rapidly drained, wrapped in a dark cotton cloth and are placed in a cloth lined sweating box. After 24 hours, the beans are removed and inspected to separate those which have not been properly killed. The next stage of sun-drying is carried out on a plot of dry, easily drained ground, to avoid contamination by dust. The killed beans are spread out on dark cloths resting on slatted platforms, constructed from bamboo and raised 70 cm above the ground. After one hour of direct exposure to the sun, the edges of the cloth are flooded over the beans to retain the heat. The cloth-covered beans are then left for a further two hours in the sun before the blanket is rolled up and taken indoors. This procedure is repeated for 6 - 8 days until the beans

become quite supple. The third stage involves slow drying in the shade for a period of 2 - 3 months. The beans are spread on racks, mounted on supports and are spaced 12 cm apart in a well-ventilated room. During this slow drying operation, the beans are sorted regularly and those which have dried to the requisite moisture content are immediately removed for conditioning. In some localities in Malagasy Republic and most commonly in Indonesia where the weather is frequently inclement during the sun and indoor-drying periods of curing, ovens set at 45 – 50^{0}C have traditionally been used. Conditioning of the beans is carried out in a similar manner in Malagasy Republic and Mexico and takes about 3 months for completion. The overall curing process for Bourbon vanilla lasts 5 - 8 months.

Grading and packaging

After conditioning, the cured beans are given an airing and are restraightened by drawing through the fingers. The beans are then subjected to a final sorting into grades and according to their length, prior to bundling and packaging for shipment. The length of the beans is an important determinant of the price. Grading system differs somewhat between producing countries, but beans are generally classified into three categories: unsplit beans, split beans and "cuts". The last type has traditionally consisted of beans which have been attacked by mold and have had the infected portion cut away. Very small and broken beans of poor aroma quality are usually combined with the "cuts" from moldy beans.

Bourbon vanilla

The vanilla of Malagasy Republic, the Comoros Islands, and Reunion is classified into five main grades of whole and split beans plus and additional category known as "bulk" which is comprised of cuts. The grades of whole and

split beans are also sub-divided according to size. The minimum acceptable length for the top five grades in the major producing area of Malagasy Republic is 12 cm while for Nossi-Be and the Comoros Islands it is 10 cm (Frere, 1954). Malagasy Republic beans are first sorted to separate beans below 12 cm in length and then the whole and split beans are classified into grades according to their aroma, moisture content, and appearance. The five main categories for Malagasy Republic whole beans are as follows: "Extra" Whole, supple, unsplit beans, free of blemishes, possessing a uniform chocolate-brown colour and an oily luster with a clean and delicate aroma. "1st" Similar to the "extra" grade but not quite so thick and of a lesser appearance. "2nd" Somewhat thinner beans with a chocolate-brown colour but with a few skin blemishes. The aroma is good. "3rd" Thinner, more rigid beans with a slightly reddish chocolate-brown colour. The aroma is fair. "4th" Rather dry beans with a reddish colour and numerous skin blemishes. The aroma is ordinary. Splits are sorted into categories corresponding to those for whole beans. Foxy splits are thin, hard, and dry, short types with a reddish-brown colour.

Spoilage of vanilla beans

Vanilla beans are quite susceptible to infection by *Penicillium* and *Aspergillus* molds and this generally occurs during the conditioning and subsequent storage (Bouriquet, 1954). In appearance, there are two types of mold: one is white at first and turns green later, while the other is black and spreads rapidly. Infection always begins at the stem end of the bean and, if left uncontrolled, the whole bean becomes wrinkled, dry and acquires a disagreeable odour. It is virtually impossible to eliminate the odour once the mold takes hold and this considerably detracts from the market value. Mold infection most

frequently occurs when immature beans are harvested and are not killed properly. They do not dry uniformly leading to excessive moisture content on conditioning.

Vanilla beans are prone to attack by mites of the *Tyrophagus* species (Chalot and Bernard, 1920; Bouriquet, 1954), which imparts a disagreeable odour to the beans. The mites infest during conditioning, shipment of subsequent storage and may be detected by the small holes which they produce in the beans. In cases of limited infestation prior to shipment, alcohol treatment or sun drying is often effective (Mallory and Cochran, 1941).

Some beans develop a creosote-like aroma which is impossible to eliminate once formed. This off-aroma generally becomes apparent at quite an early stage in the sweating process and results from an abnormal fermentation due to poor handling and curing practices. The principal cause is believed to be improper storage methods for fresh beans prior to killing. This undesirable fermentation can be avoided if fresh beans are stored in well-ventilated small piles, and by commencing curing as soon as possible. (http://www.shanks.com/vanilla/botany.asp)

Importance and Uses

The Aztecs in Mexico used vanilla to flavour cocoa before the arrival of the Spaniards. Vanilla was taken to Spain where they were used for the same purpose. Vanilla extract is obtained by macerating the cured beans in alcohol. Vanilla is used extensively to flavour ice creams, chocolates, beverages, cakes, custards, puddings and other confectioneries. The greatest use of technical grade vanillin (98% pure) is a chemical intermediate in the production (synthesis) of a number of pharmaceutical products especially as an aid for oxidation of linseed oil and as solubilising agent for riboflavin (vitamin B_2) as vanillin is an antioxidant.

Vanilla is also used in perfumery; its addition to heavy perfumery imparts a more delicate odour; it is used in soaps and other toilet preparations. The perfume industries also use vanilla as a basic or middle note because of its rich, sweet and balsamic scent (Williams, 1997). Earlier it was used in medicine as a nerve stimulant and along with other spices, as a mild sedative, to aid digestion and had a reputation as an aphrodisiac. Different processed products are made out of vanilla beans, these are vanilla extract or essence, vanilla tincture, vanilla vanillin, vanilla oleoresin, vanilla powder and vanilla absolute. During recent years, vanilla has been used in poison bait pests for such as fruit flies, grasshoppers and melon-beetles. The leaves of the plants are used as a blistering agent in some areas (CSIR, 1976; Purseglove *et al.,* 1981; Gopinath 1994).Sugar with vanilla flavour is made by putting a vanilla fruit inside a tightly closed container with sugar. The product so obtained can last for years. Chocolates, liquors, cakes and the famous ice cream are some of the delicacies made with this fragrant and delicious spice (Grieve, 1981; Norman, 1990).

Vanillism is a disease that appears in sensitive workers who deal with huge amounts of vanilla daily. It is characterized by headache, lassitude and allergic skin reactions of the face, neck and hands (Bown, 1995; Touissaint-Samat, 1994).

http://botit.botany.wisc.edu/courses/tour/Roomthree-Va.html

Flavour and fragrance

The fragrance and flavour of vanilla bean is due to numerous aromatic compounds produced during the process of curing. The unique flavour and fragrance of vanilla beans is due to vanillin ($C_8H_8O_3$), the most abundant aromatic constituent of vanilla beans. Free vanillin is not present at the time of harvest, but

is produced during curing by enzyme action on glucosides. The cured beans contain 1.5 –3.5 % vanillin (Purseglove, 1972).

Vanillin, was first isolated by Gobley (1858) by macerating the cured beans in alcohol. *Vanilla planifolia* is the principal source of natural vanillin of commerce. It was first produced artificially by Tiemann and Haarmann in 1874 for the glucoside coniferin, which occurs in the sapwood of certain conifers. Synthetic vanilla is much cheaper and is available in the market. Nevertheless, the flavour of vanilla bean from *V. planifolia* is far superior to that of synthetic vanillin due to the presence of other flavour compounds in the natural product (Purseglove *et al.,*. 1981). The mature green capsule of the plant contains on an average water 30%, protein 5%, sugars 8%, vanillin 2.5% and fibre 20% (Gopinath, 1994). The natural vanillin and other natural substances that make up the vanilla flavour are complicated and difficult to imitate. Pure vanilla extract is ageless; like a good cognac, time enhances its flavour and bouquet. Nowadays, the synthetic vanillin is obtained from lignin, a by-product from paper pulp industries (Bruneton, 1995). But the real product of *V. planifolia* is superior, probably due to the presence of subsidiary substances, including a strongly aromatic ester. This coupled with health hazards associated with synthetic vanillin seems to be the deciding factor in favour of natural vanillin.

The Indian Scenario

The genus *Vanilla* is represented by five species in India. They are *V. aphylla*, which occurs in Kerala, *V walkeriae,* which occurs in Tamil Nadu, *V. wightiana,*which occurs in Andhra Pradesh, *V. pilifera* which occurs in the North Eastern parts of India and *V. andamanica* which is indigenous to Andaman Islands. Among these *V. wightiana* and *V. andamanica* were found to have

natural seed set (Rao *et al.,* 2002). The first three of the above species, are leafless forms and the last resembles *V. planifolia* in having plain leaves. *Vanilla pilifera,* though exhibits leafless forms in juvenile stages develops flat but narrow leaves after a few years of maturity. However, none of these species yield commercial vanilla.

Vanilla planifolia was introduced into India in 1835 and its commercial cultivation is limited to the states of Kerala, Karnataka and Tamil Nadu. The area under vanilla cultivation in India is estimated to be 1000 ha with a production of 5-8 tones of cured beans (Spices Board, 2000).

Even though India has ideal agro-climatic conditions for the cultivation of vanilla, the area under this crop is very less which is not even sufficient to meet 5% of the country's requirement and hence depended on imported synthetic vanillin. Due to the high cost and non-availability of natural vanillin, most of the consumer countries including India currently depend on the low priced synthetic vanillin, which has a less pleasant odour and taste. Considering the advantages of harmless natural products in medicine and food, there is an urgent need to produce more of natural vanillin in India for internal consumption and export (Tessy Paul, 1995). In India, no organised efforts have been made to develop vanilla on large plantation scale, possibly due to the non-availability of adequate number of planting material, which is a major constraint (Rao *et al.,* 1993). There is ample scope for extending the area, notably in Kerala and Karnataka as an inter crop in coffee, cardamom, coconut and arecanut plantations requiring no additional land for its expansion (Spices Board, 2000)

Much of the planting material of vanilla originated from limited clonal source and practically no variability is available for crop improvement. This leads

to monoculture making vanilla susceptible to diseases and pests, the most common diseases being of fungal origin *viz.*, *Calospora vanillae*, leaf rot, blights, foot rot and wilting caused by *Fusarium oxysporum* and brown spots or anthracnose by *Colletotrichum gloeosporioides* (Gopinath, 1994).

Introducing vanilla from its native zone or from areas of diversity is difficult due to restriction by the host countries for germplasm exchange. This leaves us no option but to look for other sources of variability. Vanilla is suspected to be highly heterozygous with extensive genetic variations because of its cross-pollinated nature. Being a cross pollinated crop much variation could be obtained in the segregating seedling progenies. Nair and Ravindran (1994) reported mitotic association in vanilla which will give rise to further variation in seedling progenies. This resultant variation can be used in crop improvement programmes.

BIOTECHNOLOGICAL APPROACHES

Micropropagation

Micropropagation is the true-to-type propagation of a selected genotype using *in vitro* culture techniques (Debergh and Read, 1991). Following the successful rapid multiplication of orchids by shoot meristem culture (Morel, 1960; 1965) there has been increasing interest, in recent years, in the application of tissue culture techniques as an alternative means of asexual propagation of plant species (Hu and Wang, 1983). Presently there are about 1000 plant species that can be micropropagated (Murashige, 1989; George, 1996). They include, *Anthurium* spp., *Gerbera* sp., *Gladiolus* sp., *Chrysanthemum* sp., *Dianthus* sp., *Phlox* sp, *Asparagus* sp., *Cicer* sp., *Arachis* sp., *Beta* sp., *Brassica* sp., *Tectona* sp., *Dalbergia* sp., *Santalum* sp., *Lolium* sp., *Vigna* sp., *Zea* sp., *Prunus* sp., *Pyrus*

sp., *Malus* sp., *Coffea* sp., *Rosa* sp., *Grevillea* sp., *Eucalyptus* sp., *Thuja* sp., *Populas* sp., oil palm etc. Now micropropagation of plants is a multibillion-dollar industry being practiced in hundreds of small and large nurseries and biotech laboratories throughout the world (Bajaj, 1991c). Presently it is the only component of plant biotechnology, which has been commercially exploited on such a large scale. It is employed especially for propagation of ornamental and foliage plants and recently in plantation crops, medicinal and aromatic plants (Bajaj, 1988; 1989b; 1991a) and trees (Bajaj, 1986; 1989a; 1991b).

Micropropagation of vanilla using apical meristem was standardized for large scale multiplication of disease free and genetically stable plants (Cervera and Madrigal, 1981; Kononowicz and Janick, 1984; Philip and Nainar, 1986; George *et al.,* 1995; Samuel Ganesh *et al.,* 1996; Minoo *et al.,* 1997). Reports on conversion of root meristem to shoot meristem and subsequent development to plantlets were also available (Philip and Nainar, 1986).

Vanilla produces numerous minute seeds that do not germinate under natural conditions. Tissue culture technique could be used to successfully germinate the seeds. Protocols for seed and embryo culture of vanilla were standardized (Knudson 1950; Withner 1955; Minoo *et al.,* 1997).

Vanilla, a cross pollinated crop, is known to have many meiotic and post-meiotic chromosomal abnormalities (Ravindran, 1979). As a result, it is possible to get various cytotypes in the seed progenies. Culturing of seeds can thus give many genetically variant types. Preliminary studies on *in vitro* germination of vanilla seeds has been reported and the resultant progeny showed morphological and biochemical variations (Minoo *et al.,* 1997). Thus *in vitro* culture could be

used for germination of seeds and selection of useful genotypes from segregating progenies and rapid multiplication for getting disease-free planting material.

Plant regeneration

Organization can be brought about by the controlled initiation of organ primordium or embryogenesis through manipulation of the nutrient and hormonal constituents of culture medium. Usually it is the ratio of cytokinin to auxin that determines the nature of organogenesis (Narayanaswamy, 1977).

A large number of plant species have been regenerated from tissue cultures (Flick *et al.,* 1983; Ammirato, 1983; George, 1996). Plant species belonging to certain families respond readily to morphogenesis. However, monocots seem to be more recalcitrant to morphogenesis and plant regeneration. The genotype, culture environment and tissue dependant factors influence the morphogenesis.

The growth of cultured tissues and morphogenesis *in vitro* are more influenced by genotype than by other factors (George, 1993). In some cases, morphogenetic responses have been related to identifiable genetic factors like nuclear genes (Foroughchi-Wehr *et al.,* 1982; Lazar *et al.,* 1984; Rhodes *et al.,* 1986; Mathias and Fukui, 1986; Szakacs *et al.,* 1988), cytoplasmic genes (Mathias *et al.,* 1986; Narasimhulu *et al.,* 1988; Wan *et al.,* 1988) and gene interaction (Keyes and Bingham, 1979; Wan *et al.,* 1988).

The source and physiological aspects of the explant determine its regenerative potential. Various other factors like nutrient media, growth regulator combination, their concentration and culture conditions also influence plant regeneration.

Differential DNA synthesis and genomic changes that accompany tissue maturation seem to affect morphogenetic ability in long term tissue cultures. Loss of morphogenetic capacity can also be correlated with ploidy changes and age of culture (Murashige and Nakano, 1967; Torrey, 1967; Mehra and Mehra, 1974; Orton, 1980; Kibler and Neumann, 1980). Genetic changes due to continued selection for efficient growth over other non essential factors seem to affect morphogenetic potential (Orton, 1984; Simmons *et al.,* 1984).

Among the physical factors, exogenous auxin/cytokinin ratio within a particular range of concentration, largely controls organogenesis in majority of cultures while the major variable controlling embryogenesis is auxin type and concentration. 2,4-D is the most commonly used auxin for embryogenesis (Halperin, 1986).

Studies have shown other factors like explant choice, medium composition and control of physical environment also influence organogenesis (Thorpe and Patel, 1984; Brown and Thorpe, 1986; George and Sherrington, 1984; Ozias-Akins and Vasil, 1985).

There is only one report so far on successful plant regeneration from leaf and seed derived callus was also reported in vanilla (Davidonis and Knorr, 1991). The plant regeneration protocol may help in creation and exploitation of somaclonal variation.

Competence and Determination

Highly specialised cells of intact plants are never observed to change from their existing differentiated state. Similarly some cells do not become morphogenetic and are supposed to have lost the capacity to form new plants. Cells which have retained the capacity for a particular type of cellular

differentiation or morphogenesis, or have acquired it in response to an appropriate stimulus, are said to be *competent* (George, 1993). Some consider that morphogenic competence usually conveys an ability to proceed towards one particular developmental pathway *i.e.*, a cell which is competent to undergo shoot morphogenesis may not be competent for root formation. If cells of an explant are not already competent at the time of excision, they may be induced to become so by *in vitro* culture. They are then enabled to respond to the organic stimulus provided by a particular combination of growth regulators. Competence is the first step in dedication of one or more undifferentiated cells towards morphogenesis or some kind of specialised development. The next stage is the induction of *determination* in competent cells. Individual cells or groups of cells are said to be determined when they become committed to follow a particular genetically programmed developmental pathway and can continue towards that without further influence of growth regulators (Christianson, 1987). In both direct and indirect organogenesis, the competent cells, at some stage, adapt different programming (determination) which decides their subsequent pattern of development. In unorganised tissue cultures, the programme is apparently induced by the effect of growth regulator combination in the presence of correct nutritional factors. Although growth regulators help to induce morphogenesis, cells in some parts of the plant

Somaclonal variation

Larkin and Scowcroft (1981) coined the term somaclonal variation to refer the variation observed among plantlets regenerated through tissue cultures. The terms calliclonal variation, gametoclonal variation, protoclonal variation were also used for referring variations observed in plantlets derived from callus, gametes,

protoplasts etc. The variation observed in tissue cultured plants encompasses a wide array of characters (Vasil, 1986; Lal and Lal; 1990; Bajaj, 1990a).

Various reasons were suggested for the variations among micropropagated and callus regenerated plants. They are genotype of the parent material, type and physiological state of the explant, tissue culture pattern (micropropagation, callus regeneration, anther culture, protoplast culture etc.), active cycle and age of culture, nutrient media and growth regulators, culture conditions etc. (Ogura, 1990; Sibi, 1990; George, 1993). These variations can also be due to, cytogenetic factors - like polyploidy, aneuploidy, deletion, inversion, translocation, increase in heterochromatin and gene amplification; genetic factors - like point mutation, polygene trait alteration, maternally inherited mutation, organelle genome deletion and epigenetic factors- like cytokinin habituation, drought stress resistance, as a result of alteration of DNA methylation and physiological adaptation (Vasil, 1986).

A transposable element (transposon) is a genetic sequence which, under certain circumstances is capable of moving from one position in the genome to another, including between chromosomes. When a transposon becomes positioned at a new locus, a mutator element causes an unstable suppression or modification of adjacent genes. Transposable elements may become active *in vitro*, resulting in cryptic changes in the genome (Groose and Bingham, 1986; Peschke *et al.,* 1987; Phillips *et al.,* 1990).

Case studies of *in vitro* genetic variability were documented in wheat and triticale (Larkin, 1986), celery (Orton, 1986), rice (Fukui, 1986), tobacco (Dulieu, 1986), tomato (Evans, 1986), potato (SreeRamulu, 1986), oats and maize (Benzion *et al.,* 1986). The somaclonal variation was used for isolation and

characterization of plants for disease resistance, cold tolerance, salt tolerance, and auxotrophs in many crop species (Vasil, 1986).

Thus the ability to generate variability in controlled manner at some stage of the cultural process has been cited as a resource of considerable potential for the modern plant breeder. Somaclonal variation, in particular, the phenotypic variation from *in vitro* propagation, might be valuable for breeding, but is usually a problem for the propagation of clone plants. A study involving the mechanisms of the somaclonal variation is important for detecting and avoiding unfavorable variations (i.e., malformation) during the early stage of *in vitro* propagation. Many studies on somaclonal variation and the ploidy level in *Phalaenopsis alliance* were carried out on various aspects such as chromosomal number and shape, patterns of AFLP, RFLP and RAPD, DNA methylation and transposon by Tian-su Zhou (2001).

Application of somaclonal variation in crop improvement has been discussed in detail in several publications (Evans and Sharp, 1986; Brar and Kush. 1994; Karp, 1995). Few somaclonal variations, with respect to morphological characters, which have been reported to be heritable are leaf colour in tobacco (Dulicu and Barbier, 1982), fruit colour in tomato (Evans and Sharp, 1983) and seed colour in *Brassica* (George and Rao, 1983).

Heritable somaclonal variations with respect to various resistance traits have been reported. Resistance to methionine sulfoxime (Carlson, 1973) and *Pseudomonas syringae* (Thanutong *et al*, 1983), in tobacco, resistance to *Fusarium oxysporum* in tomato (Evans et al, 1984), in alfalfa (Hartman *et al*, 1984); resistance to *Helminthosporium sativum* (Chawala and Wenzel, 1987) in wheat, Xanthomonas oryzae (Ling et al, 1984) in rice are a few examples.

Interspecific hybridization

Interspecific hybridisation is a mechanism by which useful genes from wild progenitors and species can be brought into cultivated species. Cultivated types of many crop species were improved through interspecific hybridisation and backcrossing. Interspecific hybridisation is very common in orchids to produce new and novel varieties of flowering plants. As with many orchids, interspecific vanilla hybrids are relatively easy to make.

Vanilla planifolia has been crossed with other species including *V.pompona* and *V.phaeantha* which is resistant to *Fusarium* (Purseglove *et al.,* 1981). Interspecific hybridisation was also conducted in Java to develop between cultivated and wild vanilla to develop lines resistant to stem rot caused by *Fusarium oxysporum* (Mariska *et al.,* 1997).

The possibility of natural occurrence of interspecific hybrids have been reported in vanilla. Nielsen and Siegsmund (1999) reports the possible occurrence of interspecifc hybrids between *V. claviculata* and *V. barbellata* in localities where they co-exist in PuertoRico. Progenies were discovered, where some characters are morphologicaly intermediate between the two parents. Reports on successful interspecific hybridisation between *V.planifolia* and *V.phaeantha.*

Isolation and culture of protoplasts :

The 'protoplast' is a naked cell, without cell wall surrounded by plasma membrane, but is potentially capable of cell wall regeneration (in case of plants), growth and division. The absence of cell wall makes the protoplast suitable for a variety of manipulations that are not normally possible with intact cells such as

uptake of cell organelles, microorganisms, foreign genetic material to form genetically modified cell and also for production of somatic hybrid cells by fusion of two protoplasts. Thus protoplast is an important tool for parasexual modification of genetic content of cells (Vasil and Vasil, 1980).

The basic requirements for protoplast technology are the ability to isolate, culture, form cell colonies and finally regenerate into whole plants. Though the earlier workers used mechanical means for isolation of protoplasts, use of enzymatic digestion to liberate protoplasts is the most effective method employed by present workers (Cocking, 1960; Gregory and Cocking, 1965; Ruesink and Thimann, 1965). In earlier studies the cells were separated using pectinase (Macerozyme) and subsequently digest the cell wall with cellulase to release the protoplasts (Takabe *et al.,* 1968). More commonly a mixture of pectinase and cellulase is used to macerate the cells by digestion of middle lamella and also to liberate protoplasts by digesting the cell wall (Power and Cocking, 1970). For some plants such as cereals, a mixture of cellulases, pectinases, driselases and rhozyme are necessary to obtain optimum yield of viable protoplasts (Vasil and Vasil, 1980).

Protoplasts can be isolated from a variety of tissues like leaves (Kartha *et al.,* 1974; Power and Cocking, 1970), cladodes (BuiDang Ha and Mackenzie, 1973), shoot apices, fruits (Gregory and Cocking, 1965), roots (Davey *et al.,* 1973), coleoptiles (Ruesink and Thimann, 1965; Hall and Cocking, 1974), aleurone layer of cereals (Taiz and Jones, 1971), microspore mother cells (Ito and Maede, 1973), microspore tetrads (Bajaj, 1974; Bhojwani and Cocking, 1972), pollen (Bajaj, 1983a) etc. However, mesophyll tissues of young leaves, preferably from *in vitro* grown plants, are most preferable. Cells and tissues grown *in vitro*,

particularly suspensions and callus cultures are also important sources of protoplasts (Wallin and Eriksson, 1973). For callus and cell suspensions the early log phase of growth gives optimum result.

An incubation temperature of 25 -30^{0}C is optimal for protoplast release in most plants, but low incubation temperature (14^{0}C) followed by short period at 30 ^{0}C was found to be beneficial to cereals (Vasil and Vasil, 1980). Other factors like low speed agitation, brief period of vacuum and gentle pre-plasmolysis reduced protoplast damage and increased amount in release of protoplasts. Addition of L-arginine and L-lysine decreases senescence and stimulate macromolecular synthesis (Altman *et al.*, 1977).

The released protoplasts can be purified by filtering through nylon or stainless steel mesh to remove undigested tissue. This is followed by floatation and centrifugation to further purify to remove enzymes and finer debris (Gregory and Cocking, 1965).

Protoplasts released directly into standard cell culture medium will burst, hence the pressure that is mechanically supported by the plant cell wall must be replaced with an appropriate osmotic pressure. Osmotic pressure is manipulated by adding various sugars or sugar alcohols to the isolation and culture solutions. Mannitol, sorbitol, glucose, sucrose have all been frequently used. Mannitol and sorbitol separately or in combinations, have been used most often, and mannitol is preferred for isolation of protoplasts from leaf mesophyll. Mannitol at concentrations of 0.23 - 0.90 M was successfully used to isolate protoplasts (Evans and Bravo, 1983).

A density of 5000 - 100,000 cells / ml is ideal for protoplast culture. Protoplasts in culture start to generate cell wall within few hours and may take

two to several days to complete it (Pojnar *et al.,* 1967; Horine and Ruesink, 1972). Regeneration of cell wall is not a prerequisite to nuclear divisions as observed in carrot protoplasts (Reinert and Hellman, 1971). Sometimes they undergo one or several nuclear divisions to become multinucleate before cytokinesis.

Nutrient requirements of isolated protoplasts are almost similar to those of cultured cells except that the culture medium contains reduced levels of inorganic substances. In the absence of cell walls, protoplasts are more efficient in the uptake of nutrients at the same time leakage of some metabolites may also take place in the initial stage. The addition of auxins into the culture media may cause rapid bursting of the protoplasts. Protoplasts can be cultured as suspensions in drop cultures or plating in semi solid media, co-culturing, feeder layer culture or hanging droplet culture.

Protoclones were regenerated from protoplasts of various species like, rice, Hordeum vulgare, sorghum, wheat, maize, Medicago sativa, Trigonella foenum-graceum, Trifolium repens, Pennisetum americanum, potato, tomato, Brassica napus, Brassica oleracea, Nicotiana tabaccum, Glycine max etc (Davey and Kumar, 1983). Naked protoplasts offer exciting possibilities of uptake of microorganisms (Davey and Cocking, 1972), organelles (Davey and Power, 1975), chromosomes (Szabados *et al.,* 1981; Griessbach *et al.,* 1982) and foreign DNA into the protoplasts. Protoplast fusion provides a method for combining the genomes of different genera and species to overcome sexual incompatibility. Fusion products were obtained in *Nicotiana,* petunia, *Datura, Daucus, Solanum* etc. (Davey and Kumar, 1983). Protoplasts especially those of haploid plants also provide ideal system for *in vitro* mutagenesis. Several useful mutant cell lines were isolated using protoplasts. They include lines resistant to pathogens,

herbicides, environmental stress etc. Plants regenerated from tissue derived from protoplasts frequently display variability (protoclonal variation), which can be used as a source of variability.

Protoplasts can be considered ideal system for gene transfer. The freely accessible plasma membrane guarantees that DNA can reach and enter every protoplast in a given population. The efficiency of DNA uptake can be further promoted using polyethylene glycol (PEG) (Negrutiu *et al.,* 1987), by application of electric pulses (electroporation) (Fromm *et al.,* 1986) or a combination of both (Shillito *et al.,* 1985). When an appropriate protoplast-to-plant regeneration system is available, large number of transformed clones can be regenerated into fertile transgenic plants (Davey *et al.,* 1989). Regeneration of transgenic plants through protoplast transformation was reported in important cereals like rice (Shimamoto *et al.,* 1989; Datta, 1995; Wu and Shimamoto, 1995), maize (Donn *et al.,* 1990; Omirulleh *et al.,* 1995) and tobacco (Spangenberg and Potrykus, 1995).

CHARACTERIZATION OF GENETIC VARIABILITY

Morphological characterization

Morphological characterization is the most easiest and efficient way to characterize the genetic variation available in a plant type or population. Morphological features of plant, leaf, flower and fruit have invariably been used in all the plant species studied. However, the characterization based on phenotype is not always adequate since morphology reflect not only the genetic constitution of the plant, but also the interaction of the genotype with the environment (G x E) within which it is expressed (Gottieb 1977; Brown 1978; Lin and Bins 1984). This is more so in perennial crops, which take many years for phenotypic evaluation,

and in crops where the phenotypic markers are not so distinct. In such circumstances, phenotypic characterization needs to be augmented with more efficient isozyme or DNA markers.

Cytological indexing

Cytological studies can also reflect the variability existing between the genotypes, when the variability is associated with chromosomal aberrations, karyotpic changes and changes in ploidy level. However, it is difficult to detect minor changes in the genome using cytological parameters. Some of the earliest reports of genetic instability in culture were made on the basis of chromosome counts (D'Amato, 1975) but it is known that this approach will reveal only crude genetic change. Numerous studies and reviews have been made on chromosomal variation and behaviour in plant tissue culture (Sunderland, 1977; Lee and Philips, 1988). Bayliss (1980), following D'Amato (1978), comprehensively reviewed the reported instances of chromosomal variation in tissue cultures. The classes of variant cells included polyploid and aneuploid changes, structural changes in chromosome morphology, and mitotic aberrations including multipolar spindles, lagging chromosomes, fragments and asymmetric chromatid separation. Tissue cultured plants with extensive chromosome rearrangements were reported in *Lolium* sp. (Ahloowalia, 1983) and *Medicago sativa* (Reisch and Bingham, 1981).

Perturbations in the replication of heterochromatic regions during tissue culture may be the cause of chromosome alterations observed in regenerated hexaploid oat plants the presence of proximal and telomeric heterochromatic means that bridges resulting from replication problems in these regions could results in various alterations including deficiencies, duplications and translocations and can account for heterochromatin amplification, all of which

have been observed in plants that have been regenerated from tissue culture (Johnson *et al.,* 1987b). Meiotic behaviour in oat plants regenerated from tissue culture showed heteromorphic bivalents separating prematurely and lagging during anaphase I (Johnson *et al.,* 1987a).

Biochemical characterization

Markert and Muller (1959) proposed the term 'isozyme' (synonymous with 'isoenzyme') for multiple molecular forms of an enzyme sharing a catalytic activity, derived from a tissue of a single organism. Proteins are attractive for direct genetic study because they are primary products of structural genes. Changes in coding base sequence will result in corresponding changes in the primary structure proteins. Even simple amino acid substitutions, deletions or additions can have marked effects on the migration of proteins under an electric field during electrophoresis. Isozymes are different forms of an enzyme exhibiting the same catalytic activity but differing in charge and electrophoretic mobility. The advantages of isozyme profiling are, it is relatively simple and less expensive. The disadvantages are, availability of limited number of enzyme loci, and developmental and season dependent expression of activity. Isozyme profiles have been used in numerous crop species for cultivar identification, hybrid identification, characterization of genetic resources, population variations, variations in the mating system, genetic stability of *in vitro* cultures, systematic and evolutionary studies and species interrelationships etc (Simpson and Withers, 1986). A new role of isozymes as monitors of variation has arisen with the development of tissue culture methods of germplasm storage (Withers, 1980; Withers and Williams, 1982; 1985).

Molecular characterization

Molecular markers can augment phenotypic markers in a very efficient way. Moreover, one need not wait until the plant flowers for estimating genetic variability using molecular markers. These markers may reveal even a discrete variation in genome that cannot be normally detected by phenotype, karyotype or enzyme analysis. A wide variety of markers are now available for characterization and indixing of genetic variations. Some of the important molecular markers which are in extensive use for genetic characterization and detection of variations are Restriction Fragment Length Polymorphism (RFLPs), Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats or microsatellites (SSR), Cleavable Amplified Polymorphic sequences (CAP) and Single nucleotide polymorphism (SNPs). Of these, RFLP, RAPD, AFLP and microsatellites are most common. In addition these markers can be used in a wide variety of applications viz., creation of genetic maps, mapping of single traits, quantitative trait loci (QTLs) and mutations, characterization of transformants, genetic diagnostics for plant breeding, population genetics, in molecular taxonomy and evolution, identification of individuals, forensic analysis, germplasm characterization, identification of proprietary germplasm and duplicates etc.

Genetic Polymorphism

Genetic polymorphism is classically defined as the simultaneous occurrence in the same population of two or more discontinuous variants or genotypes. The first genetic polymorphism discovered as early on 1900 by Landsteiner, was the ABO blood group system. This polymorphism was analyzed at the level of gene product and not at that of the gene itself. Analysis at the genetic level became possible only after the introduction of genetic engineering in

to human genetics in the late 1970s and early 1980s. The new technology opened up the way to study a new class of polymorphic traits; namely those defined by the variation between one chromosome and another in the length of corresponding DNA fragments generated by digestion with Restriction enzyme.

The use of DNA probes (small pieces of DNA that are complementary to the region to be analyzed) which are capable of detecting in any individual a large number of highly polymorphic genetic loci simultaneously, has increased the resolution of the polymorphism much effectively. The term 'DNA finger printing' was introduced by Alec Jeffrey's in 1985 to describe the bar code like DNA fragment pattern generated by such multi locus probes after Electrophoretic separation of genomic DNA fragments. Only later was this term also used to describe the combined use of several single-locus detection systems this approach now being referred to as DNA profiling.

Molecular Basis of Polymorphism

The genetic information that makes up the genes of higher plants is stored in the DNA sequence of the nuclear chromosome and the organelle genome. Plants are able to replicate their DNA with high accuracy and rapidity, but many mechanisms are operative which cause changes in the DNA. Simple base pair changes may occur, or large-scale changes as a result of inversions, translocations, deletions or transpositions. There is such an enormous amount of DNA in a higher plant cell, that no two organisms are likely to be identical in DNA base sequence. Thus there is a tremendous amount of DNA variation present in natural population of plants. A large amount of repetitive DNA constitutes a major characteristic of higher plant genome. A great deal of variation observed between the genome of related plant species and among plants of a single species occurs in this repetitive

component. This repetitive DNA usually occur both as clustered and a 'short period' interspersed arrangement (Flavell *et al.,* 1977; Murray *et al.,* 1978). Clustered repeats include the ribosomal genes and centromeric and telomeric sequences, whereas most of the interspersed repeats are referred to as Variable Number Tandem Repeat (VNTR) or 'minisatellite' DNA (Jeffrey *et al.,* 1985a).

An Overview of Marker Systems

Usually in Plant Genetic studies, both phenotypic and molecular markers are used. The initial screening for variability are done based on the phenotypic markers such as plant height, flower colour, insect resistance etc. Classical phenotype markers were plentiful in only a few well characterized species such as maize and their utility was restricted by the difficulties involved in constructing multiple marker line by the low resolution of the map predicted and by the large amount of labour required to generate and use these markers.

But descriptions based on morphological data are fundamentally flowed in their ability to provide reliable information for calculation of genetic distance or the validation of pedigrees due to the following disadvantage of phenotype markers.

Lack of consistency in morphological variation measurements due to effect of GxE interaction and the small effects of numerous quantitative genes,

For most morphological traits, the genetic control is unknown although it is known that multiple genotypes can give phenotypes of similar outward appearance.

Therefore it is impossible to determine how completely the genome is sampled by morphological description or the extent to which similar phenotypes reflect similar genotypes. So it was concluded that morphological differences

couldn't be interpreted to provide accurate estimates of genetic differences (Smith and Smith, 1989; Smith *et al.*, 1991).

Molecular markers are based on natural variations in DNA sequence. In the vast majority of cases, they will have no phenotypic effect at all. An effectively unlimited number of Genetic markers are present in any plant and also they are advantageous over conventional markers since they simply represent the presence or absence of characteristic base sequences but not dependant on gene expression. So it less time consuming and the results are not influenced by environment in contrast to the phenotypic markers.

Each marker has a unique set of advantages and disadvantages but it is important to select the suitable marker system based on application. Other factors to be considered in selecting markers include availability of DNA, availability of markers, cost of marker, restriction in use of radioactive material etc. Markers are also classified as informative and non-informative. The more informative a given class of markers, the easier it becomes to detect a polymorphism between two individuals. A comparative study of a few molecular markers used is given in Table 2.

Table 2. A comparative study of few frequently used molecular markers

	RFLP	RAPD	SSR	AFLP	CAPS
Principle	Restriction Endonuclease Digestion, Southern blotting &Hybridization	DNA Amplification with random Primers	PCR of sample sequence repeat regions	PCR of a subset of Restriction Endonuclease fragments from extended adapter processes	Restriction Digestion of PCR Products
Nature of Polymorp-hism	Single base changes, Insertion, Deletion	Single base changes,Inserti on,Deletion	Repeat length changes	Single base changes,Insertion, Deletion	Single base changes, Insertion, Deletion
Abundance in Genome	High	Very High	Medium	High	High
Level of polymorph-ism	Medium	Medium	High	Medium	Medium
Dominance	Co-dominant	Dominant	Co-	Mixed	Co-dominant

			dominant		
DNA required	2-10µg	10-25ng	50-100ng	1-2µg	50-100ng
DNA sequence information required	No	No	Yes	No	Yes
Radio active detection	Yes/No	No	No/Yes	Yes/No	No
Development cost	Medium	Low	High	Medium	Medium/High
Startup cost	Medium/High	Low	High	Medium	High

(Rafalski *et al.*, 1993)

Random Amplified Polymorphic DNA (RAPD)

Williams *et al.*, (1990) was the first to describe such a DNA polymorphism assay based on the PCR amplified random segments with single primers of arbitrary nucleotide sequence- termed as Random Amplified polymorphic DNA. RAPD is technically simple, quick to perform, requires small amount of DNA, involves no radioactivity and is well suited for use in large sample through put systems required for breeding, population genetics and biodiversity. RAPD facilitates analysis of even badly preserved samples like field material, museum specimens, forensic samples, plant parts etc.

In this reaction a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. If these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through thermo cyclic amplification. The presence of each amplification product identifies complete or partial nucleotide sequence homology between the genomic DNA and the oligonucleotide primer, at each end of the amplified product. On average each primer will direct the amplification of several discrete loci in the genome, making the assay an efficient way to screen for nucleotide sequence polymorphism between individuals. It is also advantageous that there is no need for DNA sequence information for primer designing. But because of the

stochastic nature of DNA amplifications, with Random sequence primers, it is important to maintain consistent reaction conditions that have been optimized for reproducible DNA amplification. Factors such as template DNA concentration, Mg_2+ concentration, primer annealing temperature, primer length, primer base composition -all affect the reaction and should be controlled carefully.

RAPD polymorphism can be easily excised from the gel, re-amplified and cloned, then DNA sequenced and used as PCR primers. This Sequence Characterized Amplified Region or SCAR (Paran and Michelmore, 1992) allows high stringency amplification for the detected polymorphic region. Identification of Raspberry cultivars by SCAR analysis was done by Jean Guy Parent *et al.,* (1998). Developments of SCAR markers linked to disease resistance genes were reported in wheat (Myburg *et al.,* 1998), strawberry (Haymes *et al.,* 1997), lettuce (Paran and Michelmore, 1992). These markers will be useful for marker assisted selection and gene pyramiding. SCAR marker linked to somaclonal variations which occur frequently in tissue cultures was successfully demonstrated at the molecular level in *in vitro* germinated barley plants.(Gozukirmizri *et al.,* 1992) and in Malus (Harada *et al.,* 1993).

Identification of varieties and estimation of variability using RAPDs

RAPDs were successfully utilized in the identification of varieties (inter or intra specific variations) to distinguish wild from cultivars, the information obtained from such cases are applicable to deduce the extent of genetic relatedness and genetic diversity and hence useful in crop improvement programmes. Some of the major crop species where RAPD profiles were used in the identification of cultivars and varieties are given in Table 3.

Determination of genetic stability of micropropagated plants of ginger using RAPDs was performed by Rout *et al.,* (1998) and revealed that RAPDs are helpful for assessment of genetic stability of clonal materials. RAPDs also used to certify genetic stability of vegetatively propagating plants like *Marula* (Feiga Gutman,1999); apple root stocks (Wesley,1998); apple (Mulcahy, 1993) etc. The usefulness of RAPD is assessing genetic stability of somatic embryogenesis derived population of black spruce was demonstrated by (Isabel *et al.,* 1993). RAPD analysis was also used to identify markers linked to gene controlling somatic embryogenesis in *Medicago sativa* (Yu *et al.,* 1993). It was proved that molecular markers provide a quick simple and preliminary screening method for putative somatic hybrids in the case of *Solanum tuberosum* and *S. brevidens* (Xu *et al.,* 1993).

Table 3. Examples of successful utilization of RAPDs in identification of varieties

Crop	References
Anoectochilus sp.	Cheng KurTa *et al.,* 1998.
Apricot	Takeda *et al.,* 1998
Avocado	Lewis *et al.,* 1992
Capsicum	Rodriguez *et al.,* 1999
Chicory	Koch *et al.,* 1997
Dactylis glomerata	Kolliker *et al.,* 1999
Edible fig	Galderisi *et al.,* 1999
Elymus	Dazo *et al.,* 1999
Festuca pratensis	Kolliker *et al.,* 1999
Galicia	Vidal *et al.,* 1999
Hordeum sp.	Reddy *et al.,* 1997
Lolium perenne	Kolliker *et al.,* 1999
Lycopersicon esculentum	Williams *et al.,* 1993
Macadamia	Vithanage *et al.,* 1998
Melissa officinalis	Wolf *et al.,* 1999
Pistachio	Hormaza *et al.,* 1998
Persimon	Luo Zheng Rong *et al.,* 1998
Rice	Shuichi Fukuoka *et al.,* 1992
Rose	Torres *et al.,* 1993; Jan *et al.,* 1999
Sorghum	Tao *et al.,* 1993
Stylosanthes scabra	Liu , 1997

Tea	Wachira *et al.*, 1995
Wheat	He *et al.*, 1992, Devos *et al.*, 1992, Vierting *et al.*, 1992, Joshi *et al.*, 1993
Winter drum wheat	Sivolap *et al.*, 1998

Molecular characterization of inter and intra specific somatic hybrids of potato which eliminated the difficulty of unequivocally identifying nuclear hybrids was done by Baird *et al.*, (1992), Arti *et al.*, (1998) used RAPD fingerprinting for identifying parental lines in rice. A parentage analysis using RAPD PCR to determine both paternal and maternal DNA was demonstrated by Scott *et al.*, (1992). Finger printing was applied to paternity analysis of an apple cultivar of which the pollen parent was unknown (Harada *et al.*, 1993). RAPD analysis was also used for population genetic studies. Genetic polymorphism of 7 populations of *Capsella bursa-pastoris* was studied by Yang *et al.*, (1998). Comparison of molecular markers analysis of *Musa* breeding populations were made by Crouch *et al.*, (1994).

RAPD markers were used in construction of detailed genetic linkage maps for many important plant species. Linkage maps were constructed for many other plants also like cow pea (Menendez *et al.*, 1997) *Cucumis melo* (Liou PC *et al.*, 1998) maize (Agrama *et al.*, 1997) tomato – intra specific genetic map (Fooland *et al.*, 1993). Mapping of a T- DNA insertion site for herbicide resistance gene (bar gene) in oil seed rape was successfully investigated using RAPD markers with the help of bulk segregant analysis and comparative mapping (Baranger *et al.*, 1997). In *Allium sativum* RAPD markers related to pollen fertility was detected by Hong Chong Jian *et al.*, 1997. RAPDs linked to male sterility gene in *Brassica napus* was detected by Tu Jin Xing *et al.* Sex determination in *Actinidia* (kiwi fruit) using RAPD markers was done by Harvey *et al.*, 1997.

RAPD markers are also utilized for many purposes like taxonomic re-evaluation of Pfitzer Junipers (Alice Le Due *et al.,* 1999) finger printing of *Theobroma* clones (Wilde *et al.,* 1992) genetic diversity of coffee for genetic improvement (Anthony *et al.,* 1997) discrimination of Basidiomycetic species and strains (Yasuhiro ifo *et al.,* 1999) identification of maize in bred lines (Zhang *et al.,* 1998), molecular polymorphism between rice, root-knot nematode resistant and susceptible cult was (Bose *et al.,* 1995)

Markers that flank a gene determining a trait of agronomic interest can be used to track the trait in genetic crosses and also to estimate the genetic contribution of each parent to each member of a segregating population. Examples of such cases are RAPD assisted genetic segregation in diploid cultivated alfalfa, genetic diagnosis of F1 hybrid of *Rehmania* sp. for segregating characteristics from both parents (Hatano *et al.,* 1997)

Introgression of *Medicago* was also successfully detected using RAPD by Mc Coy *et al.,* 1993. Conte *et al.,* in 1998 reported DNA finger printing analysis by a PCR based method for monitoring the genotoxic effect of heavy metal pollution in *Arabidopsis.* The comparison between unexposed and exposed genomes showed that RAPD analysis can be used to evaluate how environmental pollutants modify the DNA structure in living organisms.

Another interesting genetic linkage mapping study was done by Tulsiram *et al.,* in 1992 in conifers using haploid DNA from mega gametophytic tissue – so that heterozygotes and dominant homozygotes can be clearly distinguished. It was also suggested that the RAPD analysis of haploid DNA might be applicable *via* anther culture for many plants.

There are no earlier reports on the use of molecular markers to study genetic polymorphism, in vanilla.

Amplified Fragment Length Polymorphism (AFLP)

The AFLP technique, originally known as selective restriction fragment amplification (SRFA) (Zabeau and Vos, 1993), produces highly complex DNA profiles by arbitrary amplification of restriction fragments ligated to double-stranded adaptors with hemi-specific primers harboring adaptor-complementary 5' termini (Vos *et al.,* 1995). The technique has been widely used in the construction of genetic maps containing high densities of DNA marker loci. The AFLP protocol amplifies restriction fragments obtained by endonuclease digestion of target DNA using "universal" AFLP primers complementary to the restriction site and adapter sequence. However, not all restriction fragments are amplified because AFLP primers also contain selective nucleotides at the 3' termini that extend into the amplified restriction fragments. These arbitrary terminal sequences result in the amplification of only a small subset of possible restriction fragments. The number of amplified fragments (generally kept around 50-100) can therefore be "tailored" by extending the number of arbitrary nucleotides added to the primer termini. Generally, the abundant restriction fragments produced from complex genomes require of AFLP primers with longer selective regions. Conversely, analysis of small genomes require of only few arbitrary nucleotides added at the primer 3' termini. The resulting AFLP fingerprints are usually a rich source of DNA polymorphisms that can be used in mapping and general fingerprinting endeavors.

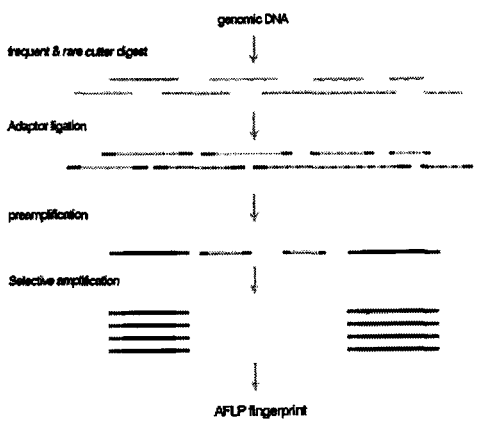
Amplification of very small "genomes" (plasmids, cosmids, BACs) requires of primers with no selective nucleotides. AFLP fingerprinting of bacteria

and fungi generally requires primers with 2 selective bases. Complex genomes require the use of more than 2 selective bases in one or both primers. In the case of complex genomes it is recommended to carry the amplification in two consecutive steps (preamplification and selective amplification) to increase specificity and the amount of initial template. The AFLP fragments are usually detected by labeling one of the two AFLP primers. For example, radioactively labeled primers can be obtained by phosphorylating the 5' ends with g-33P-ATP and polynucleotide kinase. The labelled reaction products are separated by electrophoresis using denaturing polyacrylamide gels and exposed to X-ray films to visualize the AFLP fingerprints.

The AFLP technique usually consists of the following steps

1. The restriction of the DNA with two restriction enzymes, a hexa-cutter and a tetra-cutter;
2. The ligation of double-stranded adapters to the ends of the restriction fragments;
3. The amplification of a subset of the restriction fragments using two primers complementary to the adapter and restriction site sequences, and extended at their 3' ends by 1 to 3 "selective" nucleotides *(see Figures 1 and 2);*
4. Gel electrophoresis of the amplified restriction fragments on denaturing polyacrylamide gels ("sequence gels");
5. The visualization of the DNA fingerprints by means of autoradiography, phospho-imaging, or other methods.

A schematic diagram of AFLP is given below



AFLP technique is a random amplification technique, which, in contrast to most other random amplification techniques, makes use of stringent PCR conditions. The amplification primers, known as AFLP primers, are generally 17 - 21 nucleotides in length and anneal perfectly to their target sequences; i.e. the adapter and restriction sites, and a small number of nucleotides adjacent to the restriction sites. This renders AFLP a very reliable and robust technique, which is unaffected by small variations in amplification parameters. (e.g. thermal cyclers, template concentration, PCR cycle profile). The high marker densities that can be obtained with AFLP are an essential characteristic of the technology: a typical AFLP fingerprint contains between 50 and 100 amplified fragments, of which up to 80% may serve as genetic markers. Moreover AFLP technology requires no sequence information or probe collections prior to the generation of AFLP fingerprints. This is of particular benefit when studying organisms where very little DNA marker information is available. AFLP markers usually exhibit Mendelian inheritance, indicating that they are unique DNA fragments.

AFLP can be used in a large number of applications, such as, biodiversity studies, analysis of germplasm collections, genotyping of individuals, and genetic distance analyses, identification of closely-linked DNA markers, construction of genetic DNA marker maps, construction of physical maps using genomic clones such as YACs and BACs, in the precision mapping of genes, and the subsequent isolation of these genes and the generation of "transcript profiles" for gene expression analysis.

The advantage of AFLP is its high multiplexity and therefore the possibility of generating high marker densities. In this respect, AFLP and DAF are

comparable. One limitation of the AFLP technique is that fingerprints may share few common fragments when genome sequence homology is less than 90%. Therefore, AFLP cannot be used in comparative genomic analysis with hybridization-based probes or when comparing genomes that are evolving rapidly such as those of some microbes (Janssen *et al.,* 1996). Conversely, very homogeneous genomes may not be suitable for AFLP analysis.

The molecular basis of AFLP sequence polymorphisms rests on the detection of single nucleotide changes within restriction sites or adjacent nucleotides used for AFLP primer annealing. In addition, polymorphisms can also result from deletions, insertions and rearrangements affecting the presence or restriction sites and/or adjacent sequences. Therefore, most AFLP markers will be mono-allelic with only few being di-allelic.

IN VITRO CONSERVATION

Secure genetic stocks are a fundamental requirement in plant breeding. As *in vitro* techniques are becoming more important in crop improvement through somatic cell genetics, genetic stocks are assuming more variable forms from intact plantlets to protoplasts. Atleast plant material, produced *in vitro* like callus, cell lines, protoplasts etc. will have to be conserved *in vitro*. Moreover crops, whose conservation cannot be satisfied with conventional seed storage and in field gene banks (clonal repositories) may have to be conserved *in vitro*. These included crops with recalcitrant seeds and those which are vegetatively propagated. A brief summary of major horticultural crops where *in vitro* and cryopreservation was successfully achieved is presented in Table 4.

A culture requires transfer to fresh medium every week, fortnight or month, thus presenting a tremendous workload. There is need to find alternative storage methods to reduce the workload, time and resources. By changing the growth kinetics of the cultures, the subculture intervals can be extended to quarterly, half yearly or annual and even to much longer times, upto centuries. Two basic approaches in *in vitro* conservation of germplasm are *slow growth* and *cryopreservation*, to conserve the active and base collections for short and long term conservation respectively (Withers, 1985).

Table 4. *In vitro* conservation and cryopreservation of few major horticultural crops

Application	Technique	References
Cassava		
Disease eradication	Thermotherapy and meristem culture	IPGRI / CIAT 1994
	Shoot culture	
Propagation	Plantlets at 23 ^{0}C	IPGRI / CIAT 1994
Slow growth storage	Zygotic embryos / seeds	IPGRI / CIAT 1994
Cryopreservation	Shoot apices	Marin *et al.*, 1990
	Somatic embryos	Escobar *et al.*, 1993
Distribution	In vitro plantlets	Sudarmonowati and Henshaw, 1990
		Roca *et al.*, 1984
Potato		
Disease eradication	Meristem culture, thermotherapy and chemotherapy	Cassels and Long, 1982
Propagation	Shoot cultures	Goodwin 1966
	Microtubers	Hussey and Stacey, 1981
Slow growth storage	Shoot culture at 10 ^{0}C	Mix 1984
	Microtubers	Thieme and Pett 1982
Cryopreservation	Shoot apices	Schafer-Menuhr 1995
Distribution	In vitro plantlets	Roca *et al.*, 1979
***Musa* spp.**		
Collection	Shoot meristem	FAO / IPGRI 1997
Disease eradication	Meristem culture	Gupta 1986
Propagation	Shoot culture	Vuylsteke and Swennen 1990
	Somatic embryogenesis	Novak *et al.*, 1989
Slow growth storage	Shoot culture at 10^{0}C	Van den houwe *et al.*, 1995
Cryopreservation	Zygotic embryos	Abdelnour-Esquivel *et al.*, 1992a
	Meristematic clumps	Panis 1995, Frison and Putter 1989
Distribution	In vitro plantlets	
Coconut		
Collecting	Zygotic embryos	Assy-Bah *et al.*, 1987
Propagation	Zygotic embryo culture	Assy-Bah *et al.*, 1987
Somatic embryogenesis	From leaf/inflorescence callus	Verdelland Buffard-Morel 1995
Storage	Zygotic embryo	Assy-Bah and Engelman 1993
Cryopreservation	Zygotic embryo	Assy-Bah and Engelman 1992a
Distribution	Zygotic embryo	Frison *et al.*, 1993
***Prunus* spp**		
Collecting	Shoots	Elias 1988
Slow growth storage	Shoots at 0-4 ^{0}C	Wilkins *et al.*, 1988
Cryopreservation	Shoot apices, zygotic embryos	Brizon *et al.*, 1995
Black pepper		
Disease eradication	Meristem culture	Philip *et al.*, 1992
Propagation	Shoot culture	Broome and Zimmerman, 1978
	,Leaf / root	Nirmal Babu *et al.*, 1993
Slow growth storage	In vitro plantlets at 22^{0}C	Nirmal Babu *et al.*, 1993
Cryopreservation	Seeds	Chaudhury and Chandel 1994
***Allium* spp.**		
Disease eradication	Meristem culture and thermotherapy	Conci and Nome 1991
Propagation	Shoot culture from bulb	Hussey 1978
	from inflorescence	Novak and Havel 1981
	Microbulbets	Keller 1991
Slow growth storage	Shoot culture	El-Gizawy and Ford-Lloyd 1987
	Microbulbets	Keller 1991
Cryopreservation	Apical meristem	Niwata 1995

Citrus		
Collection	Budwoods	FAO/IPGRI 1997
Disease eradication	Shoot tip grafting	Navarro 1992
	In vitro grafting	Navarro *et al.*, 1984
Propagation	Shoot culture	Duran-Vila *et al.*, 1989
	Somatic embryogenesis	Ollitrault *et al.*, 1992
Slow growth storage	In vitro plantlets	Marin and Duran-Vila 1991
Cryopreservation	Seeds	Duran Vila 1995
	Somatic embryos	Marin and Duran Vila 1992
Distribution	Nucellar callus and cell suspensions	Engelmann *et al.*, 1994
Coffee		
Collection	Cuttings	FAO / IPGRI 1997
Propagation	Shoot cultures	de Pena 1984
Slow growth storage	In vitro plantlets	Bertrand-Desbrunais and Charrier 1989
Cryopreservation	Apices	Mari *et al.*, 1995
	Zygotic embryos	Abdelnour-Esquivel *et al.*, 1992b
	Somatic embryos	Bertrand-Desbrunais *et al.*, 1988
	Seeds	Normah and Vengadasalam 1992
Colocasia esculenta		
Disease eradication and indexing	Meristem culture	Ng 1983a
	Tested for DMV (ELISA), taro bobone rhabdovirus (ISEM), TBV	Jackson 1990
Propagation	Shoot culture	Ng 1983b
	Corm tissue	Islam *et al.*, 1994
Slow growth storage	At reduced temperature	Akhond *et al.*, 1995
Cryopreservation	Somatic embryos, meristematic clumps	Islam *et al.*, 1994
***Dioscorea* spp.**		
Disease eradication and indexing	Meristem tip culture, ELISA, RT PCR	Mantell *et al.*, 1980
Propagation	Shoot culture	Ng 1992
Slow growth storage	Reduced temperature	Ng and Ng 1994
	Modified medium	Malaurie *et al.*, 1993
	Mineral overlay, reduced temperature	Takagi *et al.*, 1994
	Nodal segments at 25°C	Mandal and Chandel 1993
Cryopreservation	Shoot tips	Mandal and Chandel 1995
Distribution	In vitro plantlets	Ng 1991
	Microtubers	Malaurie *et al.*, 1993
Ipomoea batatas		
Disease eradication and indexing	Meristem culture	Green and Lo 1989
Propagation	Axillary bud culture	Jarret and Florkowski 1990
	Somatic embryos	Chee and Cantliffe 1988
Slow growth storage	Chemical and environmental inhibition	Jarret and Gawel 1991a
	ABA inhibition	Jarret and Gawel 1991b
Cryopreservation	Shoot tip vitrification	Towill and Jarret 1992
	Somatic embryo desiccation	Shimonishi *et al.*, 1994
Distribution	Axillary bud , in vitro plantlets	Jarret 1989
***Pyrus* spp.**		
Disease eradication and indexing	Thermotherapy and meristem culture	Postman 1994
Propagation	Shoot culture	Berardi *et al.*, 1993
Slow growth storage	1°C or 4°C for 1-4yr	Reed and Chang 1997
	4°C, 16h photoperiod	Wanis *et al.*, 1986
	1°C dark	Moriguchi *et al.*, 1990
Cryopreservation	Slow freezing	Reed 1990a
	Encapsulation	Scottez *et al.*, 1992
	Vitrification	Niino *et al.*, 1992
	Encapsulation and vitrification	Niino and Sakai 1992
Distribution	In vitro plantlets	Hummer 1994
Cardamom		
Propagation	Shoot buds	Nadgauda *et al.*, 1983
	Callus regeneration	Sreenivasa Rao *et al.*, 1982.
Slow growth storage	*In vitro* plantlets at 22°C	Geetha *et al.*, 1995
Cryopreservation	Seeds	Chaudhury and Chandel 1995
***Vitis* spp.**		
Disease eradication and indexing	Meristem culture	Alleweldt *et al.*, 1990
Slow growth storage	Shoot culture	Barlass and Skene 1983
Cryopreservation	Shoot tip encapsulation	Plessis *et al.*, 1991
	Slow freezing cell suspension	Dussert *et al.*, 1991

Distribution	Disease free plantlets	Cao 1990
***Xanthosoma* spp.**		
Disease eradication and indexing	Meristem culture	Ng 1983a
Propagation	Shoot culture	Ng 1983b
Slow growth storage	Reduced temperature, yearly subculture	
Distribution	*In vitro* plantlets	
***Zingiber* spp.**		
Disease eradication and indexing	Shoot cultures	Balachandran *et al.*, 1990
Propagation	Shoot buds	Hosoki and Sagawa 1977
	Inflorescence	Nirmal Babu *et al.*, 1992b
	Callus regeneration	Nirmal Babu *et al.*, 1992a
	Somatic embryogenesis	Kacker *et al.*, 1993
Slow growth storage	Shoot cultures at 22 ^{0}C	Balachandran *et al.*, 1990
	Shoot cultures under paraffin oil	Dekkers *et al.*, 1991
Distribution	Micro rhizomes	Bhat *et al.*, 1995
***Curcuma* spp.**		
Propagation	Shoot buds	Nadgauda *et al.*, 1978
	Callus cultures	Shetty *et al.*, 1982
Slow growth storage	*In vitro* plantlets at 22 ^{0}C	Geetha *et al.*, 1995
Distribution	Microrhizomes	Reghurajan *et al.*, 1997
***Vanilla* spp.**		
Disease eradication	Apical meristem	Cerevera and Madrigal 1981
Propagation	Root	Philip and Nainar 1986
Distribution	Synthetic seeds	Sajina *et al.*, 1997

*Ashmore,1997 and Nirmal Babu *et al.*, 1999.

One of the ways to induce minimal growth is to reduce culture temperature, use of hormones or osmotic inhibitors and in some cases reduction of essential nutrients. Thus by altering the growth kinetics, the subculture intervals can be increased significantly. However, only by freezing at ultra low temperatures such as -196^{0}C, the temperature of liquid nitrogen, growth in plant tissue and cell cultures can be entirely suspended. Since very few cultures have a natural resistance to freezing, special manipulations of explant and culture conditions are required to prepare a specimen with maximum freeze tolerance. Application of certain cryoprotectants like dimethyl sulphoxide (DMSO), glycol in combination with other compounds like mannitol, sorbitol, glucose, sucrose, proline or polyethylene glycol (PEG) will bring about changes in cell permeability, freezing point and response to the stresses of freezing and thawing essential for survival.

Minimal growth:

Several ways have been found to reduce the rate of growth of cultured material so that it can be kept unattended for moderate length of time. Species vary in the length of time (6-24 months) during which their cultures can be stored. Single shoots, somatic embryos, rooted plantlets are suitable for storage. Of these plantlets are most ideal. Rooted plantlets of *Mentha, Rubus*, blackberry, *Vaccinium* species could be stored for 6- 17 months under normal culture conditions in culture vessels sealed with caps or films which allow gaseous exchange but prevent escape of moisture (Gunning and Lagerstedt, 1986). Rooted plantlets of *Pestacia* species could be stored for one year (Barghchii, 1986) and those of *Coffea arabica* for more than two years (Kartha *et al.,* 1981) under normal conditions.

Interval between transfers can be greatly increased by keeping cultures in weak light or in the dark and at a temperature less than the temperature optimal for active growth. The optimum temperature and light requirements for storage, are genotype dependant. Shoots and plantlets of many temperate crops were stored successfully at temperatures close to freezing. Apple shoot tips grown *in vitro* remained viable at 1 ^{0}C to 4 ^{0}C for one year in the dark (Mullin and Schlegel, 1976). A temperature range of 0 ^{0}C to 5 ^{0}C was found suitable for conserving shoot cultures of many temperate woody species (Lundergan and Janick, 1979; Chun and Hall, 1986; Aitken-Christie and Singh, 1987). Somatic embryos can be stored at a temperature just above freezing (4^{0}C to 10^{0}C) (Durzan, 1988; Lutz *et al.,* 1985). Encapsulated embryos of *Santalum album* was stored for 45 days at 4^{0}C. Dark storage is satisfactory for some species. Shoot culture of *Prunus, Kalmia* and *Populus* can be kept in the dark at -3^{0}C and 4^{0}C respectively for one to two years (Marino *et al.,* 1985; Chun and Hall, 1986; McCulloch, 1988). The

cold storage of herbaceous species has been reviewed by Bhojwani and Razdan (1983) and that of woody plants by Aitken-Christie and Singh (1987).

Plantlets and shoot cultures of some temperate plants, many tropical and sub-tropical species loose their viability if stored at low temperature. For many of these species minimal growth is best achieved at 14^0C to 20^0C (Henshaw *et al.*, 1980). Cassava shoot cultures deteriorated if stored at temperature lower than 20^0C (Roca *et al.*, 1982). The international germplasm collection of *Musa* species is conserved as shoot cultures stored at 15^0C and a luminance of 1000 to 2000 lux. *Musa* cultures die within six weeks if kept at 5^0C and within three months at 10^0C, but at 15^0C they can be kept for 13-17 months according to their genotype (Banerjee and De Langhe, 1985). Similarly, cardamom cultures did not survive at 5^0C, 10^0C and 15^0C but could be maintained best for 12 months at 22^0C at 3000 lux (Nirmal Babu *et al.*, 1994; Geetha *et al.*, 1995).

Nitzsche (1978, 1980, 1983) reported that carrot callus can survive storage upto two years if it is pre treated with 10 mgl^{-1} of abscisic acid and dried on sterile filter paper in the air stream of laminar flow cabinet before storage. Dormancy could be induced in encapsulated somatic embryos by the use of abscisic acid followed by drying. *Medicago sativa* somatic embryos, matured in the presence of abscisic acid and dried to a moisture content of 8-15%, have remained fully viable at room temperature for twelve months (Redenbaugh *et al.*, 1991). Dried embryos may survive for longer periods if they are encapsulated in a polymer coating (Kitto and Janick, 1985).

Placing culture in an environment with a low partial pressure of oxygen appears to have potential for limiting *in vitro* growth. Caplin (1959) used mineral oil overlay to conserve callus cultures. He attributed the decrease in growth rates

to oxygen being prevented from reaching the plant tissue. Substances like autoclaved silicon (Moriguchi *et al.,* 1988), liquid paraffin oil (Mathur, 1991) were also used for the same effect.

Decreasing the carbohydrate / nutrient supply could also result in reduced growth rate (Gunning and Lagerstedt, 1986). Similar results were reported in coffee (Kartha *et al.,* 1981; Roca *et al.,* 1982). Reduction of macronutrient availability gave better results in *Vitis* shoot cultures (Moriguchi and Yamaki, 1989).

Changing the osmotic potential by increasing the sucrose levels will have an inhibitory effect on plant cell growth and thus can be used to maintain cultures in a dormant condition for one or two years (Schenk and Hildebrandt, 1972). Addition of mannitol to the culture medium can also prevent the rapid cell death (Codron *et al.,* 1979). It was suggested that mannitol preserves membrane integrity and prevents solute leakage. Addition of mannitol and increase in sucrose concentration were found to restrict the growth and enhance the viability of cells in *Cinchona* (Hunter *et al.,* 1986) and potato (Henshaw *et al.,* 1980) respectively.

Growth regulators are normally added to the culture medium to promote and regulate plant growth *in vitro*. Withdrawal of these chemicals can assist in *in vitro* storage of certain plants (Gunning and Langerstedt 1986). Certain growth regulators like abscisic acid are able to induce dormancy in plant meristems and somatic embryos of caraway (Ammirato, 1974), potato (Roca *et al.,* 1982).

Minimal growth storage is useful for the preservation of clones, which are required as stocks for continued propagation *in vivo* or *in vitro*, or as parents in plant breeding programmes. This technique is not ideal for the long term storage, because the periods during which cultures can be left unattended are relatively

short. Nevertheless, in the absence of a reliable method, minimal growth storage is being used in conjunction with shoot or node culture, to maintain genotypes in germplasm banks (George, 1993).

Synthetic seeds :

Production of artificial seeds or 'synthetic seeds', consisting of somatic embryos or shoot buds enclosed in a biodegradable protective coating, is a low cost, high volume propagation system (Redenbaugh *et al.,* 1986). Two types of artificial seeds, *hydrated* and *desiccated*, have been developed. Hydrated artificial seeds consists of somatic embryos, propagules individually encapsulated in a hydrogel such as, calcium alginate. Desiccated artificial seeds are produced by coating somatic embryos, roots and callus in polyoxyethylene glycol. The coated mixture was allowed to dry for several hours on a Teflon surface in sterile hood, before culturing for germination. At present, these methods are not completely satisfactory. Hydrated capsules are more difficult to store because of the requirement of embryo respiration, moreover these capsules dry out quickly unless they are kept in a humid environment or coated with a hydrophobic membrane (Redenbaugh *et al.,* 1987). In desiccated artificial seeds the desiccation process itself may damage the propagules.

The use of somatic embryos as artificial or synthetic seeds was long felt as a promising alternative to conventional propagule production in many crops. The main advantages of somatic embryos are that their production could be completely automated, and large quantities of embryos could be produced in closed system without much manual labour. However, somatic embryogenesis is not common in tissue cultures of many of the crops. In crop species where somatic embryogenesis is not common, attempts were made to encapsulate *in vitro*-derived vegetative

propagules in an appropriate gel and to use the encapsulated segments as artificial or synthetic seeds. This technique offers tremendous opportunities for production of large quantities of disease-free propagules from *in vitro* plantlets in protected condition within a limited time and space (Uozumi and Kobayashi, 1995 and Standardi and Piccioni, 1998).

Synthetic seeds were developed by encapsulating somatic embryos in carrot (Kitto and Janick, 1985a, b), groundnut (Padmaja *et al.*, 1995), *Eleusine coracana* (George and Eapen, 1995), *Santalum album* (Fernandes *et al.*, 1994), etc., axillary or apical shoot buds in *Morus indica* and sandalwood (Bapat *et al.*, 1987; Bapat and Rao, 1990; Bapat, 1993), alfalfa (McKersie and Bowley, 1993), *Valeriana wallichii* and *Dioscorea* sp. (Hasan and Takagi, 1995), *Coleus forskhlii* (Bhattachryya *et al.*, 2001), flower buds in citrus (Mitra and Chaturvedi, 1972) and apple root stocks (Sakamoto *et al.*, 1995; Piccioni, 1997; Capuano *et al.*, 1998; Sicurani, 2001). The encapsulation of shoot buds and production of synthetic seeds were reported in orchids like *Phaius tankervillae* (Malemnganba *et al.*, 1996) and *Spathoglottis spicata* (Nayak *et al.*,1998), *Geodorum densiflorum* (Datta *et al.*, 1999). Plant regeneration from protoplasts en-capsulated in alginate beads was reported in *Pogostemon cablin* (Kageyama *et al.*, 1995).

There is potential for the storage of artificial seeds, or the distribution of germplasm in the form of encapsulated embryos or apices (Hassan and Takagi, 1995). The advantages of synthetic seeds over sexually produced seeds are, i) they are rapidly produced at any time of the year, ii) They can be produced in large numbers, and iii) they are genetically the same as the original plant. Thus, the major interest is in the commercial production of seeds by a controlled process, with the possibility of production whenever demand occurs. There is considerable

potential for the use of synthetic seeds for germplasm exchange, and perhaps for storage of plant germplasm. The latter will require the development of synthetic seeds, which can withstand both desiccation and low temperature regimes (Senaratna and McKersie, 1989; Ashmore, 1997). Synthetic seeds can be an ideal system for low cost plant movement, propagation, conservation and exchange of germplasm.

Cryopreservation

Cryopreservation is defined as the viable freezing of biological material and their subsequent storage at ultra low temperatures, preferably at that of liquid nitrogen. Preservation of both plant and animal germplasm is an integral component of sustainable agriculture system. From a crop improvement perspective, preservation of all valuable germplasm is being accorded a high priority (IBPGR, 1985). Although several decades later, the development of cryopreservation strategy for plant cells and organs has followed the advances made with mammalian systems (Kartha and Engleman, 1994). One of the earliest report on the survival of plant tissues exposed to ultra-low temperature was made by Sakai (1956) when he demonstrated that very hardy mulberry twigs, upon induction of dehydration mediated by extra-cellular freezing are capable of survival following immersion in liquid nitrogen provided the frozen samples are subsequently re-warmed slowly at an air temperature of 0 ^{0}C. Sun (1958) achieved partial success when desiccated seedlings of *Pisum sativum* were immersed in liquid nitrogen. Quatrano (1968) reported the first record on the use of cryoprotectant for the freezing of plant cells. The first successful attempt to cryopreserve cultured cells of species is that with cell cultures of *Daucus carota* (Latta, 1971) and this has become a model species to study cryobiology of

cultured cells. Many reports were available on the cryopreservation of carrot cell cultures (Withers and Street, 1977; Dougall and Whitten, 1980; Weber *et al.*, 1983).

An array of plant material could be considered for cryopreservation as dictated by the actual needs *vis-a-vis* preservation. These include meristems, cell, callus and protoplast cultures, somatic and zygotic embryos, anthers, pollen or microspores and whole seeds (Withers, 1985; Kartha, 1985).

Plant cells contain high amounts of cellular water and freezing of plant cells implies conversion of some or all of their liquid water to ice, whereas thawing is reversal of this transition. Since most of the experimental systems (meristems, shoot tips, cultured cells etc.) contain high amount of cellular water and hence extremely sensitive to freezing injury, protection from freezing and thawing has to be imposed artificially. This involves various strategies such as the use of cryoprotectants or other manipulations (Kartha and Engleman, 1994).

However, only by freezing at ultra low temperatures such as -196^{0}C, the temperature of liquid nitrogen, growth in plant tissue and cell cultures can be entirely suspended. Since very few cultures have a natural resistance to freezing, special manipulations of explant and culture conditions are required to prepare a specimen with maximum freeze tolerance. Application of certain cryoprotectants like dimethyl sulphoxide (DMSO), glycerol in combination with other compounds like mannitol, sorbitol, glucose, sucrose, proline or polyethylene glycol (PEG) will bring about changes in cell permeability, freezing point and response to the stresses of freezing and thawing essential for survival.

Cryopreservation involves essentially three steps namely, specimen treatment and freezing, storage at ultra-low temperature and thawing and recovery. Before

commencing proper cryopreservation procedures different culture systems need to be given specific manipulations and or culture conditions in order to present a specimen with maximal freeze tolerance. An ideal cryoprotectant should protect cells from all the factors, which would affect the viability of the frozen biological sample during all these stages. Since viability loss is not expected to occur at ultra-low temperatures, protection of the cells during freezing and thawing is of paramount importance. A number of compounds such as glycerol, dimethyl sulphoxide (DMSO), ethylene glycol, polyethylene glycol (PEG), sugars and sugar alcohols either alone and in combination protect living cells against damage during freezing and thawing. Such compounds can lower the temperature at which freezing first occurs and can alter the crystal habit of ice when it separates (Withers, 1980; Kartha and Engleman, 1994). Although the exact mechanism of action of cryoprotective compounds is still poorly understood, the colligative properties of the cryoprotectants can minimize the deleterious action of excessive electrolyte concentration resulting from removal of water and conversion of water to ice (Nash, 1966).

Cryoprotectants generally used for freezing biological specimens fall into two categories namely, permeating and non-permeating. The most commonly used permeating additives are DMSO and glycerol; the former permeates rapidly and is more toxic when compared to the latter. The permeating ability of glycerol is species-specific and temperature dependent. The penetration rate of glycerol at 20°C was twice as that at 0°C. Generally a concentration of 5–10% for DMSO and 10–20% for glycerol is adequate for most material. In instances where application of a single cryoprotectant does not result in survival, a mixture of cryoprotectants has been beneficial (Finkle *et al.*, 1985; Withers, 1985). Application of

cryoprotectant to the cells and the removal of cryoprotectants from the thawed samples should always be a gradual process to alleviate the problems associated with plasmolysis and deplasmolysis, respectively (Kartha and Engleman, 1994). Cryoprotectants are usually prepared in culture medium and the material to be cryopreserved is incubated prior to their freezing (Withers, 1985; Kartha and Engleman, 1994).

