INTRA AND INTER GENERIC HYBRIDIZATION AND MOLECULAR CHARACTERIZATION IN MONOPODIAL ORCHIDS

BEENA THOMAS

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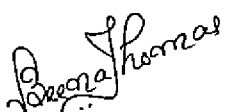
Department of Plant Breeding and Genetics,
COLLEGE OF AGRICULTURE,
VELLAYANI, THIRUVANANTHAPURAM 695 522

DECLARATION

I hereby declare that this thesis entitled **"Intra and inter generic hybridization and molecular characterization in monopodial orchids"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.



BEENA THOMAS

Vellayani,
24-10-2008.

CERTIFICATE

Certified that this thesis entitled **"Intra and inter generic hybridization and molecular characterization in monopodial orchids"** is a bonafide record of research work done independently by **Smt. Beena Thomas** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

Dr. C. LEKHA RANI

(Chairman, Advisory Committee)
Associate Professor,
Department of Plant Breeding and Genetics,
College of Agriculture, Vellayani,
Thiruvananthapuram.

Vellayani,
24-10-2008.

APPROVED BY

Chairman :

Dr. C. LEKHA RANI,
Associate Professor,
Department of Plant Breeding and Genetics,
College of Agriculture, Vellayani.

24/10/08

Members :

Dr. P. MANJU,
Professor and Head,
Department of Plant Breeding and Genetics,
College of Agriculture, Vellayani.

24/10/08

Dr. K. RAJMOHAN,
Professor and Head,
Department of Plant Biotechnology,
College of Agriculture, Vellayani.

24/10/2008

Dr. D. WILSON,
Professor,
Department of Plant Breeding and Genetics,
College of Agriculture, Vellayani.

24/10/08

Dr. VIJAYARAGHAVA KUMAR,
Professor,
Department of Agricultural Statistics,
College of Agriculture, Vellayani.

24/10/08

External Examiner :

24.10.08

In

Everloving Memory of

My Beloved Daddy

Sri. S. T. Thomas

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BEENA THOMAS

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LIST OF ABBREVIATIONS

°C	-	Degree Celsius
µg	-	microgram
µl	-	micro litre
µM	-	micro molar
2,4-D	-	2, 4-dichlorophenoxy acetic acid
ABA	-	Abscissic acid
AC	-	Activated charcoal
ACC	-	Aminocyclopropane-1 carboxylate
ANOVA	-	Analysis of variance
AFLP	-	Amplified Fragment Length Polymorphism
BA	-	Benzyl adenine
BAP	-	Benzyl amino purine
CD	-	Critical difference
CH	-	Casein hydrolysate
cm	-	Centimeter
CRD	-	Completely Randomized Design
CSTD	-	Candy Stripe x Tomie Drake
CW	-	Coconut water
DBT	-	Department of Biotechnology
DNA	-	Deoxyribonucleic acid
dNTPs	-	Deoxy nucleotide triphosphates
EDTA	-	Ethylene diamino tetra acetic acid disodium salt
et al.	-	And others
Fig.	-	Figure
GA	-	Genetic advance
GA_3	-	Gibberellic Acid
GCV	-	Genotypic coefficient of variation
H^2	-	Heritability
HCl	-	Hydrochloric Acid
IAA	-	Indole-3-acetic acid

IBA	-	Idole-3-butyric acid
KC	-	Kundson C medium
l	-	Litre
l^{-1}	-	per litre
M	-	Molar
ME	-	Malt extract
$MgCl_2$	-	Magnesium chloride
mg	-	milligram
ml	-	milli litre
mM	-	milli molar
MS	-	Murashige and Skoog medium
N	-	Normality
NAA	-	Naphthalene acetic acid
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
Ng	-	nanogram
Nm	-	nanometer
No.	-	Number
O.D.	-	Optical density
OPB	-	Operon primer of B series
OPC	-	Operon primer of C series
OPD	-	Operon primer of D series
PCR	-	Polymerase chain reaction
PCV	-	Phenotypic coefficient of variation
PLB	-	Protocorm like body
PVP	-	Polyvinyl pyrrolidone
RAPD	-	Random Amplified Polymorphic DNA
RFLP	-	Restriction Fragment Length Polymorphism
rpm	-	Rotations per minute
r_g	-	Genotypic correlation
r_p	-	Phenotypic correlation
SE	-	Standard error
TAE	-	Tris acetic acid EDTA

Tris HCl	-	Tris amino methane hydrochloride
UPGMA	-	Unweighted pair group method for arithmetic average
viz.	-	Namely
VNTR	-	Variable number of tandem repeats
VW	-	Vacin and Went (1949) medium
UV	-	Ultra violet

INTRODUCTION

1. INTRODUCTION

Orchids, the most beautiful flowers in God's creation, occupy top position among all the flowering plants valued for cut flower production and cherished as potted plants. They are the most pampered plants and are coveted for their longlasting and bewitchingly beautiful flowers which are the major players in the multibillion dollar floriculture trade in the world. Taxonomically, they belong to a highly evolved family under monocotyledons with 600-800 genera and 25,000-35,000 species (Reddy and Rao, 2008). Orchidaceae, also called the orchid family, is the largest family of the flowering plants (Batygina et al., 2003 and Walter et al., 2007).

To taxonomists and plant breeders, Orchidaceae is a unique family. Fusion of androecium and gynoecium into a single structure - the gynandrium, packaging of pollen existing as tetrads into pollinia to prevent wastage, development of an incredibly large number of ovules in the ovary which require the stimulus of pollination for completing their development and the formation of numerous, dust-like, non-endospermous miniscule seeds which germinate in nature through fungal association are some of their peculiar adaptations.

The range of variation in the size, shape, colour, fragrance and vase life of orchid flowers is tremendous. However, most of this existing variation is still unexploited. Orchids have received very little attention at the hands of geneticists untill now and the knowledge about the mode of inheritance of the economic characters in this group of plants is still inadequate. Most of the genetic diversity that exist in orchids today, has been the result of exploration, collection, introduction and conservation.

South East Asia has developed into a major supplier of orchids and the orchids cut-flower industry is a highly developed trade in the world today. Cultivation of orchids has now been recognized as a profitable occupation and it

has a great future in India. Development of new hybrids and their commercial production have expanded tremendously in other countries like Singapore, Malaysia, Thailand, USA, Europe, South America and Sri Lanka. India is blessed with a wealth of indigenous orchid flora. Hybrids of certain Indian orchids such as *Vanda coerulea*, *Dendrobium*, *Paphiopedilum* and *Cymbidium* have no peers in the world of orchids. Inspite of their commercial value, orchids have not yet gained the attention they deserve in India.

In India we have about 15,000 orchid species belonging to some 150 genera out of which about 300 species coming under 60 genera belong to peninsular India. Indian dendrobiums, cymbidiums and vandas have played a major role in the development of modern orchid industry. At present, only a few nurseries in Darjeerling, Kalimpong and Sikkim cultivate orchids, in commercial scale. Good collections of orchid species are being maintained at Orchid Sanctuary, Kalimpong (West Bengal), Indian Institute of Horticultural Research, Bangalore (Karnataka), National Orchidaria, Shillong (Meghalaya) and Yercaud (Tamil Nadu), Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram (Kerala) and Orchid Research and Development Centre, Tipi (Arunachal Pradesh). A herbarium of orchids is also being maintained at the Central National Herbarium in the Indian Botanic Garden, Howrah (West Bengal).

Orchids constitute an order of royalty among ornamental plants possessing immense horticultural importance. In tropical and temperate forests they play a very useful role in balancing the ecosystem. Commercial cultivation of orchids, both for plant sale as well as cut flower production, has developed into a vast industry in many countries.

Orchid culture has been identified as a lucrative agri-business in India. Kerala mostly has a warm, humid climate which is congenial to growing important tropical orchids without undertaking any environmental control

measures. Realizing this, Government of India has selected Kerala as a suitable zone for the development of orchid industry. With all these advantages, export oriented orchid cultivation is still in its infancy in Kerala. The main constraint is the lack of sufficient locally adapted and reasonably priced quality planting material for large scale cultivation. In spite of this, a few enterprising small scale growers in Kerala have started commercial production of orchid flowers. Yet, this is not sufficient to meet even the internal demands which are mainly concentrated in the metropolitan cities like Mumbai, Chennai, New Delhi and Kolkatha.

If properly exploited, a large number of unemployed educated youth and women can be engaged in this sector by providing remunerative employment opportunities to them. Because of immense pressure on the land due to high population density, high value orchid cultivation, utilizing mostly house terraces in urban areas otherwise unsuitable for other cultivation, can be undertaken extensively.

The present practice is to import tissue cultured planting material from countries like Thailand by certain agencies and the growers buy the plants from them at exorbitant costs. Another negative point in this system is that the planting material that a foreign country is prepared to sell to us would be hybrid varieties that have lost their competitive relevance in international markets. They always keep the newer hybrids (which are continuously being produced) for themselves. This puts the orchid growers of Kerala into considerable disadvantage as their products cannot stand up to compete with the new and novel hybrid flowers that flood the international markets. Considering the new international patent policies on plants and plant products, the import of plant materials from foreign countries will soon become an obsolete practice. As a solution to this problem, we have to develop novel varieties with commercial qualities.

Now a days orchid breeding has become a very serious agenda of all the International Orchid Societies and consistent breeding work is being undertaken

all over the world by the Royal Horticultural Society, American Orchid Society, Orchid Society of South East Asia, Australian Orchid Society, Canadian Orchid Society etc. However, in India orchid breeding is undertaken only by a few institutions. In monopodial orchids very little research has been done. Considering the seriousness of the situation, a hybridization programme including commercially important genotypes of this group, has been planned as a preliminary step to develop new hybrids which have demand from growers.

Many orchids native to our country, have already proved their worth as parents and have contributed to the production of outstanding hybrids at the international level. Due to the diversity of environmental conditions in India, it is possible to grow different types of orchids in suitable places, without having to undertake rigorous control of environment. There is tremendous scope for orchid improvement and for development of orchid industry in India. In the case of monopodial orchids, eventhough they have comparative advantages over sympodials such as easier maintenance, longer vase-life, numerous colour combinations etc., their market value is low as they lack novelty. Therefore, monopodial orchid breeding has immense significance and a bright future in our country. It is essential to produce novel and adapted quality hybrids that are acceptable in the national and international markets. Molecular characterization has become most important for establishing the identity of improved hybrids, prior to release. The proposed research programme aimed at developing new hybrids of monopodial orchids and characterizing them using molecular markers fulfills these long term objectives.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Orchids are highly priced in the international florist trade due to their intricately designed spectacular flowers, brilliant colours, delightful appearance, myriad sizes, shapes, forms and long lasting qualities. Many orchid industries have developed around the world involving this beautiful and unusual plant. In India, orchids are grown commercially (Bhattacharjee, 2006). However the export and sale of orchids in India is negligible, eventhough our country is blessed with a wealth of orchid flora. In the case of monopodial orchids, which can be more easily cultivated commercially than sympodials, novelty is lacking and market value is affected. Hence, monopodial orchid breeding is to be given importance and our own novel, adapted and quality hybrids which are acceptable in the national and international markets are to be developed.

A thorough knowledge of floral biology, pollination, compatibility, *in vitro* seed germination and development, variability and hybrid development of orchids is essential for organizing its improvement through breeding programme. Therefore, a brief review of relevant literature has been presented here, based on related research works.

2.1. Floral Biology

Orchid flowers are magnificently beautiful which are of an infinite variety. They may be large or small, showy or insignificant, long lasting or short lived, scented or non-scented and brightly coloured or white. Flowers are complete, zygomorphic, epigynous, trimerous, bracteate or ebracteate, sessile or pedicellate, mostly bisexual and rarely unisexual. Colours range from white to yellow, orange, pink, red, purple, violet, blue, brown and even green or their combinations.

2.1.1. Floral Morphology

The complex structure of the orchid flower was first described by Brown in 1831 and by Darwin in 1877. The orchid flower is modelled according to the liliaceous pattern being constituted by 15 members – 3 sepals + 3 petals + 6 stamens (in two whorls of three each) + 3 carpels (Abraham and Vatsala, 1981).

Orchid flowers are zygomorphic, mostly bisexual or rarely unisexual as in *Catasetum*. The orchid flower consists of three sepals, three petals and the column or gynostegium bearing the reproductive parts. In species like *Vanda*, the sepals are alike. But in few species of *Bulbophyllum, Renanthera, Paphiopedilum* and many species of *Oncidium*, the dorsal sepal is of different size from laterals. The sepals are narrower, coloured like petals and referred as 'petaloid' sepals. Sepals and petals are three each in number, in alternate whorls. The petals are as a rule, much more brightly coloured than the sepals. Of the three segments, the odd one is modified into the labellum or the lip. In most cases, the lip is larger, three lobed and more brightly coloured than the laterals (Sheehan and Sheehan, 1979; Abraham and Vatsala, 1981). The lip is the landing place for insects and it serves to promote pollination by insects.

Situated in the centre of the flower, opposite to the lip and facing it, is the column or the gynostegium, which is peculiar to the orchids. The gynostegium is formed by the fusion of the filaments, styles and stigmas of the flower. The only fertile anther of the flower is borne on the top of the column. In Diandrae, where two anthers are fertile, they are borne on the two sides of the column. The column is extended below beyond its attachment to the stalk of the flower, to form a structure called 'foot', in some genera of *Vanda* tribe. The lateral sepals and lip are attached to this foot and together form a sac like 'mentum', which is an important characteristic of these genera (Yadav and Bose, 1989; Abraham and Vatsala, 1981).

The anther cap (operculum) which is easily removable, provides a protective covering over the pollengrains. Pollengrains are collected in masses called the pollinia. The pollinia are contained in a cavity called the clinandrium. The pollinia, occur as two notched pollinia in *Vanda* to four pollinia applied to each other in pairs in *Arachnis, Phalaenopsis, Aerides, Renanthera* and *Angraceum*. In monopodial orchids, only the odd stamen of the outer whorl is fertile which is situated opposite to the labellum. The two lateral stamens of the inner whorl form the sides of the clinandrium and the odd stamen forms front of the column. In *Cypripediae* two stamens are fertile. Three stamens are fertile in *Apostasiae* (Abraham and Vatsala, 1981).

Just beneath the anther is the rostellum which is morphologically one of the three stigmas modified. It is the partition wall between stamen and stigma which prevents self pollination and secretes a viscid substance to hold pollinia till they mature. On the ventral side of the column, beneath the rostellum is a hollow cavity which is the functional stigma of the flower. Actually, it is the two fertile stigmas of the flower fused together. It is covered by a viscous substance which is very dear to insects. This substance helps to hold the pollinia which are deposited on it during the process of pollination. The ovary in Orchidaceae is generally tricarpellary, with three parietal placentations and innumerable ovules (Abraham and Vatsala, 1981; Mukherjee, 1990).

2.1.2. Resupination

The orchid flowers exhibits the phenomenon of resupination wherein the modified petal (labellum) becomes the lowermost segment by an extremely complicated torsion or twisting of the pedicellate ovary through an angle of 180° (Vij, 2006).

The flower of most orchids is in an upside down position or resupinated, having turned through 180° on its pedicel (Abraham and Vatsala, 1981).

According to Nyman et al. (1984) in *Dendrobium*, flowers were borne with the labellum uppermost, at inflorescence emergence. The buds became resupinate just before or during opening, by a twisting of the pedicel. The degree of twisting depended on the orientation of the inflorescence axis relative to the ground and the position of the pedicel on it. Individual flowers at successive nodes along the inflorescence alternated in twisting clockwise and counterclockwise. Resupination of orchids has also been reported by Bose and Bhattacharjee (1980).

2.1.3. Anthesis

Christenson (1992) in *Stelis argentata* reported that in sunny weather, new flowers opened primarily in the morning and during rainy weather, in the late afternoons. The flowers lasted upto nine days on the inflorescence, but most pollinia were removed (by pollinators) during the first two days of anthesis.

Anthesis occurred between 8.30 am and 5.30 pm with peaks between 9 am and 10 am and also between 3 pm and 4 pm in *Dendrobium* hybrids (Varghese, 1995). The flowers opened in acropetal succession and retained their freshness for 45-50 days on the inflorescence.

Sobhana (2000) came across a variety dependent variation in the time of anthesis in *Dendrobium* hybrids. In most of the varieties, anthesis commenced from 7.30 to 8.30 am and extended to 11.00 to 11.30 am while in some others, the onset of anthesis was delayed till 9.00 to 11.00 am and extended upto 2.30 pm.

Studies on floral biology were conducted in *Dendrobium*, selecting 14 genotypes which showed significant variation for the characters under consideration. Pollination biology was studied with respect to flower opening time, anthesis time, maximum stigma receptivity period, pollen size, pollen fertility and pollen germination (Lekha Rani and Mercy, 2008).

2.1.4. Stigma receptivity

Studies of Devi and Deka (1992) revealed that the stigma remained receptive for four consecutive days following anthesis in *Spathoglottis plicata*, for five days in *Aerides odoratum* and for six days in *Dendrobium amoenum*.

According to Varghese (1995), the stigma in *Dendrobium* hybrids remained receptive from the first day of anthesis to the ninth day. Maximum stigma receptivity was observed between the fourth and sixth day after anthesis.

Sobhana (2000) observed maximum stigma receptivity in *Dendrobium* hybrids from the second to the fifth day after anthesis. Lekha Rani et al. (2006b) observed maximum stigma receptivity in several genotypes of *Dendrobium* from third to ninth day after flower opening.

2.2. Pollen Studies

Factors like pollen viability, germination, pollen production, dissemination and fertilization affect the success of hybridization in orchids. The salient research findings on these aspects are summarized below.

2.2.1. Pollen Morphology

The pollen in Orchidaceae are found as polyads. Individual pollen grains of the group are pressed tightly in such a way that their outlines become angular (Moore and Webb, 1978). According to Sheehan and Sheehan (1979) the pollen in Orchidaceae is not powdery as in most angiosperms but agglutinated to form pollinia.

Abraham and Vatsala (1981) observed that pollen in Orchidaceae exists as tetrads, which are held together by elastic threads of tapetal origin. The tetrad

nature of pollen in orchids was also suggested by Das and Ghoshal (1988) and Varghese (1995).

Johnson and Edwards (2000) concluded from their studies that cohesive masses of pollen known as pollinia have evolved independently in two plant families *viz.*, Orchidaceae and Asclepiadaceae. They further observed that though a single hard pollinium contains more than a million pollen grains, the pollen : ovule ratio in orchids is much lower than in families with powdery pollen. This is sufficient since pollinia ensure the efficient removal of pollen from anther, minimal pollen wastage during transit and the deposition of large pollen loads on stigma to enable fertilization of the large number of ovules in orchid flowers.

In the opinion of Sobhana (2000) the pollen in *Dendrobium* existed as tetrads and were spherical to rectangular in shape. The pollen from the different hybrids and wild species were almost similar in shape but varied in size. Similar results were obtained by Lekha Rani et al. (2006b) also, in *Dendrobium*.

2.2.2. Pollen viability

A method for assessing the viability of pollen grains by mounting in acetocarmine was described by Zirkle (1937). According to him, the grains which were well – stained, plumpy and normal were viable and unstained shrivelled ones were non – viable.

In *Vanilla*, pollen viability was reduced considerably one day after anthesis. Self pollination just prior to natural flower opening resulted in normal fruit set (Nair and Mathew, 1986). Das and Ghoshal (1988) observed a low percentage of pollen fertility in *Dendrobium chrysotoxum* and *D. transparens*.

In terrestrial orchids like *Spathoglottis plicata* and *Phaius tankervilliae*, the pollen viability declined gradually after anthesis whereas in epiphytic orchids

like *Aerides odoratum* and *Dendrobium amoenum* it showed an improvement for three days after anthesis, before getting impaired (Devi and Deka, 1992).

A low percentage of pollen fertility in *Dendrobium chrysanthum* was reported by Sobhana (2000). Pollen size was comparatively large in commercial hybrids of *Dendrobium* ranging from 29.60 µ to 48.64 µ, medium in the semi - commercial hybrids with a range from 25.92 µ to 31.02 µ and small in the species *viz., D. barbatulum* (19.84 µ) and in *D. philippica* (21.44 µ) (Lekha Rani et al., 2006b).

2.2.3. Pollen Germination

Experiments by Varghese (1995) in *Dendrobium* resulted in successful germination of pollen in a medium comprising of two per cent sucrose, one per cent agar and 75 mg l^{-1} boric acid.

In a study by Latha and Namboodiri (1999) pollen of *Spathoglottis plicata* was found to germinate after 5-6 hours of incubation in Brewbaker medium with 10 per cent sucrose. Germination continued upto 30-36 hours. The pollen of *Cymbidium ensifolium* required 14 hours of incubation for germination initiation, but thereafter the rate of germination increased progressively for another 12 hours.

Sobhana (2000) concluded from her studies that pollen germination was lower in the wild species of *Dendrobium* compared to the hybrids, in a medium containing sucrose (2 %), agar (1 %) and boric acid (75 mg l^{-1}).

Lekha Rani et al. (2006b) observed that in *Dendrobium*, the flowers opened in an acropetal order and the process commenced at around 7.30 am on sunny days, but was delayed till 12.30 pm on rainy days.

2.3. Compatibility Analysis

The extent of genetical affinity of two species or hybrids used as parents is another important factor. The incompatible or genetically distant species belonging to different genera having different chromosome numbers usually cannot be crossed to produce the hybrids. Therefore, knowledge of species affinity, compatibility and chromosome number and behaviour is very important for obtaining successful hybrids (Lenz and Wimber, 1959).

It is also advisable to choose parents with identical flowering season. Selection of good and healthy plant and flower also counts to a great extent. Very young plant or seedling blooming first time should not selected as a female plant (Bose and Bhattacharjee, 1980).

Johansen (1990) demonstrated a unique incompatibility system in *Dendrobium* which also showed high incompatibility in interspecific pollination in contrast to any other orchid genus. Incompatibility response was initiated by auxin content in pollinia. The compatibility substance was specifically recognised by the eleutherocytes produced in the stigmatic mucilage.

The failure of fruit development in many reciprocal crosses hints at the operation of a unidirectional incompatibility in orchids (Devi and Deka, 1992).

Devi and Deka (1994) performed 29 interspecific and 47 intergeneric crosses in orchids to determine cross compatibility. Percentage ovary drop after initial swelling was found to be high in general. Percentage fruit set ranged form 0 to 100 in interspecific and 0 to 75 in intergeneric crosses. Parthenocarpic fruit development without seed set was observed in several cases. Out of the 13 different hybrid capsules obtained, seeds of only three cross combinations germinated.

Chen et al. (2000) reported that out of 520 hybridizations conducted with the aim of developing white Taisuco *Phalaenopsis*, only 46.2 per cent cross combinations produced viable seeds.

Melendez-Ackerman and Ackerman (2001) reported self compatibility in *Listera cordata* as all self pollinations produced fruits. Cross pollination, however, differed significantly from the selfs, registering higher number of seeds per capsule and higher percentage of fertilized ovules.

Lekha Rani (2002) performed a total 190 self and cross combinations in *Dendrobium* to determine cross compatibility out of which 84 combinations including seven selfs produced harvestable green capsules, the relative success being 44.21 per cent. Progeny from 67 hybrid combinations were established successfully in the green house out of which more than 500 plants belonging to 20 combinations flowered.

Ninitha Nath (2003) conducted compatibility studies in 116 crosses in monopodial orchids. A total of 58 combinations gave harvestable green capsules. Progeny from 24 combinations were successfully deflasked.

Lekha Rani and Udaya (2008) studied the compatibility relationships among 18 monopodial orchid genotypes comprising monogeneric and bigeneric hybrids. Capsules at seed inoculation maturity were successfully harvested from 67 out of the 324 possible combinations. These included 61 crosses and 6 selfs. The pollinated flowers / immature capsules from 257 unsuccessful combinations fell off at different stages of development immediately after pollination to just before capsule harvest depending on the relative intensity of incompatibility expressed.

2.4. Pollination Biology

Pollination is effected by insects such as wasps, bees, ants, flies, moths and butterflies or by wind. The insect lands on the labellum and during its search for honey, it comes in contact with the pollinia which stick to its body. When it enters the next flower pollinia come into contact with the stigma and pollination is effected.

Darwin (1904) described the floral modifications which have evolved to ensure cross pollination in orchids and for the proper positioning of the insect to effect pollination.