There are different freezing methods such as slow freezing, rapid freezing, droplet freezing, vitrification, etc. A number of factors such as cooling rates, pretreatment and cryoprotection, type and physiological state of the experimental material and the terminal freezing temperature, influence the success of slow freezing method. The most commonly used method of freezing meristems and cell cultures is by regulated slow cooling at a rate of 0.5 to 1.0 ^{0}C /min down to either –30^{0}C, –35^{0}C or –40 ^{0}C with the help of a programmable freezer, followed by storage in liquid nitrogen. Meristems, cell cultures and somatic embryos of a number of species have been successfully cryopreserved following slow freezing methods (Kartha, 1985; Withers, 1985).

Rapid freezing is the simplest form of cryopreservation since the procedure does not require sophisticated and expensive equipment. Rapid freezing has successfully cryopreserved meristems and somatic embryos of a few plant species. It is suggested that the viability of cells may be maintained by preventing the growth of intracellular ice crystals formed during rapid cooling by rapidly passing the tissue through the temperature zone in which lethal ice formation occurs (Seibert and Wetherbee, 1977).

The basis of droplet freezing is identical to slow freezing. This technique was originally developed for cryopreservation of cassava meristems (Kartha *et al.*,

1982). The development of vitrification technique for the cryostorage of cultured plant cells and organs is of recent origin although Sakai (1958) succeeded in obtaining the survival of hardy mulberry cortical tissue in liquid nitrogen by vitrification. In the vitrification method, cells or meristems must be sufficiently dehydrated with a highly concentrated vitrification solution at 25 ^{0}C or 0 ^{0}C without causing injury prior to immersion in liquid nitrogen. Vitrification method of cryopreservation was reported in 5 apple species or cultivars, 8 pear cultivars (Niino *et al.*, 1992a) and 13 mulberry species or cultivars (Niino *et al.*, 1992b).

For freezing of differentiated tissues and organs such as apices zygotic and somatic embryos, new techniques have been developed (Deruddre, 1992; Engelmann, 1997; 2001). They are base on the removal of most or all freezable water by physical or osmotic dehydration of explants followed by ultra-rapid freezing which results in vitrification of intracellular solutes, i.e. the formation of an amorphous glassy structure without occurrence of ice crystals which are detrimental to cellular structural integrity. Their main advantages in comparison to classical procedures are their simplicity, since they do not require the use of a programmable freezer, and their applicability to a wide range of genotypes. They include encapsulation-dehydration, vitrification, encapsulation-vitrification, desiccation, pregrowth, pregrowth-desiccation and droplet freezing (Engelmann, 1997; 2001).

The encapsulation-dehydration procedure is based on the technology developed for the production of artificial seeds. Explants are encapsulated in alginate beads, pregrown in liquid medium enriched with sucrose for several days, partially desiccated down to a water content around 20% (fresh weight basis), then frozen rapidly. Survival rates are high and growth recovery of cryopreserved

samples is generally rapid and direct, without callus formation. This technique has been developed for apices of various species from temperate origin such as apple, pear, grape, eucalyptus, and of tropical origin such as sugarcane and cassava (Dereuddre, 1992; Engelmann, 1997; Engelmann and Takagi, 2001).

Vitrification consists of placing explants in the presence of a highly concentrated cryoprotective solution, then freezing them rapidly. This technique has been experimented with cell suspensions, apices and somatic embryos of around 20 plant species (Sakai, 1993; 1997, 2001).

Encapsulation-vitrification is a combination of the above techniques, where explants are encapsulated in alginate beads and treated with vitrification solutions before freezing. it has been applied to apices of lily and wasabi (Matsumoto *et al.*, 1995; Sakai and Matsumoto, 1996).

Desiccation is the simplest procedure since it consists of dehydrating explants, then freezing them rapidly by direct immersion in liquid nitrogen. This technique is mainly used for freezing zygotic embryos or embryonic axes extracted from seeds. It has been applied to embryos of a large number of recalcitrant and intermediate seed species (Dumet *et al.*, 1997). Optimal survival rates are generally obtained when samples are frozen with a water content comprised between 10 and 20% (fresh weight basis).

Pregrowth involves preculturing the plant material on a medium containing cryoprotectants, then freezing explants rapidly. An efficient pregrowth procedure has been developed recently for meristematic clumps of *Musa* sp. (Panis and Thinh, 2001).

In a pregrowth-desiccation procedure, explants are pregrown in the presence of cryoprotectants (generally sugars such as sucrose or glucose),

dehydrated under the laminar airflow cabinet or with silica gel, then frozen rapidly. This method has currently been applied to coconut zygotic embryos and oil palm somatic embryos only (Assy-Bah and Engelmann, 1992; Dumet *et al.*, 1993). Survival rates obtained after freezing are high and growth recovery is rapid and direct.

The droplet freezing technique has been set up with potato apices. After dissection, apices are precultured with DMSO for a few hours and frozen rapidly in droplets of cryoprotective medium placed on aluminium foils. This procedure has been applied successfully to more than 150 varieties n applied successfully to more than 150 varieties with an average recovery rate of 40% (Schäfer-Menuhr, 1996).

Cryopreservation techniques have been developed for more than 80 different plant species cultivated under various forms including cell suspensions, calluses, apices, somatic and zygotic embryos (Kartha and Engelmann, 1994; Engelmann, 1997). They can be considered operational in an increasing number of cases. However, their routine utilisation is still restricted almost exclusively to the conservation of cell lines in research laboratories. The only examples of routine use with other materials are oilpalm for which more than 80 clones of somatic embryos are stored for the long term in liquid nitrogen (Dumet, 1994) and potato (Schäfer-Menuhr, 1996).

Technique of *in vitro* conservation has been made dramatic progress in the last 10 years and methods have been standardized for quite a lot of plant species (Table 7). Slow growth techniques are immediately applicable in most cases, whereas cryopreservation techniques often require further improvement before they can be applied routinely.

Many recent reports are available on slow growth storage and cryopreservation of crops such as cassava (Mabanza *et al.*, 2001); rice (Watanabe *et al.*, 1999; Wang *et al.* 2001), kiwifruit (Bachiri *et al.*, 2001; Wu, *et al.*,2001), *Zoysia* sp. and *Lolium* sp. (Chang *et al.*, 2000); orchid seed and its fungal symbiont (Wood *et al.*, 2000), *Eucalyptus* hybrid (Blakesley and Kiernan, 2001), *Auricula* (Hornung, *et al.* , 2001) etc.

Pollen cryopreservation

The pollen longevity of different species varies between minutes and years depending primarily on the taxonomic status of the plant and on abiotic environmental conditions. For a number of agronomically important taxa, including the short-lived graminaceous pollen, special storage conditions are needed to preserve the viability and fertilizing ability of pollen for a long period. There are a large number of crop species, including vegetables, fibre and fruit crops, forages and cereals, for which pollen storage strategies are desirable (Barnabas and Kovacs, 1997). According to them pollen storage has been used to satisfy the following practical needs:

1. To hybridize plants that flower at different times and locations or show nonsynchronous flowering
2. To provide a constant supply of short lived (recalcitrant) pollen
3. To facilitate supplementary pollination for improving yields
4. To eliminate the need to grow male lines continuously in breeding programmes
5. To obviate the variability incidental to the daily collection of pollen samples
6. To study pollen allergens and the mechanism of self incompatibility
7. To provide materials for international germplasm exchange
8. To ensure the availability of pollen throughout the year without using nurseries or artificial climate for plant growth

Earlier reports on storage of pollen has been reviewed by Knowlton (1922), Stanley and Linskens (1974), Shivanna and Sawhney (1997) and Towill and Walters (2000).. Various methods are available for short term as well as long-term storage of pollen. Cryogenic procedures seem to show promise for long-term preservation of pollen viability (Barnabas and Kovacs, 1997). Ultra low temperatures can be used for preserving pollen in an unaltered condition with great potential. Knowlton (1922) was the first to observe that pollen could survive extremely low temperatures. Since then numerous studies have been conducted on cryopreservation of pollen, a few of which are given in Table 5.

Table 5. Successful cryostorage of pollen from various crop species

Taxa	Storage temp. (^{0}C)	Duration of storage	Quality of stored pollen	References
Beta vulgaris	-196	1year	fertile	Hecker *et al.*, 1986
Brassica oleracea	-196	16 months	fertile	Crips and Grout, 1984
Capsicum annum	-196	42 months	fertile	Alexander *et al.*, 1991
Carya illinoensis	-196	1year	fertile	Yates and Sparks, 1990
Carica papaya	-196	485 days	viable	Ganesan, 1986
Glycine max	-192	21 days	fertile	Collins *et al.*, 1973
Gossipium hirsutum	-192	10 days	viable	Collins *et al.*, 1973
Helianthus annus	-196	4 years	fertile	Frank *et al.*, 1982
Humulus lupulus	-196	2 years	fertile	Haunold and Stanwood, 1985
Juglans nigra	-196	2 years	viable	Farmer and Barnett, 1974
L. esculentum	-190	1062 days	fertile	Visser, 1955
Narcissus cv.	-196	351 days	fertile	Bowes, 1990
Persea sp.	-196	1 year	fertile	Sedgley, 1981
Pistacia sp.	-196	1 year	fertile	VithanageandAlexander, 1985
Prunus persica	-196	1year	fertile	Jiang and Gao, 1989
Rosa spp.	-196	8 weeks	fertile	Marchant *et al.*, 1993
Solanum tuberosum	-196	24 months	fertile	Towill, 1984
Trifolium pratense	-196	24 weeks	fertile	Collins *et al.*, 1973
Vicia faba	-196	1 month	viable	Telaye *et al.*, 1990
Vitis vinifera	-196	5 years	fertile	Ganeshan and Alexander 1988

Shivanna and Sawhney, 1997

Recently Rajasekharan and Ganesan (2001) reported cryostorage of pollen in various vegetable and ornamental species such as onion, tomato, *Capsicum* spp, *Solanum* species, rose, gladiolus etc. Indian Institute of Horticultural Research, Bangalore has established a pollen cryobank which maintains more than 600 tropical pollen accessions collected over different seasons and years, kept under constant cryogenic condition. Recently Rajasekharan and Ganesan (2001) reported cryostorage of pollen in various vegetable and ornamental species such as onion, tomato, *Capsicum* spp, *Solanum* species, rose, gladiolus etc. Indian Institute of Horticultural Research, Bangalore has established a pollen cryobank, which maintains more than 600 tropical pollen accessions, collected over different seasons and years, kept under constant cryogenic condition (Rajasekharan and Ganesan, 2001)

Relevance of the present study

Vanilla planifolia cultivation in India is around 1000 hectares and production of cured beans in 1999 – 2000 was 6-8 tonnes (Spices Board, 2000). Much of the planting material of vanilla originated from limited clonal source and practically no variability is available for crop improvement. This leads to monoculture making vanilla susceptible to diseases and pests. Broadening the genetic base of vanilla by introduction is not possible.

Another major challenge is to conserve the gene pool of vanilla, which is already narrow from the onslaught of habitat destruction, over collection and climate changes. *In vitro* conservation can be a very practical approach to augment the conventional efforts adopted to conserve these plant species.

The present investigation is taken up to study the existing variability in some of the vanilla cultivars and species available in India, to broaden the

spectrum of variations in vanilla gene pool using conventional as well as biotechnological approaches, to characterize the extent of variability generated using morphological, cytological, biochemical and molecular markers and to standardize *in vitro* technologies for conservation of vanilla germplasm. A few of them were also screened for reaction against *Phytophthora meadii* and *Fusarium oxysporum,* the two major pathogens affecting vanilla plantations. Similar studies in vanilla were either not reported or scarcely reported in literature. This study gives us an opportunity to locate newer sources of variability for crop improvement in vanilla.

Materials and Methods

Minoo Divakaran “Seedling and somaclonal variation and their characterization in Vanilla ” Thesis. Indian Institute of Spices Research Calicut, University of Calicut, 2002

Materials and Methods

The materials and methods used in the study are described below.

MATERIALS

GENOTYPES STUDIED

Various species and collections maintained in the field repository of Indian Institute of Spices Research, Calicut, were used in the present study. The details of plant materials used are given below.

Species of *Vanilla*

Various species of *Vanilla,* both leafy and leafless species, were used. The leafy species included in the study are *V. planifolia, V. andamanica* and *V. tahitensis.* The leafless species included in the study are *V. aphylla, V. pilifera* and an unidentified leafless species resembling *V. aphylla.* Eight collections of *V. andamanica* were used.

Collections of *Vanilla planifolia*

In all six indigenous collections from plantations in Karnataka, Kerala and Tamil Nadu and two exotic collections one each from Madagascar and Mauritius were used.

Seedlings and Somaclones of *V. planifolia*

Vanilla planifolia was used to develop a large number of selfed seedling progenies and somaclones. A few seeds were grown in colchicine (0.002%) containing medium to increase the spectrum of variation. Few plantlets regenerated from root explants were also used. The seedlings, somaclones and root regenerated progenies used in the study are numbered as given below.

***Seedling progenies*:** Selfed seeds of *V. planifolia* were germinated *in vitro* and over 1000 seedlings were obtained. Of these, only 35 progenies numbered (with

prefix V) as V1, V2, V3, V4, V6, V7, V8, V10, V11, V12, V14, V18, V24, V32, V48, V55, V 60, V66, V67, V75, V92, V98, V101, V105, V112, V113, V120, V134, V140, V142, V144, V145, V150, V179 and V 219 were used in the study.

Callus regenerated progenies: Twenty seven plantlets of *V. planifolia* regenerated through callus, which were numbered (suffixing progeny numbers-n with n .1 for the ones generated from V and VC; prefixing VCC for ones generated *via* callus in colchicine incorporated medium) as V7.1, V 8.1, V56.1, V92.1, V98.1, V101.1, V112.1, V124.1, V142.1, V156.1, V161.1, V179.1, V258.1, VCC20, VCC21, VCC24, VCC32, VCC39, VCC40, VCC43, VCC47, VC3.1, V156.1 VC58.1, VC92.1, VC145.1 and VC153.1 and were used.

Colchicine generated progenies: Over 300 progenies were obtained when the selfed seeds of *V. planifolia* were grown on colchicine containing medium. Twenty six of them, numbered (with prefix -VC) as VC1, VC2, VC3, VC4, VC5, VC 6, VC8, VC9, VC10, VC12, VC13, VC18, VC25, VC 40; VC 65, VC 68, VC 69, VC77, VC 82, VC 86, VC 89, VC113, VC125, VC138, VC161and VC260 were used.

Root regenerated progenies: Five plantlets regenerated through conversion of root meristem into shoots were also used in the present study and were numbered as V1.R, V1.1R, V2R, V7.1R and V53R.

Interspecific hybrids

Interspecific hybridization was attempted between *V. planifolia* and *V. aphylla* and four progenies were developed. All these four numbered as VH1, VH4, VH5 and VH6, were used in the study. Though over 50 progenies were obtained in the reciprocal cross could not be characterized further due to lack of sufficient material.

MATERIALS USED FOR *IN VITRO* CULTURE

Glassware:

Borosil conical flasks and borosilicate glass bottles were used for *in vitro* culture of seeds. Borosil culture tubes were used for culture initiation and callus induction. For proliferation of callus and regeneration of callus, both culture tubes as well as 250 ml Borosil conical flasks were used. For plantlet growth and multiplication, 100-500 ml borosil conical flasks as well as borosilicate glass bottles were used. 'Hi-Media' petriplates were used for protoplast culture and cryovials from 'Nalgene' were used for cryopreservation studies.

Vessel closures:

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

Instruments:

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

For filter sterilization of thermolabile chemicals and enzymes, Millipore filter sterilization system with 0.22μ pore size filter was used. Nikon stereo zoom (SMZ-U) microscope was used to study the sequence of seed germination and morphological features of protocorms. Nikon inverted microscope was used for protoplast studies, and pollen cryopreservation and cytological studies were done in Olympus binocular microscope.

Distilled water:

Double glass distilled water and Millipore 'Milli Q' water was used for washing, stocks preparation, growth regulators, enzyme stocks, media, buffers etc.

Carbon source:

Sucrose (Qualigens, Bombay) was used as the carbon source at the rate of 20 gml^{-1} in all the experiments except *in vitro* conservation experiments, where the concentration was either increased or decreased. In *in vitro* conservation experiments, sucrose was partially replaced with mannitol.

Growth regulators:

Auxins: Two major auxins, namely ∝-naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) were used in this study at concentrations of 0-3.0 mgl^{-1}.

Cytokinins: Two cytokinins namely 6-Benzylaminopurine (BA) and 6-fufurylamino purine (kinetin) were used at a concentration of 0-3.0 mgl^{-1}.

Gelling agents:

For solidifying the culture medium, 'Qualigens' bacteriological grade agar agar was used at the concentration of 7.0 gl^{-1}. Phytagel (Sigma), 0.25 percent was used as gelling agent while plating protoplast.

Culture medium components

Basal nutrient medium:

MS (Murashige and Skoog, 1962) and Knudson (1950) medium was tried as basal medium for *in vitro* germination of seeds. MS medium was used in all other experiments. For MS medium, separate stocks were prepared for macronutrients, micronutrients, vitamins, amino acid and growth regulators (Tables 6 and 8). Sucrose at a concentration of 20 gl^{-1} and agar agar (7.0 gl^{-1}) was added directly to the medium. pH was adjusted to 5.8 before adding agar. The

agar was melted to ensure uniform distribution in the medium. The media were autoclaved at 121^{0}C at 16 psi for 20 minutes.

Growth regulators and other growth factors:

For *in vitro* seed germination, BA, NAA and IBA at 0.5 mgl^{-1} to 3.0 mgl^{-1}, singly and in combinations were used. For micropropagation, plant regeneration from callus and roots, IBA and NAA (0 to 3.0 mgl^{-1}) and BA and kinetin (0 to 3.0 mgl^{-1}) were tried in various combinations. Other growth factors and additives such as tryptone and activated charcoal were also used wherever necessary.

Table 6. Composition of Murashige and Skoog* basal medium

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

* Murashige and Skoog, 1962.

Incubation conditions:

The cultures were incubated at 22 ± 2^0C and with a photoperiod of 14 hours with a light intensity of 3000 lux, provided by 'Philips' cool white fluorescent tubes. Sometimes cultures were incubated in dark for initial two to three days for callus induction and protoplast culture.

Table 7. Details of various stock solutions for MS medium

Stock	Composition	Stock strength	Quantity@
A	Macronutrients	x 20	50 ml
	NH_4NO_3		
	KNO_3		
	$CaCl_2.2H_2O$*		
	KH_2PO_4		
	$MgSO_4.7H_2O$		
B	Micronutrients	x 100	10 ml
	H_3BO_3		
	$MnSO_4.4H_2O$		
	KI		
	$ZnSO_4.7H_2O$		
	$Na_2MoO_4.2H_2O$		
	$CuSO_4.5H_2O$*		
	$CoCl_2.6H_2O$*		
C	Micronutrients	x 100	10 ml
	Na_2EDTA*		
	$FeSO_4.7H_2O$*		
D	Vitamins	x 100	10 ml
	Thiamine HCl		
	Nicotinic acid		
	Pyridoxine HCl		
E	Amino acid	x 100	10 ml
	Glycine		
F	Myo-inositol	x 100	10 ml
	Growth regulators		
	2,4-D	50 mg / 200 ml	
	NAA	50 mg / 200 ml	
	BA	50 mg / 200 ml	
	Kinetin	50 mg / 200 ml	

* Dissolved separately before mixing in the final stock
@ For preparation of 1 litre medium

Table 8. Knudson Medium for orchid seed germination

Sl.no	Chemical	Quantity(g)
1	$Ca(NO_3)_2.4H_2O$	1.00
2	KH_2PO_4	0.25
3	$MgSO_4.7H_2O$	0.25
4	$(NH_4)_2SO_4$	0.50
5	$FePO_4.4H_2O$	0.025
6	Sucrose	20.0
7	Agar	12.0
8	Distilled water	1.0 l

(Knudson, 1950), pH adjusted to 5.5

Sterilization of culture medium

The medium was sterilized by autoclaving at 121^0C for 20 minutes at 1.08 kg/cm^2 (16 psi) pressure.

METHODS

The present study includes three major aspects, namely, 1) Production of seedlings, somaclones and interspecific hybrids, 2) Characterization of the representative progenies generated using morphological, cytological, biochemical and molecular markers, and 3) Standardization of protocols for *in vitro* conservation and cryopreservation of vanilla shoots, synthetic seeds and pollen.

PRODUCTION OF SEEDLINGS AND SOMACLONES

Hand pollination for production of seeds

The pollen was harvested from the freshly opened flowers. The pointed end of a new, clean wooden tooth pick is placed into the stigmatic cavity of the flower to be pollinated to get the toothpick wet with the stigmatic fluid. The wet end of the toothpick was touched to the pollen mass for the pollen mass to adhere.

The flower to be pollinated is grasped in the left hand and tilted it backward to expose the stigmatic cavity and the rostellum is held up to open the stigmatic cavity. The end of the toothpick carrying the pollen mass was carefully directed in to the rear end of the stigmatic cavity and was pressed firmly on the stigmatic cavity so as to leave the pollen mass there and the flower was left undisturbed for fruit set.

Interspecific hybridization

V. planifolia flowers were emasculated early in the morning and covered. Pollen mass (pollinia) was collected from freshly opened flowers of *V. aphylla* and used to hand pollinate *V. planifolia* flowers as mentioned previously. Similarly pollinia from *V. planifolia* were used to pollinate *V. aphylla* flowers in reciprocal crosses. The fruits were harvested at maturity, surface sterilized and cultured on nutrient medium.

***In vitro* seed germination**

Capsules of 4–9 months maturity were harvested and surface sterilized under aseptic conditions by dipping in 95 % ethanol and flaming it. This was repeated twice. The capsules were then split opened under the laminar airflow hood and the seeds transferred to sterilized medium. MS and Knudson media fortified with 2% sucrose were tried as basal medium for *in vitro* germination of vanilla seed. Both solid as well as liquid media were used. Medium was gelled with 0.65% agar agar. MS basal medium which was found to be better, was supplemented with auxin (2,4-D, IBA and NAA), cytokinins (BA and Kinetin) at 0.5 and 1.0 mgl^{-1} concentrations to study protocorm proliferation, multiple shoot induction and rooting.

Micropropagation, Callus regeneration and Conversion of root meristem to shoot meristem

Micropropagation was attempted in *V. planifolia, V. aphylla, V. pilifera* and *V. andamanica*. Shoot tips and nodal segments were collected from healthy plants, washed and cleaned with teepol (1ml 100 ml^{-1}) and surface sterilized with 0.1% mercuric chloride for 5-7 minutes in the laminar flow chamber. Then they were washed thoroughly with sterilized double distilled water and a fresh basal cut was made before inoculating onto a culture medium. MS medium supplemented with auxins (IBA and NAA) and cytokinins (BA and Kinetin) at 0.5 and 1.0 mgl^{-1} was tried in various combinations to study their effect.

Hardening and Planting

Micropropagated, seed germinated, callus regenerated as well as plantlets from interspecific hybrids developed in *in vitro* cultures were taken out and washed carefully for removing the traces of medium sticking to the roots. They were then dipped in 0.3% Dithane-M45, a fungicide, for five to ten minutes and transplanted in polybags containing a mixture of garden soil, sand and vermiculite in equal proportions. The transplanted plantlets were kept in a humid chamber for three to four weeks for hardening and establishment. The plantlets were then taken out and kept in the nursery for one year. In the subsequent years they were transferred to earthen pots (12 inches diameter) and planted in field with *Erythrina* as standards for evaluation

Isolation of protoplasts

Source tissue

In the present study, mesophyll tissue of *in vitro* derived leaves of *V. planifolia* and *V. andamanica* was used as source tissue. Young leaves were cut

into small pieces to ensure proper enzymatic digestion, as it is difficult to peel off the epidermis.

Enzymatic digestion

One step method of enzyme digestion was used to release the protoplasts , i.e., the tissue was digested with a mixture of macerozyme and cellulase. The various enzymes tried were Macerozyme R-10, hemicellulase, and Cellulase Onozuka R-10 at different concentrations (Table 9). The enzyme solution was prepared in Cell Protoplast Washing (CPW) medium (Table 10).

Osmoticum

During protoplast isolation osmotic strength of cytoplasm and the isolation medium needs to be balanced, to prevent plamolysis or bursting of the protoplasts. Osmotic pressure of the protoplast medium is generally manipulated by addition of sugars or sugar alcohols. In the present trials, mannitol was used at different concentrations (8%, 9% and 10%).

Incubation conditions for protoplast isolation

The leaves were incubated in CPW medium with mannitol, for pre-plasmolysis. One gram each of mechanically macerated leaves from *in vitro* cultured plants was immersed in 10 ml each of the isolation medium and incubated in dark upto 16 hours. The changes occurring during incubation were observed at hourly intervals.

Protoplast purification

A combination of filtration, centrifugation, washing and floatation centrifugation was used to purify the protoplasts. After digestion, the enzyme solution containing protoplasts was filtered through a stainless steel mesh (60

mesh size from Sigma) to remove larger particles of undigested tissues and cell clumps.

Table 9. Composition of enzyme solutions (ES) for isolation of protoplasts in vanilla*

Code No.	Mannitol (%)	Macerozyme R-10 (%)	Hemicellulase (%)	Onozuka cellulase R-10 (%)
ES -1	10	0.5	-	1.0
ES-2	9	0.5	-	1.0
ES-3	8	0.5	-	1.0
ES-4	7	0.5	-	1.0
ES-5	6	0.5	-	1.0
ES-6	5	0.5	-	1.0
ES-7	10	0.5	0.5	2.0
ES-8	9	0.5	0.5	2.0
ES-9	8	0.5	0.5	2.0
ES-10	7	0.5	0.5	2.0
ES-11	6	0.5	0.5	2.0
ES-12	5	0.5	0.5	2.0
ES-13	10	1.0	-	3.0
ES-14	9	1.0	-	3.0
ES-15	8	1.0	-	3.0
ES-16	7	1.0	-	3.0
ES-17	6	1.0	-	3.0
ES-18	5	1.0	-	3.0

* Enzyme solutions were prepared in CPW medium

A sample was observed under inverted microscope to confirm enzymatic digestion and release of protoplasts. The filtrate is distributed into sterilized screw capped centrifuge tubes and centrifuged in Beckman tabletop centrifuge for 10 minutes at 700 rpm. The protoplasts form a pellet at the bottom of the tube. The supernatant enzyme solution was removed using a Pasteur pipette without disturbing the pellet. The pellet was suspended in Cell Protoplast Washing (CPW) medium (Table 10).

Centrifugation and re-suspension in fresh medium was repeated three times so as to wash the protoplasts and remove traces of enzyme solution. After washing, the pellet of protoplasts was resuspended in 1ml of the CPW medium and layered on top of 9 ml of floatation medium (Table 10) and centrifuged at 700 rpm for 10 minutes. The live protoplasts form a band at the interphase, which was collected

with a Pasteur pipette and transferred to culture medium. Concentration of the protoplasts was estimated with the help of a haemocytometer.

Table 10. Composition of media used for protoplast isolation and culture in vanilla

Components	Cell Protoplast Washing (CPW) Medium (mgl^{-1})	Floating media (mgl^{-1})	Protoplast culture media (mgl^{-1})	
			I	II
NH_4NO_3	-	-	1650	1650
KNO3	101.0	101.0	1900	1900
$CaCl_2.2H2O$	1480.0	1480.0	440	440
$MgSO_4.7H_2O$	246.0	246.0	370	370
KH_2PO_4	27.2	27.2	170	170
KI	0.16	0.16	0.83	0.83
H_3BO_3	-	-	6.2	6.2
$MnSO_4.4H_2O$	-	-	22.3	22.3
$ZnSO_4.7H_2O$	-	-	8.7	8.7
$Na_2MoO_4.2H_2O$	-	-	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2.6H_2O$	-	-	0.025	0.025
$FeSO_4.7H_2O$	-	-	27.8	27.8
$Na_2EDTA.2H_2O$	-	-	37.3	37.3
Myo-inositol	-	-	100.0	100.0
Nicotinic acid	-	-	0.5	0.5
Thiamine Hcl	-	-	0.5	0.5
Pyridoxine HCl	-	-	0.5	0.5
Glycine	-	-	2.0	2.0
Sucrose	-	21%	2%	3%
Mannitol	7-10%	-	7%	4%
Gibberellic acid	-	-	0.5	0.5
BA	-	-	0.5	1.0
NAA	-	-	0.5	1.0
2,4-D	-	-	0.5	-

PEG mediated hybridization of the protoplasts

Two droplets of protoplast suspension from *V. planifolia* and *V. andamanica* were added to another droplet of PEG solution (Table 11), allowed to mix at room temperature for 30 minutes. They were maintained as droplet cultures on glass slides and were periodically observed for fusion of protoplasts, cell wall regeneration and cell division.

Table 11. Composition of PEG solution* for protoplast fusion

Constituents	Molar conc.	g/l
NaCl	140 mM	8.18
KCl	5 mM	0.37
Na_2HPO_4	0.75 mM	0.11
Glucose	5 mM	0.90
$CaCl_2.2H_2O$	125 mM	18.40
PEG (MW 4000)		400.00

* pH was adjusted to 5.8

Protoplast culture

Protoplasts were cultured initially in liquid medium in petri dishes. Five drops of culture medium containing protoplasts were placed in the bottom half of the petri dish, sealed with parafilm and incubated in dark at 25^0C . Fresh culture medium with low osmoticum was added after every seven days to replenish the nutrients. The samples were periodically observed for cell wall regeneration and cell division. After 40-60 days of culture they were plated on 0.25% agarose medium and monitored for microcallus formation and further development.

CHARACTERIZATION OF SEEDLINGS, SOMACLONES AND HYBRIDS

The variability existing among the somaclones was studied using morphological characterization, cytological indexing, biochemical

characterization using isozyme profiles and molecular characterization using AFLP and RAPD profiles. Some of the species and progenies were also screened for reaction to *Fusarium* and *Phytophthora.*

Morphological characterization

Morphological characters such as plant type, leaf size, growth rate, internodal length etc. of plantlets at the time of hardening and one year old plantlets in the field were recorded wherever possible. Data on the following characters were recorded

Stem Characters :	Type: Simple/Branched	(S/B)
	Shape : Cylindrical / Slender	(C/S)
	Colour of vine : Green/pink/variegated	(G/P/V)
	Vertical groove : Present/Absent	(P/A)
	Length of stem (upto 6th leaf from apex)	
	Girth of stem (at 6th leaf from apex)	
	Internode length (at 6th leaf from apex)	
Leaf characters :	Type : Succulent/nonsucculent	(S/NS)
	Sessile/petiolate	(S / P)
	Colour :Variegated/nonvariegated	(V / NV)
	Shape: Oblong/Elliptic/lanceolate	(O/ E/ L)
	Nature of tip:acute/acuminate	(A/Ac)
	Pubescence :Present/absent	(P/A)
	No.of leaves/10 cm from the apex	
	Lamina length (6th leaf from apex)	
	Lamina width	
Aerial roots:	Present/Absent	(P/A)
	No.of roots/meter of stem	
	Length of aerial root	
	Colour of aerial root	

(Abbreviations in brackets indicate its use in results- Table 27)

Cytological characterization

Chromosome indexing was done by observing root tip mitosis. For studying mitosis, actively growing root tips were collected from healthy and vigourous plants on bright sunny days between 2.00 and 3.00 p.m. The root tips were pretreated with 0.002M aqueous solution of 8-hydroxyquiloline for 2 hrs at 40^0C. Treated roots were then washed well in tap water and fixed in Clarks fluid (3 Ethyl alcohol : 1 Acetic acid). After 24 hrs of fixing the root tips were hydrolyzed for 3 minutes in 1N HCl and stained in 2% lactopropionic acid for atleast 2 hours. Observations and photographs were taken from slides on Olympus Vanox micropscope. Cells from slides were scored to assess the range of chromosome numbers observed i.e., maximum, minimum and average numbers and also for different somatic associations, if any.

Biochemical characterization

Isozyme profiles of 10 genotypes were carried out using Native PAGE in Phast system. Enzyme extracts were thoroughly homogenised using 3-3.5 g of the sample in mortar and pestle, in 5 ml of prechilled Tris extraction buffer, consisting of 0.05 M Tris HCl, pH 7.4, 0.1% cysteine, 0.1% ascorbic acids (as antioxidants and phenol inhibitors and 17% sucrose (for density) . The extract was centrifuged in refrigerated centrifuge at 2000 rpm for 15 min. the supernatant was collected and a drop of 0.1% Bromophenol blue was added to differentiate the movement of bands (Bhat *et al*, 1992).

Electrophoresis was carried out at 4^0C at 80 volts for 15 minutes and then at 250 V, till the run is completed, after which the gel is immediately immersed in staining solution. The gels were stained as per standard procedures for peroxidase

(Guikema and Sharman, 1980) and superoxide dismutase (Beauchamp and Fridovich, 1971).

The Rf values of the bands were calculated by the formula :

$$Rf = \frac{\text{Distance travelled by enzyme band on gel}}{\text{Distance travelled by the dye front}}$$

The Paired Affinity Indices (PAI) were calculated based on percentage of similarity.

Molecular characterization

Isolation and purification of genomic DNA

Modified C-TAB method was used (Ausubel *et al*, 1995) to isolate DNA from vanilla. The protocol used for extraction of DNA from vanilla leaf tissues is as follows,

1. Grind 5 g of young leaves in liquid nitrogen with a mortar and pestle and add 25 ml of preheated (65^{0}C) CTAB buffer. Add 0.2% β-Mercaptoethanol prior to use.
2. Incubate at 60^{0}C for 30 minutes.
3. Extract with equal volume of chloroform : isoamyl alcohol (24:1) at 10,000 rpm for 10 minutes at room temperature.
4. Take the aqueous phase and add 2/3 rd volume of ice-cold isopropanol.
5. Incubate at -20^{0}C for 2 hours and centrifuge (10,000 rpm, 15 minutes at 4^{0}C).
6. Discard the supernatant and invert the tube on paper towel for few minutes.
7. Dissolve the pellet and add 1.5 ml of TE buffer at room temperature over night.
8. Add 10 µg/ml of RNase A and incubate at 37^{0}C for 30 minutes.
9. Add equal volume of Tris saturated phenol, mix it well and centrifuge at 10,000 rpm for ten minutes.
10. To the aqueous phase add equal volume of phenol : chloroform : isoamyl alcohol, (25 :24 :1), shake and centrifuge at 10,000 rpm for ten minutes.
11. Take the aqueous phase and add equal volume of chloroform : isoamyl alcohol (24:1), shake and centrifuge at 10,000 rpm for ten minutes.
12. To the aqueous phase add one-tenth volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol and incubate at -20 for one hour or at -70^{0}C for 30 minutes.

13. Centrifuge at 10,000 rpm for 10 minutes and wash the pellet in 70% ethanol (10,000 rpm for 5 minutes).

14. Airdry the pellet and dissolve in 1.5 ml TE and estimate the yield.

The composition of various stock solutions and buffers used are given in Tables 12 and 13.

Quantification of DNA

The amount of DNA was estimated using Scanning Shimatzu Spectrophotometer. DNA shows a clear absorbance peak at 260 nm and the value of 1.0 OD_{260} is calculated equivalent to 50 µg/ml. DNA solution was considered pure if the value of OD_{260} : OD_{280} is 1.8. Visualize the DNA on (0.8%) agarose gel for its quality. Store the DNA at -20^{0}C.

Table12. Composition of various stock solutions for DNA isolation (Sambrook *et al*, 1989)

Solutions	Method of preparation
1M Tris (pH 8.0) 500ml	Dissolve 60.55gm Tris base (Sigma) in 300 ml distilled water. Adjust pH to 8 by adding concentrated HCl. Adjust volume to 500ml. Dispense to reagent bottles and sterilize by autoclaving.
0.5M EDTA pH 8.0	Dissolve 93.05g of EDTA-disodium salt (sigma) in 300 ml of water. Adjust pH to 8 by adding NaOH pellets. Adjust volume to 500 ml. Dispense in to reagent bottles and autoclave.
5M NaCl 500 ml.	Weigh 146.1g NaCl (Merck) add 200ml of water and mix well. When the salts get completely dissolved, adjust the final volume to 500ml. Dispense in to reagent bottles and autoclave.
3M Sodium acetate (pH5.2) 250 ml.	Dissolve 61.523g of anhydrous sodium acetate (Qualigens) in 200 ml of water and mix well. When dissolved completely adjust the pH of the solution to 5.2 with glacial acetic acid (99- 100%). Autoclave
Ethidium Bromide 10mg/ml, 100ml.	Add 1g. Ethidium Bromide to 100 ml of distilled water. Keep on magnetic stirrer to ensure that the dye has dissolved completely. Dispense to amber coloured reagent bottle and store at 4°C.
70% ethanol, 500 ml	Take 360 ml. of ethanol; mix with 140 ml of distilled water. Dispense to reagent bottle and store at 4°C.
Chloroform: isoamyl alcohol (24:1),500 ml	Measure 450 ml of chloroform and 20 ml of isoamyl alcohol. Mixed and stored in room temperature
1M $MgCl_2$, 100ml	Weigh 20.33g of $MgCl_2$, dissolve in double distilled water ,make up to 100 ml, autoclave.

Table 13. Composition of various buffers used for DNA isolation (Sambrook *et al.*, 1989)

	Buffer	Method of preparation
1	CTAB Extraction Buffer : for 1 litre 100mM Tris HCl (pH 8.0) 20mM EDTA (pH 8.0) 1.4M NaCl 2%CTAB(w/v)Merck 0.2%B-mercapto ethanol.(v/v)-Merck	Measure 100ml Tris (1M),280ml of NaCl, 40ml of EDTA(0.5M). Mix with about 400ml of hot distilled water ,add 20g of CTAB to this. Adjust final volume to 1 litre. Dispense to reagent bottles and autoclave. Just before use, add 0.2% β-mercaptoethanol.
2	TE (0.1mM) buffer ...for 100ml 100mM Tris HCl (pH 8.0) 0.1mM EDTA (pH 8.0)	Take 1ml of Tris HCl (1M),20ml of EDTA (0.5M). Mix with 99ml of sterile distilled water taken in a reagent bottle, mix thoroughly, autoclave.
3	TAE buffer 10x : for 1 litre	Weigh 48.4g of Tris base ;add 20ml of EDTA (0.5M);11.42ml of Glacial acetic acid and around 150ml d.water .Dissolve the salt and adjust volume to 1 litre . Autoclave
4	Gel loading buffer (6x) : for 100ml 0.25% Bromophenol blue (Sigma) 30% Glycerol (Merck)	Dissolve 0.25g of BPB in 99ml of 30% Glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense to reagent bottles and keep in 4 °C

Development of RAPD profiles

RAPD profiles were developed as per the method suggested by Williams *et al* (1990) with minor modifications. The dNTPs , Taq polymerases and other chemicals were procured from Amersham Pharmacia Biotech, Sweden. Sixteen arbitrary primers from Operon Technologies Inc. Alameda, California were used for PCR reaction. Each primer contains atleast 60% -70% GC content and no self-complementary ends. The primers used and their base sequences are given in Table 14.

Combinations of Variables

Different combinations and concentrations of dNTPs, Taq polymerase $MgCl_2$ and other variables were tested for good and consistent amplification of genomic DNA. It was found that 30 ng DNA, 1x assay buffer, 150 µM dNTPs, 2.0 mM $MgCl_2$ and 1 U of Taq polymerase are optimal for generating good and

consistent amplification products, at annealing temperature of 40^0C, hence used for developing RAPD profiles in vanilla.

Table 14. Operon primers, which showed polymorphism, used for developing RAPD profiles

Sl.No	Primer number	Base sequence	% of GC
1	OPA 10	5' GTGATCGCAG 3'	60
2	OPA 20	5' GTTGCGATCC 3'	60
3	OPB 02	5' TGATCCCTGG 3'	60
4	OPB 14	5' TCCGCTCTGG 3'	70
5	OPB 20	5' GGACCCTTAC 3'	60
6	OPC 09	5' CTCACCGTCC 3'	70
7	OPC 19	5' GTTGCCAGCC 3'	70
8	OPD 03	5' GTCGCCGTCA 3'	70
9	OPD 19	5' CTGGGGACTT 3'	60
10	OPE 05	5' TCAGGGAGGT 3'	60
11	OPE 09	5' CTTCACCCGA 3'	60
12	OPE 14	5' TGCGGCTGAG 3'	70
13	OPF 12	5' ACGGTACCAG 3'	60
14	OPF 03	5' CCTGATCACC 3'	60
15	OPA 04	5' AATCGGGCTG 3'	60
16	OPB 10	5' CTGCTGGGAC 3'	70

The PCR reaction mix was prepared using all the seven components in 0.2ml sterile thin walled microfuge tube. The final reaction volume was 25µl. PCR machine used was Genecycler TM from BIORAD, USA.

The reaction mixture for PCR reaction was prepared as follows

	1x
1.Sterile distilled water	11.75µl.
2.10x PCR buffer	2.5µl.
3. dNTPs (1 mM).....	3.75µl.
4. Primer (5pmoles/µl).....	2.0µl.
5. $MgCl_2$(10mM).....	1.5µl
6.Taq polymerase (2U/µl).....	0.5µl
7. Template DNA(30 ng/µl).....	3.0µl
Total reaction volume	**25µl**

Optimization of PCR Programming

The optimized PCR reaction profile is given below:

Cycles: 3

I[st] cycle:	94 °C for 2 minutes	
	40 °C for 1 minute	
	72 °C for 1 minute	Cycle repeats: 1
II[nd] cycle:	94 °C for 1 minute	
	40 °C for 30 seconds	
	72 °C for 1 minute	Cycle repeats: 30
III[rd] cycle:	94 °C for 1 minute	
	40 °C for 1 minute	
	72 °C for 15 minutes	Cycle repeats: 1

The PCR products were visualized in 2% Agarose gels and documented in BIORAD gel documentation system.

Development of AFLP profiles

AFLP profiles were developed as per the method suggested by Vos *et al.*, (1995). The various materials, primers and stock solutions used are as follows.

Primers

EcoRI and *Mse*I primers were obtained from Life Technologies , USA and was used at 50 ng /μL concentration.

*Eco*RI-primers +0 (E) : 5'-AGACTGCGTACCAATTC-3'
*Eco*RI-primers +2 (E-AC): 5'-AGACTGCGTACCAATTCAC-3'
*Eco*RI-primers +2 (E-GG): 5'-AGACTGCGTACCAATTCGG-3'
*Eco*RI-primers +2 (E-TG): 5'-AGACTGCGTACCAATTCTG-3'

*Mse*I-primers +0 (M) : 5'-GATGAGTCCTGAGTAA-3'
*Mse*I-primers +2 (M-AC) : 5'-GATGAGTCCTGAGTAAAC-3'
*Mse*I-primers +2 (M-GC) : 5'-GATGAGTCCTGAGTAAGC-3'
*Mse*I-primers +2 (M-TC) : 5'-GATGAGTCCTGAGTAATC-3'
*Mse*I-primers +2 (M-TG) : 5'-GATGAGTCCTGAGTAATG-3'

The numbers depict the number of selective nucleotides at the 3' terminus of the individual primers. The +0 nprimers were used in first amplification of restricted products and the +2 primers were used for second amplification in 4 different combinations, *i.e.*, EAC-MTG, EGG-MTC, EGG-MGC and ETG-MAC.

Corresponding adaptors

EcoRI and *Mse*I adapters were obtained from Life Technologies, USA and was used at 5 and 50 pmol /μL concentration respectively.

*Eco*RI-adapter	5'-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5'
*Mse*I-adapter	5'-GACGATGAGTCCTGAG TACTCAGGACTCAT-5'

Others

Gamma-33P-ATP (~2000 Ci/mmol) was used for radio labeling.
Double-distilled water(ddH_2O).
Buffers : 1 M Tris.HAc pH 7.5; 1 M Tris.HCl pH 8.0 and pH 8.3.
Magnesium: 0.1 mM MgCl2.
TE (10x): 100 mM Tris.HCl, 10 mM EDTA pH 8.0.
100 mM DTT.
11. 5 mM of dNTPs
10 mM ATP.
T4-buffer (10x): 250 mM Tris.HCl pH 7.5, 100 mM MgCl2, 50 mM DTT, 5 mM spermidine
Restriction-ligation buffer (10x): 50 mM Tris-HAc, 50 mM $MgCl_2$, 250 mM KAc, 25 mM DTT, 250 ng/μL, pH 7.5.
Restriction endonucleases: *Eco*RI(20 U / μL), *Mse*I(4 U/ μL) (New England Biolabs).
Enzymes: T4 DNA ligase, T4 polynucleotide kinase, *Taq* DNA polymerase
PCR buffer (10x): 100 mM Tris.HCl pH 8.3, 15 mM $MgCl_2$, 500 mM KCl.
Molecular weight standards
General reagents for polyacrylamide gel electrophoresis.

10X TBE: Tris base 108 g, Boric Acid 55g, Na_2 EDTA 9.3 g in 1 L of dd H_2O, adjust pH 8.3 and autoclave.

loading dye: same as for RAPD

Methods

Digestion of DNA

Genomic DNA (about 10 ng /μL) was digested with restriction endonucleases in a 50 μL reaction containing 25 μL DNA, 5 μL 10x RL buffer, 5 units *Eco*RI and 2 units *Mse*I, and 16 μL of dd H_2O, mixed well and incubated for 3 h at 37°C to obtain complete digestion with all the fragments below 500 bp

Adaptor ligation

Adaptors were ligated to the digested genomic DNA by adding 1 μL *Eco*RI adapter (5 pmol), 1 μL *Mse*I adapter (50 pmol), 1.2 μL 10 mM ATP, 1 μL RL buffer, 1 unit T4 DNA ligase, 0.1M dTT and 3.79 μL water to the digestion mix. Incubate another 3 h at 37°C. Overall, DNA is incubated for a total of 6 h with endonucleases, the last 3 h in the presence of T4 DNA ligase and oligonucleotide adapters.

Dilution

The ligation reaction mixture was diluted 10 times with TE (usually 10 μL in 100 μL) and the diluted reaction mixture was used directly as template DNA for the AFLP reactions. Store diluted DNA at -20°C.

AFLP Pre-amplification

Preamplification was done (50 μL total volume) with following components

5 μL ligated DNA, 1.5 μL *Eco*RI-primer +0 (75 ng), 1.5 μL *Mse*I-primer +0 (75 ng), 2 μL 5 mM dNTPs, 0.2 μL Taq polymerase (1 unit), 5 μL 10x PCR-buffer and 34.8 μL water.

This mix was preamplified for 30 cycles in PCR machine using the following regime:

30 s at 94°C; 30 s at 60°C; 60 s at 72°C with a final extension of 120 s at 72°C.

Dilution

Preamplification, 10 μL of the reaction is diluted with 190 μL of TE0.1 to 100 μL, which is sufficient for 40 AFLP-reactions +2/+2. The diluted reaction mix and the rest of the preamplification reaction are stored at -20°C. If necessary new dilutions of the preamplification reactions may be made to give additional template for the AFLP reactions.

AFLP amplification

Preparation of labelling mix

Label primers for selective AFLP amplification by phosphorylating the 5' end of the primers with gamma-33P-ATP and polynucleotide kinase. Check above for the right primer combination to use in this step. Only one of the two primers of the AFLP reaction should be labelled (e.g., the EcoRI-primer). When possible use the more expensive 33P-labelled primers because they give better product resolution in polyacrylamide gels, and are less prone to degradation due to autoradiolysis. Prepare the following primer labelling mixes (40 μL) for 100 AFLP reactions. 10 μl gamma- 33P-ATP (~2,000 Ci/mmol), 5 μL 10xT4-buffer, 2 μL T4-kinase (10 units/μL) and water to 40 μL.

Primer labeling

Add 10 μl of primer (either *Eco*RI- or *Pst*I-primers at 50 ng/μL) to 40 μL labelling mix and incubate 60 min at 37°C, followed by incubation at 70°C for 10 min for the inactivation of the kinase. This gives a labelled primer with a concentration of 10 ng/μL.

Preparation of AFLP reaction mixes

Primer and *dNTPs* mix (50 μL): 5 μL labelled primer (10 ng/μL), 6 μL unlabelled primer (50 ng/μL), 8 μL 5 mM dNTPs and 31 μL water.

Taq polymerase mix (100 μL): 20 μL 10x PCR-buffer, 0.8 μL *Taq* polymerase (4 units) and 79.2 μL water.

AFLP amplification

Assemble the reaction by adding 5 μL of the primers and dNTPs mix and 10 μL of the *Taq* polymerase mix to 5 μL of pre-amplified ligated DNA. The template DNA should be pipetted first followed by the two mixes. The reagents should be mixed by tapping the base of the tubes on the bench. Pipetting mixes is essential for the rapid start of the AFLP reactions that are assembled at room temperature (to avoid loss of AFLP fingerprint quality). Tubes are amplified in a thermocycler with the following cycle regime a first cycle of 30 s at 94°C, 30 s at 65°C and 60 s at72°C, followed by 12 cycles with a stepwise decrease of the annealing temperature in each subsequent cycle by 0.7°C, and 23 cycles of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C. The reaction is started at a high annealing temperature to obtain optimal primer selectivity. In the following steps the annealing temperature is lowered gradually to a temperature for optimal primer annealing.

Polyacrylamide gel electrophoresis of AFLP products

General: Amplification products were analyzed on 5% denaturing polyacrylamide-sequencing gels. The gels were cast at least 2 h before use and should be pre run for 30 m just before loading the samples Pre-running and running electrophoretic steps were done at 110 W. TBE (1x) was used as running buffer.

Sample loading: Mix AFLP reaction products with an equal volume (20 μL) of loading dye. Heat the samples for 3 min at 90°C, and then quickly cool on ice. Rinse the the gel wells with running buffer and push carefully two 24-well sharktooth combs about 0.5 mm into the gel surface to create the gel slots. Rinse the gel slots formed in this way with TBE and load 2 μL of each sample per well.

Post-electrophoretic procedures: Disassemble the gel cassette and remove the front glass plate with the silane-attached gel to the front dry it on gel drier.

Autoradiographic exposure of the 33P-gels to standard X-ray film for 2 to 3 days with intensifying screens give good autoradiogrammes.

Due to the cost involved, only some selfed seedling progenies of *V. planifolia* and four inter specific hybrids between *V. planifolia* and *V. aphylla* were used in developing AFLP profiles.

Studies on RAPD and AFLP polymorphism

The presence and absence of bands were scored. The similarity / differences between the genotypes was assessed using Paired Affinity Indices (PAI). The PAIs expressed as percentage indicated the similarity (%) between any two genotypes. Dendrograms were drawn using NTSYS software.

PAI was calculated by the formula = $$\mathbf{PAI = \frac{No.\ of\ similar\ bands}{Total\ no.\ of\ bands}}$$

Screening of somaclones for their reaction to *Phytophthora meadii* and *Fusarium* infection.

Thirty four accessions from the vanilla germplasm was subjected to study for their reaction to *Phytophthora meadii* and *Fusarium oxysporum* infections. Virulent strains of *P. meadii* and *F. oxysporum* isolates were obtained from Crop Protection Division of Indian Institute of Spices Research. For screening against *Phytophthora* and *Fusarium*, five-millimeter discs of *Phytophthora* cultures were

placed at the axillary bud region of vanilla plantlets maintained in glasshouse and covered with wet cotton swabs. Development of disease symptoms *viz.*, browning, development of watery spots and shrinking of stem and vine were recorded. The length of lesions developed, both on the stem surface as well as depth of the lesions (after splitting open the stem) was noted and disease incidence was scored from 0-4, based on the % of infection.

IN VITRO CONSERVATION OF THE SEEDLINGS AND SOMACLONES

Synthetic seeds

Materials

In vitro regenerated shoot buds, protocorms and regenerating calli were used in the present study as propagules for encapsulation.

Methods

Encapsulating propagules

Various concentrations (3-7.0%) of sodium alginate were made with MS basal medium and autoclaved. The propagules *i.e.*, *in vitro* developed somatic embryos or shoot buds, were placed in a gel matrix containing MS medium and sodium alginate mixture. The explants along with matrix were then dropped into a solution of calcium chloride ($CaCl_2$. $2H_2O$) solution (1.036g/150ml) using a Pasteur pipette with cut end for easy passage of propagules. The propagules were allowed to remain in the solution for 30-40 minutes on gyratory shaker for proper bead formation. The beads were recovered by decanting the $CaCl_2$ solution and later washed in one or two changes of sterile water. The synthetic seeds or 'synseeds' thus produced were stored in 50 ml flasks in sterile water or MS basal medium at 5^0C, 15^0C and 22 ± 2^0C, for storage. The synthetic seeds were transferred to germination medium at different stages of storage. Observations

were made on the germination of these seeds to assess their viability at different storage durations.

Medium term conservation *In vitro* by slow growth:

With an objective to find out suitable conditions for inducing minimal growth in vanilla cultures and to increase subculture intervals substantially, various parameters like low temperature, reduction of carbon source, reduction of basal media concentration, addition of mannitol, minimising evaporation rate using closed culture vessels (polypropylene caps and cotton plugs)l were tried. The different temperature levels tested, carbon source and mannitol concentrations used are

Low Temperatures	:	5^0C, 10^0C, 22^0C
Reduction of carbon source	:	30 gl^{-1}, 20 gl^{-1}, 15 gl^{-1} 10 gl^{-1}, 0 gl^{-1}
Use of mannitol	:	10 gl^{-1}, 15 gl^{-1}

The basal medium used was MS medium without any growth regulators. Twenty-five ml of culture medium was used per culture tube and uniform sized plantlets (2 cm) were inoculated in all the treatments tested. Observations were made on growth rate, general health of the plantlets, symptoms of necrosis and drying, exhaustion of media, ability to multiply normally after storage etc. to draw the conclusions.

Long term storage of encapsulated shoot tips and pollen by cryopreservation

Encapsulation dehydration of shoot tips and protocorms

Shoot buds of about 2mm – 5mm were dissected out from *in vitro* grown cultures of vanilla. Shoot buds were encapsulated in 4% alginate and desiccated for 1-2h in the air current of laminar flow chamber. Encapsulated shoot buds were given cryoprotection using DMSO and glucose at 5% and 2.5% concentration,

singly and in combination. The samples were kept at 4^0C and 25^0C for 24h. The cryovials along with the shoot buds were then directly plunged into LN_2 and kept for 1h. After 1h rapid thawing was done by immersing the vials in 38^0C water bath for 10m. The shoot buds were taken out of the vials and cultured on recovery medium (MS basal medium supplemented with 3% sucrose, 1 mg/l BAP and 0.5 mg/l NAA). The controls, which were not given the LN_2 treatment, were also cultured in the same medium.

The post freeze recovery was assessed initially by microscopic observation up to 2 weeks of culture. The number of shoot buds that were green and expansion of the leaf primordial were recorded. After 2 weeks, the shoot buds that emerged out of the synthetic seed coat were cultured on the same combination of solid medium for further growth responses.

Cryopreservation of pollen

In vitro pollen germination

Pollen mass (pollinia) were collected in two species of V*anilla*, *V. planifolia* and *V. aphylla* and maintained in petridishes at 25°C for 1h to dehisce. Pollen grains thus obtained were used for all the treatments in the study. Pollen germination was assessed by hanging drop technique on Brewbaker medium (Brewbaker and Kwack, 1963) incubated at 25°C in dark. Three different sucrose concentrations (5%, 10% and 15%) were tested. After 18 to 24 h counts of germinated pollen were made. Pollen germination was considered to occur *in vitro* when a pollen tube had grown at a length twice the diameter of the pollen grain.

Cryostorage of pollen

Pollinia were collected from freshly opened flowers and pollen mass were detached, placed in petridishes and maintained at 25°C for 1h to dehisce. Pollen

grains were then subjected to different pretreatments, like desiccation for 10m, 30m, 24h and desiccation combined with cryoprotection in 5 and 10% of Dimethyl sulfoxide (DMSO). The pollen samples were transferred to cryovials and plunged into liquid nitrogen. Pollen samples were cyopreserved for different durations from 1h to 1week.

Assessment of pollen viability and pollen fertility

Pollen samples were thawed by the rapid thawing process by dipping in 40°C water bath, after retrieval from cryogenic storage Viability of fresh and cryopreserved pollen samples was assessed using acetocarmine staining and also based on *in vitro* germination.

The fertility of cryopreserved pollen was tested by controlled field pollinations. Flowers of the desired female parent were emasculated. Cryopreserved pollen after thawing was applied on the receptive stigma. Pollinated flowers were covered and marked. Crosses were made with freshly collected pollen using the receptive male and female parent. Fruit formation and seed set were recorded in all the crosses after allowing for normal development and maturity. The fruits were harvested on maturity and the seed were cultured *in vitro* to assess the seed viability.

Statistical Analysis

Statistical analysis was carried out wherever possible using Duncan's multiple range test. The values of CD (Critical Difference), Mean, Standard Deviation and Standard Error were worked out and were given wherever applicable.

Organization of various work elements attempted in this study are given in Fig 2.

Results

Minoo Divakaran “Seedling and somaclonal variation and their characterization in Vanilla ” Thesis. Indian Institute of Spices Research Calicut, University of Calicut, 2002

Results

GENE POOL OF VANILLA

Vanilla planifolia germplasm in India, has a very narrow genetic base, primarily because it has been recently introduced and much of the present material arose through clonal propagation. A few species of *Vanilla*, which occur naturally in India, include *V. aphylla, V. walkeriae, V. wightiana, V. pilifera* and *V. andamanica*. The Indian Institute of Spices Research maintains a small collection of vanilla germplasm with 16 lines of *V. planifolia* collected from different vanilleries, one collection of *V. tahitensis*, two exotic collections of *V. planifolia*, from Madagascar and Mauritius and a collection of *V. planifolia* with variegated leaves. In addition, one collection each of the above mentioned species of Indian vanilla, 8 collections of *V. andamanica* and one collection of a leafless putative new species resembling *V. aphylla*, are also maintained. (Fig.4). A few of these species were used in the present study. A few other important exotic species of vanilla are *V. pompona, V. barbellata,* and *V. phaeantha* which could be included in this study, due to lack of their availability (Fig.4 c, i, j, k). Most of the studies are concentrated on *V. planifolia*, yielding the vanilla of commerce.

PRODUCTION OF SEEDLINGS AND SOMACLONES

Hand pollination and fruit set

The hand pollination experiments done in 7 consecutive years resulted in 90% fruit set. More than 80% of the fruits were with the persisting floral parts indicating the effective pollination (Fig.4d). In *Vanilla planifolia*, successful pollination could be recorded on the second day itself since perianth did not remain on the flower unless the pollination is effective. The fruits attained maturity in 9 months. The matured fruits contain numerous black powdery seeds in it.

Fig. 4 A few important species of *Vanilla*
a. *V.pilifera*, b. *V.aphylla*, c. *V.wightiana*, d. *V.planifolia*, e. *V.tahitensis*, f. *V.andamanica*, g. *V. planifolia* with variegated leaves, h. *V. planifolia*, collection from Mauritius, Other important species - i. *V.barbellata*, j. *V. phaeantha*, k. *V.pompona*

Seed Culture

In vitro seed germination and development of seedlings

Preliminary trials were conducted to germinate seeds from the second month of pollination, to find the best maturity stage for seed culture. The seeds were immature and white in colour in the second month and resembled more of the unfertilized ovules. Gradually, by the end of the third month the seeds started maturing into numerous black seeds.

Seeds could successfully be germinated *in vitro*, from the fourth month onwards. However, seeds harvested in the sixth month and cultured *in vitro* gave the maximum percentage (87%) of germination. The seeds were cultured in different basal media alone and those supplemented with different growth factors. Seed germination was observed in most of the media tried, but the percentage of germination ranged from 26% to 87% (Table 15).

Table 15. Effect of different basal media and other factors on seed germination and protocorm development* in *Vanilla planifolia*

Medium	Frequency of germination (%)	Protocorm viability (%)	Protocorm length (mm)	Callusing (%)	Shoot growth (%)
Knudson	31	12	0.91 ± 0.02	-	-
1/2MS	26	15	0.09 ± 0.35	-	-
MS basal medium	35	12.5	1.09 ± 0.04	0.2	-
MS + Charcoal (0.2%)	37	50.4	1.32 ± 0.04	2.5	-
MS + tryptone ($2gl^{-1}$)	85	74.9	2.27 ± 0.1	1.25	52
MS + BA ($0.5\ mgl^{-1}$)	82	81.7	2.71 ± 0.05	-	40
MS + BA ($1\ mgl^{-1}$). + NAA ($0.5\ mgl^{-1}$)	85	94.7	2.24 ± 0.13	85	85
MS + BA ($1mgl^{-1}$) + IBA ($0.5\ mgl^{-1}$)	81	96.4	3.3 ± 0.16	10	85
MS + Colchicine (0.002%)	87	95.6	0.87 ± 0.04	25	83

* Mean of 20 replications

Murashige and Skoog's (MS) medium gave better response than Knudson's medium, in *in vitro* cultures of vanilla. The minimum germination (26%) was observed in MS medium at half strength and maximum was recorded in full strength MS medium supplemented with $2gl^{-1}$ tryptone (85%). Though auxins and cytokinins in combination were found to be better for seed germination, there was no significant difference in germination percentage recorded in MS with BA alone or in combination with NAA or IBA (Table 15).

Germination of seeds began within 4 weeks of culture. The seed germination exhibited three different types of developmental stages for the regeneration of plantlets in various media combinations (Fig.5).

The initial stages of germination were typical of most orchids, involving swelling of the embryo followed by rupturing of the seed testa and subsequent emergence of protocorm (Fig. 5 and 6).

Addition of various supplements to enhance germination, was also tried. Seeds germinated directly into plantlets in medium with BA ($0.5\ mgl^{-1}$) alone, without any intervening callus phase (Fig.5a, b, f), hence this medium was selected for the germination and production of selfed progenies/ seedlings throughout the study. On MS medium supplemented with activated charcoal (0.2%), seeds germinated but in general showed lack of chlorophyll (Fig.5e). In medium with $2gl^{-1}$ tryptone, a growth promoting effect was observed and the germination rate was higher (85%) than MS medium with BA but the further growth and development of protocorms was better in the latter (Fig.5c,d). Germinating seeds multiplied on MS medium supplemented with BA ($1.0mgl^{-1}$) and IBA ($0.5\ mgl^{-1}$). In medium supplemented with NAA, the protocorms developed callus (Fig.5f, h).

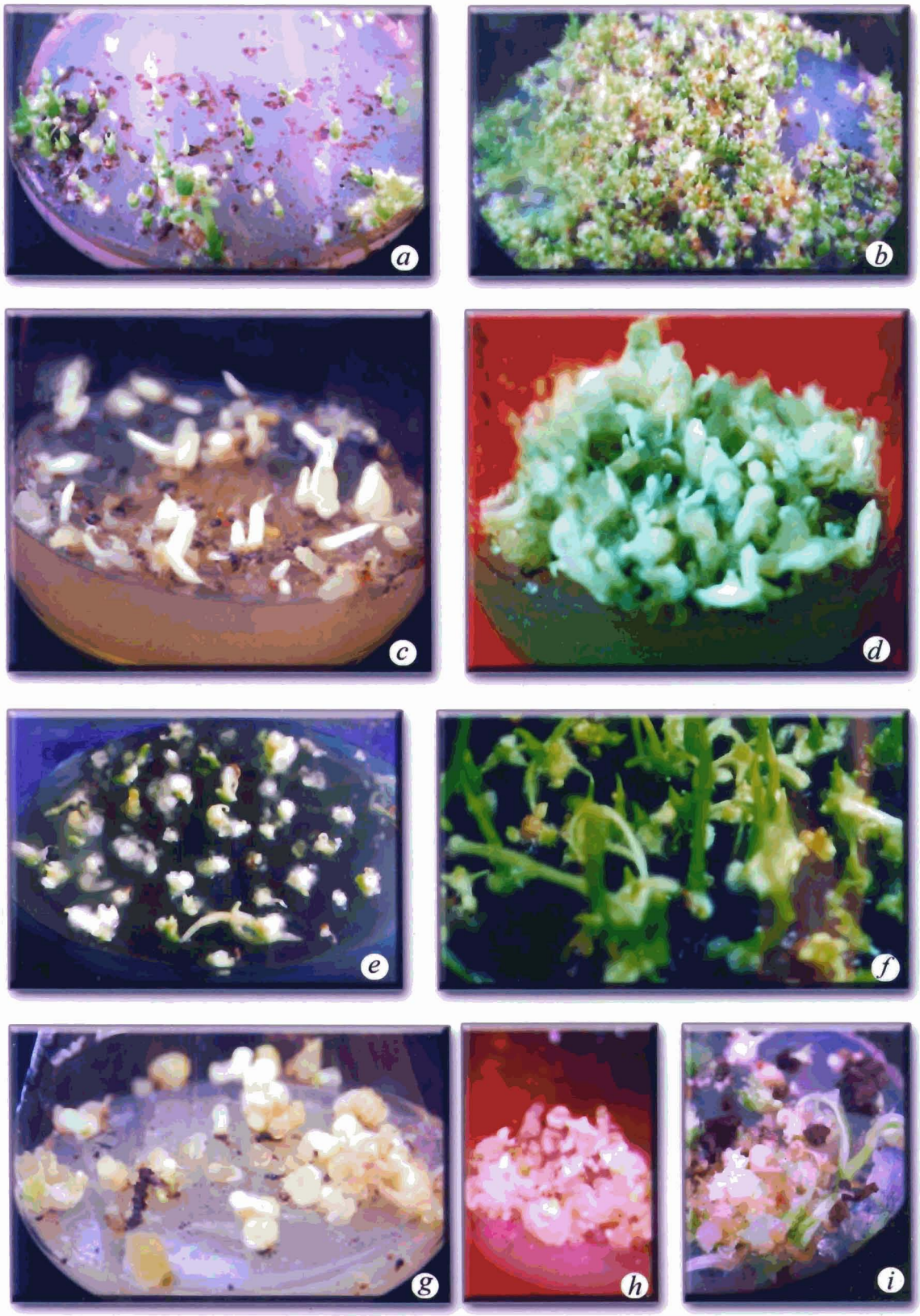


Fig.5 *In vitro* seed germination of *Vanilla planifolia*

a, b. Culture and growth of vanilla seeds on MS medium supplemented with BA (0.5mg/l), c, d. Vanilla seed culture in medium with tryptone, e. Growth of seeds on charcoal enriched medium, f. Well grown plants from seed cultures, g. Growth of seed cultures in medium incorporated with 0.002% colchicine, h, i. Induction of callus and plant regeneration from seed callus.

In medium supplemented with BA (1.0mgl^{-1}) and NAA (0.5 mgl^{-1}), regeneration of protocorm like bodies (PLBs) occurred from these calli, which multiplied and eventually developed into individual shoots and plantlets (Fig. 5 i).

In an attempt to increase variability in *Vanilla,* production of colchiploids was also attempted. MS medium supplemented with colchicine (0.002%) was used for seed germination. The frequency of seed germination was highest (87%) in this medium (Fig. 5 i) but further development of seed was slow if, left in the same medium. Most of the germinated seeds showed swelling of protocorms, which are typical of 'colchiploids' (Fig. 5g). Hence the seeds developed in colchicine medium were transferred to multiplication medium immediately after germination and the variations expressed among them were studied. Thus for production of seedlings without callus phase, MS medium with BA (0.5 mgl^{-1}) was most favourable.

Development of protocorms

In early stage of growth, the protocorms appeared as translucent white bodies with a pointed basal end, which later became swollen and gave rise to numerous tiny leaf primordial (Fig. 6 a-g). Simultaneously rhizoids also developed (Fig. 6 c-e). Protocorms then developed green colour and also increased in size (Fig. 6 e-g). In BA supplemented medium, in most of the protocorms the scale like leaf primordial developed into shoots and plants (Fig. 6 h-k).

Development of seedlings

Rapid seedling development occurred in germination medium supplemented with BA (1.0 mg/l) and IBA (0.5 mg/l). Shoot multiplication was best in this medium (Fig. 6 h, i, j), and on transfer to growth regulator free medium, the shoots developed good root system (Fig. 6 k).

Fig.6 Seed culture of *Vanilla planifolia* and development of protocorms

a. Germination of ***V.planifolia*** seed and initiation of protocorm, b, c. Various stages of development of protocorm, d. Close-up view of protocorm development into callus e,f,g . Close-up view of protocorm developing chlorophyll and multiplication , h, i. Development of plantlets from protocorms, j, k . Multiple shoot induction and development of plantlets from protocorms.

Micropropagation of *Vanilla planifolia* and its related species

Shoot tips (Fig. 7 a) and nodal explants of *V. planifolia*, were initiated in MS medium with BA (0.5 mgl^{-1}). These explants, after axillary bud break, were transferred to MS medium supplemented with auxins (NAA, IBA) and cytokinins (BA, kinetin), alone and in combinations. Of the combinations tested, BA when used alone or in combination with IBA induced multiple shoots (Table16).

Table 16. Effect of growth regulators on multiple shoot and root induction from shoot explants of *Vanilla planifolia* on MS medium*

Growth regulators				Multiple shoots frequency %	Average no. of shoots/culture±SD	Roots /culture	development
Kin	BA	NAA	IBA			No	Type
0.5	-	-	-	0.0		-	-
1.0				0.0		-	-
-	0.5	-	-	71 ± 3.45	4.18 ± 0.30	-	-
	1.0			20 ± 3.63	1.0	-	-
-	-	0.5	-	0.0	1.0	1	Velamen
		1.0		0.0	1.0	1	Velamen
-	-	-	0.5	0.0	1.0	1	Long roots
			1.0	0.0	1.0	1	Long roots
0.5	0.5			0.0	1.0	-	-
1.0	0.5			0.0	1.0	-	-
0.5	1.0			0.0	1.0	-	-
0.5		0.5		0.0	1.0	1	velamen
1.0		0.5		0.0	1.0	1	branching
0.5		1.0		0.0	1.0	1	velamen
0.5			0.5	0.0	1.0	1	-
1.0			0.5	0.0	1.0	1	-
0.5			1.0	0.0	1.0	1	-
	0.5		0.5	0.0	1.0	1	
	1.0		0.5	97 ± 6.5	15.15 ± 3.63	-	-
	0.5		1.0	65 ± 11.4	10.35 ± 3.45	-	-
0.0	0.0	0.0	0.0	0.0	1.0	1	Healthy roots

*Mean of 20 replicates

The ideal medium for multiplication was MS supplemented with BA (1mgl^{-1}) + IBA (0.5 mgl^{-1}). In this medium, an average of 15 multiple shoots were induced in 90 days of culture (Fig. 7 b-e). Nodal segments gave better response with a mean of 15 shoots per culture compared to the shoot tips (mean of 7 shoots per culture). In the present study kinetin alone or in various combinations had no effect of the induction of multiple shoots and only single shoot growth was observed.

Fig.7 Micropropagation of *Vanilla planifolia* by shoot culture

a. Shoot tip explant, b. Induction of protocorms c, d. Stages of multiple shoot induction and root, e. *In vitro* multiplied plantlets ready for hardening, f. Micropropagated plants hardened in cups, g, h. Tissue cultured plants in field.

On transfer to growth regulator free basal medium, the multiple shoots elongated (Fig. 7 e) with well developed root system producing plantlets which could be harvested for further studies and establishment in soil.

In 20% of the cultures, it was observed that roots tips were converted into shoot meristem on MS medium supplemented with cytokinins. Conversion of root tips into shoots was observed in *Vanilla planifolia* (Fig.8 a-f) and *V. aphylla* (Fig.9 f, g) on MS medium supplemented with BA (1.0 mgl^{-1}) and IBA (0.5 mgl^{-1}). These shoots, which developed into plantlets were hardened and established in soil.

An enlargement and flattening of the plant structures, called 'fasciation', was observed in about 5% of the cultures in *Vanilla planifolia* (Fig. 8 g-j) and rarely in *V. aphylla* (Fig. 9c). It occurred when BA was present in the culture medium at a concentration of atleast 1.0 mgl^{-1}, after 3-4 cycles in the same medium. The branches shortened and leaves flattened becoming unrecognizable as leaves.

Many species of *Vanilla* are endangered, *viz.*, *V. aphylla* and *V. walkeriae*, hence standardization of *in vitro* micropropagation protocols for related species was attempted. The culture media and conditions favourable for micropropagation of *V. planifolia* was suitable for other related species like *V. andamanica, V. aphylla* and *V. pilifera* also. MS medium supplemented with BA (1.0 mgl^{-1}) and IBA (0.5 mgl^{-1}) induced multiplication in all the species studied. However, the number of shoots induced in different species varied (Table 17). About 12-15 shoots / culture could be induced in *V. planifolia*, followed by *V. aphylla* (8-10 shoots). Among the species studied, the lowest multiplication rate was observed in *V. pilifera* (Fig. 9k) with only 2-4 shoots in 120 days. *Vanilla andamanica* also responded in the same multiplication medium with 5-7 multiple shoots per culture.

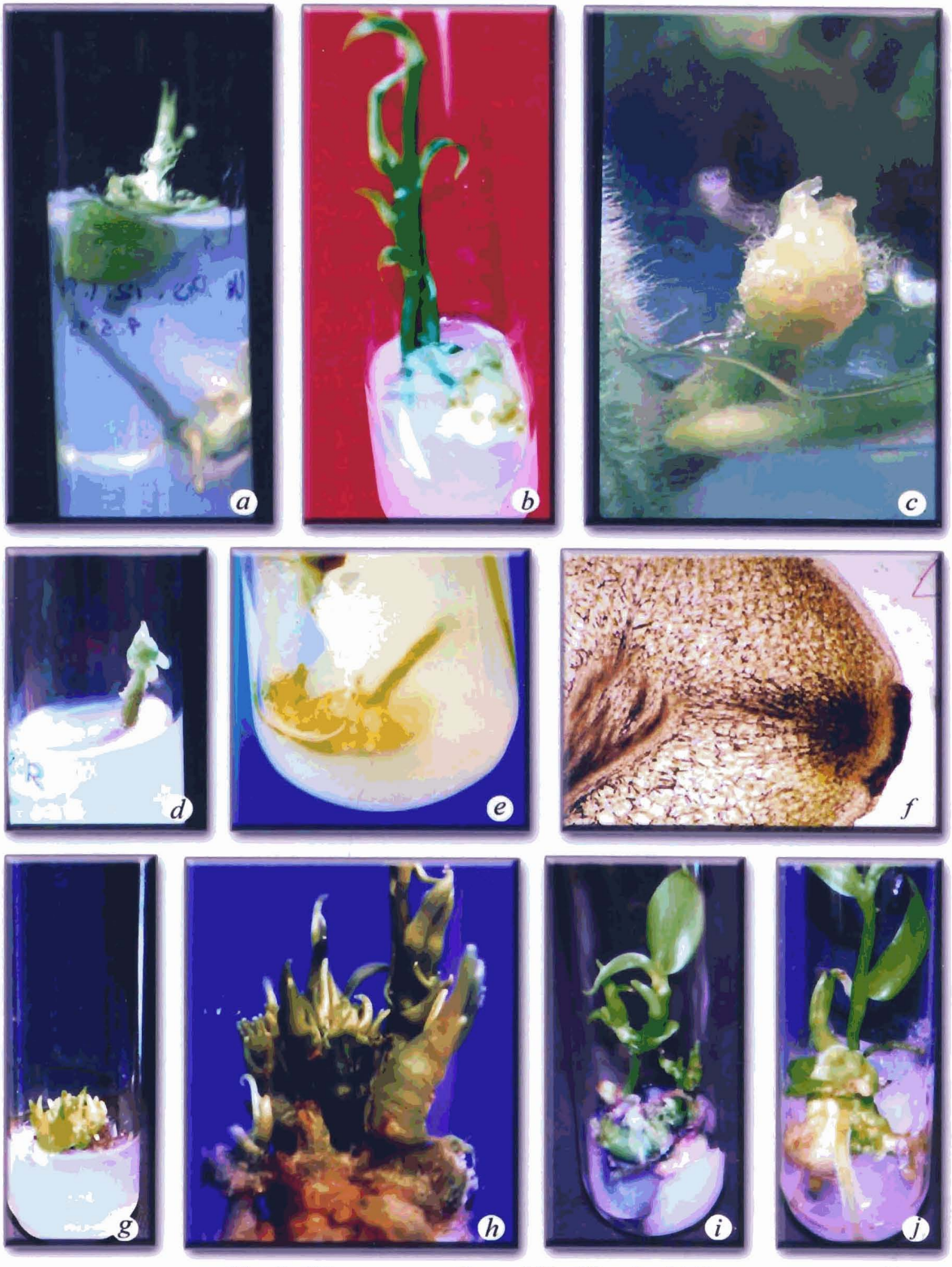


Fig. 8 Micropropagation of *Vanilla planifolia*

a. Root tip conversion into shoots in germinating seeds, b, c, d, e. Various stages showing conversion of root meristem into shoot and development of plantlets, f. Anatomical details of the root tip about to convert to shoot meristem, g, h. Different manifestations of fasciation and i, j. development of plantlets

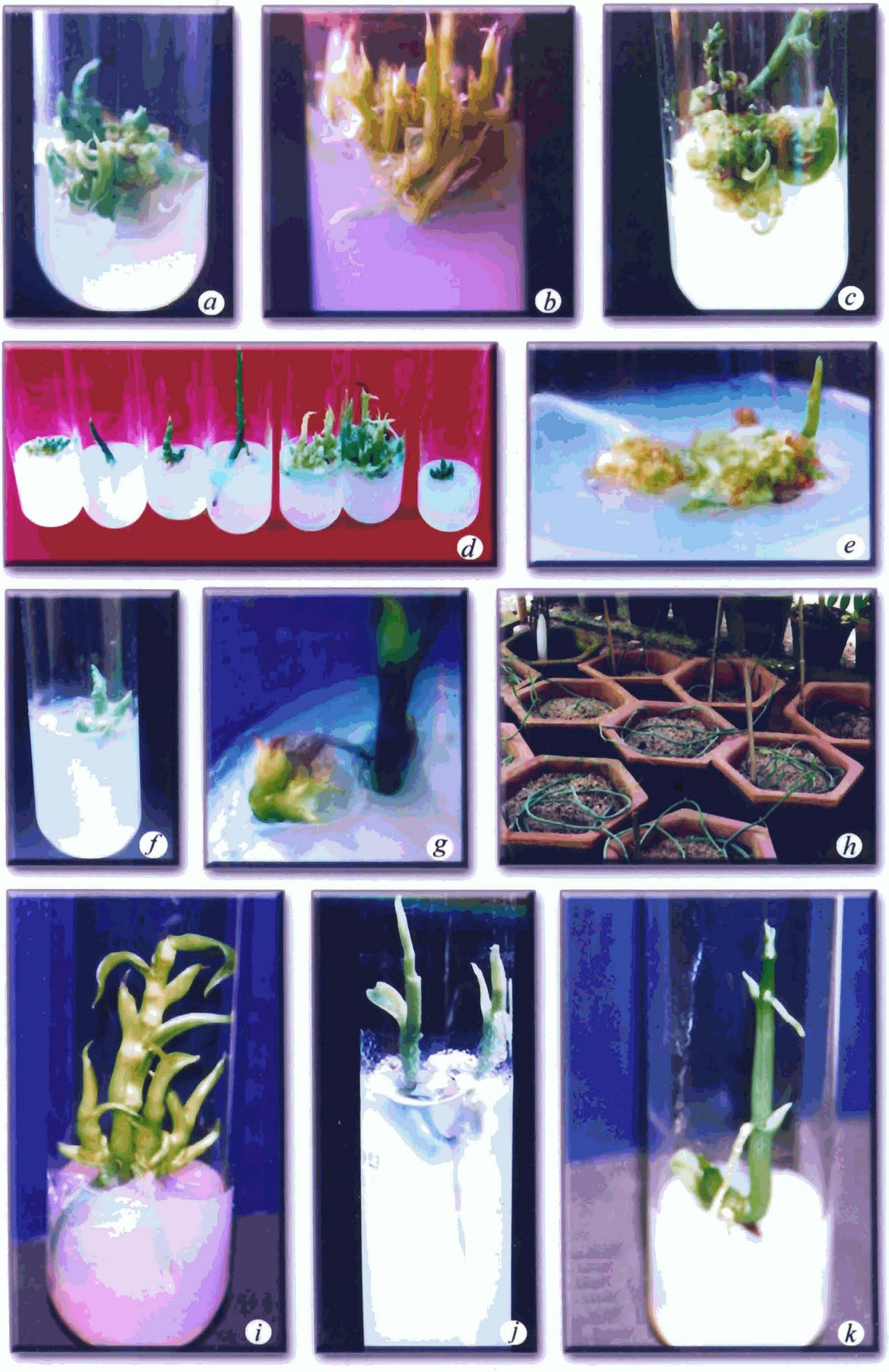


Fig.9 Micropropagation of *Vanilla* species

a, b. Induction of multiple shoots in *V.aphylla*, c. Fasciation in cultures of *V.aphylla*, d. Stages of micropropagation in *V.aphylla*, e. Callus regeneration in *V.aphylla*, f, g. Conversion of root meristem to shoot meristem in *V.aphylla*, h. Micropropagated plants of *V.aphylla* in pots, i,j. Induction of multiple shoots and roots in *V.andamanica*, k . Micropropagation of *V.pilifera*

Table 17. Comparison of *in vitro* responses in different species of Vanilla *

Growth regulators	*In vitro* responses			
	V.planifolia	***V.andamanica***	***V.aphylla***	***V.pilifera***
Kinetin	Single shoot	Single shoot	Single shoot	Single shoot
BA	Multiple shoots (3-4)	Multiple shoots (3-4)	Multiple shoots	Single shoot
NAA	Root induction	Root induction	Root induction	Root induction
BA + Kin	Single shoot	Single shoot	Single shoot	Single shoot
Kin + IBA	Single shoot	Single shoot	Single shoot	Single shoot
BA + IBA (1.0 + 0.5 mgl-1)	Multiple shoot induction (12-15 nos., in 10 days of culture)	Multiple shoot induction (5-7 in 90 days)	Multiple shoot induction (8-10 in 90 days)	Multiple shoot induction (2-4 in 120 days)
BA + NAA	Callusing and plant regeneration	Multiple shoots	Callusing and plant regeneration	Single shoot
Kin + NAA	Single shoot	Single shoot	Single shoot	Single shoot
Basal medium	Single shoot elongation and development of roots	Single shoot elongation, and development of roots	Single shoot elongation and development of roots	Single shoot elongation and development of roots

* All growth regulators were supplemented on MS basal medium at 0.5 to 1.0 mgl-[1]

In Vanilla, the shoots developed *in vitro*, elongated and produced good root system, when transferred from multiplication medium to growth regulator free medium. Hence, multiple shoots were transferred to basal medium for 30 days, before well-grown plantlets could be harvested for hardening and transfer to soil (Fig. 7f-h and Fig. 8h).

Callus regeneration and production of somaclones

Callus induction and plant regeneration

Shoot explants were cultured on the different media for callus induction and regeneration (Table 18). Callus was induced in media supplemented with NAA (0.5 mgl^{-1}) (Fig. 10 a-c). When BA and NAA was used in combination, plants regenerated. The most suitable medium was MS medium with NAA (0.5 mgl^{-1}) and BA (1.0 mgl^{-1}). In this medium, an average of 10 plants could be regenerated in 80% of the cultures (Fig. 10 d-g).

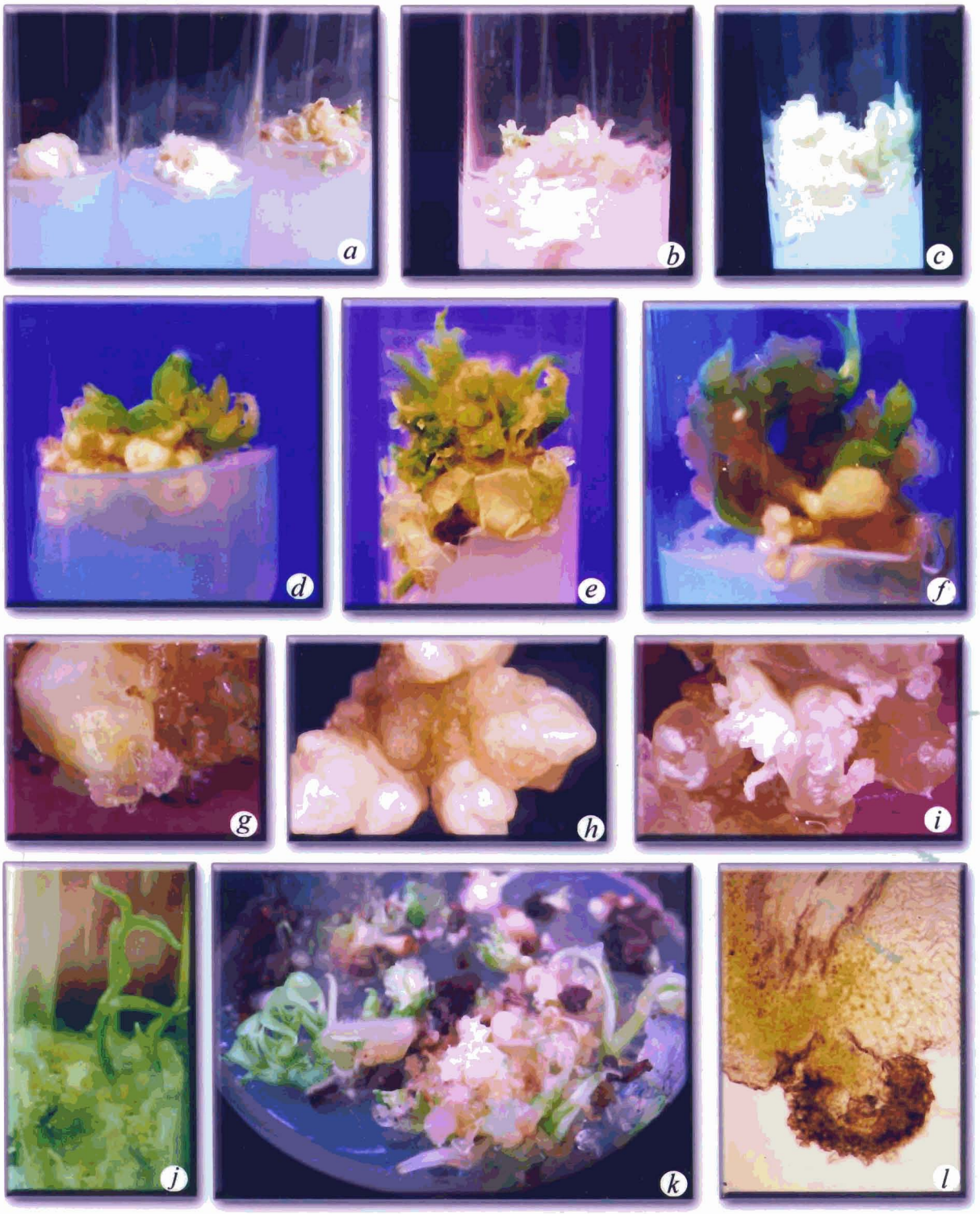


Fig.10 Plant regeneration from callus cultures of *Vanilla planifolia*

a. Induction of callus, b, c. Morphogenic callus, d, e, f. Various stages of plant regeneration from callus cultures and development of plantlets, g. Protocorms developing into callus, h, i. Close-up view of shoot bud initiation from callus, j. Plant regeneration from callus cultures, k. Plant regeneration from seed derived callus, l. Anatomical details of shoot initiation from embryogenic calli.

Table 18. Effect of NAA and BA on callus induction and plant regeneration from shoot explants of *Vanilla planifolia* on MS medium

Growth regulators (mgl^{-1})*		Nature of response	Percentage of response**	Mean no. of shoots / culture**
NAA	BA			
0.0	0.0	Only single shoot elongation	100	1
0.5	0.0	Callus induction and proliferation	20	-
1.0	0.0	Callus induction	10	-
0.0	0.5	No callus, only multiple shoot induction	80	6
0.0	1.0	No callus, multiple shoots	70	3
0.5	1.0	Callus formation and regeneration of shoots	80	10
1.0	0.5	Single shoot	100	1
0.5	0.5	Single shoot	100	1

*Mean of 20 replicates

In medium supplemented with NAA ($0.5mgl^{-1}$), it was found that sudden disorganization of the germinating seeds occurred to form a callus tissue in 80% of the cultures. The use of NAA and BA alone did not induce plant regeneration. In medium supplemented with BA ($1.0mgl^{-1}$) and NAA ($0.5mgl^{-1}$), 80% of the cultures developed embryogenic calli with 90% of them giving rise to a mean of 10 plants / culture (Table 19 and Fig. 10 h-m). Anatomical studies confirmed the origin of the regenerating shoots.

Table 19. Influence of growth regulators on callus induction and plant regeneration in seed cultures of *Vanilla planifolia* on MS medium*

Growth regulators (mgl^{-1})	Callusing (%)	Shoot Regeneration (%)	No. of shoots/ culture
0	0	0	0
NAA (0.5)	80	0	0
BA (1.0)	0	-	-
BA (1.0) + NAA (0.5)	80	90	10
BA (0.5) + NAA (1.0)	0	-	-
BA (1.0) + IBA (0.5)	10	60	6
BA (0.5)+ IBA (1.0)	0	-	-

- Mean of 20 replicates

The plants induced from callus tissue were transferred to growth regulator free medium for root induction. MS basal medium induced rooting in over 95% of the shoots, in contrast to the BA supplemented media.

Hardening and Planting out

The plantlets were removed from culture vessels, washed, treated with fungicide, and transferred to polybags containing potting mixture (sand, soil and vermiculite). They were hardened for 30 days under controlled conditions. The plantlets established in soil with over 80% success and were field planted with *Glyrcidia* standards for proper shade and support (Fig. 7 f, g and h).

Interspecific hybridization between *V. planifolia* and *V. aphylla*

Interspecific hybridization was attempted between *V. planifolia* and its related species to increase the spectrum of variations by bringing desirable characters from wild species into the cultivated vanilla. Pollinia from *V. aphylla* flowers were used to pollinate *V. planifolia* flowers. Eleven crosses were made, but after 4 days, perianth of six of the flowers fell off indicative of unsuccessful pollination. The remaining five fruits were left to mature and harvested at different maturity periods to study germination of the 'hybrid' embryos. Only six seeds germinated of which two were albinos and did not survive. The remaining four expressed characters segregating between the two parents. *V. aphylla* is a wild leafless type while *V. planifolia* is a cultivated plain leafed type (Fig. 12). Male character was expressed in 50 % of progenies, indicating successful pollination and the hybrid nature of the progenies. The distinctive features of *V. planifolia* and *V. aphylla* and the variation exhibited by their interspecific progenies are given (Tables 20, 21). Reciprocal crosses were also made, but not used for further study.

Fig.11 Interspecific hybridization between *Vanilla planifolia* and *V. aphylla*
a. Inflorescence of *V. planifolia*, b. Inflorescence of *V. aphylla*, c. Floral parts of *V. planifolia*, Inset : Pollinia of *V. planifolia*, d. Dissected parts of *V. aphylla* flower, e. Fruit set in *V. planifolia* pollinated with *V. aphylla* pollen, f. Fruit set in *V. aphylla* in reciprocal crosses.

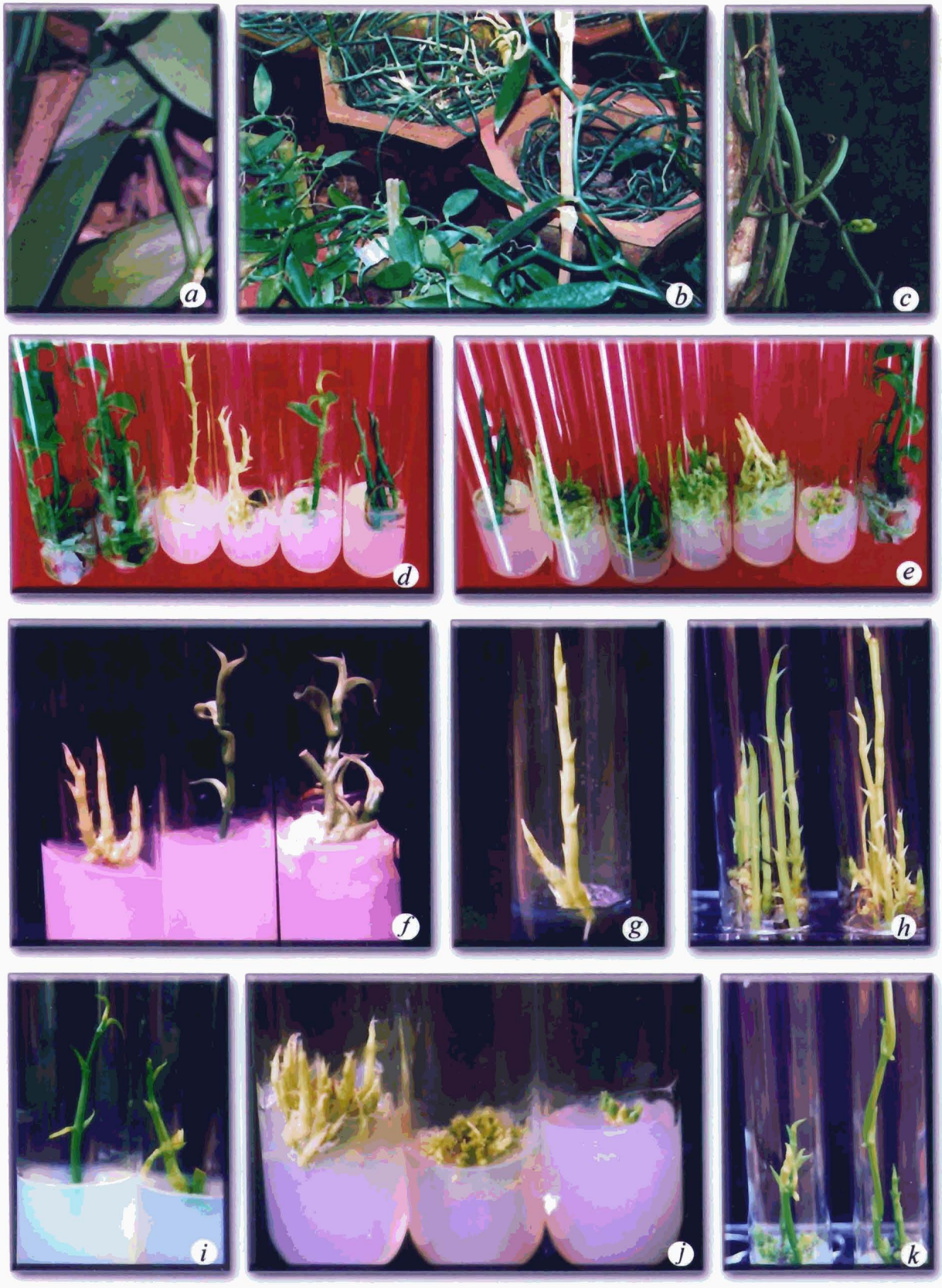


Fig. 12 Interspecific hybridization between *Vanilla planifolia* and *V.aphylla*

a. *V. planifolia* branch, b. Parent plants in pots, c. *V. aphylla*, d. Progenies developed from *V. planifolia* (♀) and *V. aphylla* (♂) crosses, e. Progenies developed from *V. aphylla* (♀)and *V. planifolia* (♂)crosses, f, g, h, i. Interspecific hybrids developed from *V. planifolia* (♀) and *V. aphylla* (♂) crosses, showing segregation of characters, expression of male parent leaf characters (note plain leaf and eafless forms) and absence of chlorophyll development, j, k. Interspecific hybrids of the reciprocal cross, showing lack of chlorophyll.

Table 20. Distinctive features of *V. planifolia* and *V. aphylla*

Species	Leaf	Flower	Chromosome no.
V.planifolia	Subsessile	Pale greenish yellow	2n = 32
V.aphylla	Leafless	Greenish yellow, tuft of violet tipped hairs on throat of lip	2n = 64

Table 21. Important morphological and cytological characters of *V. planifolia* and *V. aphylla* and their hybrids

No	Characters	*V.planifolia*	*V.aphylla*	Interspecific hybrids
1	Fruit set (%)	95	90	80
2	Fruit size (cm)	12-15	8-10	9
3	Maturity time (months)	6-9	6-9	-
4	No. of seeds / cm of fruit	2500	2000	< 1000
5	Germination rate (%)	87	80	0..2
6	Multiplication rate (seed)/60days	1: 9	1 : 9	1 : 6
7	Development of chlorophyll	Normal	Normal	50%-achlorphyllous
8	Chromosome nunmber	32	64	18-58

The progenies exhibited segregation of both the parental characters in that VH1, VH6 were 'planifolia' types in leaf characters while VH4, VH5 are 'aphylla' types. These progenies were indexed cytologically and using molecular markers.

PROTOPLAST ISOLATION AND CULTURE

Isolation of protoplasts from *in vitro* leaf tissues

Protoplast isolation was attempted in two species of Vanilla, viz., *V.planifolia* and *V.andamanica*. *In vitro* leaves were plasmolysed in a solution containing CPW salts with 9% mannitol prior to enzymatic digestion. Since it was difficult to peal off lower epidermis in vanilla, the plasmolysed leaf tissue was mechanically macerated by scraping the lower surface of leaf with a sharp blade and incubating in different concentrations and combinations of enzyme solutions (Table 4). Periodical microscopic observations showed the liberation of cell clusters and individual cells after 2 hrs of incubation in enzyme solution (Fig 13 a and b).

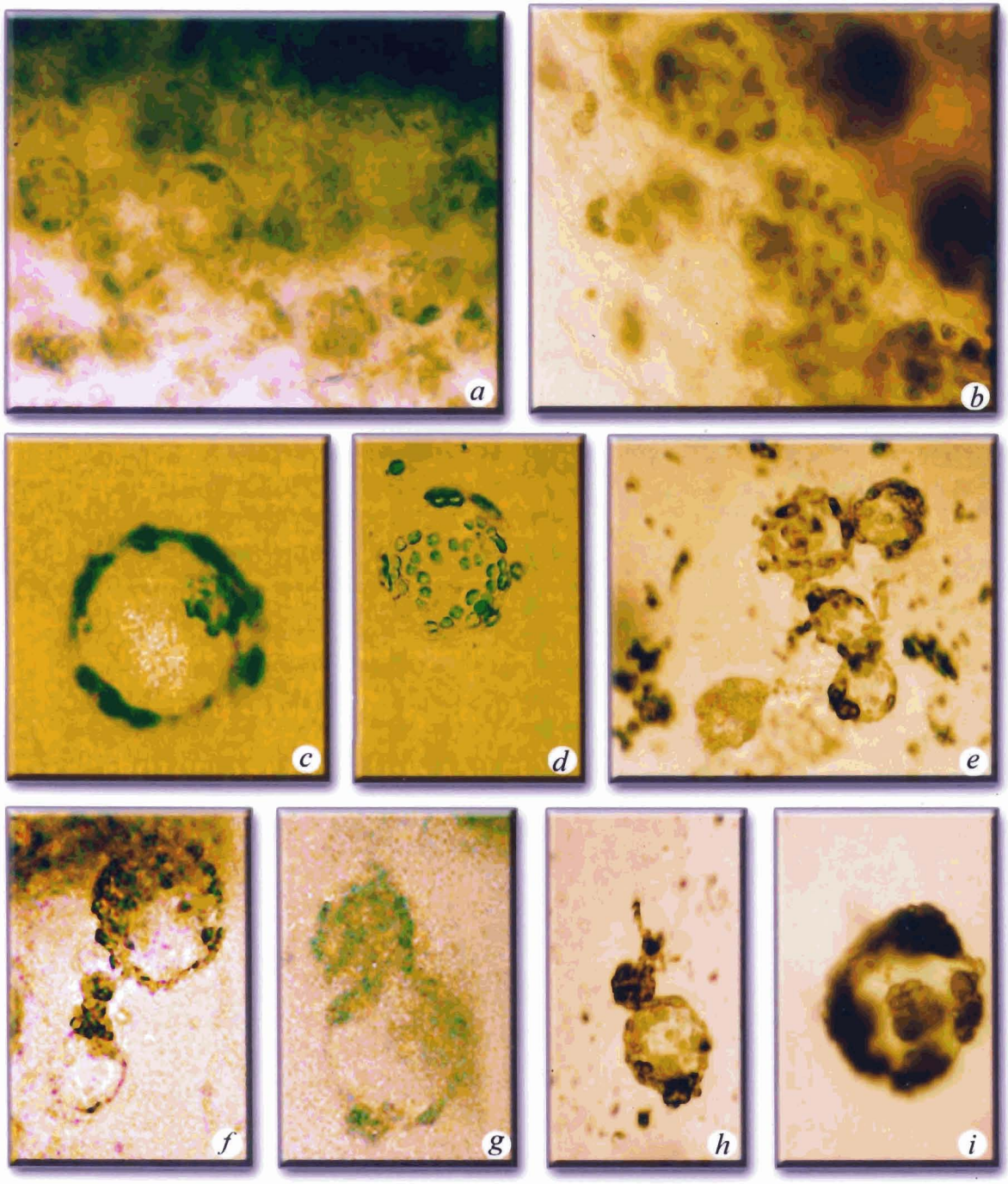


Fig.13 Isolation and fusion of protoplasts in *Vanilla*.

a. Release of protoplast from *V. planifolia* leaf tissue, b. Release of protoplast from *V. andamanica* leaf tissue, c. Isolated protoplasts from *V. planifolia* (note the concentration of chloroplasts in margins of protoplasts), d. Isolated protoplasts from *V. andamanica* (note presence of chlorophyll in the center of the protoplasts, e. Protoplasts coming closer in PEG incorporated medium, f. Formation of a channel for transfer of cell contents from one protoplast to another, g, h. Fusion of protoplasts of *V. planifolia* and *V. andamanica*, i. A heterokaryon - the fusion product of*V. planifolia* and *V. andamanica* (Identified by the size and arrangement of chloroplasts along margins and center),.

In vanilla, macerozyme at 0.5% was sufficient to digest the middle lamella separate the cells. Among the enzyme solutions tried, only the enzyme solution containing macerozyme R10 (0.5%) and cellulase Onozuka R10 (2%) was found to digest the cell wall and liberate protoplasts after incubation for 8 hours.

The tissues were incubated initially for 8h at 30^0C in dark (Table 22). Among the cellulases tested only Cellulase Onozuka R10 was found to be effective in releasing the protoplasts. The isolation solution containing 9% mannitol was found necessary for releasing and maintaining viable protoplasts.

The isolated protoplasts were round and were filled with chloroplasts (Fig.13 c, d). The protoplast yield was 2.5 x 10^5 per gram of leaf tissue. The protoplast viability as assessed by FDA staining was 72% in *V.planifolia* and 55% in *V.andamanica.*

Table 22. Effect of enzyme concentration and incubation conditions on yield of protoplasts

Species	Enzyme solution	Incubation conditions	Protoplast yield	Viability (%)
V.planifolia	0.5% Macerozyme R 10 + 2% Onozuka cellulase R10	8 h at 30^0C in dark	$2.5x10^5$/g of leaf	72
V.andamanica	1% Macerozyme R10 + 3% Hemicellulase + 6% Onozuka cellulase R10	8 h at 30^0C in dark	$1x10^5$/g of leaf	55

Protoplast fusion and culture

Protoplasts were isolated from *V. planifolia* and *V. andamanica.* The protoplasts of the two species were visibly different (Fig 13 c and d). *V. planifolia* protoplasts were bigger in size (0.031mm) than *V. andamanica* (0.022 mm) and in the former, chloroplasts were arranged around the periphery whereas in the

latter chloroplasts remained scattered within. When subjected to PEG mediated fusion, the protoplasts of the two species came nearer and form a passage gradually leading to the transfer of the cell contents of one species into another (Fig 13 f,g and h). The fusion product or heterokaryon has chloroplasts both at the periphery and in the middle making its selection easier(Fig 13 i). This can be very useful in gene transfer of useful traits, especially the natural seed set and disease tolerance observed in *V. andamanica* to *V. planifolia*. The fused protoplasts were cultured as droplet cultures in MS liquid medium with 0.5 mgl $^{-1}$ BA, 0.5 mgl $^{-1}$ IBA supplemented with 3% sucrose and 7% mannitol for 20 days. The cell wall development around the fusion product was observed after 36 hours. The contents became dense by one week and fresh medium need to be added at 7-day interval.

CHARACTERIZATION OF SELFED PROGENIES AND SOMACLONES

The collections, species available, selfed seed progenies, somaclones, interspecific hybrids and colchicine treated progenies were characterized using morphological features, isoenzyme profiles, molecular markers and chromosome indexing wherever possible to estimate the extent of variations between them.

Morphological characterisation

Progenies of vanilla at the time of planting out

The selfed progenies of *V. planifolia* obtained through ovule culture have shown variations in plant, leaf and stem characters. These variations were evident both *in vitro* as well as after planting out. *In vitro* variations were observed as low and high multiplying types, somaclone with slender to stout plant types with short and long internodes, dark, light green and variegated leaves and leaf shape from lanceolate, oblong or elliptic with acute or acuminate leaf tips (Fig.14, 15, 16, 17).

Fig.14 Phenotypic variations observed in seed cultures and somaclones of *Vanilla planifolia*
a. Tissue cultured plantlets with long internodes. b.Tissue cultured plantlets with short internodes but good growth, c. 3 morphotypes showing plants with long leaves and normal internodes (left), plants with small leaves and short internodes (middle) and plants with long leaves and long internodes (right), d. somaclone with long internodes, e. somaclones with variegated leaves, f. Somaclones with narrow leaves, g, h. Variations among narrow leaved somaclones, i, j, k, l.Variations in the rate of multiplication, *in vitro*, among somaclones, *in vitro*, m. Bulging of axillary buds in colchicine incorporated medium, n. Colchicine derived plants

Fig. 15 Morphological variations among six month old seedlings and somaclones of *Vanilla planifolia*

a. Hardened seedling and somaclones b, d. Large population of hardened seedlings and somaclones, c, e, f. Growth variations among somaclones, g. Leaf variations among six month old selfed progenies, h. Leaf variations among seedling progenies (L-R : V48, V211, V7, V150, V24, V51, V69, V1, V59, V221), i. Leaf variations among colchicine generated progenies (VC89, VC25, VC65, VC64, VC59, VC49, VC3, VC40, VC1 and V41, V211), j. Stem variations Note : High variations in internodal length, leaf shape, size viz., somaclones with shorter nodes, long broad leaves, long narrow leaves

Fig. 16 Morphological variations among one year old seedlings and somaclones of *Vanilla planifolia*

a. *Variant* somaclone with broad leaves, b, c, d, e, f. Variations observed among selfed progenies and its callus regenerated progeny, g, h, i. Distinct leaf variations among somaclones, j, k. Plants in nursery ready for field planting

Fig. 17 Morphological variations among two year old seedlings and somaclones of *Vanilla planifolia* established in field

a. Plants established in pots in field, b. Somaclone with shorter internodes and broad leaves, c. Somaclone with very short internodes, d. Selfed progeny showing a different plant type, e. Leaf shape variations among field established species, collections and selfed progenies (L-R : *V.planifolia*, Mauritius collection, *V.tahitensis*, V7, V211, V69, V221), f. Long leaved somaclone, g. Normal leaved somaclone (V1), h. Small leafed somaclone with good vegetative growth (V8), i. Selfed progeny with smaller internodes (V32),j, k. Colchicine generated progenies (VC 25 and VC 26) with shorter internodes seeming as opposite arrangement of leaves as compared to alternate arrangement in *V.planifolia*

The morphological variations observed among the progenies of *V. planifolia* in the nursery and at the time of field planting are given in Tables 23, 24, 25 and 27 and are graphically represented in Figures 18, 19, 20 and 21. Among the selfed progenies V113 showed highest plant height (41 cm), internodal length, as well as leaf breadth. Leaves of V66 were ovate in shape. Though V98 was a slow growing progeny with lesser height and smaller internodes, it has long narrow leaves and highest leaf length (Figs. 14, 15, 16, 17).

Table 23. Variations in plant characters observed at the nursery stage among a few selfed progenies and somaclones of *V. planifolia* (six months after planting out)

No.	Progenies	Plant height (cm) ±S.D	Internodal length (cm) ±S.D	Leaf size (cm) Length ±S.D	Leaf size (cm) Breadth ±S.D
	Selfed seedlings				
1	V66	23.7±2.51	2.43 ±0.49	0.67±0.32	1.82±0.47
2	V67	24.6±1.08	2.65±0.43	1.72±0.29	0.68±0.32
3	V75	12.29±1.6	1.81±0.41	2.32±2.49	1.72±0.29
4	V98	5.64±0.76	0.88±0.24	**5.64**±0.64	1.69±0.30
5	V105	22.3±2.37	1.99±0.43	3.72±0.41	1.52±0.37
6	**V113**	**41.4**±3.1	**2.96**±0.22	2.83±0.26	**1.82**±0.37
7	V134	20.4±2.31	2.22±0.54	2.84±0.36	0.68±0.32
8	V140	24.3±1.36	2.74±0.3	3.79±0.30	1.68±0.32
9	V142	17.9±2.04	1.89±0.29	3.50±0.38	0.76±0.21
10	V144	13.8±2.03	2.16±0.34	2.19±0.43	0.62±0.24
11	V145	17.5±2.46	2.88±0.31	2.42±0.41	1.82±0.62
	Colchicine treated				
12	VC1	**24.1**±2.2	2.54±0.32	2.59±0.31	1.83±0.39
13	VC2	10.0±1.33	1.75±0.35	1.97±0.32	**1.90**±0.4
14	VC3	17.6±1.3	**2.97**±0.51	1.99±0.48	1.72±0.39
15	VC4	11.5±0.98	1.59±0.26	2.89±0.36	1.46±0.52
16	VC5	13.0±1.55	1.94±0.33	1.26±0.48	1.46±0.52
17	VC 6	6.03±1.56	1.22±0.33	1.22±0.48	1.59±0.40
18	VC8	5.3±1.04	1.78±0.38	2.44±0.37	1.73±0.40
19	VC9	11.2±1.31	1.55±0.36	3.03±0.27	1.86±0.46
20	VC10	25.5±3.3	2.48±0.59	1.95±0.23	1.83±0.52
21	VC12	5.50±0.71	0.83±0.42	0.98±0.28	0.87±0.24
22	VC13	3.7±0.71	1.1±0.32	**4.34**±0.55	1.18±0.30
23	VC18	17.5±1.6	2.04±0.35	1.6±0.38	1.44±0.30
	Callus regenerated				
24	VCC21	14.35±1.0	1.05±0.29	**3.86**±0.30	1.25±0.23
25	VCC24	18.4±2.03	**2.55**±0.40	3.1±0.72	**2.50**±0.24
26	VCC32	9.8±1.27	0.86±0.52	1.54±0.34	2.24±0.35
27	VCC39	12.03±1.8	1.82±0.45	2.02±0.42	1.72±0.41
28	VCC40	**19.64**±1.45	2.21±0.39	1.82±0.36	1.70±0.41
29	VCC43	14.19±2.06	2.07±0.34	2.02±0.31	1.62±0.34
30	VCC47	7.3±1.01	1.66±0.37	1.22±0.42	1.23±0.33
31	VC3.1	11.6±1.26	1.57±0.38	2.82±0.70	0.75±0.23
32	*V.planifolia*	22.3±1.44	2.03±0.29	3.39±0.30	2.11±0.29

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

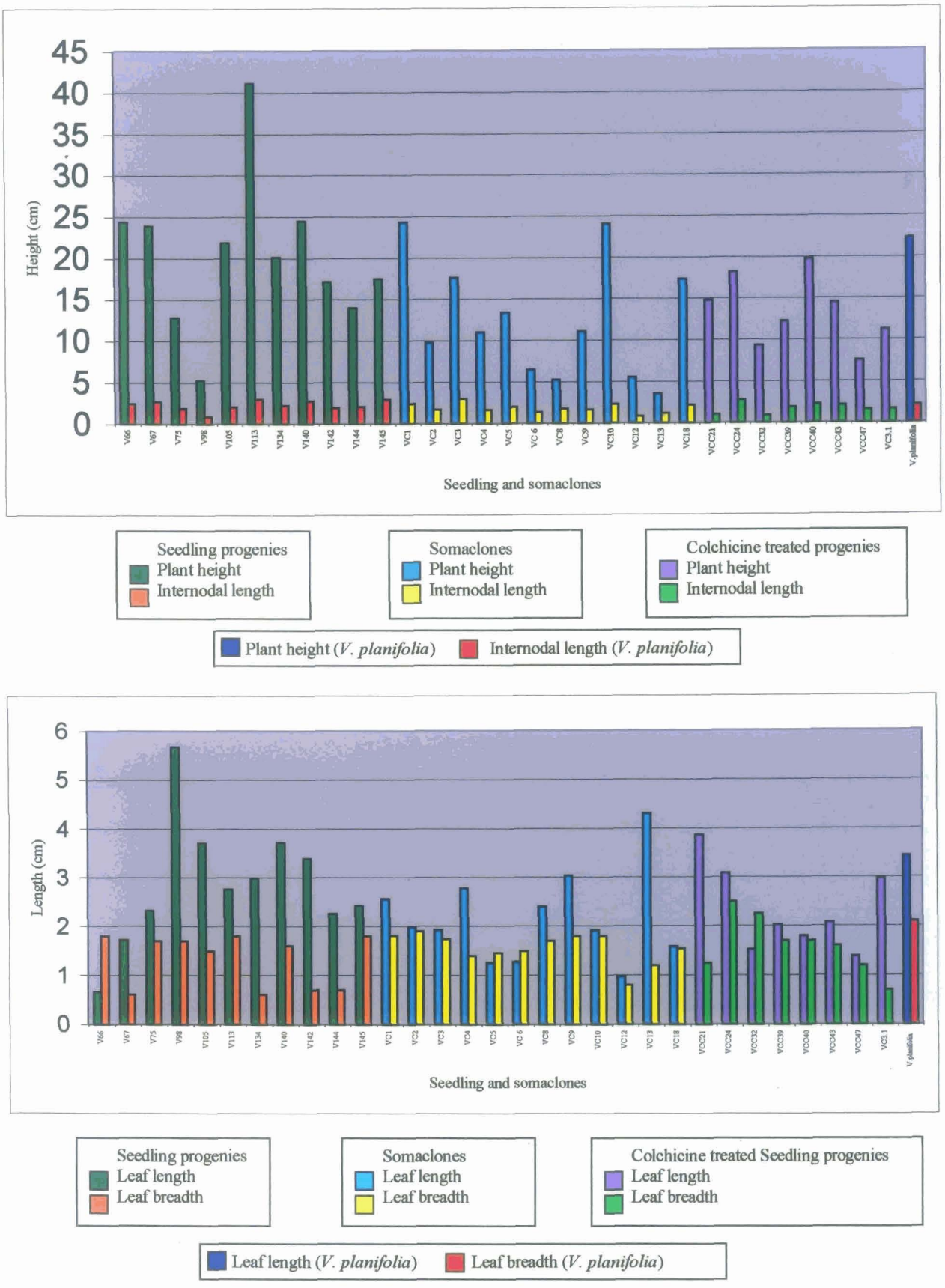


Fig 18. Variations in plant height, internodal length, leaf length and leaf breadth in seedling progenies, somaclones and colchicine treated progenies of *Vanilla planifolia*

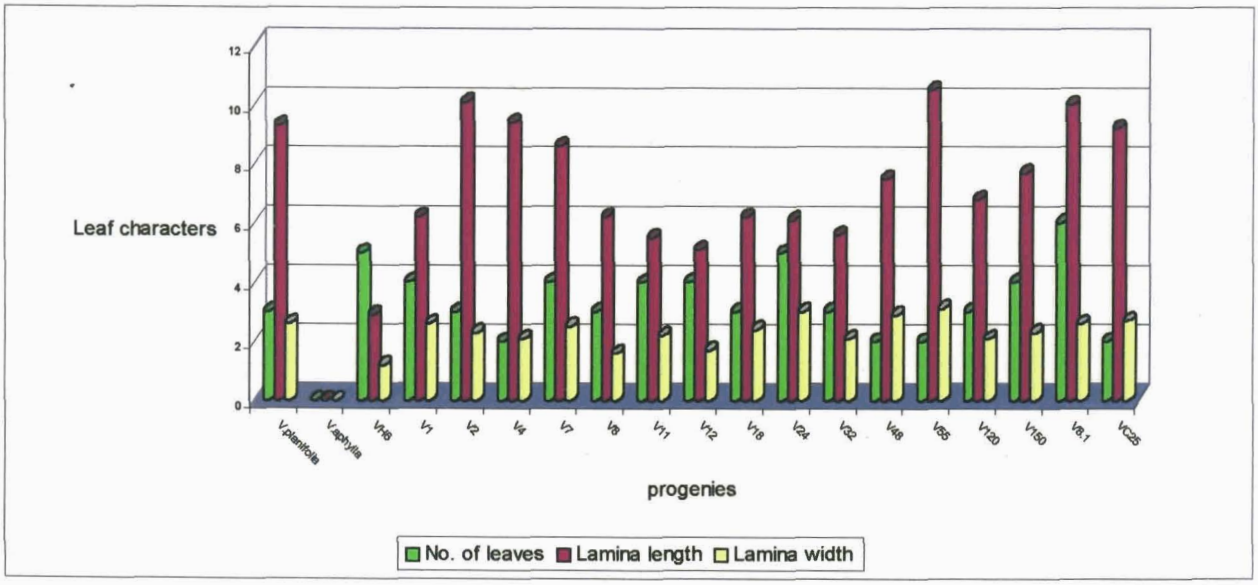


Fig. 19 Variations in leaf characters of one year old seedling progenies of *Vanilla planifolia*

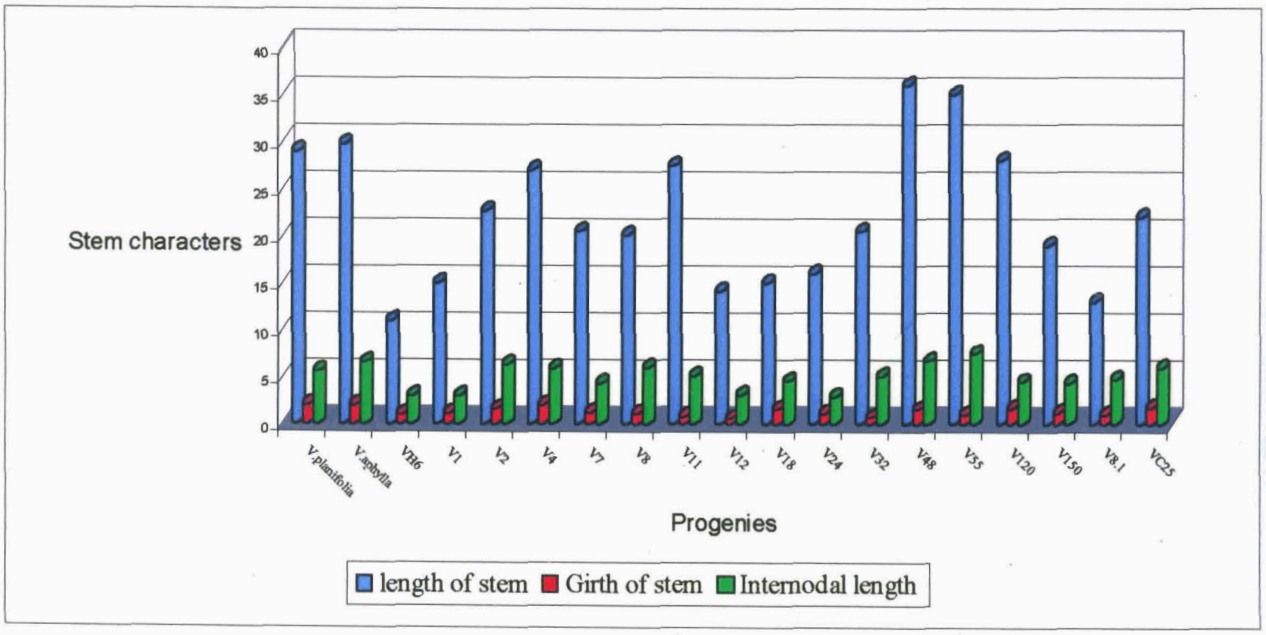


Fig 20. Variations in stem characters of oneyear old seedling progenies of *Vanilla planifolia*

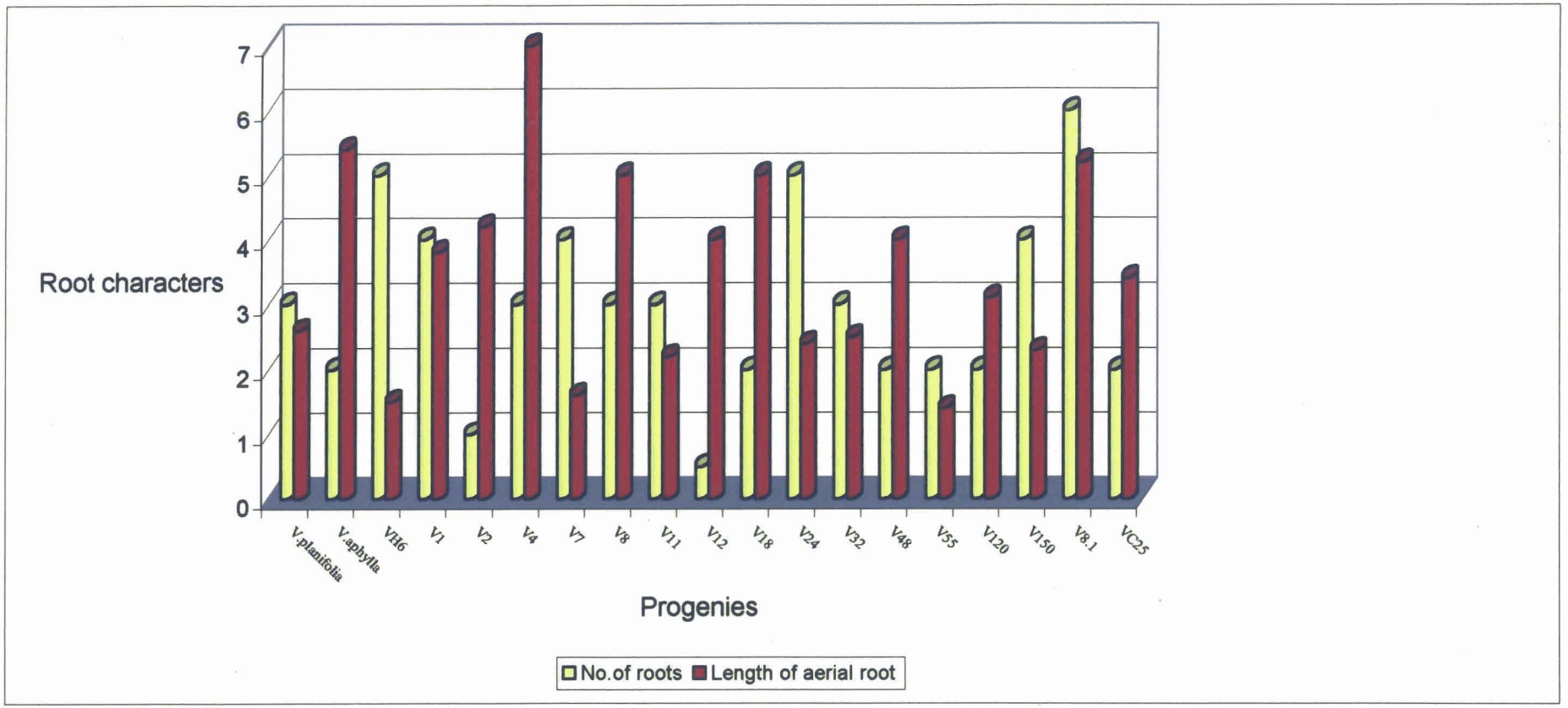


Fig. 21 Root characters of 1 year old seedling progenies of *Vanilla planifolia*

Table 25. Variations in plant characters observed in progenies of *V. planifolia* after one year of field planting

No.	Progenies	Plant height (cm) ±S.D	Internodal length (cm) ±S.D	Leaf size*	
				Length (cm) ±S.D	Breadth (cm) ±S.D.
1	V1	145.0± 18.38	7.5± 1.41	9.5± 0.99	3.0± 0.71
2	V2	120.0± 2.83	6.5± 2.26	10.5± 0.99	3.0± 0.28
3	V4	55.0± 9.90	5.5± 2.12	12.0± 2.83	3.6± 0.57
4	V6	5.0± 1.41	2.5± 0.71	5.5± 1.13	1.7± 0.28
5	V7	105.0± 21.21	5.5± 0.28	7.5± 0.85	1.9± 0.14
6	V8	170.0± 19.8	6.5± 0.71	8.5± 0.99	2.5± 0.07
7	V10	90.0± 11.31	5.0± 2.83	12.5± 0.85	3.2± 0.14
8	V 60	109.0± 4.24	4.7± 2.24	12.8± 0.28	3.2± 0.42
9	V 219	39.0± 9.90	2.8± 0.42	7.8± 0.99	2.9± 0.42
10	VCC20	88.0± 5.66	4.5± 0.99	9.2± 1.48	2.6± 0.57
11	VC 86	62.0± 8.49	5.3± 0.42	9.7± 0.14	3.4± 0.42
12	VC 82	188.0± 1.41	6.9± 1.41	13.5± 2.12	3.6± 0.85
13	VC 69	51.5± 3.54	2.5± 0.57	5.2± 1.27	2.6± 0.57
14	VC 68	112.0± 9.9	5.6± 0.99	8.5± 0.17	2.2± 0.57
15	*V.planifolia*	122.0± 12.73	4.2± 0.42	12.0± 1.98	3.2± 0.14

- Data taken from the sixth leaf onwards; S.D : Standard Deviation

Comparison of plant characters among interspecific hybrids between *Vanilla planifolia* and *V. aphylla* along with their parents at the time of hardening are given in the Table 26.

Table 26. Plant characters of interspecific hybrids between *Vanilla planifolia* and *V. aphylla*, at the time of hardening

No	Interspecific parents/hybrids	Plant type similar to	Plant height (cm)	Internodal length (cm)	Leaf size	
					Length (cm)	Breadth (cm)
1	V.planifolia	-	7.00	1.36	2.36	1.28
2	V.aphylla	-	6.62	1.53	0.30*	0.30*
3	V. H1	Vp	5.85	1.59	2.25	1.12
4	V. H4	Va	8.57	1.61	0.6*	0.3*
5	V. H5	Va	9.25	1.50	0.5*	0.2*
6	V. H6	Vp	4.26	1.40	3.05	1.65

* Scale leaves

The hybrids showed segregation of parental characters between them. Hybrids VH1 and VH6 resemble *V. planifolia* and VH4 and VH5 resemble *V.aphylla,* in leaf and stem characters (Fig.12).

The progenies generated in colchicine incorporated medium (VC), were characterized by their short internodes and broader but smaller leaves (Fig.17), distinct from all other somaclones. The average internode length among colchicine treated progenies was 1.6 cm while that of selfed progenies was 2.3 cm. Among them, VC3 had the longest internodes (2.97 cm) whereas VC 12 had the shortest internodal length (0.78 cm). VC1 and VC10 were the most vigorous in growth in comparison to VC8, a slow growing somaclone (Tables 23). The average leaf width among VC progenies was 1.3 cm while the average among selfed progenies was 1.5 cm whereas the average leaf length among VC progenies was 2.16 cm while it was 2.9 cm at the time of planting out.

The callus regenerated progenies exhibited higher variations among themselves. After a year of planting out in pots in fields, V8 (among the selfed progenies) and VC82 (among the somaclones) showed higher plant growth rate. VC82 was characterized by good growth of the plant as well as leaf size and internodal length. Among the selfed progenies, V48 and V55 showed better growth when compared to *V. planifolia.*

Thus morphological variants among the seedlings, somaclones and interspecific hybrids of vanilla were manifested in plant, stem and leaf characters (Table 27). Some of these variants could be detected in the culture stage itself and others at nursery stage and after planting out. This indicates the existence of high genetic variability in the seedling, somaclones and interspecific hybrids in Vanilla.

Table 27 Morphological variations in field planted progenies of *V. planifolia* (selfed progenies and somaclones)

Plant Characters	V.pl	V.aph	VH6	V1	V2	V4	V7	V8	V11	V12	V18	V24	V32	V48	V55	V120	V150	V8.1	VC 25
Stem type Simple/Branched (S / B)	S	S	S	S	S	B	B	S	S	S	S	B	S	S	B	S	B	B	S
Shape Cylindrical / Slender*	C	C	C	C	C	C	C	C	S	S	C	C	C	C	C	C	C	C	C
Color of vine Green/pink/variegated	G	DG	G	DG	G	G	G	G	DG	G	G	G	DG	G	G	G	G	DG	DG
Vertical groove Present/Absent	A	P	A	A	A	A	A	A	P	A	A	A	A	A	A	A	A	A	A
Length of stem	29	29.8	11	15	22.7	27	20.5	20.1	27.5	14.1	15	16	20.5	36	35	28	19	13	22
Girth of stem	2	2	1.0	1.1	1.6	2.0	1.2	1.1	0.8	0.6	1.6	1.1	0.7	1.6	1.0	1.3	1.2	1	1.8
Internode length (from 10th leaf fromapex)	5.6	6.6	3.0	3	6.3	5.9	4.3	6	5.1	3.1	4.6	2.9	5.1	6.7	7.5	4.5	4.3	4.8	6
Leaves type Succulent/nonsucculent	S	-	N	NS	NS	S	S	NS	NS	NS	NS	S	NS	NS	NS	NS	NS	S	S
Sessile/petiolate (S / P)	P	-	P	S	P	P	P	P	P	P	S	S	S	P	P	P	P	P	P
Variegated/nonvariegated	Nv	-	Nv	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV
Shape: Oblong/Elliptic/lanceolate	L	-	L	O	L	L	L	O	O	OL	L	O	O	L	L	L	E	L	L
Nature of tip:acute/acuminate	Ac	-	Ac	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
Pubescence :Present/absent	A	-	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
No.of leaves/10cm	3	-	5	4	3	2	4	3	4	4	3	5	3	2	2	3	4	6	2
Lamina length (6th leaf from apex)	9.3	-	2.9	6.2	10.1	9.4	8.6	6.2	5.5	5.1	6.2	6.1	5.6	7.5	10.5	6.8	7.7	10	9.2
Lamina width	2.6	-	1.2	2.6	2.3	2.1	2.5	1.6	2.2	1.7	2.4	3.0	2.1	2.9	3.1	2.1	2.3	2.6	2.7
Aerial roots Present/Absent	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
No.of roots/meter	3	2	5	4	1	3	4	3	3	4	2	5	3	2	2	2	4	6	2
Length of aerial root	2.6	5.4	1.5	3.8	4.2	7	1.6	5	2.2	0.5	5	2.4	2.5	4	1.4	3.1	2.3	5.2	3.4
Color of aerial root	CG	G	G	LG	G	G	CG	C	B	DG	CG	G	CG	C	C	B	CB	DG	G
Diseases																			

*** See p.94 Materials and Methods for explanation of the abbreviations**

Cytological indexing of selfed progenies, somaclones and interspecific hybrids

Cells from both selfed as well as somaclones showed great deviations from the normal reported somatic chromosome number 2n= 32 (Heusser, 1938). Metaphase stage chromosomes exhibited both structural as well as numerical variations. Numerical changes were observed as a result of a high rate of somatic associations. The different chromosome numbers observed ranged from 24 to 36 among the somaclones. *V.planifolia* itself showed a high frequency of variations (78.18%). Among the selfed progenies, V219 had the highest rate of chromosomal aberrations (82.85%) whereas V10 showed the most stable chromosome numbers with only 14.28% variations from the normal somatic number (Fig.22 and 23).

Contrary to the concept that root regenerated plants are highly stable, V1R showed a high frequency of variations (44.76%). Among the cells observed, callus regenerated progeny V 8.1 did not have any cell with the normal somatic compliment. Colchicine generated progeny VC 3 showed a chromosome number 36, which was higher than the normal somatic chromosome number in *V.planifolia.* Since all the other somaclones of *V.planifolia,* showed variable chromosome numbers lesser than 2n= 32 which may be due to associations, the increased number of VC3 is probably due to the colchicine treatment resulting in higher ploidy level (Table 28). Structural abnormalities like end-to-end pairing, bridge formation, tripolar and tetrapolar unequal segregation at late metaphase, clumping were observed (Fig. 22 and 23).

Hybrids showed somatic associations and segmental allopolyploidy, indicating the hybrid origin of the progenies and that the parents are related closely. VH6 had chromosome numbers nearer to *V.planifolia*, but no cell with 2n=32.

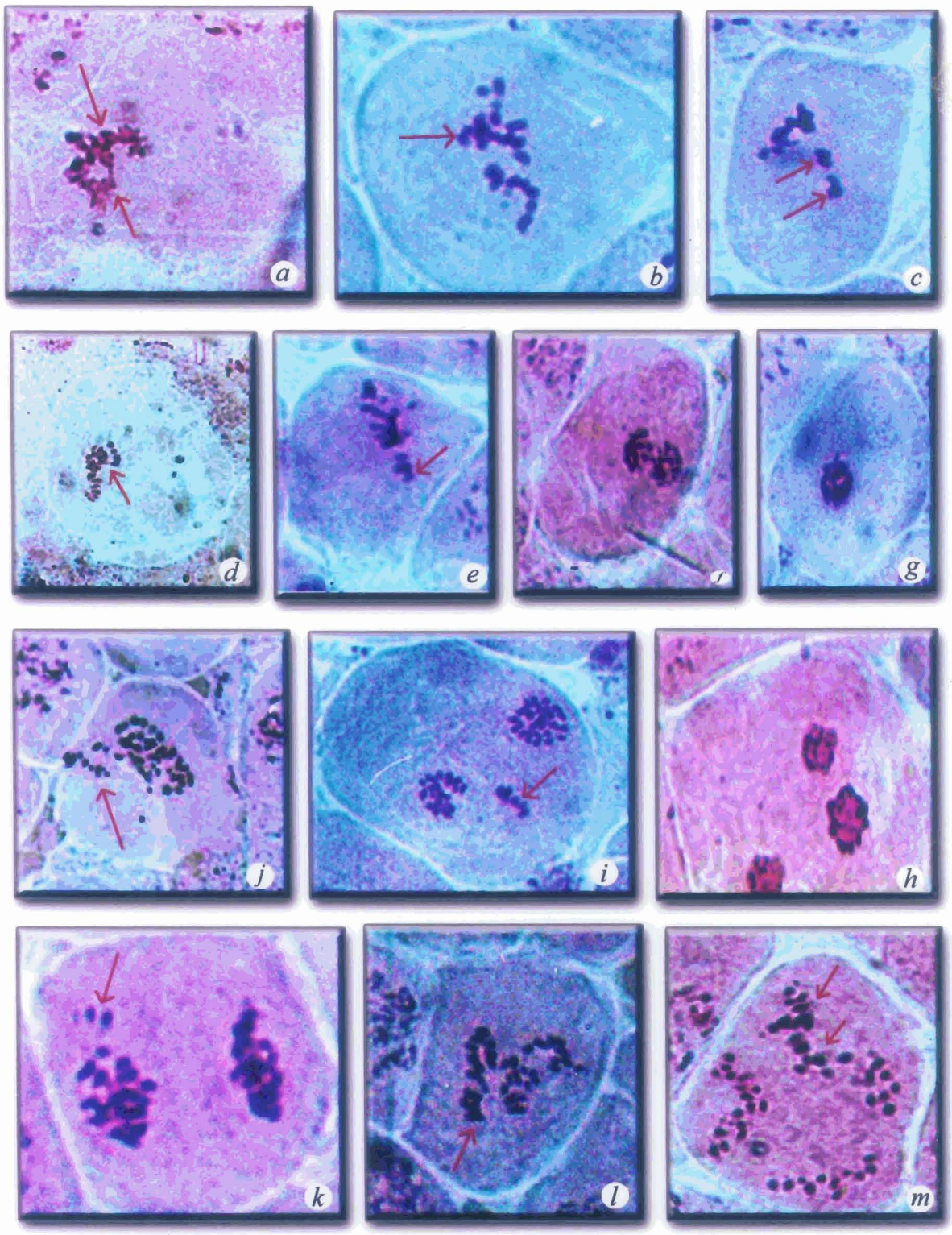


Fig.22 Structural aberrations during mitosis in Vanilla somaclones

a. Ring formation in selfed progeny V7, b - e. Abnormal metaphase formation and separation in somaclones, f. Anaphase bridge formation in V13, g,h. Clumping of chromosomes, i, j, k. Laggards at anaphase, l. tripolar segregation in VC3, m. Tetrapolar segregation in V60.

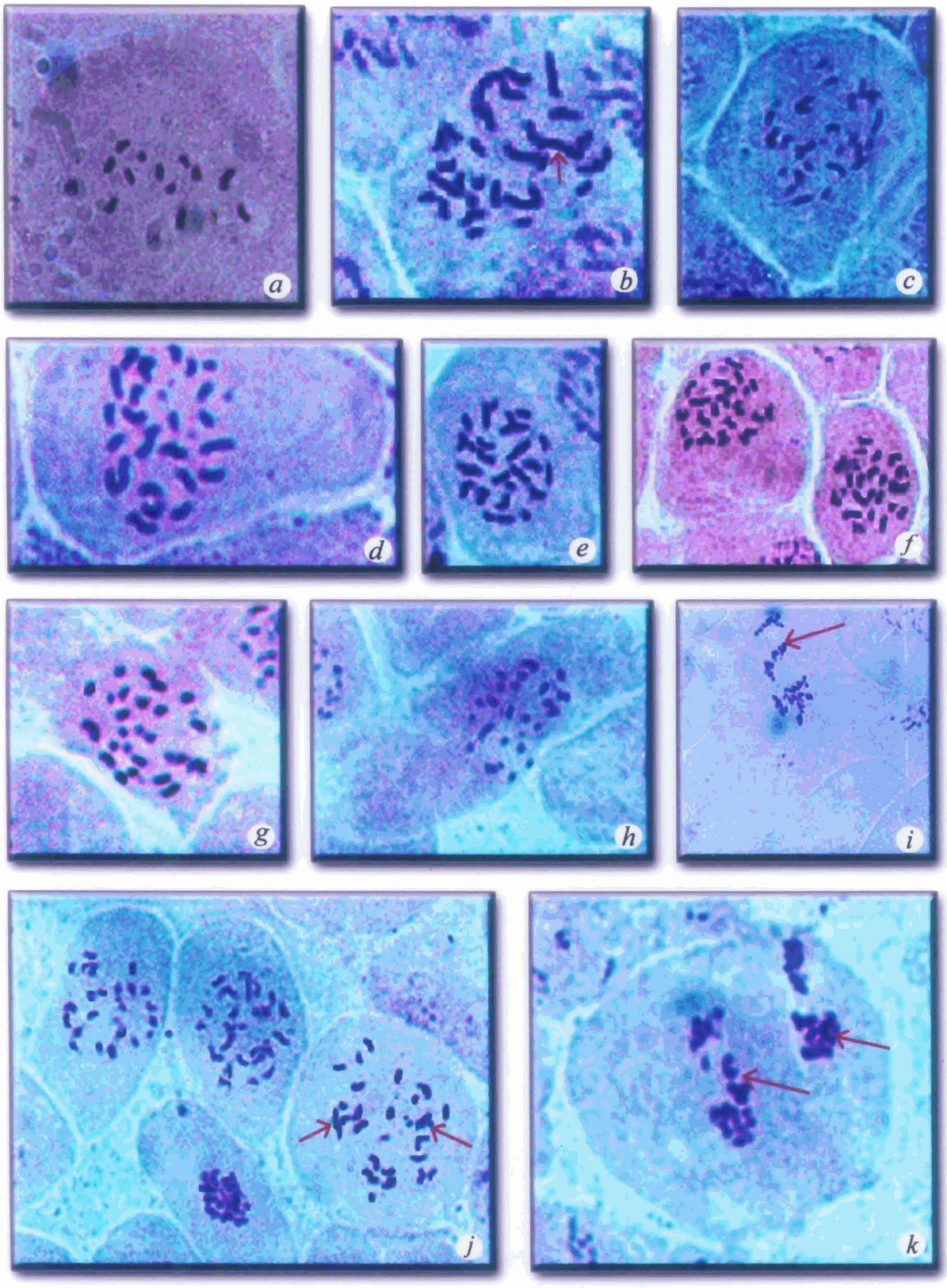


Fig. 23 Numerical changes during mitotis in *Vanilla planifolia*

a. Chromosome associations leading to chromosome number 12 in interspecific hybrid VH4, b-h. Variations in chromosome numbers in *V. planifolia* somaclones due to associations during metaphase, i. Abnormal separation after metaphase in colchicine generated progeny, VC3, j.Variation observed in adjacent cells during metaphase in V10, kAssociations seen after metaphase

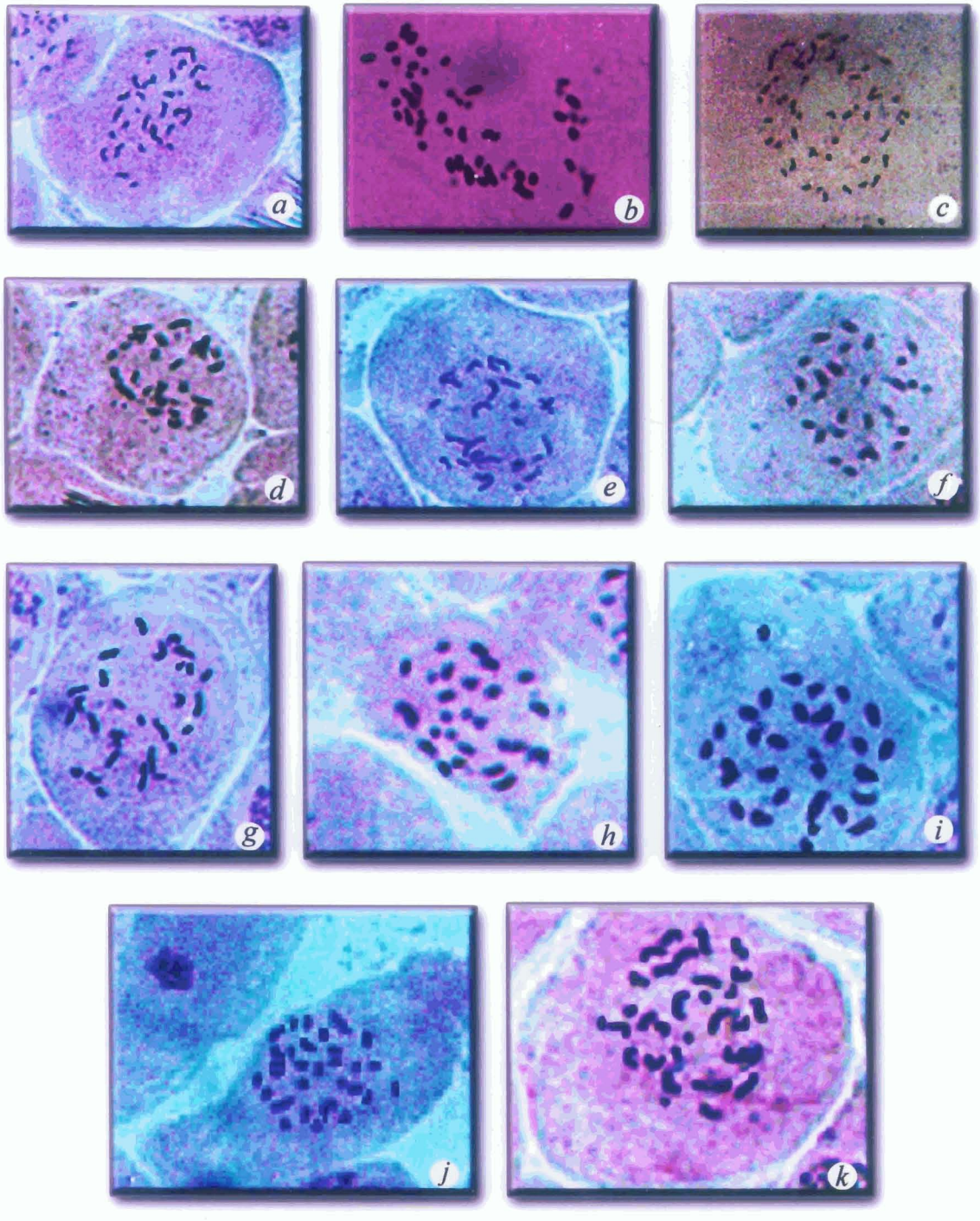


Fig. 24 Mitotis in Vanilla -Cells at metaphase

a. *V.planifolia*, b. *V. aphylla* (2n=64), c. interspecific hybrids, VH5 (2n=50+)
d - k. Normal divisions showing 2n = 30, 32, among the selfed progenies and somaclones,

Table 28 Mitotic behaviour in vanilla (*V.planifolia, V.aphylla* and interspecifc hybrids)

Sl.no	Plant	Metaphase stage (No. of cells)+		Variation observed		Chromosome aberrations observed
		Total	Normal	Chromosome numbers	Frequency (%)	
1.	*V.planilfolia*	110	32 (24)*	22, 24, 26, 28, 30	78.18	Unequal separation, clumping, lagging chromosomes
Seedling progenies						
2	**V10**	140	32 (120)	36, 30	**14.28**	Clumping in 21.4% cells and coiling
3	V18	190	32 (40)	24, 28, 26, 30	78.94	Bridge formation, end to end sticking, tetrapolar segregation, mosaic
4	V32	230	32 (70)	26, 28, 30	69.56	Clumping and sticking
5	V60	120	32 (100)	30	16.67	Unequal tetrapolar segregation and clumping
6	V219	350	32 (60)	30, 24, 28, 26	82.85	End-to end sticking and clumping
Somaclones						
7	V1.R	105	32 (58)	30	44.76	clumping
8	VC 3	43	32 (2)	36, 34, 24	95.34	Tripolar segregation
9	**V8.1**	49	32 (0), 30 (?)	24, 26, 28	**100.00**	Lagging (2%), clumping (2%)
10	VC156.1	30	32 (2)	18, 24, 30	93.0	End-to-end pairing
11	*V.aphylla*	50	64 (7)	60, 58, 54	86.0	Clumping, unequal segregation, end-to-end associations
Interspecific hybrids						
12	VH1	13	32 (5)	28, 30, 32	61.53	Unequal segregation
13	VH4	11	32 (2)	58, 54, 48, 42, 36, 32, 24, 20, 18	81.82	Lagging chromosomes
14	VH5	8	32 (1)	54, 46, 36	87.5	Chromosome clumping, unequal segregation, end to end associations
15	VH6	9	32 (0)	28, 30, 36,48	100	Chromosome clumping

* Figures in parenthesis indicate number of cells observed with 2n = 3

VH 1 chromosome numbers were nearest to *V. planifolia*, however it also showed a variation frequency of 61.53 %. VH 4 and VH5 were morphologically similar to *V.aphylla* with higher chromosome numbers indicating their nearness to *V.aphylla* and its associations indicate the possibility of it being a true hybrid (Fig. 24).

Biochemical characterization of the progenies

Isozyme profiles of superoxide dismutase (SOD) and peroxidase (PRX) were studied in 10 selfed progenies of *V. planifolia*. The profiles (Fig. 25) clearly indicated differences among progenies as expressed by the presence and absence of specific bands. Paired affinity indices were calculated (Table 29) to estimate the amount of variability and it was found that high variability existed among the selfed progenies. The maximum similarity that these progenies exhibited was 47.37% indicating high segregation and heterozygosity existing in *V. planifolia*.

Table 29. Paired affinity indices (% similarity), based on biochemical characterization among selfed progenies of vanilla

	V1	V2	V3	V4	V7	V8	V10	V11	V14	V24
V1	-	28.57	34.70	36.37	40.91	47.37	36.84	39.13	33.33	42.11
V2		-	28.57	45.45	31.82	26.32	31.58	36.09	37.50	36.84
V3			-	30.43	43.48	35.00	25.00	37.50	38.00	35.00
V4				-	37.50	33.00	38.09	32.00	42.31	42.86
V7					-	38.09	38.09	36.00	34.62	42.86
V8						-	33.33	36.36	34.78	38.89
V10							-	31.82	34.78	44.44
V11								-	40.74	36.36
V14									-	34.78
V24										-

Among the progenies studied, it was found that V1 and V8 were the most similar with 47.37% similarity whereas V3 and V10 were the least similar with 25% similarity.

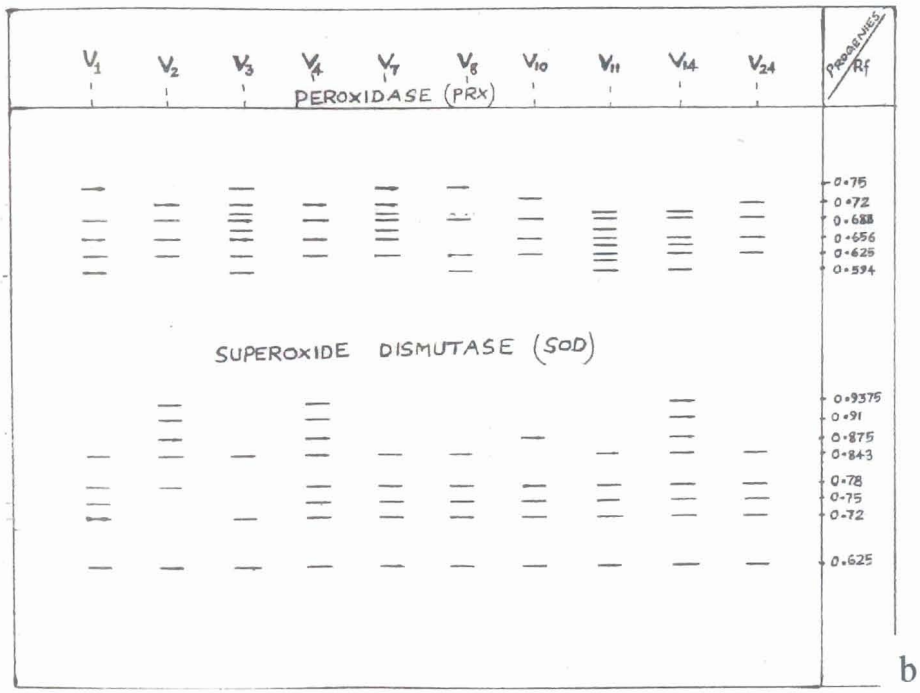


Fig. 25 Biochemical characterisation of seedlings of *V.planifolia*

a. Leaf variations among seedlings

b. Zymogram of Superoxide Dismutase and Peroxidase of seedlings

Molecular characterization

Random Amplified Polymorphic DNA (RAPD)

Isolation and purification of genomic DNA

Using the CTAB method, genomic DNA was successfully isolated from young fresh leaves of *V. planifolia,* its related species, selfed progenies, somaclones, interspecific hybrids. The extracted DNA samples were dissolved in TE buffer and its quality was tested on 0.8% agarose gel. The DNA obtained was of reasonably good quality (Fig. 26). The amount of DNA in the isolated and purified samples was calculated by comparing with standard λDNA marker on agarose gels and are given in Table 30.