All the known informations on orchid flower pollination were summarized by Pijl and Dodson (1966). A list of pollinators of several orchid species was given, placing bees as the dominant pollinator. Other pollinators included were a variety of insects, including flies, wasps, mosquitoes, moths, butterflies, beetles, ants, spiders and even birds.

Orchids are known to attract pollinators by means of offer of food and smell (good or bad) as well as mimicry of prey, food and antagonists. *Vanda tricolor* attracts the pollinator by the fragrance of its flower and visual light image (Arditti, 1966).

Pollination mechanism works only when an insect of right size and shape enters the flower. This relationship between orchids and pollinators is marvellous. The pollinia are so positioned that they get stuck to the pollinator insect as it leaves the flower. When it visits the next flower and moves down to the nectary, pollen is deposited on the stigma (Northen, 1970).

The various contrivances for allogamy in orchids were reviewed by Abraham and Vatsala (1981). In the genus *Ophrys*, mimicry is the mechanism.

The lip resembles the female of the pollinator wasp to the minute details including the odour, in a species of this genus. In another one, it mimics the common wasp, the natural prey of the pollinator.

Warren (1981) described different pollination mechanisms in orchids which varied widely depending on the floral morphology of the genera concerned.

Eventhough orchid flowers have evolved so that cross pollination is ensured in most orchid species, a considerable number of species have been identified to be autogamous (Abraham and Vatsala, 1981; Ridley, 1888).

Lekha Rani (2002) reported a high percentage of pollen fertility in *Dendrobium* Candy Stripe X *D.* Tomie Drake.

As in other deceit-pollinated orchids, natural selection in *Myrmecophila christinae*, favours individuals flowering early or late in relation to population peak flowering (Parra - Tabla and Vargas, 2004).

2.4.1. Artificial Pollination and Hybridization

In orchids, artificial hybridization was attempted much later than in other angiosperm families. There was lack of understanding of the method of pollination due to the complexity of flower structure in orchids. Rev.Williams Herbet, Dean of Manchester was the first person to attempt orchid hybridization. He crossed *Orchis* and *Ophrys* and produced pods, but the seedlings died after a few days (Lenz and Wimber, 1959). Now even multigeneric hybrids are being successfully produced.

Before one embarks on seed production, the best time for pollinating the flower should be decided. Usually pollination is done after it has been open for a few days. But in species where flowers last only for a day or two, they must be

pollinated immediately. In general, half way the life of a flower is the best time for pollination (Mercy and Dale, 1997). When there are a number of flowers in an inflorescence, mature fresh flowers towards the base must be pollinated. If inflorescence is large several flowers can be pollinated in the same spray. One important factor is that when several capsules are developing simultaneously their maturation time may be increased.

2.4.2. Storing pollen

To make hybrids, flowers can be pollinated with previously collected and stored pollen also. In case female parent flowers later, one has to store the pollen in viable condition for future use. The pollens were collected and placed in small pieces of tissue paper and after folding carefully they were kept in small tubes, placed in a dessicator having calcium chloride. These dessicators were then kept in refrigerator. By this way pollen could be stored for at least six months. (Northen, 1970). Dehydrants were found to reduce pollen viability. Pollen stored at 7.2 °C germinate better than those stored at 22.2 °C. The viability varied with species. Pollen of *Dendrobium phalaenopsis* retained its viability for 4-6 months whereas pollen of *Dendrobium undidatum* remained viable for more than 12 months at 7.2 °C.

2.4.3. Reciprocal Hybridization

Reciprocal crosses between species and cultivars are often difficult to accomplish successfully. There may be structural, functional or genic barriers. Hybridization between long styled and short styled plants becomes successful only when the long styled plant was used as pollen parent. The pollen tubes of short styled species might not be able to traverse a long style. The sensitivity of one of the parents to the environment would block the genes while it would not affect the other. As a result, development of the capsule could be checked.

Plants that are infertile due to irregularities at meiosis may produce viable egg cells whereas the pollen could be non functional (Lenz and Wimber, 1959).

Wallbrunn (1988) concluded that reciprocal hybridization within the hybrids yielded progeny with remarkable variation in flower characters.

2.4.4. Backcrossing and Selfing

Orchid breeders avoid backcrossing and selfing techniques, now a days. This may be due to the reason that inbreeding leads to inferior forms and reduction in vigour. However, in the long run it will produce superior types. By selfing *Paphiopedilum insigne* var. Sanderae several plants having colours were produced. A pure white and several coloured cultivars were produced from the same capsule obtained by selfing *Cattleya gaskelliana* (Lenz and Wimber, 1959).

D. nobile Verginale was selfed and several hundred plants were raised having white flowers. Selection and inbreeding in amphidiploid *Dendrobium* were also found effective in increasing the flower size and improving the colour purity (Bobisud and Kamemoto, 1982).

The long continued inbreeding of *Phalaenopsis sanderiana* is a famous example leading eventually to superior clones, one of which in the seventh generation received a gold medal award of American Orchid Society. *Cymbidium* Alexanderi 'Westonbirt' was both selfed and used for back-crossing and in both cases, flowers far superior to the original were developed. *Phalaenopsis* Princess Kaiulani was a hybrid of *P. violacea* and *P. amboinensis*. When it was crossed back to *P. violacea* the very variable *P.* Princess Violet was produced (Wallbrunn, 1988).

Alcorn (1990) carried out selfing in *Lycaste* Macama Jocelyn and obtained surprising variations in colours and shapes.

2.5. Post Pollination Phenomena

Pollination not only shortens the life of flowers, but also induces numerous and remarkable changes in morphology and colouration. The pollinated plant must be left undisturbed in normal maintenance conditions. Successful fertilization is indicated by the fading of the petals and greening and thickening of the ovary region of the flower within a week. The perianth lobes shrivel and arch over the stigma indicating successful crossing. At this time, paper bags should be removed and withered parts of the flower may carefully be clipped. Capsule ripening time differs in different genera but will be similar in related species and may vary between 2 to 12 months.

Once the flower is pollinated, it fades rapidly and the ovary begins to enlarge. The ovules get fertilized and the ovary starts developing. Fruits mature in 2 to 12 months depending on the genera, and when ripe, open along the three sutures releasing innumerable tiny seeds. The fruit is a capsule and seeds are usually non-endospermic with an undifferentiated embryo. Seeds are very light and are dispersed by wind. All the seeds may not be viable and fertile.

Other post pollination phenomena include stigmatic closure, increase in fresh and dry weights of ovaries, hormone production, synthesis and destruction of pigments, deresupination, nastic movements, new biochemical pathways and cessation of scent evolution (Arditti, 1977).

Yadav and Bose (1989) and Slater (1991) explained in detail the post pollination phenomena in orchids.

Nadeau et al. (1993) observed that the activity of the ACC oxidase which catalyses the conversion of ACC to ethylene increased in the stigma after pollination.

Porat (1994) reported a rapid acceleration of the wilting processes following successful pollination in several orchid genera. He also observed that wilting of flowers was accompanied by a loss of moisture from the cells of the upper layer of petals, leading to their upward folding.

Successful seed production in orchids is preceded by complex sequences of physiological and morphological events initiated by pollination (Guha et al., 2006). According to him the post pollination phenomenon in orchids serves three basic functions.

a) To protect the pollinia , ensure a close contact between them and the stigmatic surface and provide a favourable environment for pollen germination and tube growth. This is achieved by the swelling of the column and stigmatic closure.

b) To recycle substances from senescing organs into those that became the centre of new activities.

c) To render the pollinated flowers no longer attractive to pollinators there by conserving pollinator-power and increasing the chance that unpollinated flowers will be visited by the pollinators.

Even when a cross appears successful there may be incompatibility between the parents which will be revealed later with the death of the pollinated flower several days or weeks after pollination. Sometimes development will proceed till the ripening of the capsule but the seeds will be sterile.

2.6. Harvesting of Capsules for Culture

Harvesting of capsules should be done before they dehisce. When the capsule starts becoming brownish or yellowish it is a sign of maturity. Generally,

it takes 4-10 months for capsule to mature and ripen. The time taken for maturity of pods also depends upon species crossed.

Very young as well as fully mature ovules do not form good explants *in vitro* due to dormancy, pH, inhibitory and other metabolic factors (Withner, 1953).

Green capsule / pod culture was a major advancement in increasing the germination of orchid seeds *in vitro* and reducing the time to reach flowering stage. Immature ovules from young pods of orchids have been cultured *in vitro* in nutrient media to give rise to plants (Withner, 1943; Withner, 1959).

Abraham and Vatsala (1981) suggested to harvest the pods earlier which prevented contamination of the seeds with fungal or bacterial spores and resulted in good germination. The highest percentage of germination obtained in *Bletilla striata, Calanthe discolor, C. furcata, C. cardiosa* and *Phaius minor* was with seeds harvested when embryos were almost mature (Nagashima, 1982).

For dry seed culture, fully ripe pods or nearly ripe pods are collected. Pods may be put in a sterile jar or paper cover and can be stored in the refrigerator, upto even 6 months without loss of viability. As seeds can grow only in culture, they must be sterilized carefully which may cause damage to some of the seeds. For green capsule culture, pods can be collected at 60-75 per cent pod maturity. At this stage, the green pods can be surface sterilized more easily during culture without damaging the seeds inside.

Johansen (1990) found that the variation in maturation period for the capsules in different species was very much pronounced, ranging from 43 to 441 days in *Dendrobium salaccense* and *D. heterocarpum*, respectively. It was further observed that in reciprocal crosses, capsules of the same parents differed in size at maturity.

Seaton (1994) suggested harvest of seed capsules just a few days prior to the onset of dehiscence, the stage at which seeds will be fully mature and highly viable.

Maturity time for harvesting of orchid pods of some genera were studied (Singh, 1995). For *Cattleya* it was 180 days and for *Vanda* and *Phalaenopsis* it was 170 and 110 days respectively.

2.6.1. Capsule Maturity

Assessment of the correct maturity stage is a major deciding factor in green capsule culture. Sagawa and Valmayor (1966) found that the earliest culture of *Dendrobium nobile* capsule was possible only 80-85 days after pollination.

In the opinion of Sauleda (1976) the pistillate parent was mainly responsible for determining the harvesting time when crosses were made between parents with different harvesting times.

Green capsules of *Paphiopedilum* harvested four months after pollination and that of *Cattleya*, *Cymbidium*, *Phalaenopsis* and *Eulophia* harvested eight to nine months after pollination germinated satisfactorily (Rosa and Laneri 1977).

Pods of *Cypripedium reginae* harvested a week before dehiscence germinated well. Very early harvest reduced germination greatly (Harvais, 1982).

Nagashima (1982) obtained the highest germination in orchid genera such as *Cymbidium goeringii* and *Paphiopedilum insigne* var. *sanderae*, when the green pods were harvested at 115-120 days and 195-200 days, respectively after pollination.

Hegde (1984) found that the pods of *Dendrobium* species matured in nine to 17 months.

Yadav and Bose (1989) considered capsules turning yellowish or brownish as a sign of maturity.

2.6.2. Capsule Culture

Green pod culture trials conducted on a wide range of orchids led to the conclusion that success is possible only after fertilization (Valmayor and Sagawa, 1967).

Abraham and Vatsala (1981) observed that good seed germination without fungal and bacterial contamination was obtained when the green capsules were harvested earlier.

Arditti et al. (1982) could germinate both mature and immature capsules of *Epipactis* asymbiotically. However, seeds from immature capsules germinated well and rapidly.

In the opinion of Harvais (1982) the capacity of *Cypripedium reginae* seeds to germinate varied from capsule to capsule, place to place or from season to season.

Experimental results obtained by Mitra (1986) indicated that seeds obtained from unripe capsule germinated readily in several orchid species. Reduced germination at capsule maturity was due to dormancy factors and changes in enzyme compliments. He further suggested that identification of the critical stage at which dormancy sets in would be beneficial.

Ballard (1987) in *Cypripedium reginae* found that fertilization remained incomplete even beyond the sixth weeks after pollination, delayed perhaps by weather conditions.

The technique of green pod culture reduces the time lapse between germination and sowing of seeds, saves them from exposure to sterilizing agents and favours production of large number of seedlings (Pathak et al., 1992).

According to Singh (1993) depending on genera the difference in harvesting time between the dry seed culture process and the green pod culture process varied by as much as six to eight months.

Results of the studies of Nagashima (1993) using 47 orchid species indicated that germination was fast in seeds in which embryogenesis was almost complete.

Sharma (1998) could observe a decrease in the germination of fully mature *Vanda* seeds with progressive age.

2.7. *In Vitro* Seed Culture

Due to lack of any nutritive tissue to make the germinating seed self supporting, germination of the seed and further growth of the seedling in orchids is an extremely slow process. Contact with suitable mycorrhizal fungi of orchids under natural conditions or presence of a sugar containing medium, gives the seed the necessary stimulus for germination. Eventually the growing seedlings develop chlorophyll and become tiny independent green plants.

In 1909, Bernard isolated the root infecting fungus which promoted the germination of orchid seeds. Subsequently the work of Knudson, clarified that the fungi were responsible for breaking down starch into simple sugars needed for

germination. He formulated a medium known as 'Knudson C' in 1946 to provide the balanced organic and inorganic nutrients for the developing seedlings without mycorrhyza. He also showed that it was possible to grow orchid seeds *in vitro* without any fungal associations.

Another medium was proposed by Vacin and Went in 1949 which was equally good. In 1962, a new medium was formulated by Murashige and Skoog which was also utilized for growing orchid seedlings. Thus the era of growing orchids *in vitro* in an industrial scale was initiated. Now it is possible to get about 90 per cent of the seeds successfully germinated into seedlings through the use of these media.

Immature embryos of *Vanda coerulea* were inoculated in both liquid and semi-solid VW media, supplemented with vitamins and growth regulators like NAA, BAP and kinetin. When the semisolid VW medium was enriched with 20 per cent coconut water, germination of embryos occurred in both liquid and semi-solid media. However, higher percentage of and faster rate of germination were observed in VW liquid medium as compared to that in VW semi-solid medium. The differentiation of the protocorms into seedlings varied with the concentration and combination of growth regulators in VW semi-solid medium, enriched with 15 per cent coconut water (Devi et al., 1998).

Nagaraju et al. (2002) found that embryos collected at 270 days after pollination and cultured in KC medium supplemented with BAP required a minimum number of days for germination. Higher number of protocorms was formed in MS medium. Addition of activated charcoal assisted in stimulating growth.

Das et al. (2004) reported Gamborgs medium to be better than the other media for swelling and greening of embryo of *Cattleya labiata* x *C. aurantiaca.*

2.7.1. Nature of Orchid Seed

In Orchidaceae, hybrid production is confronted with several hurdles at each step. The nature of orchid seed is one such hurdle. The first published description of an orchid seed is by Theophrastus (Salisbury, 1804). The seeds in orchids are very minute, without a functional endosperm and with specific nutritional requirements which have to be provided *in vitro* in hybrid development, through well-balanced culture media.

The orchid seeds though produced in very large numbers do not readily germinate in nature as they possess immature embryo (Zeigler et al., 1967) and because of the specific nutritional requirements of these embryos.

The series of events leading to the development of egg is a post pollination phenomenon in orchids and the time interval from pollination to fertilization may range from ten days to six months (Sagawa and Valmayor, 1966).

Orchid seeds are unique in several aspects. They are minute, measuring from 0.25 to 1.20 mm in length (Hoene, 1949), 0.090 to 0.270 mm in width (Arditti, 1967) and weighing from 0.30 to 14.0 g (Harley, 1951).

Orchid seeds are produced in large numbers, ranging from 1,300 to 4,000,000 per capsule and the great majority of species have nonendospermous seeds with relatively undifferentiated embryos (Arditti, 1967).

The colour of orchid seeds may be white, cream, pale green, reddish orange or dark brown (Arditti, 1967).

The orchid embryo lies within a testa and consists of 80-100 cells which are relatively undifferentiated and mostly isodiametric with dense, granulated cytoplasm. The embryo is attached to the testa at the posterior end by means of a

suspensor, having very large, vacuolated, dead cells (Arditti, 1979). Single-celled suspensors were observed in certain species (Muralidhar and Mehta, 1986).

As the orchid seed matures, the suspensor shrinks, followed by changes in the structure and organization of integuments. Cells of the testa are dead at maturity and are thick in epiphytes and in terrestrial orchids (Vijayaraghavan et al., 1986).

2.7.2. Seed Germination

Although produced in very large numbers, orchid seeds lack metabolic machinery and functional endosperm, with the result that only 0.2-0.3 per cent seeds germinate in nature, with the association of mycorrhiza (Abraham and Vatsala, 1981).

Burgeff (1959) carried detailed studies and demonstrated the association of various fungal mycelia with orchid roots at different stages of germination and plant growth.

Higher percentage of and faster seed germination rate in liquid culture of orchid seeds as compared to that cultured in semi-solid VW medium has been reported by Vacin and Went (1949). Arditti (1966) reported that organic extracts like coconut water, tomato juice, banana extract etc. favoured orchid seed germination and development of plantlet. Olivia and Arditti (1984) compared *in vitro* seed germination in seeds of unbursted and bursted capsules of orchids. Seeds of bursted capsules revealed poor germination response as compared to those from unbursted capsules.

Stimulation of germination and growth of seedlings with low concentration of IAA and IBA were reported by Chung et al. (1985) in *Aerides japonicum*.

Immature seed culture has been succcessfully accomplished in several orchid genera including *Phalaenopsis, Dendrobium* and *Cymbidium* (Vij and Pathak, 1988). Pathak et al. (1992) also reported that green pod culture has been successfully utilized in obtaining seedlings *in vitro* from immature seeds in several species of orchids, both epiphytic and terrestrials.

The immature seeds from unripe green capsules of *Dactylorhiza hatagirea*, collected 16 weeks after pollination successfully germinated on agar-gelled modified KC medium containing selective growth adjuncts. A combination containing yeast extract (1 g l^{-1}) and 6-furfuryl amino purine (1 mg l^{-1}) proved very useful during germination and seedlings complete with a leaf and tuberous root were obtained in 38 weeks (Vij et al., 1995).

Seeds of *Dendrobium transparens* were inoculated on B5 medium supplemented with 6- furfuryl amino purine (KN) and Naphthalene acetic acid (NAA) by Hazarika and Sarma (1995). The seeds showed signs of swelling and the embryos which emerged out, developed into globular, yellowish-green protocorms within 25 days of culture.

The process of orchid seed germination, symbiotic or asymbiotic, essentially remains the same and differes from that of any other angiosperm. During germination, the embryo imbibed moisture, enlarged and burst out of the testa as an ovoid, top-shaped protocorm (Arditti et al., 1981).

Depending on the genotype of seed, its quality and culture conditions, protocorms developed chlorophyll within 10-30 days after inoculation (Shoushtari et al., 1994).

The protocorm differentiated into shoot and root meristems in opposite directions. A scale leaf developed first, followed by foliage leaves. Single celled

rhizoids developed from the protocorm for absorption. After the two leaf stage, the protocorm and rhizoid lost their nutritive function and real roots were formed endogenously (Mitra, 1971).

2.7.3. Changes During Germination

In the protocorms of *Vanda*, the parenchymal cells accumulated substantial quantities of lipid, protein and carbohydrate reserves, which disappeared gradually with the senescence of the parenchymatous region (Ricardo and Alvarez, 1971).

In *Cattleya*, lipid reserves were utilized slowly when seeds were on medium lacking carbohydrate source, but utilized rapidly on medium containing sucrose (Harrison, 1977).

Further studies of Harrison and Arditti (1978) indicated that during germination of *C. aurantiaca*, the levels of chlorophyll and specific activity of ribulsoe 1-5 diphoshate carboxylase increased in sucrose containing media.

Sangama (1986) was of the opinion that germinating orchid seeds utilized lipids, proteins and carbohydrates, in that order.

In the protocorms of *Vanilla planifoia* the cells were heavily laden with proteins and starch grains. Protein bodies disappeared during differentiation of meristem. Bipolar differentiation of meristem produced the shoot and after formation of a few leaves the first root differentiated endogenously from the base of the meristem (Philip and Nainar, 1988).

Krishnan et al. (1993) found that the stored lipids and proteins were entirely adequate for the development of protocorms in *Spathoglottis* during initial stages. The accumulated starch was used up for organogenesis during later stages.

Raghavan and Goh (1994) reported that the regulatory events in the embryo prior to seed maturity determined the fate of its proximal and distal parts during germination. Synthesis of DNA and cell division were confined to the proximal end, whereas cells at the distal end underwent enlargement.

Pyati and Murthy (1995) obtained optimum *in vitro* seed germination of *Dendrobium ovatum* on KC medium. Additional use of growth adjuncts such as coconut milk, cane juice, yeast extract, casein hydrolysate, peptone and nicotinic acid markedly enhanced germination and differentiation of protocorms.

In an endangered species *Vanda coerulea* seeds from capsules (pods) at various stages of development were cultured asymbiotically *in vitro*. The germination percentage increased with the capsule age ranging from 180 days to 270 days. The seeds obtained from 270 days old capsules revealed the maximum seed germination on Knudson C medium (Sharma, 1998).

Paphiopedilum species and hybrids are the only commercially grown orchids not currently cloned by tissue culture, largely because of the difficulty in removing bacterial and fungal contamination in explants derived from greenhouse plants.

Huang (2001) reported that they have overcome the contamination problem by using aseptically established seedlings as explants. They developed a mericloning protocol that results in multiplication and rooting of *Paphiopedilum* orchids in one step or in the same culture medium. The one-step protocol produces an average of 12 new plants per culture in 12 weeks which extrapolates to 100 plants from each culture per year.

Jamir et al. (2002) recorded that earliest and maximum germination of *Cymbidium* sp. was obtained in seeds procured from 120 days old capsules cultured in Nitsch medium.

Sobhana and Rajeevan (2002) conducted studies for the refinement of culture media for growing *Dendrobium* New Pink x Emma White seedlings. High rate of germination was observed in all the five media, viz., MS full strength, ½ strength, ¼ strength, Vacin and Went and Knudson C. MS at ¼ strength, Vacin and Went and Knudson C media showed better response in respect of seedling growth.

Seeds of *Spiranthes sinensis* were cultured on quarter strength MS basal medium supplemented with 20 g sucrose l^{-1}, 50 g banana pulp l^{-1} and 1 g activated charcoal l^{-1}. Green protocorms were then observed after one month. Seedlings were grown to plantlets with shoots and roots after five months of culture, and plantlets were then subcultured onto quarter strength MS salt basal medium containing 20 g sucrose l^{-1}, 30 g potato pulp l^{-1}, 1 g activated charcoal l^{-1}, and 150 mg coconut milk l^{-1} (Chang et al., 2003).

Talukdar and Ahmed (2003) observed highest seed germination and subsequent growth and protocorm development of *Spathoglottis plicata* on Knudson C media supplemented with CW 150 ml l^{-1}, ME 0.5 g l^{-1}, KN 0.5 mg l^{-1} and IAA 0.5 mg l^{-1} and *Rhynchostylis retusa* on Knudson C media supplemented with ME 0.5 g l^{-1}, NAA 1 mg l^{-1} and BAP 1 mg l^{-1}.

In vitro seed germination studies were carried out in *Dendrobium* hybrids by Sobhana and Rajeevan (2003). Very high germination was noticed in the crosses Emma White (LW) x New Pink (NP), EW x Hieng Beauty (FIB), FIB x Candy Stripe (CS), FIB x NP, NP x Sonia-28, NP x FIB, NP x LW, NP x CS and Sonia-28 x NP.

Asymbiotic cultures of the dove orchid *Peristeria alata* was raised by Bejoy et al. (2004) from seeds obtained from 50-55 days old green capsules in liquid. In Mitra et al. (1976) medium supplemented with 1 g l^{-1} peptone, protocorms differentiated in 5-6 weeks.

Talukdar and Deka (2004) observed earlier germination and protocorm formation and higher germination percentage for the hybrid seeds of a cross between *Spathoglottis plicata* and *Dendrobium densifolium* in Nitsch medium. The protocorms when subcultured in Nitsch medium supplemented with NAA- 1.0 mg l^{-1} + kinetin- 1.0 mg l^{-1} produced earlier leaf and root initials.

2.7.4. Seed/Capsule Sterilization

Sterilization prior to inoculation is inevitable as orchid seeds are cultured under completely aseptic conditions. Since mature orchid seeds have tough seed coats, chemical treatments for sterilization can be safely employed (Jordan, 1965).

Mature seeds of *Vanda* Miss Joaquim pretreated with 5 per cent chlorox for 10 minutes and rinsed with sterile water prior to inoculation produced seedlings in 10-12 weeks whereas mature seeds without pretreatment were lost due to contamination. Green pod culture proved to be the best, since seeds directly transferred to the medium without exposure to the outside germinated well and produced strong seedlings within 8-10 weeks (Rao and Avadhani, 1964).

Mitra (1971) used chlorine water to sterilize capsules and seeds. Pods were dipped in absolute alchohol (12 seconds) and chlorine water (45 minutes) whereas seeds folded in filter paper were dipped in chlorine water for 10 minutes and rinsed with three changes of sterile water.

Rosa and Laneri (1977) successfully used 70 per cent ethanol and 1.7 per cent sodium hypochlorite for sterilizing pods and 7.0 per cent calcium hypochlorite for sterilizing seeds of five orchid genera. Immature capsules of *Epipactis*, when sterilized by immersing in saturated calcium hypochlorite solution (7 g 1000 ml^{-1} water) for 10 minutes gave good germination without contamination (Arditti et al., 1982).

Pyati and Murthy (1995) achieved pod sterilization in *Dendrobium ovatum* by dipping in alcohol followed by flaming. Pod sterilization of *Vanda coeridea* was effected by pre-treatment in 0.1 per cent mercuric chloride for five minutes followed by alcohol dip and flaming.

2.7.5. Seed germination and development *in vitro*

Curtis (1943) studied germination and seedling development in five species of *Cypripedium*. It was observed that the protocorms were initially non-chlorophyllous and chlorophyll development was initiated in the leaf tips.

Knudson (1946) showed that the seeds of *Cattleya, Laelia* and *Epidendrum* germinated freely on sugar and mineral containing agar medium under aseptic conditions without fungal association.

Arditti (1979) reported in four orchid genera including *Dendrobium* that only a few apical cells of protocorms divided to form a promeristem which gave rise to shoot apex and structure homologous to cotyledons.

Olivia and Arditti (1984) found that roots and shoots generally appeared together in most *Cypripedium* seedlings.

According to Mathews and Rao (1985), the differentiated protocorms had to be subcultured within a period ranging from 70 to 80 days for proper *in vitro* growth. Overcrowding without transfer resulted in stunted growth.

Muralidhar and Mehta (1986) reported that *Cymbidium longifolium* embryos exhibited a prominent zone of pro-meristermatic cells by the fiftieth day of culture from which an unequal pair of first embryonic photosynthetic leaves developed. Simultaneously, marginal cells gave rise to unicellular rhizoids.

Yam and Weatherhead (1988) considered the seeds to have germinated when protocorms, either green or white, were observed in cultures.

Rubulo et al. (1989) defined germination as the presence of protocorms with one leaf primordium one month after culture. Adult plantlet stage *in vitro* was attained when the seedlings developed pseudobulbs, roots and leaves of at least 30 mm length.

Pathak et al. (1992) reported that in *Goodyera biflora*, protocorms were white and hairy. Inherent features in a species has role in differentiation (Reddy et al.,1992).

Singh (1992) reported that depending upon genotype, the seeds develop chlorophyll within 10-20 days on the nutrient medium.

According to Krishnan et al. (1993) protocorm differentiation occurred in second or third week of culture. Terminal bud of protocorm elongated downward in *Cymbidium* (Nagashima, 1994).

From their studies on four orchid genera, Reddy et al. (1992) found that the inherent genetic and physiological features in a species play a direct role in the differentiation of organs.

Requirements of light and photoperiod vary in different species. Several genera like *Paphiopedilum, Cymbidium* and *Phalaenopsis* are known to grow and develop better on darkened media. *Paphiopedilum ciliolare* seeds germinate only when darkness is given for first 3 months (Singh, 1992).

Singh (1993) found that inoculation of seeds into a nutrient medium under *in vitro* conditions not only improves the percentage of germination, but also

reduces the time for differentiation of orchid seeds, both biochemically and morphologically.

Krishnan et al. (1993) observed visible protocorm formation from the embryos by the second and third weeks of culture in *Spathoglottis plicata*. He also found that the first leaf primordium was initiated between the fifth and sixth weeks of culture.

Nagashima (1994) reported in temperate *Cymbidium* species that the terminal bud of the protocorm elongated downward and formed the rhizome. After the elongation of rhizome, the terminal bud grew upward and differentiated into shoots and roots.

Hazarika and Sarma (1995) reported that immature seeds of *Dendrobium transparens* showed signs of swelling 16-18 days after inoculation. The basal medium MS half strength was found to be the best for early germination and rapid *in vitro* development as compared to MS quarter strength and MS, KC and VW full strengths (Lekha Rani, 2002).

Sivamani (2004) observed that the best *in vitro* medium for the rapid culture establishment of *Dendrobium* hybrids was VW with kinetin 4.0 mg l^{-1}+ IAA 4.0 mg l^{-1}+ coconut water 200 ml l^{-1}.

Half strength MS medium supplemented with BA (1.0 mg l^{-1}) and coconut water (7.5 %) was found to be ideal for growth of *Dendrobium* cv. Sonia -17 (Sheela et al., 2004).

2.8. Culture media, components and media supplements

Culture media and culture conditions are important in the development of hybrids in orchids. Since the plants are exacting in their requirements, very often,

modifications are to be made to suit specific situations. Several attempts were made in the past for developing and refining *in vitro* culture techniques for seedling production in orchids. The pertinent information gathered in these aspects through research works is reviewed below.

2.8.1. Effect of Culture Media on Seed Germination

Some media like Knudson C, Vacin and Went and Mitra et al. medium are broad spectrum, while media like Burgeff N_3f are exclusively for *Paphiopedilum* seeds.

Orchid seeds utilize disaccharides such as sucrose for initial germination and subsequent growth. Some species of *Cymbidium* prefer glucose to sucrose, while *Phalaenopsis* prefers fructose to glucose. Thiamine appears to be essential and growth promoting. Calcium and nitrogen promotes germination of orchid seeds.

Many media have been used for the axenic germination of terrestrial and epiphytic orchids. However, none of these media is universal. Nagaraju and Upadhyaya (2001) observed that Nitsch medium was the best for growth of *Cymbidium*. The addition of 0.3 % activated charcoal to the medium had a marked effect on shoots and roots.

The commonly used nutrient media for orchid seed culture are those proposed by Knudson (1946) (KC), Vacin and Went (1949) (VW), Murashige and Skoog (1962) (MS), Raghavan and Torrey (1964), Nitsch (1969), Mitra et al. (1976) and Rosa and Laneri (1977) (RL).

Seed germination and morophogenesis studies in *Epidendrum radicans* and *Dendrobium* Jaquelyn Thomas clearly indicated the superiority of MS medium over KC and VW media (Sangama, 1986).

Devi et al. (1990) pointed out that the preferred medium for *Dendrobium* seed germination varied with the species. *D. farmeri* and *D. primulinum* gave 50-60 per cent higher germination on VW medium. On the other hand, Nitsch medium gave better results with *D. moschatum* and *D. fimbriatum* as compared to other media.

Kumaria and Tandon (1991) were of opinion that high ionic concentration of nutrient salts and vitamins in the medium was inevitable for the germination of *Dendrobium fimbriatum var.maculatum* seeds. On inoculating four-month seeds, highest germination (91 %) was obtained on Nitsch medium followed by MS (85 %). Protocorm stage was reached in four to five weeks on MS and VW media.

In vitro studies on seed germination and seedling development in four species of South Indian tropical orchids showed a significant interaction between the media and the species. *Dendrobium crepidatum* yielded better results in MS and RL media than in KC medium (Reddy et al., 1992).

Nagashima (1993) recorded that out of the forty seven orchid species tested, some were found to respond better to hyponex medium whereas others gave better germination and seedling growth in MS medium.

The effect of media, however, was found to be inconsistent by Pauw and Remphrey (1993). They found that it varied depending on the year and season of collection of capsules as well as the species cultured.

Hazarika and Sarma (1995) conducted *in vitro* germination studies in *Dendrobium transparens* Lindl. and reported that best growth of seedlings was obtained in supplemented MS medium.

Since MS medium contained high ionic concentration of nutrient salts Zhang et al. (1993) found that half strength MS could adequately support rapid protocorm proliferation in orchids.

Bhasker (1996) found that supplemented quarter strength MS could produce seedlings with maximum number of shoots, leaves and roots in *Phalaenopsis* after a 12 week culture period.

Nagaraju and Upadhyaya (2001) suggested that Nitsch medium + 0.3 % charcoal was the best for *Cymbidium.*

Lekha Rani (2002) conducted studies on effect of media on *in vitro* seed germination and growth of seedlings in *Dendrobium* using media such as MS (1/4 strength), MS (1/2 strength), MS (full strength), VW (full strength) and KC (full strength). It was observed that basal medium MS half strength was the best.

Hormone free MS medium was best for early germination, protocorm formation and their further differentiation in *Aerides odorata*, "Fragrant Orchid" of Nepal. Its ground fruit is used for healing of wounds and juice of fleshy leaves for the healing of boils in ear and nose. *In vitro* seed culture was suggested for its rapid propagation by Pant and Gurung (2005) .

Embryo culture and artificial seed production of *Spathoglottis plicata* (a sympodial terrestrial orchid) and *Rhynchostylis retusa* (a monopodial orchid) were successfully done by Madhumita and Ahmed (2005). *S. plicata* and *R. retusa* seeds (30 days and 120 days respectively) were inoculated onto Knudson C medium modified with compiled organic additives .The highest seed germination and its subsequent growth and protocorm development of *Spathoglottis plicata* were optimum on Knudson C medium supplemented with CW 150 ml l^{-1}, ME 0.5 g l^{-1}, KN 0.5 mg l^{-1}, IAA 0.5 mg l^{-1} and TJ 50 ml l^{-1}. *R. retusa* showed

highest frequency of germination and protocorm development in Knudson C medium supplemented with ME 0.5 g l^{-1}, NAA 1 mg l^{-1} and BAP 1 mg l^{-1}.

Sheela et al. (2008) reported beneficial effect of half strength MS medium supplemented with BAP at 0.5 mg/l and 1.5 mg l^{-1} in *Dendrobium* cv. Sonia -17.

2.8.2 Effect of Complex Additives on Seed Germination

McIntyre et al. (1974) found that addition of coconut water (15 %) to KC medium led to increased growth of both epiphytic and terrestrial orchids. Vigorous root resulted in epiphytes.

Flamee (1978) concluded that no one substance stimulated germination and there were variations in germination between species on the same medium.

The approximate composition of coconut water and banana pulp has been provided (Arditti and Ernst, 1993).

Sahid (1980) reported that growth rate of *Dendrobium* hybrids could be improved by adding potato and pea extracts to KC medium.

Mitra (1986) stated that a growth-stage dependent variation in nutritional requirement was observed in orchid cultures. The nutritional requirement of the seedling stage differed from those of germination and protocorm stages.

Addition of peptone along with vitamins and casein hydrolysate to VW medium enhanced germination of *Acampe praemorsa* (Krishnamohan and Jorapur, 1986).

Bopaiah and Jorapur (1986) found that a nutrient medium supplemented with coconut water (CW), peptone, casein hydrolysate and banana pulp along

with vitamins and kinetin was most suitable for normal and healthy growth in *Cymbidium aloifolium.*

Pierik (1987) observed that supplemented VW medium (CW 15 % + NAA 10 ppm) produced rapid protocorm proliferation followed by enhanced seedling growth in *Dendrobium* Jaquelyn Thomas.

According to Rubulo et al. (1989), supplementing KC medium with 10 per cent coconut water gave the best germination in *Bletia urbana.* All seedlings developed to the adult stage, forming leaves, pseudobulbs and roots after 90 days of *in vitro* culture.

Das and Ghoshal (1989) found that modified KC medium when supplemented with NAA (1 mg l^{-1}) could promote further growth of *Dendrobium chrysotoxum* and *D. pierardii* x *D. crepidatun* seedlings at two leaves stage.

For *Cattleya, Encyclia* and *Oncidium*, 25 per cent CW and for *Stanhopea*, 60 g l^{-1} BP were the best additives (Villolobos and Munoz, 1994).

Bhasker (1996) has pointed out the beneficial effects of peptone and CW on *in vitro* seedling growth in *Phalaenopsis.* Peptone (1000 mg l^{-1}) along with BA (20 mg l^{-1}) and NAA (1 mg l^{-1}) maximized shoot, leaf and root production after 12 weeks of culture. Foliar growth was enhanced by addition of CW.

Coconut water (CW) promotes cell division in non dividing cells, morphogenesis and mass multiplication of protocorms in orchids. Banana pulp, orchid salep, peptone, tomato juice, honey, yeast and bean extracts were also used.

Half strength MS medium supplemented with BA (1.0 mg l^{-1}) and coconut water (7.5 %) was ideal for growth of *Dendrobium* cv Sonia-17 (Sheela et al., 2004).

Coconut water 200 ml l^{-1} was proved to be the best in terms of early protocorm differentiation and rapid seedling growth of *Dendrobium* hybrids (Lekha Rani et al., 2005).

2.8.2. Effect of Growth Regulators on Seed Germination

In order to promote seed germination and seedling growth in orchids, many plant growth regulators have been tried.

It was reported that IAA 1 mg l^{-1} or 2 mg l^{-1} effectively promoted growth of orchid seedlings (Hayes, 1969).

Kinetin enhanced shoot characters and chlorophyll formation but suppressed the root characters of *Dactylorrhiza* protocorms (Harvais, 1972).

Fonnesbech (1972a) opinioned that the protocorms of *Cymbidium* proliferated when grown on a medium supplemented with IAA whereas 2,4-D inhibited protocorm formation.

Payawal and de Guzman (1972) suggested that the optimal concentration of NAA in *Vanda* to be 1.25 mg l^{-1} . NAA 0.1 mg l^{-1} was sufficient in *Cattleya* cultures for inducing optimum growth (Ichihashi and Kako, 1973).

Strauss and Reisinger (1976) found that the growth regulator NAA stimulated germination and seedling growth in several species like *Cattleya aurantiaca, Cymbidium maddidum* and *Bletilla* spp.

Kusumoto (1978) observed that the combined effect of 2,4-D and kinetin or BAP favours protcorm formation in some orchid species.

Arditti (1979) reported that experiments with auxins, cytokinins and gibberellins on orchid seed germination have given inconsistent and therefore inconclusive results. According to him 2,4-D inhibited germination and induced callus formation in *Vanda* Miss Joaquim, 2 mg l^{-1} of IAA and NAA induced callus formation and reduced the percentage of normal seedlings.

Various parameters controlling the growth of orchid protocorms were investigated in the hybrid embryos of *Vanda* by Mathews and Rao (1985). All the auxins except 2,4-D favoured protocorm multiplication in the concentration range of 0.5 to 4.0 mg l^{-1}. IAA was found to be the most favourable auxin for protocorm multiplication.

Muralidhar and Mehta (1986) found that in *Cymbidium longifolium* VW medium supported 80 per cent seed germination when supplemented with IAA (0.2 mg l^{-1}) and kinetin (0.4 mg l^{-1}) in addition to vitamins and aminoacids.

A kinetin : NAA ratio of 10:1 favoured early germination and subsequent growth in *Coelogyne punctulata* (Sharma and Tandon, 1986).

Lim *et al.* (1993) found that IBA and kinetin gave enhanced shoot production in the seed cultures of *Dendrobium*.

A combination of kinetin and IBA (0.5 ppm each) produced best seedling development of *Bletilla striata*. Best results were obtained in *Spiranthes* with 3 ppm kinetin (Suner, 1995).

Hazarika and Sarma (1995) found that a combination of kinetin, IBA (0.1 mg l^{-1} each) and NAA (1.0 mg l^{-1}) was best for enhanced germination and seedling growth in *Dendrobium transparens* Lindl.

Maximum number of shoots and leaves in *Phalaenopsis* cultures resulted from a combination of 8 ppm adenine and 16 ppm BA (Bhasker, 1996). IAA 0.1 mg l^{-1} enhanced seed germination in *Cymbidium mastersii* up to 80 per cent. GA_3 is rarely used in seed germination as it caused abnormal elongation of emergent shoot. IAA, IBA and NAA enhance seed germination. IAA at very low concentration (0.1 mg l^{-1}) promoted germination of seeds in *Cymbidium mastersii* upto 80 % . The same auxin was effective at 0.25 mg l^{-1} in vandaceous taxa.

The auxin NAA 1mg l^{-1} enhanced germination and accelerated seedling growth *in Dendrobium fimbriatum and D. moschatum* (Devi et al.,1990).

Nayak et al. (1997) successfully encapsulated protocorm like bodies (PLBs) of a terrestrial orchid *Spathoglottis plicata* in calcium alginate beads containing the nutrients of Murashige and Skoog medium supplemented with 2 mg l^{-1} NAA and 0.5 mg l^{-1} BA.

2.8.4. Effect of Carbon Source on Seed Germination

Orchids must have an external supply of carbohydrates to continue their growth and differentiation. Orchid seeds and young seedlings have the ability to utilize various carbohydrates. However, different species have their own preference (Arditti, 1967).

Glucose, fructose, or oligosaccharides containing these sugars could adequately satisfy the energy requirements of *Phalaenopsis* protocorms (Ernst et al., 1971). In *Dactylorrhiza purpurella*, the results with dextrose and sucrose were essentially similar (Harvais, 1972).

Of the sugars tested on the growth of *Cymbidium* protocorms, sucrose was better then maltose, glucose and fructose. The optimum concentrations of sucrose ranged from 3.0 to 4.0 per cent (Fonnesbech, 1972b).

Harrison and Arditti (1978) found that sucrose induced germination and enhanced chlorophyll development in certain species that failed to germinate on sugar-free medium. Sucrose could be replaced by glucose.

In hybrid *Vanda*, Mathews and Rao (1985) tested different carbon sources and found that 2.0 per cent sucrose was the best source. Absence of sucrose stopped growth of protocorms and 10.0 per cent sucrose caused tissue necrosis.

Ballard (1987) in *Cypripedium raginae* reported that sucrose was better compared to dextrose and 2.0 per cent sucrose was better compared to 1.5 or 1.0 per cent.

In *Paphiopedilum ciliolare*, Pierik et al. (1988) concluded that an extra ordinary low sugar concentration was optimal for germination, higher concentration being inhibitory.

High sucrose concentration (4.0 %) reduced germination in *Bletia urbana*, but no significant difference could be observed in the response between 2.0 per cent and 3.0 per cent sucrose (Rubulo et al., 1989).

Among various carbon sources tested, sucrose, fructose and glucose at 2.0 to 3.0 per cent gave best germination and seedling growth in *Cymbidium elegans* and *Coelogyne* spp. In sugar-free medium, germination and growth were negligible (Sharma and Tandon, 1990).

Ernst and Arditti (1990) recorded that although *Phalaenopsis* seeds and seedlings can utilize many sugars as carbon source, seedling fresh weight and survival decreased with the increased polymerization and increased molecular weight.

Bhattacharjee et al. (1999) demonstrated that sucrose at 20g l^{-1} was optimum for protocorm formation and development in *Phalaenopsis.*

Wang et al. (2000) found that sucrose level of 20 to 35 g l^{-1} in culture medium was ideal for *Dendrobium officinale.*

Effect of carbon source on protocorm development and further growth of *Cattleya* hybrids was studied in detail by Prakash and Thejeswari (2008). Early development of protocorm was recorded at 2 per cent sucrose in VW medium.

2.8.5. Effect of Charcoal on Seed Germination

Ernst (1974) recorded that *Paphiopedilum* seedlings grew well on culture media to which activated charcoal was added.

According to Rosa and Laneri (1977), the addition of charcoal to the culture medium helped in the rapid and better development of shoots and roots in seedlings of *Phalaenopsis.*

Fridborg et al. (1978) attributed the beneficial effects of activated charcoal to its adsorption of inhibitory phenolic and carboxylic compounds produced by the tissues in culture. They further observed that charcoal has the tendency to absorb hormones and vitamins and thereby inhibit growth. Hence it should be used with caution in culture media.

The initial formulation of charcoal containing medium for orchid seed germination gained wide acceptance (Yam and Weatherhead, 1988).

Pierik et al. (1988) found that significant increase of shoot and root development resulted in *Paphiopedilum ciliolare* when activated charcoal at a concentration of 2 g l^{-1} was added to the medium after protocorm formation, but was inhibitory during seed germination.

According to Hinnen et al. (1989) activated charcoal strongly enhanced the growth and development of *Phalaenopsis* seedlings.

Yam et al. (1990) observed that activated charcoal exerted a beneficial effect on culture media by adsorption and removal of phytotoxic metabolites. They further pointed out that it can also be detremental due to the removal of additives such as auxins and /or cytokinins.

2.8.6. Effect of pH of Media on Seed Germination

Knudson (1951) noted inability of *Cattleya* seeds to germinate it the initial pH of medium is below 4.5.

Dendrobium nobile germinated better within a pH range of 4.0-5.0 (Ito, 1955) whereas many orchid species responded favourably to media with pH between 5.0 and 6.0 (Scott and Arditi, 1959; Kotomori and Murashige, 1965).

Maintaining the pH at 5.2 to 5.5 was favourable for successful germination in *Cymbidium mastersii* (Prasad and Mitra, 1975).

Rosa and Laneri (1977) observed that a pH of 5.2 for *Cattleya* and *Phalaenopsis* and 6.0 for *Cymbidium* and *Paphiopedilum* was satisfactory for germination.

Reyburn (1978) recorded that in *Cymbidium* germination in the dark was optimal at pH 5.5-6.0 and pH of 7.0 was strongly inhibitory.

Orchid seeds germinated well within a pH range of 4.8 to 5.2 with germination commencing at pH 3.6 and tapering off at 7.6 (Arditi, 1979).

Maximum germination and optimal growth of protocorms at pH 5.0 was reported in *Dendrobium chrysanthum* and *Sarcanthus pallidus* (Raghuwanshi et al., 1986). Optimal germination of *Paphiopedilum ciliolare* occurred at a pH of 6.0 (Pierik et al., 1988).

Ichihashi (1990) obtained good germination of *Bletilla striata* seeds when the pH was adjusted to 5.1. George (1997) found that optimal growth of protocorms in *Dendrobium osterholt* resulted when the pH was adjusted to 5.8. For best germination the pH should be in range of 4.8-5.2. Seedlings can tolerate low pH upto 3.5.

Raghuwanshi et al. (1986) reported that for *Dendrobium* species, optimum germination and seedling growth was on pH range 4.0-6.0, due to greater uptake of water and nutrients. Decreased growth at extreme pH values 3.0 and 10.0 was due to injury to root cells.

2.9. Deflasking, Planting Out and Acclimatization

The embryo / seed sown on the nutrient media start turning green after 10-20 days, depending upon different genera and develop the chlorophyll for photosynthesis. It is recommended to sub culture the seedlings 2-3 times in *in vitro* conditions before they are big enough to be transplanted.

Once the seedlings are developed *in vitro*, congenial environment is to be provided to acclimatize them to the harsh external conditions. Post deflasking

mortality can be considerably reduced by minimizing transplanting shock. Here again, special skill and care is the key to success. The salient research findings on these aspects are brifly summarized below.

Dunstan and Turner (1984) were of opinion that post deflasking mortality was mainly caused by desiccation.

Phalaenopsis hybrid seedlings got easily established within two weeks of planting out, recording 100 per cent survival. Broken tiles was found to be the best medium followed by charcoal, cassava pith and rubber seed husk in that order (Seeni and Latha, 1990).

Sureshkumar (1992) reported that *Dendrobium* hybrid seedlings registered the best survival in pure charcoal, followed by cassava pith and rubber seed husk. They got established in one month after transplanting.

A mixture of brick and charcoal in equal proportions was used as potting medium for *Dendrobium fimbriatum* and *D. moschatum* plantlets and obtained 20 and 40 per cent survival, respectively by Lakshmidevi (1992).

Lim et al. (1993) found that for *Dendrobium moniliforme* peat moss was the best medium for aerial growth of seedlings where as perlite was best for increasing root numbers.

A potting mixture comprising of broken bricks, broken charcoal, tree-fern, bark pieces, leaf mould and dry sphagnum in 1:1:1:1:1:2 ratio with green sphagnum on top supported maximum survival in *Dendrobium* (Sharma and Chauhan, 1995).

The medium should be changed with each pricking, reducing the level of sucrose with advancement of growth (Singh, 1995). After 3-4 months, the

seedlings were taken out of flasks and washed carefully in running water to remove all traces of agar and planted in community pots in a mixture of 1:1 tree fern fibre (soft boiled) and charcoal. Seedlings generally grow better in group than singly due to community effect.