Optimization of PCR

RAPD profiles were developed as by Williams *et al.,* (1990) with modifications.

Combinations of Variables

Different combinations and concentrations of dNTPs, Taq polymerase $MgCl_2$ and other variables were tested for good and consistent amplification of genomic DNA. It was found that 30 ng DNA, 1x assay buffer, 150 µM dNTPs, 2.0 mM $MgCl_2$ and 1 U of Taq polymerase are optimal for generating good and consistent amplification products, at annealing temperature of 40 ^{0}C, hence was used for developing RAPD profiles in vanilla. The PCR reaction profile which gave good amplification products is given below:

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

Table 30. DNA concentrations of various species, collections, selfed progenies, callus regenerated, colchicine generated and interspecific hybrids of Vanilla

Sl .no	**Species and collections**	Conc (ng/µl)	Sl. no	**Selfed progenies**	Conc (ng/µl)	Sl. .no	**Selfed progenies**	Conc (ng/µl)	Sl. no	**Callus regenerated**	Conc (ng/µl)	Sl .no	**Cochicine treated**	Conc (ng/µl)	Sl.no	**Hybrids**	Conc (ng/µl)	Sl. no	**Root egenerated**	Conc (ng/µl)
1	*V. planifolia* (variegated)	100	1	V1	200	17	V55	100	1	V8.1	50	1	VC25	50	1	VH1	200	1	V1.1R	50
2	*V, planifolia* IISR	150	2	V2	250	18	V67	50	2	V56.1	100	2	VC40	125	2	VH4	200	2	V2.1R	100
3	*V.planifolia* (Madagascar)	100	3	V4	150	19	V92	50	3	V92.1	100	3	VC65	125	3	VH5	350	3	V7.1R	50
4	*V.planifolia* (Coimb)	100	4	V6	250	20	V112	100	4	V98.1	100	4	VC69	125	4	VH6	200	4	V53.R	150
5	*V.planifolia* (Mauritius)	100	5	V7	250	21	V115	125	5	V101.1	100	5	VC77	100						
6	***V.tahitensis***	75	6	V8	250	22	V120	50	6	V112.1	150	6	VC89	100						
7	***V.pilifera***	200	7	V10	250	23	V124	50	7	V115.1	150	7	VC113	50						
8	***V.aphylla***	250	8	V11	250	24	V150	100	8	V124.1	250	8	VC125	150						
9	*V.andamanica*	150	9	V12	150	25	V156	50	9	V142.1	150	9	VC138	150						
10	*V.andamanica*	100	10	V18	50	26	V179	125	10	V156.1	150	10	VC160	50						
11	*V.andamanica*	100	11	V20	50	27	V221	125	11	V161.1	250	11	VC260	50						
12	*V.andamanica*	200	12	V24	50	28	V223	50	12	V179.1	50	12	VC58.1	125						
13	*V.andamanica*	100	13	V32	50	29	V233	125	13	V258.1	250	13	VC92.1	75						
14	*V.andamanica*	100	14	V44	50	30	V408	125				14	VC145.1	50						
15	*V.andamanica*	150	15	V48	100	31	V422	100				15	VC153.1	75						
16	*V.andamanica*	150	16	V53	100							16	VH5.C	50						
17	New spp.	100																		

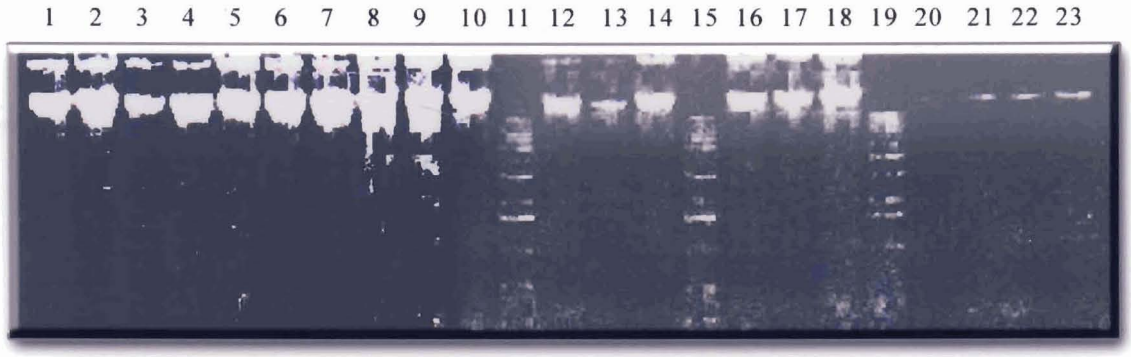


Fig. 26 Genomic DNA isolated from *V.planifolia, V.aphylla* and their progenies
1,V1, V2, V4, V6, V7, V8, V10, V11, V12, V24, 1kb ladder, *V.planifolia, V.aphylla1, V.aphylla2*, VH1, VH4, VH5, 1kb ladder, 10ng/l DNA, 20ng/l DNA, 30ng/l DNA, 50ng/l DNA,

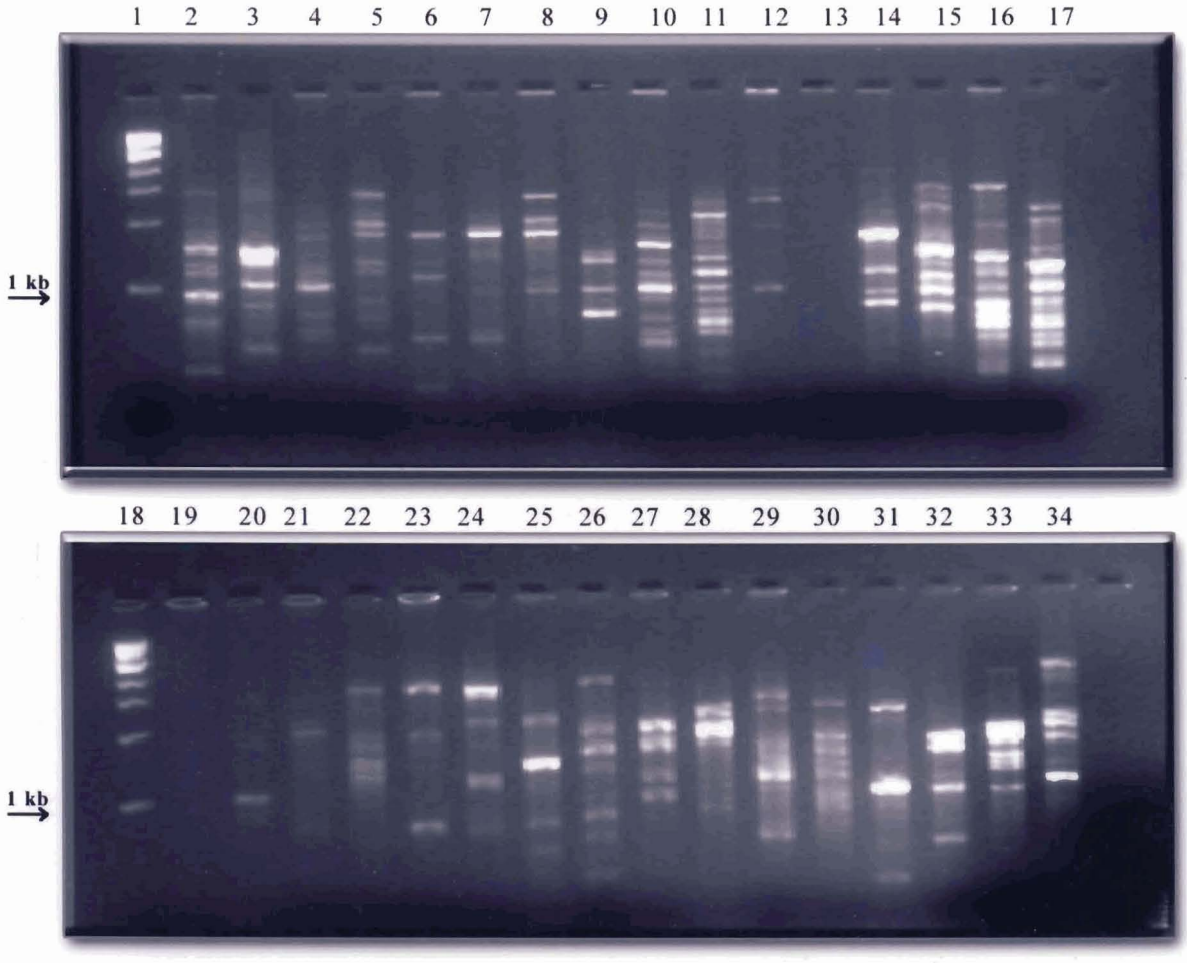


Fig. 27 Screening of various primers for detection of polymorphism in Vanilla
1,17 : 1kb ladder, Lanes : 2, 4,6,8,10,12,14,16,18,2022, 24, 26, 28, 30, 32,34- *V.planifolia,* Lanes : 3,5,7,9,11,13,15,17,19,21,23,25,27,29,31,33 - *V.aphylla,* (2-3)OPA10, (4-5)OPA20, (6-7)OPB02, (8-9)OPB14, (10-11)OPB20, (12-13)OPC09, (14-15)OPC19, (16-17)OPD03, (19-20)OPD19, (21-22)OPE05, (23-24)OPE09, (25-26)OPE14, (27-28)OPF12, (29-30)OPF03, (31-32)OPA04, (33-34)OPB10.

Screening for primers

Sixteen primers were tested for their efficiency in detecting polymorphism between the genotypes. Most of them gave good amplification products (Fig. 27). Only the primers, which gave maximum polymorphism, were used to develop RAPD profiles.

RAPD profiles of different species and collections

RAPD profiles were developed using 9 polymorphic primers viz., OPA 10, OPB 14, OPE 14, OPB 20, OPD 19, OPD 03, OPE 05, OPA 20, OPF 12 in various collections and species of *V.planifolia* (Fig, 28, 29, 30). The genotypes used in this study are five collections of *V,planifolia* from IISR, Madagascar, Coimbatore, one each of *V.tahitensis, V.pilifera, V.andamanica* and a putative new species, 8 collections of *V.andamanica,* 3 interspecific hybrids and a line each of vanilla somaclone(V161 .1) and colchicine treated (V161c).

Of the 9 primers tested, primers OPB14, OPE14, OPD 03 gave excellent amplification products and clear polymorphisms to differentiate this species. The polymorphism observed between the collections and the species as expressed by presence and absence of bands are presented in Table 31.

The primer OPB 14, gave good amplification products in all the genotypes studied (Fig 28b). Clear cut groupings were noticed in the vanilla samples studied. In general all the five collections of *V. planifolia* collected from different regions and *V, tahitensis* exhibited similar patterns. *V. tahitensis* differed only in the presence of one band at 2Kb region. Similar band was also noticed in NBPGR collection of *V. planifolia.* Among the eight collections of *V. andamanica* the collection 3 has a different banding pattern, while the rest of them are exactly

same. A putative new species of *Vanilla* and *V. aphylla* have similar RAPD profiles, but the profiles of *V. pilifera* and *V. aphylla* differed. The four interspecific hybrids displayed banding pattern intermediate to *V. planifolia* and *V. aphylla.* The banding pattern of colchicine treated *V. planifolia* is similar to that of its mother species. The V161.1 showed a banding pattern different from that of *V. planifolia,* but similar that of *V. andamanica.*

The RAPD profiles developed by primer OPE 14 gave indications of clear-cut groupings of the genotypes studied (Fig 28c). All the five collections of *V. planifolia* collected from different regions exhibited similar patterns. This primer could not detect polymorphism between the 8 collections of *V. andamanica* except the collections 2 and 5, which seems to miss a band at 2.1 Kb region. A putative new species of *Vanilla* and *V. aphylla* have similar RAPD profiles, but the profiles of *V. pilifera* and *V. aphylla* differed in the absence of a band at 0.75 kb region. The four interspecific hybrids displayed banding pattern intermediate to *V. planifolia* and *V. aphylla.* The banding pattern of colchicine treated and callus regenerated *V. planifolia* is in general similar to that of *V. planifolia.*

The other primers, OPA 10, OPA 20, OPB 20, OPD 03, OPD 19, OPE 05 and OPF 12, though gave polymorphism between genotypes did not give clear cut groupings.

Paired affinity indices indicating the percentage of similarity between the genotypes studied are given in Table 32. The dendrograms drawn using NTSyS software are given in Fig. 31.

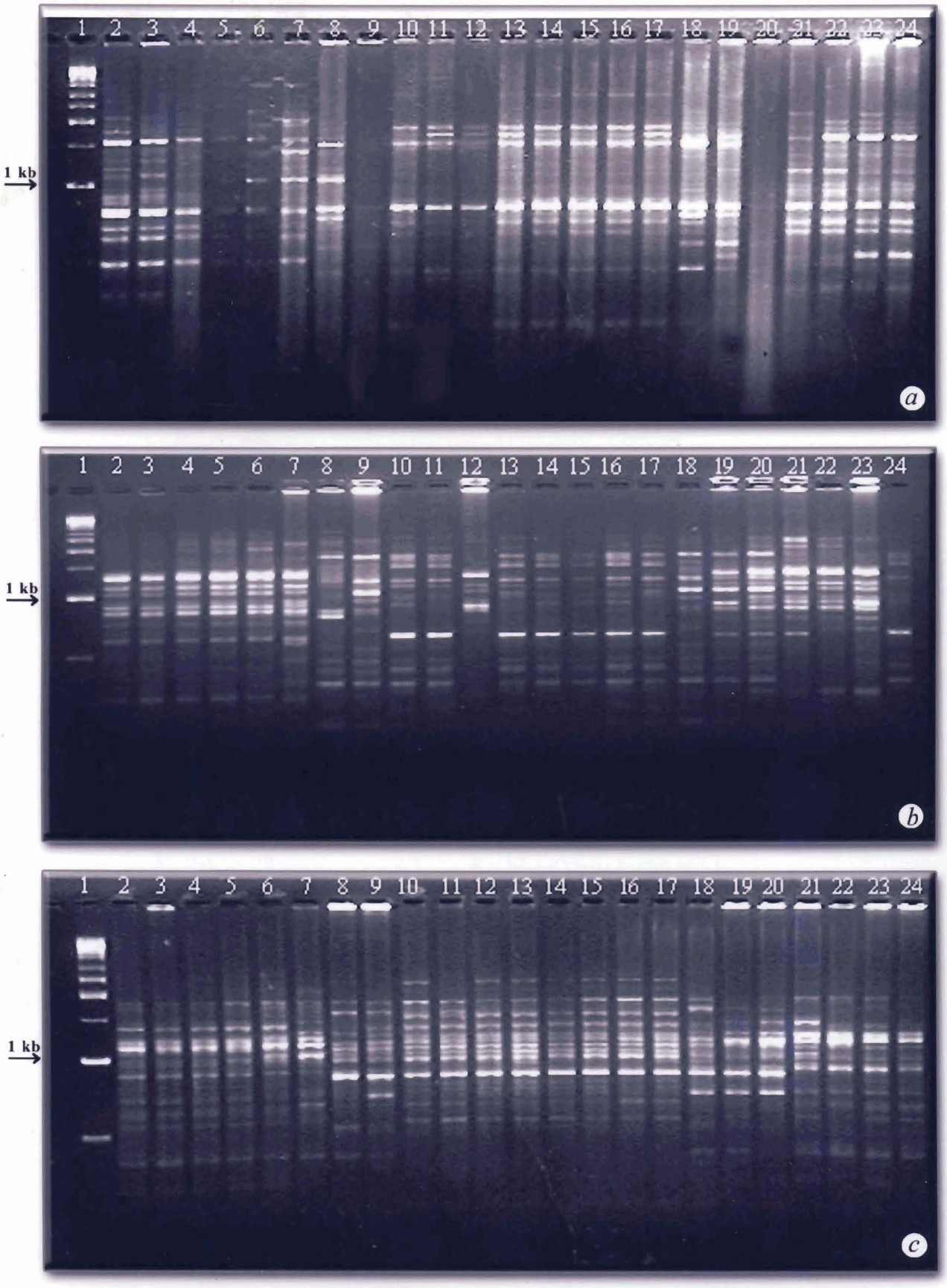


Fig. 28 RAPD profiles of collections, species, selfed progenies and inter specific hybrids of vanilla using OPERON primer a. OPA10, b. OPB14, c. OPE 14

1. 1 kb Ladder, 2.*Vanilla planifolia* variegated 3 : *V. planifolia* IISR, 4 : *V. planifolia* Madagascar, 5. *V. planifolia* IAHS, Coimbatore, 6 : *V. planifolia* NBPGR, 7 : *V. tahItensis*, 8 : *V. pilifera*, 9 : *V. aphylla*,10. *V. andamanica* 1, 11.*V. andamanica* 2, 12.*V. andamanica* 3, 13.*V. andamanica* 4, 14. *V. andamanica* 5, 15. *V. andamanica* 6, 16.*V. andamanica* 17, *V. andamanica* 8, 18. Vanilla spp. (new), 19. Interspecific hybrid (VH1), 20. Interspecific hybrid (VH4), 21. Interspecific hybrid (VH5), 22. Interspecific hybrid (VH6), 23. V161c (*V. planifolia* colchicine treated), 24. V161.1 (*V. planifolia* callus regenerated progeny)

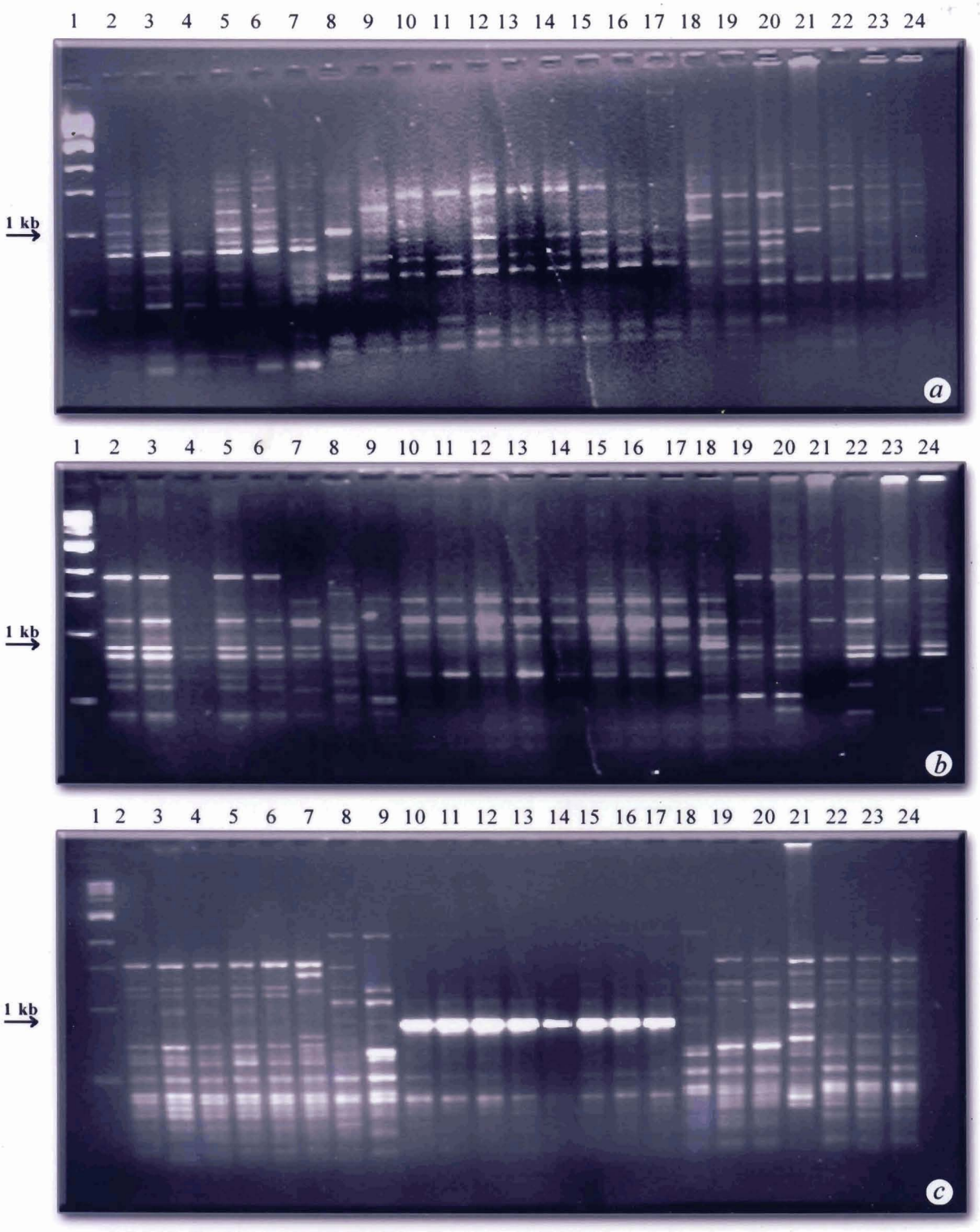


Fig. 29 RAPD profiles of collections, species, selfed progenies and inter specific hybrids of vanilla using OPERON primer a :OPB20, b : OPD19, c : OPD03

1.1 kb Ladder, 2 :*Vanilla planifolia* variegated 3 : *V. planifolia* IISR, 4 : *V. planifolia* Madagascar, 5 : *V. planifoli*a IAHS, Coimbatore, 6 : *V. planifolia* NBPGR, 7 : *V. tahItensis,* 8 : *V. pilifera,* 9 : *V. aphylla,*10 : *V. andamanica* 1, 11.*V. andamanica* 2, 12.*V. andamanica* 3, 13.*V. andamanica* 4, 14. *V. andamanica* 5, 15. *V. andamanica* 6, 16.*V. andamanica* 17, *V. andamanica* 8, 18. Vanilla spp. (new), 19. Interspecific hybrid (VH1), 20. Interspecific hybrid (VH4), 21. Interspecific hybrid (VH5), 22. Interspecific hybrid (VH6), 23. V161c (*V. planifolia* colchicine treated), 24. V161.1 (*V. planifolia* callus regenerated.

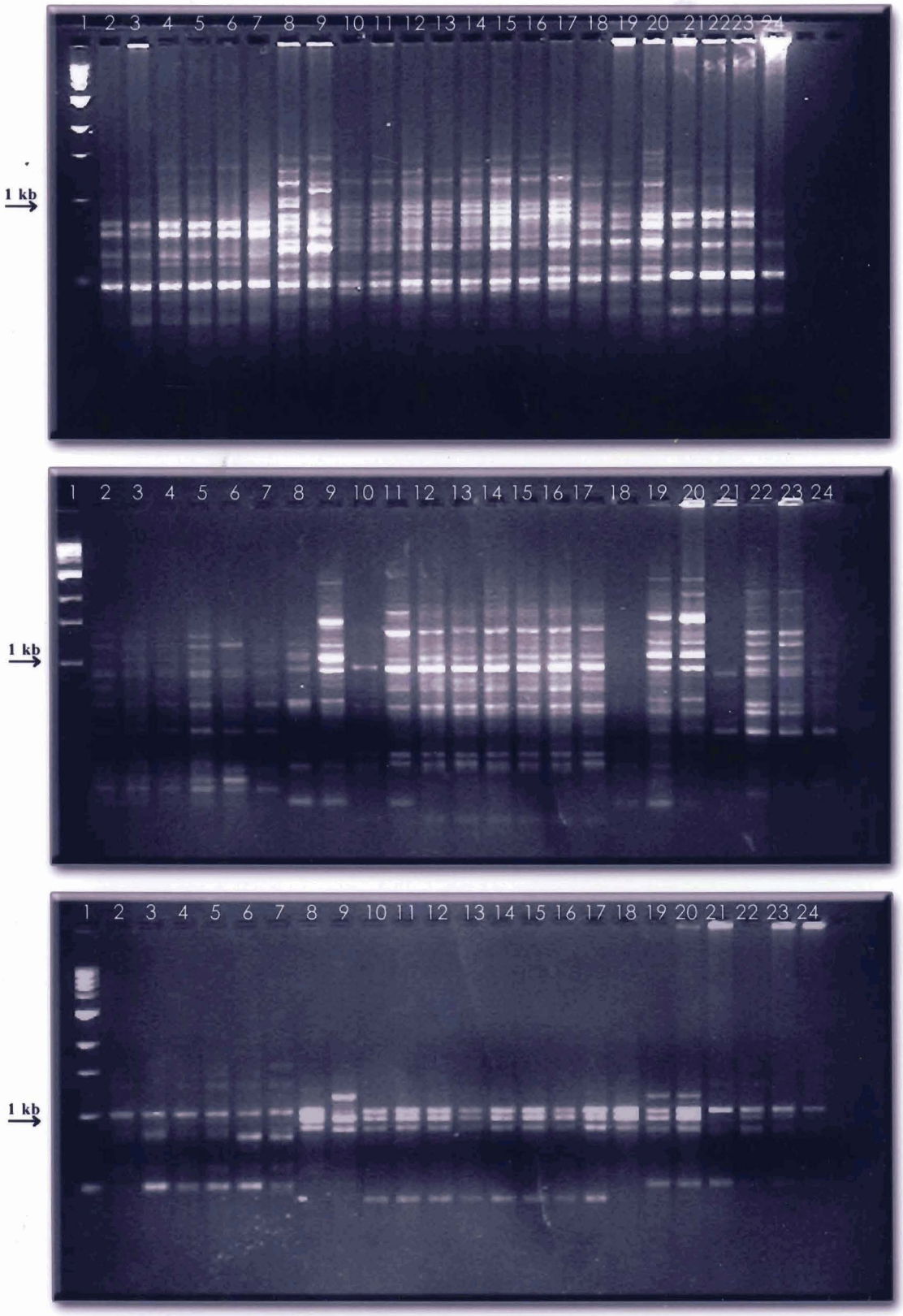


Fig. 30 RAPD profiles of collections, species, selfed progenies and inter specific hybrids of vanilla using OPERON primer a : OPE 5; b : OPA20 ; c : OPF121 kb Ladder, 2 :*Vanilla planifolia* variegated 3 : *V. planifolia* IISR, 4 :V. *planifolia* Madagascar,5 : *V. planifolia* IAHS, Coimbatore,6: *V. planifolia* NBPGR, 7 : *V. tahltensis*, 8 : *V. pilifera*, 9 : *V. aphylla*,10 : *V. andamanica* 1, 11.*V. andamanica* 2, 12.*V. andamanica* 3, 13.*V. andamanica* 4, 14. *V. andamanica* 5, 15. *V. andamanica* 6, 16.*V. andamanica* 7 17, *V. andamanica* 8, 18. *Vanilla* spp. (new), 19. Interspecific hybrid (VH1), 20. Interspecific hybrid (VH4), 21. Interspecific hybrid (VH5), 22. Interspecific hybrid (VH6), 23. V161c (*V. planifolia* colchicine treated), 24. V161.1 (*V. planifolia* callus regenerated

Table 31 : RAPD profile data of collections, species, selfed progenies and interspecific hybrids

No	Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	Size kb
1	OPA10-1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1	2.1
2	OPA10-2	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	2.0
3	OPA10-3	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.6
4	OPA10-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.5
5	OPA10-5	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.4
6	OPA10-6	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1.35
7	OPA10-7	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1.2
8	OPA10-8	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0.9
9	OPA10-9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.8
10	OPA10-10	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0.75
11	OPA10-1'	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0.7
12	OPA10-12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0.6
13	OPA10-13	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0.55
14	OPA10-14	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5
15	OPB14-1	0	0	0	0	0	0	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1.7
16	OPB14-2	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	0	1	1	0	0	0	1.5
17	OPB14-3	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1.4
18	OPB14-4	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1.2
19	OPB14-5	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0	1	0	1	1	1	1	1	0.9
20	OPB14-6	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	1	1	0	0	1	0.6
21	OPE14-1	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1	1	0	0	0	0	0	0	0	2.1
22	OPE14-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1.9
23	OPE14-3	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1.6
24	OPE14-4	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1.45
25	OPE14-5	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1.4
26	OPE14-6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.3
27	OPE14-7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.2
28	OPE14-8	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1.0
29	OPE14-9	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.9
30	OPE14-10	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0.75
31	OPE 05-1	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1.35
32	OPE 05-2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1.3
33	OPE 05-3	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1.0
34	OPE 05-4	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0.95
35	OPE 05-5	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0.9
36	OPE05-6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.8
37	OPE05-7	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0.75
38	OPE05-8	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0.5
39	OPE05-9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.45
40	OPE05-10	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0.4
41	OPA20-1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	1.75
42	OPA20-2	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1.5
43	OPA20-3	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1.4
44	OPA20-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.0
45	OPA20-5	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.9
46	OPA20-6	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0.75
47	OPA20-7	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0.5
48	OPA20-8	0	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.45
49	OPA20-9	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0.4
50	OPA20-10	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0.35
51	OPF12-1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1.25
52	OPF12-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.00
53	OPF12-3	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0.9
54	OPF12-4	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0.8
55	OPF12-5	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.75
56	OPF12-6	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0.5

57	OPF12-7	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0.4
58	OPB20-1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1.5
59	OPB20-2	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1.4
60	OPB20-3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1.0
61	OPB20-4	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0.9
62	OPB20-5	0	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0.8
63	OPB20-6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0.7
64	OPB20-7	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0.5
65	OPB20-8	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0.4
66	OPD19-1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	2.0
67	OPD19-2	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1.5
68	OPD19-3	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1.2
69	OPD19-4	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1.0
70	OPD19-5	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	0.75
71	OPD19-6	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0.7
72	OPD19-7	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0.6
73	OPD19-8	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0.5
74	OPD03-1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1.5
75	OPD03-2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.45
76	OPD03-4	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1.25
77	OPD03-5	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1.10
78	OPD03-6	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1.0
79	OPD03-7	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0.75
80	OPD03-8	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0.7
81	OPD03-9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.5
82	OPD03-10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.4

0 : Band Absent, 1 ; Band Present; Fig..... for details

1-5: Collections of *V.planifolia*; 6: *V.tahitensis*, 7 : *V.pilifera*, 8 : *V.aphylla*, 9-16 : Collections of *V,andamanica*, 17 : new species , 18-20 : Interspecific hybrids, 23 and 24. Somaclones of V.*planifolia*.

The results indicate that among the different species and collections of *Vanilla*, the percentage of similarity ranged from 98.8–42.7.%. Most of the collections of *V. planifolia* are very similar to each other but with minor variations. Among the collections, that from Mauritius, is more divergent from the rest of the group.

The leafless forms of vanilla viz., *V. aphylla, V. pilifera* and the new species are placed in the same cluster. As a species, *V. andamanica* is the most divergent from the rest of the species studied as it formed a separate and unique cluster. There is also reasonable variability within the V. *andamanica* collections indicating the possibility of a natural seed set.

Table 32. Paired affinity indices based on RAPD profiles exhibited by species, collections, selfed progenies and interspecific hybrids of vanilla

	V.pl.varieg	V.pl.IISR	Madagas.col	Coimb.coll	Mauritius	*V.tahitensis*	*V.pilifera*	*V.aphylla*	*V.andaman1*	*V.andaman2*	*V.andaman3*	*V.andaman4*	*V.andaman5*	*V.andaman6*	*V.andaman7*	*V.andaman8*	Newsp	VH1	VH4	VH5	VH6	V161.C	V161.1
V.plvarieg	x																						
V.plIISR	97.5	x																					
Madag	96.3	98.8	x																				
Coimb	97.6	97.6	96.3	x																			
Maurit	91.5	89.0	90.2	91.5	x																		
V.tahit	81.7	84.1	82.9	81.7	85.3	x																	
V.pili	50.0	50.0	51.2	50	56.1	56.1	x																
V.aphylla	53.6	53.6	54.9	56.1	57.3	57.3	76.8	x															
V.and1	42.7	45.1	46.3	42.7	43.9	48.8	61.0	64.6	x														
V.and2	42.7	45.1	46.3	42.7	43.9	48.8	58.5	62.2	95.1	x													
V.and3	43.9	46.3	47.6	43.9	45.1	50.0	62.1	61.0	91.4	91.4	x												
V.and4	43.9	46.3	47.6	43.9	45.1	50.0	57.3	63.4	96.3	96.3	92.7	x											
V.and5	45.1	47.6	48.8	45.1	46.3	51.2	58.5	64.6	95.1	97.6	91.4	98.8	x										
V.and6	45.1	47.6	48.8	45.1	46.3	51.2	56.1	62.2	95.1	95.1	94.0	98.8	97.6	x									
V.and7	43.9	46.3	47.6	43.9	45.1	50.0	57.3	63.4	96.3	96.3	92.7	100	98.8	98.8	x								
V.and8	43.9	46.3	47.6	43.9	45.1	50.0	59.7	63.4	93.9	93.9	92.7	97.6	96.3	96.3	97.6	x							
New sp	61.0	61.0	62.2	63.4	62.2	62.2	71.9	87.8	59.7	57.3	56.1	58.5	59.8	57.3	58.5	58.5	x						
VH1	69.5	69.5	68.3	71.9	65.1	63.4	61.0	69.5	48.7	48.7	45.1	47.6	48.8	46.3	47.6	50.0	76.8	x					
VH4	71.7	72.0	70.7	74.4	70.7	65.8	58.5	76.8	48.7	51.2	45.1	50.0	51.2	48.7	50.0	50.0	74.4	85.4	x				
VH5	84.1	84.1	82.9	84.1	78.0	70.7	51.2	59.7	46.3	46.3	42.7	47.6	48.8	46.3	47.6	47.6	59.8	63.4	70.7	x			
VH6	82.9	80.5	79.3	82.9	76.8	67.1	59.8	63.4	50.0	50.0	51.2	51.2	52.4	50.0	51.2	51.2	65.9	67.1	69.5	81.7	x		
V161.C	86.6	84.1	82.9	86.6	80.5	73.1	60.9	62.2	46.3	46.3	47.6	47.6	48.8	46.3	47.6	47.6	62.2	63.4	68.3	82.9	91.5	x	
V161.1	85.4	83.0	81.7	85.4	79.3	74.4	59.7	61.0	47.6	47.6	46.3	48.8	50.0	47.6	48.8	48.8	61.0	67.1	69.5	81.7	87.8	96.3	x

See Figures 28, 29, 30 for details

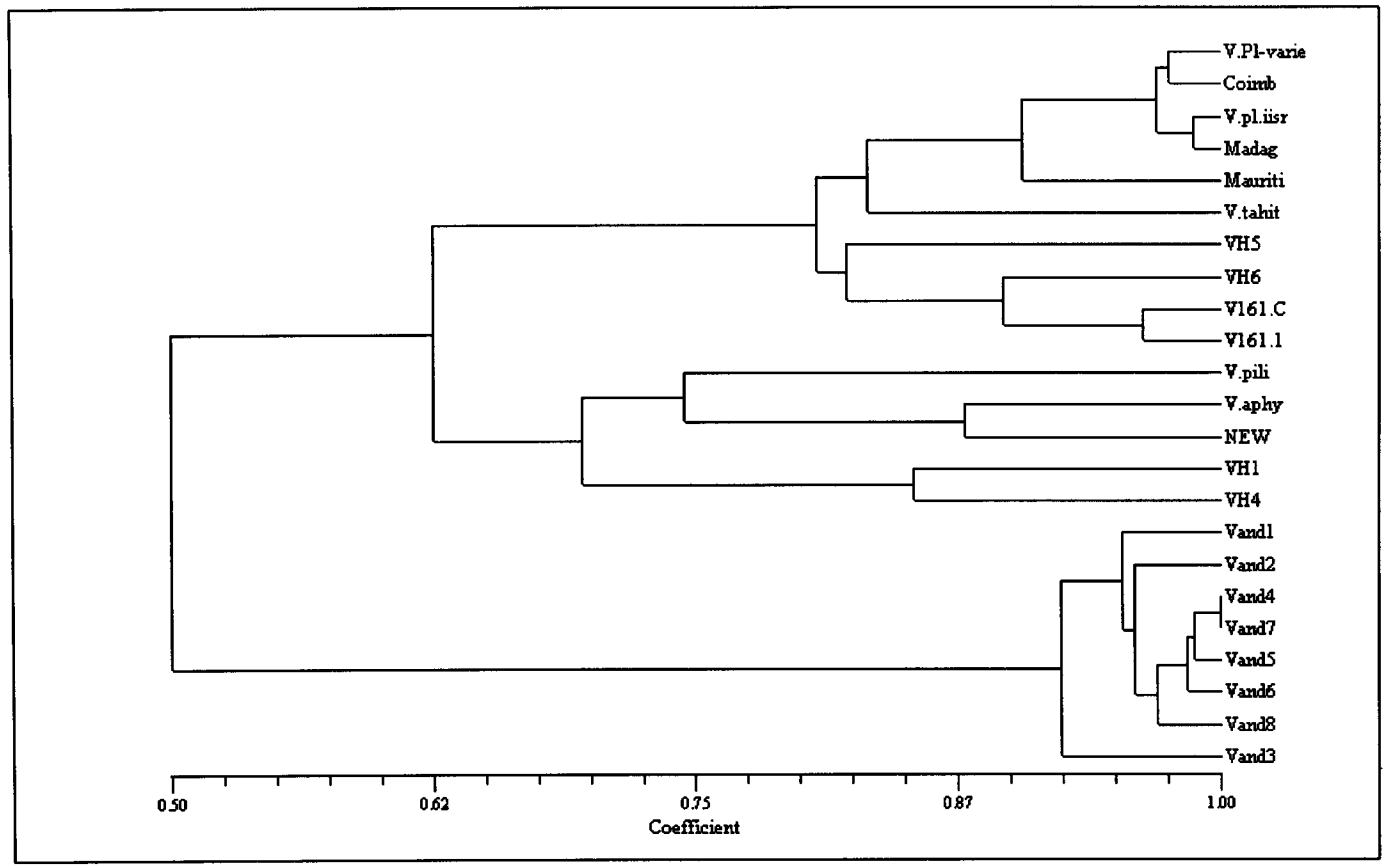


Fig. 31 Dendrogram of divergence in species, collections, interspecific hybrids and somaclones of *Vanilla*

Among the interspecific hybrids, VH4 and VH5 are clustered with *V. aphylla* parents whereas VH1 and VH6 are clustered with *V.planifolia* parent. Among the species studied, *V. tahitensis* is nearest to *V. planifolia* with an average similarity index of 81.7%, while *V. andamanica* is the farthest. Callus regenerated (V161.1) and colchicine treated (V161c) seedling progeny, though very similar to *V. planifolia,* was placed in a slightly different cluster indicating induction of certain amount variability through tissue culture.

RAPD profiles of seedling progenies of Vanilla

RAPD profiles were developed for 23 seedling progenies using 9 operon primers viz., OPA10, OPA 20, OPB 02, OPB14, OPB 20, OPC 19, OPD 03, OPE 09 and OPE 14, to estimate the extent of variability between the seed progenies. All the primers gave good amplified products and polymorphism is observed between the progenies in certain primers. Primers OPC19 and OPE14 are generally monomorphic with minor differences (Fig. 32, 33 and 34).

The polymorphism observed between the seedling progenies is expressed by presence and absence of bands are presented in Table 33. The Paired Affinity Indices were calculated based on Similarity index and presented in Table 34. Dendrograms were drawn based on NTSyS software to study the extent of variability (Fig. 35).

The studies indicate that all the progenies tested were variable when compared to each other.

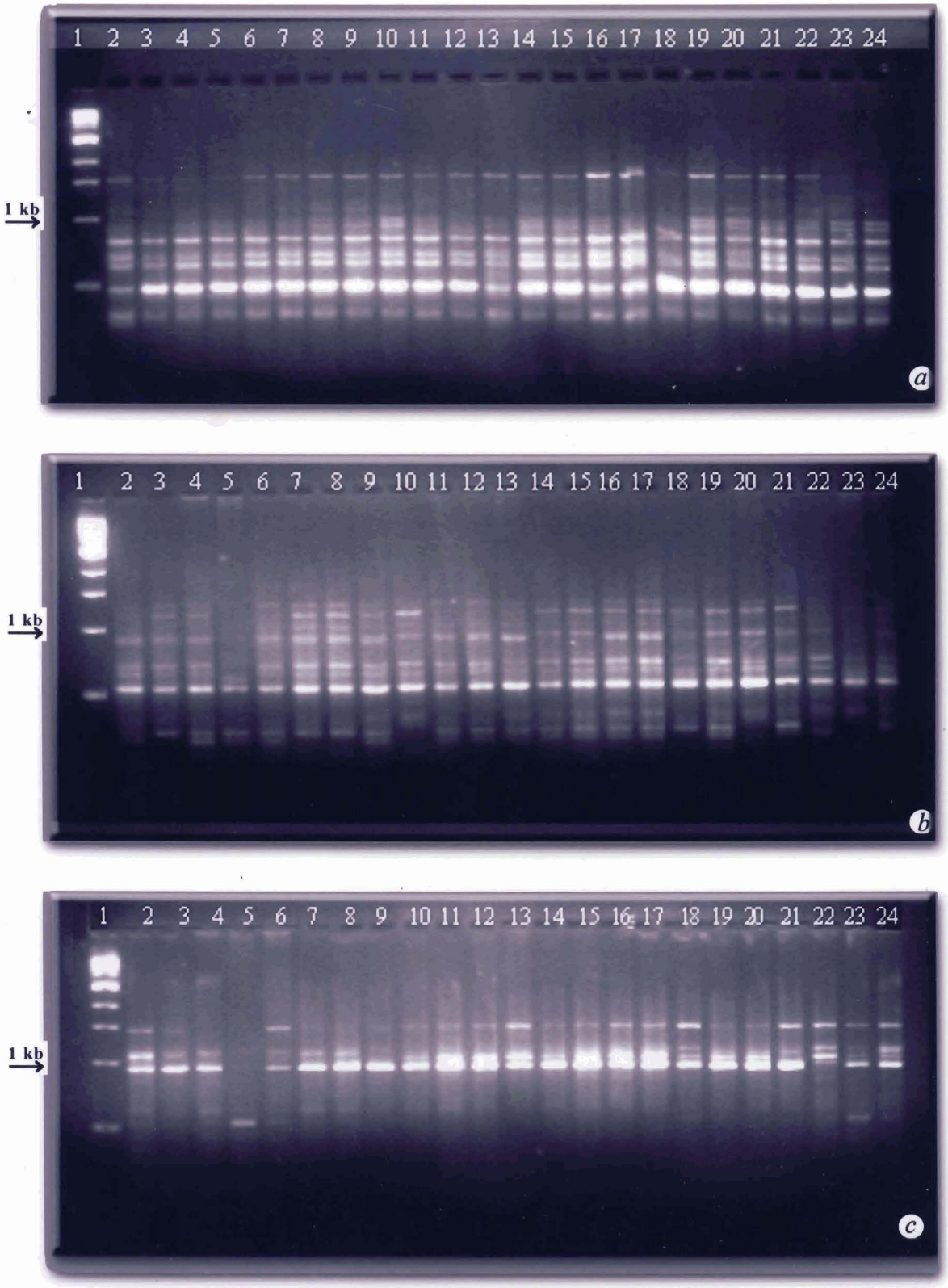


Fig. 32 RAPD profiles of selfed/seedling progenies of vanilla using OPERON primers a. OPA10, b : OPA 20 and c : OPB 14

1 : 1 kb ladder, 2 : *V.planifolia*, 3 : V12, 4 : V18, 5 : V20, 6 : V24, 7 : V32, 8 : V44, 9 : V48, 10 : 53, 11 : V55, 12 : V92, 13 : V112, 14 : V115, 15 : V120, 16 : V124, 17 : V150, 18 : V156; 19 : V179, 20 : V221, 21 : V225, 22 : V233, 23 : V422, 24 : V408

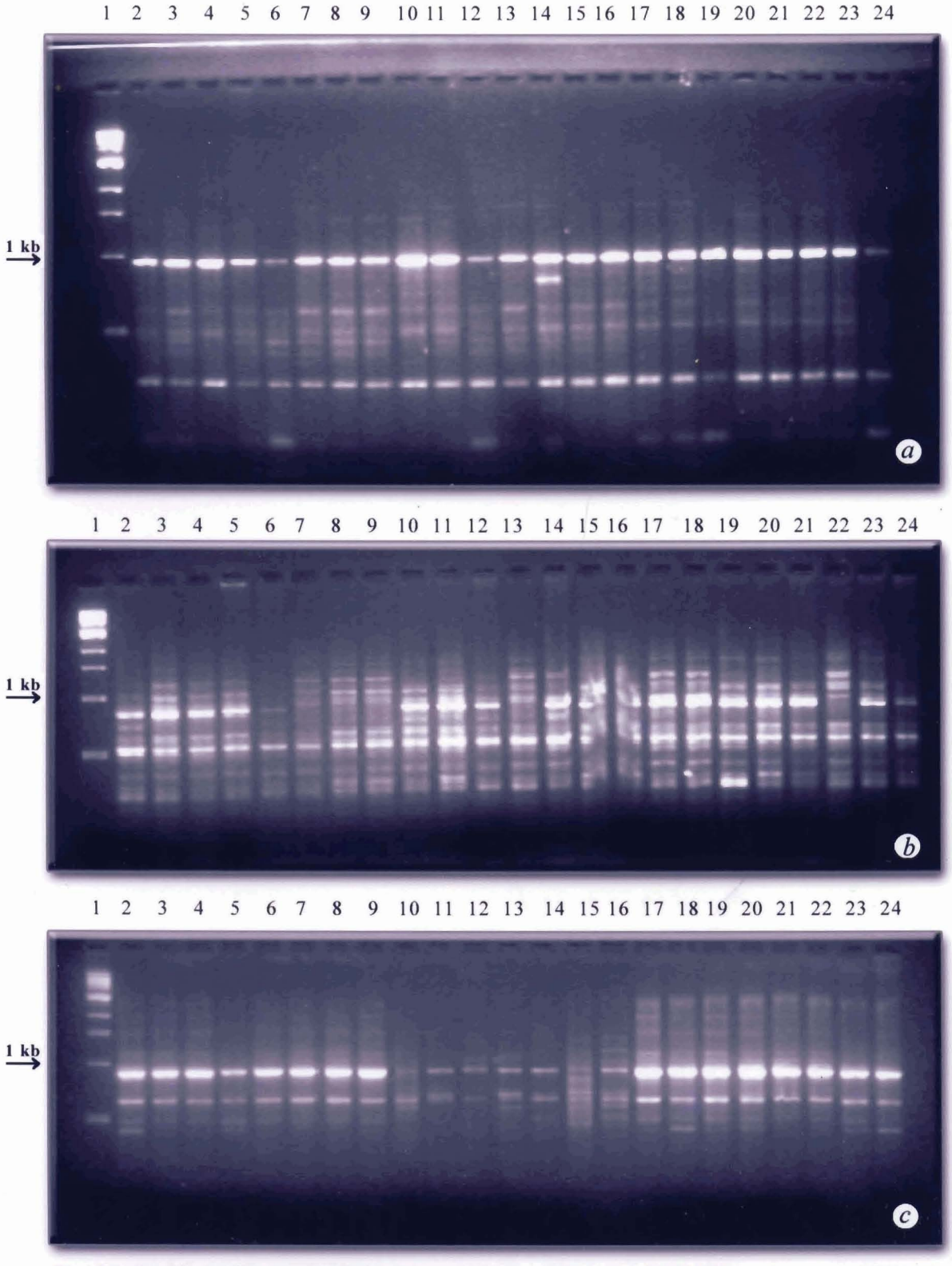


Fig. 33 RAPD profiles of seedling progenies of vanilla using OPERON primers a.OPB0 02, b ; OPB20, c : OPC19

1 : 1 kb ladder, 2 : *V.planifolia*, 3 : V12, 4 : V18, 5 : V20, 6 : V24, 7 : V32, 8 : V44, 9 : V48, 10 : 53, 11 : V55, 12 : V92, 13 : V112, 14 : V115, 15 : V120, 16 : V124, 17 : V150, 18 : V156; 19 : V179, 20 : V221, 21 : V225, 22 : V233, 23 : V422, 24 : V408

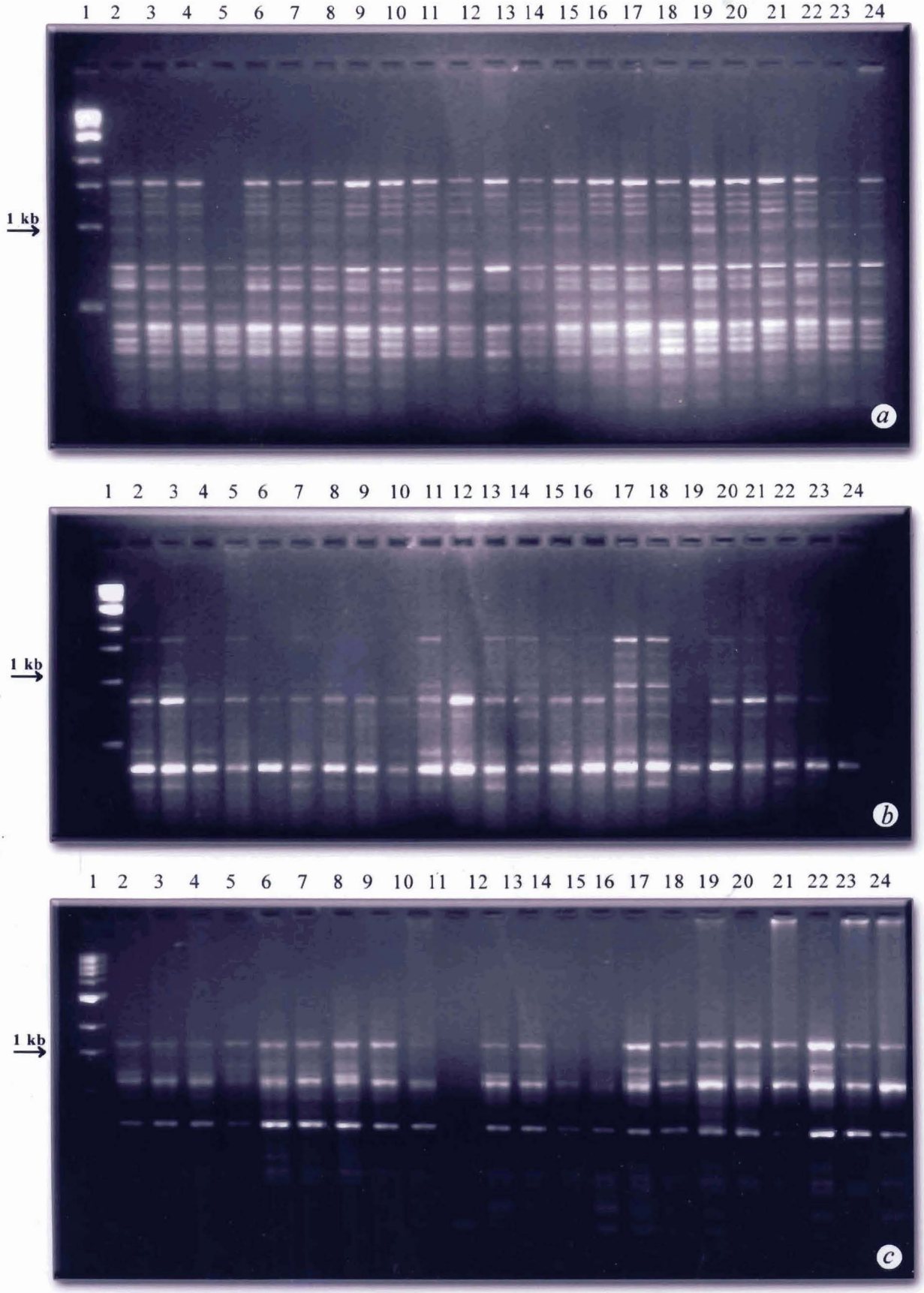


Fig 34 RAPD profiles of seedling progenies of vanilla using OPERON primers a.OPD 03 , b ; OPE09, c : OPE14

1 : 1 kb ladder, 2 : *V.planifolia*, 3 : V12, 4 ; V18, 5 : V20, 6 : V24, 7 : V32, 8 : V44, 9 : V48, 10 : 53, 11 : V55, 12 : V92, 13 : V112, 14 : V115, 15 : V120, 16 : V124, 17 : V150,18 : V156; 19 : V179, 20 : V221, 21 : V225,22 : V233,23 : V422, 24 : V408

Table 33 . RAPD profile data on selfed progenies of *Vanilla planifolia*

No.	Marker	.pl	V12	V18	20	V24	V32	V44	V48	V53	55	V92	V112	V115	V120	V124	V150	V156	V179	V221	V225	V233	V422	V408	Size
1	OPA10-1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1.5
2	OPA10-2	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0.9
3	OPA10-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.8
4	OPA10-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.7
5	OPA10-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.6
6	OPA10-6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.5
7	OPA10-7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.4
8	OPA20-1	0	0	1	0	0	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	0	1.3
9	OPA20-2	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1.0
10	OPA20-3	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0.6
11	OPA20-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.55
12	OPA20-5	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0.3
13	OPA20-6	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	1	0	1	0	0	1	0	0	0.25
14	OPA14-1	1	0	0	0	1	0	0	0	0	1	1	1	0	1	1	1	1	0	0	1	1	1	1	1.5
15	OPA14-2	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1.1
16	OPA14-3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	1	1.0
17	OPA14-4	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0.9
18	OPA14-5	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0.5
19	OPB02-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.0
20	OPB02-2	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	0	0.95
21	OPB02-3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0.8
22	OPB02-4	0	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	1	0	1	1	1	1	0	0.6
23	OPB02-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.3
24	OPB20-1	1	1	0	1	0	1	1	1	0	0	1	1	1	0	0	1	1	0	0	0	1	0	0	1.25
25	OPB20-2	0	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1.0
26	OPB20-3	1	1	1	1	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	0.9
27	OPB20-4	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0.6
28	OPB20-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.5
29	OPB20-6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.4
30	OPB20-7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.3
31	OPB20-8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.2
32	OPC19-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.9
33	OPC19-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.65
34	OPD03-1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1.5
35	OPD03-2	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.4
36	OPD03-3	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.3
37	OPD03-4	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1.2
38	OPD03-5	1	1	1	0	0	1	1	0	1	0	0	1	1	1	0	1	0	1	1	1	1	1	1	1.0
39	OPD03-6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.75
40	OPD03-7	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0.6
41	OPD03-8	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0.5
42	OPD03-9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.4
43	OPD03-10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.35
44	OPD03-11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.3
45	OPD03-12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.25
46	OPE09-1	1	1	0	1	0	0	0	0	0	1	0	1	1	0	0	1	1	0	1	1	1	0	0	1.6
47	OPE09-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1.5
48	OPE09-2	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	1.0
49	OPE09-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0.9
50	OPE09-4	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.45
51	OPE09-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.4
52	OPE09-6	1	1	0	0	0	1	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0.35
53	OPE14-1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1.1
54	OPE14-2	1	0	0	0	1	1	1	0	0	0	1	1	0	0	1	1	1	0	0	1	1	0	1	0.65
55	OPE14-3	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1	1	0.6
56	OPE14-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.3

0 : Band Absent, 1 ; Band Present;V.pl : *V. planifolia*, V12-V408 : Seedling progenies of *V. planifolia*

Table 34. Paired affinity of seedling progenies of *V. planifolia*

	V.pl	V12	V18	V20	V24	V32	V44	V48	V53	V55	V92	V112	V115	V120	V124	V150	V156	V179	V221	V225	V233	V422	V408
V.pl	x																						
V12	85.7	x																					
V18	83.9	87.5	z																				
V20	67.8	67.8	69.6	x																			
V24	83.9	73.2	82.1	66.1	x																		
V32	87.5	83.9	89.3	62.5	85.7	x																	
V44	85.7	85.7	94.6	67.8	83.9	94.6	x																
V48	83.9	87.5	89.3	69.6	85.7	89.3	91.1	x															
V53	78.6	85.7	94.6	64.3	76.8	83.9	89.3	83.9	x														
V55	82.1	78.6	80.3	71.4	76.8	69.6	75.0	76.8	78.6	x													
V92	85.7	78.6	80.3	71.4	83.9	76.8	82.1	80.3	75.0	89.2	x												
V112	87.5	80.3	78.5	69.6	75.0	82.1	83.9	82.1	73.2	80.3	83.9	x											
V115	83.9	80.3	85.7	66.1	71.4	82.1	83.9	78.6	87.5	80.3	76.8	78.6	x										
V120	80.3	76.8	89.3	62.5	75.0	78.6	83.9	78.6	91.1	83.9	80.3	78.6	89.3	x									
V124	83.9	73.2	85.7	62.5	82.1	82.1	83.9	78.6	83.9	80.3	83.9	78.6	85.7	92.8	x								
V150	85.7	78.6	76.8	64.3	69.6	76.8	78.6	73.2	75.0	82.1	82.1	80.3	80.3	83.9	83.9	x							
V156	85.7	82.1	80.3	64.3	73.2	80.3	82.1	80.3	75.0	78.6	82.1	80.3	76.8	80.3	83.9	89.3	x						
V179	76.8	76.8	89.3	69.6	78.6	78.6	83.9	78.6	87.5	76.8	80.3	71.4	82.1	89.3	85.71	80.3	73.2	x					
V221	78.6	85.7	91.1	67.8	73.9	80.3	85.7	80.3	92.8	76.8	71.4	73.2	83.9	87.5	83.9	82.1	78.6	87.5	x				
V225	85.7	82.1	91.1	67.8	83.9	83.9	89.2	80.3	89.3	78.6	82.4	80.3	83.9	87.5	87.5	82.1	82.1	87.5	89.2	x			
V233	78.6	78.6	768	67.8	76.8	76.8	82.1	80.3	78.6	75.0	75.0	80.3	76.8	80.3	80.3	82.1	75.0	76.8	85.7	82.1	x		
V422	71.4	78.6	80.3	71.4	73.2	73.2	75.0	73.2	82.1	67.8	67.8	69.6	69.6	76.8	73.2	64.3	67.8	73.2	78.6	78.6	71.4	x	
V408	80.3	73.2	78.6	66.1	82.1	75.0	76.8	71.4	76.8	69.6	76.8	71.4	71.4	78.6	82.1	73.2	69.6	85.7	76.8	83.9	73.2	80.3	x

V.pl : *V.planifolia*; V12 – V408 : Seedling progeneis

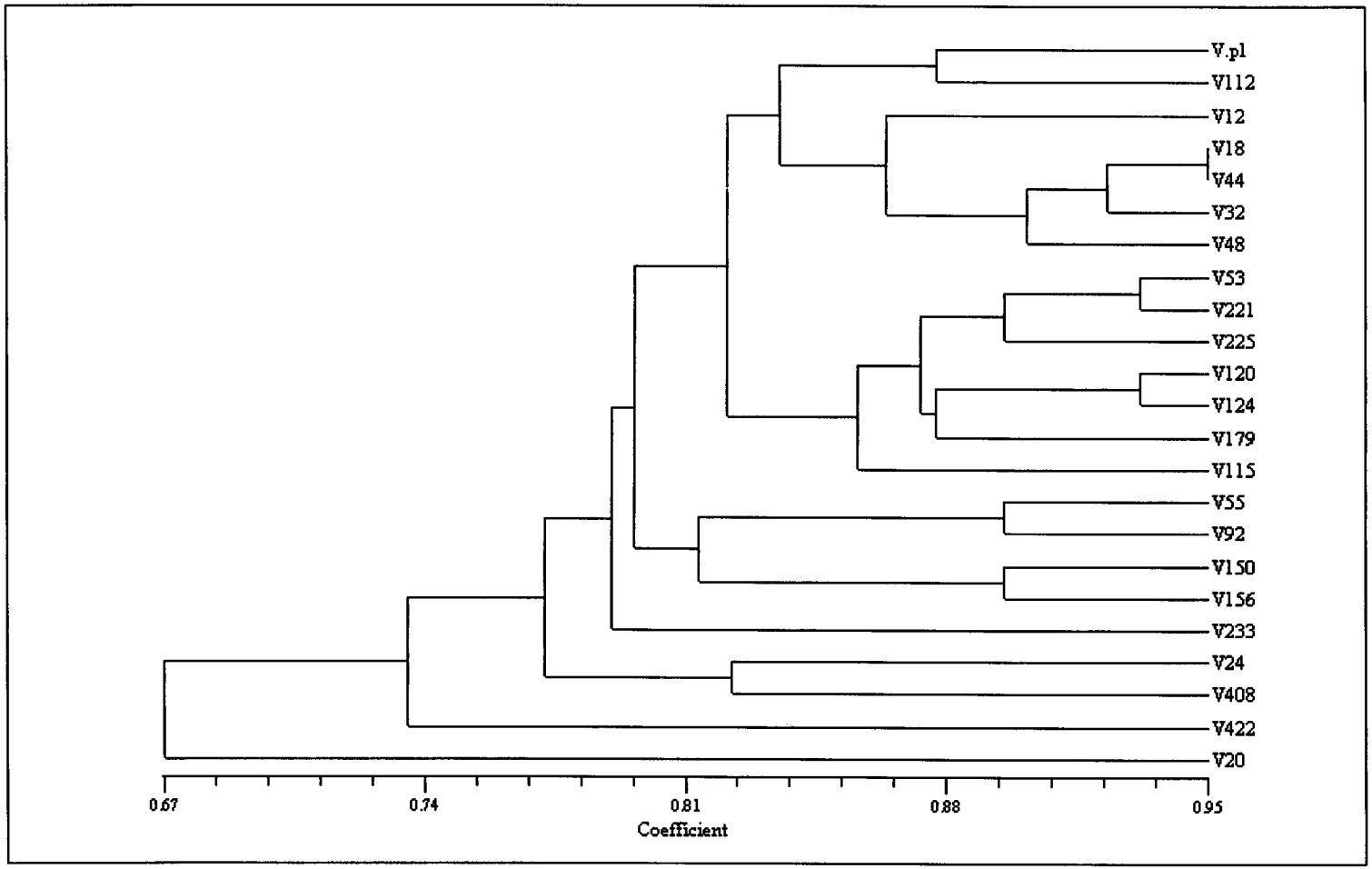


Fig. 35 Dendrogram of divergence of seedling progenies of *Vanilla planifolia*

The Paired Affinity Index indicated that the similarity of *V. planifolia* and its progenies ranged from 94.6 to 62.5%. V112 is the closest to *V. planifolia* while V20 is the farthest. V18 and V44 are exactly similar to each other. Thus the present study indicates highly variability among the seedling progenies.

RAPD profiles of callus and root regenerated progenies of Vanilla

RAPD profiles were developed for 23 callus and root regenerated progenies by 9 operon primers viz., OPA 20, OPB14, OPE 09, OPB 20, OPD 03, OPF12, OPA10, OPD19 and OPC19, to estimate the extent of somaclonal variation induced in callus and root regenerated progenies. All the primers gave good amplified products but OPB20, OPA10 and OPD03 and OPB20 gave good polymorphism (Fig. 36,37 and 38). The other profiles are generally monomorphic.

The polymorphism observed between the somaclones are presented in Table 35. The Paired Affinity Indices were calculated based on Similarity index and presented in Table 36. Dendrograms were drawn based on NTSyS software to study the extent of variability (Fig.39).

Studies indicated that all the somaclones tested are variable when compared to each other. The Paired Affinity Index ranged from 97.5 to 52.5%. The dendrogram of divergence has indicated that all the somaclones tested are variable when compared to each other and the variability in PAI between them is indicative of somaclonal variation as the possible cause for this variation.

Plants regenerated from root were variable in their RAPD profiles, indicating that conversion of conversion of root meristem to shoots may also induce variations in vanilla and differs from earlier understanding that the roots are highly stable and the plants cloned from roots are also genetically uniform.