Gangaprasad (1996) observed that seedlings and tissue cultured plants of different orchid species such as *Dendrobium aquem, Ipsea malabarica, Vanda spathulata* etc. got established well within three to six months of transplanting into a brick + charcoal medium.

The best potting medium was observed to be broken tiles + charcoal + soilrite (2:2:1) favouring high survival and balanced growth of seedlings along with the easy availability of all ingredients (Lekha Rani et al., 2006 a).

Sobhana et al. (2008) concluded that tile bits, charcoal and coconut pith significantly improved flowering and floral characters in *Dendrobium* hybrids. Regarding spike production and length of spike, highest values were obtained in the medium containing tile bits, coconut husk and charcoal in *D.* Emma White. Coconut pith produced maximum number of flowers per spike as well as maximum longevity of spike on the plant and tile bits and charcoal yielded largest flowers.

In vitro hardening efforts were made in *Dendrobium* hybrid seedlings of two leaf stage in half strength Nitsch basal media lacking in sucrose, vitamins and organic component, but having sterilized brick pieces, charcoal and coconut husk. After six months they were transferred into community pots. The results revealed cent per cent survivability in the treatments of brick pieces + charcoal and brick pieces + coconut husk after six months of planting out (Devadas et al., 2008).

2.10. Genetic Analysis and Variability Studies

Genetic analysis of *Dendrobium* aggregate and certain other species grown in the plains of West Bengal was done by Rehman et al. (1993). High degree of genetic variance was recorded for length of inflorescence, number of flowers per inflorescence and flower size. Heritability and genetic advance estimates were also high for these characters indicating that selection based on these characters would be successful.

Genetic analysis studies were conducted by Sobhana (2000) in *Dendrobium* hybrids. High genetic variability was observed for flowers per spike, days for opening of florets and shoots per plant. Heritability was moderate to high for most of the characters. Flower size exhibited the highest heritability. Length of inflorescence exhibited high positive correlation with height of shoots and number of leaves.

2.11. Breeding of Hybrids

An orchid hybrid is a man-made or natural cross between two or more different orchid species and /or members of orchid genera. At present, there are approximately 115,000 registered man made orchid hybrids (http:www.clanorchids.com/store/os.html).

The first man-made hybrid *Calanthe* Dominyi was produced by John Dominy in 1852, in England, by crossing *Calanthe masuca* to *C. furcata.* It flowered for the first time in 1856. In 1863, he created the first bigeneric *Laeliocattleya exoniensis,* crossing *Cattleya mossiae* with *Laelia crispa* (Dressler, 1981). Following him, several others successfully produced interspecific and intergeneric hybrids. However, for many years the only method to produce plantlets from the seeds was to sprinkle the seeds around the base of the mother plant and just wait for success.

Evidences of natural hybridization occurring among wild members of the family have been noticed by Lindley as early as 1853 (Abraham and Vatsala, 1981).

Hundreds of intergeneric, interspecific or intraspecific natural hybrids of *Dendrobium, Odontoglossum, Cattleya, Laelia, Oncidium, Phalaenopsis* etc. have been reported from different parts of the world (Abraham and Vatsala, 1981).

One of the earliest scientific accounts on orchid hybrids was presented by Hurst (1898). Out of the 800 hybrids then on record, about 500 were primary hybrids, including 100 intergeneric hybrids. A total of 270 secondary hybrids and about 30 tertiary hybrids had flowered till then. Primary hybrids were found to be intermediate between, yet specifically distinct from either parents in morphological characters. Secondary hybrids showed a far wider range of character variation.

The majority of commercially grown orchids today are bigeneric and multigeneric hybrids derived from sympodial and monopodial orchid genera such as *Arachnis, Vanda, Renanthera, Ascocentrum, Cymbidium, Cattleya, Dendrobium, Oncidium, Phalaenopsis* and *Paphiopedilum* (Mercy and Dale, 1997).

Many Indian species have earned world-wide recognition in breeding programmes due to their inherent attractiveness coupled with their ability to transmit these characters to the hybrids. Some of the leading species are *Aerides multiflorum, Cymbidium devonianum, C. lowianum, C. traceanum, C. elagans, Dendrobium aggregatum, D. chrysotoxum, D. formosum, D. nobile, Paphiopedilum ventusum, Vanda coerulea* etc. (Bose and Bhattacharjee, 1980).

Vacherot and Lecouffle of France were the pioneers of *Dendrobium* breeding. The nobile type (narrow petals) dendrobiums of Eastern Himalayas and *D. phalaenopsis* (rounded petals) of Eastern Asia were the most frequently used parents. Colour has always been of prime importance in *Dendrobium* breeding, ranging from chalky white to yellow brown and intense crimson (Abraham and Vatsala, 1981).

Bobisud and Kamemoto (1982) evaluated inbred progenies developed from amphidiploid *Dendrobium* Jaquelyn Thomas through selfings, sibmating and back crosses. Selection and inbreeding were effective in increasing flower size and improving colour purity. The characters like flower size, flower colour, flower production, vase life and bud drop were primarily influenced by parental genotypes since inbreeding decline was not apparent.

While breeding with yellow *Phalaenopsis*, Singh (1982) observed that the farther from the species, the better becomes the flower shape, flower size and number of flowers per spike, but the lighter becomes the colour.

Kamemoto (1983) evaluated the *Dendrobium* cultivars Louis Bleriot and Pompadour. Data on spray yields of five seed propagated amphidiploid cultivars were tabulated.

Ando (1983) conducted breeding trials involving reciprocal crosses between *Dendrobium nobile* and eight species and between *D. moniliforme* and 21 other species. It was found that *D. nobile* was reciprocally compatible only with *D. haniffii*, *D. linowianum* and *D. moniliforme*. With *D. moniliforme* all crosses were fertile, the seed set varying between 17 and 100 per cent.

McConnel and Kamemoto (1983) reported that reciprocal crosses involving two accessions of *Dendrobium canaliculatum* yielded offsprings differing in cane height, pseudobulb production and flower yield. Reciprocal crosses of *Dendrobium schilleri* and *D.* Sunset differed in flower quality, whereas

reciprocal mating of two amphidiploid *D.* Jaquelyn Thomas selections did not differ in vegetative or floral characters.

Singh (1984) observed that fragrance is a character most sought after by *Cymbidium* breeders since majority of the species lack that attribute. The scented *C. munronianum* has been used as parentsin several breeding programmes.

Cheng et al. (1985) concluded from a study on 22 species and six hybrids of diploid and triploid *Dendrobium* that no correlation existed between chromosome number and flower size.

Singh (1986) has described the *Dendrobium* hybrids IIHR 38 (*D. pompadour* x *D. superbiens*) and the *Vanda* hybrid IIHR 164 (*V. rothschildianum* x *V. coerulea*). The *Dendrobium* hybrid was robust with 35-40 cm long flower spikes bearing 12-15 flowers per spike. The flowering season was between mid February and late May.

Philips (1986), while breeding with *Paphiopedilum rothschildianum* noted that the species can add immense desirable floral qualities to the hybrids.

Stewart (1986) found that the intergeneric hybrid *Eulocymbidiella* (*Eulophiella* x *Cymbidiella*) was intermediate in shape between the two parents. In colouration, the flowers were quite different from either parent.

Thammasiri et al. (1987) found that colour fading in yellow flowered *Dendrobium* hybrids was due to the degradation of flower pigments at different stages of maturity, from bud stage.

Das and Ghoshal (1988) studied the breeding behaviour of *Dendrobium crysotoxum, D. crepidatum, D. pierardii, D. primulinum* and *D. transparens*. Successful reciprocal crosses were achieved between *D. crepidatum* and

D. transparens while unidirectional crosses were successful with *D. primulinum* x *D. crepidatum* and *D. transparens* x *D. pierardii.*

Atwood (1989) identified a natural hybrid in *Paphiopedilum* and observed the barriers to hybridization occasionally, even among remotely related genera. The flowers were more massive than either parent, probably due to hybrid vigour. The hybrid also possessed several other attributes that were not observed in either parent.

Kamemoto et al. (1989) evaluated 16 seed propagated amphidiploid *Dendrobium* progenies for cut flower production. Variations were observed in flowering season, flower yield, plant height and floral characters. Bud percentage varied from 2.5 to 10.5 and vase life from 12.8 to 22.2 days. A cross displaying several desirable characteristics was released under the number UH 800 as a white, seed propagated, amphidiploid cultivar.

Luer and Escobar (1989) identified natural hybrids within the genus *Dracula* in a cultivated collection. Ten natural hybrids of horticultural potential were described.

Porter (1989) reported that many strains of the primary hybrid *Paphiopedilum* M. Pearman have been developed and they have produced flowers larger than either parent with very full bloom, white colour and beautiful raspberry stippling.

Takassaki (1989) reported that out of the hybrids obtained by crossing the striped *Phalaenopsis* Kathleen Ali with the spotted *P.* Frisson, some had good spots while others had stripes with spots.

Wallbrunn (1988) concluded that reciprocal hybridization within the hybrids yielded progeny with remarkable variation in flower characters.

Alcorn (1990) experimented with selfing in *Lycaste* Macama Jocelyn and obtained surprising variations in colours and shapes.

McDonald (1991) has pointed out that any hybridization program in orchids aims at increase in terms of flower size, flower number and spike length as well as improvement in flower quality and extended flowering season. Scent may be transmitted to the progeny by the careful selection of pod parents. Vase life is most often attributable to the substance of the flower and tetraploids are usually of thicker substance. Vegetative vigour of hybrids is important as it results in bigger, better blooms and more floriferous hybrids with greater flower substance.

Oakeley (1991) observed that *Lycaste cruenta* was found not to transmit its cinnamon fragrance to its hybrid *L. imshootiana*, although the fragrance was transmitted to its hybrid with *Lycaste* Brugesin, *viz., L.* Hera.

Rogerson (1991) reported that the higher order hybrid, *Paphiopedilum* F.C. Puddle with six species in its parentage plays a predominant role in breeding for white flower colour in the genus. The high fertility of the variety, its tendency to inhibit and suppress colours in hybrids and the relatively recessive nature of its poor shape mainly account for its popularity as a parent.

Rittershausen (1991) has described *Dendrobium phalaenopsis* and its four hybrids. In most cases, the plants have been line bred from *D. phalaenopsis* and its varieties like *Bigibbum, Schroederae* and *Hololeuca* with no other species added.

Behar (1993) succeeded in combining the distinctive inflorescence shape of the miniature species *Lepanthopsis floripecten* with the bright red flower colour

and better flower shape of *Lepenthes cochlearifolia* in the hybrid which flowered *in vitro*.

Wing (1993) summarized the positive and negative attributes of *Vanda* Miss Joaquim and evaluated its role in hybridization. Miss Joaquim was a cross between *Vanda hookeriana* and *V. teres* and was the first *Vanda* hybrid to be registered.

Davidson (1994) conducted intergroup hybridization between phalaenopsis type dendrobiums and those in section spathulata. The objective was to extend the flowering season, to expand the range of flower colours and shapes and to increase flowering in phalaenopsis type dendrobiums. Some outstanding intermediate hybrids with good flowering characteristics were obtained.

Moses (1994) described the progressive development of semi alba *Phalaenopsis* with their full, flat flowers and white sepals and petals contrasting with the deep red lips. Their breeding began in 1986 by crossing the lavender pink lipped *P. equestris* with the solid white *P. aphsodite*.

Nash (1995) presented historical information on the breeding of hybrid *Cattleya*, including the importance of *Brassavola* (*Rhyncolaelia*) *digbyana* as a parental species.

Yam (1994) discussed the progress made in improving flower colour in the genus *Paraphalaenopsis* by considering intra and intergeneric hybridizations.

Chen et al. (1995) conducted an extensive varietal improvement programme in *Phalaenopsis* using 29 wild species and 873 varieties. They succeeded in releasing 35 new hybrid varieties. Studies on protoplast fusion, isoenzyme electrophoresis and DNA finger printing to assist in varietal identification were performed.

Griesbach (1995) described peloric mutations in orchids where by the flower becomes actinomorphic by the replacement of the labellum by a petal . Inheritance of peloria was studied and details of prize winning peloric orchids were enumerated.

The details of selective hybridization that resulted in the development of *Vanda* Motes Gold Flake were published by Motes (1995).

While breeding for yellow colour in *Phalaenopsis*, Norton et al. (1995) observed that only a limited number of hybrids proved fertile enough to continue the breeding programme.

Coleman and Glicenstein (1995) found the pale whitish – green flower colour of a natural primary hybrid to be intermediate between its parents, the pure white *Platanthera dilatata* and green *P. hyperborea.* The shape of lip was also intermediate between the two parents.

Fuchs (1997) reported that *Vanda sanderiana* and *V. coerulea* were two important *Vanda* species found in the background of most of the vandaceous hybrids. *V. sanderiana* gives full form, whereas *V. coerulea* imparts the rich blue-violet colouration, lovely tessellation as well as the long inflorescence.

According to Mercy and Dale (1997), majority of the commercially grown orchids today are hybrids derived from *Arachnis, Vanda, Renanthera, Ascocentrum, Cymbidium, Cattleya, Dendrobium, Oncidium, Phalaenopsis* and *Paphiopedilum.* They also observed that when species of extremely different flower sizes were crossed, the hybrid did not reach the mid point or average of the two parents but exhibited a size closer to their geometric means. When the flower sizes of the two parents were closer to each other, this difference was not apparent.

Tippit (1997) reported that in a new hybrid, certain characteristics such as growth habit, natural spread of the flowers, number of blooms, length of inflorescence etc. were the geometric means of the two parents involved in the cross with genetically dissimilar parents; wide variations in colour and form resulted regardless of the genus.

Islam et al. (1998) analyzed floral pigments in 18 *Calanthe* species and hybrids and pointed out that such data can be successfully used to raise progenies with desirable flower shades using species as parents.

Sharma et al. (1998) evaluated the possibility of sustainable commercial exploitation of orchids and observed that several wild species possess blossom characters good enough to compete with the best hybrids and can be used as breeding material for the production of attractive, novel varieties.

Catling and Brownell (1999) observed that natural hybrids between *Platanthera lacera* and *P. leucophaea* were intermediate between the two parental species for 11 quantitative characters.

Chen et al. (2000), while discussing the breeding behavior of *Phalaenopsis equestris* observed that hybrids with compact, multiple branched inflorescence grew faster compared to those with large flowers. Studies on the influence of parents on fertility and the inheritance of pink and white floral colours have been presented. They further reported that during the first ten years of the breeding programme till 1998, a total of 30 hybrids could be registered with the Royal Horticultural Society. Currently, 12 hybrids are in production for the market.

Knyasev et al. (2000) confirmed the hybrid status of *Cypripedium ventricosum* by statistical analysis of morphological characters, which were intermediate in expression, between the two parents. Allozyme data was fully consistent with hybrid origin.

Motes (2001), while hybridizing with lesser known vandas, commented on the potentiality of *V. denisoniana* to confer full form on its progeny, making it a pre eminent species for breeding successful hybrids.

Lekha Rani (2002) performed a total 190 cross combinations in *Dendrobium* to determine cross compatibility out of which 84 combinations including seven selfs produced harvestable green capsules, the relative success being 44.21 per cent. Progeny from 67 hybrid combinations were established successfully in the greenhouse out of which more than 500 plants belonging to 20 combinations flowered.

Ninitha Nath (2003) conducted compatibility studies in 116 crosses in monopodial orchids. A total of 58 combinations gave harvestable green capsules. Progeny from 24 combinations were successfully deflasked.

Beena Thomas and Lekha Rani (2008) analysed biological traits in 15 monopodial orchid genotypes, belonging to the genera *Aranda, Aranthera, Kagawara, Mokara, Renanthera* and *Vanda,* as a preliminary step for developing novel hybrids of commercial value.

2.12. Molecular Characterization

Molecular characterization has become most important for establishing the identity of improved hybrids, prior to release.

2.12.1. Molecular Markers

Molecular markers have revolutionized the various biological fields such as plant breeding, genetic engineering, embryology, taxonomy and medical science. DNA markers are molecular markers that reveal polymorphism at the DNA level. They are widely used in genome analysis. They provide an

opportunity to characterize genotypes and measure genetic relationships more precisely than other markers (Soller and Beckmann, 1983).

Various types of molecular markers are utilized to evaluate DNA polymorphism. Polymerase chain reaction (PCR) based markers are the most important among them. These led to the development of marker based gene tags, marker assisted selection of desirable genotypes, phylogenetic analysis, variability studies, synteny mapping etc. The time span for the development of novel improved varieties can be reduced by these techniques.

2.12.2.Polymerase Chain Reaction based DNA Markers

In orchids, molecular characterization is possible using a wide range of characters and molecular markers. Random Amplified Polymorphic DNA (RAPD), AFLP, minisatellite and microsatellite are the important PCR based marker techniques used in orchids.

2.12.3.Random Amplified Polymorphic DNA (RAPD)

This method was first developed by Welsh and McClelland (1990) and Williams et al. (1990). The advantage of RAPD when compared to RFLP is that RAPD is less labour intensive, requires smaller quantities of genomic DNA, is less costly and is quicker than RFLP (William et al., 1990). It can be used to detect even single gene mutations RAPD was reported to be used for population studies by Astley (1992).

RAPD was used for identification of genome specific markers and other uses by Williams et al. (1990) and Erlich et al. (1991).

RAPD analysis in particular has proven to be a rapid and efficient means for genome mapping (Williams et al., 1990). RAPD is well suited for genetic resource characterization (Anderson and Fairbanks, 1990).

DNA marker polymorphism analyses are advantageous for genetic resource characterization, since DNA markers represent only genetic variation and are not subject to environmental influence (Anderson and Fairbanks, 1990).

Several authors have applied the RAPD technique to investigate genetic variability and found the technique very efficient and reliable (Brown et al., 1993).

RAPD technique has been used for the identification of hybrids and determination of their parents. Wang et al. (2003) proposed RAPD fingerprinting as a convenient tool for the identification, protection and parentage determination of plant hybrids.

The DNA markers are extremely useful for testing the clonal fidelity as well as identification (Paden et al., 1996). According to Ben-Meir et al., (1997) advances in the use of molecular genetic markers have enabled research on genetic variation at the DNA level.

RAPD markers are commonly used for molecular characterization studies despite discrepancies in reliability (Peteira et al., 1999).

Rout et al. (2006) opined that RAPD relies on the repeatable amplification of DNA sequence amplified using arbitrary primers to provide DNA fingerprints.

2.12.4. RAPD in Orchids

RAPD technique was used to identify wild *Phalaenopsis* species and to study their relationship (Fu et al., 1994).

Benner et al. (1995) analysed DNA polymorphism within the genus *Cattleya* using RAPD. They observed a high level of molecular variability among

the eight species, with each of them exhibiting an unique DNA fingerprint with 9 out of 10 arbitrary primers used in single primer RAPD reactions.

The compatability of Korean native *Cymbidium goeringii* with other *Cymbidium* species was studied using RAPD analysis. It was detected that the taxonomic relationship between *Cymbidium goeringii* and either *Cymbidium aloifolium* was distant, with no compatibilily and even more distant in the case of *Dendrobium* or *Phalaenopsis* which have different chromosome numbers from the *Cymbidium* species (Choi et al.,1998).

Gurunanger et al. (1998) characterized the genetic material of seven Italian population of the *Ophrys bertolonii* using RAPD markers. They reported a high genetic variability occurring within the same populations.

Okeyo and Kako (1998) reported that RAPD technique was used to study genetic diversity and to identify *Cymbidium* cultivars. A total of 132 RAPD bands, 78 per cent of which were polymorphic were produced from 15 arbitrary primers.

Lim et al. (1999) used RAPD markers to study the genetic closeness of various species of *Vanda*. They reported that five strap-leaved *Vanda* species (including *Vanda sanderiana and Ascocentrum miniatum*) were more closely related to each other than to the terete-leaved *Vanda* species. On the basis of RAPD analysis, terete-leaved *Vanda* species and *Vanda hookeriana* could be grouped in a separate genus, *Papilionanthe,* while *Vanda sanderiana* stood in the genus *Vanda*.

Molecular (RAPD) analysis of some taxa of the *Ophrys bertolonii* aggregate (Orchidaceae) was done by Caporali et al. (2001). The RAPD methodology was utilized for analyzing the genetic material for four allopatric populations of the *Ophrys bertolonii formis* type, as well as of two populations of *Ophrys bertolonii* together with *Ophrys fucflora* as out group. Significant genetic

diversity was observed for six taxa under examination, thus suggesting separation at species (or subspecies) level.

Phenetic relationship and identification of subtribe *Oncidinae* genotype by RAPD markers was carried out by Taai et al. (2002). The study was conducted to generate random amplified polymorphic DNA markers for 24 accessions of subtribe *Oncidinae* (one of the diverse groups in the orchid family) and to determine the phenetic relationship among them. Eighty decamer primers were screened and 14 primers producing clear and reproducible DNA patterns were selected. In total, 263 bands were scored in which 251 revealed polymorphism. Cluster analysis based on molecular data of band distribution in all the samples showed six major clusters and one independent cluster.

Fujii et al. (2001) studied the effects of explants and plant hormones on putative variability in cloned *Cymbidium* plantlets using random amplified polymorphic DNA (RAPD) analysis. A total of 113 distinct major RAPD bands were consistently generated from 18 primers. The appearance of polymorphic bands in cloned plantlets clearly demonstrates somaclonal variation. This also suggests that the appearance of putative variability may relate to the type of explants used and the plant hormone added for plant tissue culture.

Screening for genetic markers in a *Dendrobium* hybrid, White Angel x (Luciwi Pink x Black Spider), a potential cutflower variety was done by Hong et al. (2003). Results of the analysis yielded more than 24 markers that could be linked to such phenotypes as overall yield, inflorescence length, vase life, days to flowering and flower colour.

Pillai (2003) reported molecular characterization of fifteen *Dendrobium* varieties using RAPD technique. The primers OPA-19, OPB-02, OPB-04 and OPB-10 yielded good resolution bands out of 40 decamer primers tested. These primers amplify 44 RAPD markers of which 39 were polymorphic and five were monomorphic. The 15 varieties were divided into six clusters in the dendrogram.

RAPD analysis was used to characterize genetic variability and relationships among 12 cultivars of *Dendrobium* at molecular level. The primers OPB-11, OPB-12 and OPB-17 were identified for RAPD analysis based on their performance in DNA amplification, reproducibility and production of highest number of polymorphic bands as well as intense bands. The three primers together produced 27 bands. Among these, 24 were polymorphic (Krishnapriya, 2005).

Chakrabarti (2005) used RAPD technique to study the genetic variability of three morphologically distinct groups of orchids to include them in subfamily Epidendroideae and tribe Dendrobeae and Cymbideae. A total of 227 distinct major RAPD bands, of which 97 per cent were polymorphic, were generated from 15 arbitrary primers. The molecular analysis grouped all the species into five groups. The polymorphic pattern generated by RAPD profiles showed different degrees of genetic relationship among the species studied and the RAPD markers were found to be an useful tool for detecting genetic variation within the species of three important genera of orchids.

RAPD markers were utilized for molecular authentication of eight wild population of *Dendrobium officinale.* A total of 104 amplified bands were generated, 95 were polymorphic, corresponding to 91.35 per cent genetic polymorphism. Primer S 412 was used to authenticate eight wild population completely (Ding et al., 2005).

The genetic diversity of an epiphytic orchid, *Dendrobium moniliforme* was investigated using RAPD. UPGMA dendrogram based on RAPD data showed two principal clusters differing each other with genetic variability (Kobayashi and Iwami, 2005).

Rahana (2007) reported that the cluster based on RAPD analysis using eight primers clearly demonstrates the existence of genetic variation within the 40 selected *Dendrobium* hybrids. Polymorphism obtained in the study will be useful in fingerprinting of these selected *Dendrobium* hybrids

Rahana et al. (2007) observed a total of 69 scorable bands (average of 8.63 bands per primer) generated by the selected eight primers of which three were monomorphic and the remaining 66 were polymorphic (95.65 per cent) in selected *Dendrobium* hybrids. The number of amplification products ranged from four to twelve with an average of two per primer.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present research programme was undertaken in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani, during 2005-2008. The parent materials for investigations were maintained in the green house and observations on vegetative and floral characters were taken. Hybridization and compatibility studies were also conducted here. Embryo culture and molecular work were done in Plant Biotechnology and Molecular Biology Laboratory of the college. For hardening of hybrid seedlings humidity chamber fabricated within the green house was utilized.

The major studies conducted are given below.

1. Evaluation of parent material
2. Hybridization and compatibility studies
3. Immature embryo (green pod) culture of hybrid seeds
4. Hardening techniques of hybrid seedlings
5. Evaluation of hybrid material
6. Molecular characterization

3. 1. EVALUATION OF PARENT MATERIAL

3.1.1. Experimental Material

The experimental material comprised of fifteen monopodial orchid genotypes belonging to six genera, *viz.*, *Aranda, Aranthera, Kagawara, Mokara, Renanthera* and *Vanda*, with good cut flower qualities and high demand in the market. *Vanda* and *Renanthera* are monogenerics whereas *Aranda* and *Aranthera* are bigenerics. *Kagawara* and *Mokara* which are gaining more popularity recently in the national and international markets are multigeneric grexes. The single flower and inflorescence of these parents, which are listed here, are shown in Plate 1 and 2 respectively.

P_1 *Aranda* Salaya Red

P_2 *Aranthera* Annie Black

P_3 *Aranthera* James Storei

P_4 *Kagawara* Christielow

P_5 *Mokara* Calypso

P_6 *Mokara* Chak Kuan Pink

P_7 *Mokara* Lumsum Sunlight

P_8 *Mokara* Singapore Red

P_9 *Mokara* Thailand Sunspot

P_{10} *Mokara* Walter Oumae White

P_{11} *Renanthera coccinia*

P_{12} *Vanda* John Clubb

P_{13} *Vanda* Popoe Diana

P_{14} *Vanda* Ruby Prince

P_{15} *Vanda spathulata*

The sources of parental genotypes used in the experiment are given in Table 1.

The terminal cuttings of these monopodial orchid parents, with three to five velamen roots, were used as planting material. These were planted in linear trenches prepared on ground using coconut husk, brick pieces and wood shavings in December, 2005. Proper management practices were undertaken as per the Package of Practices Recommendations of Kerala Agricultural University (KAU, 2002).

Design	:	Completely Randomized Design (CRD)
Treatments	:	15
Replications	:	10
(Single plant per treatment)		

Table 1. Sources of parental monopodial orchid genotypes

Parental orchid genotype	Source of planting material
1. *Aranda* Salaya Red 2. *Kagawara* Christielow 3. *Mokara* Calypso 4. *Mokara* Chak Kuan Pink 5. *Mokara* Lumsum Sunlight 6. *Mokara* Singapore Red 7. *Mokara* Thailand Sunspot 8. *Mokara* Walter Oumae White	Asiatic Blooms, Thiruvananthapuram
9. *Aranthera* Annie Black 10. *Aranthera* James Storei 11. *Renanthera coccinia* 12. *Vanda* John Clubb 13. *Vanda* Popoe Diana 14. *Vanda* Ruby Prince 15. *Vanda spathulata*	Department of Plant Breeding and Genetics, College of Agriculture, Vellayani

3.1.2. Experimental Methods

The selected materials were evaluated by recording observations on their vegetative and floral characters (both quantitative and qualitative) six months after planting.

Vegetative Characters

1. Length of Shoot (cm)

The height of each mature shoot was measured from its base to the tip.

2. Number of Leaves per Shoot

Total number of laminate leaves per shoot was recorded at maximum leaf stand.

3. Length of Aerial Roots (cm)

The length of aerial root was measured from the base of the root to its tip.

4. Thickness of Shoot (cm)

Thickness of shoot was measured as the diameter at the widest point using vernier calipers.

5. Length of Internode (cm)

The length of internode i.e., distance between two consecutive leaves was measured for the seventh, eighth and ninth internodes.

6. Length of Leaf (cm)

The length of mature leaves was measured from the base to the tip from five leaves per plant and average was calculated.

7. Width of Leaf (cm)

The width of mature leaves was measured at the widest region from five leaves per plant and average was taken.

8. Thickness of Leaf (cm)

The thickness of leaf was measured at the widest region on the leaf at a point equidistant from the margin and the midrib using vernier calipers.

9. Leaf Area (cm^2)

The leaf area was measured graphically from five leaves per plant and average was taken.

Quantitative Floral Characters

1. Days to First Flower Opening from Inflorescence Emergence

Number of days was counted from visible emergence of inflorescence to the opening of first flower and recorded.

2. Days of Last Flower Opening from First Flower Opening

Number of days from opening of first flower to the last flower opening in the inflorescence was counted and recorded.

3. Number of Spikes per Shoot

Total number of spikes produced per cane during an year was recorded.

4. Length of Inflorescence (cm)

The length of inflorescence was measured from the base of a fully opened inflorescence to the tip of the axis.

5. Length of Scape (cm)

The distance from the base of a fully opened inflorescence to the first flower was measured and recorded.

6. Diameter of Inflorescence Axis (cm)

Diameter of inflorescence axis was measured at the widest region using vernier calipers.

7. Number of Flowers per Inflorescence

Total number of flowers produced per inflorescence was counted and recorded.

8. Length of Internode of Inflorescence (cm)

Length of internode i.e., distance between two consecutive flowers was measured for the entire inflorescence and the mean was computed.

9. Length of Flower (cm)

Flower length was measured at the widest region from the tip of the labellum to the tip of the odd sepal and recorded.

10. Width of Flower (cm)

Flower width was measured at the widest region and was recorded.

11. Fullness Value

Fullness value of flower was calculated using the formula developed by Leonhardt (1977).

$$F = \frac{6\,W}{2S+2P+L+DS}$$

F	=	Fullness value
W	=	Width o f flower
S	=	Width of lateral sepal
P	=	Width of lateral petal
L	=	Width of labellum
DS	=	Width of dorsal sepal

12. Vase Life (days)

Vase life of the cut inflorescence in water as the holding solution was noted. Fading of the first flower was recorded as the end of vase life.

Floral Biology

Floral biology studies were carried out in detail in all the fifteen parental genotypes. The following observations were made:

1. Flower opening time

Mature flower buds were tagged individually at full-bud stage when the buds attained maximum size. The buds were observed at hourly intervals till initiation of flower opening.

2. Anthesis

The pollen in orchids are aggregated into sticky masses called 'pollinia' which are held within the anther cavity and covered over by an anther cap. So anthesis time cannot be defined by the emergence of anther and shedding of pollen. Hence the time at which pollen achieved maturity enough to effect successful fertilization was taken as the correct time of anthesis.

To judge the time of maturity of pollen and time of anthesis, pollinia were extracted in the morning (8-10 am) and immediately used for hand-pollinating selected flowers. This was continued for five consecutive days from the first day of flower opening. Initiation of capsule development following pollination was considered as indicative of pollen maturity and the day on which pollen extraction was done was taken as the day of anthesis.

3. Stigma receptivity

The flowers were hand pollinated for ten consecutive days starting from the day of anthesis to find out the period of stigma receptivity. Pollination was done three times during the day, *viz.*, morning, noon and evening. Initiation of fruit development following pollination denoted stigma receptivity.

4. Pollen Characteristics

1. Pollen Size (μ)

Pollinia were collected from the fully opened orchid flowers, and pollen grains were stained in 1: 1 glycerine-acetocarmine solution (2 %). The diameter of atleast 100 normal-shaped and well-stained pollen grains was measured at random using a standard occular micrometer after calibrating occular division under the high power (10 x 40x) of a microscope. Then frequency was found. The mean, standard deviation and standard error were calculated from frequency table. Average diameter was computed and was recorded in microns.

2. Pollen Fertility (%)

Acetocarmine staining technique was employed for studying pollen fertility. It was estimated by counting fertile and sterile pollen grains separately in the microscope field from a smear under the low power (10 x 10x) of a microscope. Pollen grains which were well-stained, normal-shaped and plumpy were considered as fertile. Unstained, small or shrivelled pollen grains were considered as sterile (Zirkle, 1937). Three slides were prepared and five random fields from each slide were observed in each parent genotype. Fertility of pollen grains was expressed as percentage of the total number observed which was not less than 300 tetrads per treatment.

3. Pollen Germination (%)

Pollen germination was tested on a medium containing sucrose (2 %) + agar (1 %) + 75 ppm boric acid (Varghese,1995) for a better assessment of pollen fertility. Pollinia were collected on the third day after flower opening. A drop of germination medium was placed on a cover glass. Fresh pollengrains from the pollinia were introduced into the medium. The medium was then allowed to rest as a hanging drop by inverting the coverslip on a cavity slide. Prepared slides were kept at room temperature for incubation in a dessicator containing water.

After 24 hours of incubation, germination counts were taken under the low power (10 x 10x) of a microscope. The observations were made in five different microscope fields on not less than 100 tetrads per treatment and the mean percentage of germination was worked out.

Qualitative Floral Characters

1. Flowering Nature – Free Flowering/ Seasonal

Flowering time was observed and recorded as free flowering i.e., flowering all round the year or seasonal i.e., flowering at specific seasons.

2. Nature of Inflorescence Axis

Nature of inflorescence axis was observed and recorded as

a) erect — inflorescence axis held erect (0-30°C)

b) arching — inflorescence axis held at an angle of 30-60°C

3. Mode of Display of Flowers

Mode of display of flowers was recorded, based on orientation of individual flowers, as given below.

a) Flowers arranged alternately on either sides of the inflorescence axis facing the opposite side so that flowers appear in two parallel rows.

b) Flowers arranged all around the peduncle so that inflorescence shows a bunched appearance.

4. Shape of Flower

Shape of flower was observed and recorded as given below.

a) Full, flat, broad sepals and petals

b) Flat, narrow, spatulate sepals and petals

3.1.1.3. Statistical Analysis

The collected data were subjected to the analysis of variance to test for significant difference among the fifteen monopodial orchid genotypes selected, following Panse and Sukhatme (1967). Genetic parameters *viz.*, variability, heritability and genetic advance, correlation and selection index were also estimated.

3.2. HYBRIDIZATION AND COMPATIBILITY STUDIES

All possible self and cross combinations including reciprocals ($n^2 = 15^2 = 225$) were done to conduct compatibility analysis. Self compatibility was assessed in the genotypes by using the pollen of the same plant. Following the conventional practice in orchid hybridization, the first and last flowers of each inflorescence were not used for crossing. As the pollinia are held within the clinandrium and covered by operculum which prevents self pollination effectively, emasculation of flowers was not attempted. The parent plants used for crossing were protected by insect-proof netting, to prevent pollination through insect pollinators. After pollination, flowers were tagged properly for identification.

3.2.1. Observations

1. Post Pollination Floral Changes (days)

Time required for the following post pollination floral changes was noted and recorded.

i. Drooping of perianth
ii. Closure of stigma by overgrowth of column tip
iii. Covering of stigma by wilted sepals and petals
iv. Complete drying of sepals and petals

2. Stages of Capsule Development (days)

The various stages of capsule development till harvest were sequenced as listed below and recorded.

i. Greening of ovary with slight swelling
ii. Swelling of ovary into capsule
iii. Prominent ribbing of capsule
iv. Slight flattening of capsule rib
v. Bursting of capsule beginning from tip

3. Days to Green Capsule Harvest in Successful Crosses

Number of days from pollination to green capsule harvest in compatible crosses was observed and recorded.

4. Length of Capsule (cm)

Length of capsule from base to tip was measured and recorded.

5. Width of Capsule (cm)

Width of capsule at the widest region was noted and recorded.

6. Percentage of Capsule Set

Number of green capsules harvested to total number of pollinations made was recorded and the percentage was computed.

7. Percentage of Capsules with Germinating Seeds

Number of capsules with seeds that germinated on inoculation to the total number of capsules harvested was computed.

3.3. IN VITRO CULTURE OF HYBRID SEEDS

The capsules from all successful parental combination harvested at the green capsule stage formed the experimental material.

3.3.1. Stage of Green Capsule Harvest

From the successful combinations green capsules were harvested at 70-90 per cent maturity. The best time to harvest a pod is when the tip of capsule starts to turn yellow.

3.3.2. Preparation and Cleaning of Capsule

Preparation of harvested green capsules were carried out by removing extra length of pedicel and adhering wilted perianth parts. Initially, they were washed in running tap water. For proper cleaning of intact capsules soaking in one percent solution of laboline detergent in distilled water was practised for 20 minutes. Then the capsules were rinsed thoroughly three to four times with distilled water.

3.3.3. Surface Sterilization, Inoculation and Incubation

As the immature seeds cannot be stored, all seeds must be sown immediately after harvest. The pod was surface sterilized inside a laminar air flow chamber, first by immersing in 0.1 % mercuric chloride solution for 10 minutes. Then it was again immersed in 70 per cent ethyl alcohol for three to five

minutes and then was 'flamed' by passing through the flame of a spirit lamp. (If the pods are too much mature, it will split open at this time). Then it was cut open under sterile conditions. The immature embryos were scrapped out and sown in sterile flasks containing the culture medium.

The first sown flasks were the master flasks. The cultures were incubated in a culture room for further development. Controlled conditions of light, temperature and humidity were provided.

Culture racks were covered with black muslin cloth to provide darkness initially, after inoculation. Once germination was initiated, a 12 – hour photoperiod with a light intensity of 3000 lux under fluorescent tube lights was provided. A uniform temperature of 26 $\pm$ 2 °C and a relative humidity of 95 per cent was maintained in the culture room.

3.3.4. Sub-culture

Sub-culturing was done periodically to prevent overcrowding of seedlings in the flasks. All sub-culture operations were carried out in the laminar airflow chamber.

During first sub-culture, approximately equal numbers of developing protocorms from each master flask were transferred into three to 12 new flasks. The developing seedlings were then taken through two to three further subculture passages, as and when needed.

3.3.5. Selection of Culture Media

Effect of various culture media on the *in vitro* growth of monopodial orchid seedlings were studied. The hybrid combination used for the study was *Mokara* Walter Oumae White x *Aranthera* Annie Black. The green capsules

were harvested at 75 % maturity and proper sterilization procedures were carried out as explained above.

The seeds were inoculated in five different media compositions, *viz.*, KC (Knudson, 1946) full strength, MS (Murashige and Skoog, 1962) quarter strength (25 % concentration of inorganic salts), half strength (50 % concentration of inorganic salts), full strength and VW (Vacin and Went, 1949) full strength and incubated. The basic chemical composition of these media is given in Appendix I. Coconut water 200ml l^{-1} was used as organic additive in all the cases. Activated charcoal was also added at a concentration of 1g l^{-1}, to all the media studied.

Observations on height of seedling, number of leaves, length of the longest leaf, number of roots and length of the longest root were recorded after six months of inoculation and the data were statistically analysed.

3.3.6. Refinement of culture media

To improve the *in vitro* growth of hybrid seedlings in monopodial orchids, different combinations of growth hormones were added to the identified best culture medium and their effect was studied. The basal medium selected was MS half strength. The various treatments included in this research work were as listed below.

T_1 – Control (no growth regulators)
T_2 – 8 mg l^{-1} BA + 2 mg l^{-1} IAA
T_3 – 8 mg l^{-1} BA + 2 mg l^{-1} NAA
T_4 – 2 mg l^{-1} IAA + 8 mg l^{-1} NAA
T_5 – 8 mg l^{-1} IAA + 2 mg l^{-1} NAA
T_6 – 10 mg l^{-1} IAA
T_7 – 10 mg l^{-1} NAA

The study was conducted using the hybrid combination *Mokara* Walter Oumae White x *Aranthera* Annie Black. At correct stage of maturity (75 %), the green capsules were harvested for embryo culture. After proper sterilization techniques, hybrid seeds were inoculated in these various treatments and incubated properly as described above. Six months after inoculation, observations on height of seedling, number of leaves, length of the longest leaf, number of roots and length of the longest root were recorded and the data were subjected to statistical analysis.

3.3.7. Observations on Seed Germination and Seedling Growth of Successful Hybrid Combinations

The following observations were recorded for seed germination and seedling growth at appropriate stages from seed inoculation in MS half strength basal medium to deflasking.

1. Days for Initiation of Germination

Number of days from inoculation to swelling and glistening of embryos *in vitro*, prior to protcocorm formation was recorded.

2. Days for Protocorm Development

Number of days from inoculation to protocorm development was noted.

3. Days for Greening of Protocorms

Number of days from inoculation to pigment synthesis in germinating embryos/protocorms was recorded.

4. Days for First Leaf Initiation

Number of days from inoculation to the visible emergence of leaf was recorded.

5. Days for First Shoot Initiation

Number of days from inoculation to the visible differentiation of shoot was recorded.

6. Days for First Root Initiation

Number of days from inoculation to development of first root was recorded.

7. Days for Deflasking

Number of days from inoculation to deflasking was recorded.

3.4. *EX VITRO* ESTABLISHMENT OF HYBRID SEEDLINGS

3.4.1. Deflasking

The hybrid seedlings from successful combinations were deflasked for planting out and hardening. Deflasking was done when the *in vitro* raised hybrid seedlings attained sufficient growth for transplanting. Flasks containing seedlings with atleast two to three leaves and roots were selected. The selected culture flasks were transferred and kept in the preparation room under natural light, temperature and humidity conditions for one week prior to deflasking, in order to reduce the transplanting shock.

For deflasking, each flask was opened and half filled with tap water. The rooted hybrid seedlings were loosened by gently shaking the flask. The contents were then poured into a clean glass vessel and the seedlings were separated out, washed clean in tap water without damaging the roots.

The deflasked seedlings were soaked in tap water for half an hour to remove all the adhering portions of medium. Seedlings were then treated with the fungicide solution Indofil M-45 (2 %) for twenty minutes prior to planting out onto unsterilized potting medium. Observations on seedling morphology were recoded before planting out.

3.4.2. Observations on Seedling Morphology at Deflasking

The hybrid seedling of the different parental combinations were deflasked at 2-3 leaves and 2-4 roots stage. Growth measurements were made at deflasking.

1. Height of Seedling (cm)

Height of seedling was measured using graph paper.

2. Number of Leaves

Number of leaves produced per seedling was counted.

3. Length of the Longest Leaf (cm)

Length of the longest leaf from base to tip was measured using graph paper.

4. Breadth of the Longest Leaf (cm)

Breadth of the longest leaf was measured at the widest region using graph paper.

5. Number of Roots

Number of roots per seedling was counted.

6. Length of the Longest Root (cm)

Length of the longest root was recorded with the aid of graph paper.

7. Thickness of the Longest Root (cm)

Thickness of the longest root was recorded using graph paper.

3.4.3. Planting out

The deflasked seedlings were transplanted in plastic cups with holes to ensure adequate drainage. Potting media used was a mixture of broken tiles + charcoal + coconut husk (each item as 2 x 2 x 2 cm pieces) in 2 : 2 : 1 ratio.

3.4.4. Acclimatization

Seedlings planted out were kept in a humidity controlled chamber for acclimatization. This was done by fabricating an improvised, tunnel shaped humidity chamber (6 x 1.5 x 1.5 m) made of curved iron frame tightly covered with clear polythene sheet. The chamber floor had a single layer of loose bricks arranged in a zig-zag manner and covered over by a 5 cm thick layer of loose sand.

The temperature inside the chamber was within the range of 28 to 35 °C. When the sand was thoroughly moistened and the chamber kept covered with the polythene sheet, a relative humidity ranging from 85 to 95 % could be maintained. The hybrid seedlings were kept in the chamber and irrigation was provided on every day using a hand sprayer with fine mist nozzle. To maintain relative humidity, the sand flooring was moistened as and when required. One month period in humidity chamber was needed for acclimatization.

3.4.5. Hardening

Seedlings after acclimatization were transferred to the net house (30 x 12 x 4 m) in 50 % shade (under plastic black agro shade net) and placed on orchid stands of 75 cm height, for hardening. After four months of deflasking, these seedlings were repotted in small clay pots with holes using the same initial potting media i.e., mixture of broken tiles + charcoal + coconut husk (each item as 2 x 2 x 2 cm pieces) in 2 : 2 : 1 ratio.

Timely management practices were followed as per the Package of Practices Recommendations of Kerala Agricultural University (KAU, 2007). The hardened monopodial orchid hybrid seedlings were maintained in the net house for further growth.

3.4.6. Observations on Seedling Morphology after Four Months of Planting Out

After four months of deflasking, when the acclimatized hybrid seedlings were repotted, measurements on seedling growth were taken. The observations on height of seedling, number of leaves, length of the longest leaf, breadth of the longest leaf, number of roots, length of the longest root and thickness of the longest root were recorded as done at deflasking.

3.5. MOLECULAR CHARACTERIZATION

3.5.1. Materials

In the present study, Random Amplified Polymorphic DNA markers were used for the molecular characterization of twenty monopodial orchid hybrids, belonging to four grex combinations, developed under the DBT project, entitled "Breeding for Commercial Orchid Hybrids" and maintained in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani. These combinations are *Aranthera* James Storei x *Arachnis* Red Ribbon (H-1 to H-6), *Arachnis* Maggie Oei Red Ribbon x *Aranthera* Beatrice Ng (H-7 to H-11), *Aranthera* James Storei x *Arachnis flos-aeris* (H-12 to H-16) and *Aranthera* Maggie Oei Red Ribbon (selfed) (H-17 to H-20).

3.5.2. Methods

3.5.2.1. Isolation of Genomic DNA

The equipments and glasswares used in the procedure including mortar and pestle, conical flasks, reagent bottles, eppendorf tubes, spatula, tips of micropipettes and glass rods were washed with Labolene solution, rinsed with distilled water and autoclaved.

Leaf samples were collected from young new leaves of selected monopodial orchid hybrids for the isolation of genomic DNA. After removing the tip and midrib, the leaves were chopped into small pieces. 0.5 g of sample was first washed in running tap water, then in distilled water and dried using tissue paper. The sample was homogenated with 1 ml C-TAB buffer and pulverized by rapid grinding with a pinch of PVP (Polyvinyl pyrrolidone) in a pre-cooled mortar . The leaf material was transferred to a 2 ml eppendorf tube and incubated in water bath at 65 °C for 45 minutes with occasional gentle shaking. It was cooled to room temperature. To this, same volume of chloroform : isoamyl alcohol (24:1) was added and shaken well for 10 minutes.

The mixture was then subjected to centrifugation at 10,000 rpm for 5 minutes. Three layers were formed. The supernatant (upper layer) obtained was collected (400 µl) into a 2 ml eppendorf tube and the remaining matter was discarded. 3µl of RNase was added to the supernatant and kept at room temperature for half an hour. Then 400 µl of chloroform was added to the eppendorf tube, shaken well for five minutes and centrifuged at 10,000 rpm for 5 minutes. Then 200 µl of the supernatant was collected and to this equal volume of chloroform was added.

The mixture was shaken for 5 minutes and again centrifuged at 10,000 rpm for 5 minutes. From this also 200 µl of supernatant was collected and equal volume of ice-cold pure ethanol was added. The mixture was refrigerated for five minutes. Centrifugation was repeated at 4,000 rpm for five minutes for pelleting the DNA . The pellet obtained was washed in 200 µl of 80 per cent ethanol. The pellet was air dried and then dissolved in 60 µl of sterile distilled water and stored at -20°C.

3.5.2.2. Quantification of DNA

Before the amplification of DNA by PCR , its quantification is necessary. DNA quantification was undertaken with the help of UV-visible spectrophotometer (Spectronic Genesys 5). Distilled water was taken in a cuvette to calibrate the Spectrophotometer at 260 and 280 nm wavelength. The optical density (O.D.) of the samples dissolved in the buffer was recorded at both 260 and 280 nm. The quantity of DNA in the sample was estimated by employing the following formula:

Amount of DNA ($\mu g\ \mu l^{-1}$) = (A_{260} x 50 x dilution factor) / 1000, where, A_{260} is the absorbance at 260 nm .

The quantity of DNA could be judged from the ratio of the O.D. values recorded at 260 and 280 nm. The A_{260} / A_{280} ratio between 1.8 and 2.0 indicates good quality of DNA, where A_{280} is the absorbance at 280 nm.

3.5.2.3. Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out, to analyse the bands, in a horizontal gel electrophoresis unit (GENEI, Bangalore). The required amount of agarose was weighed out (0.8 per cent for visualizing the genomic DNA and 1.4 per cent for visualizing the amplified products) and melted in 1 x TAE buffer (0.04 mM Tris acetate, 0.001 mM EDTA, pH 8) by boiling. At about 50 °C, ethidium bromide was added to the buffer (6 µl 50 ml^{-1}). Then, it was poured to a preset template with appropriate comb. The comb and the sealing tapes were removed, after solidification.