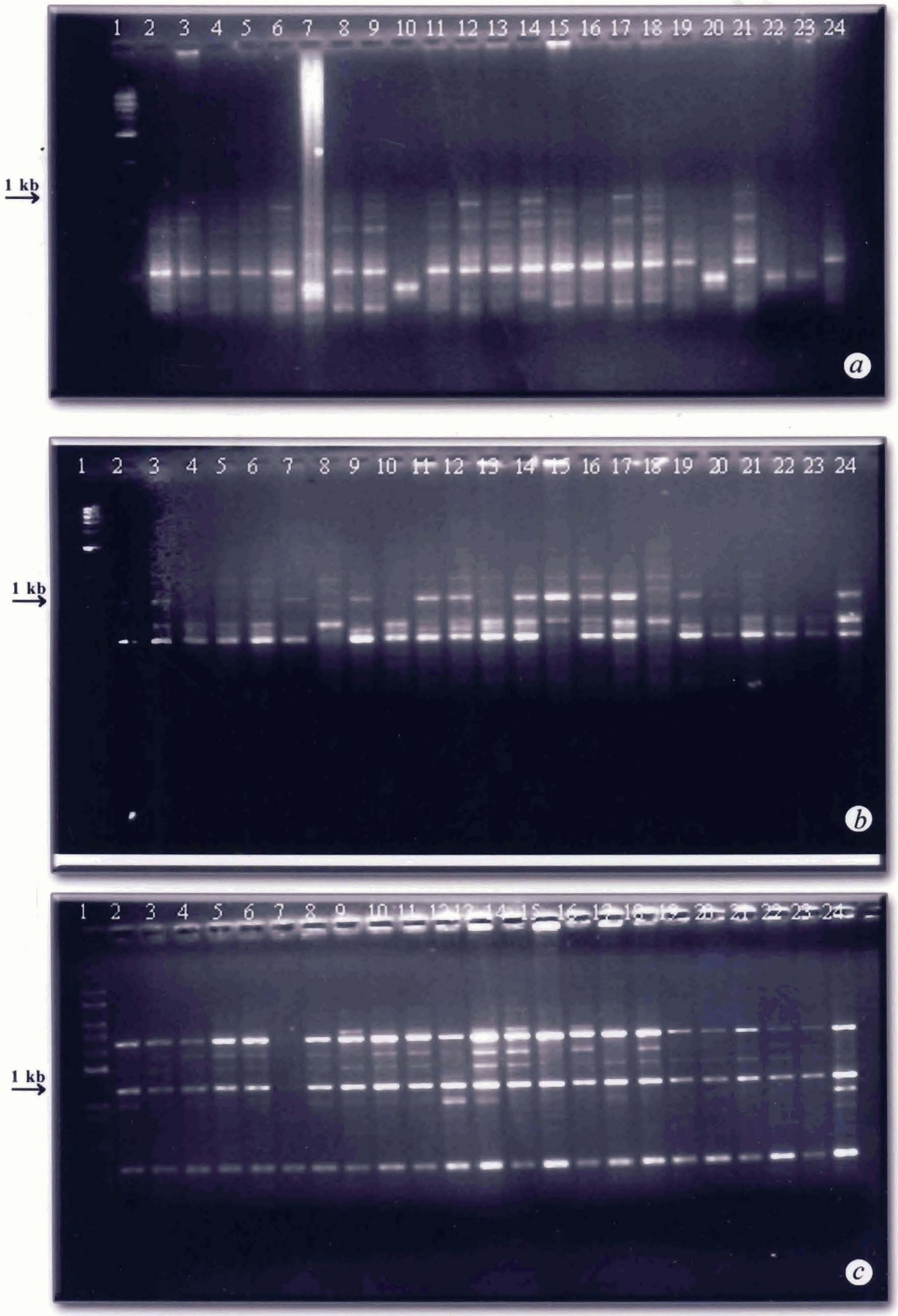


Fig.36 RAPD profiles of callus regenerated (2-18) and root regenerated (19-23) progenies of vanilla using OPERON primers
a : OPA 20; b : OPB 14; c : OPE 09
1 . 1 kb ladder 2 :V8.1; 3 : V56.1; 4 : V92; 5 : V92.1; 6 : V98.1; 7 : V101; 8 : V101.1; 9 : V112; 10: V112.1; 11 ; 115.1; 12 : V124.1; 13 : V142.1; 14 : V156.1; 15 ; V161.1; 16 : V179; 17 : V179.1; 18 : V258.1; 19 : V1.1R; 20 : V2R; 21 : V7.1; 22 : V7.1R; 23 : V53R ; 24: V.planifolia (Control)

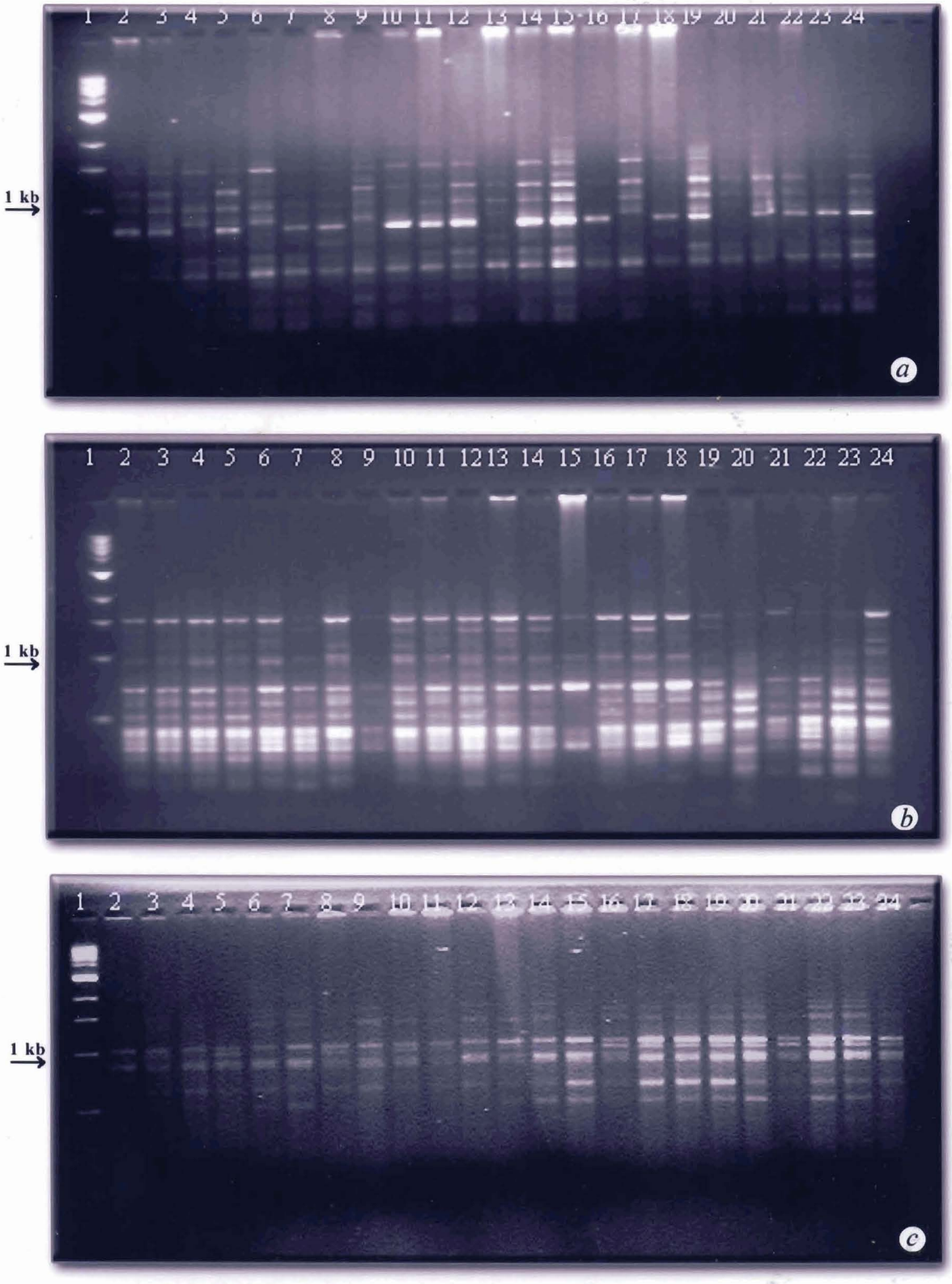


Fig. 37 RAPD profiles of callus regenerated (2-18) and root regenerated (19-23) progenies of vanilla using OPERON primers. a : OPB 20; b : OPD 03, c : OPF 12

1 . 1 kb ladder 2 :V8.1; 3 : V56.1; 4 : V92; 5 : V92.1; 6 : V98.1; 7 : V101; 8 : V101.1; 9 : V112; 10: V112.1; 11 ; 115.1; 12 : V124.1; 13 : V142.1; 14 : V156.1; 15 ; V161.1; 16 : V179; 17 : V179.1; 18 : V258.1; 19 : V1.1R; 20 : V2R; 21 : V7.1; 22 : V7.1R; 23 : V53R ;24: *V.planifolia* (Control)

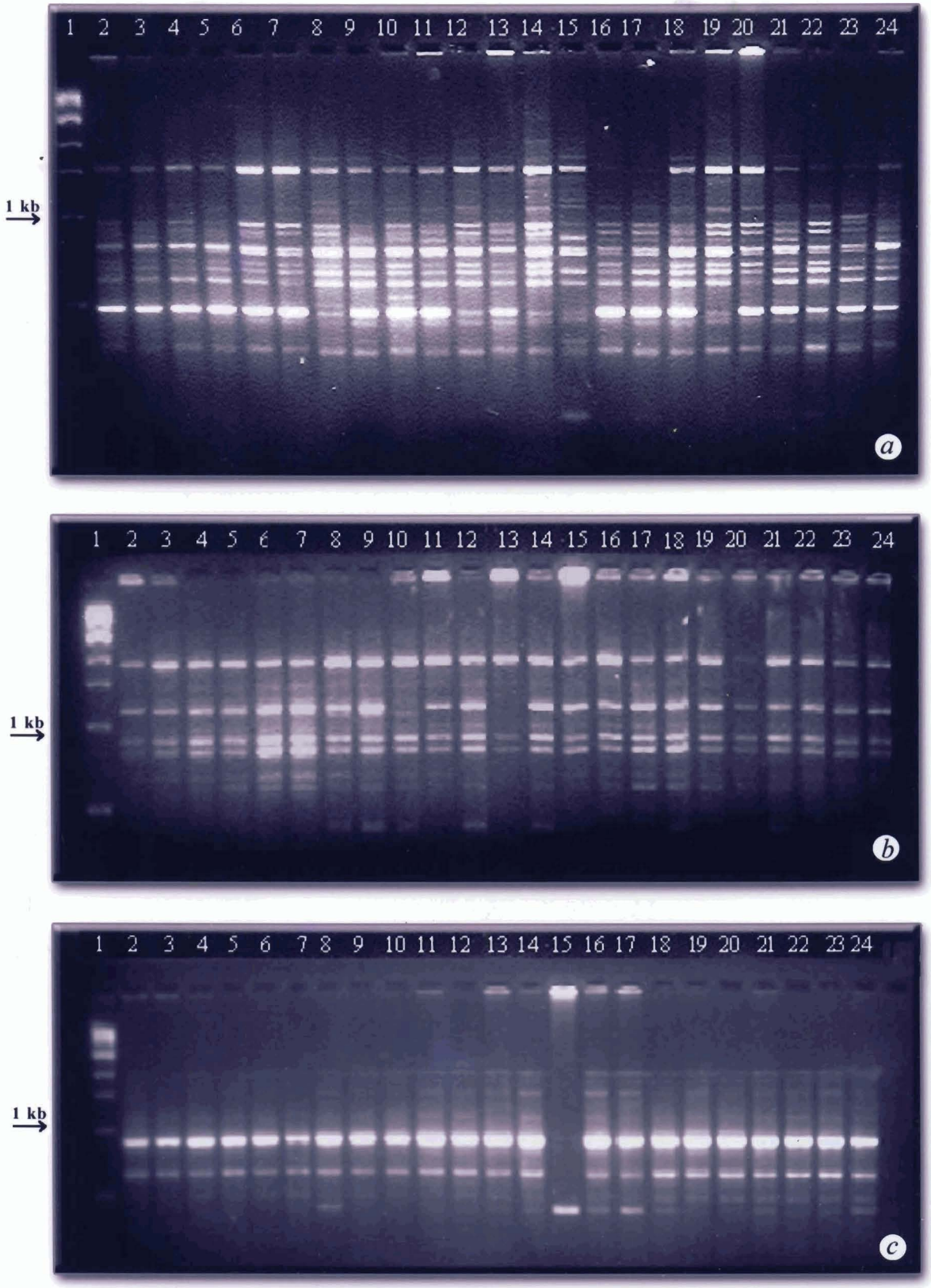


Fig 38 RAPD profiles of callus regenerated (2-18) and root regenerated (19-23) progenies of vanilla using OPERON primers a : OPA10, b : OPD 19, c : OPC 19 1 . 1 kb ladder 2 :V8.1; 3 : V56.1; 4 : V92; 5 : V92.1; 6 : V98.1; 7 : V101; 8 : V101.1; 9 : V112; 10: V112.1; 11 ; 115.1; 12 : V124.1; 13 : V142.1; 14 : V156.1; 15 ; V161.1; 16 : V179; 17 : V179.1; 18 : V258.1; 19 : V1.1R; 20 : V2R; 21 : V7.1; 22 : V7.1R; 23 : V53R ;24: V.planifolia (Control)

Table 35 . RAPD profile data of callus and root regenerated progenies of vanilla

No	Primer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	size
1	OPA20-1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0.6
2	OPA20-2	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0.5
3	OPA20-3	1	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0	1	0	0	0	0.4
4	OPB14-1	0	0	0	0	0	1	0	1	0	1	1	0	1	1	0	1	0	1	0	0	0	0	1	1.0
5	OPB14-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.9
6	OPB14-3	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	0	1	0	0	0	0	1	0.7
7	OPB14-4	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0.65
8	OPE09-1	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1.5
9	OPE09-2	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.45
10	OPE09-3	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	0	1	0	0	0	0	0	0	1.3
11	OPE09-4	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	1	1	1	1	1	1	0	0	1.2
12	OPE09-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.0
13	OPE09-6	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0.9
14	OPE09-7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.5
15	OPB20-1	0	1	1	0	1	0	0	1	1	1	1	0	1	1	0	1	0	1	0	0	0	0	0	1.5
16	OPB20-2	0	1	0	1	0	0	0	1	1	1	1	0	1	1	0	1	0	1	0	1	1	0	1	1.25
17	OPB20-3	1	1	1	1	1	0	0	1	0	1	1	1	1	1	0	1	0	1	0	1	0	0	0	1.0
18	OPB20-4	1	1	0	1	0	1	1	0	1	1	1	0	1	1	1	0	1	1	0	1	1	1	1	0.9
19	OPB20-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.5
20	OPD03-1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1	1	1	1	0	0	0	1	1	1.5
21	OPD03-2	0	0	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1.0
22	OPD03-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0.7
23	OPD03-4	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0.5
24	OPD03-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0.4
25	OPF12-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.0
26	OPF12-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.8
27	OPF12-3	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1	1	1	0	1	0	0	0.7
28	OPA10-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	1.5
29	OPA10-2	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1.0
30	OPA10-3	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0.8
31	OPA10-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.7
32	OPA10-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.5
33	OPA10-6	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	0	0.4
34	OPA10-7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.3
35	OPD19-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	2.0
36	OPD19-2	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1.1
37	OPD19-3	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.8
38	OPC19-1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1.0
39	OPC19-2	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0.7
40	OPC19-3	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1	0.5

0 : Band Absent, 1 ; Band Present;

Lanes 1-17 (V8.1 – V258.1) : Callus regenerated progenies, Lanes 18-22 (V1.1R - V53.1R) : Root regenerated progenies, 23 (V.pl) : *V.planifolia*

Table 36. Paired Affinity Index of callus and root regenerated progenies of *V.planifolia*

	V8.1	V56.1	V92	V92.1	V98.1	V101	V101.1	V112	V112.1	V115	V124.1	V142.1	V156.1	V161.1	V179	V179.1	V258.1	V1.1R	V2.1R	V7.1	7.1R	53.1R	V.pl
V8.1	x																						
V56.1	90.0	x																					
V92	90.0	90.0	x																				
V92.1	92.5	92.5	87.5	x																			
V98.1	85.0	85.0	95.0	87.5	x																		
V101	77.5	72.5	72.5	75.0	72.5	x																	
V101.1	75.0	70.0	75.0	77.5	80.0	77.5	x																
V112	77.5	82.5	82.5	80.0	77.5	70.0	57.5	x															
V112.1	70.0	80.0	75.0	77.5	80.0	67.5	70.0	72.5	x														
V115	80.0	85.0	85.0	87.5	90.0	72.5	75.0	82.5	85.0	x													
V124.1	75.0	80.0	80.0	77.5	80.0	72.5	70.0	77.5	75.0	90.0	x												
V142.1	77.5	72.5	82.5	80.0	87.5	65.0	72.5	80.0	77.5	82.5	77.5	x											
V156.1	70.0	80.0	75.0	77.5	80.0	62.5	65.0	82.5	85.0	90.0	85.0	82.5	-										
V161.1	60.0	65.0	65.0	62.5	65.0	62.5	65.0	67.5	60.0	75.0	75.0	57.5	70.0	-									
V179	85.0	80.0	85.0	87.5	90.0	77.5	85.0	67.5	80.0	85.0	75.0	82.5	75.0	60.0	-								
V179.1	65.0	70.0	75.0	67.5	75.0	57.5	60.0	77.5	70.0	80.0	85.0	77.5	80.0	75.0	70.0	-							
V258.1	75.0	70.0	75.0	77.5	80.0	72.5	85.0	67.5	80.0	80.0	75.0	82.5	80.0	70.0	85.0	70.0	-						
V1.1R	80.0	85.0	80.0	82.5	80.0	72.5	65.0	87.5	80.0	90.0	90.0	77.5	90.0	75.0	75.0	85.0	80.0	-					
V2.1R	62.5	62.5	62.5	60.0	62.5	70.0	57.5	60.0	67.5	52.5	52.5	60.0	57.5	47.5	62.5	52.5	67.5	62.5	-				
V7.1	82.5	82.5	77.5	85.0	77.5	80.0	72.5	85.0	77.5	82.5	77.5	80.0	77.5	72.5	77.5	72.5	82.5	87.5	70.0	-			
V7.1R	67.5	72.5	62.5	70.0	62.5	75.0	62.5	70.0	82.5	67.5	67.5	65.0	72.5	62.5	72.5	67.5	77.5	77.5	80.0	85.0	-		
V53.1R	75.0	75.0	70.0	72.5	70.0	77.5	75.0	57.5	75.0	65.0	60.0	62.5	60.0	55.0	80.0	60.0	70.0	65.0	97.5	72.5	82.5	-	
V.pl	82.5	87.5	82.5	85.0	77.5	75.0	72.5	75.0	77.5	82.5	82.5	75.0	77.5	62.5	82.5	72.5	72.5	77.5	60.0	75.0	70.0	72.5	-

Lanes 1-17 (V8.1 – V258.1) : Callus regenerated progenies,
Lanes 18-22 (V1.1R - V53.1R) : Root regenerated progenies,
23 (V.pl) : *V.planifolia*

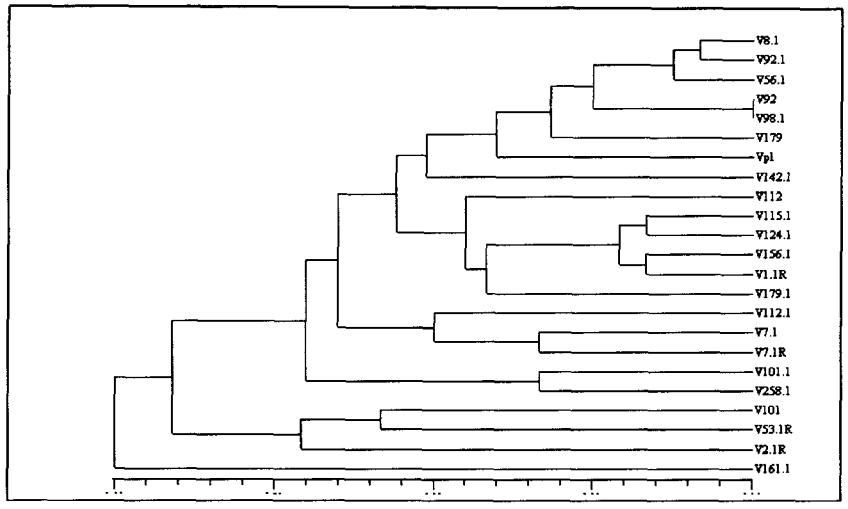


Fig. 39 Dendrogram of callus and root regenerated progenies of *V. planifolia*

RAPD profiles of colchicine generated progenies of Vanilla

RAPD profiles were developed for 17 colchicine generated progenies and were compared with those of *V. planifolia*, using 9 operon primers viz., OPA10, OPB 20, OPD19, OPA 20, OPD 03, OPE 09, OPE 05, OPE14, OPB 14. All the primers gave good amplified products. Of these, primers OPE05, OPA 20 and OPB20 gave good polymorphism. The other profiles are generally monomorphic with minor differences. (Fig. 40, 41 and 42).

The polymorphism between the colchicine generated progenies was scored and Paired Affinity Indices were calculated (Tables37, 38). Dendrograms were drawn based on NTSyS software to study the extent of variability (Fig.43). Studies indicated that most of the colchicines generated progenies are variable to each other and with *V. planifolia.* The Paired Affinity Index ranged from 96.0 to 44.0 % indicating high degree of variability. The dendrogram of divergence has indicated that VH5C is the nearest to *V. planifolia* while VC25 and VC153.1 are the farthest. There was no difference between VC92.1 and VC145.1,but VC89 and VC138 are exactly similar.

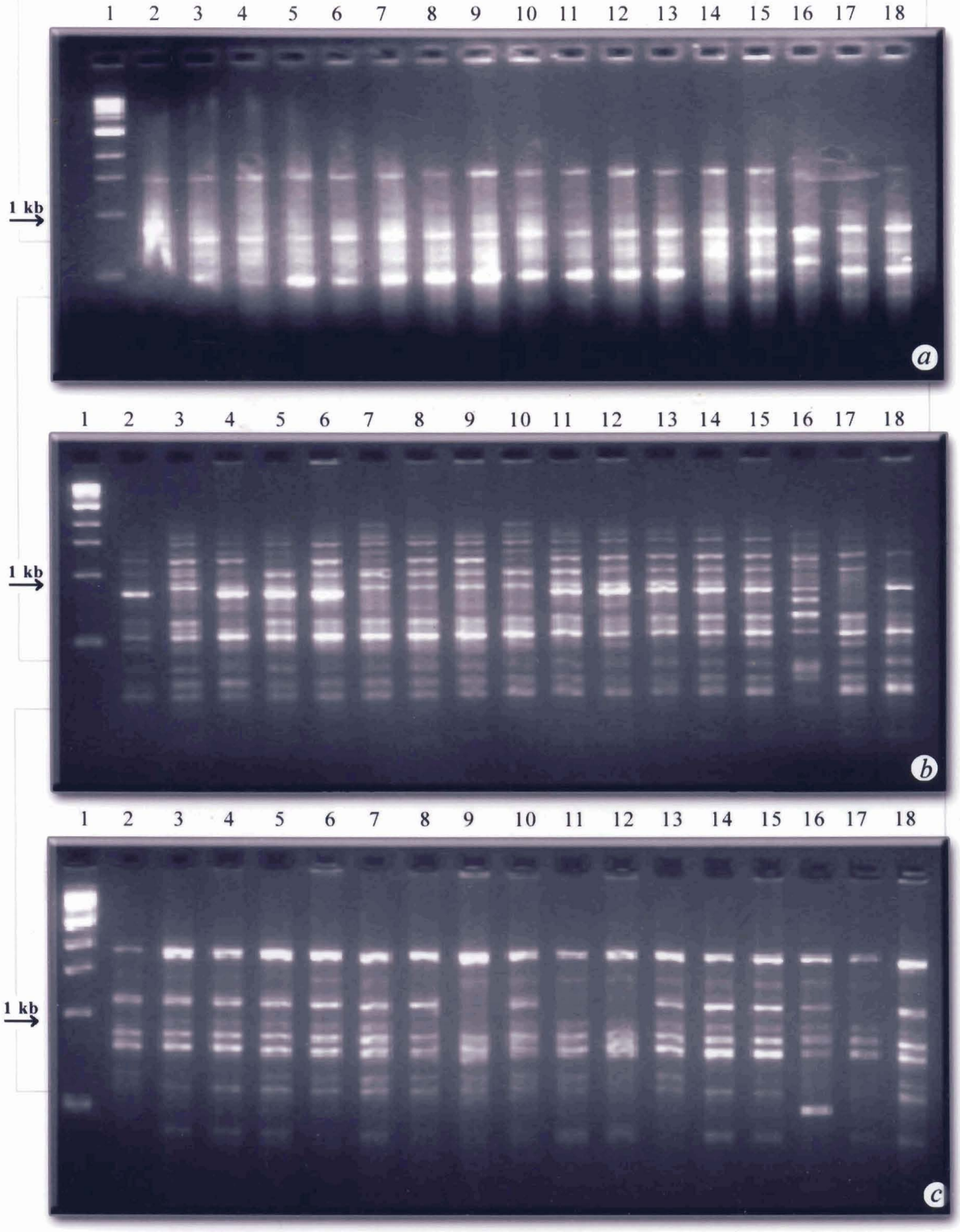


Fig. 40 RAPD polymorphism as expressed by OPERON primers in colchicine treated seedling progenies of *Vanilla planifolia* a: OPA 10; b : OPB 20; c ; OPD 19

Lanes : 1 : 1kb ladder; 2 : VC25; 3 : VC 40; 4 : VC 65; 5 : VC 69; 6 : VC77; 7: VC 89; 8 : VC113; 9: VC125; 10: VC138; 11:VC161; 12:VC260; 13:VC58.1; 14:VC 92.1; 15:VC145.1; 16: VC153.1; 17 :VH5; 18 :*V.planifolia*

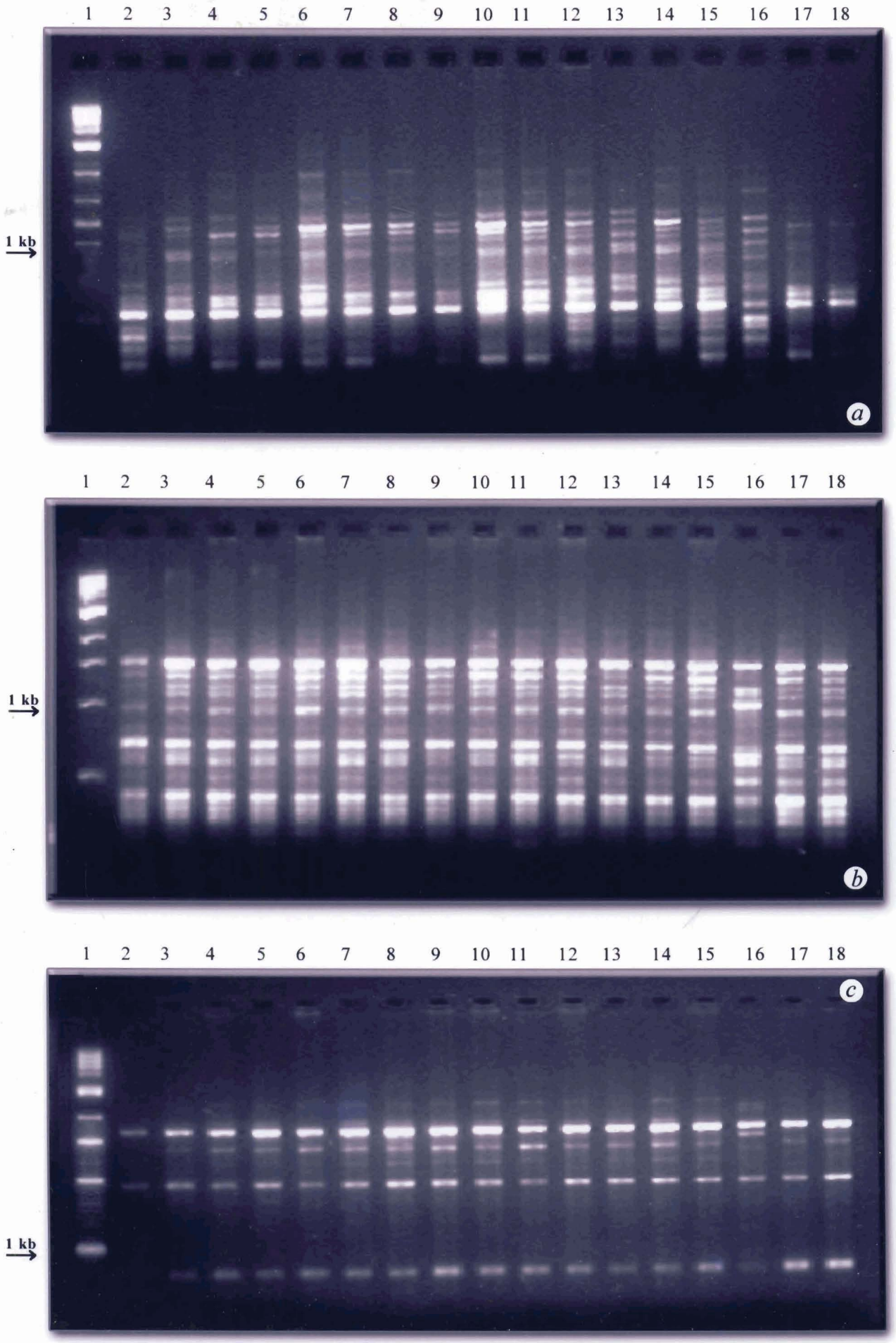


Fig. 41. RAPD polymorphism as expressed by OPERON primers in colchicine treated seedling progenies of *Vanilla planifolia*

a : OPA 20; b : OPD 03; c : OPE 09

Lanes : 1 : 1kb ladder, 2 : VC25, 3 : VC 40, 4 : VC 65, 5 : VC 69, 6 : VC77, 7: VC 89, 8:VC113, 9: VC125, 10: VC138, 11:VC161, 12:VC260, 13:VC58.1, 14:VC 92.1, 15:VC145.1, 16:153.1,

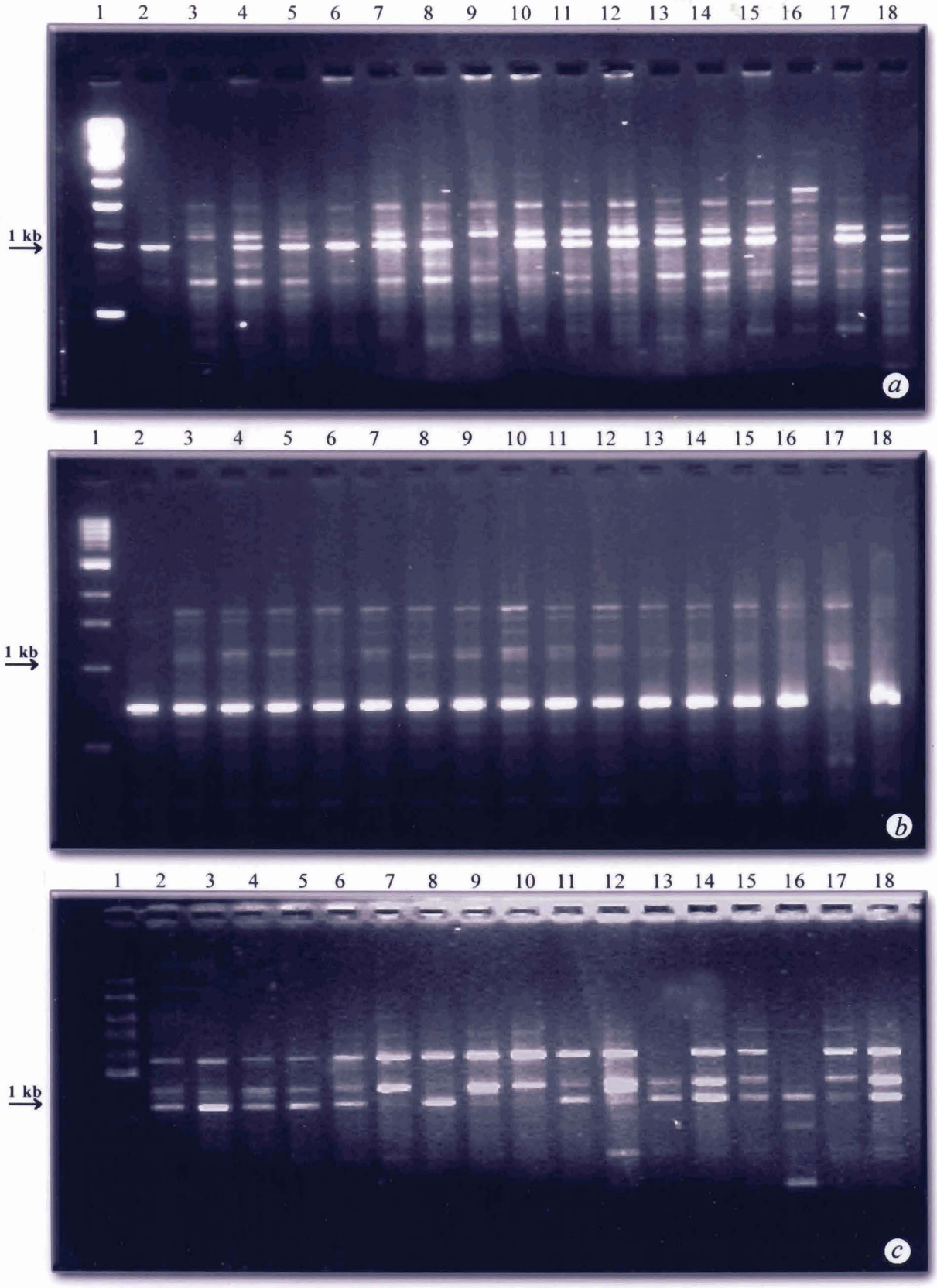


Fig. 42 RAPD polymorphism as expressed by OPERON primers in colchicine treated progenies of *Vanilla planifolia*
a. OPE 05, b. OPE 14, c. OPB 14

Lanes : 1 : 1kb ladder; 2 : VC25; 3 : VC 40; 4 : VC 65; 5 : VC 69; 6 : VC77; 7: VC 89; 8:VC113; 9: VC125; 10: VC138; 11:VC161; 12:VC260; 13:VC58.1; 14:VC 92.1; 15:VC145.1; 16:153.1; 17 :VH5; 18 :*V. planifolia*

Table 37. RAPD profile data on colchicine generated progenies of *Vanilla planifolia*

No	Primer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	size
1	OPA10-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.5
2	OPA10-2	0	0	0	0	1	1	1	1	1	0	0	0	1	1	0	1	1	0.8
3	OPA10-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.75
4	OPA10-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0.7
5	OPA10-5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0.6
6	OPA10-6	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0.5
7	OPB20-1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1.5
8	OPB20-2	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1.25
9	OPB20-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.0
10	OPB20-4	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0.9
11	OPB20-5	1	0	1	1	1	0	0	0	0	1	1	1	1	1	1	0	1	0.85
12	OPB20-6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0.6
13	OPB20-7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.5
14	OPB20-8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0.3
15	OPD19-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2.0
16	OPD19-2	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	0	1	1.2
17	OPD19-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.9
18	OPD19-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.8
19	OPD19-6	0	0	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0.6
20	OPD19-7	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0.5
21	OPA20-1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1.5
22	OPA20-2	0	1	1	0	1	1	1	0	1	1	1	1	1	1	1	0	1	1.2
23	OPA20-3	1	1	0	0	1	0	0	0	1	1	1	1	1	1	1	0	0	0.6
24	OPA20-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.55
25	OPA20-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.5
26	OPA20-6	1	0	1	1	1	1	0	0	1	1	0	0	0	1	0	1	0	0.3
27	OPE09-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.6
28	OPE09-2	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1.5
29	OPE09-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.0
30	OPE09-4	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0.4
31	OPD03-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.5
32	OPD03-2	0	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1.4
33	OPD03-4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1.1
34	OPD03-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1.0
35	OPD03-6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0.7
36	OPD03-7	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0.6
37	OPD03-8	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0.5
39	OPD03-9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.4
40	OPE14-1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1.6
41	OPE14-2	0	1	1	1	0	1	1	1	1	1	1	0	1	1	0	0	1	1.1
42	OPE14-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0.75
43	OPE05-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1.6
44	OPE05-2	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.5
45	OPE05-3	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1.1
46	OPE05-4	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1.0
47	OPE05-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.65
48	OPB14-1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1.2
49	OPB14-2	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	0.8
50	OPB14-3	1	1	1	0	0	1	0	1	1	0	1	0	1	1	1	0	0	0.7
51	OPB14-4	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	0.6

0 : Bands absent, 1 : Band present, 1-17 : Colchicine generated progenies, 18 : *V.planifolia*

Table 38. Paired affinity Indices of colchicines generated progenies of *V.planifolia*

	VC25	VC40	VC65	VC69	VC77	VC89	VC113	VC125	VC138	VC161	VC260	VC58.1	VC92.1	VC145.1	VC153.1	VH5C	V.pl
VC25	x																
VC40	66.0	x															
VC65	72.0	86.0	x														
VC69	66.0	80.0	90.0	x													
VC77	70.0	80.0	86.0	88.0	x												
VC89	60.0	82.0	88.0	90.0	86.0	x											
VC113	56.0	82.0	84.0	90.0	86.0	92.0	x										
VC125	62.0	80.0	82.0	84.0	80.0	90.0	86.0	x									
VC138	64.0	86.0	88.0	86.0	90.0	96.0	88.0	90.0	x								
VC161	66.0	84.0	90.0	92.0	92.0	86.0	86.0	80.0	90.0	x							
VC260	66.0	88.0	90.0	84.0	84.0	82.0	82.0	80.0	86.0	92.0	x						
VC58.1	68.0	82.0	84.0	86.0	90.0	80.0	84.0	78.0	84.0	90.0	86.0	x					
VC92.1	66.0	88.0	90.0	88.0	92.0	90.0	90.0	88.0	94.0	92.0	92.0	90.0	x				
VC145.1	70.0	88.0	94.0	88.0	92.0	90.0	86.0	84.0	94.0	92.0	92.0	86.0	96.0	x			
VC153.1	66.0	64.0	58.0	48.0	56.0	46.0	50.0	44.0	50.0	52.0	60.0	62.0	56.0	56.0	x		
VH5c	70.0	68.0	74.0	76.0	76.0	74.0	74.0	72.0	74.0	76.0	76.0	70.0	72.0	76.0	52.0	x	
V.pl	68.0	74.0	80.0	78.0	78.0	76.0	80.0	74.0	76.0	78.0	82.0	76.0	82.0	82.0	54.0	82.0	x

VC 25 – VH5C : Colchicine generated progenies, V.pl : *V.planifolia*

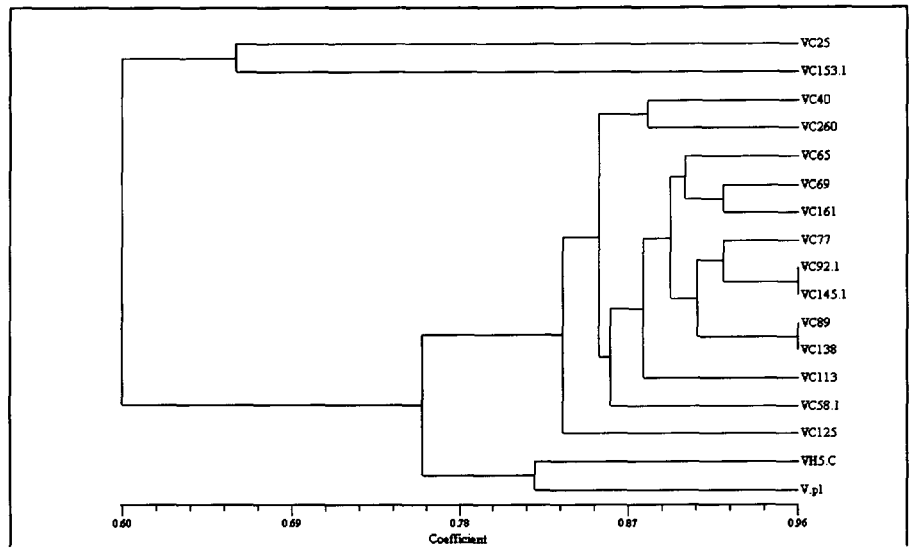


Fig. 43 Dendrogram of colchicine generated progenies of *V.planifolia*

***RAPD profiles of selfed and interspecific hybrid progenies of* V .planifolia**

A separate experiment was conducted to study the variability between *V. planifolia* and its selfed progenies. RAPD profiles were developed for 7 selfed seedling progenies of *V. planifolia,* four interspecific hybrids and their parents. Nine operon primers viz., OPC09, OPD 03, OPE 14, OPA 10, OPE 09, OPA 20, OPD 19, OPB 14, OPB 20 were used. All the primers gave good RAPD profiles. Of these, primers OPB 20, OPD 19, OPA 20, OPA10, OPD 03 and OPE 14 gave good polymorphism. The other profiles are generally monomorphic with minor differences (Fig. 44, 45 and 46).

The polymorphism observed between the seedlings and hybrids are presented in Table 39. The Paired Affinity Indices were calculated based on Similarity index and presented in Table 40. Dendrograms were drawn based on NTSyS software to study the extent of variability (Fig.47).

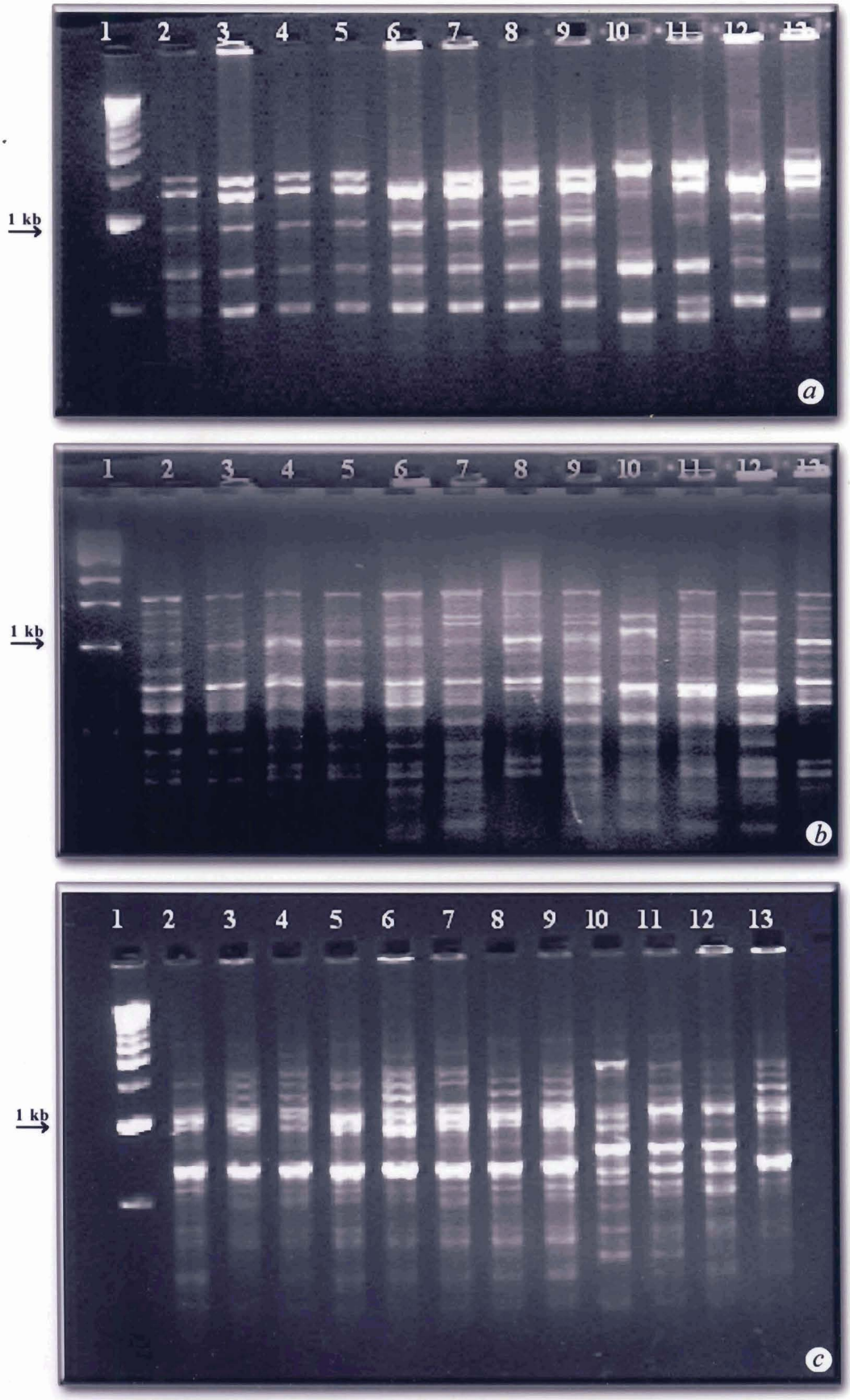


Fig. 44 RAPD profiles of selfed and inter specific hybrids of vanilla using operon primer

a : OPC 09; b : OPD 03 ; c : OPE 14

1 : 1 kb ladder, 2 : V1, 3 : V2, 4 : V4, 5 : V6, 6 : V7, 7 : V8, 8 : V10, 9 : *V.planifolia*, 10 : *V.aphyl*la, 11 : VH1, 12 : VH4, 13 : VH5, 14 : VH6.

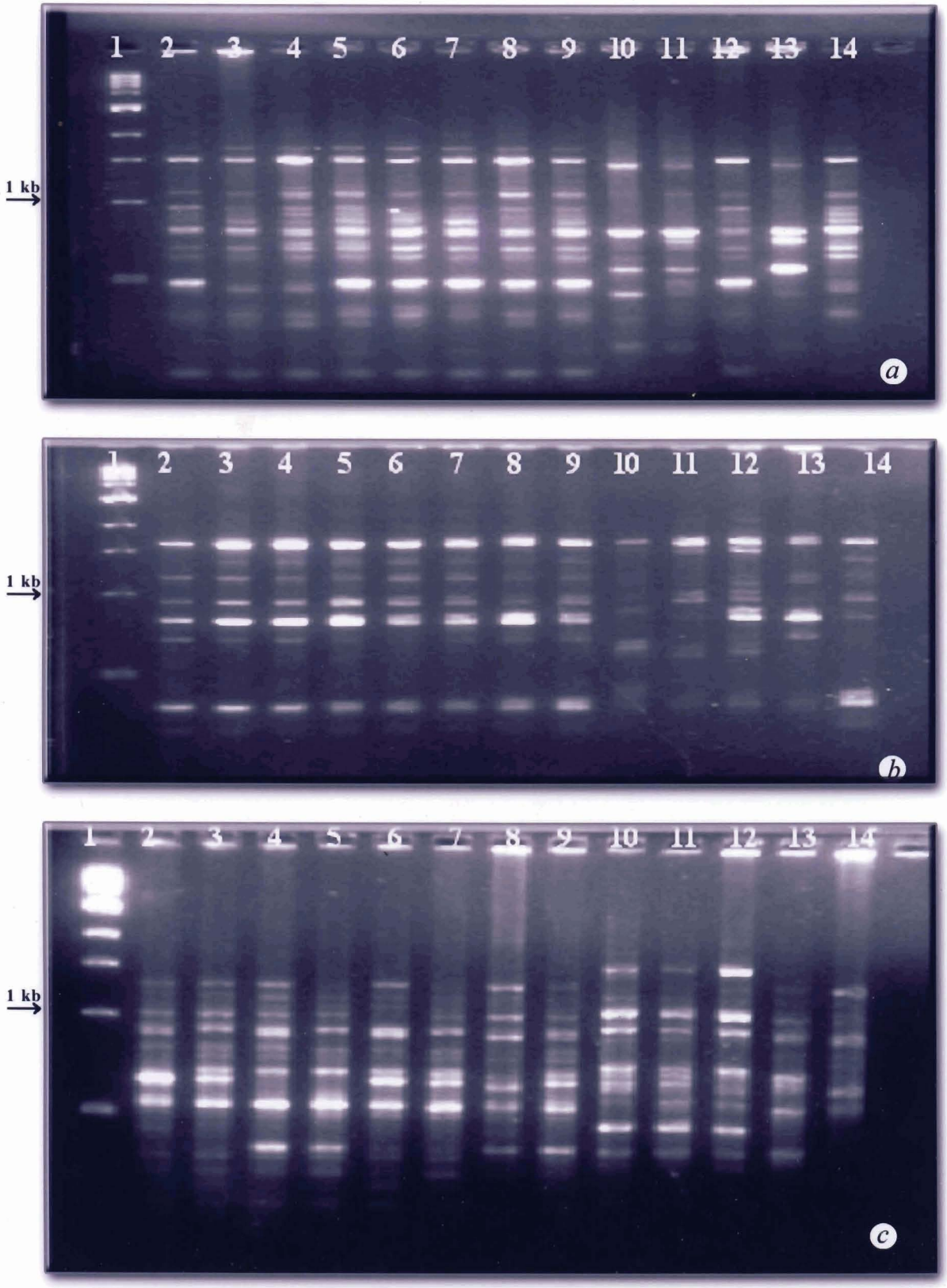


Fig. 45 RAPD profiles of selfed and interspecific hybrids of vanilla using OPERON primer OPA 10; OPE 09; OPA 20

1 : 1 kb ladder, 2 : V1, 3 : V2, 4 : V4, 5 : V6, 6 : V7, 7 : V8, 8 : V10, 9 : *V.planifolia*, 10 : *V.aphylla*, 11 : VH1, 12 : VH4, 13 : VH5, 14 : VH6.

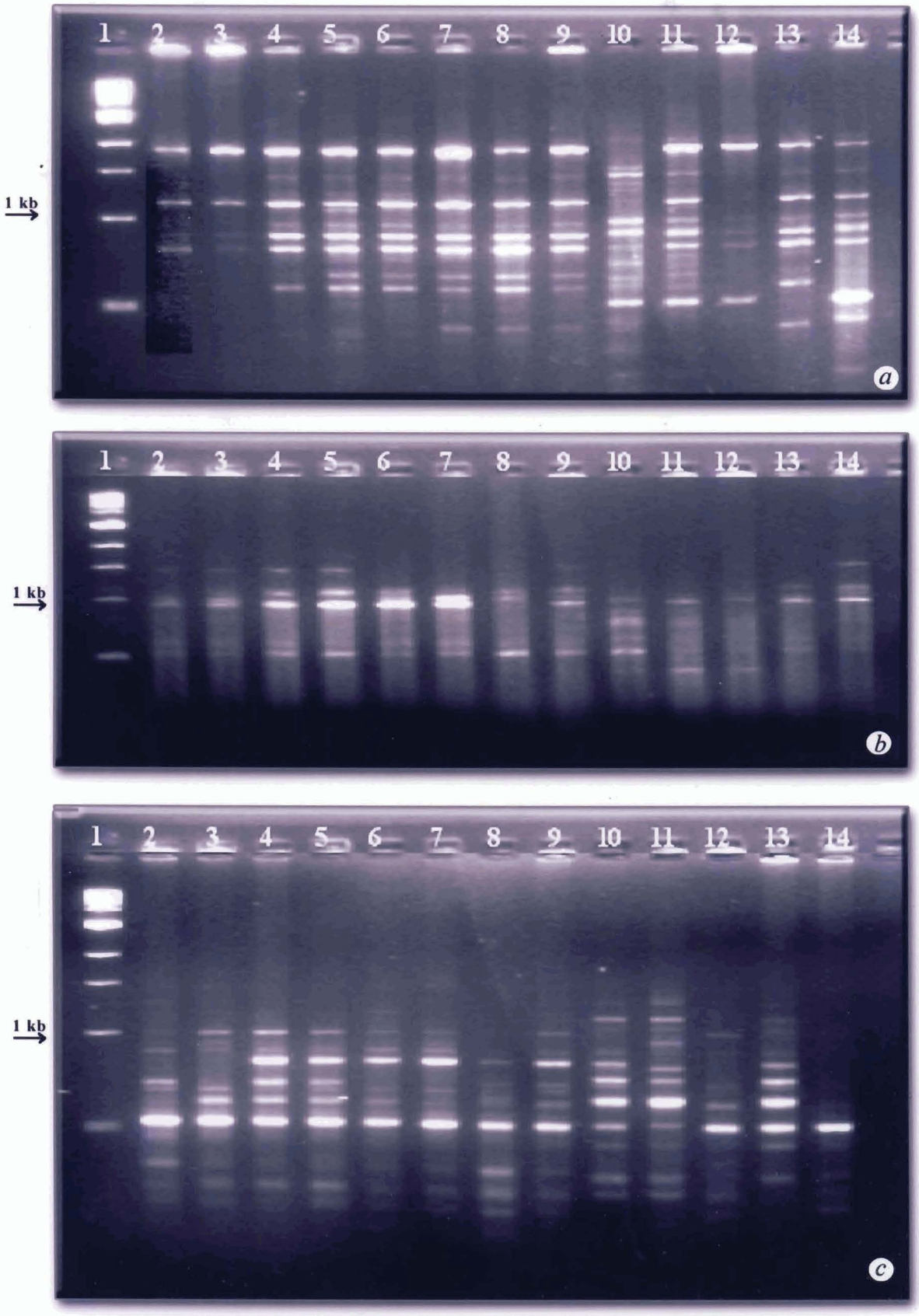


Fig. 46 RAPD profiles of selfed and interspecific hybrids of vanilla using OPERON primer a OPD 19, b : OPB 14, c : OPB20,

1 : 1 kb ladder, 2 : V1, 3 : V2, 4 : V4, 5 : V6, 6 : V7, 7 : V8, 8 : V10, 9 : *V.planifolia,* 10 : *V.aphylla,* 11 : VH1, 12 : **VH4, 13 : VH5, 14 : VH6.**

Table 39: RAPD profile data on selfed progenies and interspecific hybrids of vanilla

No.	Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	Size
1	OPC09-1	1	1	1	1	0	1	1	1	1	1	0	1	1	1.5
2	OPC09-2	1	1	1	1	1	1	0	1	1	1	1	1	1	1.4
3	OPC09-3	1	1	1	1	1	1	1	1	0	1	1	1	1	1.0
4	OPC09-4	1	1	1	1	1	1	1	1	1	1	1	1	1	0.7
5	OPC09-5	1	1	1	1	1	1	1	1	0	1	1	0	1	0.5
6	OPC09-6	0	0	0	0	0	0	0	0	1	1	0	1	0	0.4
7	OPD03-1	1	1	1	1	1	1	1	1	0	1	1	1	1	1.6
8	OPD03-2	1	0	0	0	0	1	1	1	0	0	0	1	0	1.4
9	OPD03-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1.3
10	OPD03-5	1	1	1	1	1	1	0	1	0	1	0	0	1	1.2
11	OPD03-6	0	0	0	0	0	0	0	0	1	0	1	0	0	1.1
12	OPD03-7	1	1	1	1	1	1	1	1	0	1	0	1	1	1.0
13	OPD03-8	1	1	1	1	1	1	1	1	0	0	1	1	.1	0.75
14	OPD03-9	0	1	1	0	1	1	1	1	1	1	1	1	1	0.7
15	OPD03-10	1	1	1	1	1	1	0	1	1	1	1	0	1	0.5
16	OPD03-11	1	1	1	1	1	1	0	1	1	1	1	0	1	0.4
17	OPD03-12	1	1	1	1	1	1	0	1	1	0	1	0	1	0.3
18	OPD03-13	1	1	1	1	1	1	1	1	1	1	1	1	1	0.25
19	OPE14-1	0	1	1	1	1	1	1	1	1	1	1	1	1	1.6
20	OPE14-2	0	1	1	1	1	1	1	1	0	1	0	1	1	1.5
21	OPE14-3	0	1	1	0	1	0	1	1	0	1	0	1	0	1.25
22	OPE14-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1.2
23	OPE14-5	1	1	1	1	1	1	1	1	1	0	0	1	0	1.0
24	OPE14-6	0	0	0	0	0	0	0	0	1	1	1	0	0	0.75
25	OPE14-7	1	1	1	1	1	1	1	1	0	0	0	1	1	0.7
26	OPE14-8	1	1	1	1	1	1	1	1	1	1	1	1	1	0.65
27	OPE14-9	1	1	1	1	1	1	1	1	0	1	1	0	1	0.5
28	OPE14-10	1	1	1	1	1	1	1	1	1	1	1	1	1	0.4
29	OPA10-1	1	1	1	1	1	1	1	1	0	0	0	0	0	1.6
30	OPA10-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1.5
31	OPA10-3	1	1	1	1	1	1	1	1	0	0	1	0	1	1.1
32	OPA10-4	1	1	1	1	1	1	1	1	0	0	1	0	1	0.9
34	OPA10-5	1	1	1	1	1	1	1	1	0	0	1	0	1	0.8
35	OPA10-6	1	1	1	1	1	1	1	1	1	1	1	1	1	0.75
36	OPA10-7	1	1	1	1	1	1	1	1	0	0	1	0	1	0.7
37	OPA10-8	0	0	0	0	0	0	0	0	1	1	0	1	0	0.6
38	OPA10-9	1	1	1	1	1	1	1	1	0	0	1	0	1	0.5
39	OPA10-10	1	1	1	1	1	1	1	1	0	0	0	0	1	0.4
40	OPA10-11	0	0	0	0	0	0	0	0	1	1	0	1	0	0.3
41	OPA10-12	1	1	1	1	1	1	1	1	0	0	1	0	0	0.25
42	OPE05-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.6
43	OPE05-2	0	0	0	0	0	0	0	0	0	1	1	0	0	1.5
44	OPE05-3	1	1	1	1	1	1	1	1	0	0	1	1	1	1.2
45	OPE05-4	1	1	1	1	1	1	1	1	0	1	1	1	1	0.9
46	OPE05-5	1	1	1	1	1	1	1	1	0	0	1	1	0	0.8
47	OPE05-6	1	1	1	0	0	1	1	1	0	0	0	1	0	0.6
48	OPE05-7	0	0	0	0	0	0	0	0	1	1	1	0	0	0.55
49	OPE05-8	1	1	1	1	1	1	1	1	0	0	0	0	1	0.4
50	OPF12-1	0	0	0	0	0	0	0	0	1	1	1	0	0	1.5
51	OPF12-2	1	1	1	0	1	0	1	1	0	0	1	1	1	1.4
52	OPF12-1	1	1	1	1	0	0	1	1	1	1	1	1	1	1.0
53	OPF12-3	1	1	1	1	1	1	1	1	1	1	1	1	1	0.9
54	OPF12-4	1	1	1	1	1	1	1	1	0	0	0	0	1	0.8
55	OPF12-5	0	1	1	1	1	1	0	1	1	1	1	0	0	0.7

56	OPF12-6	1	1	0	0	1	1	1	1	1	1	1	1	0	0.6
57	OPF12-7	1	1	1	1	1	1	1	1	0	1	1	1	1	0.55
58	OPF12-8	0	0	0	0	0	0	0	0	1	1	1	0	0	0.4
59	OPF12-9	0	0	1	1	0	0	1	1	1	1	1	1	0	0.3
60	OPD19-1	1	1	1	1	1	1	1	1	0	1	1	1	1	2.0
61	OPD19-2	0	0	0	0	0	0	0	0	1	0	0	0	0	1.6
62	OPD19-3	0	0	0	0	0	0	0	0	1	1	0	1	1	1.5
63	OPD19-4	1	1	1	1	1	1	1	1	0	1	0	1	1	1.2
64	OPD19-5	0	0	1	1	1	1	1	1	0	0	0	0	0	1.0
65	OPD19-6	1	1	1	1	1	1	1	1	1	1	1	1	1	0.8
66	OPD19-7	1	1	1	1	1	1	1	1	1	1	1	1	1	0.7
67	OPD19-8	0	0	1	1	1	1	1	1	1	0	0	1	0	0.6
68	OPD19-9	0	0	0	0	0	0	0	0	1	1	1	1	0	0.5
69	OPD19-10	0	0	0	0	0	1	1	1	0	0	0	1	1	0.4
70	OPB14-1	1	1	1	1	0	0	0	0	0	0	0	0	1	1.5
71	OPB14-2	0	0	1	1	1	1	1	0	0	0	0	1	1	1.1
72	OPB14-3	1	1	1	1	1	1	0	1	1	1	1	1	1	1.0
73	OPB14-4	0	0	0	0	0	0	0	0	1	0	0	0	0	0.8
74	OPB14-5	1	1	1	1	1	1	1	1	1	0	0	0	0	0.5
75	OPB20-1	0	0	0	0	0	0	0	0	1	0	1	0	1	1.25
76	OPB20-2	0	1	1	1	1	1	1	1	1	1	1	1	0	1.0
77	OPB20-3	1	1	1	1	1	1	0	0	1	0	0	1	0	0.9
78	OPB20-4	0	0	1	1	1	1	1	1	1	1	0	1	1	0.85
79	OPB20-5	1	0	1	1	0	0	0	0	1	1	0	1	0	0.7
80	OPB20-6	0	1	1	1	1	0	0	0	1	0	1	1	0	0.6
81	OPB20-7	1	1	1	1	1	1	1	1	1	1	1	1	1	0.5
82	OPB20-8	1	1	1	1	1	1	1	1	1	1	1	1	1	0.4
83	OPB20-9	1	1	1	1	1	1	1	1	1	1	1	0	1	0.3

0 : Band Absent, 1 ; Band Present;
1 to 7 : Seedling progenies of *V.planifolia*, 8 . *V.planifolia*, 9 : *V.aphylla*, 10-13. Interspecific hybrids

Table 40. Paired Affinity Indices of selfed progenies and interspecific hybrids in Vanilla

	V1	V2	V4	V6	V7	V8	V10	V.pl	V.aph	VH1	VH4	VH5	VH6
V1	x												
V2	89.0	x											
V4	82.9	91.5	x										
V6	82.9	86.6	95.1	x									
V7	79.3	90.2	91.5	89.0	x								
V8	82.9	86.8	87.8	87.8	91.5	x							
V10	75.6	79.3	82.9	78.0	81.7	85.4	x						
V.pl	82.9	89.0	90.2	85.4	89.0	92.7	90.2	x					
V.aph	40.2	43.9	45.1	47.6	43.9	42.7	35.4	42.7	x				
VH1	51.2	57.3	56.1	56.1	54.9	53.7	51.2	58.5	72.0	x			
VH4	63.4	69.5	63.9	63.4	67.1	61.0	58.5	65.8	62.2	68.3	x		
VH5	57.3	61.0	64.6	59.7	61.0	62.2	69.5	64.6	60.9	69.5	52.4	x	
VH6	70.7	69.5	68.3	68.3	67.1	68.3	68.3	68.3	50.0	58.5	65.9	59.8	x

V1-V10 : Selfed progenies of V.planifolia; V.pl : V.planifolia, V.aph : V.aphylla, VH1-VH5 : Interspecific hybrids of V.planifolia x V.aphylla

Studies indicated that most of the seed progenies are variable when compared to each other and with *V. planifolia.*

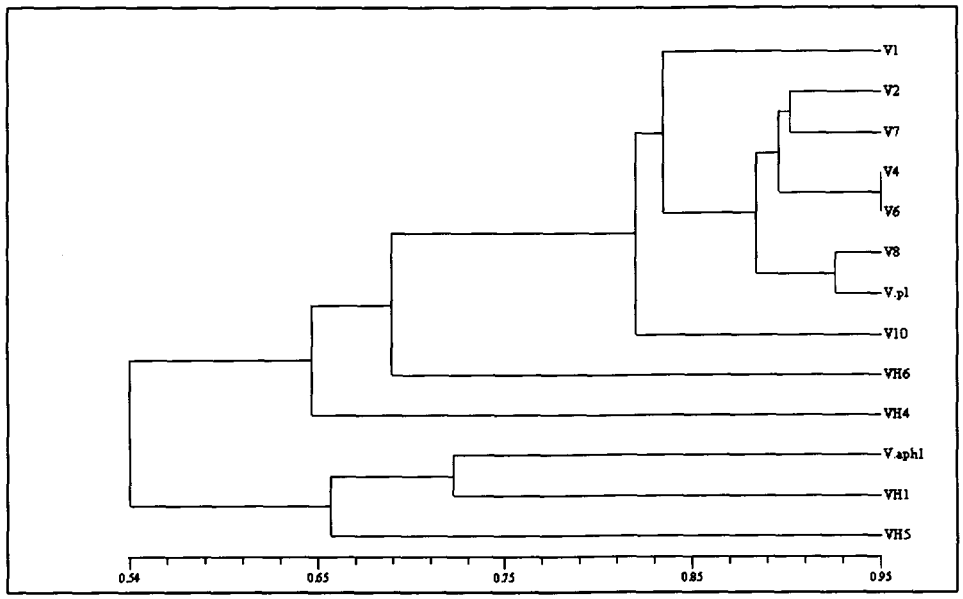


Fig. 47 Dendrogram showing linkage groups between selfed progenies and interspecific hybrids as expressed by RAPD markers

The result indicates the progenies of vanilla are more similar to their parent *V. planifolia* and to each other. The percentage similarity ranged from 35.4 – 95.1. Among progenies V4 and V6 are nearest to each other with 95.1% similarity. Among the progenies, V1 is farthest (82.9%), while V8 is the nearest to *V. planifolia*, with 92.7% similarity. Studies confirm that *V. aphylla* is the farthest from *V. planifolia* with 42.7% similarity. When the progenies are compared with their parent *V. planifolia*, the % of similarity ranged from 75.6 – 95.1. The present study thus indicated a good amount of variation among the selfed progenies of *V. planifolia* as expressed by RAPD polymorphisms.

The interspecific hybrids VH6 and VH4 are grouped with *V. planifolia* while VH1 and VH5 are grouped with *V. aphylla*. In general, the hybrids were equidistant from *V. planifolia* and *V.aphylla*, indicating their hybrid nature

Amplified Fragment Length Polymorphism (AFLP)

The AFLP profiles were developed for the first time in *V.planifolia*, *V.aphylla*, interspecific hybrids and a few selfed progenies of *V. planifolia* (Fig. 48, 49, 50 and 51).

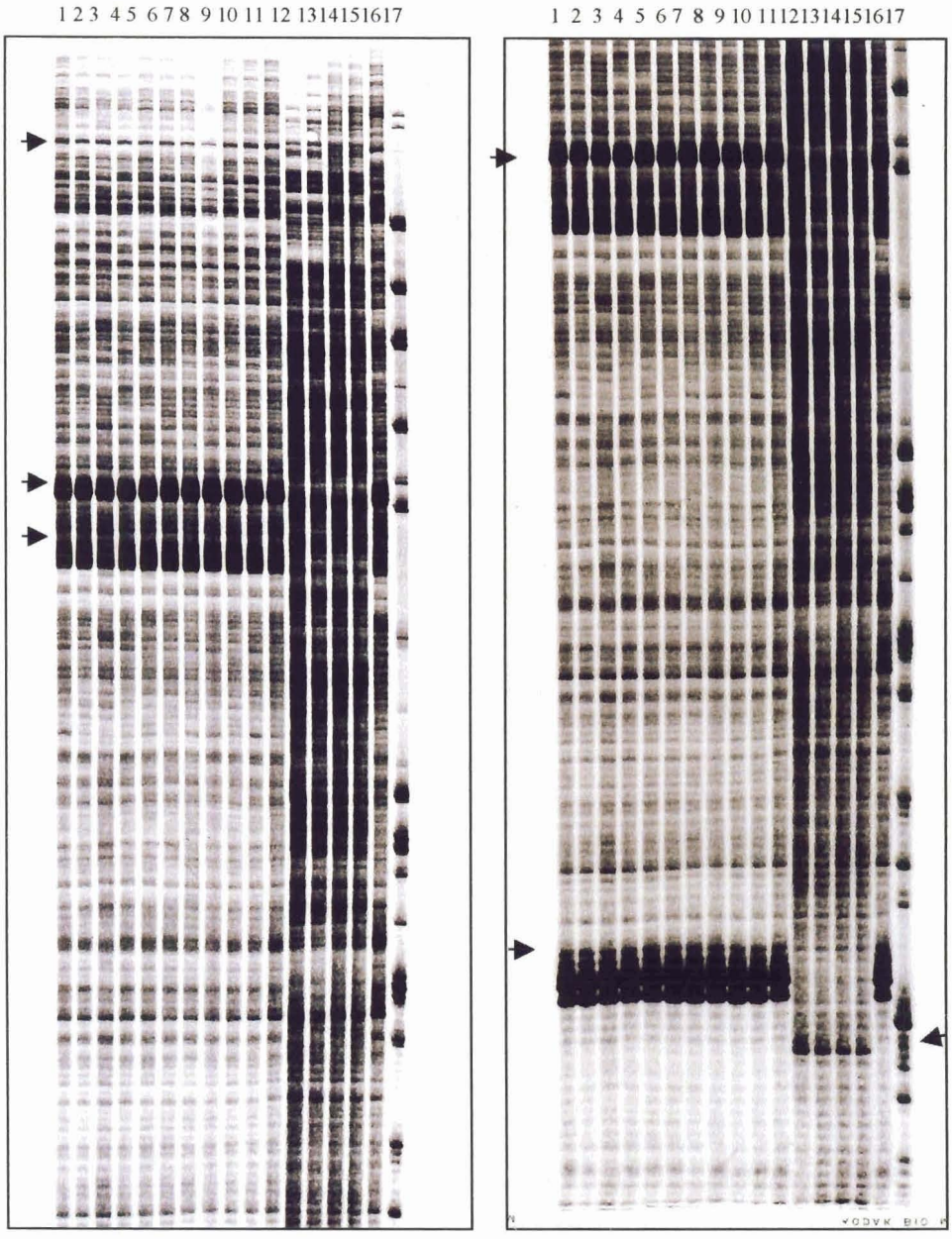


Fig. 48 AFLP profiles of vanilla seedling progenies and interspecific hybrids developed by primer combination EAC-MTG. (lanes 1-10 : Seedling progenies of *V.planifolia*, 14-16 : Interspecific hybrids of *V.planifolia* and *V.aphylla*)

1 : V1, 2 : V2, 3 : V4, 4 : V6, 5 : V7, 6 : V8, 7 : V10, 8 : V11, 9 : V12, 10 : V24, 11 : *V.planifolia*, 12 : *V.aphylla1*, 13 : *V.aphylla2* , 14 : VH1, 15 : VH4, 16 : VH5, 17 : Water control. → Arrows indicate species specific bands

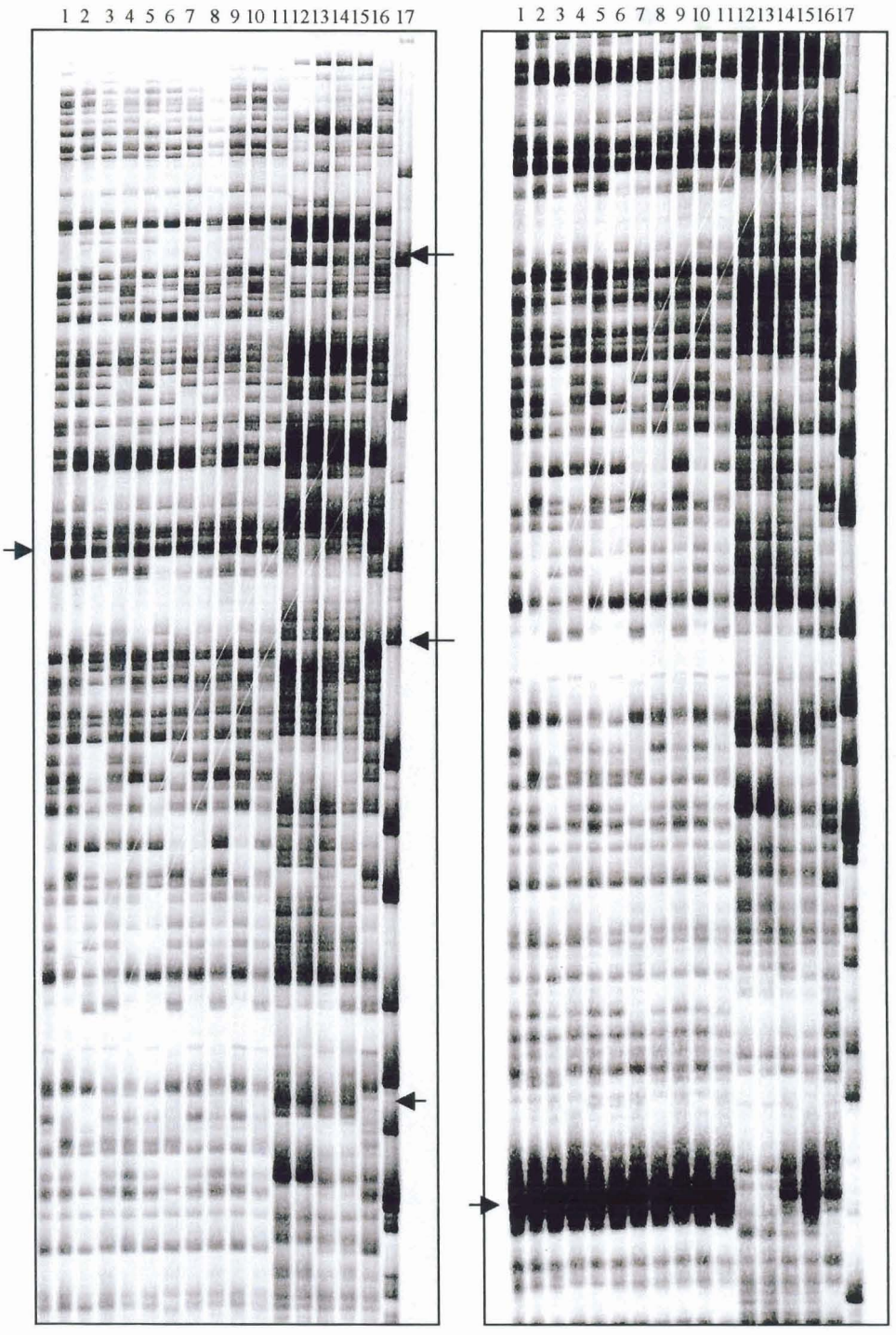


Fig 49 AFLP profiles of vanilla seedling progenies and interspecific hybrids developed by primer combination EGG-MGC. (lanes 1-10 : Seedling progenies of *V.planifolia*, 14-16 : Interspecific hybrids of *V.planifolia* and *V.aphylla*)

1 : V1, 2 : V2, 3 : V4, 4 : V6, 5 : V7, 6 : V8, 7 : V10, 8 : V11, 9 : V12, 10 : V24, 11 : *V.planifolia*, 12 : *V.aphylla1*, 13 : *V.aphylla2* , 14 : VH1, 15 : VH4, 16 : VH5, 17 : Water control. → Arrows indicate species specific bands

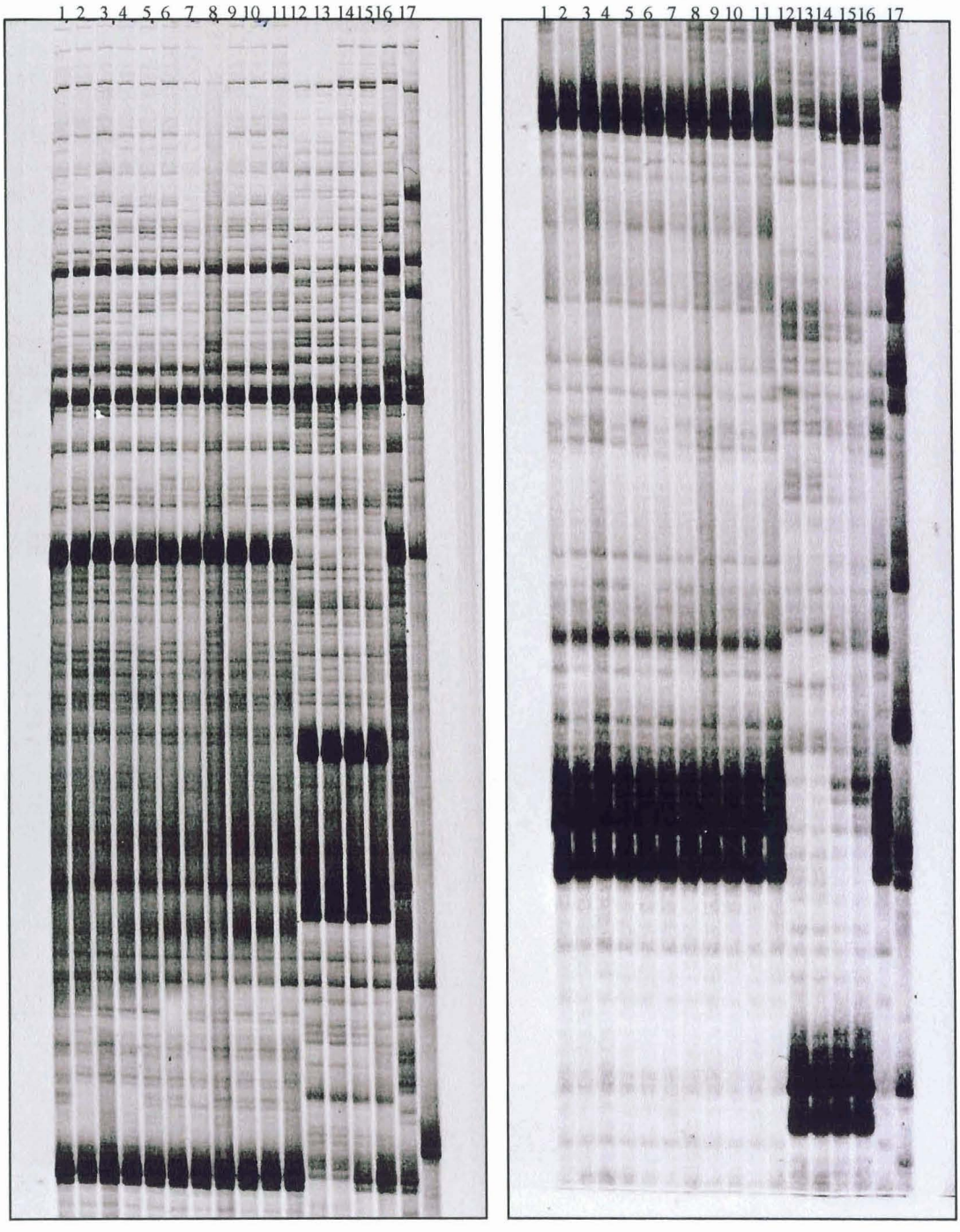


Fig. 50 AFLP profiles of vanilla seedling progenies and interspecific hybrids developed by primer combination EGG-MTG. (lanes 1-10 : Seedling progenies of *V.planifolia*, 14-16 : Interspecific hybrids of *V.planifolia* and *V.aphylla*)

1 : V1, 2 : V2, 3 : V4, 4 : V6, 5 : V7, 6 : V8, 7 : V10, 8 : V11, 9 : V12, 10 : V24, 11 : *V.planifolia*, 12 : *V.aphylla1*, 13 : *V.aphylla2* , 14 : VH1, 15 : VH4, 16 : VH5, 17 : Water control. . Arrows indicate species specific bands

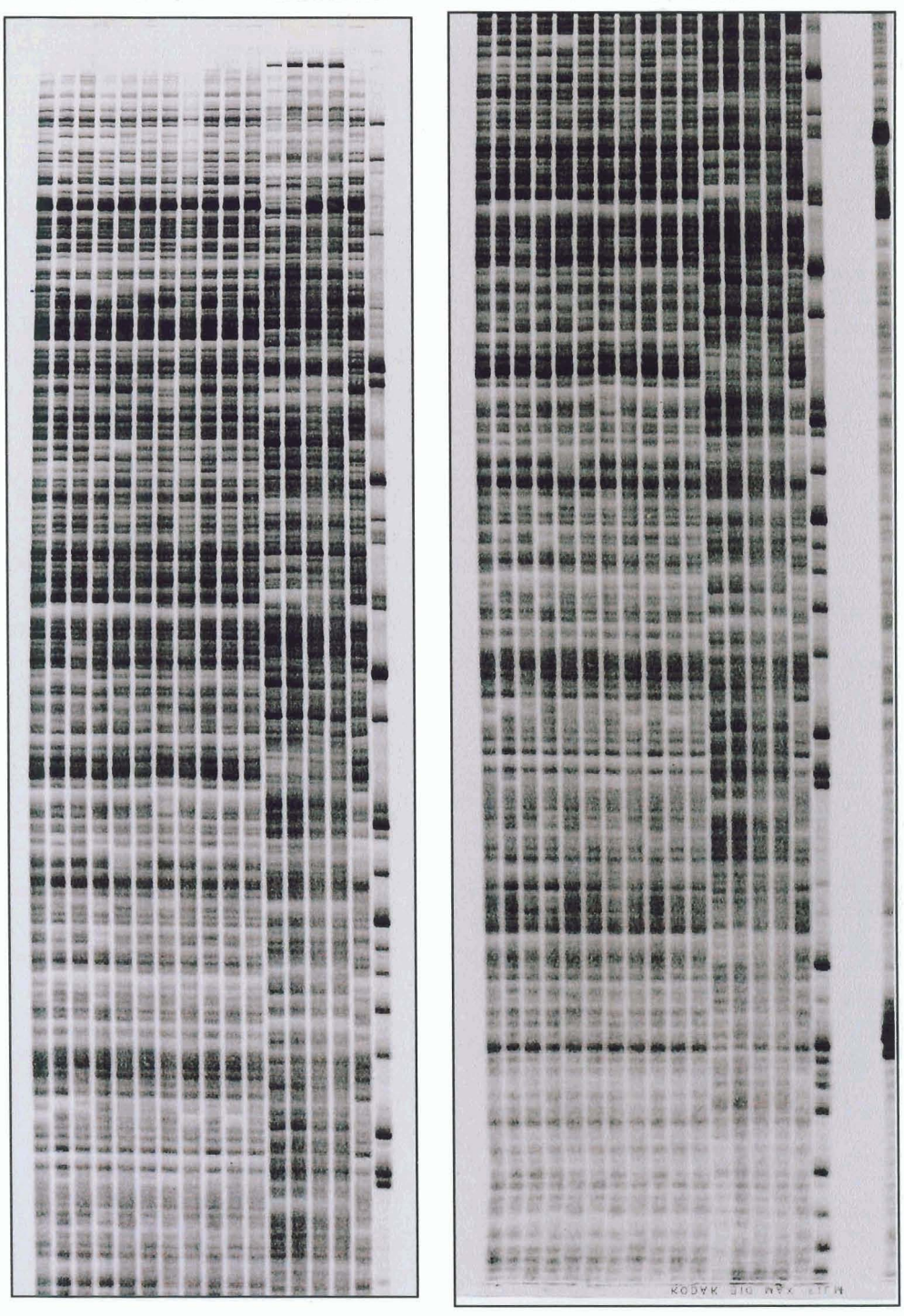


Fig. 51 AFLP profiles of vanilla seedling progenies and interspecific hybrids developed by primer combination ETG-MAC. (lanes 1-10 : Seedling progenies of *V.planifolia*, 14-16 : Interspecific hybrids of *V.planifolia* and *V.aphylla*)

1 : V1, 2 : V2, 3 : V4, 4 : V6, 5 : V7, 6 : V8, 7 : V10, 8 : V11, 9 : V12, 10 : V24, 11 : *V.planifolia,* 12 : *V.aphylla1,* 13 : *V.aphylla2* , 14 : VH1, 15 : VH4, 16 : VH5, 17 : Water control. . Arrows indicate species specific bands

Four different primer combinations *viz.*, EGG-MTG, EGG-MGC, ETG-MAC and EAC-MTG were used to develop autoradiograms. The polymorphism observed between the seedlings and hybrids (Table 41). The Paired Affinity Indices was calculated based on Similarity index (Table 42). Dendrograms were drawn (NTSyS software) to study the extent of variability (Fig.52).