The gel was mounted in an electrophoresis tank filled with 1 x TAE buffer. The gel was immersed in the buffer completely. The DNA sample was mixed with the required volume of gel loading buffer (30 per cent glycerol and 0.25 per cent Bromophenol blue dye). Each well was loaded with 8 µl for genomic DNA and 12 µl for amplified products of sample. One of the wells were loaded with 5.0 µl of molecular weight marker along with 1.5 µl of gel loading buffer. Electrophoresis was performed at 45 volts until the loading dye reached $^{3/4th}$ of the length of the gel. Using the gel documentation system (Bio-Rad, USA) the gel was visualized and documented.

3.5.2.4. Random Amplified Polymorphic DNA (RAPD) Analysis

Seventy random decamer primers (Operon Technologies, Inc., USA) were screened on template DNA from the hybrids so as to identify those giving good and scorable amplification products. Each reaction mixture (25 µl) for PCR amplification consisted of 2.5 µl 10 x PCR buffer, 2.5 µM $MgCl_2$, 0.5 µl each of dNTPs, 0.2 units of Taq polymerase (GENEI, Bangalore), 1 µl of primer (Operon

Technologies, USA) and 35 ng genomic DNA template. Amplification was performed in a Programmable Thermal Controller (PTC 100, MJ Research Inc.).

Amplification reaction

Initial denaturation at 95 °C for one minute was followed by 45 cycles of denaturation at 95 °C for one minute, primer annealing at 35 °C for two minutes and extension at 72 °C for 10 minutes. After the reaction, the amplification products were cooled at 4 °C. In each reaction set, a negative control containing sterile water, instead of template, was included.

The PCR product was size fractioned on a 1.4 per cent agarose gel prepared in I x TAE buffer.Then, it was stained with ethidium bromide. The bands were visualized and documented using gel documentation system. The RAPD bands were represented as '1'for presence and '0' for absence and recorded. Finally, eight primers were selected for further analysis.

*3.5.2.5. **Data Analysis***

The bands obtained from RAPD analysis for each primer were scored by visual observation. Reproducible bands were scored for their presence (1) or absence (0) for the 20 selected monopodial orchid hybrids. Jaccard's Similarity coefficient values were calculated, from this RAPD marker data, for each pair-wise comparison between hybrids and a similarity coefficient matrix was constructed (Jaccard, 1908).

$Sij = a / (a+b+c)$

Where,

Sij = similarity coefficient between i^{th} and j^{th} pair

a = number of bands present in both the genotypes in a pair

b = number of bands present in the first genotype, but not in the other

c = number of bands present in the second genotype, but not in the other

The similarity matrix was subjected to cluster analysis using Unweighted Pair Group Method for Arithmetic Averages Analysis (UPGMA).

A dendrogram was constructed, based on the similarity coefficient, with the help of the software package 'NTSYS' (Version 2.02). From the dendrogram, association between the genotypes was found out.

RESULTS

4. RESULTS

The results of the investigations on 'Intra and inter generic hybridization and molecular characterization in monopodial orchids' carried out are presented below:

4.1. Evaluation of parent material

4.1.1. Comparison of performance of parents

4.1.2. Estimation of genetic parameters of parents

4.1.3. Floral biology of parents

4.2. Hybridization and compatibility / incompatibility studies

4.3. *In vitro* culture of hybrid seeds

4.4. *Ex vitro* establishment of hybrid seedlings

4.5. Molecular characterization

4.1. EVALUATION OF PARENT MATERIAL

Fifteen monopodial orchid genotypes belonging to six genera *Aranda, Aranthera, Kagawara, Mokara, Renanthera* and *Vanda* were evaluated in the greenhouse, each being replicated five times.

4.1.1. Comparison of Performance of Parents Based on Quantitative Characters

The mean performance of the parental genotypes for vegetative and quantitative floral characters are presented (Tables 2 and 3; Fig. 1). A wide range of variation was noticed for all the characters among the parental genotypes.

4.1.1.1. Vegetative Characters

1. Length of Shoot

Length of shoot was seen significantly high for P_3 (83.4 cm) followed by P_2 (75.54 cm) and P_{13} (74.98 cm). The mean shoot length was recorded the

Table 2. Mean performance of 15 monopodial orchid parental genotypes for vegetative characters

Parents	Length of shoot (cm)	Number of leaves / shoot	Number of aerial roots	Length of aerial roots (cm)	Thickness of shoot (cm)	Length of internode (cm)	Length of leaf (cm)	Width of leaf (cm)	Thickness of leaf (cm)	Leaf area (cm^2)
P_1	47.08	30.0	4.8	21.42	1.424	2.360	16.30	1.84	0.212	44.28
P_2	75.54	25.6	3.4	35.78	0.926	2.668	10.00	2.32	0.158	27.34
P_3	83.40	31.0	3.6	31.98	1.252	2.476	12.08	3.10	0.118	32.06
P_4	64.02	30.4	4.4	23.70	1.408	2.512	21.10	2.16	0.148	43.24
P_5	47.64	21.4	4.6	39.02	1.248	2.856	15.68	2.20	0.134	33.96
P_6	57.76	27.8	6.4	27.46	1.618	2.132	16.60	3.36	0.184	50.72
P_7	55.76	18.8	4.6	26.06	1.266	2.376	21.00	2.06	0.108	58.82
P_8	67.98	33.6	7.2	13.66	1.312	2.868	17.00	2.98	0.210	46.34
P_9	63.10	29.2	5.0	36.20	1.572	2.564	17.66	3.34	0.176	47.76
P_{10}	57.04	18.6	5.2	38.98	1.340	2.284	17.48	2.60	0.116	43.80
P_{11}	61.98	25.8	14.8	28.96	0.790	3.056	6.14	1.80	0.090	16.80
P_{12}	63.70	20.4	7.0	34.78	1.126	3.460	16.12	0.68	0.486	11.60
P_{13}	74.98	22.4	9.2	14.44	0.902	3.016	7.78	0.36	0.356	2.96
P_{14}	65.38	20.0	14.6	31.90	1.112	3.764	12.14	0.54	0.512	5.12
P_{15}	46.92	25.2	4.4	25.32	1.002	1.788	8.34	2.20	0.120	16.14
CD(0.05)	1.914	1.907	1.078	3.986	0.049	0.073	0.558	0.150	0.011	1.257
SE_m	0.677	0.674	0.381	1.409	0.014	0.024	0.197	0.053	0.000	0.444

Table 3. Mean performance of 15 monopodial orchid parental genotypes for quantitative floral characters

Parents	Days to first flower opening from inflorescence emergence	Days to last flower opening from first flower opening	Number of spikes per shoot	Length of inflorescence (cm)	Length of scape (cm)	Thickness of inflorescence axis (cm)	Number of flowers per inflorescence	Length of internode of inflorescence (cm)	Length of flower (cm)	Width of flower (cm)	Fullness value	Vase Life (days)
P_1	33.22	16.84	5.4	32.68	14.72	0.730	17.0	1.060	5.98	5.18	3.03	7.8
P_2	37.90	12.94	5.8	49.64	23.72	0.566	14.6	1.802	5.92	4.20	5.33	14.2
P_3	36.34	14.24	1.6	42.9	21.54	0.586	17.6	1.224	5.70	4.18	5.11	13.4
P_4	35.16	15.36	4.4	37.42	11.98	0.704	23.0	1.154	4.26	4.46	3.28	7.4
P_5	31.88	14.12	5.2	35.18	22.60	0.694	10.2	1.254	5.30	5.60	3.53	7.0
P_6	30.96	15.50	7.0	40.22	21.36	0.776	9.2	2.080	9.00	7.68	4.24	8.2
P_7	30.72	17.02	4.4	26.66	15.58	0.540	8.8	1.266	6.22	5.48	3.13	7.6
P_8	31.20	14.52	4.8	32.92	15.30	0.572	10.4	1.420	6.88	6.02	3.13	7.2
P_9	32.08	18.44	7.2	36.40	19.60	0.836	14.2	1.196	7.04	6.52	3.23	6.8
P_{10}	35.70	15.72	5.0	21.78	14.20	0.670	9.6	0.798	6.88	6.38	3.88	6.4
P_{11}	27.40	17.20	1.2	38.16	11.78	0.510	24.2	1.078	4.76	2.34	4.01	8.4
P_{12}	28.90	8.52	2.0	26.10	17.20	0.546	5.4	1.662	7.88	8.06	2.60	6.4
P_{13}	28.30	7.58	4.4	13.04	9.10	0.522	4.2	0.944	6.98	7.32	3.17	5.6
P_{14}	28.96	8.72	4.6	22.54	13.56	0.510	4.4	2.068	7.30	6.34	2.74	6.0
P_{15}	34.92	10.74	1.2	51.22	32.24	0.486	10.0	1.904	3.84	3.96	2.82	5.8
CD (0.05)	0.754	0.394	0.894	2.265	2.188	0.030	2.487	0.252	0.255	0.328	0.129	0.852
SE_m	0.267	0.139	0.316	0.801	0.774	0.014	0.879	0.089	0.091	0.116	0.046	0.301

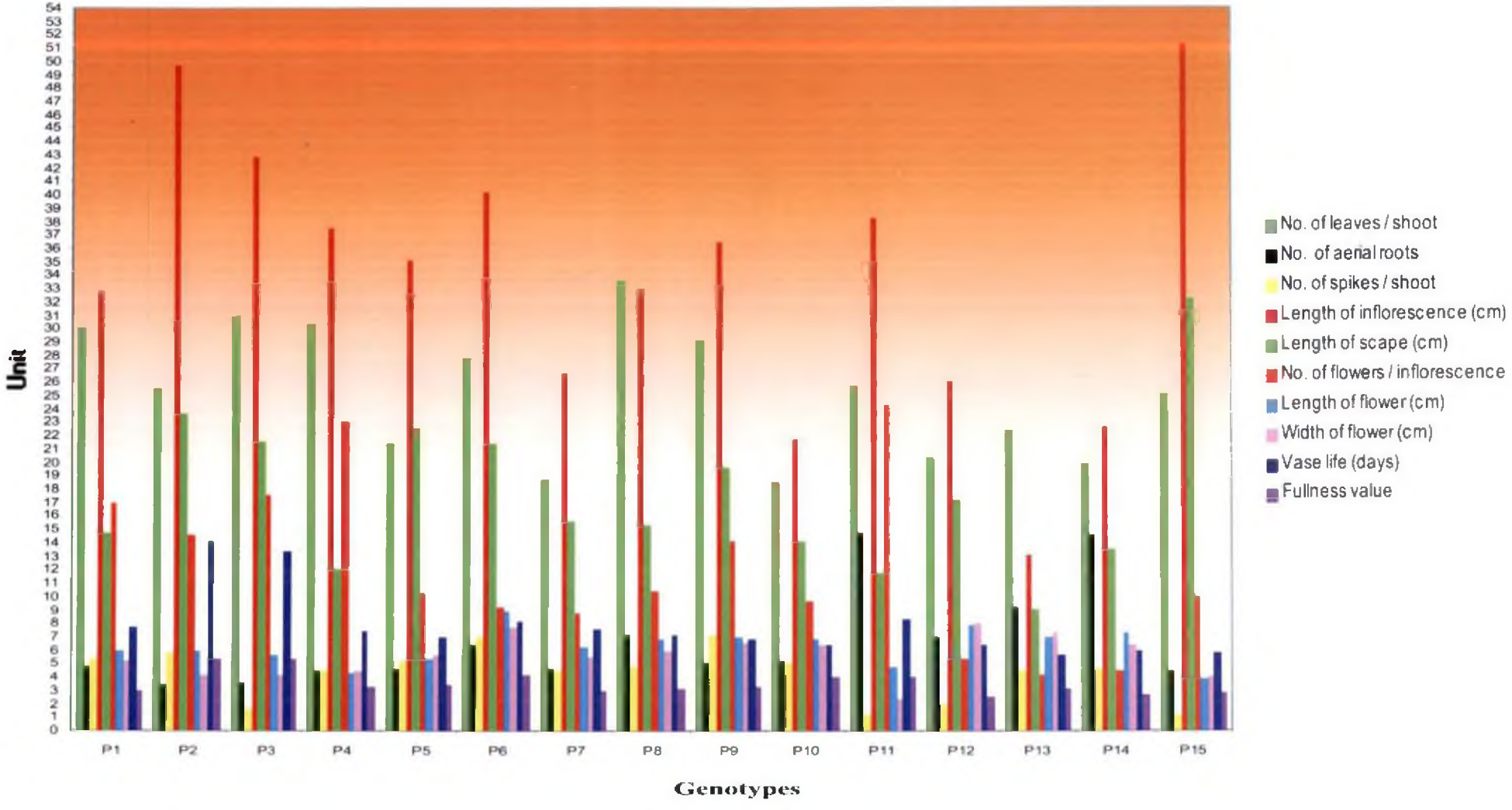


Fig.1. Mean performance of 15 monopodial orchid parental genotypes for important characters

lowest for P_{15} (46.92 cm) which was on par with P_1 (47.08 cm) and P_5 (47.64) also.

2. Number of Leaves per Shoot

The maximum number of leaves per shoot was recorded for P_8 (33.6) which was significantly high . For P_4 (30.4) and P_1 (30.0) also comparatively higher number of leaves were recorded. The minimum values for this trait was for genotypes P_{10} (18.6) and P_1 (18.8) which was found to be on par with P_{12} (20.4) and P_{14}(20.0).

3. Number of Aerial Roots

The number of aerial roots was observed to be the highest in P_{11} (14.8) which was recorded to be statistically on par with P_{14} (14.6). The lowest numbers of aerial roots were recorded in P_2 (3.4) and P_3 (3.6).

4. Length of Aerial Root

The mean aerial root length was maximum in P_5 (39.02 cm). This was on par with P_{10} (38.98 cm) and P_9 (36.2 cm). The lowest mean aerial root length was recorded in P_8 (13.66 cm) ; for P_{13} it was 14.44 cm.

5. Thickness of Shoot

The mean thickness of shoot ranged from a maximum value in P_6 (1.618 cm) to a minimum in P_{11} (0.790 cm). P_9 was assessed to have a value (1.572 cm) which was statistically on par with the highest thickness.

6. Length of Internode

The length of internode was observed to be the highest in P_{14} (3.764 cm) which was significantly high. Higher values were found for the genotypes P_{11}

(3.056 cm) and P_{13} (3.016 cm). The lowest mean internodal length was recorded in P_{15} (1.788 cm).

7. Length of Leaf

For P_4 the mean leaf length was recorded to be the highest with a value of 21.1 cm. The lowest mean length was for P_{11} (6.14 cm) followed by P_{13} (7.78 cm).

8. Width of Leaf

The width of leaf ranged from a maximum value of 3.36 cm in P_6 to a minimum of 0.36 cm in P_{13}. P_8 had a leaf width of 3.34 cm which was statistically analyzed to be on par with the highest width.

9. Thickness of Leaf

The thickness of leaf ranged from a significantly high value of 0.512 cm in P_{14} to 0.09 cm in P_{11}. P_{12} and P_{13} were observed to have comparatively thicker leaves with values of 0.486 cm and 0.356 cm respectively.

10. Leaf Area

The leaf area was observed to be significantly high in P_7 (58.82 cm^2) followed by P_6 (50.72). The lowest mean leaf area was seen in P_{13} (2.96 cm^2).

4.1.1.2. Quantitative Floral Characters

1. Days to First Flower Opening from Inflorescence Emergence

The genotype P_2 exhibited maximum time interval from inflorescence emergence to first flower opening of 37.91 days which was statistically on par with P_3 (36.34 days) and P_4 (35.16 days). The shortest time interval was recorded

in P_{11} (27.40 days). This period was less in P_{14} (28.96 days) which was statistically on par with P_{12} (28.90 days) and P_{13} (28.30 days).

2. Days to Last Flower Opening from First Flower Opening

The time interval from the opening of the first flower to the last in the inflorescence was recorded to be significantly high in P_9 (18.44 days) followed by P_{11} (17.2 days) and low in P_{13} (7.58 days), P_{14} (8.72days) and P_{12} (8.52 days).

3. Number of Spikes per Shoot

The highest mean total number of spikes per shoot was significantly high in P_9 (7.2) followed by P_6 (7.0) and P_2 (5.8). The lowest mean number of spikes per shoot recorded in P_{11} and P_{15} with a value of 1.2 which was on par with P_3 (1.6).

4. Length of Inflorescence

P_{15} produced the longest inflorescence (51.22 cm) which was statistically assessed to be on par with P_2 (49.64 cm). Lowest mean length of inflorescence was observed in P_{13} (13.04 cm) .

5. Length of Scape

The mean length of scape was also the highest in the species P_{15} (32.24 cm), which was significantly high, followed by P_2 (23.72 cm). It was noted to be the lowest in P_{13} (9.1 cm).

6. Thickness of Inflorescence Axis

The thickness of inflorescence axis was significantly high in P_9, with maximum diameter of 0.836 cm, followed by P_6 (0.776 cm). The inflorescence axis diameter was the lowest in P_{15} (0.486 cm) . The genotypes P_{11} and P_{14} also produced inflorescences with thin axis both having a diameter of 0.51 cm.

7. Number of Flowers per Inflorescence

The species P_{11} produced 24.2 flowers per inflorescence which was the highest. P_4 was found to be on par with this, producing a high mean number of flowers i.e., 23.0. The lowest mean number of flowers per inflorescence was expressed in P_{13} and P_{14} (4.20 and 4.40 respectively).

8. Length of Internode of Inflorescence

The highest length of internode of inflorescence was observed in P_6 (2.08 cm) which was statistically on par with P_{14} (2.068 cm) and P_{15} (1.904 cm). The lowest internodal length was observed in P_{10} (0.798 cm). This was on par with P_{13} (0.944cm) and P_1 (1.06 cm).

9. Length of Flower

Length of flower ranged from 9.0 cm in P_6 to 3.84 cm in species P_{15}. Significantly long flowers were observed in P_{12} (7.88 cm) and P_{14} (7.3 cm) also.

10. Width of Flower

The width of flower was recorded to be the maximum in P_{12} (8.06 cm) which was on par with P_6 (7.68 cm) and was followed by P_{13} (7.32 cm). This trait was registered to be the minimum in the species P_{11} (2.34 cm).

11. Fullness Value

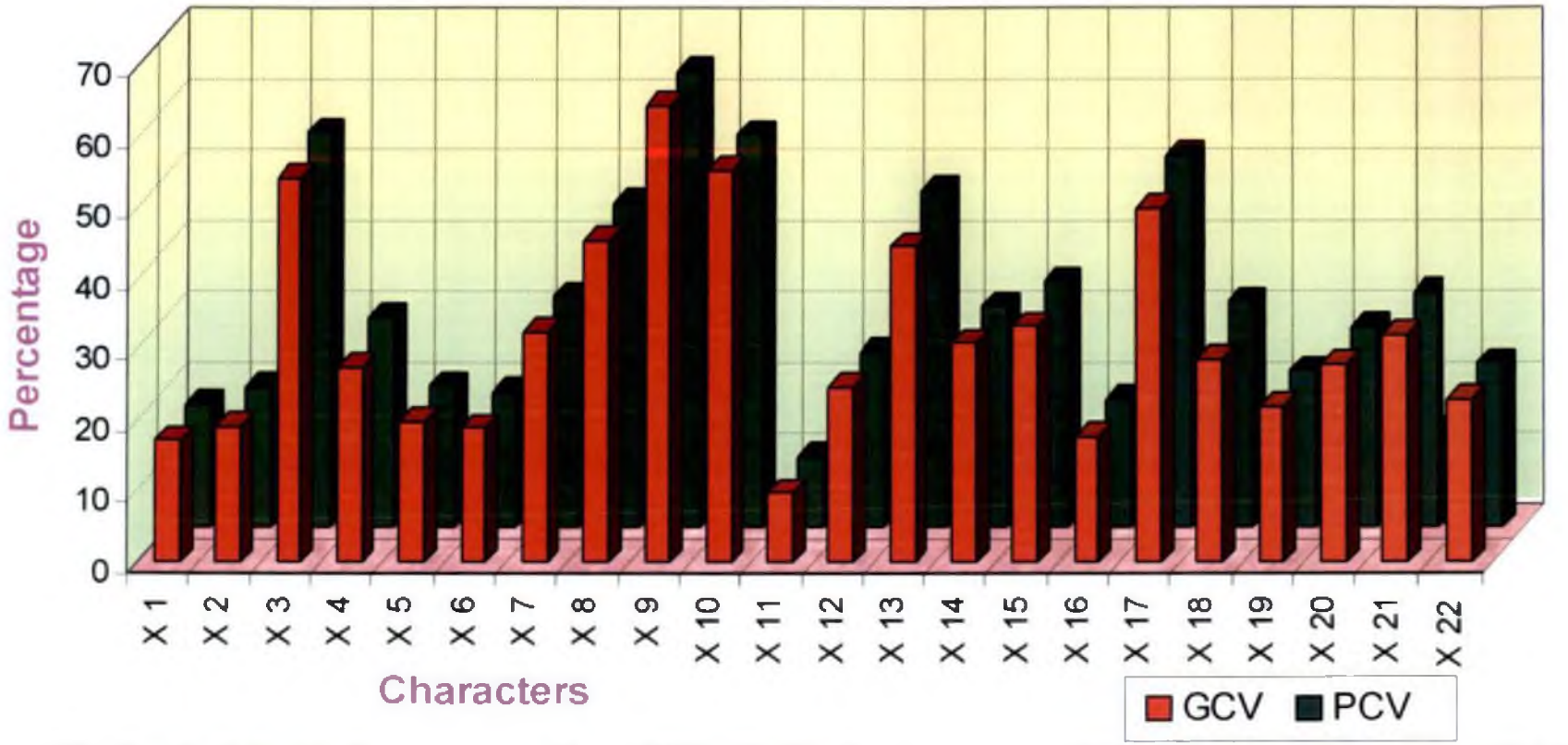
Fullness value indicates the degree of fullness of a flower and the lower the value, the greater will be the fullness. Fullness values were significantly high in P_2 (5.33) followed by P_3 (5.11). This value was significantly low in P_{12} (2.60) followed by P_{14} (2.74) which was statistically found to be on par with P_{15} (2.82).

Table 4. Variability parameters for morphological characters in 15 parental genotypes of monopodial orchids

Sl.No	Characters	Genotypic variance $\sigma^2 g$	Phenotypic variance $\sigma^2 p$	Environmental variance $\sigma^2 e$	Genotypic coefficient of variation GCV(%)	Phenotypic coefficient of variation PCV(%)
1	Length of shoot (cm)	114.009	116.298	2.289	17.180	17.351
2	No of leaves/ shoot	23.411	25.684	2.273	19.089	19.995
3	Number of aerial roots	12.943	13.670	0.727	54.400	55.906
4	Length of aerial roots (cm)	62.587	72.515	9.928	27.619	29.729
5	Thickness of shoot (cm)	0.059	0.060	0.001	19.868	20.118
6	Length of internode (cm)	0.259	0.262	0.003	18.998	19.120
7	Length of leaf (cm)	21.888	22.082	0.194	32.577	32.721
8	Width of leaf (cm)	0.916	0.930	0.014	45.508	45.858
9	Thickness of leaf (cm)	0.018	0.018	0.000	64.442	64.573
10	Leaf area (cm^2)	316.248	317.235	0.987	55.464	55.551
11	Days to first flower opening from inflorescence emergence	10.181	10.537	0.356	9.896	10.067

Table 4. Continued

Sl. No	Characters	Genotypic variance $\sigma^2 g$	Phenotypic variance $\sigma^2 p$	Environmental variance $\sigma^2 e$	Genotypic coefficient of variation GCV(%)	Phenotypic coefficient of variation PCV(%)
12	Days to last flower opening from first flower opening	11.765	11.862	0.097	24.801	24.902
13	Number of spikes per cane	3.659	4.159	0.500	44.692	47.648
14	Length of inflorescence (cm)	108.909	112.116	3.207	30.884	31.336
15	Length of scape (cm)	34.676	37.668	2.992	33.398	34.808
16	Thickness of inflorescence axis(cm)	0.012	0.012	0.001	17.682	18.097
17	Number of flowers per inflorescence	37.304	41.170	3.867	50.118	52.651
18	Length of internode of inflorescence	0.160	0.200	0.040	28.720	32.070
19	Length of flower (cm)	1.895	1.936	0.041	21.982	22.217
20	Width of flower (cm)	2.436	2.503	0.067	27.965	28.347
21	Vase life(days)	6.405	6.859	0.453	32.118	33.235
22	Fullness value	0.671	0.682	0.010	23.092	23.271



X1	Length of shoot (cm)	X8	Width of leaf (cm)	X16	Thickness of inflorescence axis (cm)
X2	No of leaves/ shoot	X9	Thickness of leaf (cm)	X17	Number of flowers per inflorescence
X3	Number of aerial roots	X10	Leaf area (cm^2)	X18	Length of internodes of inflorescence
X4	Length of aerial roots (cm)	X11	Days to first flower opening from inflorescence emergence	X19	Length of flower (cm)
X5	Thickness of shoot (cm)	X12	Days to last flower opening from first flower opening	X20	Width of flower (cm)
X6	Length of internodes (cm)	X13	Number of spikes per shoot	X21	Vase life (days)
X7	Length of leaf (cm)	X14	Length of inflorescence (cm)	X22	Fullness value
		X15	Length of scape (cm)		

Fig. 2. Variability parameters for morphological characters in 15 parental genotypes of monopodial orchids

12. Vase Life

The highest vase life was recorded in P_2 with a significantly high value of 14.2 days which was on par with P_3 (13.4 days). The lowest vase life of 5.6 days was observed in P_{13}. Vase life was comparatively low for P_{15} (5.8 days), P_{14} (6.0 days), P_{10} (6.4 days) and P_{12} (6.4 days) which were on par with the lowest value.