Table 41: AFLP profile data on selfed progenies and interspecific hybrids of vanilla with +2, +2 primers

No	Primers	V1	V2	V4	V6	V7	V8	V10	V11	V12	V24	V.Pl	V.apl	V.ap2	VH1	VH4	VH5
1	EGG-MTG1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0
2	EGG-MTG 2	1	1	1	1	1	1	1	0	1	1	1	0	0	1	1	1
3	EGG-MTG 3	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
4	EGG-MTG 4	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
5	EGG-MTG 5	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
6	EGG-MTG 6	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
7	EGG-MTG 7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	EGG-MTG 8	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
9	EGG-MTG 9	1	1	0	1	1	1	0	1	1	1	1	0	0	0	0	1
10	EGG-MTG 10	0	0	0	1	0	0	1	0	0	0	1	0	0	1	1	0
11	EGG-MTG 11	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	1
12	EGG-MTG 12	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
13	EGG-MTG 13	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14	EGG-MTG 14	0	0	1	0	1	1	1	0	1	0	1	0	0	1	1	0
16	EGG-MTG 15	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
17	EGG-MTG 16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18	EGG-MTG 17	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
19	EGG-MTG 18	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
20	EGG-MTG 19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	EGG-MTG 20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
22	EGG-MTG 21	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
23	EGG-MTG 22	1	0	1	0	0	0	0	0	0	1	1	1	1	1	1	0
24	EGG-MTG 23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
25	EGG-MTG 24	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
26	EGG-MTG 25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
27	EGG-MTG 26	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
28	EGG-MTG 27	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
29	EGG-MTG 28	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
30	EGG-MTG 29	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
31	EGG-MTG 30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
32	EGG-MTG 31	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0
33	EGG-MTG 32	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
34	EGG-MTG 33	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0
35	EGG-MTG 34	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
36	EGG-MTG 35	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
37	EGG-MTG 36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
38	EGG-MTG 37	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1
39	EGG-MTG 38	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
40	EGG-MTG 39	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
41	EGG-MTG 40	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
42	EGG-MTG 41	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0
43	EGG-MTG 42	1	0	1	1	0	1	0	1	0	1	1	0	0	0	0	1
45	EGG-MTG 43	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1
46	EGG-MTG 45	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
47	EGG-MTG 46	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
48	EGG-MTG 47	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

49	EGG-MTG 48	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
50	EGG-MTG 49	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
51	EGG-MTG 50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
52	EGG-MTG 51	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
53	EGG-MTG 52	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
54	EGG-MTG 53	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
55	EGG-MTG 54	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
56	EGG-MTG 55	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
57	EGG-MTG 56	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
58	EGG-MTG 57	0	1	1	1	1	0	1	1	1	1	1	0	0	0	0	1
59	EGG-MTG 58	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	1
60	EGG-MTG 59	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
61	EGG-MTG 60	0	1	0	0	1	0	0	0	0	0	0	1	1	1	1	0
62	EGG-MTG 61	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
63	EGG-MTG 62	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
64	EGG-MTG 63	0	0	0	1	1	0	1	1	1	1	0	0	0	0	0	0
65	EGG-MTG 64	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	1
66	EGG-MTG 65	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
67	EGG-MTG 66	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
68	EGG-MTG 67	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
69	EGG-MTG 68	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
70	EGG-MTG 69	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
71	EGG-MTG 70	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
72	EGG-MTG 71	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
73	EGG-MTG 72	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
74	EGG-MTG 73	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
75	EGG-MTG 74	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
76	EGG-MTG 75	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
77	EGG-MTG 76	0	1	1	0	1	0	1	1	0	0	1	1	1	1	1	1
78	EGG-MTG 77	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
79	EGG-MTG 78	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1
80	EGG-MTG 79	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
81	EGG-MTG 80	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
82	EGG-MTG 81	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
83	EGG-MTG 82	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
84	EGG-MTG 83	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1
85	EGG-MTG 84	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
86	EGG-MTG 85	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0
87	EGG-MTG 86	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
88	EGG-MTG 87	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
89	EGG-MTG 88	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
90	EGG-MTG 89	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
91	EGG-MTG 90	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
92	EGG-MTG 91	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
93	EGG-MGC1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0
94	EGG-MGC 2	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
95	EGG-MGC 3	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
96	EGG-MGC 4	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	1
97	EGG-MGC 5	0	0	1	0	1	1	0	0	1	0	0	1	1	1	1	1
98	EGG-MGC 6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
99	EGG-MGC 7	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1
100	EGG-MGC 8	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
101	EGG-MGC 9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
102	EGG-MGC 10	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
103	EGG-MGC 11	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
104	EGG-MGC 12	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
105	EGG-MGC 13	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0	1
106	EGG-MGC 14	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
107	EGG-MGC 15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
108	EGG-MGC 16	0	1	1	1	1	0	1	0	1	0	1	1	1	1	1	0
109	EGG-MGC 17	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
110	EGG-MGC 18	0	0	1	1	0	0	1	0	1	0	1	0	0	0	0	1
111	EGG-MGC 19	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0

112	EGG-MGC20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
113	EGG-MGC21	1	1	1	1	0	1	1	1	1	1	1	0	0	1	0	1
114	EGG-MGC22	1	0	0	1	0	0	1	1	0	1	1	0	0	1	1	1
115	EGG-MGC23	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
116	EGG-MGC24	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
117	EGG-MGC25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
118	EGG-MGC26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
119	EGG-MGC27	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
120	EGG-MGC28	1	1	1	0	1	0	1	1	1	1	1	0	0	0	1	1
121	EGG-MGC29	1	1	0	1	1	1	0	1	0	1	1	0	0	0	0	1
122	EGG-MGC30	1	1	1	1	1	1	0	1	0	1	1	0	0	1	1	1
123	EGG-MGC31	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
124	EGG-MGC32	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0
125	EGG-MGC34	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
126	EGG-MGC35	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
127	EGG-MGC36	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
128	EGG-MGC37	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0
129	EGG-MGC38	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
130	EGG-MGC39	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
131	EGG-MGC40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
132	EGG-MGC41	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
133	EGG-MGC42	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	1
134	EGG-MGC43	1	1	1	0	0	0	1	1	1	1	1	0	0	1	1	1
135	EGG-MGC44	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
136	EGG-MGC45	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0
137	EGG-MGC46	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
138	EGG-MGC47	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
139	EGG-MGC48	0	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1
140	EGG-MGC49	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
141	EGG-MGC50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
142	EGG-MGC51	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
143	EGG-MGC52	1	1	0	1	1	1	1	1	1	1	1	0	0	0	0	0
144	EGG-MGC53	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
145	EGG-MGC54	1	1	0	1	1	0	1	1	0	1	0	0	0	0	1	1
146	EGG-MGC55	1	1	0	1	1	1	0	1	1	1	1	0	0	0	0	1
147	EGG-MGC56	1	1	1	1	0	1	1	1	1	1	1	0	0	0	0	0
148	EGG-MGC57	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
149	EGG-MGC58	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
150	EGG-MGC59	0	1	1	1	1	1	0	0	1	0	1	0	0	0	0	0
151	EGG-MGC60	0	1	1	1	1	1	0	0	1	0	1	1	1	1	1	0
152	EGG-MGC61	0	1	0	0	1	1	1	0	1	0	0	0	0	0	0	1
153	EGG-MGC62	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
154	EGG-MGC63	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
155	EGG-MGC64	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
156	EGG-MGC65	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0
157	EGG-MGC66	1	0	1	1	0	0	1	1	0	1	1	1	1	1	1	0
158	EGG-MGC67	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
159	EGG-MGC68	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
160	EGG-MGC69	0	0	1	1	0	0	1	0	1	0	1	1	1	1	1	1
161	EGG-MGC70	0	0	1	1	0	0	1	0	1	0	1	0	0	0	1	1
162	EGG-MGC71	1	0	1	1	0	1	0	1	0	1	1	1	1	1	0	1
163	EGG-MGC72	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
164	EGG-MGC73	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
165	EGG-MGC74	1	0	0	1	1	1	1	1	1	1	0	1	1	1	1	0
166	EGG-MGC75	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
167	EGG-MGC76	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1
168	EGG-MGC77	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
169	EGG-MGC78	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
170	EGG-MGC79	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
171	EGG-MGC80	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
172	EGG-MGC81	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
173	EGG-MGC82	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
174	EGG-MGC83	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

175	EGG-MGC84	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
176	EGG-MGC85	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
178	EGG-MGC 86	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
179	EGG-MGC 87	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1
180	EGG-MGC 88	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
181	EGG-MGC 89	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
182	EGG-MGC 90	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
183	EGG-MGC 91	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1
184	EGG-MGC 92	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0
185	EGG-MGC 93	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
186	ETG-MAC1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1
187	ETG-MAC 2	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
188	ETG-MAC 3	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
189	ETG-MAC 4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
190	ETG-MAC 5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
191	ETG-MAC 6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
192	ETG-MAC 7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
193	ETG-MAC 8	1	0	0	0	1	1	0	0	1	1	1	0	0	0	0	1
194	ETG-MAC 9	0	1	1	1	1	0	1	0	1	0	1	0	0	0	0	1
195	ETG-MAC 10	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
196	ETG-MAC 11	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
197	ETG-MAC 12	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
198	ETG-MAC 13	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
199	ETG-MAC 14	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	0
200	ETG-MAC 15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
201	ETG-MAC 16	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
202	ETG-MAC 17	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
203	ETG-MAC 18	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1
204	ETG-MAC 19	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
205	ETG-MAC 20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
206	ETG-MAC 21	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
207	ETG-MAC 22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
208	ETG-MAC 23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
209	ETG-MAC 24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
210	ETG-MAC 25	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
211	ETG-MAC 26	0	1	1	1	1	1	1	0	1	0	1	0	0	0	0	1
212	ETG-MAC 27	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
213	ETG-MAC 28	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
214	ETG-MAC 29	1	1	0	0	0	0	1	1	1	1	0	0	1	1	1	0
215	ETG-MAC 30	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
216	ETG-MAC 31	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
217	ETG-MAC 32	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1
218	ETG-MAC 33	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	1
219	ETG-MAC 34	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
220	ETG-MAC 35	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
221	ETG-MAC 36	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
222	ETG-MAC 37	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
223	ETG-MAC 38	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
224	ETG-MAC 39	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
225	ETG-MAC 40	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1
226	ETG-MAC 41	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
227	ETG-MAC 42	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
228	ETG-MAC 43	0	1	1	1	1	1	0	0	1	1	1	0	0	0	0	1
229	ETG-MAC 44	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
230	ETG-MAC 45	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
231	ETG-MAC 46	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
232	ETG-MAC 47	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
234	ETG-MAC 48	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
235	ETG-MAC 49	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
236	ETG-MAC 50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
237	ETG-MAC 51	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
238	ETG-MAC 52	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
239	ETG-MAC 53	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

240	ETG-MAC 54	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
241	ETG-MAC 55	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
242	ETG-MAC 56	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0
243	ETG-MAC 57	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
244	ETG-MAC 58	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
245	ETG-MAC 59	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
246	ETG-MAC 60	1	1	0	1	1	1	1	0	1	1	1	0	0	0	0	0
247	ETG-MAC 61	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
248	ETG-MAC 62	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
249	ETG-MAC 63	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
250	ETG-MAC 64	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
251	ETG-MAC 65	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
252	ETG-MAC 66	0	1	1	0	1	1	0	1	1	0	0	0	0	0	0	1
253	ETG-MAC 67	1	0	1	1	1	0	1	1	1	1	1	1	1	1	0	1
254	ETG-MAC 68	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
255	ETG-MAC 69	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
256	ETG-MAC 70	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
257	ETG-MAC 71	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
258	ETG-MAC 72	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
259	ETG-MAC 73	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
260	ETG-MAC 74	0	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0
261	ETG-MAC 75	1	0	0	1	1	1	0	1	0	1	1	1	1	1	1	1
262	ETG-MAC 76	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
263	ETG-MAC 77	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
264	ETG-MAC 78	1	1	1	1	0	1	1	1	0	1	1	0	0	0	0	0
265	ETG-MAC 79	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
266	ETG-MAC 80	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
267	ETG-MAC 81	1	0	1	0	1	0	0	1	1	1	1	0	0	0	0	0
268	ETG-MAC 82	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
269	ETG-MAC 83	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
270	ETG-MAC 84	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
271	ETG-MAC 85	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
272	ETG-MAC 86	0	1	1	1	1	1	1	0	1	0	1	1	1	0	0	0
273	ETG-MAC 87	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
274	ETG-MAC 88	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
275	ETG-MAC 89	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
276	ETG-MAC 90	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
277	ETG-MAC 91	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
278	ETG-MAC 92	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
279	ETG-MAC 93	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
280	ETG-MAC 94	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
281	ETG-MAC 95	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
282	EAC-MTG1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
283	EAC-MTG 2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
284	EAC-MTG 3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
285	EAC-MTG 4	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
286	EAC-MTG 5	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
287	EAC-MTG 6	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
288	EAC-MTG 7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
289	EAC-MTG 8	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
290	EAC-MTG 9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
291	EAC-MTG 10	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
292	EAC-MTG 11	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
293	EAC-MTG 12	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
294	EAC-MTG 13	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
295	EAC-MTG 14	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
296	EAC-MTG 15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
297	EAC-MTG 16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
298	EAC-MTG 17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
299	EAC-MTG 18	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
300	EAC-MTG 19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
301	EAC-MTG 20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
302	EAC-MTG 21	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1

303	EAC-MTG 22	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
304	EAC-MTG 23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
305	EAC-MTG 24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
306	EAC-MTG 25	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
307	EAC-MTG 26	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
308	EAC-MTG 27	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
309	EAC-MTG 28	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
310	EAC-MTG 29	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
311	EAC-MTG 30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
312	EAC-MTG 31	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
313	EAC-MTG 32	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
314	EAC-MTG 33	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
315	EAC-MTG 34	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
316	EAC-MTG 35	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
317	EAC-MTG 36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
318	EAC-MTG 37	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
319	EAC-MTG 38	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0

0 : Band absent, 1 : Band present

Clear polymorphism was detected in inter-specific hybrids, selfed progenies and the parents- *V.planifolia* ♀ X *V. aphylla* ♂. The profiles of *V. planifolia* and *V. aphylla* indicated that they are widely distant and banding patterns consistent with the species, were also observed indicating that these bands are species-specific.

Table 42. Paired affinity indices of selfed progenies and interspecific hybrids as expressed by AFLP markers

	V1	V2	V4	V6	V7	V8	V10	V11	V12	V24	V.pl	V.ap1	V.ap2	VH1	VH4	VH5
V1	x															
V2	89.2	x														
V4	89.2	90.7	x													
V6	89.8	91.1	91.4	x												
V7	88.8	93.3	89.8	92.0	x											
V8	90.7	92.0	91.1	92.6	93.6	x										
V10	88.5	88.5	88.8	90.4	88.2	87.6	x									
V11	94.6	88.2	86.6	88.2	88.5	87.3	88.2	x								
V12	88.8	90.7	90.4	90.7	93.0	91.7	92.0	87.3	x							
V24	97.7	90.1	88.5	91.4	90.4	89.8	88.8	95.5	90.4	x						
V.pl	91.7	91.7	93.3	93.6	91.4	91.4	90.4	88.2	92.0	92.0	x					
V.aph1	45.7	43.1	44.7	43.8	44.1	44.7	46.3	44.7	43.4	43.4	44.4	x				
V.aph2	46.0	44.1	44.4	44.1	44.4	44.4	47.3	45.7	44.4	44.4	44.7	99.0	x			
VH1	59.3	56.1	57.1	56.8	55.8	56.5	60.6	59.0	56.5	57.7	58.0	83.8	84.7	x		
VH4	60.0	59.3	59.0	58.0	58.4	56.7	63.8	59.0	59.0	58.4	60.0	80.6	81.5	93.6	x	
VH5	85.0	85.7	86.0	85.0	86.6	85.3	84.4	86.0	86.0	86.0	86.9	48.6	48.8	59.6	60.9	x

V1-V24 : Selfed progenies of *V.planifolia*; V.pl : *V.planifolia*; V.aph1, V.aph2 : *V.aphylla*, VH1-VH5 : Interspecific hybrids of *V.planifolia*♀ x *V.aphylla* ♂.

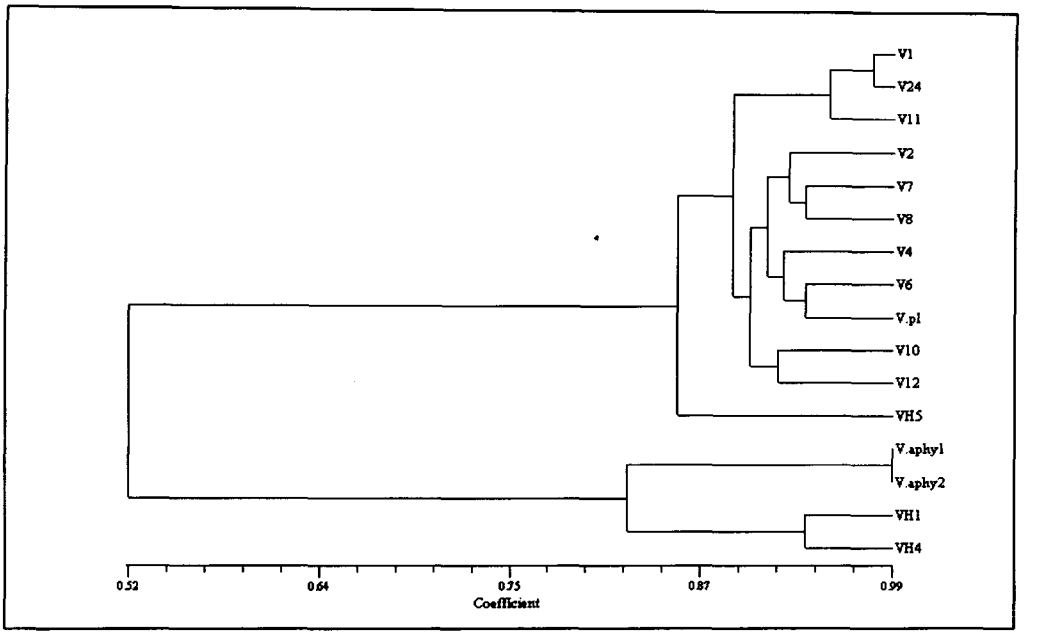


Fig. 52. Dendrogram showing linkage groups between selfed progenies and interspecific hybrids as expressed using AFLP markers

The selfed progenies of *V. planifolia* showed the banding pattern similar to that of *V.planifolia* parent and did not show the species specific bands of *V.aphylla*. These progenies did show variation in banding pattern within them indicating that there is considerable variation among the selfed progenies. Interspecific hybrids showed the banding patterns inbetween the parents in that segregation of species specific bands were noticed among the progenies further confirming hybrid nature of the progenies.

Screening of selfed progenies and somaclones for disease resistance

Thirty four lines of vanilla, including species, collections of *V. planifolia*, somaclones, seedlings, interspecific hybrids was screened against the infection of *Phytophthora meadii* (Fig. 53) and *Fusarium oxysporum* (Fig. 54), the causal agents of foot rot and wilt diseases in vanilla. The pathogen was inoculated at the

leaf axil region of the stem. The disease development was manifested as browning and water soaked patches at the axil spreading out onto the either sides of the internode and at certain times into the leaf finally leading to death of the portion of stem above the inoculation point. Reaction of the vanilla collections to infection was scored as 0 to (++++), based on the depth of penetration and disease spread (Table 36). The resistant was scored as '0' and the susceptible reaction as '+', the most susceptible reaction being scored as '++++'.

Table 43. Reaction of a few vanilla collections against infection to *Phytophthora* and *Fusarium*

Sl. No	Plant No.	Disease incidence (%)	
		Phytophthora meadii	***Fusarium oxysporum***
		Disease index	Disease index
1.	V1	++	+
2.	V2	++++	0
3.	V4	++	++
4.	V7	++++	++
5.	V3	0	+
6.	V11	+++	++++
7.	V12	++++	++++
8.	V18	+++	0
9.	V24	0	++++
10.	V32	+	0
11.	**V48**	0	0
12.	V55	0	++++
13.	V120	+	0
14.	V150	++	++++

Sl. No	Plant No.	Disease incidence (%)	
		Phytophthora meadii	*Fusarium oxysporum*
		Disease index	Disease index
15.	V8.1	+++	0
16.	VC25	++	+
17.	*V.planifolia*	++++	+
18.	*V.aphylla*	++++	0
19.	*V.andamanica1*	0	0
20.	*V.andamanica2*	0	0
21.	*V.andamanica* 3	0	0
22.	*V.andamanica* 4	0	0
23.	*V.andamanica* 5	0	+
24.	*V.andamanica* 6	0	0
25.	*V.andamanica* 7	0	0
26.	*V.andamanica* 8	++++	0
27.	Madagascar collection	0	++++
28.	Mauritius collection	0	0
29.	V. aphylla 2	++++	0
30.	Coimbatore collection	+++	++++
31.	*V.tahitensis*	++++	+++
32.	Calicut Univ.coll.	++	0
33.	Saklespur colln.	0	++++
34.	VH6	++++	++++

Index is scored as 0 : 0 % infection, + : 0-25% infection
++ : 25-50% infection, +++ : 50-75% infection
++++ : 75-100% infection

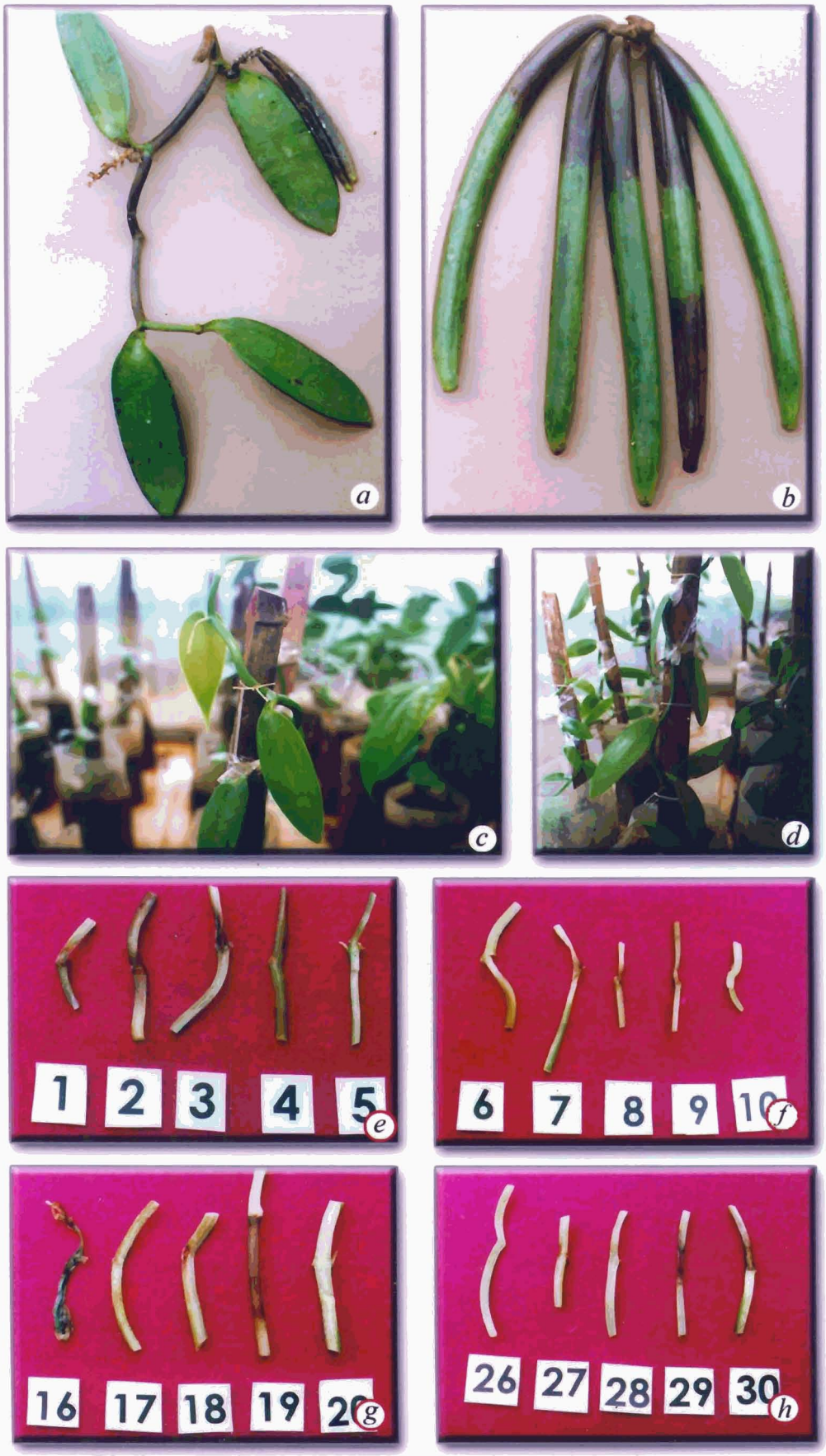


Fig. 53 Screening of Vanilla species, collections, seedling and somaclones against *Phytophthora meadii*

a. Symptoms of *Phytophthora* on adult branch, b. Symptoms of *Phytophthora* on pods, c.Population of Vanilla to be screened d.Inoculation of *P. meadii* discs on the axils of the population, e, f, g, h. Sections of nodal segments of the genotypes screened indicating the infection spread, leading to susceptibility (Note on Infection spread in nos., 20, 27, 28)

Fig.54 Screening of Vanilla species, collections, seedling and somaclones against *Fusarium oxysporum*

a. Population of Vanilla to be screened, b. Inoculation of *F. oxysporum* discs on the axils of the population, c, d. Sections of nodal segments of the genotypes screened indicating the infection spread, leading to susceptibility e, f, g. Variations in degrees of infection spread on different genotypes (Note variations viz., no infection, superficial infection, infection spreading to leaves and complete susceptibility)

The various genotypes screened showed varying degree of tolerance / susceptibility to disease incidence. In general, the plants were susceptible to infection but a few showed low disease incidence while others escaped infection..

Among the species, *V. andamanica* was most tolerant to both the pathogens. Most of the collections of *Vanilla andamanica,* gave resistant reaction to both the pathogens. *V. andamanica* 8 was susceptible to *Phytophthora* while *V. andamanica* 5 was partially susceptible to *Fusarium*. *V. aphylla* is though susceptible to *Phytophthora* did not take up infection of *Fusarium*. *V. planifolia* collections are generally susceptible but an exotic collection of *V. planifolia* from Mauritius did not take up infection to both these pathogens. Five seedling (selfed) progenies *viz.*, V8, V24, V48 and V55 were promising, as they did not take up any infection of *Phytophthora meadii* during screening. (Fig. 53). Selfed progenies V2, V18, V32, V48, V120 and callus regenerated progeny V 8.1 showed tolerance to infection *Fusarium oxysporum* (Fig. 54). Only one seedling progeny, V48, gave resistance reactions to both the pathogens. The interspecific hybrid did not show any tolerance even though its male parent *V. aphylla* showed tolerance to *Fusarium*. In the absence of any resistant line to *Phytophthora* and *Fusarium* in the exisiting vanilla germplasm, the disease escapes identified from this study could be considered as potential candidates to be exploited for identification of disease resistant lines.

IN VITRO CONSERVATION STRATEGY FOR VANILLA GERMPLASM

Synthetic seeds

In vitro regenerated shoot buds, protocorms and regenerating calli were used in the present study as propagules for encapsulation. Sodium alginate at 4% was sufficient to produce good quality rigid beads in vanilla, higher

concentrations were not suitable as they produced very hard matrix which hindered the emergence of shoot buds and thereby affecting the rate of germination and recovery. Lower concentrations of alginate were also not suitable because it was difficult to handle. Shoot buds of 0.4 - 0.5 cm size were suitable for encapsulation as smaller buds failed to survive the storage and lost their viability within a month.

The synthetic seeds were stored at 5^0C, 15^0C and 22^0C to study the effect of temperature on their storage and viability. Low temperatures (5^0C and 15^0C) were not suitable for synthetic seed storage as they lost their viability within thirty days. But at 22 ± 2^0C, synthetic seeds could be stored successfully upto ten months in sterile water. The beads germinated normally when transferred to MS medium and multiplication of the buds was observed when transferred to MS medium supplemented with BAP and IBA (Fig. 55g).

When cultured on MS medium supplemented with BAP (1 mgl^{-1}) and IBA (0.5 mgl^{-1}), maximum germination (80%) was observed in two weeks. The buds multiplied in the same medium in 60 days of culture.

The plants derived from these encapsulated buds were apparently healthy and developed into normal plantlets. Synthetic seeds are ideal for germplasm conservation and exchange, especially since there is no natural seed set.

***In vitro* conservation by slow growth**

Three temperature regimes (5^0C, 10^0C, 22^0C), four types of culture vessel closures (cotton plugs, screw caps, aluminium foil, polypropylene caps), MS basal medium in half or full strength, mannitol (osmoticum) at two levels (10 gl^{-1},15 gl^{-1}), sucrose (carbon source) at five levels (30 gl^{-1},20 gl^{-1}, 15 gl^{-1},10 gl^{-1},and0 gl^{-1})

Fig. 55 *In vitro* conservation of Vanilla

a. Induction of slow growth in *V. planifolia*, b. Induction of slow growth in *V. aphylla*, c. Comparison of shoot tip in minimal growth medium (left) to that in normal growth medium (right), d. Cultures of *V. planifolia* under slow growth, e. Vanilla germplasm (Species, Collections, Selfed progenies, Interspecific hybrids and somaclones) conserved *in vitro*, f. Synthetic seeds of Vanilla shoot buds encapsulated in sodium alginate beads, g. Germinating synthetic seeds of *V. planifolia* after six months of storage

in various combinations, were tested for their efficiency in inducing minimal growth and increasing subculture interval in vanilla.

Effect of temperature on longevity of *in vitro* cultures

Of the 3 temperature regimes tested only 22±2^{0}C was found suitable for growth of vanilla cultures. Temperatures below 22^{0}C were deleterious and not suitable for plant growth. At 5^{0}C and 10^{0}C, all the cultures turned yellow and died within 20 days (Table 44). This indicates that low temperatures are detrimental to vanilla cultures and 22±2^{0}C is ideal for maintaining vanilla cultures over longer periods.

Table 44 . Effect of temperature on survival of vanilla shoot cultures*.

Temperature (^{0}C)	Survival period (days)@	Survival (%)@	Growth response
5	20	0	No growth , cultures died
10	20	0	No growth, cultures died
22	360	80	Minimal growth

* ½ MS + 15 gl^{-1} each of sucrose and mannitol, @ Mean of 20 replicates

Effect of various closure types on longevity of cultures :

Different types of closures *i.e.*, cotton plugs, screw caps, aluminium foil and polypropylene caps were used to study their effect on the longevity of cultures (Table 45).

Table 45. Effect of vessel closures on growth and survival of vanilla shoot cultures@

Closure types	Survival period (days)*	Survival (%)*	Plant height (cm)*	No. of shoots / tube*
Cotton plugs	200	70	7.4±1.48	1
Screw caps	360	80	6.7±0.87	1
Aluminium foil	360	90	7.7±0.98	1
Polypropylene caps	360	80	7.8±1.24	1

@ : ½ MS + 15 gl^{-1} each of sucrose and mannitol, * Mean of 20 replicates

Though the cotton plugs allowed better gaseous exchange, it resulted in moisture loss leading to drying up of cultures. But when the culture tubes were closed with screw caps, polypropylene caps or aluminium foil, the moisture loss was minimal resulting in healthy and fresh cultures even after 360 days in the medium, which induced slow growth (Fig. 56 c, d). One thousand and seventy five accessions of vanilla germplasm have been conserved in the *in vitro* repository of Indian Institute of Spices Research, using the method described.

Effect of various media combinations on induction of minimal growth in vanilla:

The effect of nutrient medium and sucrose at various levels, separately and in combination with mannitol, on growth of vanilla cultures are given in Table 46.

Table 46. Effect of various media components on induction of minimal growth in vanilla cultures[@]

Media components			Growth rate*		Storage period (days)*
Basal medium	Sucrose (gl^{-1})	Mannitol (gl^{-1})	Plant ht (cm)	No. of shoot/tube	
Full MS	0	0	2.4±0.46	1.0±0.62	30
	10	0	4.7±0.73	1.2±0.71	200
	20	0	9.3±0.80	1.8±0.65	200
	30	0	11.3±1.03	2.2±0.82	180
	20	10	9.0±0.68	1.2±0.11	280
	15	15	8.2±0.87	1.2±0.08	360
	10	10	7.7±0.56	1.5±0.10	360
½ MS	0	0	2.5±0.72	1.1±0.01	20
	10	0	3.5±0.54	1.2±0.12	120
	20	0	9.2±2.02	1.1±0.14	240
	30	0	9.2±0.76	1.2±0.88	180
	20	10	8.2±0.84	1.0	360
	15	15	7.8±0.93	1.0	360
	10	10	6.7±0.87	4.0	360

* Mean of 20 replicates, Initial plant ht = 2 cm, [@]:In screw capped tubes at 22±2°C

MS medium when used in half strength supported 'normal' growth and development of plantlets. The rate of growth was higher when full strength MS

medium was used. High concentration of sucrose ($30gl^{-1}$) increased culture growth substantially resulting in exhaustion of culture medium.

In full strength MS medium with 30 gl^{-1} sucrose, the cultures grew faster and filled the culture vessel within 180 days resulting in drying up of cultures. When the concentration of sucrose was reduced to 20 gl^{-1} and nutrient concentration to half, the cultures could be maintained for much longer period of 240 days with a survival percentage of 80 in sealed culture tubes. In all media containing full strength MS salts, the cultures could be stored only upto a maximum of 200 days with 70% survival, when the culture vessels were closed with cotton plugs, irrespective of the other parameters. Addition of mannitol (10-15 gl^{-1}) and reduction of sucrose to lower levels (15-10 gl^{-1}) induced slow growth and subsequently 80-90% of the cultures could be maintained for a period of 360 days, when the culture vessels were closed with screw caps, aluminium foil or polypropylene caps. The best result was observed when mannitol and sucrose were added in equal proportions at $10gl^{-1}$ or 15 gl^{-1}, the cultures could be maintained for 1 year (Fig.55a -e). Thus out of 14 different combinations tested only five *i.e.*, full or half strength MS medium supplemented with 10 or 15 gl^{-1} each of sucrose and mannitol and ½ MS with 20 gl^{-1} sucrose and 10 gl^{-1} mannitol, allowed the cultures to be maintained for 360 days. In all these, the closure used was aluminium foil. The plantlets maintained in this medium showed reduced growth rate and maximum survival. In general, culture tubes covered with aluminium foil rendered longer duration of subculture interval compared to cotton plugs due to minimisation of water loss in the former.

The conserved material was transferred to the multiplication medium (MS + 30 gl^{-1} sucrose and 1 mgl^{-1} NAA) for normal growth. The small sized plantlets

kept in the conservation medium for over one year showed good growth and developed into normal sized plants with good multiplication rate (1:5). These plantlets were transferred to soil (garden soil : sand : perlite in equal proportions) and established easily with 80 percent success when kept in humid chamber for 20-30 days after transfer. They developed into normal plants without any deformities and deficiency symptoms and exhibited apparent morphological similarities to the mother plants.

Long term conservation by cryoconservation

Cryopreservation of vanilla shoot tips by encapsulation dehydration

Shoot buds of about 2mm – 5mm were dissected out from *in vitro* grown cultures of vanilla. Shoot buds desiccated for 1-2h in the air current of laminar flow chamber. Shoot buds were given cryoprotection using DMSO and glucose at 5% and 2.5% concentration, singly and in combination. The samples were kept at 4^0C and 25^0C for 24h. The cryovials along with the shoot buds were then directly plunged into LN_2 and kept for 1h. After 1h rapid thawing was done by immersing the vials in 38^0C water bath for 10m. The shoot buds were taken out of the vials and cultured on recovery medium (MS basal medium supplemented with 3% sucrose, 1 mg/l BAP and 0.5 mg/l NAA). The controls, which were not given the LN_2 treatment were also cultured in the same medium. After 1 week the beads were broken and the shoot buds were cultured on same combination of solid medium for further growth responses.

Shoot tips, which were desiccated for 1h exhibited 90% recovery in the control, whereas the LN_2 treated ones showed only 20% viability. Desiccation for 2h was lethal and shoots dried up. Shoot tips which were precultured in 5% DMSO at 25^0C for 24h, showed 90% viability in terms of retaining the green

colour immediately after thawing and initiation of growth after 2 weeks of culture. The same treatment at 4^0C, was not promising, as it showed vitrification even though remain green soon after reculture. In all other treatments the shoot tips turned white and / vitrified and no favourable growth response was observed.

Pollen cryopreseravation

Effect of sucrose on pollen germination

The germination capacity of the fresh pollen grains collected from flowers of three different maturity levels were assessed by *in vitro* germination in Brewbaker and Kwack (BK) medium with three different sucrose concentrations (5%, 10%, 15% and 20%). Pollen germination was considered to occur *in vitro* when a pollen tube had grown at a length twice the diameter of the pollen grain. The results indicated that pollen germination required the addition of sucrose to the medium (Table 47). The highest percentage of pollen germination was observed at 10% sucrose for both the species of *Vanilla* and in all other experiments BK medium with 10% sucrose was used. The pollen collected from opened flowers showed maximum percentage of germination and there was no significant difference in germination of pollen collected from flowers one day prior to opening. There is drastic reduction in germination percentage of pollen collected from flowers 2 days prior to opening. In all other cryopreservation experiments pollen were collected from freshly opened flowers.

Table 47. Effect of sucrose and stage of pollen on percentage pollen germination

Sucrose concentration (%)	Percentage germination					
	V. planifolia			*V. aphylla*		
	Opened flower	1d prior to opening	2d prior to opening	Opened flower	1d prior to opening	2d prior to opening
0	6.0±2.31	5.4	0	7.1 ± 4.01	5.2	0
5	11.9±3.6	11.2	0	10.8 ±3.2	11.0	0
10	89.4±8.0	80.1	0.15	95.1± 2.0	92.2	0
15	73.6±5.91	73.0	0	40.5± 2.0	82.0	0

SE: 4.98; CV: 12.9, CD: 9.088

Effect of cryogenic preservation on in vitro germination

The pollen samples of vanilla responded well to the 1h to 7d cryogenic exposure. In all the different treatments tried all the pollen samples survived freezing. The maximum percentage of germination was observed in pollen desiccated for 5 minutes in the air current of laminar air flow followed by desiccation for 5 minutes and cryoprotection with 5% DMSO. In this case the pollen germination was 73.4 % and 82.1 % respectively in *V. planifolia* and 75% and 75.4% in *V.aphylla.* In all other treatments viability was drastically reduced, and abnormalities in pollen germination such as bulging, bursting of pollen tube tip, branching of pollen tube, feeding bottle type of pollen tube development etc were observed in 9.5 % of the pollen (Fig.56). The percentage of normal pollen germination in treatments without any cryoprotection was 91.4 % in *V. planifolia* and 74.01 % in *V. aphylla* (Table 48).

Table 48. Pollen viability in vanilla after cryopreservation as indexed by *in vitro* germination

Sample	*V. planifolia*	*V. aphylla*
	Germination (%)	**Germination (%)**
1. Fresh pollen	91.40	74.01
Cryopreserved pollen		
2. 5 m dessication	73.4	75.0
3. 1h desiccation	56.0	35.0
4. 24h desiccation	48.0	20.8
5. 5% DMSO + LN2	2.4	0.65
6. 5m desiccation + 5% DMSO + LN2	82.1	75.40
7. 10m desiccation + 5% DMSO + LN2	38.2	53.33
8. 15m desiccation + 5% DMSO + LN2	40.5	68.89

Effect of cryopreservation on pollen fertility and hybridisation

Fertility of cryopreserved pollen was tested by controlled field pollinations. Flowers of the desired female parent pollinated with cryopreserved pollen after thawing. It was observed that the pollen cryopreserved for various intervals was fertile. Pollination with this pollen induced fruit set and seed set. This indicated the retention of pollen fertility after cryogenic storage. Cryopreserved pollen from all the treatments induced fruit set even though there was very limited percentage of normally developed pollen grains observed in *in vitro* germination of some of the cryopreserved samples. The seed were germinated in culture and gave rise to normal PLBs and subsequently seedlings were developed.

Cryopreservation of pollen from two *Vanilla* species, *V. planifolia* and *V. aphylla* was successfully achieved. The normal pollen viability is 80-90% in both *V. planifolia* and *V. planifolia* (Fig.56). In the cryopreservation trials, vanilla pollen desiccated for 5 m and treated with 10% DMSO for 10m gave about 80% germination in *V. planifolia and V. aphylla*). In *Vanilla* species the pollination using cryopreserved pollen was also successful and fruit set was achieved (Fig. 56). The seeds from these fruits germinated in culture.

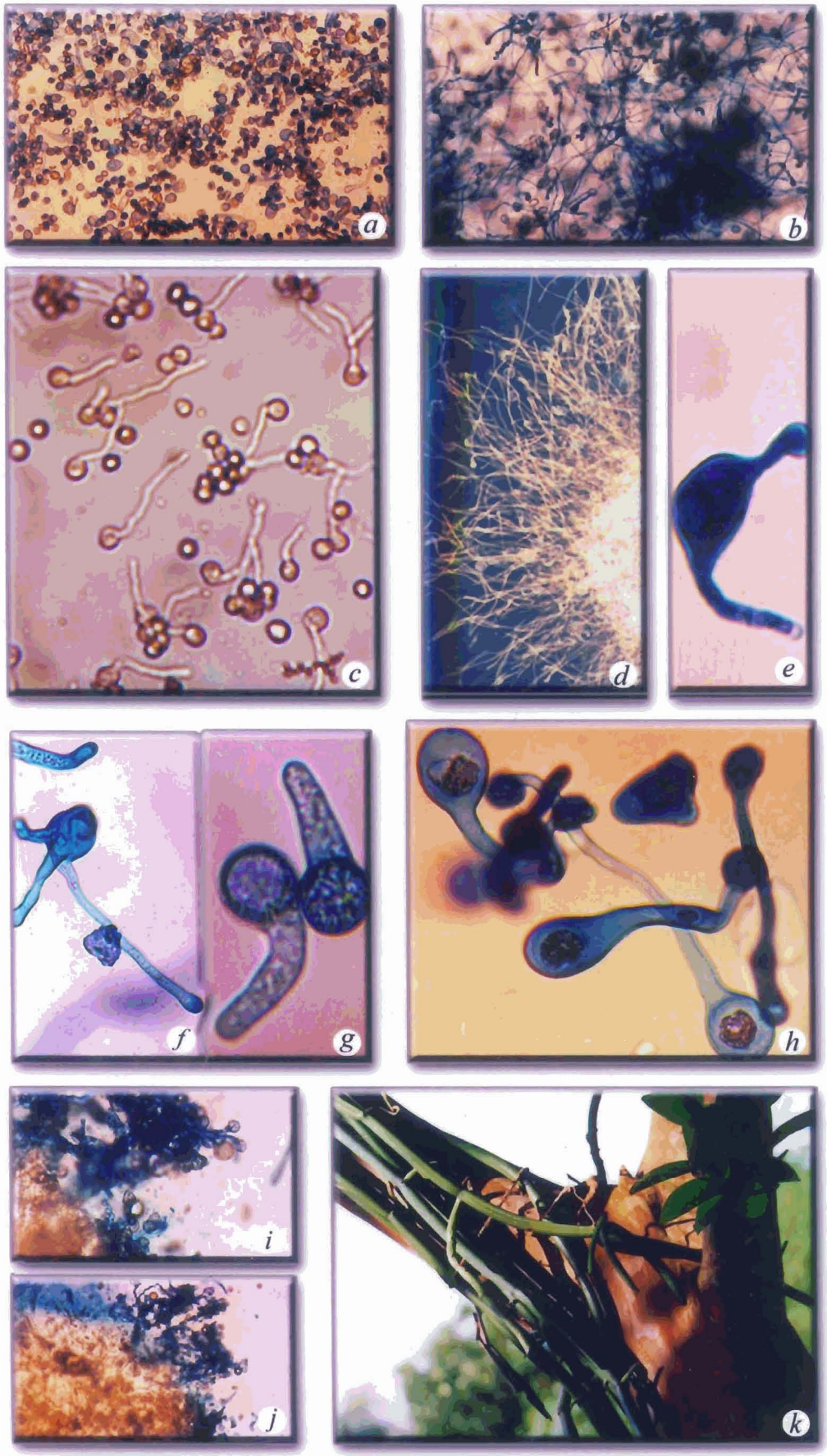


Fig.56 Cryopreservation of pollen in Vanilla and utilization in interspecific hybridization

a.Germination of pollen in *V. planifolia*, b.Germination in *V. aphylla*, c.Germination in cryopreserved pollen of *V. planifolia*, d. Germination in cryopreserved pollen of *V. aphylla*, e, f, g, h. Pollen germination in Vanilla (feeding bottle type, pollen tube branching, normal germination and tube tip bursting, i, j. Pollen germination on stigmatic surface, k. Fruit set after interspecific hybridization (*V. planifolia* pollen introduced into *V. aphylla*)

Discussion

Minoo Divakaran “Seedling and somaclonal variation and their characterization in Vanilla ” Thesis. Indian Institute of Spices Research Calicut, University of Calicut, 2002

Discussion

Vanilla, an important spice, is the fermented and cured fruit of the orchid, *Vanilla planifolia* Andrews (syn. *V. fragrans* Salisb.). *V. planifolia* is a tropical climbing orchid, indigenous to wet low land forests of South East Mexico, Gautemala and Central America. It is now cultivated in other parts of the tropics, especially in the Malagasy Republic, Indonesia, Reunion and the Comoro Islands (Purseglove *et al.*, 1981).

The germplasm base of *V. planifolia* is narrow due to limited breeding and recent domestication and is further threatened by deforestation and over exploitation. Since the narrow primary gene pool is evidently threatened, the secondary gene pool that is, the close relatives of *V. planifolia*, becomes more important as a source of desirable traits like self-pollination and disease resistance. Some of these traits are found in its near relatives (Rao *et al.*, 2000).

Vanilla was introduced into India in the 18th century and practically no variability is available in cultivated vanilla. This leads to monoculture making vanilla susceptible to diseases and pests. Vanilla is suspected to be highly heterozygous because of its cross-pollinated nature and variations could be obtained in the segregating seedling progenies. Nair and Ravindran (1994) reported mitotic association in vanilla, which could give rise to further variation in seedling progenies. Somaclonal variation can also form an important source of novel genetic variability. Interspecific hybridization will help in bringing together certain useful characters into cultivated vanilla from wild relatives. The resultant variations in the progenies, somaclones and hybrids could be used in crop improvement especially to develop high quality, self-pollinating and disease resistant genotypes.

The main objectives of the present investigation are to study the existing variability in some of the *Vanilla* species and cultivars available in India; to broaden the spectrum of variations in vanilla gene pool using conventional as well as biotechnological approaches; to characterize the extent of variability generated and to standardize protocol for *in vitro* conservation of vanilla germplasm. Attempts were made to standardize micropropagation, callus regeneration and protoplast culture protocols and also to produce large number of selfed progenies, somaclones and interspecific hybrids. Attempts were also made to characterize some of the species, progenies, somaclones and hybrids using morphological, cytological, biochemical and molecular markers, wherever possible. A few of them were also screened for reaction against *Phytophthora meadii* and *Fusarium oxysporum,* the two major pathogens affecting vanilla plantations.

PRODUCTION OF SEEDLINGS (SELFED PROGENIES) AND SOMACLONES

Hand pollination and fruit set

Successful hand pollination with 90–100% fruit set was achieved in experiments done during 7 consecutive years. More than 80% of the fruits were with the persisting floral parts indicating effective pollination (Fig. 4d). Fruits are of normal size and attained maturity in 9 months. The matured fruits contained numerous black powdery seeds.

Professor Charles Morren was the first to develop hand pollination technique in vanilla (Purseglove *et al.,* 1981). Bhat and Sudharshan (2000) reported that the size of the bean depends on the pollen load and efficiency of pollination.

In vitro Seed Culture

In vitro seed germination

Orchid seeds are characterized by their minute size and lack of any storage tissues, which is required during germination of seeds and development of seedlings. In nature, association with a specific fungal partner is a pre-requisite for orchid seed germination (Arditti, 1967; Mitra, 1986).

In the present study seeds could successfully be germinated *in vitro*, from the fourth month of fruit setting onwards. However, seeds harvested in the 6^{th} month and cultured *in vitro* gave the maximum (87%) germination.

Among various media combinations tested, though seed germination was observed in all the media, the percentage of germination ranged from 26% to 87%. The minimum germination was observed in MS medium at half strength and maximum was recorded in MS medium supplemented with 2 gl^{-1} tryptone. Though combination of cytokinin and auxin was found to be better for seed germination, there was no significant difference in germination recorded in MS with BA alone.

Germination of seeds began within 4 weeks of culture and the initial stages of germination were typical of most orchids, such as swelling of the embryo followed by rupturing of the seed testa and subsequent emergence of protocorms. Seeds germinated directly into plantlets in the medium supplemented with BA (0.5 mgl^{-1}) alone, without any intervening callus phase, hence this medium was selected for the germination of vanilla seeds and production of selfed progenies/seedlings throughout the study. Germinating seeds multiplied when cultured on MS medium supplemented with BA (1 mgl^{-1}) and IBA (0.5 mgl^{-1}).

In MS medium supplemented with NAA, callus was induced. In MS medium supplemented with BA (1.0 mgl^{-1}) and NAA (0.5 mgl^{-1}), regeneration of protocorm like bodies (PLBs) occurred from these calli. About 5-20 PLBs were formed from each mass and each of them was capable of regenerating into individual shoot and plantlet. These somaclones were studied in comparison with vanilla and its seedlings.

Production of colchiploids was also attempted as a tool to further increase variability in *Vanilla*. In MS medium supplemented with colchicine (0.002%) the seed germination was as high as 87%. Further development of seedlings was slow if, left in the same medium and hence the seeds developed in colchicine medium were transferred to MS medium supplemented with BA (1.0 mgl^{-1}) and NAA (0.5 mgl^{-1}) immediately after germination.

The use of BA, with or without addition of NAA, was inhibitory to seed germination and lowered frequency significantly. Presence of exogenous cytokinin has been found to improve, inhibit or at times produce no effect on germination of orchid seeds. Orchid seeds that do not require exogenous cytokinin for germination are known as cytokinin autonomous, since they contain sufficient endogenous cytokinin (De Pauw, 1995; Mereier and Kerbauy, 1991). The requirement of cytokinin for germination is considered to be related to the utilization of lipids that constitute primary storage material in most orchid seeds and it has been observed that unless storage lipid is utilized, germination does not continue (De Pauw, 1995; Manning and van Staden, 1987). In many terrestrial species like *Cypripedium reginae* and *C .candidum*, a definite cytokinin preference has been reported (De Pauw, 1995; Harvais, 1982; van Waes and

Debergh, 1986). In all these species a low level of cytokinin has been found to be optimum. Inhibitory effect of BA in *Geodorum densiflorum* was reported and was evident by the high frequency of germination in its absence (Roy and Banerjee, 2001). Interactions between auxin and cytokinin has been reported in seed cultures of *Cypripedium reginae* where auxin could counteract the inhibitory effect of supra-optimal cytokinin on seed germination (Harvais, 1982).

In the present study, seed culture in different basal media has indicated that *Vanilla* seeds have no stringent nutritional requirements for initiation of germination (Table 15 and Figs. 5, 6) unlike some terrestrial orchids of temperate climate, which show better responses in low salt media as reported earlier (Fast, 1982; van Waes and Debergh, 1986). In addition the germination efficiency is excellent. Previous studies with *in vitro* culture of orchid seeds have shown that different species require different and often specific nutrient media, organic additives and plant growth regulators (Arditti, 1967; De Pauw *et al.,* 1995; Mitra, 1986; Oliva and Arditti, 1984; Roy and Banerjee, 2001). Seed culture in vanilla was earlier reported by (Withner, 1950, 1955)

Development of protocorms

The protocorms appeared as translucent white bodies, in early stage of growth and development, with a pointed basal end, which later became swollen and gave rise to numerous tiny leaf primordials. Simultaneously rhizoids also developed, protocorms then developed green colour and also increased in size. In treatments with BA, most of the protocorms remained the same with the scale like leaf primordial and developing into shoots whereas treatment with auxin supplements showed gradual disorganization of the protocorms into callus.

Wherever there is high protocorm development mortality of protocorms were noticed. Maximum protocorm death occurred in media that induced faster growth and higher germination, this could be due to depletion of nutrients in the culture media and could be overcome by less denser inoculation of the seeds. Generally necrosis of orchid seeds of developing protocorms is a common phenomenon and is found to be associated with excessive phenolic production in *Cypripedium reginae*. This is due to induction of polyphenol oxidase activity, usually brought about by certain cultural conditions (Harvais, 1982). Protocorm mortality is also reported to be due to improper nutrients and lack of essential growth stimulating substances (Stoutamire, 1964) or also presence of non-viable embryos that enlarge and breakthrough the seed testa following imbibition of moisture, as in *C. candidum* (De Pauw *et al.,* 1995). In *Geodorum densiflorum*, high death rate was found in medium supplemented with NAA and may be accorded to the toxic effect of this auxin (Harvais, 1982; Roy and Banerjee, 2001).

The present study indicated that addition of tryptone brought about a growth promoting effect on the size and development of protocorm irrespective of the basal medium to which it was added. In general, reports on tryptone usage in orchid culture media are not yet well established, however, a few species have shown improvement in germination and growth (Curtis, 1947; Krishnamohan and Jorapur, 1986). Earlier authors suggested that tryptone could be a source of organic nitrogen, comprising primarily of various amino acids, thus supplying ample exogenous amino acids, especially in the early stages of development, when endogenous biosynthesis of amino acids may not be adequate for healthy and faster growth (Roy and Banerjee, 2001).

Development of seedlings

Rapid seedling development occurred in germination medium supplemented with BA and IBA. Shoot multiplication was best in this medium, however, on transfer to growth regulator free medium, the shoots developed good root system, in contrast to the BA supplemented media and growth of existing shoots only was observed with rare axillary branching.

The shoots developed good root system when transferred to growth regulator free medium, in contrast to the BA supplemented media. Inhibitory effects of many cytokinins on seedling development have been reported in angiospermic plants (Nielsen *et al.,* 1993; Sutter, 1996). NAA supplemented medium was found to induce thick fleshy velamen like roots, which were of a disadvantage in the hardening stages (Minoo *et al.*, 1997; Roy and Banerjee, 2001).

Micropropagation

Micropropagation was standardized for *V. planifolia, V. aphylla, V. andamanica* and *V. pilifera.* MS supplemented with BA ($1mgl^{-1}$) + IBA ($0.5\ mgl^{-1}$) was the most efficient in inducing multiple shoots in all the species studied. The culture media and conditions favourable for micropropagation of *V. planifolia* was suitable for other related species like *V. andamanica, V. aphylla* and *V. pilifera* also. However, the number of shoots induced in different species varied (Table 17). About 12-15 shoots / culture could be induced in *V. planifolia*, followed by *V. aphylla* (8-10 shoots). Among the species studied, the lowest multiplication rate was observed in *V. pilifera* (Fig. 9k) with only 2-4 shoots in 120 days. *Vanilla andamanica* also responded in the same multiplication medium with 5-7 multiple

shoots per culture.

Micropropagation of *V. planifolia* was reported earlier by various workers (Cervera and Madrigal, 1981; Kononowicz and Janick, 1984; Philip and Nainar, 1986; Duang and Hong, 1989; George *et al.*, 1995; Samuel Ganesh *et al.*, 1996; Minoo *et al.*, 1997). However, the protocol developed in the present study is more efficient in the production of more multiple shoots, and hence can be used for large scale multiplication of disease free plants in all these species. Earlier reports are not available for micropropagation of *V. aphylla, V. andamanica* and *V. pilifera.*

Callus regeneration and production of somaclones

Among the various media combinations tried for callus induction and regeneration, the most suitable medium was MS medium fortified with NAA (0.5 mgl^{-1}) and BA (1.0 mgl^{-1}) in which 75% of the cultures developed callus and an average of 10 plants could be regenerated from them (Table 18). In certain cases the regeneration was *via* development of protocorms like bodies (PLBs) from callus. Only callus was induced in media supplemented with NAA (0.5 mgl^{-1}), but no regeneration was obtained. Both seedlings as well as PLBs showed the same response. The regenerated plants induced from callus tissue produced roots in growth regulator free medium. This induced rooting in over 90% of the shoots.

Potential of protocorm derived calli to regenerate PLBs in developing a callus associated micropropagation system in *Geodorum densiflorum* has been discussed by Roy and Banerjee (2001). Successful plant regeneration from callus was also reported in vanilla (Xju *et al.*, 1987; Davidonis and Knorr, 1991). Development of callus regeneration systems was also reported in other orchids

like *Cymbidium* (Chang and Chang, 1998), *Phalaenopsis* (Chen *et al.*, 2000), *Paphiopedilium* (Lin *et al.*, 2000) and these morphogenesis systems are being used to explore the control process on reducing juvenility and precocious flowering *in vitro*. This is the first report of plant regeneration of vanilla through callus from India. This efficient regeneration system can be utilized for creation and exploitation of somaclonal variation.

Other in vitro responses

Conversion of root tips into shoots was observed in *V. planifolia* (Fig. 8) and *V. aphylla* (Fig 9) when cultured on MS medium supplemented with BA (1.0 mgl^{-1}) and IBA (0.5 mgl^{-1}). These shoots developed into plantlets and were hardened and established in soil. Conversion of root meristem in to shoots in *in vitro* cultures of vanilla was earlier reported (Philip and Nainar, 1988).

Fasciation, the term derived from the Latin fasces or fasciculum, meaning a bundle or packet, is used to describe an unusual anomalous sort of growth or meristems side by side. When these multiple meristems then produce more tissues or organs, the structures are often abnormal, crowding together in band like or clumped configurations, often with prized or bizarre shapes.

An enlargement and flattening of the plant structure especially the stem was observed in *V. planifolia* (Fig. 8) and *V. aphylla* (Fig. 9) cultures. It occurred when BA was present in the culture medium at a concentration of atleast 1.0 mgl^{-1}. The branches shortened and the leaves became so modified they were usually no longer recognizable as leaves. This could possibly be due to a change or mutation taken place during the development of a meristem and before the meristematic cells was channeled into various paths of differentiation. This is the first report of

occurrence of fasciation in vanilla cultures and could be of use as a source of variability.

Interspecific hybridisation between *V. planifolia* and *V. aphylla*

Interspecific hybridization between *V. planifolia* and its related species *V. aphylla* was successfully achieved which can be used to increase the spectrum of variations by bringing desirable characters from wild species into the cultivated vanilla. Only 4 plantlets could be obtained from the 11 crosses made. The interspecific hybrids exhibited segregation of both the parental characters in that VH1 and VH6 are planifolia types (leafy) while VH4 and VH5 are aphylla types (leafless). The expression of male parent character - leafless type, indicates the hybridity of the progenies.

Development of interspecific hybrids and utilisation of RAPD markers to characterise them in *Carica* has been made by Drew *et al.* (1998) and Magdalita *et al.* (1998) respectively. Only a few cases of genetic incompatibility (Sanford, 1964; 1967) between closely related species in orchids have been reported. Occurrence of natural interspecific hybridization was suggested between *V. barbellata*, *V. claviculata* and *V. dilloniana* based on the enzyme assay of the vanilla populations in Caribbean Islands by Nielssen and Siegismund (1999). This is the first report of successful interspecific hybridization and production of hybrids between *V. planifolia* and *V. aphylla.*

Ex vitro *establishment of seedlings*

Substantial number of tissue-cultured plants does not survive transfer from *in vitro* conditions to green house or field environments, due to exposure to substantially lower relative humidity, higher light and septic environment. The

'delicate' nature of plants raised *in vitro* results in high mortality if they are transferred directly from the culture vessel to the natural environment. Most plant species grown *in vitro* require a gradual acclimatization and hardening for survival and growth in natural environment (Preece and Sutter, 1991; George, 1996).

The survival of *in vitro* plants depends upon their ability to withstand water loss and carry out photosynthesis. The ability of shoots to withstand water loss ultimately depends upon the presence of roots capable of absorbing water from soil. The death of *in vitro* induced roots was observed in many plants during hardening. The possible reason for this may be due to an intercalary callus phase between roots and shoots and adventitious roots produced *in vitro* are from primary vascular system with poor conductance. Under these circumstances plant survives the acclimatization process only by the development of new roots *ex vitro*. Plants with good root system only can be more easily acclimatized.

In the present study, the survival rate of transferred plants was over 80% during hardening process. Initiation of new growth occurred through development of the axillary branch. These plants were successfully transferred to soil after initial hardening period of 3 weeks.

Protoplast isolation and culture

Isolation of protoplasts from* in vitro *leaf tissue

The techniques of protoplast isolation and fusion are important because of the far reaching implications in studies of plant improvement by cell modification and somatic hybridisation. Since the first report on the regeneration of complete plants from isolated protoplasts (Takebe *et al.*, 1971) tremendous progress has been made and a number of crops of commercial importance such as potato,

tomato, tobacco, rice, linseed, alfalfa, cucumber, eggplant, lettuce, *Brassica* species etc., have been routinely regenerated and this paved the way for their genetic manipulation. The refinement in the technology has also enabled the successful culture of somatic hybrids/cybrids at interspecific and intergeneric levels with far reaching implications in crop improvement programmes for widening the pool of genetic resources (Bajaj, 1989c, 1989d). The successful isolation of protoplasts, their culture and subsequent regeneration into complete plants depend on a number of factors, the important ones being genotype, tissue from which protoplasts are isolated, the physiological conditions under which the plant cultures have been raised, purity of the enzymes and the osmoticum, period of incubation, culture media and growth regulators, milieu of protoplasts/plating density, method of culture (liquid/solid), incubation conditions etc.

The enzymatic (cellulase) isolation of protoplasts was first reported in tomato from root tips by Cocking (1960). The easy availability of commercial purified enzymes such as cellulase, cellulysin, pectinase, macerozyme, driselase, rhozyme, hemicellulase etc. has now resulted in increase in the yield and viability of protoplasts and their subsequent response in the culture medium. The period of treatment is reduced and thus deleterious effects on plasma membrane are avoided. Usually a combination of pectinase and cellulase is used to macerate cells and also liberate protoplasts in a single cell (Power and Cocking, 1970). The concentration and the combination of enzymes required depend upon a number of factors such as age, genotype and stage of differentiation of the tissue from which the protoplasts were to be isolated. Though protoplasts can be isolated from a variety of tissues, young *in vitro* grown plants (Bajaj, 1972), tissues and explants

such as root tips (Xu *et al.*, 1982), hypocotyl (Glimelius, 1984), cotyledons (Hammatt *et al.*, 1987) and shoots (Russell and McCown, 1986) require low concentrations of enzymes and relatively short period of treatment as compared to leaves from old or mature plants. Cells and tissues grown *in vitro* particularly suspension and callus cultures in exponential growth stage are also important sources of protoplasts (Wallin and Eriksson, 1973).

Protoplasts were successfully isolated from *V. planifolia* and *V. andamanica* when incubated in an enzyme solution containing macerozyme R10 (0.5%) and cellulase Onozuka R10 (2%) for 8 hours at 30°C in dark. The isolation solution containing 9% mannitol was found necessary for releasing and maintaining viable protoplasts. The isolated protoplasts were round and were filled with chloroplasts. The protoplast yield was 2.5 x 10^5 per gram and 1.0 x 10^5 gram of leaf tissue and protoplast viability was 72% and 55% in *V. planifolia* and *V. andamanica* respectively. Protoplasts of *V. planifolia* were bigger in size (0.031 mm) than *V. andamanica* (0.022 mm). The protoplasts can be further distinguished by the arrangement of chloroplasts. Chloroplasts were arranged around the periphery in *V. planifolia* whereas in *V.andamanica* they remained scattered in the center.

The present study indicated the requirement of lower concentration of macerozyme R10 and Onozuka cellulase R10 and shorter incubation period for release of protoplasts. The visibly distinguishable nature of protoplast can be exploited for the purpose of genetic transformation in these species.

Protoplast fusion

Protoplast fusion was attempted in the two species of *Vanilla.* The

protoplasts of the two species were visibly different (Fig. 13) due to their size and arrangement of chloroplasts and thus providing an useful method to differentiate the protoplasts of the two species. When subjected to PEG mediated fusion, the protoplasts of the two species come adjacent and formed a passage gradually leading to the transfer of the cell contents of one species into another. The fusion product (heterokaryon) could easily be identified due to the difference in chloroplast arrangement. This can be very useful in gene transfer for useful traits, especially the natural seed set and disease tolerance observed in *V. andamanica* to *V. planifolia*. The fusion product was cultured on MS liquid medium with 0.5 mgl $^{-1}$ BA, 0.5 mgl $^{-1}$ IBA supplemented with 3% sucrose and 7% mannitol for 20 days. The cell wall development around the fusion product was observed after 36 hours.

The possibility of protoplast systems in spice crops such as cardamom, ginger and vanilla was studied by (Triggs *et al.*, 1995, Geetha *et al.*, 2000).

This is the first report of protoplast isolation in *V. andamanica* and PEG mediated fusion in *V. planifolia* and *V. andamanica.*

CHARACTERISATION OF SELFED PROGENIES AND SOMACLONES

Morphological characterization

The success of any *in vitro* culture technique depends either on the ability to clone the genotypes for production of uniform planting material, or the ability to bring about variations which can be exploited in crop improvement programmes. Plants regenerated from callus or cell suspension cultures may include a varying proportion showing structural or physiological abnormalities depending upon the species, origin and the age of culture (Yeoman, 1986), various

other factors like growth regulators (Singh and Harvey, 1975; D'Amato, 1978; Zakhlenyuk and Kunakh, 1987), composition of the culture medium (Bayliss, 1977; Feng and Quyang, 1988), culture conditions (Cerutti, 1985; Jackson and Dale, 1988) and culture method (Wilson *et al.*, 1976) which influence somaclonal variation.

The selfed progenies of *V. planifolia* obtained through seed culture have shown variations in morphological characters such as plant height, internodal length, leaf length and leaf breadth (Figs. 14,15,16, 17). Variations were evident in *in vitro* cultures, as well as after planting out. *In vitro* cultures could be categorized into low and high multiplying types; plantlets with short and long internodes, dark and light green leaves, lanceolate, oblong, elliptic leaves with acute and acuminate leaf tips. These morphological variations were observed among the progenies of *V. planifolia* at the time of hardening also (Table 27). Among the selfed progenies, V113 showed maximum plant height, internode length as well as leaf breadth whereas leaves of V66 were ovate in shape. Though V98 was a slow growing progeny with lesser height and smaller internodes, it has long narrow leaves and highest leaf length. V422 and V408 were distinct in *in vitro* cultures by their leaf variegations, V422 with green colour only around the midrib region and V408 with yellow margins

The progenies generated in colchicine-incorporated medium (VC), have exhibited short internodes and broader but smaller leaves (Fig. 14), distinct from all other seed progenies and somaclones. These characters coupled with occurrence of cells with 2n chromosome number 36 in VC 3, may be indicative of their polyploid nature.

The callus regenerated progenies exhibited higher variations among themselves. After a year of planting out in fields, V8 (among the selfed progenies) and VC82 (among the somaclones) showed higher plant growth rate. VC82 was characterized by good growth of the plant as well as leaf size and internodal length. Among the selfed progenies, V48 and V55 showed better growth when compared to *V. planifolia.*

The success of any crop improvement programme depends on the extent of genetic variability in the base population. However, there is a lack of existing genetic variability in most of the agricultural crops, which hampers their improvement through conventional plant breeding methods. In this context, *in vitro* technology is a powerful tool for the induction of much-needed genetic variability. Heritable somaclonal variations, which have been reported in tobacco leaf colour (Dulicu and Barbier, 1982), fruit colour in tomato (Evans and Sharp, 1983) and seed colour in *Brassica* (George and Rao, 1983). Already plants showing tolerance / resistance to phytotoxins (Gengenbach *et al.*, 1977), herbicides (Chaleff and Ray, 1984), nematodes and viruses (Wenzel and Uhrig, 1981), salt (Bajaj and Gupta, 1987), high yielding (Ogura *et al.*, 1988), rich in protein (Schaeffer and Sharpe, 1987) and sugar contents (Liu and Chen, 1976) and male fertile (Ling *et al.*, 1987), have been obtained, and are being incorporated in crop improvement programmes. In important agricultural crops such as wheat, rice, maize, potato, sugarcane, *Brassica* etc. these techniques have already yielded positive results and of new cultivars are being released (Bajaj, 1990).

Cytological indexing of selfed progenies, somaclones and interspecific hybrids

Some of the earliest reports of genetic instability in culture were made on

the basis of chromosome counts (D'Amato, 1975), but it is known that this approach will reveal only crude genetic change. Bayliss (1980), comprehensively reviewed the reported instances of chromosomal variation in tissue cultures. The classes of variant cells included polyploid and aneuploid changes, structural changes in chromosome morphology, and mitotic aberrations including multiplolar spindles, lagging chromosomes, fragments and asymmetric chromatid separation. Tissue cultured plants with extensive chromosome rearrangements were reported in *Lolium* sp. (Ahloowalia, 1983) and *Medicago sativa* (Reisch and Bingham, 1981).

Cells from both selfed as well as somaclones of vanilla showed great deviations from the normal reported somatic chromosome number (2n=32). Metaphase stage chromosomes exhibited both structural as well as numerical variations. High rate of somatic associations was observed making counting extremely difficult in most of the cells (Figs. 22, 23, 24). The different chromosome numbers observed ranged from 24 to 36 among the somaclones. Callus regenerated progeny V 8.1 did not have any cell with the normal somatic compliment of 2n=32. Colchicine generated progeny VC 3 showed a chromosome number 36, which was higher than the normal somatic chromosome number in *V. planifolia*. Since all the other somaclones of *V. planifolia,* showed variable chromosome numbers lesser than 2n=32 which may be due to associations, the increased number of VC3 is probably due to the colchicines treatment resulting in higher ploidy level. Structural abnormalities like end-to-end pairing, bridge formation, tripolar and tetrapolar unequal segregation at late metaphase, clumping were observed (Fig. 22).

Cytological studies of the hybrids indicated somatic associations and segmental allopolyploidy, indicating the hybrid origin of the progenies and also that the parents are related closely. Among the hybrids VH6 (morphologically planifolia type) did not show any cell with 2n=32 but had chromosome numbers nearer to *V. planifolia*. VH1 chromosome numbers were nearest to *V. planifolia*, however it also showed a variation frequency of 61.53%. VH4 and VH5 were not only morphologically similar to *V. aphylla* but also showed higher chromosome numbers indicating their nearness to *V. aphylla*. Reports on karyotype variations in orchids due to accumulation of heterochromatin have been reported (Kao *et al.*, 2001) Vanilla is reported to have chromosomal associations both in somatic cells and pollen. Many meiotic and post-meiotic chromosomal abnormalities have been reported by Ravindran (1979) and somatic associations by Nair and Ravindran (1994). As a result, it is possible to get various cytotypes and genetically variant types in the seed progenies. The present study supports these findings.

Biochemical characterization of the progenies using isozyme profiles

Isozyme technology offers a unique opportunity of estimating the degree of genetic variability/stability in *in vitro* cultures (Withers and Williams, 1985). Very little attention has been given to isozyme studies in *in vitro* culture and the studies revealed that the cell suspensions and callus cultures tend to differ considerably from the intact plant (Simpson and Withers, 1986).

Isozyme profiles of superoxide dismutase (SOD) and peroxidase (PRX) were studied in 10 selfed progenies of *V. planifolia*. The profiles (Fig. 25) clearly indicated differences among progenies as expressed by the presence or absence of specific bands. The maximum similarity that these progenies exhibited was

47.37% indicating high segregation and amount of heterozygosity existing in *V. planifolia.*

Variations in isozyme profiles were used to estimate genetic variability in many crop plants (Fedak and Rajhathy, 1972; Yndgaard and Iloskuldsson, 1985; Jarret and Litz, 1986). Arnison and Boll (1975) reported isozyme differences between stock suspension cultures derived from single seedling cultures of bush bean (*Phaseolus vulgaris*). Stejskal and Griga (1995) have made a comparative analysis of some isohyets and proteins in somatic and zygotic embryos of soybean.

Molecular characterization of species, selfed progenies and somaclones

DNA markers like RAPD, RFLP, AFLP and micro-satellites have very efficiently supplemented the phenotypic markers in the estimation of genetic variability and characterizing the plant types or germplasm. In the absence of many classical phenotypic markers, in perennial crops like vanilla, molecular markers which are more reliable can very effectively help in identification of genetic variations especially in the early stages of plant development.

Assessment of genetic stability of vegetatively propagating plants like *Marula* (Feiga Gutman, 1999, apple root stocks (Wesley, 1998), apple (Mulcahy, 1993), ginger (Rout G.R *et al* 1998,) black spruce (Isabel *et al.,* 1993), *Medicago sativa* (Yu *et al.,* 1993), etc. was reported earlier. It was proved that molecular markers provide a quick simple and preliminary screening method for putative somatic hybrids in the case of *Solanum tuberosum* and *S. brevidens* (Xu *et al.,* 1993).

Isolation and purification of genomic DNA

Isolation of high molecular weight DNA is a prerequisite for any studies on molecular markers. Many protocols are available for isolation of DNA from plant tissues (Draper and Scott, 1988; Ausubel *et al.*, 1995). The CTAB method of DNA isolation is widely used in plants because of its adaptability. The total genomic DNA has been reported to be isolated from many genera adopting these techniques and with certain modifications (Murray and Thompson, 1980; de la Cruz *et al.*, 1997; Porebski, *et al.*, 1997).

In the present study, genomic DNA was successfully isolated from young and fresh leaves of 105 different vanilla plants using modified CTAB method. The DNA obtained was of reasonably good quality (Fig. 26). The yield of DNA ranged from 50–250 ng/µl indicating good yield. Thus efficient protocol for isolation of high molecular weight DNA from leaf tissues was standardized in *Vanilla* species.

Optimization of PCR components

Different combinations and concentrations of dNTPs, Taq polymerase $MgCl_2$ and other variables were tested for good and consistent amplification of genomic DNA. It was found that 30 ng DNA, 1x assay buffer, 150 µM dNTP's, 2.0 mM $MgCl_2$ and 1 U of Taq polymerase are optimal for generating good and consistent amplification products, at annealing temperature of 40 ^{0}C, hence was used for developing RAPD profiles in vanilla.

The PCR reaction profile given below gave good amplification products in vanilla and is used in all the studies.

Ist cycle: 94 °C for 2 minutes
40 °C for 1 minute
72 °C for 1 minute Cycle repeats: 1

II[nd] cycle:	94 °C for 1 minute	
	40 °C for 30 seconds	
	72 °C for 1 minute	Cycle repeats: 30
III[rd] cycle:	94 °C for 1 minute	
	40 °C for 1 minute	
	72 °C for 15 minutes	Cycle repeats: 1

Screening for primers

Sixteen primers were tested for their efficiency in detecting polymorphism between the genotypes. Most of them gave good amplification products (Fig. 27). Only the primers, which gave maximum polymorphism, were used to develop RAPD profiles.

Development of RAPD profiles

RAPD profiles were developed in various collections, species, seedling progenies, somaclones and interspecific hybrids using 16 Operon primers (Figs. 28–46). Paired affinity indices indicating the percentage of similarity between the genotypes were also studied. The dendrograms were drawn using NTSyS software.

***Vanilla* species**

The results indicate that among the different species and collections of *Vanilla*, the percentage of similarity ranged from 98.8–42.7 %. The leafless forms of vanilla viz., *V. aphylla, V. pilifera* and the new species are placed in the same cluster. As a species, *V. andamanica* is the most divergent from the rest of the species studied as it formed a separate and unique cluster. There is also reasonable variability within the V. *andamanica* collections indicating the possibility of a natural seed set. *V. tahitensis* is nearest to *V. planifolia* with an average similarity index of 81.7%, while *V. andamanica* is the farthest.

Among the different collections of *V. andamanica* the percentage of similarity ranged from 91.4–100, with highest percentage of resemblance between *V. andamanica*-4 and *V. andamanica*-7 and the lowest percentage between *V. andamanica*-3 and *V. andamanica*-1. This indicates considerable variability among different collections of *V. andamanica* supporting the probability that this species would have originated in Andaman Islands and the collections may be of seedling origin. This also points to natural seed set in its native habitat. Natural seed set in *V. wightiana* was reported earlier (Rao *et al.*, 2000).

The only earlier study on molecular taxonomy of *Vanilla* species was that of Cameron *et al.*, (1999) who did phylogenetic analysis of orchidaceae based on rbcL nucleotide sequences. This is the first report of this kind on Indian vanilla.

Collections of V. planifolia

Most of the collections of *V. planifolia* are very similar to each other but with minor variations. The collection from Mauritius, is more divergent from the rest of the group. The present study indicated that various collections of *V. planifolia* from different geographical locations did not show major differences in RAPD profiles, except for the Mauritius collection. This shows low levels of genetic variability and hence a narrow genetic base in cultivated *Vanilla.* This may be due to the fact that most cultivated vanilla in India may be of single clonal origin from very few genotypes. This narrow genetic base is maintained through continuous vegetative propagation. The *V. planifolia* collection with variegated leaves could not be demarcated from other collections by RAPD profiles.

RAPD profiles of callus and root regenerated progenies of vanilla

Studies indicated that all the somaclones tested are variable when

compared to each other. The Paired Affinity Index ranged from 97.5 to 52.5 % (Table 35). The dendrogram of divergence (Fig.39) has indicated that all the somaclones tested are variable when compared to each other and the variability in PAI between them is indicative of somaclonal variation as the possible cause for this variation.

Plants regenerated from root were also found to be variable in their RAPD profiles. This indicates regeneration of plantlets through conversion of root meristem to shoots may also induce variations in vanilla. This observation differs with earlier understanding that the roots are highly stable and the plants cloned from roots are also genetically uniform(Philip *et al.,* 1989).

Colchicine treated and callus derived progenies of *V. planifolia*

Studies based on RAPD profiles indicated that most of the colchicine generated progenies tested are variable when compared to each other and with *V. planifolia* (Fig. 43 and Table 38). The Paired Affinity Index ranged from 96.0 to 44.0 % indicating high degree of variability. The dendrogram of divergence has indicated that VH5C is the nearest to *V. planifolia* while VC25 and VC153.1 are the farthest. No differences could be observed between VC92.1 and VC145.1. VC89 and VC138 are exactly similar.

There are reports on the use of RAPD markers to detect somaclonal variation in different species such as *Lolium* and *Festuca* (Valles *et al.,* 1993); wheat (Brown *et al.*, 1993); *Picea* (Isabel *et al.,* 1993); sugarbeet (Munthali *et al.*, 1996); ginger (Rout *et al.,* 1998), *Allium* sp. (Al Zahim *et al.*, 1999), *Lilium* sp. (Varshney *et al.*, 2001), turmeric (Salvi *et al.*, 2001).

Interspecific hybrids of *V.planifolia* x *V. aphylla*

Both RAPD and AFLP data indicate similar patterns of interrelationships between the parents selfed progenies and interspecific hybrids (Tables 40 and 42). The interspecific hybrids VH1, VH4, VH5 and VH6 in general showed equidistance from *V. planifolia* and *V. aphylla*. Of the four, VH1 and VH4 clustered along with *V. aphylla* indicating their similarity, while VH5 and VH6 clustered along with *V. planifolia* (Figs. 47 and 52). Morphologically VH-1 and VH-6 are *V. planifolia* types and VH-4 and VH5 are aphylla types, with regard to leaf nature. RAPD and AFLP profiles do not entirely agree with this and two of the interspecific hybrids, VH4 and VH5 are clustered with *V. aphylla* parents whereas VH1 and VH6 are clustered with *V. planifolia* parent. Thus the RAPD profiles coupled with morphological characters indicate the true hybrid nature of these four genotypes. This is the first report of this kind in vanilla.

Ajmone *et al.* (1998) studied genetic diversity and its relationship to hybrid performance in maize by RFLP and AFLP markers.

V. planifolia and its selfed progenies

The RAPD and AFLP profiles indicate that all the progenies tested were variable when compared to each other. The Paired Affinity Index indicated that the similarity of *V. planifolia* and its progenies ranged from 94.6 to 62.5% .V112 is the closest to *V. planifolia* while V20 is the farthest (Figs. 47 and 52; Tables 40 and 42). V18 and V44 are the only ones which showed very close similarity to each other. Thus the present study indicates that the seedling progeneis of *Vanilla planifolia* are highly variable and hence can be used in crop improvement. The other morphological, cytological and biochemical data supports this view. Earlier studies by Minoo *et al.* (1997) also supported this view. This variation is expected

because vanilla is a cross-pollinated crop and variations are bound to occur in the segregating progenies. In addition mitotic associations reported in root tissues (Nair and Ravindran, 1994) and during pollen mitosis (Ravindran, 1979) further contribute to the spectrum of variation.

V. planifolia* and *V. aphylla

The RAPD and AFLP study indicated that *V. aphylla* is the most divergent from *V. planifolia* with a similarity index of only 44.4% in AFLP profiles in various other studies. This wide variation between these two species is expected because *V. planifolia* has originated in Central America while *V. aphylla* originated in South Asia with very wide geographical isolation.

Interspecific hybrids of *Vanilla planifolia* X *V.aphylla*

The interspecific hybrids VH1, VH4 and VH5 in general showed equidistance from *V. planifolia* and *V. aphylla*. Of these three, VH4 is more nearer to *V. planifolia* while VH5 seems to be more nearer to *V. aphylla*. However, morphologically VH4 and VH5 resemble aphylla types, while VH1 resembles planifolia types with regard to its leaf structure. Between the interspecific hybrids, VH1 and VH4 are more similar with 58.3% similarity while VH1 and VH5 has 25% similarity and VH4 and VH5 showed only 16.7% similarity. Thus, the RAPD profiles coupled with morphological characters indicate the hybridity of these 3 genotypes.

In conclusion, the morphological, cytological, biochemical, and molecular data indicate that:

1. There is very limited variability among the cultivated collections of *V. planifolia* cultivated in India.

2. *Vanilla planifolia* from Mauritius is the most variable among the *V. planifolia* collections studied.
3. *Vanilla tahitensis* is nearest to *V.planifolia* and *V.aphylla* is the farthest, significant variations occur among the collections of *V. andamanica* indicting natural seed set and seedling origin of the collections.
4. There are significant variations among the selfed seed progenies of *V.planifolia.* This variation further increased when the plant regeneration is through callus or when grown in colchicines containing medium.
5. The progenies obtained from crosses between *V. planifolia* and *V. aphylla* are truly hybrids and
6. *In vitro* technology can be used for generation of variability and its utility in crop improvement in vanilla.

Molecular characterization of inter and intraspecific somatic hybrids of potato which eliminated the difficulty of unequivocally identifying nuclear hybrids was done by Baird *et al.* (1992), Arti *et al.* (1998) used RAPD fingerprinting for identifying parental lines in rice. A parentage analysis using RAPD PCR to determine both paternal and maternal DNA was demonstrated by Scott *et al* (1992). Finger printing was applied to paternity analysis of an apple cultivar of which the pollen parent was unknown (Harada *et al.,* 1993). RAPD analysis was also used for population genetic studies. Genetic polymorphism of 7 populations of *Capsella bursa-pastoris* was studied by Yang *et al.* (1998) Comparison of molecular markers analysis of *Musa* breeding populations were

made by Crouch *et al.* (1994). Development of interspecific hybrids and utilisation of RAPD markers to characterise them in *Carica* has been made by Drew *et al.* (1998) and Magdalita *et al.* (1998) respectively. Markers that flank a gene determining a trait of agronomic interest can be used to track the trait in genetic crosses and also to estimate the genetic contribution of each parent to each member of a segregating population. Examples of such cases are RAPD assisted genetic segregation in diploid. Cultivated alfalfa, genetic diagnosis of F1 hybrid of *Rehmania* sp. for segregating characteristics from both parents (Hatano *et al.*, 1997).

Development of AFLP profiles

The AFLP profiles were developed for the first time in *V. planifolia, V. aphylla,* interspecific hybrids and a few selfed progenies of *V. planifolia,* using 4 different primer combinations (Figs.4, 5). Clear polymorphism was detected in inter-specific hybrids, selfed progenies and the parents- *V. planifolia*♀ X *V. aphylla*♂ in all the primers tested. The profiles of *V. planifolia* and *V. aphylla* indicated that they are widely distant and banding patterns consistent with the species, were also observed indicating that these bands are species-specific. The selfed progenies of *V. planifolia* showed the banding pattern similar to that of *V. planifolia* parent and did not show the species specific bands of *V. aphylla.* These progenies did show variation in banding pattern within them indicating that there is considerable variation among the selfed progenies. Interspecific hybrids showed the banding patterns in between the parents in that segregation of species specific bands were noticed among the progenies further confirming the hybrid nature of the progenies.

The dendrogram of divergence also supported the above findings (Fig .3).

Similar studies using RAPDs, have been reported earlier in many crop species. RAPD polymorphism was used to study species interrelationships in junipers (Alice *et al.,* 1999), cocoa (Wilde *et al.*, 1992), coffee (Antony *et al.,* 1997). RAPD polymorphism was used to study the inbred lines in maize (Zhang *et al.,* 1995) and to differentiate between root knot nemetode resistant and susceptible lines of rice (Bose *et al.,* 1995), and to detect introgression in *Medicago* (Coy *et al.,* 1993). RAPD polymorphism was also used to study somaclonal variation in ginger (Rout *et al.,* 1998), black spruce (Isabel *et al.,* 1993), *medicago sativa* (Yu *et al.,* 1993) and *solanum tuberosum* (Xu *et al.,* 1993).

Screening of selfed progenies and somaclones for disease resistance

A population of vanilla, including species, collections, somaclones, seedlings, interpspecific hybrids was screened against the infection of *Phytophthora meadii* and *Fusarium oxysporum,* the causal agents of foot rot and wilt diseases in vanilla. The pathogen was inoculated at the axil region of the stem. The disease development was manifested as browning and water soaked patches at the axil spreading out onto the either sides of the internode and at times into the leaf. Reaction of the somaclones to infection was scored as 0-4 scale, based on the percentage of infection spread.

High degree of variability in resistance to fungal pathogen was noticed among the 34 species, somaclones and progenies tested. Among the species, a few collections of *V. andamanica* showed no infection. A few of the somaclones also showed no disease incidence. The lines which showed resistance reaction to

Phytophthora meadii are seven collections of *V. andamanica, V. planifolia* collections from Madagascar and Mauritius and four seedling progenies of *V.planifolia* viz., V8, V24, V48 and V55. The lines which gave resistant reaction to *Fusarium* are seven collections of *V. andamanica, V. planifolia* collection from Mauritius, *V. aphylla*, selfed progenies of *V. planifolia* viz., V18, V32, V48 and V120 and one somaclone of *V. planifolia* V8.1. Of these, *V. andamanica* collections 1,2,3,4, 6 and 7, *V. planifolia* collections from Mauritius and *Vanilla* seedling progeny V48 were resistant to both the pathogens. *V. aphylla* gave resistant reactions to *Fusarium oxysporum* while it is susceptible to *Phytophthora.*

Application of somaclonal variation in crop improvement has been discussed in detail (Evans and Sharp, 1986; Brar and Kush. 1994; Karp, 1995). Heritable somaclonal variations with resistance traits have been reported. Resistance to methionine sulfoxime (Carlson, 1973) and *Pseudomonas syringae* (Thanutong *et al*, 1983), in tobacco, resistance to *Fusarium oxysporum* in tomato (Evans et al, 1984), in alfalfa (Hartman *et al*, 1984); resistance to *Helminthosporium sativum* (Chawala and Wenzel, 1987) in wheat, Xanthomonas oryzae (Ling *et al.*, 1984) in rice are a few examples.

In the absence of genotypes resistant to *P. meadii* and *F.oxysproum*, the present information is of very high relevance in that for the first time, a few genotypes resistant to these two major pathogens have been identified. These genotypes will play an important role in developing high yielding disease resistant cultivars of vanilla.

IN VITRO CONSERVATION STRATEGY FOR VANILLA GERMPLASM (SELFED PROGENIES AND SOMACLONES)

Synthetic seeds

In vitro regenerated shoot buds, protocorms and regenerating calli were used in the present study as propagules for encapsulation. Sodium alginate at 4% was sufficient to produce good quality rigid beads in vanilla, higher concentrations were not suitable as they produced very hard matrix which hindered the emergence of shoot buds and thereby affecting the rate of germination and recovery. Shoot buds of 0.4-0.5 cm size were suitable for encapsulation as smaller buds failed to survive the storage and lost their viability within a month. Encapsulated synthetic seeds were stored at 4^0C, 8^0C and 22^0C to study the effect of temperature on their storage and viability. Low temperatures (4^0C and 8^0C) were not suitable for synthetic seed storage as they lost their viability within thirty days. But at 22 ± 2^0C, synthetic seeds could be stored for ten months. When cultured on MS medium supplemented with BA (1 mgl^{-1}) and IBA (0.5 mgl^{-1}), maximum germination (80%) was observed after two weeks (Fig. 64).

Addition of activated charcoal (1%) did not have any significant effect on storage and germination of the synthetic seeds (Fig. 65). The plants derived from these encapsulated buds were apparently healthy and developed into normal plantlets.

Clonal propagation of *V. planifolia* using encapsulated shoot buds have been reported by George *et al.*, 1995.

Synthetic seeds are ideal for germplasm conservation and exchange, especially in vanilla, where there is no natural seed set.

Medium term conservation *in vitro* by slow growth

Three temperature regimes (5^0C, 10^0C and 22^0C), four types of culture

vessel closures (cotton plugs, screw caps, aluminium foil and polypropylene caps), MS basal medium in half or full strength, mannitol (osmoticum) at two levels (10 $gl^{-1}$and 15 gl^{-1}), sucrose (carbon source) at five levels (30 gl^{-1}, 20 gl^{-1}, 15 gl^{-1}, 10 gl^{-1} and 0 gl^{-1}) in various combinations, were tested for their efficiency in inducing minimal growth and increasing subculture interval in vanilla.

Effect of various media combinations on induction of minimal growth in vanilla:

The effect of nutrient medium and sucrose at various levels, separately and in combination with mannitol, on growth of vanilla cultures are given in Table 46. MS medium when used in half strength supported normal growth and development of plantlets. The rate of growth was higher when full strength MS medium was used. High concentration of sucrose (30 gl^{-1}) increased culture growth substantially resulting in exhaustion of culture medium.

In full strength MS medium with 30 gl^{-1} sucrose, the cultures grew faster and filled the culture vessel within 180 days resulting in drying up of cultures. When the concentration of sucrose was reduced to 20 gl^{-1} and nutrient concentration to half, the cultures could be maintained for much longer period of 200 to 240 days with a survival percentage of 75 to 81 depending upon the closure used. In all media containing full strength MS salts, the cultures could be stored only upto a maximum of 200 days with 75% survival, when the culture vessels were closed with cotton plugs, irrespective of the other parameters. Addition of mannitol (10-15 gl^{-1}) and reduction of sucrose to lower levels (15-10 gl^{-1}) induced slow growth and subsequently 73-80% of the cultures could be maintained for a period of 360 days, when the culture vessels were closed with aluminium foil. The

best result was observed when mannitol and sucrose were added in equal proportions at 10 gl^{-1} or 15 gl^{-1}, the cultures could be maintained for 1 year (Fig. 55c). Thus out of 14 different combinations tested only five *i.e.*, full or half strength MS medium supplemented with 10 or 15 gl^{-1} each of sucrose and mannitol and ½ MS with 20 gl^{-1} sucrose and 10 gl^{-1} mannitol, allowed the cultures to be maintained for 360 days. In all these, the closure used was aluminium foil. The plantlets maintained in this medium showed reduced growth rate and maximum survival. In general, culture tubes covered with aluminium foil rendered longer duration of subculture interval compared to cotton plugs due to minimization of water loss in the former.

Of the 3 temperature regimes tested only 22±2^{0}C was found suitable for growth of vanilla cultures. Temperatures below 22^{0}C were deleterious and not suitable for plant growth. At 5^{0}C and 10^{0}C, all the cultures turned yellow and died within 20 days (Table 26). This indicates that low temperatures are detrimental to vanilla cultures and 22±2^{0}C is ideal for maintaining vanilla cultures over longer periods.

Different types of closures *i.e.*, cotton plugs, screw caps, aluminium foil and polypropylene caps were used to study their effect on the longevity of cultures (Table 45).

Though the cotton plugs allowed better gaseous exchange, it resulted in moisture loss leading to drying up of cultures. But when the culture tubes were closed with screw caps, polypropylene caps or aluminium foil, the moisture loss was minimal resulting in healthy and fresh cultures even after 360 days in the medium, which induced slow growth (Fig.66). One thousand five hundred accessions of vanilla

germplasm have been conserved in the *in vitro* repository of Indian Institute of Spices Research, using the method described (Fig.67). Other report on vanilla conservation was that of Jarret and Fernandez 1992 who has reported storage of vanilla shoot tips as tissue cultures for 10 months and Philip (1989) has discussed the possibility of use of root cultures for conservation of vanilla germplasm. *In vitro* conservation of *V. walkeriae* using slow growth method was reported by Agrawal *et al.*, (1992) and effects of polyamines on *in vitro* conservation of *Vanilla planifolia* has been reported by Thyagi *et al.*, (2001).

The conserved material was transferred to the multiplication medium (MS + BA 1.0mgl $^{-1}$ and 0.5 mgl $^{-1}$ IBA) for normal growth. The small sized plantlets kept in the conservation medium for over one year showed good growth and developed into normal sized plants with good multiplication rate (1:5). These plantlets were transferred to soil (garden soil : sand : perlite in equal proportions) and established easily with 80% success when kept in humid chamber for 20-30 days after transfer. They developed into normal plants without any deformities and deficiency symptoms and exhibited apparent morphological similarities to the mother plants.

Thus a efficient protocol has been standardized for conservation of Vanilla and its related species, *in vitro,* for a period of more than one year. This method is specifically useful for conservation of endangered species like *V. aphylla.*

Long-term conservation by cryopreservation

Cryopreservation of vanilla shoot tips by encapsulation dehydration

Shoot tips, which were desiccated for 1h exhibited 90% recovery in the control, whereas the LN_2 treated ones showed only 20% viability. Desiccation for 2 h resulted in drying up of shoot tips. Shoot tips which were precultured in 5% DMSO at 25^0C for 24 h, showed 90% viability in terms of retaining the green colour immediately after thawing and initiation of growth after 2 weeks of culture. The same treatment at 4^0C, was not promising, as it showed vitrification even though remain green soon after reculture. In all other treatments the shoot tips turned white and / vitrified and no favourable growth response was observed.

The encapsulation-dehydration technique is based on the technology for the production of synthetic seeds where somatic embryos or apices are encapsulated in a bead of hydro soluble gel (Redenbaugh *et al.*, 1991). Cryopreservation of shoot apices by encapsulation-dehydration method was reported in *Solanum* spp. (Fabre and Deruedre, 1990), grapes (Plessis *et al.*, 1991), *Malus* spp., *Pyrus* spp. and *Morus* spp. (Niino and Sakai, 1992) etc. The encapsulation-dehydration technique has been successfully extended to several genotypes or varieties in four crops namely, pear, apple, sugarcane and potato.

Thus cryopreservation protocol was standardized for the first time for long term conservation of vanilla shoot tips with limited success.

Pollen cryopreservation

Pollen banks make easy and ready availability of pollen, mainly for facilitating crosses in breeding programmes, distribution and exchange of germplasm and preserving nuclear genes of germplasm.

Cryopreservation of pollen from two species of *Vanilla* viz., *V. planifolia* and *V. aphylla* was attempted. The germination studies of the fresh pollen grains collected from flowers of three different maturity levels indicated that pollen collected from opened flowers showed maximum percentage of germination and there was no significant difference in germination of pollen collected from flowers one day prior to opening. Drastic reduction in germination percentage of pollen collected from flowers 2 days prior to opening was observed. Hence pollen was collected from freshly opened flowers in all cryopreservation experiments. Pollen germination required the addition of sucrose to the medium and the highest percentage of pollen germination was observed at 10% sucrose for both the species of *Vanilla*

Among the different treatments tried all the pollen samples survived freezing with maximum percentage of germination in pollen desiccated for 5 minutes in the air current of laminar flow and cryoprotected with 5% DMSO. In this case the pollen germination was 82.1% in *V. planifolia* and 75.4% in *V. aphylla* respectively. In all other treatments various viability percentages were observed, but abnormalities in pollen germination such as bulging busting of pollen tube tip, branching of pollen tube, feeding bottle type of pollen tube development etc were observed.

Fertility of cryopreserved pollen was tested by controlled field pollinations. Flowers of the desired female parent were pollinated with cryopreserved pollen after thawing. It was observed that the pollen cryopreserved for various intervals was fertile. Pollination with this pollen induced fruit set and seed set. This indicated the retention of pollen fertility after cryogenic storage.

Cryopreserved pollen from all the treatments induced fruit set even though there was very limited percentage of normally developed pollen grains observed in *in vitro* germination of some of the cryopreserved samples. The seeds germinated in culture and gave rise to normal PLBs and subsequently seedlings were developed.

The role of sucrose as an osmoticum (Visser, 1995) and a source of metabolic energy (O'Kelley, 1995) was proved to be essential for germination of pollen grain of all plant species. Present study has shown that pollen grains from vanilla can be successfully recovered after cryogenic storage for various intervals (1h to 7 days) without significant loss of viability. The ability of cryopreeserved pollen to effect fertilization does not appear to be adversely affected by cryopreservation as is evident from the fruit set and seed formation among the crosses made using cryostored pollen.

The retention of higher rates of viability and functionality is the ultimate aim of any pollen storage. Pollen viability is usually measured by metabolic activity, membrane semipermiability, germination and seed set. *In vitro* germination is commonly used for assessing the viability. Pollen germination on the stigma and growth in the style are also used as viability tests. Fertility is an absolute measurement of the ability of the pollen grain to germinate and set seed. The FDA test is generally considered the most rapid and accurate staining test for viability.

Viability and fertility assessment of cryopreserved pollen from *Vanilla* species have shown that it is possible to use cryogenic methods for conservation and management of the haploid gene pool in this species. Field pollination with cryogenic stored pollen successfully produced fruit and seed set. Long-term

cryogenic storage of vanilla pollen could enhance breeding efficiency. Pollen parents could be made available throughout the breeding programme, ensuring supply at the time of peak stigma receptivity. Genetic diversity conservation at haploid level is another advantage of pollen cryobank. Such a protocol made available to breeders would help to improve the efficiency of breeding, in terms of identifying potential pollen parents and preserving them. Pollen storage is being used to supplement classic clonal preservation methods (Conner and Towill, 1993). Through a pollen cryobank, pollen of desired species or variety could be obtained for breeding without any seasonal or geographic constraints. In vanilla the problem of no synchronous flowering in different species could be overcome by the cryostorage of pollen.

The technologies developed during the present investigation can be effectively used for production of seedlings and somaclones broadening the spectrum of genetic variations in vanilla, thus overcoming a major bottleneck in vanilla breeding and crop improvement programmes. The protoplast isolation and fusion technology developed can be used in transfer of useful traits through the production of somatic hybrids thus, making way for genetic manipulations in vanilla. The characterization of vanilla species, collections, seedlings, somaclones and interspecific hybrids, proved the existence and extent of genetic variations that is available and brought by biotechnological tools. The *in vitro* conservation methods, through synthetic seed, slow growth and cryopreservation, standardized in the present study will form an integral and important part of overall conservation strategy in genetic recourses management of vanilla germplasm.

Summary and Conclusions

Minoo Divakaran "Seedling and somaclonal variation and their characterization in Vanilla " Thesis. Indian Institute of Spices Research Calicut, University of Calicut, 2002

Summary and Conclusions

Cultivated vanilla, *Vanilla planifolia* Andrews (syn. *V. fragrans* Salisb.), is a tropical climbing orchid, native to Mexico and Central America. Vanilla, an important and popular flavouring material and spice, is the fermented and cured fruit of the orchid, *V. planifolia*. Vanilla is used extensively to flavour ice creams, chocolates, beverages, cakes, custard, puddings and other confectionery. It is also used in perfumery and to a small extent in medicine as nerve stimulant. The fragrance and flavour of vanilla beans are mainly due to vanillin. The widest use of highly purified vanillin is as a chemical intermediate in the synthesis of numerous pharmaceutical products. Vanilla is the second most expensive spice traded on the world market and the world wide annual consumption was 1900 tonnes in 1995. The total area under vanilla cultivation in the world during 1999 was 37,525 ha with a production of 4,403 MT. Indonesia is the largest producer of vanilla.

The area under vanilla cultivation in India is estimated to be around 1000 ha and production of cured beans in 1999 – 2000 was 6-8 tonnes. The narrow primary gene pool of *V. planifolia* is threatened due to various factors such as recent domestication, limited breeding, deforestation and over-collection. The secondary gene pool, the close relatives of *V. planifolia*, is more important as a source of desirable traits like self-pollination, higher fruit set and disease resistance. Some of these traits are found in its near relatives. Vanilla is suspected to be highly heterozygous because of its cross-pollinated nature and variations could be obtained in the segregating seedling progenies. The most common diseases are of fungal origin *viz.*, foot rot and wilting caused by *Phytophthora meadii*, *Fusarium oxysporum, Calospora vanillae, Sclerotium* rot.

The main objectives of the present investigation are to study the existing variability in some of the vanilla cultivars and species available in India, to broaden

the spectrum of variations in vanilla gene pool using conventional as well as biotechnological approaches, to characterize the extent of variability generated using morphological, cytological, biochemical and molecular markers, and to standardize protocol for *in vitro* conservation of vanilla germplasm. A few of the somaclones were screened for reaction against *Phytophthora meadii* and *Fusarium oxysporum,* the two major pathogens affecting vanilla plantations.

PRODUCTION OF SEEDLINGS (SELFED PROGENIES) AND SOMACLONES

Successful hand pollination with 90–100% fruit set was achieved. The matured fruits contained numerous black powdery seeds. In the present study seeds could be successfully germinated *in vitro*, from the fourth month of fruit setting onwards. Seeds germinated directly into plantlets in the medium supplemented with BA (0.5 mgl^{-1}) alone, without any intervening callus phase, hence this medium was selected for the germination of vanilla seeds and production of selfed progenies/seedlings throughout the study. Germinating seeds multiplied when cultured on MS medium supplemented with BA (1 mgl^{-1}) and IBA (0.5 mgl^{-1}).

Plant regeneration from callus was induced in MS medium fortified with NAA (0.5 mgl^{-1}) and BA (1.0 mgl^{-1}) in which 75% of the cultures developed callus The regenerated plants induced from callus tissue produced roots in growth regulator free medium. This is the first report of plant regeneration of vanilla through callus from India. This efficient regeneration system can be utilized for creation and exploitation of somaclonal variation.

Production of colchiploids was attempted and seed were germinated in MS medium supplemented with colchicine (0.002%). Further development and multiplication of seedlings was achieved in MS medium with BA (1.0mgl^{-1}) and

NAA (0.5 mgl^{-1}). Rapid seedling development occurred in medium supplemented with BA and IBA. Shoot multiplication was best in this medium, however, on transfer to growth regulator free medium, the shoots developed good root system. These somaclones were studied in comparison with vanilla and its seedlings.

Micropropagation was standardized for *V. planifolia, V. aphylla, V. andamanica* and *V. pilifera*. MS supplemented with BA ($1mgl^{-1}$) + IBA (0.5 mgl^{-1}) was the most efficient in inducing multiple shoots in all the species studied. The protocol developed in the present study is very efficient in the production of more multiple shoots, and hence can be used for large scale multiplication of disease free plants in all these species. Earlier reports are not available for micropropagation of *V. aphylla, V. andamanica* and *V. pilifera.*

Conversion of root tips into shoots was observed in *V. planifolia* and *V. aphylla* when cultured on MS medium supplemented with BA (1.0 mgl^{-1}) and IBA (0.5 mgl^{-1}). These shoots developed into plantlets and were hardened and established in soil. Fasciation, an enlargement and flattening of the plant structure especially the stem was observed in *V. planifolia* and *V. aphylla* cultures, when BA was present in the culture medium at a concentration of atleast 1.0 mgl^{-1}. This is the first report of occurrence of fasciation in vanilla cultures and could be of use as a source of variability.

Interspecific hybridization between *V. planifolia* and its related species *V. aphylla* was successfully achieved, which can be used to increase the spectrum of variation by bringing desirable characters from wild species into the cultivated vanilla. Only 4 plantlets could be obtained from the 11 crosses made. The interspecific hybrids exhibited segregation of both the parental characters in that VH1 and VH6 are planifolia types (leafy) while VH4 and VH5 are aphylla types

(leafless). The expression of male parent character - leafless type, indicates the hybridity of the progenies. This is the first report of successful interspecific hybridization and production of hybrids between *V. planifolia* and *V. aphylla.*

Selfed seedling progenies, somaclones, micropropagated plants and interspecific hybrids were hardened and planted in the field with over 80% success.

The techniques of protoplast isolation and fusion are important because of the far reaching implications in studies of plant improvement by cell modification and somatic hybridization. Protoplasts were successfully isolated from *V. planifolia* and *V. andamanica* when incubated in an enzyme solution containing macerozyme R10 (0.5%) and cellulase Onozuka R10 (2%) for 8 hours at 30°C in dark. The protoplast yield was 2.5 x 10^5 per gram and 1.0 x 10^5 gram of leaf tissue and protoplast viability was 72% and 55% in *V. planifolia* and *V. andamanica* respectively. Protoplasts of *V. planifolia* were bigger in size (0.031 mm) than *V. andamanica* (0.022 mm). The protoplasts can be further distinguished by the arrangement of chloroplasts. Chloroplasts were arranged around the periphery in *V. planifolia* whereas in *V.andamanica* they remained scattered in the center.

The visibly distinguishable nature of protoplast can be exploited for the purpose of genetic transformation in these species. Protoplast fusion was attempted in these two species of *Vanilla.* PEG mediated protoplasts fusion between *V. andamanica* and *V. planifolia* was successful and the fusion product (heterokaryon) could easily be identified due to the difference in chloroplast arrangement. This can be very useful in gene transfer for useful traits, especially the natural seed set and disease tolerance observed in *V. andamanica.* The cell wall development around the fusion product was observed after 36 hours. This is the first report of protoplast

isolation in *V. andamanica* and PEG mediated fusion in *V. planifolia* and *V. andamanica.*

CHARACTERISATION OF SELFED PROGENIES AND SOMACLONES

Attempts were made to characterise the species, collections, selfed seedling progeneis, somaclone and interpspecific hybrids using morphological, cytological, biochemical and molecular markers, wherever possible.

The selfed progenies, somaclones and interspecific hybrids of *V. planifolia* obtained through seed culture have shown variations in morphological characters such as plant height, internodal length, leaf length and leaf breadth. Variations were evident in *in vitro* cultures, as well as after planting out. *In vitro* cultures could be categorized into low and high multiplying types; plantlets with short and long internodes, dark and light green leaves, lanceolate, oblong, elliptic leaves with acute and acuminate leaf tips. These morphological variations were observed among the progenies of *V. planifolia* at the time of hardening also.

Cytological indexing showed great deviations from the normal reported somatic chromosome number (2n=32). Metaphase stage chromosomes exhibited both structural as well as numerical variations. The different chromosome numbers observed ranged from 24 to 36 among the somaclones. Callus regenerated progeny V 8.1 did not have any cell with the normal somatic compliment of 2n=32. Colchicine generated progeny VC 3 showed a chromosome number 36, which was higher than the normal somatic chromosome number in *V. planifolia.* Cytological studies of the hybrids indicated somatic associations and segmental allopolyploidy, indicating the hybrid origin of the progenies and also that the parents are related closely. Among the hybrids VH6 (morphologically planifolia type) did not show any cell with 2n=32 but had chromosome numbers nearer to *V. planifolia.*

Isozyme technology offers a unique opportunity of estimating the degree of genetic variability/stability in *in vitro* cultures. Isozyme profiles of superoxide dismutase (SOD) and peroxidase (PRX) were studied in 10 selfed progenies of *V. planifolia*. The profiles clearly indicated differences among progenies as expressed by the presence or absence of specific bands. The maximum similarity that these progenies exhibited was 47.37% indicating high segregation and amount of heterozygosity existing in *V. planifolia*.

In the absence of many classical phenotypic markers, in perennial crops like vanilla, molecular markers such as RAPD and AFLP, which are more reliable can very effectively help in identification of genetic variations especially in the early stages of plant development.

Isolation of high molecular weight DNA is a prerequisite for any studies on molecular markers. In the present study, genomic DNA was successfully isolated from young and fresh leaves of 105 different vanilla plants using modified CTAB method. The yield of DNA ranged from 50–250 ng/µl indicating good yield. Thus efficient protocol for isolation of high molecular weight DNA from leaf tissues was standardized in *Vanilla* species. Sixteen primers were tested for their efficiency in detecting polymorphism between the genotypes and were used to develop RAPD profiles.

Among the different species and collections of *Vanilla*, the percentage of similarity ranged from 98.8–42.7 %. The leafless forms of vanilla viz., *V. aphylla, V. pilifera* and the new species are placed in the same cluster. As a species, *V. andamanica* is the most divergent from the rest of the species studied as it formed a separate and unique cluster. There is also reasonable variability within the V. *andamanica* collections indicating the possibility of a natural seed set. *V. tahitensis*

is nearest to *V. planifolia* with an average similarity index of 81.7%. This is the first report ot species interrelationship studies in vanilla.

Most of the collections of *V. planifolia* are very similar to each other but with minor variations. The collection from Mauritius, is more divergent from the rest of the group. The present study indicated that various collections of *V. planifolia* from different geographical locations did not show major differences in RAPD profiles, except for the Mauritius collection. This shows low levels of genetic variability and hence a narrow genetic base in cultivated *Vanilla.*

Studies indicated that all the somaclones tested are variable when compared to each other and somaclonal variation as the possible cause for this variation. The Paired Affinity Index ranged from 97.5 to 52.5 %. Plants regenerated from root were also found to be variable in their RAPD profiles. This indicates regeneration of plantlets through conversion of root meristem to shoots may also induce variations in vanilla. Studies based on RAPD profiles indicated that most of the colchicine generated progenies tested are variable when compared to each other and with *V. planifolia.* The Paired Affinity Index ranged from 96.0 to 44.0 % indicating high degree of variability.

Both RAPD and AFLP data indicate similar patterns of interrelationships between the parents selfed progenies and interspecific hybrids. The interspecific hybrids VH1, VH4, VH5 and VH6 in general showed equidistance from *V. planifolia* and *V. aphylla.* Of the four, VH1 and VH4 clustered along with *V. aphylla* indicating their similarity, while VH5 and VH6 clustered along with *V. planifolia.* Morphologically VH1 and VH6 are *V. planifolia* types and VH-4 and VH5 are aphylla types, with regard to leaf nature. RAPD and AFLP profiles do not entirely agree with this and two of the interspecific hybrids, VH4 and VH5 are

clustered with *V. aphylla* parents whereas VH1 and VH6 are clustered with *V. planifolia* parent. Thus the RAPD profiles coupled with morphological characters indicate the true hybrid nature of these four genotypes. This is the first report of this kind in vanilla.

The RAPD and AFLP profiles indicate that all the progenies tested were variable when compared to each other. The Paired Affinity Index indicated that the similarity of *V. planifolia* and its progenies ranged from 94.6 to 62.5% . Thus the present study indicates that the seedling progeneis of *Vanilla planifolia* are highly variable and hence can be used in crop improvement.

The RAPD and AFLP study indicated that *V. aphylla* is the most divergent from *V. planifolia* with a similarity index of only 44.4% in AFLP profiles in various other studies. This wide variation between these two species is expected because *V. planifolia* has originated in Central America while *V. aphylla* originated in South Asia with very wide geographical isolation.

In conclusion, the morphological, cytological, biochemical, and molecular data indicate that:

1. There is very limited variability among the cultivated collections of *V. planifolia* cultivated in India.
2. *Vanilla planifolia* from Mauritius is the most variable among the *V. planifolia* collections studied.
3. *Vanilla tahitensis* is nearest to *V.planifolia* and *V.aphylla* is the farthest, significant variations occur among the collections of *V. andamanica* indicting natural seed set and seedling origin of the collections.

4. There are significant variations among the selfed seed progenies of *V.planifolia.* This variation further increased when the plant regeneration is through callus or when grown in colchicines containing medium.
5. The progenies obtained from crosses between *V. planifolia* and *V. aphylla* are truly hybrids and
6. *In vitro* technology can be used for generation of variability and its utility in crop improvement in vanilla.

A population of vanilla, including species, collections, somaclones, seedlings, interpspecific hybrids was screened against the infection of *Phytophthora meadii* and *Fusarium oxysporum,* the causal agents of foot rot and wilt diseases in vanilla. High degree of variability in resistance to fungal pathogen was noticed among the 34 species, somaclones and progenies tested. The lines which showed resistance reaction to *Phytophthora meadii* are seven collections of *V. andamanica, V. planifolia* collections from Madagascar and Mauritius and four seedling progenies of *V.planifolia* viz., V8, V24, V48 and V55. The lines which gave resistant reaction to *Fusarium* are seven collections of *V. andamanica, V. planifolia* collection from Mauritius, *V. aphylla,* selfed progenies of *V. planifolia* viz., V18, V32, V48 and V120 and one somaclone of *V. planifolia* V8.1. Of these, *V. andamanica* collections 1,2,3,4, 6 and 7, *V. planifolia* collections from Mauritius and *Vanilla* seedling progeny V48 were resistant to both the pathogens. *V. aphylla* gave resistant reactions to *Fusarium oxysporum* while it is susceptible to *Phytophthora.*

In the absence of genotypes resistant to *P. meadii* and *F.oxysproum,* the present information is of very high relevance in that for the first time, a few genotypes resistant to these two major pathogens have been identified. These

genotypes will play an important role in developing high yielding disease resistant cultivars of vanilla.

IN VITRO CONSERVATION STRATEGY FOR VANILLA GERMPLASM

Various approaches such as production of synthetic seeds, storage of shoot cultures by slow growth method and cryopreservation was attempted for conservation. *In vitro* regenerated shoot buds, protocorms and regenerating calli were successfully encapsulated in 4% sodium alginate and stored in sterile water for ten months, at 22±2^{0}C. When cultured on MS medium supplemented with BA (1 mgl^{-1}) and IBA (0.5 mgl^{-1}), the synthetic seeds germinated (80%) after two weeks.

Slow growth method of *in vitro* conservation was also achieved in vanilla. Vanilla shoot cultures could be stored for 1 year, without subculture in full or half strength MS medium supplemented with 10 or 15 gl $^{-1}$ each of sucrose and mannitol and ½ MS with 20 gl^{-1} sucrose and 10 gl $^{-1}$ mannitol in sealed culture vessels at 22±2^{0}C. The conserved material could be multiplied normally in MS medium with 1.0mgl $^{-1}$ BA and 0.5 mgl $^{-1}$ IBA. The small sized plantlets kept in the conservation medium for over one year showed good growth and developed into normal sized plants with good multiplication rate (1:5). One thousand five hundred accessions of vanilla germplasm have been conserved in the *in vitro* repository of Indian Institute of Spices Research, using the method described

Thus a efficient protocol has been standardized for *in vitro* conservation of *Vanilla* and its related species. This method is specifically useful for conservation of endangered species like *V. aphylla*.

Vanilla shoot tips were successfully cryopreserved in liquid nitrogen with 90% viability, when desiccated for 1 hour and precultured in 5% DMSO at 25^{0}C for

24 h. Thus cryopreservation protocol was standardized for the first time for long term conservation of vanilla shoot tips with limited success.

Pollen banks make easy and ready availability of pollen, mainly for facilitating crosses in breeding programmes, distribution and exchange of germplasm and preserving nuclear genes of germplasm. Cryopreservation of pollen from two species of *Vanilla* viz., *V. planifolia* and *V. aphylla* was attempted. Among the different treatments tried all the pollen samples survived freezing with maximum percentage of germination in pollen desiccated for 5 minutes in the air current of laminar flow and cryoprotected with 5% DMSO. In this case the pollen germination was 82.1% in *V. planifolia* and 75.4% in *V. aphylla* respectively. Fertility of cryopreserved pollen was tested by controlled field pollinations and results indicated the retention of pollen fertility after cryogenic storage. In vanilla the problem of no synchronous flowering in different species could be overcome by the cryostorage of pollen.

The technologies developed during the present investigation can be effectively used for production of seedlings and somaclones, thus broadening the spectrum of genetic variations in vanilla. The protoplast isolation and fusion technology developed can be used in transfer of useful traits through the production of somatic hybrids thus, making way for genetic manipulations in vanilla. The characterization of vanilla species, collections, seedlings, somaclones and interspecific hybrids, proved the existence and extent of genetic variations that is available and brought by biotechnological tools. The *in vitro* conservation methods, through synthetic seed, slow growth and cryopreservation, standardized in the present study will form an integral and important part of overall conservation strategy in genetic recourses management of vanilla germplasm.

References

Abdelnour–Esquivel A, Mora A and Villalobos V. 1992a. Cryopreservation of *Musa acuminata* (AA) and *M. balbisiana* (BB). *CryoLetters* 13: 159–164.

Abdelnour–Esquivel A, Villalobos V and Engelmann F. 1992b. Cryopreservation of zygotic embryos of *Coffea* spp. *CryoLetters*. 13: 297–302.

Agrama H A, Tare C G and Housing S F.1997. Genetic linkage maps in Maize based on RAPD and RFLP markers. *Journal of Genetics and Breeding* 51(3): 225–233.

Agrawal D C, Morwal G C and Mascarenhas A F. 1992. *In vitro* propagation and slow growth storage of shoot cultures of *Vanilla walkeriae* Wight, an endangered orchid. *Lindleyana* 7 (2) : 95-99.

Ahloowalia B S. 1983. Spectrum of variation in somaclones of triploid rye grass. *Crop Sci.* 23:1141–1147.

Aitken-Christie J and Singh A P. 1987. Cold storage in tissue cultures. *In.* Bonga and Durzon (eds.) pp : 285-304.*Cell and Tissue Culture in Forestry*. Vol. 2. Specific Principles and Methods; Growth and Developments. Martinus-Nijhoff Publishers, Dordrecht.

Ajmone Marsan P A, Castiglioni P, Fusari F, Kuiper M and Motto M. 1998. Genetic diversity and its relationship to hybrid performance in maize as revealed by RFLP and AFLP markers. *Theoretical and Applied Genetics.* 96 : 219-227.

Al Zahim M A, Ford Loyd B V and Newbury H J. 1999. Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. *Plant Cell Rep.* 18: 473–477.

Alec Jeffreys, Wilson V, and Thin S L.1985. Hyper variable 'minisatellite' regions in human DNA. *Nature* 314: 67–73.

Alexander M P, Ganeshan S and Rajasekharan P E. 1991. Freeze preservation of capsicum pollen (*Capsicum annuum*) in liquid nitrogen (–196^{0}C) for 42 months: effect on viability and fertility. *Plant Cell Incompatibility Newsletter* 23 : 1–4.

Alice Le Due, Robert P Adams, Ming Hong. 1999. Using RAPD for a Taxonomic Re evaluation of Pfitzer Junipers. *Hort Science* 34 (b): 1123–1125.

Alleweldt G, Speigel-Roy P and Reisch B. 1990. Grapes (*Vitis*). In Genetic Resources of temperate fruits and nuts. *ActaHortic.* 290 : 291–327.

*Altman A, Kaur-Sawhney R. and Galston A W. 1977. *Plant Physiol.* 60: 570–574.

Ammirato P V. 1974. The effects of abscisic acid on the development of somatic embryos from the cells of caraway (*Carum carvi* L.). *Bot. Gaz.* 135 : 328–337.

Ammirato P V. 1983. Embryogenesis. pp : 82-123. *In.* Evans *et al* (eds.), 1983 (*q.v.*).

*Anthony F, Bertrand B, Cashmeres P and Charier A. 1997. Molecular Biology in support of Arabic Coffee genetic improvement. La biology molecular en appoint a l'

amelioration gene' toque due Caffeine Arabica. Plantations, Recherche, Developpment (6): 369–376.

Arditti J 1967. Factors affecting the germination of orchid seeds. *Bot. Rev.* 33: 1.

Arnison P G and Boll W G. 1975 Isozymes in cell cultures of bush bean (*Phaseolus vulgaris* cv. Contender): Isozyme differences between stock suspension cultures derived from a single seedling. *Can. J. Bot.* 53 : 261–271.

Arti P, Shenoi V V and Sarma N P 1998. Identifying parental lines in hybrid rice by RAPD finger printing. *International Rice Research Notes* 23 (1): 13.

Ashmore S E. 1997. Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. International Plant Genetic Resources Institute, Rome, Italy. 67 p.

Assy–Bah B and Engelmann F. 1992b. Cryopreservation of mature embryos of coconut (*Cocos nucifera* L.) and subsequent regeneration of plantlets. *CryoLetters* 13 : 117–126.

Assy-Bah B and Engelmann. F. 1992a. Cryopreservation of immature embryos of coconut (*Cocus nucifera* L.) *Cryoletters* 13: 67–74.

Assy-Bah B and Engelmann. F. 1993. Medium term conservation of mature embryos of coconut. *Plant Cell Tiss. Org. Cult.* 33: 19–24.

Assy-Bah B, Durrand-Gasselin T and Pannetier C. 1987. Use of zygotic embryo culture to collect germplasm of coconut (*Cocos nucifera* L.). FAO / IPGRI *Plant Gen. Res. Newsl.* 71: 4–10.

Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J, Smith J A and Smith K 1995 Current protocols in molecular biology Vol. 1, John Wiley and Sons, Inc. pp 2.3.1 to 2.3.7.

Bachiri Y, Song G Q, Plessis P, Shoar–Ghaffari A, Rekab T and Morisset C. 2001 Routine cryopreservation of kiwifruit (*Actinidia* spp.) germplasm by encapsulation–dehydration: Importance of plant growth regulators. *CryoLetters* 22 : 61–74.

Baird E, Cooper-Bland S, Waugh R, Demaine M and Powell W 1992. .Molecular characterization of Inter and Intra specific somatic hybrids of Potato using RAPD markers. *Molecular and General Genetics* 233 (3) : 469–475.

Bajaj Y P S (ed.) 1990a. Biotechnology in Agriculture and Forestry Vol. 11 - Somaclonal variation in Crop Improvement I. Springer-Verlag, Berlin.

Bajaj Y P S 1974. Isolation and culture studies on pollen tetrad and pollen mother cell protoplast. *Plant Sci. Lett.* 3 : 93–99.

Bajaj Y P S 1983a. Haploid Protoplast. *In.* Giles, K.L. (ed.) Plant Protoplast. *Int. Rev. Cytol* (Suppl.). 16 : 113–141.

Bajaj Y P S 1988. Biotechnology in Agriculture and Forestry. Vol.4. Medicinal and Aromatic Plants 1. Springer–Verlag, Berlin.

Bajaj Y P S 1989a. Biotechnology in Agriculture and Forestry. Vol.5. Trees II. Springer–Verlag, Berlin.

Bajaj Y P S 1989b. Biotechnology in Agriculture and Forestry. Vol.7. Medicinal and Aromatic Plants II. Springer–Verlag, Berlin.

Bajaj Y P S 1991a. Biotechnology in Agriculture and Forestry. Vol.15. Medicinal and Aromatic Plants III. Springer–Verlag, Berlin.

Bajaj Y P S 1991b. Biotechnology in Agriculture and Forestry. Vol.16. Trees III. Springer–Verlag, Berlin.

Bajaj Y P S 1991c. Automated micropropagation for *en masse* production of plants. *In.* Bajaj Y P S (ed.) Biotechnology in Agriculture and Forestry. Vol.17. pp: 3–16. High–Tech and Micropropagation I. Springer–Verlag, Berlin.

Bajaj Y P S and Gupta, P.K. 1987. Plants from salt tolerant cell lines of napier grass *Pennisetum purpureum* Schum. *Ind. J. Exp. Biol.* 25: 58-60.

Bajaj Y P S. 1972. Protoplast culture and regeneration of haploid tobacco plants. *Amer. J. Bot.* 59 (6) : 647.

Bajaj Y P S. 1986. Biotechnology for tree improvement for rapid propagation and biomass energy production. In. Bajaj Y.P.S. (ed.) Biotechnology in Agriculture and Forestry. Vol. 1. pp. 1–23. Trees–1. Springer–Verlag, Berlin..

Balachandran S M, Bhat S R and K P S Chandel. 1990. *In vitro* clonal multiplication of turmeric (*Curcuma* spp.) and ginger (*Zingiber officinale* Rosc.). *Plant Cell Reports.* 8 : 521–524.

Banerjee N and De Langhe E. 1985. A tissue culture technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (banana and plantain). *Plant Cell Rep.* 4 : 351–354.

Bapat V A 1993. Studies on synthetic seeds of sandalwood (*Santalum album* L.) and Mulberry (*Morus indica* L.). In. Redenbaugh K. (ed.) pp. 381–407Synseeds: Applications of Synthetic Seeds to Crop Improvement. CRC press, Boca Raton. FL.

Bapat V A and Rao P S 1990. *In vivo* growth of encapsulated axillary buds of mulberry (*Morus indica* L.). *Plant Cell Tiss. Org. Cult.* 20 : 69–70.

Bapat V A, Mhatre N and Rao P S 1987. Propagation of *Morus indica* L. (mulberry) by encapsulated shoot buds. *Plant Cell Rep.* 6 : 393–395.

Baranger A, Delourme R, Foisset N, Eber R, Burret P, Dupuis P, Renard M and Chevre A M 1997. Wide mapping of a T-DNA insertion site in oil seed rape using bulk segregant analysis and Comparative mapping. *Plant Breeding* 116 (6) : 553–560.

Barlass M and Skene K G M. 1983. Long term storage of grape *in vitro*. *Plant Genet Resources Newsletter*. 53 : 19–21.

Barnabas B and Kovacs G 1997. Storage of pollen. In. Shivanna K R and Sawhney V K (eds.) pp. 293–314. Pollen biotechnology for crop production and improvement. Cambridge University Press.

Bayliss M W. 1980. Chromosomal variation in plant tissue in culture. In Vasil I K (ed.) Perspectives in Plant Cell and Tissue Culture. *Int. Rev. Cytol.* (Suppl.) IIA: 113.

Beauchamp C and Fridovich I 1971. Superoxide dismutase : improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry*. 44 : 276–287.

Benzion G, Phillips R L and Rines H W 1986 Case Histories of Genetic Variability *In Vitro* : Oats and Maize. *In.* I K Vasil (ed.) pp : 435-448. 1986 (*q.v.*).

Berardi G, Infante R and Neri D. 1993. Micropropagation of *Pyrus calleryana* Den. from seedlings. *Sci. Hort.* 53 : 157–165.

Bertrand-Desbrunais A and Charrier A 1989. *Conservation des ressources genetiques cafeieres en vitrotheque*. pp.438–447. In. Proc. 13th ASIC Conference, Paipa, Colombia.

Bertrand-Desbrunais A, Fabre J, Engelmann F, Dereuddre J and Charrier A. 1988. *Reprise de l'embryogenese adventive d'embryons somatiques de cafeier (Coffea arabica) apres leur congelation dans l'azote liquide*. C. R. Acad. Sci. Paris. 307, Serie III : 795-801.

Bhat K V, Bhat S R and Chandel K P S. 1992. Survery of isozyme polymorphism for clonal identification in *Musa. II* Peroxidase, Superoxide dismutase, Shikimate deehydrogenase and Malate deehydrogenase. *J. Hort.Science*. 67 (6) : 737–743.

Bhatt S S and Sudharshan M R. 2000. Effect of pollen load on growth and development of vanilla (*Vanilla planifolia* Andr.) fruits. In. Spices and aromatic plants: challenges and opportunities in the new century. Centennial conference on spices and aromatic plants, Calicut, Kerala, India, 20 23 September. 13-16.

Bhattacharyya R, Mandal B and Bhattacharya S. 2001. *In vitro* conservation and encapsulation of *Coleus forskolii. Indian J. Plant Genetic Resources*. 14 (2): 313–315.

Bhojwani S S and Razdan M K .1983. *Plant Tissue Culture: Theory and Practice*. p 502. Elsevier Science Publishers B.V. Amsterdam.

Bhojwani S S and Cocking E C 1972. Isolation of protoplasts from pollen tetrads. *Nature New Biol.* 239 : 29–30.

Blakesley D and Kiernan R J 2001. Cryopreservation of axillary buds of a *Eucalyptus grandis* x *Eucalyptus camaldulensis* hybrid. *CryoLetters.* 22 : 13–18.

Boisvert, C; Hubert, A. 1998. *L´ABCdaire des Épices*. Flammarion, Paris.

Bose L K, Sahu S C, Mishra C D and Ratho S N 1998. Molecular polymorphism between Rice root-knot nematode resistant and susceptible cultivars. *Oryza* 35 (2) : 190–192.

Bouriquet G. 1954 (ed.) *Le vanillier et la Vanille dans le Monde,* Paris: Editions Paul Lechavalier.

Bowes S A 1990. Long term storage of *Narcissus* anthers and pollen in liquid nitrogen. *Euphytica* 48 : 275–278.

Bown D. 1995. The Royal Horticultural Society Encyclopedia of Herbs and Their Uses. Dorling Kindersley, London.

Brar and Karp

Brewbaker J L and Kwack B H. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. *American Journal of Botany.* 50 : 859-865.

Brizon M, de Boucaud M T, Rissler P Y, Pierronnet A and Dosba F. 1995. Incidence of cryopreservation for improving the sanitary state of a plum poxvirus infected *Prunus. Cryobiology* 32 : 558.

Broome O C and Zimmerman R N. 1978. *In vitro* propagation of black pepper. *Hort Sci.* 43 : 151–153.

Brosse J, Nantet B, Touchard M C, Beauthéac N and Touissaint-Samat, M. 1989. A Rota das Especiarias. Edições Inapa, Lisboa.

Brown A H D 1978. Isozymes in plant population structure and genetic construction. *Theoretical and Applied Genetics* 52 : 145–157.

Brown D C W and Thorpe T A 1986. Plant regeneration by organogenesis. pp: 49–65. *In.* Vasil I K. (ed.) 1986 *(q.v).*

Brown P T H, Lang F, Kranz E and Lorz H. 1993. Analysis of single protoplast and regenerated plants by PCR and RAPD technology. *Mol. Gen. Genet.* 237 : 313–317.

Bruneton, J. 1995. Pharmacognosie. Technique et Documentation . Lavoisier, Paris.

Bui Dang Ha D and Mackenzie I A 1973. The division of protoplast from *Asparagus officinalis* L. and their growth and differentiation. *Protoplasma.* 78 : 215-221.

Cameron, K M and Chase M W 1999 Nuclear 18S rDNA sequences of Orchidaceae confirm the subfamilial status and circumscription of Vanilloideae. D. Morrison

and K. Wilson (eds.), Proceedings of the Monocots Conference. CSIRO Publishing, Syndey.

Cao Z. 1990. Grape : Micropropagation. pp : 312-328. In. *Hand Book of Plant Cell Culture* Vol. 6. Perennial Crops (Z.Chen, D A Evans, W R Sharp, P V Ammirato and M R Sohndahl, eds.) McGraw Hill, New York.

Caplin S M. 1959. Mineral oil overlay for conservation of plant tissue cultures. *Amer. J. Bot.* 46 : 324–329.

Capuano G, Piccioni E and Standardi A 1998. Effect of different treatments on the conversion of M.26 apple rootstock synthetic seeds obtained from encapsulated apical and axillary micropropagated buds. *J. Hort. Sci. Biotech.* 73 (3) : 299–305.

Carlson P S. 1973. Methonine sulfoxamine-resistant mutants of tobacco. *Science.* 180: 1366-1368.

Cassels A C and Long R D. 1982. The elimination of potato viruses X, Y, S and M in meristem and explant cultures of potato in the presence of Virazole. *Potato Res.* 25 : 165–173.

Cerutti, P. A. 1985. Peroxidant states and tumor promotion. *Science* 227 : 375–381.

Cervera E and Madrigal R. 1981. *In vitro* propagation (*Vanilla planifolia* A.) *Environmental and Experimental Biology.* 21: 441.

Chaleff, R.S. and Ray, T.B. 1984. Herbicide - resistant mutants from tobacco cell cultures. *Science.* 223 : 1148-1151.

Chalot C and Bernard U. 1920. Culture et Preparation de la Vanille. Paris : E Larose.

Chang C and Chang W C. 1998. Plant regeneration from callus culture of *Cymbidium ensifolium* var misericors. *Plant Cell Rep.* 17 : 251–255.

Chang Y, Barker R and Reed B M. 2000. Cold acclimation improves recovery of cryopreserved grass (*Zoysia* and *Lolium* sp.). *CryoLetters* 21 : 107–116.

Chaudhury R and Chandel K P S. 1994. Germination studies and cryopreservation of seeds of black pepper (*Piper nigrum* L.) a recalcitrant species. *CryoLetters* 15: 145 – 150.

Chawla H S and Wenzel G 1987. *In vitro* selection of barley and wheat for resistance against *Helminthosporium sativum. Theor Appl Genet.* 74 : 841-845.

Chee R E and Cantcliffe D J. 1988. Somatic embryony patterns and plant regeneration in *Ipomoea batatas* Poir. *in vitro. Cell Develop. Biol.* 24 : 955–958.

Chen Y C, Chang C and Chang W C 2000. A reliable protocol for plant regeneration from callus culture of *Phalaenopsis. In Vitro/Plant* 36 : 420–423.

Cheng Kur Ta, Fu LiChung, Wang Chang Shang, Hsu Fenglin and Tsay Hsinshen 1998. Identification of *Anoectochilis formosanus* and *A. Koshunensis* species with RAPD. *Planta Medica* 64 (1) : 46–49.

Christianson M 1987. Casual effects in morphogenesis. *In.* Green, C.E., Sommers, D.A., Hackett, W.P. and Biesboer, D.D. (eds.) . pp : 45–55. Plant Tissue and Cell Culture, Allan R Liss, Inc., New York.

Chun Y W and Hall R B. 1986. Low temperature storage of *in vitro* cultured hybrid poplar, *Populus Alba* X *P.grandidendata* plantlets. p. 13. *In* Abst. VIth *Int. Cong. Plant Tissue and Cell Culture,* Minneapolis, Minn.

Cocking E C. 1960. A method for the isolation of plant protoplasts and vacuoles. *Nature.* 187 : 962–963.

Codron H, Latche A, Pech J C, Nebie B and Fallot J. 1979. Control of quiescence and viability in auxin deprived pear cells in batch and continuous cultures. *Plant Sci. Lett.* 17 : 29–35.

Collins F C, Lertmongkol V and Jones J P 1973. Pollen storage of certain agronomic species in liquid nitrogen. *Crop Sci.* 13: 493– 494.

Conci V C and Nome S F. 1991. Virus free garlic (*Allium sativum* L.) plants obtained by thermotherapy and meristem tip culture. *J. Phytopathol.* 132 : 186–192.

Connor K F and Towill L E. 1993. Pollen handling protocol and hydration / dehydration characterization of pollen for application to long term storage. Euphytica 68 (1/2) : 77 84.

Conte C, Mutti I, Puglisi P, Ferrarini A, Regina G, Maestri E, Marmiroli N .1998 DNA fingerprinting analysis by a PCR based method for monitoring the genotoxic effects of heavy metals pollution. *Chemosphere.* (14-15) : 2739-49.

Correll D S. 1953. Vanilla – its botany, history, cultivation and economic importance. *Econ. Bot.* 7 : 291–358.

Crips P and Grout B W W. 1984. Storage of broccoli pollen in liquid nitrogen. *Euphytica* 33 : 819–823.

Crouch J H, Crouch H K, Constandt H, Van Gysel A, Breyne P, Van Montagu M, Jarrot R L and Ortiz R. 1994. Comparison of PCR based molecular marker analyses of *Musa* breeding populations. *Molecular Breeding* 5 : 233–244.

Cruz de la M, Fabiola R and Hector H. 1997. DNA isolation and amplification from cacti. Plant *Molecular Biology Reporter.* 15 : 319 325.

CSIR 1976. The Wealth of India – A dictionary of Indian Raw Materials and Industrial Products. Raw Materials – Vol. X: Sp-W. Publication and Information Directorate, Council of Scientific and Industrial Research. (CSIR), New Delhi p: 432–436.

Curtis J T. 1947. Studies on the nitrogen nutrition of orchid embryos, I, complex nitrogen sources. *Amer. Orch. Soc. Bull.* 16 : 654.

D'Amato F. 1975. The problem of genetic stability in plant tissue and cell cultures. *In.* Frankel O and Hawkes J G (eds.) Crop Genetic Resources for Today and Tomorrow. Cambridge University Press. pp: 333–348.

D'Amato F. 1978. Chromosome number variation in cultured cells and regenerated plants. pp: 287–295. *In.* Thorpe T A (ed.) Frontiers of Plant Tissue Culture. Int. Assoc. Plant Tissue Culture, Calgary, Canada.

Datta K B, Kanjilal B and De Sarker D. 1999. Artificial seed technology. Development of a protocol in *Geodorum densiflorum* (Lam) Schltr. – An Endangered Orchid. *Curr. Sci.* 76 : 1142–1145.

Datta S K. 1995. Polyethylene-glycol-mediated direct gene transfer to indica rice protoplast and regeneration of transgenic plants. pp: 66–74. *In.* Potrykus I and Spangenberg G (eds.). (*q.v.*).

Davey M R and Cocking E C 1972. Uptake of bacteria by isolated higher plant protoplasts. *Nature* (London) 239 : 455–456.

Davey M R and Kumar A. 1983. Higher Plant Protoplast-Retrospect and Prospect. *In.* Giles K L (ed.) Plant Protoplasts. *Int. Rev. Cytol.* (Suppl.) 16 : 219–299.

Davey M R and Power J B 1975. PEG-induced uptake of microorganisms into higher plant protoplasts: an ultrastructural study. *Plant Sci. Lett.* 5 : 269–274.

Davey M R, Cocking E C and Bush E. 1973. Isolation of legume root nodule protoplasts. *Nature.* 244 : 460–461.

Davey M R, Rech E L and Mulligan B J. 1989. Direct DNA transfer to plant cells. *Plant Mol. Biol.* 13 : 273–285.

Davidonis G and Knorr D 1991. Callus formation and shoot regeneration in *Vanilla planifolia. Food Biotechnology* 5 (1): 59–66.

De Pauw M A, Remphrey W R and Palmer C E 1995. The cytokinin prefernce for *In vitro* germination and protocorm growth of *Cyperidium candidum. Ann. Bot.* 75: 267.

Debergh P C and Read P E 1991. Micropropagation. In. *Micropropagation. Technology and Application.* Debergh P C and Zimmerman R H (eds.). pp: 1–13. Kluwer Academic Publishers, Dordrecht. The Netherlands.

Dekkers A J, Rao A N and Goh C J 1991. *In vitro* storage of multiple shoot cultures of gingers at ambient temperatures of 24 – 20^{0}C. *Sci. Hort* . 47: 157–167.

Dereuddre J 1992. Cryopreservation of *in vitro* cultures of plant cells and organs by vitrification and dehydration. In Reproductive Biology and Plant Breeding. Dattée Y, Dumas C. and Gallais A. (eds.), Springer Verlag, Berlin, pp. 291–300.

Devos K M and Gale M D 1992 The use of RAPD markers in wheat. *Theoretical and Applied Genetics* 84 (5-6) : 567–572.

Diaz.O. 1999. Genetic diversity in *Elymus* sps. (Triticeae) with emphasis on the Nordic region. Thesis Swedish University of Agriculture Sciences, Sweden Acta Universitatis Agriculturae Sueciae–Agraria. No. 166: 61 pp.

Donn G. Nilges M and Morocz S. 1990. Stable transformation of maize with a chimeric, modified phosphinotrycin - acyltransferase gene from *Streptomyces viridocheomogenes*. pp : 53. *In.* Nijkamp H J J, Van der Plas L H W and Aartrijk (eds.) Progress in Plant Cellular and Molecular Biology. Kluwer, Dordrecht.

Dougall D K and Whitten G H. 1980. The ability of wild carrot cell cultures to retain their capacity for anthocyanin synthesis after storage at –140^{0}C. *Planta Medica* (Suppl.), pp. 129–135.

Draper J. and Scott R. 1988. The isolation of plant nucleic acids. *In.* Draper, J., Scott, R. and Armitage, P. (eds.). pp: 201–236. Plant Genetic Transformation and Gene Expression. Blackwell Scientific Publications, Oxford.

Drew R A, Magdalita P M, O'Brien C M. 1998. Development of *Carica* interspecific hybrids ***In*** International symposium on Biotechnology of Tropical and Subtropical species Part 2.ISHS. *Acta Horticulturae* : 461.

Duang J and Hong H. 1989. Clonal propagation of *Vanilla planifolia. Acta Bot. Yunnanica* 11 (1) : 107-109.

Dulieu H and Barbier M. 1982. High frequencies of genetic variant plants regenerated from cotyledons of tobacco. In. L Earle and Y Demarly (eds.), pp 211-229. *Variability of Regenerated Plants from Tissue Culture*. New York, Praeger.

Dulieu H. 1986. Case histories of genetic variability *in vitro* : tobacco. pp : 399-416. *In.* Vasil I K (ed.) 1986 *(q.v.)*

Dumet D 1994. Cryoconservation des massifs d'embryons somatiques de palmier à huile (*Elaeis guineensis* Jacq.) par déshydratation–vitrification. Etude du rôle du saccharose pendant le prétraitement. Thèse d'Université, Univ. Paris 6: 125 p.

Dumet D, Berjak P and Engelmann F 1997. Cryopreservation of zygotic and somatic embryos of tropical species producing recalcitrant or intermediate seeds. In. Razdan M.K. and Cocking E.C. (eds.), p. 153–174.Conservation of Plant Genetic Resources *In vitro Vol. 1:* General Aspects. Science Publishers Inc., USA.

Dumet D, Engelmann F, Chabrillange N. and Duval Y. 1993. Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) somatic embryos involving a desiccation step. *Plant Cell Rep.* 12 : 352–55.

Duran-Vila N, Ortega V and Navarro L. 1989. Morphogenesis and tissue cultures of three citrus species. *Plant Cell Tiss. Org. Cult.* 16 : 123–133.

Duran-Vila N. 1995. Cryoconservation of germplasm of Citrus. In. Y P S Bajaj, (ed.) pp. 70-86. Biotechnology in Agriculture and Forestry. Vol.32 Springer-Verlag, Berlin, Heidelberg.

Durzan D J. 1988. Rooting in woody perennials: problems and opportunities with somatic embryos and artificial seeds. *Acta. Hort.* 227 : 121–125.

Dussert S, Mauro M C, Deloire A, Hamon S and Engelmann F. 1991. Cryopreservation of grape embryogenic cell suspensions. 1. Influence of pretreatment, freezing and thawing conditions. *CryoLetters* 13 : 12–22.

El-Gizawy A M and Ford-lloyd B V. 1987. An *in vitro* method for the conservation and storage of garlic (*Allium sativum*) germplasm. *Plant Cell Tissue Organ Culture*. 9 : 147–150.

Elias K. 1988. *In vitro* culture and plant genetic resources. A new approach : *in vitro* collecting. Lettere d'Informazione. Instituto Agronomico Mediterraneo (Valenzano, Italy) 3: 33–34.

Engelmann F 2000. Importance of cryopreservation for the conservation of plant genetic resources. In. Engelmann F and Takagi H (eds.). pp. 8–20. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for agricultural Sciences, Japan/International Plant genetic Resources Institute, Rome, Italy.

Engelmann F and Takagi H. 2000. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for agricultural Sciences, Japan/International Plant genetic Resources Institute, Rome, Italy. 496 p.

Engelmann F, Dambier D and Ollitrault P. 1994. Cryopreservation of cell suspensions and embryogenic calluses of citrus using a simplified freezing process. *CryoLetters* 15 : 53–58.

Engelmann F. 1997. *In vitro* conservation methods. In. . Callow J A. Ford–Loyd B V. and Newbury H J (eds.). pp. 119–161.Biotechnology and Plant Genetic Resources: Conservation and UseBiotechnology in Agriculture Series, No. 19. CAB International. UK.

Escobar R, Mafla G and Roca W M. 1993. Cryopreservation of cassava shoot tips. pp.166-121. In. W M Roca and A M Thro (eds.). Proc. First Intl Meeting of the Cassava Biotechnology Network, Cartagena, Columbia.

Evans D A .1986. Case histories of genetic variability *in vitro:* Tomato. pp : 419-434. *In.* Vasil, I.K. (ed.) 1986 *(q.v.).*

Evans D A 1986. Case histories of *genetic variability in vitro:* Tomato. pp : 419-434. *In.* Vasil I K (ed.) 1986 *(q.v.).*

Evans D A and Bravo J E 1983. Plant protoplast isolation and culture. pp. 124-176. *In.* Evans *et al* (eds.) 1983 *(q.v.).*

Evans D A and Sharp W R 1983. Single gene mutations in tomato plants regenerated from tissue culture. Science. 221: 949-951.

Evans D A, Sharp W R and Medina-Filho A P 1984. Somaclonal and Gametoclonal variation. *Am. J. Bot.* 71 : 759- 774.

Fabre J and Dereuddre J. 1990. Encapsulation–dehydration: a new approach to cryopreservation of *Solanum* shoot tips. *CryoLetters* 11: 413–426.

FAO / IPGRI. 1997. Tecnicas *in vitro* para la Colecta de Germoplasma Vegetal. (Sandoval J A and Villalobos V M (eds.). Food and Agriculture Organization of the United Nations, Rome.

Farmer R E and Barnett P E. 1974. Low temperature storage of black walnut pollen. *Cryobiology* 11: 366–367.

Fast G. 1982. Orchid seed germination and seedling culture – a manual : European terrestrial orchids (symbiotic and asymbiotic methods). In. p.309.Orchid Biology: Reviews and Perspectives. II. Arditti J (ed.). Cornell Univeristy Press, New York.

Fedak G. and Rajhathy T 1972 Esterase Isozyme variants in Canadian barley cultivars. *Can.J.Plant.Sci.* 52 : 507-510

Feiga Gutman, Avinoam Nerd, Yosef Mizrahi, Dudy Bar–Zvi and Dina Raveh 1999. Application of RAPD markers for Identification of *Morula* Genotype. *HortScience* 34 (7) : 1256–1258.

Feng, G.H. and Ouyang, J. 1988. The effects of KNO_3 concentration in callus induction medium for wheat anther culture. *Plant Cell Tiss. Org. Cult.* 12 : 3-12.

Fernandes P, Bapat V A and Rao P S 1995. Effect of crushed seed homogenate on germination of synthetic seeds of *Santalum album* L. *Ind. J. Exp. Biol.* 32 : 840–841.

*Ferrão J E M 1992. A Aventura das Plantas e os Descobrimentos Portugueses. Comissão Nacional para a Comemoração dos Descobrimentos Portugueses, Lisboa, Portugal.

*Ferrão J E M 1993. Especiarias. Instituto de Investigação Científica Tropical, Lisboa, Portugal.

Finkle B J, Zavala M E and Ulrich J M 1985. Cryoprotective compounds in the viable freezing of plant tissues. In. K.K. Kartha (ed.), pp. 75–113. Cryopreservation of Plant Cells and Organs. CRC press, Boca Raton, Florida.

Flavell R et al. 1977. Repeated sequence DNA relationships in four cereal genomes *Chromosoma* 63 : 205–222

Flick C E, Evans D A and Sharp W R. 1983. Organogenesis. pp : 13-81. *In.* Evans *et al* (eds.) 1983 (*q.v.*).

Fooland M R; Jones R A and Rodieiguez R L 1993. RAPD markers for constructing Intra specific tomato genetic maps. *Plant Cell Reports* 12 (5) : 293–297.

Forougchi-Wehr B, Friedt W and Wenzel G. 1982. On the genetic improvement of androgenic haploid formation in *Hordeum vulgare* L. *Theoretical and Applied Genetics.* 62 : 233-239.

Frank J, Barnabas B, Gal E and Farkas J 1982. Storage of sunflower pollen. *Z.* Pflanzenziichtg. 89 : 341–343.

Frere J 1954. Le Conditionnement', in Le Vanillier et la Vanille. Bouriquet G (ed.). Paris Editions Paul Lechavalier.

Frison E A and Putter C A J (eds.). 1989. FAO / IPGRI Technical Guidelines for the Safe Movement of Musa Germplasm. Food and Agriculture Organization of the United Nations, Rome / International Board for Plant Genetic Resources, Rome.

Frison E A, Putter C A J and Diekmann M (eds.). 1993. FAO / IPGRI Technical Guidelines for the safe Movement of Coconut Germplasm. Food and Agriculture Organization of the United Nations, Rome / International Board for Plant Genetic Resources, Rome.

Fromm M E, Taylor L P and Walbot V 1986. Stable transformation of maize after gene transfer by electroporation. *Nature* 319 : 791–793.

Fukui K. 1986. Case histories of genetic variability *in vitro*: Rice. pp: 385–398. *In.* Vasil I K (ed.) 1986 (*q.v.*).

Galderisi U, Cipollaro M, Di Bernardo G,.De Masi L, Galano G and Cascino A 1999. Identification of the edible Fig 'Bianco del Cilento'by RAPD analysis. *Hort Science* 34 (7) : 1263–1265.

Ganeshan S and Alexander M P 1988. Fertilizing ability of cryopreserved grape (*Vitis vinifera* L.) pollen. *Genome* 30 (suppl. 1) : 464.

Ganeshan S. 1986. Cryogenic preservation of papaya pollen. *Sci. Hort.* 28 : 65–70.

Geetha S P, Manjula C and Sajina A. 1995. *In vitro* conservation of genetic resources of spices. In pp. 12-16Proc. Seventh Kerala Science Congress, State Committee on Science, Technology and Environment, Kerala.

Geetha S P, Nirmal Babu K, Rema J, Ravindran P N and Peter K V. 2000. Isolation of protoplasts from cardamom (*Elettaria cardamomum* Maton.) and ginger (*Zingiber officinale* Rosc.). *J. Spices and Aromatic Crops.* 9 (1) : 23 – 30.

Gengenbach, B J, Green, C E and Donovan C M. 1977. Inheritance of selected pathotoxin-resistance in maize plants regenerated from cell cultures. *Proc. Nat. Acad. Sci.* USA. 74 : 5113-5117.

George C 1996. Vanilla development in India. *Indian Cocoa, Arecanut and Spices Journal* 20 (2) : 47 – 49.

George E F 1993. *In.* Plant Propagation by Tissue Culture. Part I The Technology, Exegenetics Ltd., Edington, England.

George E F 1996. Plant Propagation by Tissue Culture. Part 2. In Practice, Exegenetics Ltd., Edington, England.

George E F and Sherington P D 1984. Plant Propagation by Tissue Culture. Exegetics, Eversley, England

George L and Eapen S. 1995. Encapsulation of somatic embryos of finger millet, *Eleusine coracana* Gaertn. *Ind. J. Exp. Biol.* 33 : 291–293.

George L and Rao P S. 1983. Yellow seeded variants in *in vitro* regenerants of mustard (*Brassica juncea* coss var RAI –5) *Plant Sci Lett.* 30 : 327-330.

Glimelius K. 1984. High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. *Physiol. Plant.* 61 : 38-44.