4.1.2. Estimation of Genetic Parameters of Parents

Genetic parameters were estimated for the parents under the following heads:

4.1.2.1. Variability studies
4.1.2.2. Heritability and genetic advance
4.1.2.3. Correlation analysis

4.1.2.1. Variability Studies

The genotypic, phenotypic and environmental variances and coefficients of variation at genotypic and phenotypic levels were studied in monopodial orchids (Table 4; Fig. 2).

The vegetative characters such as thickness of leaf (GCV = 64.442%, PCV = 64.573%), leaf area (GCV = 55.464 %, PCV = 55.551 %) and number of aerial roots (GCV = 54.4 %, PCV = 55.906 %) in the descending order exhibited the highest estimates of both genotypic and phenotypic variances.

Among the floral traits, number of flowers per inflorescence (GCV = 50.118 %, PCV = 52.651 %), number of spikes per cane (GCV = 44.692 %, PCV = 47.648 %) and vase life (GCV = 32.118 %, PCV = 33.235 %) showed highest genotypic and phenotypic variances in the decreasing order.

4.1.2.2. Heritability and Genetic Advance

Estimates of heritability in the broad sense and genetic advance in parental material were recorded (Table 5; Fig. 3 and 4).

Heritability per cent was categorised as suggested by Allard (1960) into low (<30), moderate (30-70) and high (>70). Accordingly, all the vegetative characters and floral traits exhibited high heritability.

A wide range of genetic advance (at 0.05) was exhibited by the characters studied. This was highest for leaf area (36.58) followed by length of shoot (21.78) and length of inflorescence (21.19).

Genetic advance (% mean) was high (>70) for six characters considered. It was exhibited in the range of 30-70 per cent by sixteen characters analysed.

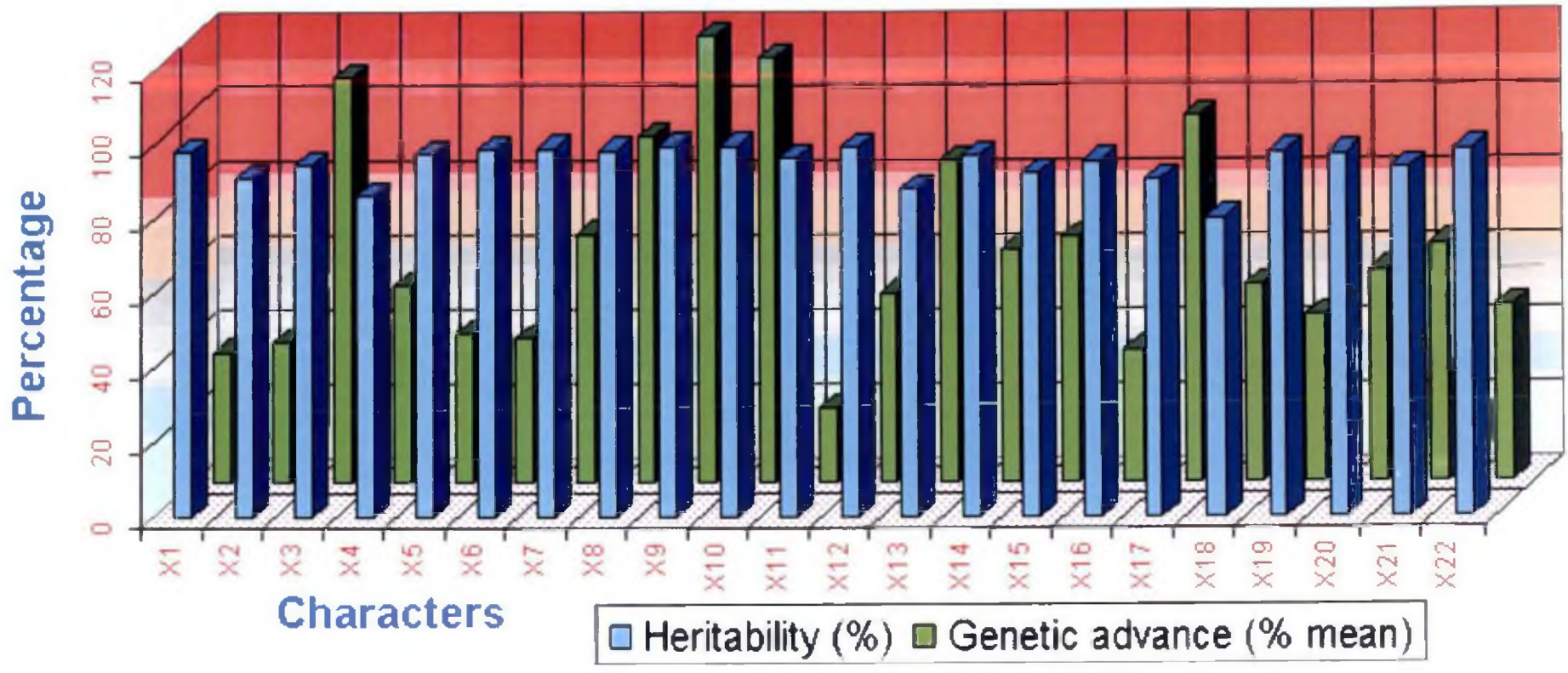
High heritability (>70 %) combined with genetic advance greater than 70 per cent was observed for number of aerial roots, width of leaf, thickness of leaf, leaf area, number of spikes per shoot and number of flowers per inflorescence.

High heritability (>70 %) combined with 30-70 per cent genetic advance was observed for length of shoot, number of leaves per shoot, length of aerial roots, thickness of shoot, length of internode, length of leaf, days to last flower opening from first flower opening, length of inflorescence, length of scape, thickness of inflorescence axis, length of internode of inflorescence, length of flower, width of flower, pollen size, vase life and fullness value.

Although days to first flower opening from inflorescence emergence and pollen fertility showed high heritability, their genetic advance was found to be less than 30 per cent.

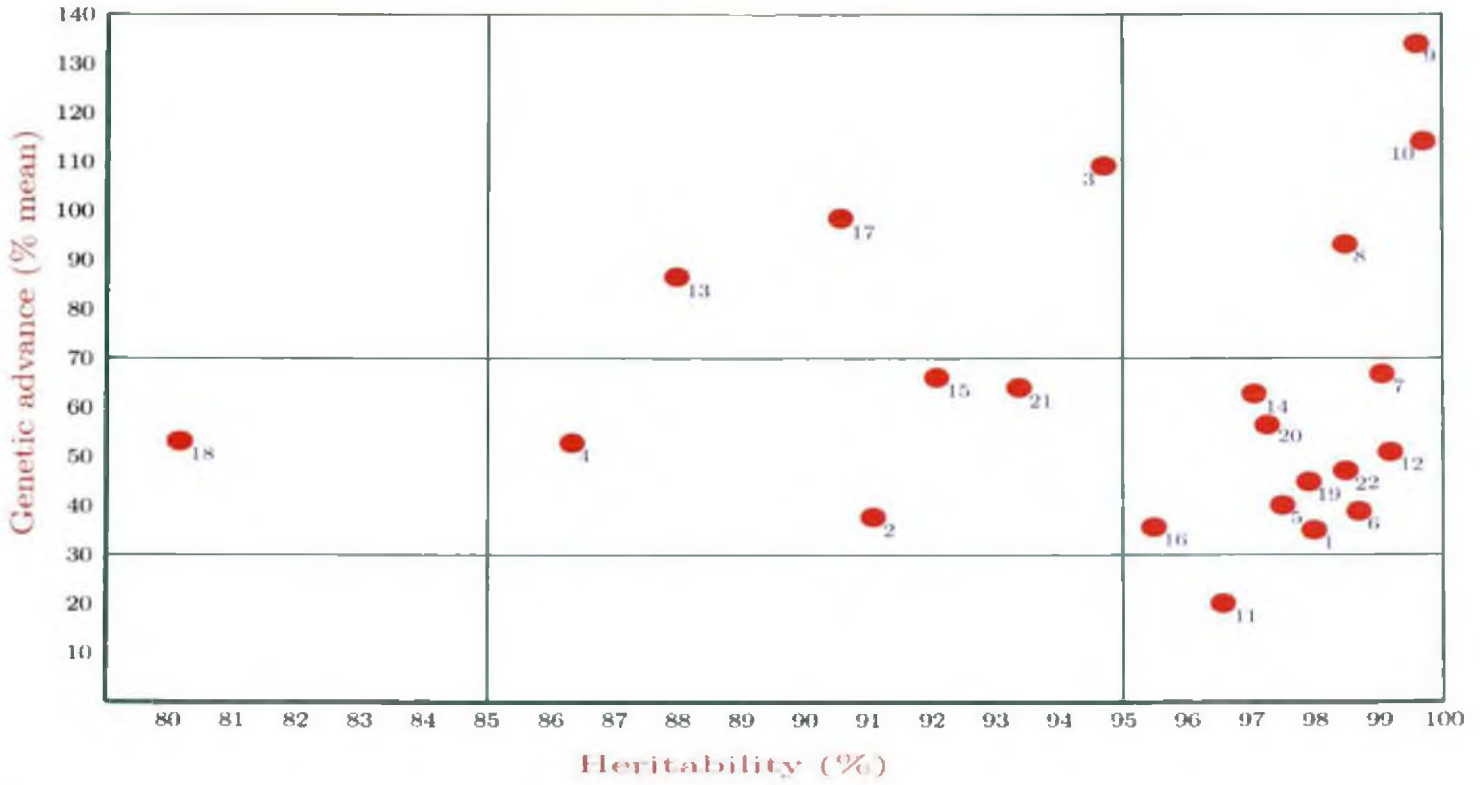
Table 5. Heritability and genetic advance for morphological characters in 15 parental genotypes of monopodial orchids

Sl. No	Characters	Heritability (%)	Genetic advance at 0.05	Genetic advance (% mean)
1	Length of shoot (cm)	98.0	21.78	35.04
2	No of leaves/ shoot	91.1	9.52	37.56
3	Number of aerial roots	94.7	7.21	109.03
4	Length of aerial roots (cm)	86.3	15.14	52.86
5	Thickness of shoot (cm)	97.5	0.49	40.16
6	Length of internode (cm)	98.7	1.04	38.82
7	Length of leaf (cm)	99.1	9.60	66.85
8	Width of leaf (cm)	98.5	1.96	93.20
9	Thickness of leaf (cm)	99.6	0.28	133.97
10	Leaf area (cm^2)	99.7	36.58	114.09
11	Days to first flower opening from inflorescence emergence	96.6	6.46	20.04
12	Days to last flower opening from first flower opening	99.2	7.04	50.90
13	Number of spikes per cane	88.0	3.70	86.45
14	Length of inflorescence (cm)	97.1	21.19	62.71
15	Length of scape (cm)	92.1	11.64	66.02
16	Thickness of inflorescence axis(cm)	95.5	0.22	35.66
17	Number of flowers per inflorescence	90.6	11.98	98.30
18	Length of internode of inflorescence	80.2	0.74	53.08
19	Length of flower (cm)	97.9	2.81	44.87
20	Width of flower (cm)	97.3	3.17	56.80
21	Vase life(days)	93.4	5.04	63.96
22	Fullness value	98.5	1.67	47.04



X1	Length of shoot (cm)	X8	Width of leaf (cm)	X16	Thickness of inflorescence axis (cm)
X2	No of leaves/ shoot	X9	Thickness of leaf (cm)	X17	Number of flowers per inflorescence
X3	Number of aerial roots	X10	Leaf area (cm^2)	X18	Length of internodes of inflorescence
X4	Length of aerial roots (cm)	X11	Days to first flower opening from inflorescence emergence	X19	Length of flower (cm)
X5	Thickness of shoot (cm)	X12	Days to last flower opening from first flower opening	X20	Width of flower (cm)
X6	Length of internodes (cm)	X13	Number of spikes per shoot	X21	Vase life (days)
X7	Length of leaf (cm)	X14	Length of inflorescence (cm)	X22	Fullness value
		X15	Length of scape (cm)		

Fig. 3. Heritability and genetic advance for morphological characters in 15 parental genotypes of monopodial orchids



X1	Length of shoot (cm)	X8	Width of leaf (cm)	X16	Thickness of inflorescence axis (cm)
X2	No of leaves/ shoot	X9	Thickness of leaf (cm)	X17	Number of flowers per inflorescence
X3	Number of aerial roots	X10	Leaf area (cm^2)	X18	Length of internodes of inflorescence
X4	Length of aerial roots (cm)	X11	Days to first flower opening from inflorescence emergence	X19	Length of flower (cm)
X5	Thickness of shoot (cm)	X12	Days to last flower opening from first flower opening	X20	Width of flower (cm)
X6	Length of internodes (cm)	X13	Number of spikes per shoot	X21	Vase life (days)
X7	Length of leaf (cm)	X14	Length of inflorescence (cm)	X22	Fullness value
		X15	Length of scape (cm)		

Fig. 4. Character distribution in terms of heritability and genetic advance

4.1.2.3. Correlation Studies

Among the different quantitative characters studied in parental monopodial orchid genotypes, ten important biological traits were selected for the genotypic, phenotypic and environmental correlation studies. The ten characters selected for study were number of leaves per shoot, number of aerial roots, leaf area, number of spikes per shoot, length of inflorescence, length of scape, number of flowers per inflorescence, length of flower, width of flower and vase life. The significance of both genotypic, phenotypic and environmental correlations was tested and the results are presented (Tables 6, 7 and 8; Figs. 5, 6 and 7).

Highest significant positive correlation was exhibited by width of flower with length of flower (r_g = 0.8517, r_p = 0.8422). Width of flower recorded significant positive correlation with number of spikes per shoot (r_g = 0.4786, r_p = 0.4540) also. Length of inflorescence showed maximum significant positive correlation with length of scape ((r_g = 0.7638, r_p = 0.7596).

Highly significant negative correlation with number of aerial roots was expressed by leaf area (r_g = -0.5746, r_p = -0.5602), length of scape (r_g = -0.5211, r_p = -0.4919)and length of inflorescence (r_g = -0.4015, r_p = -0.3871).

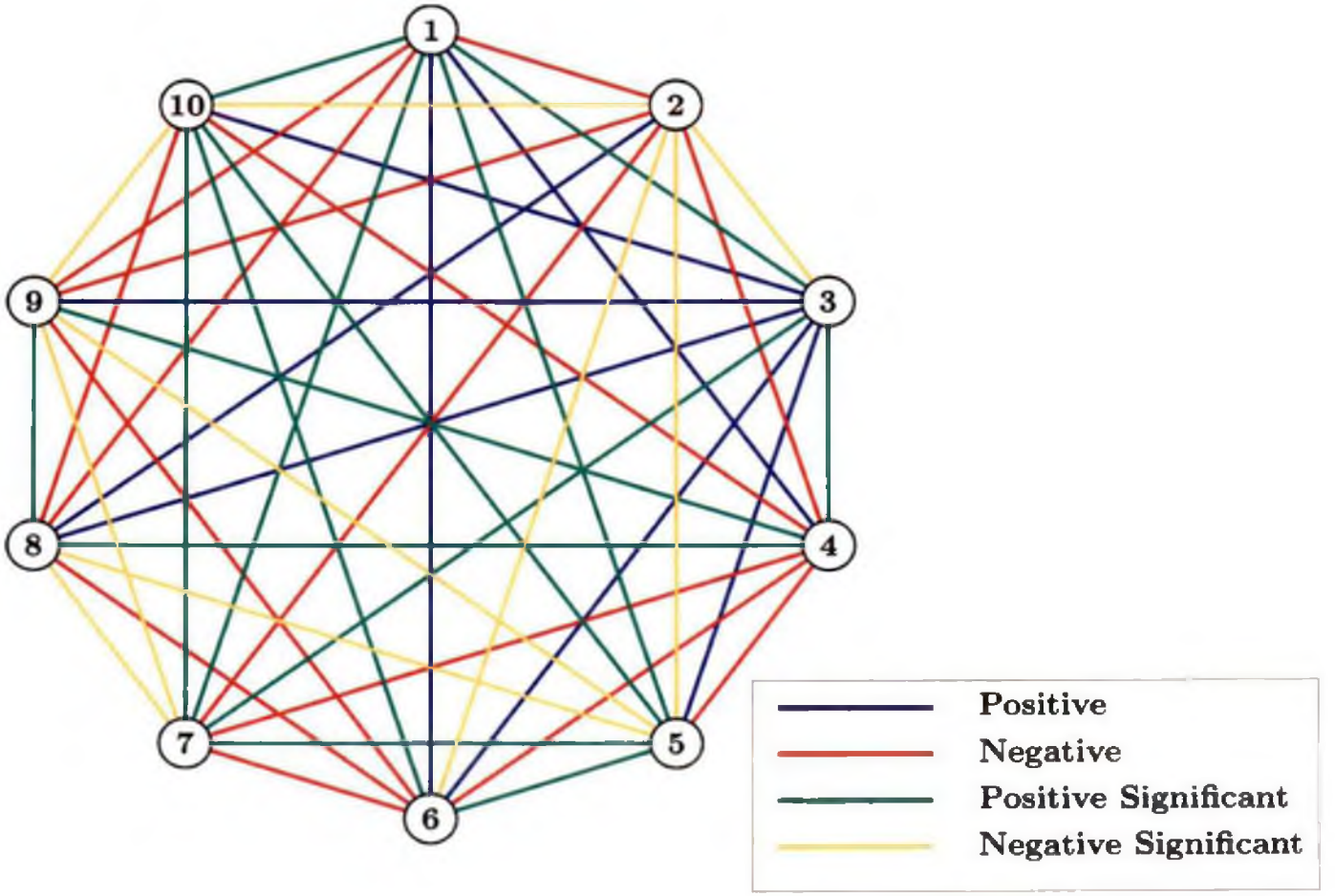
Length of flower expressed positive correlation with number of spikes per shoot (r_g = 0.5089, r_p = 0.4822). However, length of inflorescence (r_g = -0.4705, r_p = -0.4594) and number of flowers per inflorescence (r_g = -0.6100, r_p = -0.5696) showed highly significant negative correlation with this trait.

The character number of spikes per shoot recorded significant positive correlation with leaf area (r_g = 0.5371, r_p = 0.5037). Number of flowers per inflorescence was positively correlated with number of leaves per shoot (r_g = 0.5961, r_p = 0.5338) and length of inflorescence (r_g = 0.7479, r_p = 0.5091) .

Table 6. Phenotypic correlation among selected traits in 15 monopodial parental genotypes

Characters	No.of leaves/ Shoot	No.of aerial roots	Leaf area	No. of spikes / shoot	Length of inflorescence	Length of scape	No. of flowers / inflorescence	Length of flower	Width of flower	Vase Life
No.of leaves / shoot	1									
No.of aerial roots	-0.2073	1								
Leaf area	0.3200**	-0.5602**	1							
No. of spikes / cane	0.1046	-0.2304	0.5037**	1						
Length of inflorescence	0.4876**	-0.3871**	0.1869	-0.1378	1					
Length of scape	0.0875	-0.4919**	0.0505	-0.1046	0.7596**	1				
No. of flowers / inflorescence	0.5338**	-0.0852	0.2649*	-0.1656	0.5091**	-0.066	1			
Length of flower	-0.1574	0.1613	0.0906	0.4822**	-0.4594**	-0.2145	-0.5696**	1		
Width of flower	-0.2721	-0.0252	0.0229	0.454**	-0.5844**	-0.1724	-0.7327**	0.8422**	1	
Vase life	0.3193*	-0.3232**	0.1341	-0.031	0.5546**	0.2703*	0.4258**	-0.1238	-0.4274**	1

* Significant at 5% level, ** Significant at 1% level



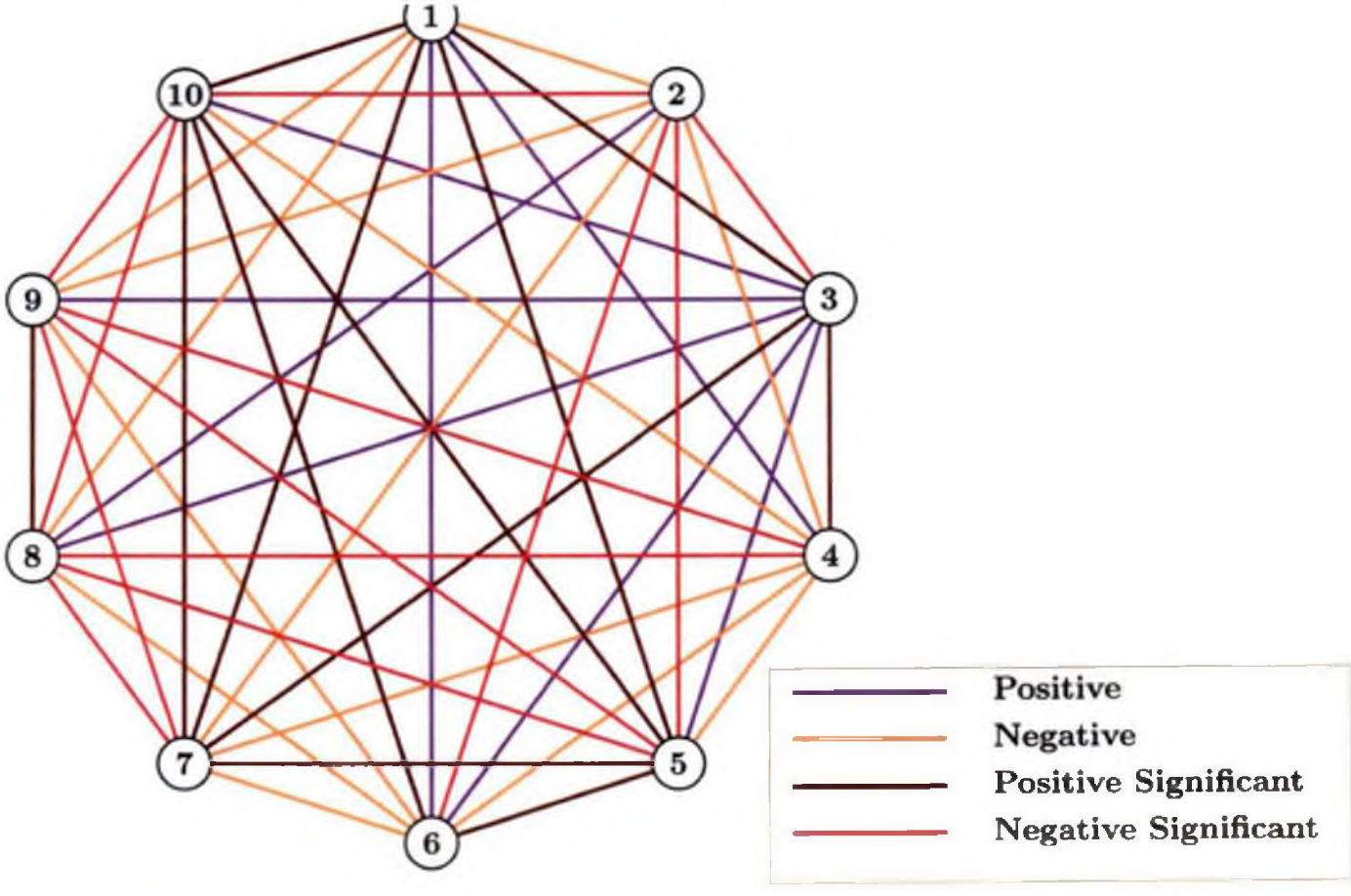
1. Number of leaves per shoot
2. Number of aerial roots
3. Leaf area
4. Number of spikes/shoot
5. Length of inflorescence
6. Length of scape
7. Number of flowers per inflorescence
8. Length of flower
9. Width of flower
10. Vase life