Gobley T W 1858. 'Recherches sur la principe odorant de la vanille'. *J. Pharm. Chim.*, 111 (34) : 401-5.

Goodwin P B. 1966. An improved medium for the rapid growth of isolated potato buds. *Exp. Bot.* 17 : 590–595.

Gopinath C. 1994 Secret of vanilla – Farmer's notebook on vanilla. Indian Spice Associates, Puttur, Karnataka.p.31.

Gottieb L D 1977. Electrophoretic evidence and plant systematics. *Ann.Mol. Bot. Gard.* 64 : 161–180.

Gozukirmizii N, Are S, Gürel F, Gümüsel F and Ci Rakôghi 1992. Finger printing barely genome using PCR with Arbitrary primer in barely plants regenerated from tissue culture. In Biotechnology in Agriculture: Proceedings of the first Asia- Pacific conference of Agriculture Biotechnology Beijing China, 20-24 August 1992 Current Plant Science and Biotech in Agriculture Vol.15.

Green S K and Lo C Y. 1989. Elimination of sweet potato yellow dwarf virus (SPYDV) by meristem tip culture and by heat treatment. *J. Plant Dis. Protection* 96 : 464–469.

Gregory D W and Cocking E C 1965. The large scale isolation of protoplast from immature tomato fruits. *J. Cell Biol.* 24 : 143–146.

Gregory L E, Murray H G and Colberg C 1967 Parthenocarpic pod development by *Vanilla planifolia* Andrews induced with growth regulating chemicals. *Economic Botany* 21: 351-7.

Griessbach R J, Malmberg R L and Carlson P S 1982. *J. Heredity.* 73: 151–152.

Grieve M 1981. A Modern Hebal. Dover Publications, New York.

Groose R W and Bingham E T 1986. An unstable anthocyanin mutation recovered from tissue culture of alfalfa (*Medicago sativa*). 1- High frequency of reversion upon reculture. *Plant Cell Rep.* 5: 104–107.

*Guikema J A and Sherman L A. 1980. Electrophoretic profiles of cyanobacterial membrane polypeptides showing heme-dependant peroxidase activity. *Biochem. Biophys. Acta.* 637 : 189 – 201.

Gunning, J. and Lagerstedt, H.B. 1986. Long term storage techniques for plant germplasm. *Comb. Proc. Int. Plant Crop Soc.* 1985. **35**: 199–205.

Gupta P P 1986. Eradication of mosaic disease and rapid clonal multiplication of bananas and plantains through meristem tip culture. *Plant Cell Tiss. Org. Cult.* 6 : 33–39.

Hall M D and Cocking E C 1974. The response of *Avena* coleoptile protoplast to indole 3-acetic acid. *Protoplasma.* 19 : 225–234.

Halperin W. 1986. Attainment and retention of morphogenetic capacity *in vitro*.pp 549-567. *In.* Vasil I K (ed.) 1986 *(q.v.).*

Hammatt N, Kim H I, Davey M R, Nelson R S. and Cocking E C. 1987. Plant regeneration from cotyledon protoplasts of *Glycine canascens* and *G. glandestina. Plant Sci.* 48 : 129-135.

Harada T, Matsukawa K, SatoT, Ishikawa R, Niizaki M, SaiTok 1993. DNA – RAPDs detect genetic variation & Paternity in *Malus. Euphytica* 65 (2) : 87–91.

Hartman C L, McCoy T J and Knous T R 1984. Selection of alfalfa (*Medicago sativa*) cell lines and regeneration of plants resistant to toxin(s) produced by *Fusarium oxysporum* f. sp. *medicaginis. Plant Sci Lett.* 34 : 183-194.

Harvais G. 1982. An improved culture medium for growing the orchid *Cypripedium reginae* axenically. *Can.J. Bot.* 60 : 2547.

Harvey C F, Fraser LG and Gill G P 1997. Sex determination in *Actinidia* – In Proc. of the III[rd] International Symposium on Kiwi fruit – Thessaloniki, Greece 19–22 September 1995 *Acta Horticulture* 444 : 85–88.

Hassan S M Z and Takagi H. 1995. Alginate coated nodal segments of yam (*Dioscirea* spp.) for germplasm exchange and distribution. IPGRI/FAO *Plant Genetic Resources Newsletter.* 103 : 32–35.

Hatano M, Nakai R, Kawanishi F, Kedo Kand Shoyama 1997 Genetic Diagnosis of *Rehmannia* species micropropagated by tip tissue culture and an F_1 hybrid by RAPD analysis. *Plant breeding* 116 (6):

Haunold A and Stanwood P C 1985. Long-term preservation of hop pollen in liquid nitrogen. *Crop Sci.* 25 : 194–196.

Havkin-Frenkel D and Dorn R. 1996. Vanilla. In Spices: Flavour chemistry and antioxidant properties. Sara J R and Chi-Tang Ho (eds.) p.223

Havkin-Frenkel D, Podstoski A and Knorr D. 1996. Effect of light on vanillin precursors formation by *in vitro* cultures of *Vanilla planifolia. Pl. Cell Tissue and Organ Culture* 45 : 133–136.

Haymes K M, Weg W E Van De, Arens P, Vosman B and Nijs A P M. Den 1997. Molecular mapping and construction of SCAR markers of the strawberry RPFI resistance gene to *Phytophthora trayarial* and their use in breeding programmes. In proc. of the IIIrd International Strawberry symposium, Veldhoren, Netherlands, 29 April – 4 May 1998. *Acta Horticulturae* 1997 439 : 845–851.

He S, Ohm H and Mackenzie S 1992. Detection of DNA sequence polymorphisms among wheat varieties. *Theoretical and Applied Genetics.* 84 (5-6) : 573–578.

Hecker R J, Stanwood P C and Soulis C A 1986. Storage of Sugarbeet pollen. *Euphytica* 35 : 777–783.

Henshaw G G ,O'Hara J F and Westcott R J. 1980. Tissue culture methods for the storage and utilisation of potato germplasm. In Ingram, D. S. and Helgeson, J. P. (eds.). pp: 71–76. Tissue Culture for Plant Pathogens, Blackwell Scientific, Oxford

*Heusser V C 1938. Chromosomen verhaltnisse-bei schweizerischen basitonen Orchideen. Ber. Schweiz. *Bot Gewell* 48 : 562-605.

Hong Chong Jian, Eto HT, Landry B and Matsuzoe N 1997. RAPD markers related to pollen fertility in Garlic (*Allium satirum* L). *Breeding Science* 47 (4): 359–362.

Horine R.K and Ruesink A W. 1972. Cell wall regeneration around protoplasts isolated from *Convolvulus* tissue culture. *Plant Physiol.* 50: 438–445.

Hormaza J I, Pinney K and Polito V S 1998.Genetic Diversity of *Pistachio* Germplasm based on RAPD markers. *Economic Botany* 52 (1): 78–87.

Hornung R, Holland A, Taylor H F and Lynch P T. 2001. Cryopreservation of *Auricula* shoot tips using the encapsulation/dehydration technique. *CryoLetters* 22 : 27–34.

Hosoki T and Sagawa Y. 1977. Clonal propagation of ginger (*Zingiber officinale* Rosc.) through tissue culture. *Hort. Sci.* 12 : 451–452

Hu C Y and Wang P J 1983. Meristem, shoot tip and bud cultures. In. Handbook of Plant Cell Culture Vol. 1 Techniques for Propagation and Breeding, pp: 177–227. Evans D A, Sharp W R, Ammirato P V and Yamada Y (eds.) Macmillan Publishing Company, New York.

Hummer K E. 1994. Genetic resources of *Pyrus* and related genera at the Corvallis repository. *Acta Hort.* 367 : 64–71.

Hunter, C.S., Champion, L.N. and Saul, J. 1986. Photosynthesis by *Cinchona* shoots *in vitro*: Effects of CO_2 concentration. *In.* Abst. VI th *Int. Cong. Plant Tissue and Cell Culture.* Int. Assoc. Plant Tissue Culture. Somers *et al* (eds.). Minneapolis, Minn. pp. 410

Hussey G and Stacey N J. 1981. *In vitro* propagation of potato (*Solanum tuberosum* L.) *Ann. Bot.* 48 : 787–796.

Hussey G. 1978. *In vitro* propagation of the onion *Allium cepa* by axillary and adventitious shoot proliferation. *Sci. Hortic.* 9 : 227–236.

IBPGR. 1985. *In vitro* conservation. IBPGR Research Highlights 1984 – 85. pp. 1–21. In *Hordeum* Forougchi-Wehr B, Friedt W and Wenzel G 1982. On the genetic improvement of androgenic haploid formation *vulgare* L. *Theor. Appl. Genet.* 62 : 233-239.

IPGRI / CIAT. 1994. Establishment and operation of a pilot *in vitro* active genebank. Report of a CIAT-IPGRI Collaborative Project using Cassava (*Manihot esculenta* Crantz.) as a model. IPGRI, Rome.

Isabel N, Tremblay L, Michand M, Tremblay F M, Bousquet J 1993. RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis desired population of *Picea mariana. Theor. Appl. Genet.* 80 (1) : 81–87.

Islam M O, Takagi H and Senboku T. 1994. Cryopreservation of taro germplasm. pp. 33-34. In. Proc. BAAS Conference.Section II, Dhaka, Bangladesh.

Ito M and Maeda M 1973. Fusion of meiotic protoplast in liliaceous plants. *Exp. Cell. Res.* 80 : 453–456.

*Jackson G V H. 1990. Pathogen-tested taro. FAO Plant Protection Bull. 38 : 145–150.

Jackson, J.A. and Dale, P.J. 1988. Callus induction, plant regeneration and an assessment of cytological variation in regenerated plants of *Lolium multiflorum* L. *J. Plant Physiol.* 132: 351–355.

Jan C H, Byrne D H, Manhost J and Wilson H. 1999 Rose Germplasm analysis with RAPD markers. *Hort Science* 34 (2) : 341–345.

Janssen P, Coopman R, Huys G, Swings J, Bleeker M, Vos P, Zabeau M and Kersters K. 1996. Evaluation of the DNA-fingerprinting method of AFLP as a new tool in bacterial taxonomy. *Microbiology* 142 (7) : *1881-1893*

Jarret R L and Fernandez Z. 1992. Shoot tip vanilla culture for storage and exchange (FAO Publication). pp 25-27.

Jarret R L and Florkowski W. 1990. *In vitro* active vs. field genebank maintenance of sweet potato germplasm: Major costs and considerations. *HortSci.* 25 : 141–146.

Jarret R L and Gawel N. 1991a Abscisic acid induced growth inhibition of sweet potato (*Ipomoea batatas* L.) *in vitro. Plant Cell Tiss. Org. Cult.* 24 : 13–18.

Jarret R L and Gawel N. 1991b. Chemical and environmental growth regulation of sweet potato (*Ipomoea batatas* (L.) Lam.) *in vitro. Plant Cell Tiss. Org. Cult.* 25 : 153-159.

Jarret R L. 1989. A repository for sweet potato germplasm. *Hort.Sci.* 24 : 141–150.

Jarret, R. L. and Litz, R. E. 1986. Isozymes as genetic markers in bananas and plantains. *Euphytica* 35 : 539–549.

Jean-Guy Parent, Daniele Page 1998. Identification of Raspberry Cultivars by sequence characterized amplified Region DNA analysis. *HortScience* 33 (1) : 146–142.

Jiang Y S and Gao Z J 1989. Ultra low temperature (–196^{0}C) storage of peach and pear pollen. Acta Agriculturae Shanghai 5 : 1–8.

Johnson S S, Philips R L and Rines H W. 1987a. Meiotic behaviour in progeny of tissue culture regenerated oat plants (*Avena sativa* L.) carrying near telocentric chromosomes. *Genome* 29 : 431– 438.

Johnson S S, Philips R L and Rines H W. 1987b. Possible role of heterochromatin in variation induced by tissue culture in oats (*Avena sativa* L.). *Genome* 29 : 439-446.

Joshi C P, Nguyen H T 1993. Application of the RAPD technique for the detection of polymorphism among wild and cultivated tetraploid wheat. *Genome* 36 (3): 602–609.

Kacker A, Bhat S R, Chandel K P S and Malik S K. 1993. Plant regeneration *via* somatic embryogenesis in ginger. *Plant Cell Tiss. Org. Cult.* 25 : 153 – 159.

Kageyama Y, Honda Y and Sugimura Y. 1995. Plant regeneration from patchouli protoplasts encapsulated in alginate beads. *Plant Cell Tiss. Org. Cult.* 41 : 65–70.

Kao Y Y, Chang S B, Lin T Y, Hsieh C H, Chen Y H, Chen W H, and Chen C C 2001. Differential accumulation of heterochromatin as a cause of karyotype variation in *Phalaenopsis* orchids. *Ann. Bot.* (UK) 87 (3): 387–395.

Karp, A. 1990 .Somaclonal variation in potato.pp 379-399. *In.* Bajaj, Y.P.S. (ed.) 1990a *(q.v.).*

Kartha K K 1985. In. pp. 243–267. Cryopreservation of Plant Cells and Organs. Kartha K K (ed.) CRC Press, Boca Raton, Florida.

Kartha K K and Engelmann F. 1994. Cryopreservation and germplasm storage. In. Vasil I K and Thorpe T A (eds.), pp. 195–230. Plant Cell and Tissue Culture. Kluwer Academic Publishers, Dordrecht/Boston/London.

Kartha K K, Leung N L and Mronginski L A 1982. *In vitro* growth responses and plant regeneration from cryopreservation meristems of cassava (*Manihot esculenta* Crantz.). *Z. Pflanzenphysiol.* 107 : 133–140.

Kartha K K, Michayluk M R, Kao K N, Gamborg O L and Constabel F 1974. Callus formation and plant regeneration from mesophyll protoplast of rape plant (*Brassica napus* L. cv. Zephyr). *Plant Sci. Lett.* 3 : 265–271.

Kartha K K, Mroginski L A , Pahl K and Leung N L.1981. Germplasm preservation of coffee (*Coffea arabica*) by *in vitro* culture of shoot apical meristems. *Plant Sci. Lett.* 22 : 301–307.

Keller J 1991. *In vitro* conservation of haploid and diploid germplasm in *Allium cepa* L. *Acta Hortic.* 289 : 231–232.

Keyes G J and Bingham E T 1979. Heterosis and ploidy effects on the growth of alfalfa callus. *Crop. Sci.* 19 : 473–476.

Kibler R and Neumann K H 1980. On cytogenetic stability of cultured tissue and cell suspensions of haploid and diploid origin. pp: 59–65. *In.* Sala F, Parisi B, Cella R and Ciferri O (eds.) Plant Cell Cultures: Results and Perspectives. Elsevier/North-Holland Biomedical Press, Amsterdam.

Kitto S L and Janick J 1985a. Production of synthetic seeds by encapsulating asexual embryos of carrot. *J. Amer. Soc. Hort. Sci.* 110: 277–282.

Kitto S L and Janick J 1985b. Hardening treatments increase survival of synthetically–coated asexual embryos of carrot. *J. Amer. Soc. Hort. Sci.* 110: 283–286.

Knowlton E H. 1922. Studies in pollen with special reference to longevity. Cornell Univ. Agric. Exp. Stat. Memoir. 52 : 747–794.

Knudson L 1950. Germination of Seeds of *Vanilla. American Orchid Soc. Bull.* 41: 443–445.

Koch G and Jung C. 1997. Phylogenetic relationship of Industrial Chicory Varieties revealed by RAPDs and AFLPs. *Agronamie* 17(6/7): 323–333.

Kölliker R, Stadelmann F J, Reidy B, Nosberger J 1999. Genetic variability of forage grass cultivars: A comparison of *Fesfuca–pratensis* Hinds.; *Lolium perenne* L; *Dactylis glomerata* L *Euphytica* 106 (3): 261–270.

Kononowicz H and Janick J 1984. *In vitro* propagation of *Vanilla planifolia. HortScience.* 19: 58–59.

Krishnamohan P T and Jorapur S M 1986. *In vitro* seed culture of *Acampe praemorsa* (Roxb.) Blatt and McC. In Vij S P (ed) q.v. p. 437.

Kuruvilla K M, Radhakrishnan V V, Madhusoodanan K V and Potti S N 1996. Floral biology of *Vanilla. Spice India* : 20-22

Lal R and Lal S 1990. Somaclonal Variation in Crop Improvement. *In.* Rup Lal and Sukanya Lal (Auth.) Crop Improvement and Biotechnology, CRC Press, Florida.

Larkin P J 1986. Case histories of genetic variability *in vitro*: wheat and triticale. pp: 367–384. *In.* Vasil I K (ed.) 1986 (*q.v.*).

Larkin P J and Scowcroft W R 1981. Somaclonal Variation - A novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* 60 : 197–214.

Latta R. 1971. Preservation of suspension cultures of plant cells by freezing. *Can. J. Bot.* 49 : 1253–1254.

Lavarack B 1996. *Vanilla planifolia.* Austral. *Orchid Rev.* 61(3): 15.

Lazar M D, Baenziger P S and Schaeffer G W 1984. Combined abilities and heritability of callus formation and plantlet regeneration in wheat (*Triticum aestivum* L.) anther culture. *Theoretical and Applied Genetics.* 68: 131–134.

Lee M and Phillips R L 1988. The chromosomal basis of somaclonal variation. *Ann. Rev. Plant Physiol. Plant Mol Biol.* 39 : 413–437.

Lewis E 1992. Identification of Avocado cultivars with RAPD markers Inligtingsbulletin – Instituut Vir Tropiece en subtropiece Gewasse South Africa. No. 241 7–9

Lin C S, Bins M R 1984. The precision of cultivar traits. *Can. J. Plant Sciences* 64 : 587–591.

Lin Y H, Chang C and Chang W C 2000. Plant regeneration from callus culture of a *Paphiopedilum* hybrid. *Plant Cell Tiss. Org. Cult.* 62 : 21–25.

Ling D H., Ma Z R., Chein W Y and Chein M F. 1987. Male sterile mutant from somatic cell culture of rice. *Theor. Appl. Genet.* 75 : 127-131.

Lionnet J F G. 1958. 'Seychelles Vanilla', World Crops. 10 : 441–444.

Liou P C, Chang Y M, Hsu W S, CHeng Y H, Chang H R, Hsiao C H 1998. Construction of a Linkage map in *Cucumis melo* (L) using RAPD markers. *Acta Horticulturae* 461: 123–131.

Liu C J 1997.Geographical distribution of genetic variation in stylosanthes Scabra revealed by RAPD analysis. *Euphytica* 98 (1/2): 21-27.

Liu C J. 1997. Geographical distribution of genetic variation in *Stylosanthes scabra* revealed by RAPD analysis. *Euphytica* 98 (1/2) : 21–27.

Lundergan C and Janick J. 1979. Low temperature storage of *in vitro* apple shoots. *Hort. Sci.* 14 : 514.

Luo Zheng Rong, Yone Mori K and Sugiura A. 1998 Study on the genetic relationship among *Persimmon* cultivars by RAPD. *J. Fruit Science* 15(4) : 311–316.

Lutz J D, Wong J R, Rowe J., Tricoli D M. and Lawrence R H. Jr. 1985. Somatic embryogenesis for mass cloning of crop plants. pp: 105–116. *In.* Henke *et al* (eds.). Tissue Culture in Forestry and Agriculture. Plenum Press, New York, London.

*Mabanza J, Otabo F R and Moussouami C 2001. Conservation *in vitro* du germplasme de cultivars Africans de manioc (*Manihot esculenta* Crantz). *Plant Genetic Resources Newsletter,* 125: 29–32.

Mabberley D J 1993. The Plant-Book, a Portable Dictionary of the Higher Plants. Cambridge University Press, Cambridge.

Magdalita P M and R A Drew 1998. An efficient interspecific hybridisation protocol for *Carica papaya* L. x *C. cauliflora* Jacq. *Australian Journal of Experimental Agriculture* 38 (5): 523-530.

Malaurie B, Pungu R, Dumont R and Trouslot M F 1995. Effect of growth regulators concentration on morphological development of meristem tips in *Dioscorea cayenensis–D.rotundata* complex and *D. praehensilis. Plant Cell. Tiss. Org. Cult.* 41 : 229–235.

Malemnganba H, Ray B K, Bhattacharya S and Deka P C 1996. Regeneration of encapsulated protocorms of *Phaius tankervillae* stored at low temperature. *Indian J. Exp. Biol.* 34 : 802–805.

Mallory L D and Cochran W P. 1941. Mexican vanilla production and trade. USDA, *Foreign Agric.,* 5 : 469–488.

Mandal B B and Chandel K P S 1993. Conservation of genetic diversity of sweet potato and yams using *in vitro ex situ* strategies. p.11. In. Abst. Intl. Symp.Tropical Tuber Crops, Trivandrum, India

Mandal B B and Chandel K P S 1995. Cryopreservation of encapsulated shoot tips of yams (*Dioscorea* spp.) for long term conservation. IPGRI Newsletter for Asia The Pacific and Oceania 19: 14.

Manning J C and van Staden J 1987. The development and mobilization of seed reserves in some African orchids. *Aust. J. Bot.* 35: 343.

Mantell S H, Haque S Q and Whitehall R P. 1980. Apical meristem-tip culture for eradication of flexous rod viruses in yam (*Dioscorea alata*). Trop. Pest Manage. 26 : 170–179.

Marchant R, Power J B, Davey M R, Chartier–Hollis J M and Lynch P T 1993. Cryopreservation of pollen from two rose cultivars. *Euphytica* 66 : 235–241.

Marin M L and Duran-Vila 1992. Cryopreservation of somatic embryos of 'Washington Navel' sweet orange. pp. 313–317. In Proc. Intl. Soc. Citriculture. VII Int. Citrus Congress.

Marin M L and Duran-Vila. 1991. Conservation of citrus germplasm *in vitro*. *J. Am. Soc. Hort. Sci.* 116 : 740–746.

Marin M L, Mafla G, Roca W M and Withers L A. 1990. Cryopreservation of cassava zygotic embryos and whole seeds in liquid nitrogen. *CryoLetters* 11: 257–264.

Marino G, Rosati P. and Sagrati F. 1985. Storage of *in vitro* cultures of *Prunus* rootstocks. *Plant Cell Tiss. Org. Cult.* 5 : 73-78.

Mariska I Hobir, Husni A, Kosmiatin M, Rusyadi Y.1997. *In vitro* culture of hybrid seeds cultivated and wild *Vanilla*. Breeding to increase competitiveness of Indonesian agriculture commodities.pp.471–480.

Markert C L and Muller F 1959. Multiple forms of enzymes: Tissue, autogene and species specific patterns. Proc. National Acad. Sciences. USA. 45 : 753–763.

Mathew K M, Rao Y S, Kumar K P, Madhusoodanan K J, Potty S N, Kishor P B K. 1999. *In vitro* culture systems in *Vanilla*. In. Proc. Symp. Plant tissue culture and biotechnology: emerging trends, Hyderabad, India, 29-31 January, 171-179.

Mathew M K, Rao Y S, George G L, Lakshmanan P, and Madhusoodanan K J 2000. *In vitro* propagation of *Vanilla tahitensis* Moore. *J. Spices and Aromatic Crops* 9 (2): 171–173.

Mathias R J and Fukui K 1986. The effect of specific chromosome and cytolplasmic substitution on the tissue culture response of wheat (*Triticum aestivum*) callus. *Theor. Appl. Genet.* 71 : 797–800.

Mathias R J and Fukui K. 1986. The effect of specific chromosome and cytolplasmic substitution on the tissue culture response of wheat (*Triticum aestivum*) callus. *Theor. Appl. Genet.* 71 : 797-800.

Mathur J. 1991. Enhanced somatic embryogenesis in *Selenum candolii* DC. under a mineral oil overlay. *Plant Cell Tiss.Organ. Cult.* 27: 23–26.

Matsumoto T, Sakai A, Takahashi C and Yamada K 1995. Cryopreservation of *in vitro*–grown apical meristems of wasabi (*Wasabia japonica*) by encapsulation–vitrification method. *CryoLetters* 16 : 189–196

McCoy T J and Echt C S 1993. Potential of tri-species bridge crosses and RAPD markers for introgression of *Medicago daghestanica* and *M. pironae* germplasm in to alfalfa (*Medicago sativa*) *Genome* 36 (3): 394–601.

McCulloch S M. 1988. Commercial micropropagation of *Kalmia.* Comb. *Proc. Int. Plant Prop. Soc.* 1987. 37: 107-109.

McKersie B D and Bowley S R 1993. Synthetic seeds of alfalfa. In. Redenbaugh K (ed.) Synseeds: Applications of Synthetic Seeds to Crop Inprovement. CRC press, Boca Raton. FL. pp. 231–255.

Mehra A and Mehra P N 1974. Organogenesis and plantlet formation *in vitro* in almond. *Bot. Gaz.* (Chicago) 135: 61–73.

Menendez C M, Hall A E, Gepts P A 1997. Genetic linkage map of cow Pea developed from a cross between 2 inbred domesticated lines. *Theor. Appl. Genet.* 95 (8): 1210–1217.

Mereier H and Kerbauy G B. 1991. Effects of nitrogen source on growth rates and levels of endogenous cytokinins and chlorophyll in protocorms of *Epidendrum fulgens.* J. Plant Physiol. 138 : 195.

Minoo D, Sajina A, Nirmal Babu K and Ravindran P N 1997. Ovule culture of vanilla and its potential in crop improvement. In. Edison S, Ramana K V, Sasikumar B, Nirmal Babu K and Santhosh J. Eapen (eds.). Biotechnology of Spices, Medicinal and Aromatic Plants, Indian Society for Spices, Calicut, India, p. 112–118.

Mitra G and Chaturvedi H 1972. Embryos and complete plants from un-pollinated ovaries and from ovules of *in vitro* grown encapsulated flower buds of citrus species. *Bull. Torrey Bot. Club.* 99 : 184–189.

Mitra G C 1986. *In vitro* culture of orchid seeds. In. Biology, conservation and culture of orchids. Vij S P (ed.), Affiliated East-West Press Private Ltd., New Delhi. 401.

Mix G 1984. Long-term storage *in vitro* of potato gene material. *Plant Res. Develop.* 19: 122–127.

Morel G M 1960. Producing virus–free Cymbidiums. *Amer. Orchid. Soc. Bull.* 29 : 495–497.

Morel G M 1965. Clonal propagation of orchids by meristem culture. *Cymbidium Soc. News.* 20: 3–11.

Moriguchi T, Kozaki I, Matsuta N and Yamaki S.1988. Plant regeneration from grape callus stored under a combinationof low temperature and silicon treatment. *Plant Cell Tiss.Org. Cult.* 15 : 67-71.

Moriguchi T and Yamaki S. 1989. Prolonged storage of grape nodal culture using a low concentration of ammonium nitrate. *HortScience.* 24 : 370-373.

Moriguchi T, Kozai I, Yamaki S and Sanada T 1990. Low temperature storage of pear shoots *in vitro. Bull. Fruit Tree Research Station.* 17 : 11–18.

Mulcahy D L, Cresti M, Sansavini S, Donglan G C, Linskens H F, Bergamini Mulcahy G, Vignani R and Pancaldi M. 1993. Use of RAPDs to fingerprint apple genotype. *Scientia Horticulturae* 54 (2) : 89–96.

Mullin R H and Schlegel D E. 1976. Cold storage maintenance of strawberry meristem plantlets. *HortScience.* 11: 100–101.

Mullin R H and Schlegel D E. 1976. Cold maintenance of strawberry meristem explants. *HortScience* 11: 100–101.

Mullis K B and Faloona F A 1987. Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. *Methods in enzymology* 155 : 335–351.

Munthali M T, Newbury H J and Ford–Lloyd B V. The detection of somaclonal variants of beet using RAPD. *Plant Cell Rep.* 15 : 474–478.

Murashige T 1989. Plant propagation by tissue culture practice with unrealized potential. In. Handbook of plant cell culture. Vol. 5. Ornamental species, Ammirato P V, Evans D A, Sharp W R and Bajaj Y P S (eds) McGraw–Hill, New York. pp: 3–9.

Murashige T and Nakano R 1967. Chromosome complement as a determinant of the morphogentic potential of tobacco cells. *Amer. J. Bot.* 54 : 963–970.

Murashige T and Skoog F 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15 : 473–497.

Murray M G and Thompson W F. 1980. Rapid isolation of high- molecular-weight plant DNA. *Nucleic Acids Res.* 8 : 4321-4325.

Murray M G and Thompson W F 1980. Rapid Isolation of high molecular weight plant DNA. *Nucleic Acid Research* 8: 4321–4325.

Myburg A A, Cawood M, Wingfield B D and Botha A M 1998. Development of RAPD and SCAR markers linked to the Russian wheat Aphid resistance gene Dn 2 in wheat. *Theor. Appl. Genet.* 96 (8) : 1162–1169.

Nadgauda R S, Mascarenhas A F, Hendre R R and Jagannathan V 1978. Rapid clonal multiplication of turmeric, *Curcuma longa* L. plantlets by tissue culture. *Indian J.Exp.Biol.* 16 : 120–122.

Nair R R. and Ravindran P N 1994 Somatic association of chromosomes and other mitotic abnormalities in *Vanilla planifolia* (Andrews). *Caryologia* 47 : 65–74.

Narasimhulu S B, Prakash S and Chopra V L 1988. Comparative shoot regeneration responses of diploid Brassicas and their synthetic amphidiploid products. *Plant Cell Rep.* 7: 525–527.

Narayanaswamy S. 1977. Regeneration of plant from tissue cultures. pp: 179–248. *In.* Reinert J and Bajaj Y P S (eds.) Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. Springer-Verlag, Berlin, Heidelberg.

Nash T 1966. Chemical constitution and physical properties of compounds able to protect living cells against damage due to freezing and thawing. In. Cryobioogy, Meryman H Y (ed.), pp. 197–211. Academic Press, New York,.

Navarro L, Juarez J, Pina J A and Ballester J F 1984. The *Citrus* quarantine station in Spain. In Proc 9th Conf. Int. Organization Citrus Virol. (IOVC) (Garnsey S M, Timmer L W and Dodds J A (eds.) Riverside.

Navarro L. 1992. Citrus shoot tip grafting *in vitro*. pp.327–338. In. Biotechnology in Agriculture and Forestry. Vol. 18 Bajaj Y P S (ed.). Springer-Verlag, Berlin.

Nayak N R, Rath S R, Patnaik S N 1998. High frequency plant regeneration from alginate encapsulated protocorm like bodies of *Spathoglottis plicata* BL., a terrestrial orchid. *Phytomorphology* 48: 179–186.

Negrutiu I, Shillito R D, Potrykus I, Biasini G and Sala F. 1987. Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. *Plant Mol. Biol.* 8 : 363–373.

Ng N Q and Ng S Y C. 1994. Approaches for yam germplasm conservation. pp. 97–102. In. Root Crops for Food Security in Africa, Akoroda M O (ed.). ISTRC-AB/CTA/IITA.

Ng S Y C 1992. Micropropagation of white yam (*D. rotundata* Poir.). pp.135–159. In. Biotechnology in Agriculture and Forestry, vol. 19 Bajaj Y P S (ed.). Springer-Verlag, Berlin.

Ng S Y C. 1983a. Virus elimination in sweet potato, yam and cocoyam. pp. 97–102 In. Global Workshop on Root and Tuber Crops Propagation. CIAT Columbia.

Ng S Y C. 1983b. Rapid propagation technique for sweet potato, yam and cocoyam. pp. 117–18 In. Global Workshop on Root and Tuber Crops Propagation. CIAT Columbia.

Ng S Y C. 1991. *In vitro* conservation and distribution of root and tuber crop germplasm. pp. 95-106. In. Crop Genetic Resources of Africa. Vol. II Ng N Q, Perrino P, Attere F and Zedan H (eds.). IITA / IBPGR/ UNEP/ CNR.

Nielsen J M, Brandt K and Hansen J 1993. Long-term effects of thidiazuron are intermediate between benzyladenine, kinetin or isopentyladenine in *Miscanthus sinensis*. *Plant Cell Tiss. Org. Cult.* 35 : 173.

Nielsen R L and Siegismund H R. 1999. Interspecific differentiation and hybridization in *Vanilla* species (Orchidaceae). *Heredity* 83 : 560–567.

Niino T and Sakai A. 1992. Cryopreservation of alginate coated *in vitro* grown shoot tips of apple, pear and mulberry. *Plant Sci.* 87: 199–206.

Niino T and Sakai A. 1992. Cryopreservation of alginate coated *in vitro* grown shoot tips of apple, pear and mulberry. *Plant Sci.* 87: 199–206.

Niino T, Sakai A, Enomoto S, Magosi J and Kato K. 1992b. Cryopreservation of *in vitro* grown shoot tips of mulberry by vitrification. *Cryoletters* 13 : 379–388.

Niino T, Sakai A, Yakuwa H and Nojori K 1992a. Cryopreservation of *in vitro* grown shoot tips of apple and pear by vitrification. *Plant Cell Tiss. Org. Cult.* 28 : 261–266.

Nirmal Babu K ,Geetha S P, Manjula C, Ravindran, P N and Peter K V. 1994. Medium term conservation of cardamom germplasm - an *in vitro* approach. *In.* Abst. Second Asia-Pacific Conference on Agricultural Biotechnology, Madras, India. p. 57.

Nirmal Babu K, Geetha S P, Minoo D, Ravindran P N and Peter K V 1999. *In vitro* conservation of germplasm. pp :106–129, In. In Ghosh S P (ed) Biotechnology and its application in Horticulture. Narosa Publishing House, New Delhi.

Nirmal Babu K, Ravindran P N and Peter K V 1997. Protocols for Micropropagation of Spices and Aromatic Crops. Indian Institute of Spices Research, Calicut, Kerala. 35 p.

Nirmal Babu K, Rema J, Ravindran P N and Peter K V. 1993. Micropropagation of black pepper and related species – its potential in crop improvement. In. Golden Jubilee Symposium on Horticultural Research – Changing scenario, Hortiuclture Society of India. Abst. p. 250.

Nirmal Babu K, Samsudeen K and Ratnambal M J. 1992a. *In vitro* plant regeneration from leaf derived callus in ginger, *Zingiber officinale* Rosc. *Plant Cell Tiss. Org. Cult.* 29 : 71–74.

Nirmal Babu K, Samsudeen K and Ravindran P N. 1992b. Direct regeneration of plantlets from immature inflorescences of ginger (*Zingiber officinale* Rosc.) by tissue culture. *J. Spices and Aromatic Crops* 1: 43 –48.

Nitzsche W 1978. Conservation of viability in dried callus. *Z. Pflanzenphysiol.* 87: 469–472

Nitzsche W. 1980. One year storage of dried carrot callus. *Z. Pflanzenphysiol.* 100 : 269–272.

Nitzsche W. 1983. Germplasm preservation. pp.782-805. In. Evans *et al* (eds.) 1983 (q.v.). *Z. Pflanzenphysiol.* 87: 469– 472

Niwata E. 1995. Cryopreservation of apical meristems of garlic (*Allium sativum* L.) and high subsequent plant regeneration. *CryoLetters* 16 : 102–107.

Normah M N and Vengadasalam M. 1992. Effects of moisture content on cryopreservation of Coffea and Vigna seeds and embryos. *CryoLetters.* 13 : 99–208.

Norman J. 1990. The Complete Book of Spices. Dorling Kindersley, London.

Novak F J and Havel L. 1981. Shoot production from *in vitro* cultured flower heads of *Allium porrum* L. *Biol. Plant.* 23 : 266–269.

Novak F J, Afza R, Van Duren M, Perea-Dallos M, Conger B V and Xiaolang T. 1989. Somatic embryogenesis and plant regeneration in suspension cultures of dessert (AA and AAA) and cooking (ABB) bananas (*Musa* spp.). *Bio/Technology* 7: 154–159.

O'Kelley J C 1995 . External carbohydrates in growth and respiration of pollen tubes *In vitro. Amer. J. Bot.* 42 : 322–327.

Ogura H, Kyozuka J, Hayashi Y and Shimamoto K. 1988. Yielding ability and phenotypic uniformity in the selfed progeny of protoplast derived rice plants. *Jap. J. Breed.* 39 : 47-56.

Ogura H 1990. Chromosome variation in plant tissue culture. pp: 49–84. *In.* Bajaj Y P S (ed.) 1990a *(q.v.).*

Oliva A P and Arditti J. 1984. Seed germination of North American orchids. II. Native California and related species of *Aplectrum*, *Cypripedium* and *Spiranthes*. *Bot. Gaz.* 145 : 495.

Ollitrault P, Ollitrault F and Cabasson C. 1992. Induction de cals embryogenes d'agrumes par culture d'ovules : determination isoenzymatique de I'origine tissulaire des embryons. *Fruits* 47 : 204–212.

Omirulleh S, Morocz S and Dudits D 1995. Regeneration of transgenic maize plants from embryogenic protoplasts after polyethylene glycol-mediated DNA uptake. pp : 99–105. *In.* Potrykus, I. and Spangenberg, G. (eds.) (*q.v.*).

Orton T J 1980. Chromosomal variability in tissue cultures and regenerated plants of *Hordeum. Theor. Appl. Genet.* 56 : 101–112.

Orton T J 1984. Genetic variation in somatic tissue-method or madness? *Adv. Plant. Pathol.* 2 : 153–189.

Orton T J. 1986. Case histories of genetic variability *in vitro:* Celery. pp 345–366. In Vasil I K (Ed.) (q.v.).

Ozais-Akins P and Vasil I K 1985. Nutrition of plant tissue cultures. pp : 129–147. *In.* Vasil I K (ed.) Cell Culture and Somatic Cell Genetics of Plants, Vol.2. Academic Press, Florida.

Padmaja G, Reddy L R. and Reddy G M 1995. Plant regeneration from synthetic seeds of groundnut, *Arachis hypogea* L. *Ind. J. Exp. Biol.* 33 : 967–971.

Panis B and Thinh N T 2001. Cryopreservation of *Musa* germplasm. INIBAP Technical Guideline 5 Escalant J V and Sharrock S (eds.). International Network for the Improvement of Banana and Plantain, Montpellier, France. pp. 44.

Panis B. 1995. Cryopreservation of Musa germplasm. *InfoMusa* 4 : 17–20.

Paran I and Michelmore R W 1993. Development of reliable PCR based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet.* 85 : 985–993.

Paran I and R W Michelmore 1993.Development of reliable PCR based markers linked to downey mildew resistance genes in lettuce. *Theor. Appl. Genet.* 85 : 985-993.

Paran I, Kesseli R and Michelmore R W 1991. Identification of RFLP and RAPD markers linked to downy mildew resistance genes in lettuce using near isogenic lines. *Genome* 34 (6):1021–1027.

*Pena M de. 1984. Somatic embryo induction and plant regeneration from *Coffea canephora* and *C. arabica.* pp. 493–512. In. Symposium sobre Ferrugens do Cafeeiro. Comunicacoes. Centro de Investigacao da Ferrugens do Cafeeiro, Oeiras, Portugal.

Peschke V M, Phillips R L and Gegenbach B G. 1987. Discovery of transposable element activity among tissue culture derived maize plants. *Science.* 238 : 804–807.

Philip V J 1989 Use of tissue culture technology in *Vanilla* and possibilities of germplasm conservation. *Indian Journal of Plant Genetic Resources* 2 (2): 114–121.

Philip V J and Nainar S A Z 1986. Clonal propagation of *Vanilla planifolia* (Salisb.) Ames using tissue culture. *J. Plant Physiol.* 122 : 211–215.

Philip V J and Nainar S A Z 1988. *In vitro* transformation of root meristem to shoot and plantlets in *Vanilla planifolia. Ann. Bot.* 61: 193–199.

Philip V J and Nainar S A Z. 1986. Clonal propagation of *Vanilla planifolia* (Salisb.) Ames. using tissue culture. *J. Plant Physiol.* 122 : 211– 215.

Philip V J, Joseph D, Triggs G S and Dickinson N M. 1992. Micropropagation of black pepper (*Piper nigrum* L.) through shoot tip cultures. *Plant Cell Reports.* 12: 41–44.

Piccioni E. 1997. Plantlets from encapsulated micropropagated buds of M.26 apple rootstock. *Plant Cell Tiss. Org. Cult.* 47 : 256–260.

*Plessis P, Leddet C and Dereuddre J. 1991. Resistance to dehydration and to freezing in liquid nitrogen of alginate coated shoot tips of grape vine (*Vitis vinifera* L.cv.Chardonnay). C.R. Acad. Sci (Paris) 313, Ser III: 373 – 380.

Plessis P, Leddet C, Collas A. and Dereuddre J. 1993. Cryopreservation of *Vitis vinifera* L.cv.Chardonnay shoot tips by encapsulation–dehydration: effect of pretreatment, cooling and postculture conditions. *CryoLetters* 14 : 309– 320.

Pojnar, E., Willison, J.H.M. and Cocking, E.C. 1967. Cell wall regeneration by isolated tomato fruit protoplasts. *Protoplasma.* 64: 460-475.

Porebskii S. l. Bailey, G. and Baum, R. B. 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Rep.* 12: 8–15.

Postman J D. 1994. Elimination of virus from clonal pear germplasm. *Acta Hortic.* 367 : 72-75.

Power J B. and Cocking E C. 1970. Isolation of leaf protoplast: macro-molecule uptake and growth substance response. *J. Exp. Bot.* 21 : 64-70.

Preece J E and Sutter E G. 1991. Acclimatization of micrpropagated pants to the greenhouse and field. *In.* Debergh, P.C. and Zimmerman, R.H. (eds.). *Micropropagation.* Kluwer Academic Publishers, Netherlands. pp: 71– 93.

Purseglove J W, Brown E G, Green C l and Robbins S R J 1981. Spices. Vol. 2. Tropical Agricultural Series. Longman Inc., New York. p 644-735.

Purseglove J W. 1972. Tropical Crops : Monocotyledons. Longman, London

Quatrano R S. 1968. Freeze preservation of cultured flax cells using DMSO. *Plant* 43: 2057– 2061.

Rafalski J A and Scott V. Tingey. 1993. Genetic diagnostics in plant breeding – RAPDs, microsatellites and machines. *Trends in Genetics* 9(8).

Rafalski J A, Scott V, Tingey S and Williams J G K. 1994. Plant Molecular Biology Manual H4: 1-8.

Rafalski J A, Tingey S, Williams J G K 1991. RAPD markers – a new technique for genetic map and plant breeding. *Agribiotech News & Information* 3: 645-648.

Raghu Rajan V. 1997. Micropropagation of turmeric (Curcuma longa L.) by *in vitro* microrhizomes. In. (S Edison, K V Ramana, B Sasikumar, K Nirmal Babu and Santhosh J E eds.) Biotechnology of Spices, Medicinal and Aromatic Plants. pp.25-28. Indian Society for Spices, India

Rajasekharan P E and Ganeshan S. 2001. Pollen cryopreservation of vegetable and ornamental species: Retrospects and prospects. *Indian J. Plant Genet. Resources* 14 (2) : 294–295.

Ranadive A S. 1994. In Spices, herbs and edible fungi. Charalambous G (Ed.) Elsevier Science B.V. Amsterdam.

Rao Y S, Mary Mathew K, Madhusoodanan K J and Naidu R. 1993. Multiple shoot regeneration in Vanilla (*V. planifolia* Andrews). J. plantation Crops 21: 351-354.

Rao Y S, Mathew K M, Madhusoodanan K J, Sudharshan M R, Kumar V K, Potty S N. 2000. Natural fruit set in *Vanilla wightiana* Lindl., an endangered species from Andhra Pradesh, India. *J. Spices and Aromatic Crops.* 9 (1): 77.

Rao Y S, Mathew K M, Pradip Kumar K, Lakshmanan R and Madhusoodanan K J. 2002. Occurrence of useful genes in *Vanilla wightiana* and attempts to transfer them into *V. planifolia.* In. Abst. National Seminar on Strategies for Increasing production and export of Spices. 24-26 October. Indian Society for Spices.p8-9

Rao Y S, Mathew M K, Madhusoodanan K J, Sudarshan M R, Krishna Kumar V and Potty S N 2000. Natural fruit set in *Vanilla wightiana* Lindl., an endangered species from Andhra Pradesh, India. *J. Spices and Aromatic Crops.* 9 (1) : 77

Ravindran P N 1979. Nuclear behavior in the sterile pollen of *Vanilla planifolia* (Andrews). *Cytologia* 44 : 391-396.

Reddy P V, Soliman K M. 1997. Identification of wild & Cultivated Hordeum species using two- primer RAPD fragments. *Biologica Plantarum* 39 (4) : 543-552.

Redenbaugh K, Fijii J A and Slade D 1991. Synthetic seed technology. pp 35-74. In. Vasil I K (ed.)Scale up and automation in plant propagation. Cell culture and somatic Cell Genetics of Plants. Vol.8. Academic Press. Inc. San Diego, New York.

Redenbaugh K, Fujii J A and Slade D. 1991. Synthetic seed technology. *In.* Vasil, I.K. (eds.) 1991.Scale up and automation in plant propagation. *Cell Culture and Somatic Cell Genetics of Plants.* Vol.8. Academic Press. Inc. San Diego, New York. pp: 35–74.

Redenbaugh K, Paasch B D, Nichol J W, Kossler M E, Viss P R and Walker KA. 1986. Somatic seeds: Encapsulation of asexual plant embryos. *Bio/Technology.* 4 : 79–83.

Redenbaugh K, Slade D, Viss P R and Fujii J. 1987a. Encapsulation of somatic embryos in synthetic seeds coats. *HortScience.* 22 : 803–809.

Redenbaugh K, Viss P, Slade D and Fijii J. 1987b. Scale – up: artificial seeds. pp: 473–493. *In Plant Tissue and Cell Culture.* Green, C., Somers, D., Hackett, W., Biesboer, D. (eds.). Allan Liss, New York.

Reed B M and Chang Y. 1997. Medium and long-term storage of *in vitro* cultures of temperate fruit and nut crops. In Conservation of Plant Genetic Resources *In vitro.* Vol.1 (MK Razdan and EC Cocking eds.). M/S Science Publishers, Inc. USA.

Reed B M. 1990a. Survival of *in vitro* grown apical meristems of Pyrus following cryopreservation. *HortSci.* 25 : 111 – 113.

Reinert J and Hellman S. 1971. Mechanism of formation of polynuclear protoplasts from cells of higher plants. *Natur wissenschaften.* 58 : 419.

Reisch B and Bingham E T. 1981. Plants from ethionine resistant alfalfa tissue cultures : variation in growth and morphological charaaacteristics. *Crop. Sci.* 21 : 783-788.

Rhodes C A, Green C E and Phillips R L 1986. Factors affecting tissue culture initiation from maize tassels. *Plant Science*. 46 : 225-232.

Roca W M, Bryan J E and Roca M R. 1979. Tissue culture for the international transfer of zygotic embryos of potato genetic resources. *Am. Potato J.* 56 : 1 –10.

Roca W M, Rodriguez J, Beltran J, Roa J. and Mafla G. 1982. Tissue culture for the conservation and international exchange of germplasm. *In.* Fujiwara, A. (ed.). pp: 771–772.Plant Tissue Culture . Proc. Vth. Int. Cong. Plant Tissue and Cell Culture, Japan, Jap. Assoc. Plant Tissue Culture, Tokyo. .

Rodriguez J M, Berke T, Engle L, Nienhuis J. 1999. Variation among and within *Capsicum* sps revealed by RAPD markers. *Theoretical and Applied Genetics* 99 : 147 – 156.

Rout G R, Das P, Goel S and Raina S N. 1998. Determination of Genetic stability of micropagated plants of Ginger using RAPD markers. *Botanical Bulletin of Academia Sincia* 39 (1) : 23-27.

Roy J and Banerjee N 2001. Cultural requirements for *in vitro* seed germiantion, protocorm growth and seedling development of *Geodorum densiflorum* (Lam.) Schltr. *Ind. J. Exp. Biol.* 39 : 1041-1047.

Ruesink A W and Thimann K V. 1965. Protoplast from the *Avena* coleoptile. *Proc. Nat. Acad. Sci.* U.S.A. 54 : 56-64.

Russell J A. and McCown B H 1986. Culture and regeneration of *Populus* leaf protoplasts isolated from non-seedling tissue. *Plant Sci.* 46 : 133-142.

Sajina A, Minoo D, Geetha S P, Samsudeen K, Rema J, Nirmal Babu K and Ravindran P N. 1997. Production of synthetic seeds in few spice crops. In. (S Edison, K V Ramana, B Sasikumar, K Nirmal Babu and Santhosh J E eds.) Biotechnology of Spices, Medicinal and Aromatic Plants. pp. 65-69. Indian Society for Spices, India.

Sakai A. 1993. Cryogenic strategies for survival of plant cultured cells and meristems cooled to –196°C. In *Cryopreservation of Plant Genetic Resources*: Technical Assistance Activities for Genetic Resources Projects, *JICA*, p. 5–30.

Sakai A. 1997. Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems. In Razdan, M.K. and Cocking, E. C. (eds.). pp. 53–66. *Conservaton of Plant Genetic Resources In Vitro. Vol. 1: General Aspects*. Science Publishers Inc. USA. Oxford and IBH Publishing Co. Pvt Ltd., New Delhi.

Sakai A. 1997. Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems. In. Razdan, M.K. and Cocking, E. C. (eds.). *Conservaton of Plant Genetic Resources In Vitro. Vol. 1: General Aspects*. Science Publishers Inc. USA. Oxford and IBH Publishing Co. Pvt Ltd., New Delhi. pp. 53–66.

Sakai A. 1993. Cryogenic strategies for survival of plant cultured cells and meristems cooled to –196°C. In *Cryopreservation of Plant Genetic Resources*: Technical Assistance Activities for Genetic Resources Projects, JICA, p. 5–30.

Sakai A. 1956. Survival of plant tissues at super low temperatures, 1. Low Temp. *Sci. Ser. E.* 14: 17–23.

Sakai A. 1958. Survival of plant tissues at super low temperatures, II. Low Temp. *Sci. Ser. B.* 16: 41–53.

Sakai A. 2000. Development of cryopreservation techniques. In. Engelmann, F. and Takagi, H. (eds.) *Cryopreservation of Tropical Plant Germplasm.* Current Research Progress and Application. Japan International Research Centre for agricultural Sciences, Japan/International Plant genetic Resources Institute, Rome, Italy. pp. 1–7.

Sakai A and Matsumoto T. 1996. A novel cryogenic procedure for cryopreservation of *in vitro* grown meristems of temperate crops – encapsulation–vitrification. In *Proc. Intl. Workshop on In Vitro Conservation of Plant Genetic* Resources (1995)., Kuala Lumpur, Malaysia.

Sakamoto Y, Onishi N and Hiroshima T. 1995. Delivery systems for tissue culture by encapsulation. In. Aitken–Christie, J., Kozai, T. and Smith, M. A. l. (eds.) pp. 215–243. Automation and environmental control in Plant tissue culture, Kluwer Academic Publishers, Dordrecht.

Salvi N D, George L and Eapen S. 2001. Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. *Plant Cell Tissue and Organ Culture.* 66 : 113–119.

Sambrook J, Fritsch E F and Maniatis T. 1989. Molecular cloning : A Laboratory manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. New York.

Samuel Ganesh D, Sreenath H L and Jayashree G. 1996. Micropropagation of vanilla through node culture. *J. Plantation Crops.* 24 (1) : 16-22.

Sanford W W 1964. Sexual compatibility relationship in *Oncidium* and related genera.*Am.Orchid.soc.Bull*, 36 : 114-122.

Sasikumar B, Rema J and Ravindran P N 1992. *Vanilla. Indian Cocoa, Arecanut & Spices.* 16 (1) : 6-10.

Schaeffer G W and Sharpe E T. 1987. Increased lysine and seed protein in rice plants recovered from calli selected with inhibitory levels of lysine plus threonine and S-(2-aminoethyl) cysteine. *Plant Physiol.* 84 : 509-515.

Schafer-Menhur A. 1995. Refinement of cryopreservation techniques for potato. Year-end IPGRI Project Report. International Plant Genetic Resources Institute, Rome, Italy.

Schafer–Menuhr A. 1996. Storage of old potato varieties in liquid nitrogen. IPGRI Newsl. for Europe 7: 8.

Schenk R U and Hildebrandt A C. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cecll cultures. *Can J Bot* 50 : 199-204.

Scott M P, Haymes K M, Williams S M. 1992. Parentage analysis using RAPD – PCR. *Nucleic Acid Research* 20 (20): 4493.

Scottez C, Chevreau E, Godard N, Arnaud Y, Durand M and Dereuddre J. 1992. Cryopreservation of cold-acclimated shoot tips of pear *in vitro* cultures after encapsulation-dehydration. *Cryobiology*. 29 : 691-700.

Sedgley M 1981. Storage of avocado pollen. *Euphytica* 30: 595–599.

Seibert M and Wetherbee P M. 1977. Increased survival and differentiation of frozen herbaceous plant organ cultures through cold treatment. *Plant Physiol.* 59 : 1043–1046.

Seneratna T and McKersie 1989. Artificial seeds for germplasm preservation, exchange and crop improvement. *Diversity* 44.

Shaw C R and Prasad R. 1970. Starch gel electrophoresis of enzymes - a compilation of recipes. *Biochem. Genet.* 4 : 297-320.

Shillito R J, Saul M W, Paszkowski J, Muller M and Potrykus I. 1985. High frequency direct gene transfer to plants. *Bio/Technology.* 3 : 1099-1103.

Shimamoto K, Terada, R., Izawa, T. and Fujimoto, H. 1989. Fertile rice plants regenerated from transformed protoplast. *Nature*. 338 : 274-276.

Shimoni M and Reuveni R 1988. A method for staining and stabilizing peroxidase activity in polyacrylamide gel electrophoresis. *Annal Biochem.* 175 : 35.

Shivanna K R and Sawhney V K. 1997. Pollen Biotechnology for Crop Production and Improvement. Cambridge University Press, UK. 447 pp.

Shuichi Fukuoka, Kazuyoshi, Hosaka & Osamu Kamijima. 1992. Use of RAPDs for identification of Rice accessions. *Japanese Journal of Genetics* 67 : 243-252.

Sibi M. 1990. Genetic basis of variation from *in vitro* tissue culture. pp 112-113. *In.* Bajaj Y.P.S. (ed.) 1990a *(q.v.).*

Sicurani M, Piccioni E, and Standardi A. 2001. Micropropagation and preparation of synthetic seeds in M.26 apple rootstock l: Attempts towards saving labour in the production of adventitipous shoot tips suitable for encapsulation. *Plant Cell Tiss. Org. Cult.* 66 : 207–216.

Simmons R A, Nabors M W and Lee C W. 1984. A model of mutant selection in plant suspension cultures. *J. Plant Physiol.* 116 : 95-102.

Simpson M J A and Withers L A. 1986. Documentation of genetic resources. Characterisation using isozyme electrophoresis: A guide to literature. International Board for Plant Genetic Resources, Rome. pp. 102.

Simpson M J A and Withers L A. 1986. Documentation of genetic resources-Characterization using isozyme electrophoresis: A guide to literature. International Board for Plant Genetic Resources, Rome. pp. 102.

Singh, B.D. and Harvey, B.L. 1975. Selection for diploid cells in suspension cultures of *Haplopappus gracilis*. *Nature*. 253 : 453.

Sivolap Yu M, Kutsevich L I, Palamarchuk A I and Totsky V N. 1998. Molecular Genetic polymorphism of winter durum wheat determined by PCR with arbitrary primers Russian Agricultural Science No: 1 : 9-13.

Smith J S C 1989. The characterisation & assessment of genetic diversity among maize (*Zea mays* L.) hybrids that are widely grown in France, chromatographic data & Isozyme data. *Euphytica* 40 : 73-85.

Smith J S C, Smith O S, Bowen S L, Tenborg R A, Walls S J 1991. The description & assessment of distances between inbred lines of maize. III – A revised scheme for the testing of distinctiveness between inbred lines utilising DNA RFLPs. *Maydica* 36. 213 –226.

Spangenberg, G. and Potrykus, I. 1995. Polyethylene-glycol-mediated direct gene transfer to tobacco protoplasts and regeneration of transgenic plants. pp : 58-65. *In.* Potrykus, I. and Spangenberg, G. (eds.) (*q.v.*).

Spices Board. 2000. Vanilla Status Paper. Spices Board, Cochin, India p.33.

Sreenivasa Rao N K, Narayanaswamy S, Chacko E K and Doraiswamy R. 1982. Regeneration of plantlets from callus of *Elettaria cardamomum* Maton. Proc. India. Aca Sci. (*Plant Sci.)* 91 : 37 – 41.

SreeRamulu, K. 1986. Case histories of genetic variability *in vitro* : Potato. pp : 449-474. *In.* I.K. Vasil (ed.). 1986 *(q.v.).*

Standardi A and Piccioni E. 1998. Recent perspectives on the synthetic seed technology using non–embryonic *in vitro* derived explants. *Intern. J. Plant Sci.* 159(6), 968–978.

Stanley R G and Linskens H F. 1974. *Pollen: Biology, Biochemistry and Management.* Berlin, Springer–Verlag.

Stejskal J and Griga M. 1995. Comparative analysis of some isozymes and proteins in somatic and zygotic embryos of soybean (*Glycine max* [L.] Merr.). *J. Plant Physiol.* 146: 497–502.

Stern W L and Judd W S 1999. Comparative vegetative anatomy and systematics of Vanilla (Orchidaceae). *Botanical Journal of the Linnean Society*. Dec. 131 (4): 353-382. {a} Department of Botany, University of Florida, Gainesville, FL, 32611-8256, USA.

Stoutamine W P. 1964. Seeds and seedlings of native orchid. *Michigan Bot* 3:107

Sudarmonowati E and Henshaw G H. 1990. Cryopreservation of cassava somatic embryos. p. 378 In. Abst. VIIth Intl. Cong. Plant Tissue and Cell Culture, Amsterdam.

Sun C N 1958. The survival of excised pea seedlings after drying and freezing in liquid nitrogen. *Bot. Gaz.* (Chikago) 19 : 234–236.

Sunderland N. 1977. Nuclear cytology.pp : 177-205. *In.* Street, H.E. (ed.) *Plant Tissue and Cell Culture. Bot.* Monographs. Blackwell Scientific Publications, Oxford, London.

Suseela B and Thomas J 2000. *Phytophthora* rot – a new disease of vanilla (*Vanilla planifolia* Andrews) in India. *J. Spices and Aromatic Crops* 9 (1) : 73-75.

Sutter E G 1996. General laboratory requirements, media and sterilization methods. In . Plant Tissue Culture concepts and laboratory exercises. (Trigiano RN & Gray DJ (Eds.). CRC press, Inc., New York. p.11.

Sza bados L, Hadlaczky G Y and Dutis D. 1981.*Planta.* 151 : 141-145

Szakacs E, Kovacs G, Janos P and Barnabas B. 1988. Substitution analysis of callus induction and plant regeneration from anther culture in wheat (*Triticum aestivum* L.). *Plant. Cell. Rep.* 7 : 127-129.

Taiz I and Jones R L. 1971. The isolation of barley-aleurone protoplast. *Planta.* 101 : 95-100.

Takabe I, Otsuki Y and Aoki S. 1968. Isolation of tobacco mesophyll cells in intact and active state. *Plant Cell Physiol.* 9 : 115-124.

* Takabe, I., Labib, G. and Melchers, G. 1971. Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. *Naturwissenschaften.* 58 : 318-320.

Takagi H, Otoo E, Islam M O and Senboku T. 1994. *In vitro* preservation of germplasm in root and tuber crops : 1. Preliminary investigation of mid and long term preservation of yams (Dioscorea spp.) and Taro (Colocasia esculenta Schott). Breeding Science Japan. 44 : 273.

Takeda T, Shimada T, Nomura K, Ozaki T, Haji T, Yamaguchi M, Yoshida M. 1998. Classification of Apricot varieties by RAPD analysis. *Journal of Japanese Society for Horticulture Science* 67 (1) 21-27.

Tao Y, Manner J M, Ludlow M M Henzel R G. 1993. DNA polymorphism in grain sorghum (S. bicolor). *Theoretical and Applied Genetics* 86 (6) : 679-688.

Telaye A, Bemiwal S P S and Gates P. 1990. Effect of desiccation and storage on the viability of *Vicia faba* pollen. *FABIS Newsletter*. 26 : 6–10.

Tessy Paul P 1995. Large scale production and field performance of tissue culture Vanilla. Proc. 7th Kerala Science Congress, Jan. Palakkad, pp. 316-317.

Thanutong P, Furusawa I and Yamamoto M. 1983. Resistant tobacco plants from tolerant cell lines. Plant Sci. 13: 105-111.

Thieme R and Pett B. 1982. Erzeugung und Anwendung von *in vitro* – Knollen bei der Anlage eines Kartoffeldepots. Arch. Zuchtungsforsch 12 : 257-262.

Thomas J and Suseela B. 2000. *Sclerotium* rot – a new disease of vanilla (*Vanilla planifolia* Andrews) in India. *J. Spices and Aromatic Crops* 9 (2) : 175-176.

Thorpe T A and Patel K R 1984. Clonal propagation: Adventitious buds. pp : 49-60. *In.* I.K. Vasil (ed.) *Cell Culture and Somatic Cell Genetics of Plants*,Vol.1 Academic Press, New York,

Thyagi R K Yusuf A, Jeyaprakash P and Poonam Dua. 2001. Effects of polyamines on *in vitro* conservation of *Vanilla planifolia* (Salisb.) Ames. *Indian J. Plant genetic Resources.* 14 (2): 300–302.

Tian-su Zhou, Shuichi Sakaguchi 2001.Somaclonal variation and the ploidy level in *Phalaenopsis Alliance.* In. Proceedings of the Seventh Asia Pacific conference APO C7 March 15-21, Nagoya, Aichi, Japan.

Torres A M, Millan T and Cubero J I. 1993. Identifying Rose cultivars using RAPD markers. *Hort Science* 28 (4) : 333-334 .

Torrey J G. 1967. Morphogenesis in relation to chromosomal constitution in long term plant tissue cultures. *Physiol. Plant.* 20 : 265-275.

Touissaint-Samat M. 1994. *History of Food.* Blackwell, Oxford.

Towill L E and Jarret R L. 1992. Cryopreservation of sweet potato (*Ipomoea batatas* (L.) Lam.) shoot tips by vitrification. *Plant Cell Rep.* 11 : 175-178.

Towill L E and Walters C. 2000. Cryopreservation of pollen. In. Engelmann, F. and Takagi, H. (eds.), pp. 115–129. Cryopreservation of tropical plant germplasm–Current research progress and application. Japan International Research Centre for Agricultural Sciences, Tsukuba, Japan.

Towill L E. 1984. Seed set with potato pollen stored at low temperatures. *American Potato J.* 61: 569–575.

Triggs H M, Triggs G S, Lowe K C, Davey M R and Power J B 1995. Protoplast systems for spice crops: Cardamom (*Elettaria cardamomum*) and vanilla (*Vanilla planifolia*). *Journal of Experimental Botany*, 46 (Suppl.) : 48-49.

Tu Jin Xing Zheng Younghian and Fu Ting Dong 1997. RAPD markers linked to a genetic male sterility gene in *Brassica napus*. *Journal of Huazong Agricultural University* 16 (2) : 112-117.

Tulsieram L K, Glaubitz J C, Kiss G, Carlson J E. 1992. Single tree genetic linkage mapping in conifers using haploid DNA from mega gametophytes. Bio/Technology 10 (6) : 86-690.

Uozumi N and Kobayashi T 1995. Artificial seed production through encapsulation of hairy roots and shoot tips. In. Bajaj, Y. P. S (ed.) pp. 170–180. .*Biotechnology in Agriculture and Forestry.* Vol. 30. Somatic embryogenesis and Synthetic seed l. Springer, Berlin, Heidelberg.

Valles M P, Wang Z Y, Montovon P, Potrykus I and Spangenberg G. 1993. Analysis of genetic stability of plants regenerated from suspension cultures and protoplasts of meadow fescue (*Festuca pratensis* Huds.). *Plant Cell Rep.* 12: 101–106.

Van den houwe I, De Smet K, Tezenas du Montcel H and Swennen R. 1995. Variability in storage potential of banana shoot cultures under medium term storage conditions. *Plant Cell Tiss. Org. Cult.* 42 : 269-274.

Van Waes J M and Debergh P C. 1986. *In vitro* germination of some Western European orchids. *Physiol. Plant.* 67 : 253.

Varshney A, Lakshmikumaran M, Srivastava P S and Dhawan V. 2001. Establishment of genetic fidelity of *in vitro* raised *Lilium* bulblets through RAPD markers. *In vitro Cell. Dev. Biol. –Plant* 37: 227– 231.

Vasil I and Vasil V. 1980. Isolation and culture of protoplast. In. Vasil, I.K.(ed.) Perspectives in plant cell and tissue culture. *Int. Rev. Cytol* (Suppl.) IIB : 10-19.

Vasil I K and Vasil V. 1980. Isolation and culture of protoplast. *In.* Vasil, I.K.(ed.) Perspectives in plant cell and tissue culture. *Int. Rev. Cytol* (Suppl.) IIB : 10-19.

Vasil I K. (ed.). 1986. *Cell Culture and Somatic Cell Genetics of Plants*. Vol.3 Plant Regeneration and Genetic Variability. Academic Press, Inc, New York.

Vidal J R, Moreno S, Gogorcena Y, Masa A, Ortiz J M.1999. On the Genetic Relationships & Origins of six Grape cultivars of Galicia (Spain) using RAPD makers. *American Journal of Enology and Viticulture* 50 : 1.

Vierting R A and Nguyen H T. 1992. Use of RAPD markers to determine the genetic diversity of diploid, wheat Genotypes. *Theoretical and Applied Genetics* 84 (7-8) : 835-838.

Visser T. 1955. Germination and storage of pollen. Meded. Landbouwhogenschool Wageningen, Holland, 55: 1–68.

Vithanage H I M V and Alexander D M. 1985. Synchronous flowering and pollen storage techniques as aids to artificial hybridization in pistachio (*Pistacia* spp.). *J. Hort. Sci.* 60 : 107–113.

Vithanage V, Hardner C, Anderson K L, Meyers N, Mc Conchie C and Peace C (1998). Progress made with molecular markers for genetic improvement in Macadamia. *Acta Horticulturae* No: 461.199-207. In proceedings of the International Symposium on biotechnology of Tropical and subtropical Species Part II Brisbane, Queensland,Australia29 Sept-3 Oct 1997.

VithanageV, Hardner C, Anderson K L, Meyers N, Mc Conchie C, Peace C 1998. Progress made with molecular markers for genetic improvement of *Macadamia. Acta Horticulturae* No : 461 : 199-207. In proceedings of the International Symposium on biotechnology of Tropical & Subtropical Species Part II Brisbane, Queensland, Australia 29 Sept–3 Oct 1997.

Vos P, Hogers R, Bleeker M, Reijans M, Vande Lee T, Hornes M, Frijters A, Hornes M, Frijters A, Pot J, Pelman J, Kuiper M and Zabeau M. 1995. AFLP : A new technique for DNA fingerprinting. *Nucleic Acid Research* 23 : 4407 – 4414.

Vuylsteke D R and Swennen R. 1990. Somaclonal variation in African plantains. *IITA Research* 1 : 4-10.

Wachira F N, Waugh R, Hack eH C A and Powell W. 1995. Detection of Genetic Diversity in Tea (*Camellia sinensis*) using RAPD markers. *Genome* 38 : .201-210.

Wallin A and Eriksson T. 1973. Protoplast cultures from cell suspensions of *Daucus carota. Physiol. Plant.* 28 : 33-39.

Wan Y, Sorensen E L and Liang G H. 1988. Genetic control of *in vitro* regeneration in alfalfa (*Medicago sativa* L.). *Euphytica* 39 : 3-9.

Wang J H, Bian H W, Zhang Y X and Cheng H P. 2001. The dual effect of antifreeze protein on cryopreservation of rice (*Oryza sativa* L.) embryogenic suspension cells. Cryo–Letters 22 175–182.

Wanis W H, Callow J A and Withers L A. 1986. Growth limitations for the conservation of pear genotypes. Pp. 285-290. In. Plant Tissue Culture and its Agricultural Applications. (LA Withers and Alderson PJ eds.). Butterworths. London.

Watanabe K, Kuriyama A, Kawai F and Kanamori M. 1999. Effect of cryoprotectant treatment and post–thaw washing on the survival of cultured rice (*Oryza sativa* L.) cells after cryopreservation. *CryoLetters* 20 : 377–382

Weber G, Roth E J and Schweiger H G. 1983. Storage of cell suspensions and protoplasts of *Glycine max* (L.) Merr. , *Brassica napus* (L.), *Datura innoxia* (Mill.) and *Daucus carota* (L.) by freezing. *Z. Planzenphysiol.* 10 : 23–29.

Welsh J and Mc Clelland M 1990. Finger printing genomes using PCR with Arbitrary primers. *Nucleic Acid Research* 18 : 7213-7218.

Wenzel G and Uhrig H. 1981. Breeding for virus and nematode and virus resistance in potato via anther culture. *Theoretical and Applied Genetics*. 59 : 333-340.

Wesley R A, James R S, David C F, Richard G and David L M. 1998. Application of RAPDs to DNA extracted from Apple Root stock. *Hort Science* 33 (2) : 333– 355.

Wilde J, Waugh R and Powell W. 1992 Genetic finger printing of *Theobroma* clones using RAPD markers. *Theoretical and Applied Genetics* 83 (6-7) : 871-877.

Wilkins C P, Newbury H J and Dodds J H. 1988. Tissue culture conservation of fruit trees. FAO/IBPGR. *Plant Gen. Res. Newsl.* 73/74 : 9-20.

Williams C E and ST Clair D A. 1993 Phenetic relationship and levels of variability detected by RFLP and RAPD analysis of cultivated and wild accessions of *Lycopersicon esculentum. Genome* 36 (3) : 619-630.

Williams D G. 1997. *The Chemistry of Essential Oils.* Micelle Press, Weymouth, England.

Williams J G K, Hanafey M K, Rafalsky J A and Tingey S V 1993.Genetic analysis using random amplified polymorphic DNA markers. *Methods. Enzymol.* 218 : 705-740.

Williams J G K, Kubelik A R, Livak R J, Rafalski J A and Tingey S V 1990. DNA Polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research* 18: 6531-6535.

Wilson R M, Eisa M Z and Irwin S W B. 1976. The effects of agitated liquid medium on *in vitro* cultures of *Hevea brasiliensis. Physiol. Plant.* 36 : 399-402.

Withers L A and Street H E. 1977. Freeze preservation of cultured plant cells, 111: The pregrowth phase. *Physiol. Plant.* 39 : 171–178.

Withers L A and Williams J T. 1985. Research on long term storage and exchange of *in vitro* plant germplasm. In. Biotechnology in International Agricultural Research, International Rice Research Institute, Manila, pp 11–24.

Withers L A and Williams J T. 1985. Research on long term storage and exchange of *in vitro* plant germplasm. In. Biotechnology in International Agricultural Research, International Rice Research Institute, Manila, pp 11–24.

Withers L A. 1980. Tissue culture storage for genetic conservation. IBPGR Technical Report. International Board for Plant Genetic Resources, Rome. p. 91.

Withers L A. 1985. Cryopreservation of cultured cells and meristems., In *Cell culture and somatic cell genetics of plants.* Vol.2: Cell growth, nutrition, cytodifferentiation and cryopreservation. (I. K. Vasil Ed.). Academic Press, Orlando, Florida. pp. 253–316.

Withers L A. 1985d. Cryopreservation and gene banks. In. *Plant Cell culture Technology.* M. M. Yeoman (ed.), Oxford Blackwell.

Withner 1950. Germination of seeds of Vanilla. *American.Journal of Botany* 37 : 241-247.

Withner C L 1955. Ovule culture and growth of *Vanilla* Seedlings. *American Orchid. Soc. Bull.* 24 : 381 – 392.

Wolf H T, Berg T V, Czygan F C, Mosandl A, Winckler T, Zundorf J and Dingermann T. 1999. Identification of *Melissa officinalis* sub species by DNA finger printing. *Planta Medica* 65 (1) : 83-85.

Wood C B, Pritchard H W and Miller A P. 2000. Simultaneous preservation of orchid seed and its fungal symbiont using encapsulation–dehydration is dependent on moisture content and storage temperature. *CryoLetters* 21: 125–136.

Wu C and Shimamoto K. 1995. Electroporation-mediated gene transfer to japonica rice protoplast and regeneration of transgenic plants. pp : 93-98. *In.* Potrykus, I. and Spangenberg, G. (eds.) (*q.v.*).

Wu Y, Zhao Y, Engelmann F and Zhou M. 2001. Cryopreservation of Kiwi shoot tips. *Cyoletters* 22 : 277–284.

Xju Z, Arditti J and Nyman I P. 1987. *Vanilla planifolia* – callus induction and plantlet production *in vitro*. *Lindleyana* 2 : 88-90.

Xu Y S, Clark M S and Pehu T 1993. Use of RAPD markers to screen somatic hybrids between *Solanum tuberosum* and *S. brevidens*. *Plant Cell Reports*. 12 (2) : 107-109.

Xu Z H, Davey M R and Cocking E C. 1982. Organogenesis from root protoplasts of the forage legumes *Medicago sativa* and *Trigonella foenum-graecum*. *Z. Pflanzenphysiol.* 107 : 231-235.

Yang Y W, Kuo H J W and Wong T H. 1998. Genetic Polymorphism of 7 populations of *Capsella bursa pastoris* based on RAPD markers. *Botanica Bulletin of Academia Sinica* 39 (1): 17-21.

Yasuhiro I T O and Sonoe O Y. 1999 Discrimination of Basidiomycete species and strain by RAPD analysis. *JARQ* 33: 149-154.

Yates I E and Sparks D. 1990. Three–year old pecan pollen retains fertility. *J. Amer. Soc. Hort. Sci.* 115 : 359–363.

Yeoman M M 1986. *Plant Cell Culture Technology.* Botanical Monograph. pp: 7– 25.Vol. 23. Blackwell Scientific Publications. Oxford.

Yndgaard and Iloskuldsson A 1985. Electrophoresis: A tool for gene banks. FAO/IBPGR *Pl.Gen.Res.Newsletter.*63 : 34-40.

Yu K F and Pauls K P.1993. Rapid estimation of genetic- relatedness among heterologous population of alfalfa by Random amplification of bulked genomic DNA samples. *Theoretical and Applied Genetics* 86 (6) : 788-794.

Yu K F, Pauls K P. 1993. Identification of a RAPD marker associated with somatic embryogenesis in alfalfa. *Plant Molecular Biology* 22 (2): 269-277.

Zabeau M, and Vos, P 1993. Selective restriction fragment amplification: A general method for DNA fingerprinting. European patent application number 92402629.7, Publication number 0 534 858 A1.

Zakhlenyuk O V and Kunakh V A 1987. Cytophysiological and cytogenetic effects of adenine derivatives in *Haplopappus gracilis* tissue culture. *Fiziol. Rast.* 34 : 584–594.

Zhang C L, Sun Z L, Jin D M, Sun S M, Guo B T and Wang B. 1998. Identification of maize inbred lines & Validation of genetic relationship among maize inbred lines using RAPD markers. *Maize Genetics Cooperation Newslett.* No: 72 : 9-10.