Fig. 5. Phenotypic correlation among selected traits in 15 monopodial parental genotypes

Table 7. Genotypic correlation among selected traits in 15 monopodial parental genotypes

Characters	No.of leaves/ shoot	No.of aerial roots	Leaf area	No. of spikes / shoot	Length of inflorescence	Length of scape	No. of flowers / inflorescence	Length of flower	Width of flower	Vase life
No.of leaves/ shoot	1									
No.of aerial roots	-0.2361	1								
Leaf area	0.332**	-0.5746**	1							
No. of spikes / cane	0.0891	-0.2433	0.5371**	1						
Length of inflorescence	0.5217**	-0.4015**	0.189	-0.1434	1					
Length of scape	0.1022	-0.5211**	0.0529	-0.1052	0.7638**	1				
No. of flowers / inflorescence	0.5961**	-0.0916	0.2809*	-0.1803	0.5387**	-0.0708	1			
Length of flower	-0.1724	0.1608	0.0928	0.5089**	-0.4705**	-0.2332	-0.61**	1		
Width of flower	-0.293	-0.0289	0.0246	0.4786**	-0.6043**	-0.1865	-0.7781**	0.8517**	1	
Vase life	0.3578**	-0.3324**	0.1419	-0.039	0.5746**	0.2821*	0.4495**	-0.1328	-0.4547**	1

* Significant at 5% level, ** Significant at 1% level



1. Number of leaves per shoot
2. Number of aerial roots
3. Leaf area
4. Number of spikes/shoot
5. Length of inflorescence
6. Length of scape
7. Number of flowers per inflorescence
8. Length of flower
9. Width of flower
10. Vase life

Fig. 6. Genotypic correlation among selected traits in 15 monopodial parental genotypes

Table 8. Environmental correlation among selected traits in 15 monopodial parental genotypes

Characters	No.of leaves/ shoot	No.of aerial roots	Leaf area	No. of spikes / shoot	Length of inflorescence	Length of scape	No. of flowers / inflorescence	Length of flower	Width of flower	Vase life
No.of leaves/ shoot	1									
No.of aerial roots	0.1764	1								
Leaf area	0.2111	-0.157	1							
No. of spikes / cane	0.2408	-0.1051	0.0327	1						
Length of inflorescence	-0.0653	-0.0518	0.0946	-0.0898	1					
Length of scape	-0.0731	-0.0818	-0.0097	-0.1017	0.7831**	1				
No. of flowers / inflorescence	-0.0866	-0.006	-0.1203	-0.0432	0.0718	-0.0155	1			
Length of flower	0.1271	0.1937	-0.133	0.1962	-0.026	0.1675	0.11	1		
Width of flower	0.0785	0.068	-0.1448	0.1966	0.1174	0.0881	-0.0393	0.4598**	1	
Vase life	-0.1412	-0.18	-0.2007	0.049	0.1672	0.1208	0.1561	0.0859	0.1453	1

** Significant at 1% level

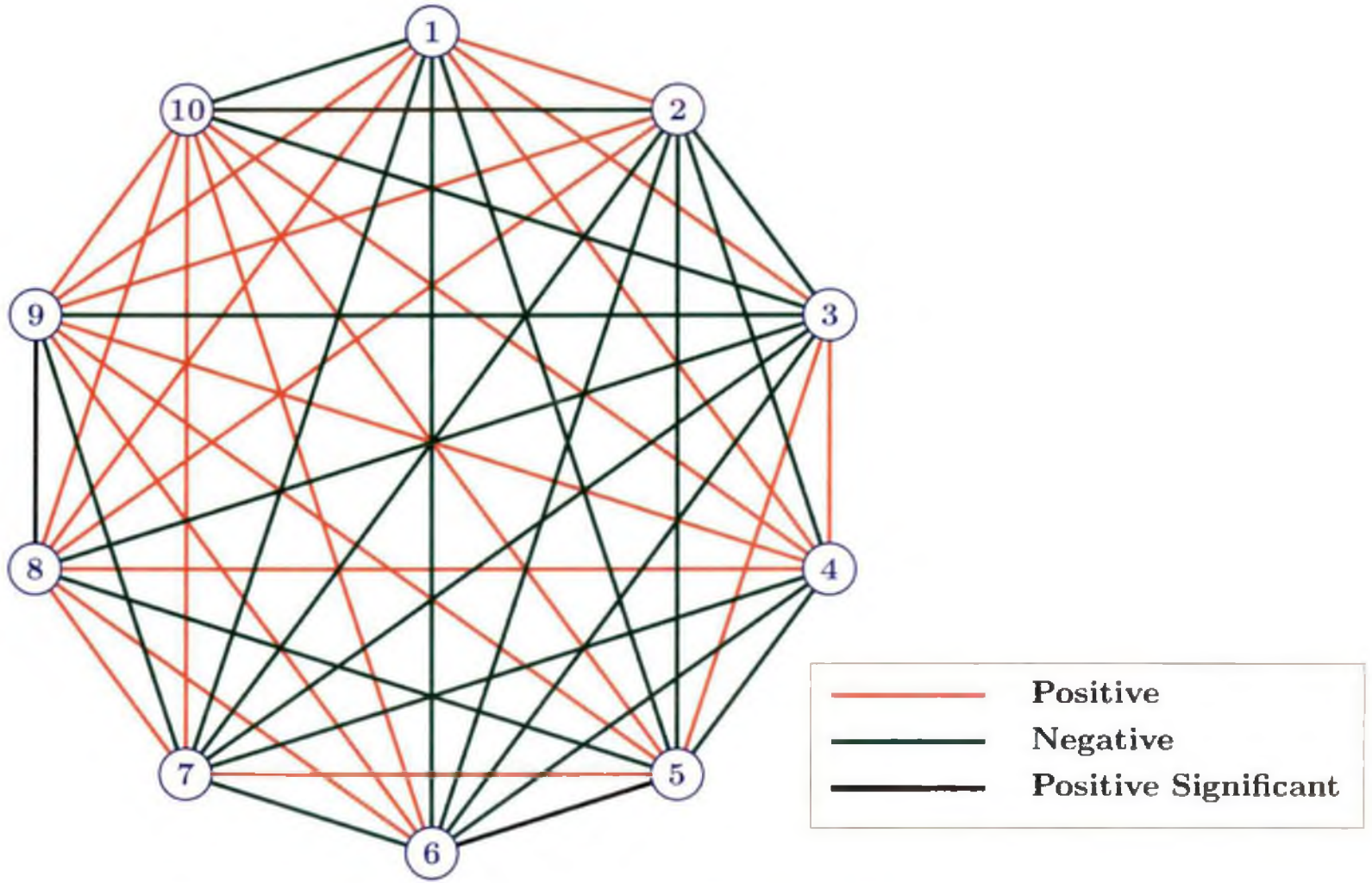


Fig. 7. Environmental correlation among selected traits in 15 monopodial parental genotypes

Vase life showed the highest positive correlation with length of inflorescence (r_g = 0.5746, r_p = 0.5546) followed by number of flowers per inflorescence (r_g = 0.4495, r_p = 0.4258) and number of leaves per shoot (r_g = 0.3578, r_p = 0.3193). This trait showed highly significant negative correlation with width of flower (r_g = -0.4547, r_p = -0.4274) and number of aerial roots (r_g = -0.3324, r_p = -0.3232).

Environmental correlation was found to be low in comparison with genotypic and phenotypic correlations for most of the character combinations. In the case of length of inflorescence, its environmental correlation with length of scape (r_e = 0.7831) was positive and significant which was observed to be higher than the genotypic and phenotypic correlations. Length of flower also showed significant positive environmental correlation with width of flower.

4.1.3. Floral Biology of Parents

Floral biology of parents was studied with respect to the following heads:

4.1.3.1. Flowering and floral morphology (Plate 1, 2 and 3)

4.1.3.2. Pollen characters (Plate 4)

4.1.3.1. Flowering and Floral Morphology

Flowering and qualitative floral characters in the parental monopodial orchid genotypes were analysed in detail (Table 9).

1. Flowering Nature—Free-flowering / Seasonal

Out of the fifteen genotypes studied, ten *viz.*, P_1, P_2, P_3, P_4, P_5, P_6, P_7, P_8, P_9 and P_{10} exhibited free-flowering nature i.e., flowering throughout the year. Seasonal flowering was observed mainly from June to December in P_{12}, P_{13}, P_{14} and P_{15} whereas it was confined to two seasons, from February to March and August to October in the species P_{11}.

Table 9. Qualitative floral characters of 15 monopodial orchid parental genotypes

Parents	Flowering nature	Nature of inflorescence axis	Mode of display of flowers	Shape of flower
P_1	Free-flowering	Erect	Whorled	Full,flat,broad sepals and petals
P_2	Free-flowering	Arching	Alternate and facing opposite sides	Flat, narrow, spatulate sepals and petals
P_3	Free-flowering	Arching	Alternate and facing opposite sides	Flat, narrow, spatulate sepals and petals
P_4	Free-flowering	Erect	Whorled	Full,flat,broad sepals and petals
P_5	Free-flowering	Erect	Whorled	Full,flat,broad sepals and petals
P_6	Free-flowering	Erect	Whorled	Full,flat,broad sepals and petals
P_7	Free-flowering	Erect	Whorled	Full,flat,broad sepals and petals
P_8	Free-flowering	Erect	Whorled	Full,flat,broad sepals and petals
P_9	Free-flowering	Erect	Whorled	Full,flat,broad sepals and petals
P_{10}	Free-flowering	Erect	Whorled	Full,flat,broad sepals and petals
P_{11}	Seasonal February-March August-October	Arching	Alternate and facing opposite sides	Flat, narrow, spatulate sepals and petals
P_{12}	Seasonal June-December	Erect	Alternate and facing opposite sides	Full,flat,broad sepals and petals
P_{13}	Seasonal June-December	Erect	Alternate and facing opposite sides	Full,flat,broad sepals and petals
P_{14}	Seasonal June-December	Erect	Alternate and facing opposite sides	Full,flat,broad sepals and petals
P_{15}	Seasonal June-December	Erect	Whorled	Full,flat,broad sepals and petals

Plate 1. Floral characteristics of parental genotypes of monopodial orchids used in the hybridization programme (from left to right, row- wise) – Single flower

Aranda Salaya Red (P_1)	*Aranthera* Annie Black (P_2)	*Aranthera* James Storei (P_3)	*Kagawara* Christielow (P_4)
Mokara Calypso (P_5).	*Mokara* Chak Kuan Pink (P_6)	*Mokara* Lumsum Sunlight (P_7)	*Mokara* Singapore Red (P_8)
Mokara Thailand Sunspot (P_9)	*Mokara* Walter Oumae White (P_{10})	*Renanthera coccinia* (P_{11})	*Vanda* John Clubb (P_{12})
Vanda Popoe Diana (P_{13})	*Vanda* Ruby Prince (P_{14})	*Vanda spathulata* (P_{15})	

Plate 1

Plate 2. Floral characteristics of parental genotypes of monopodial orchids used in the hybridization programme (from left to right, row- wise) – Inflorescence

Aranda Salaya Red
(P_1)

Aranthera Annie Black
(P_2)

Aranthera James Storei
(P_3)

Kagawara Christielow
(P_4)

Plate 2

Plate 2. Continued

Mokara Calypso

(P_5)

Mokara Chak Kuan Pink

(P_6)

Mokara Lumsum Sunlight

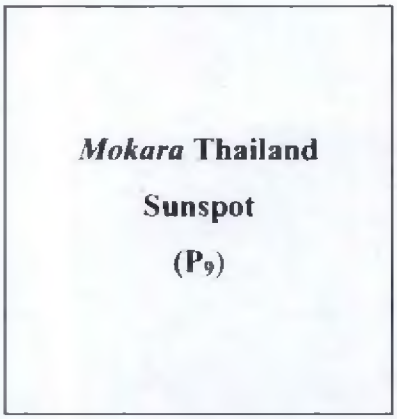
(P_7)

Mokara Singapore Red

(P_8)

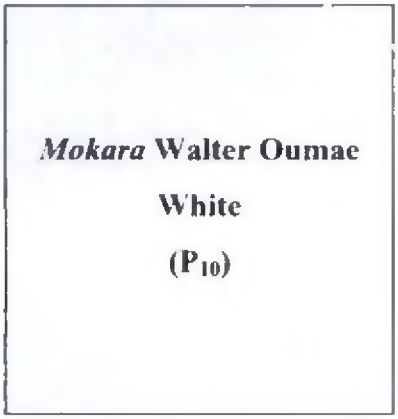
Plate 2 Continued

Plate 2. Continued



***Mokara* Thailand Sunspot**

(P_9)

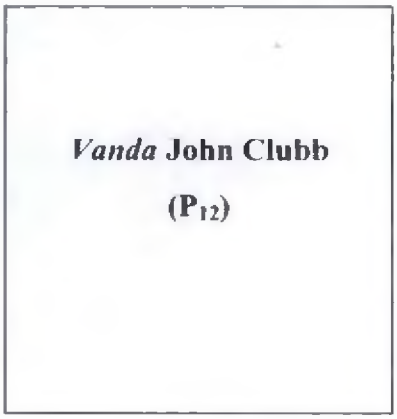


***Mokara* Walter Oumae White**

(P_{10})

Renanthera coccinia

(P_{11})

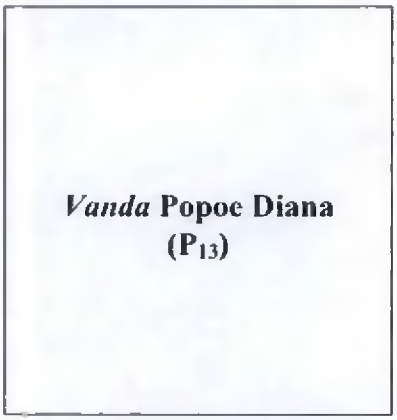


***Vanda* John Clubb**

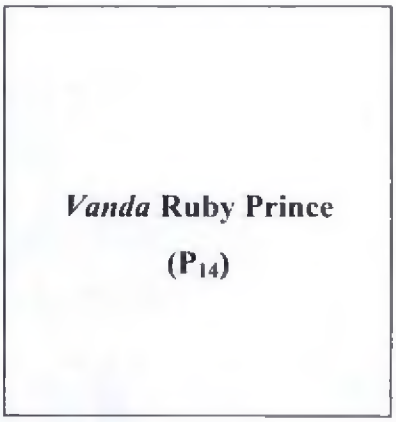
(P_{12})

Plate 2 Continued

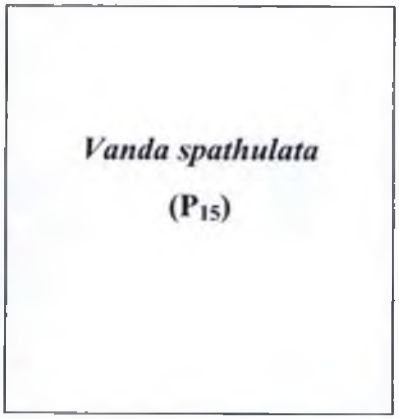
Plate 2. Continued



***Vanda* Popoe Diana**
(P_{13})



***Vanda* Ruby Prince**
(P_{14})



Vanda spathulata
(P_{15})

Plate 2 Continued

Plate 3. Field view and Floral biology

General view of field

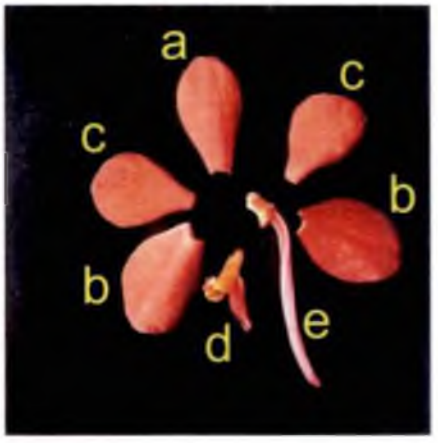
Dorsal view of single flower of *Aranda* Salaya Red

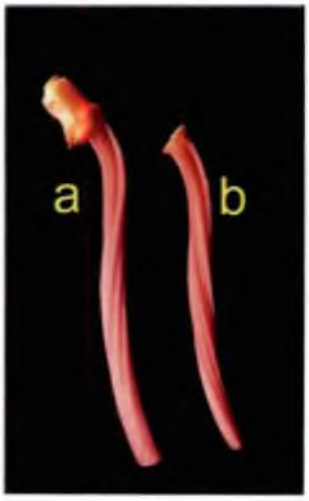
Single flower dissected showing
a) Dorsal sepal
b) Lateral sepals
c) Lateral petals
d) Odd petal or labellum
e) Column and ovary

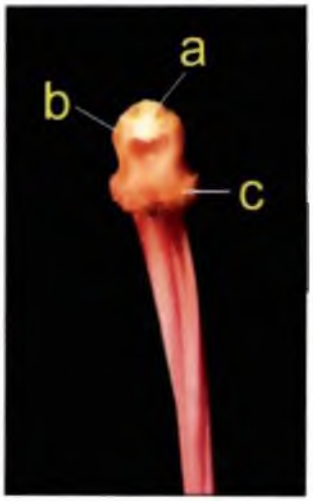
a) Ovary attached to column
b) Ovary

Column – ventral view showing
a) Operculum
b) Rostellum
c) Stigmatic cavity
(Sepals and petals removed)

Column tip showing
a) Clinandrium
b) Operculum
c) Pollinia







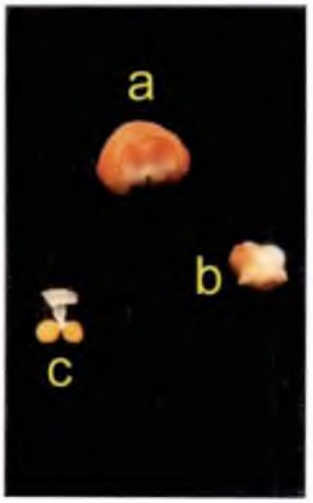


Plate 3

Plate 4. Pollination, pollen morphology and hybrid seeds

Process of pollination

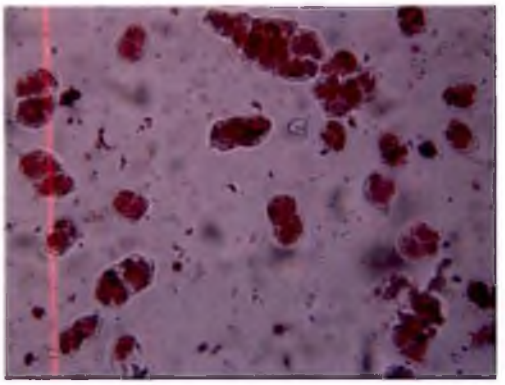
Pollinated flowers protected with insect proof net

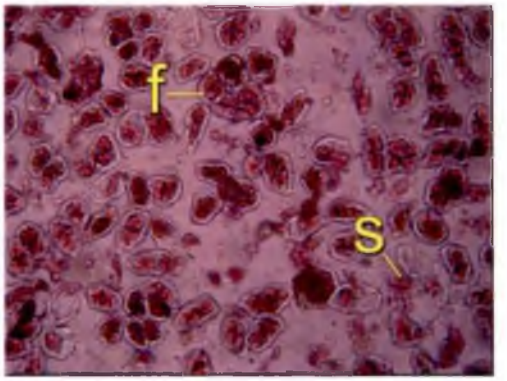
Pollen tetrads in *Aranthera* Annie Black 400 x

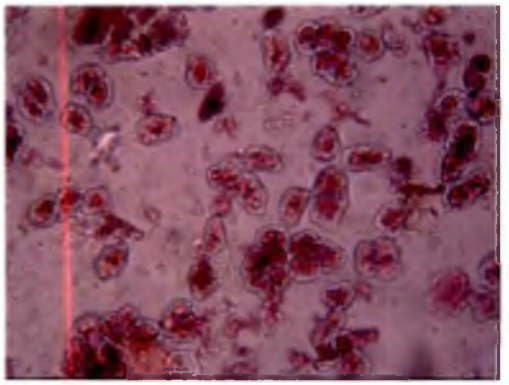
Pollen tetrads in *Vanda* Ruby prince 400 x
f- fertile
s- sterile

Pollen tetrads in *Mokara* Thailand sunspot 400 x

Hybrid seeds of *Vanda* Ruby Prince x *Vanda* John Clubb 200 x







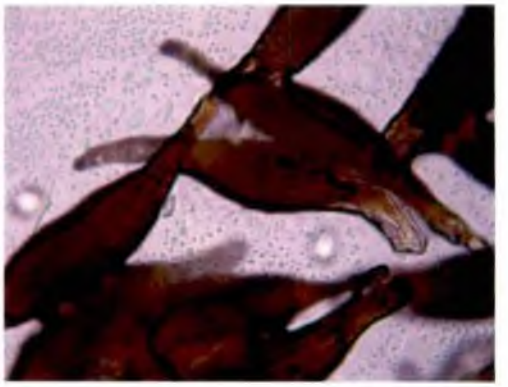


Plate 4

2. Nature of Inflorescence Axis

Inflorescence axis was found to be arching in P_2, P_3 and P_{11} while the rest of the genotypes produced erect inflorescence axis.

3. Mode of Display of Flowers

Nine of the genotypes such as P_1, P_4, P_5, P_6, P_7, P_8, P_9, P_{10} and P_{15} produced bunch type inflorescences. The remaining six *viz*., P_2, P_3, P_4, P_{12}, P_{13} and P_{14} exhibited the mode of display of flowers as alternate and facing opposite directions.

4. Shape of Flower

The flowers were full and flat with broad sepals and petals in almost all the parental genotypes except P_2, P_3 and P_{11} where the flowers presented a flat appearance with narrow spatulate sepals and petals with the tips slightly incurved.

4.1.3.2. Flower Opening, Anthesis and Stigma Receptivity

4.1.3.2.a Flower OpeningTime

In all the monopodial genotypes studied, flowers opened during the day-time, in acropetal succession (Table 10; Fig. 8). Each flower in an inflorescence opened almost during the same time of the day at a uniform time interval. Flower opening commenced from 7.30 am on sunny days and was delayed till 12.30 pm on rainy days in the present investigation. The process started between 7.30 and 9.30 am in P_{15} whereas in P_8, P_{12} and P_{13} it took place between 7.30 and 11.00 am. In P_{14} flowers opened between 7.30 and 11.00 am. Among the fifteen parentals tested, flower opening was delayed till 9.00 am to12.30 pm in P_4 and P_9. It was between 9.00 am and 12.00 noon in P_1.

Table10. Flower opening time, anthesis time and stigma receptivity period in 15 parental monopodial orchid genotypes

Parental genotype	Flower opening time	Anthesis time (days)	Maximum stigma receptivity period
P_1	9.00 am – 12.00 noon	2.5	2nd – 5th day
P_2	8.00 am – 11.30 am	3.3	3rd – 9th day
P_3	8.30 am – 12.00 noon	3.5	4th – 10th day
P_4	9.00 am – 12.30 am	2.6	3rd – 7th day
P_5	8.00 am – 11.00 am	2.8	2nd – 6th day
P_6	8.00 am – 11.00 am	2.9	3rd – 8th day
P_7	8.30 am – 11.30 am	2.4	3rd – 9th day
P_8	7.30 am – 11.00 am	3.3	3rd – 9th day
P_9	9.00 am – 12.30 am	3.5	4th – 10th day
P_{10}	8.30 am – 12.30 am	3.7	3rd – 9th day
P_{11}	8.00 am – 12.00 noon	3.8	4th – 10th day
P_{12}	7.30 am – 11.00 am	2.5	2nd – 6th day
P_{13}	7.30 am – 11.00 am	2.7	2nd – 6th day
P_{14}	7.30 am – 11.30 am	2.8	2nd – 6th day
P_{15}	7.30 am – 9.30 am	2.0	2nd – 4th day

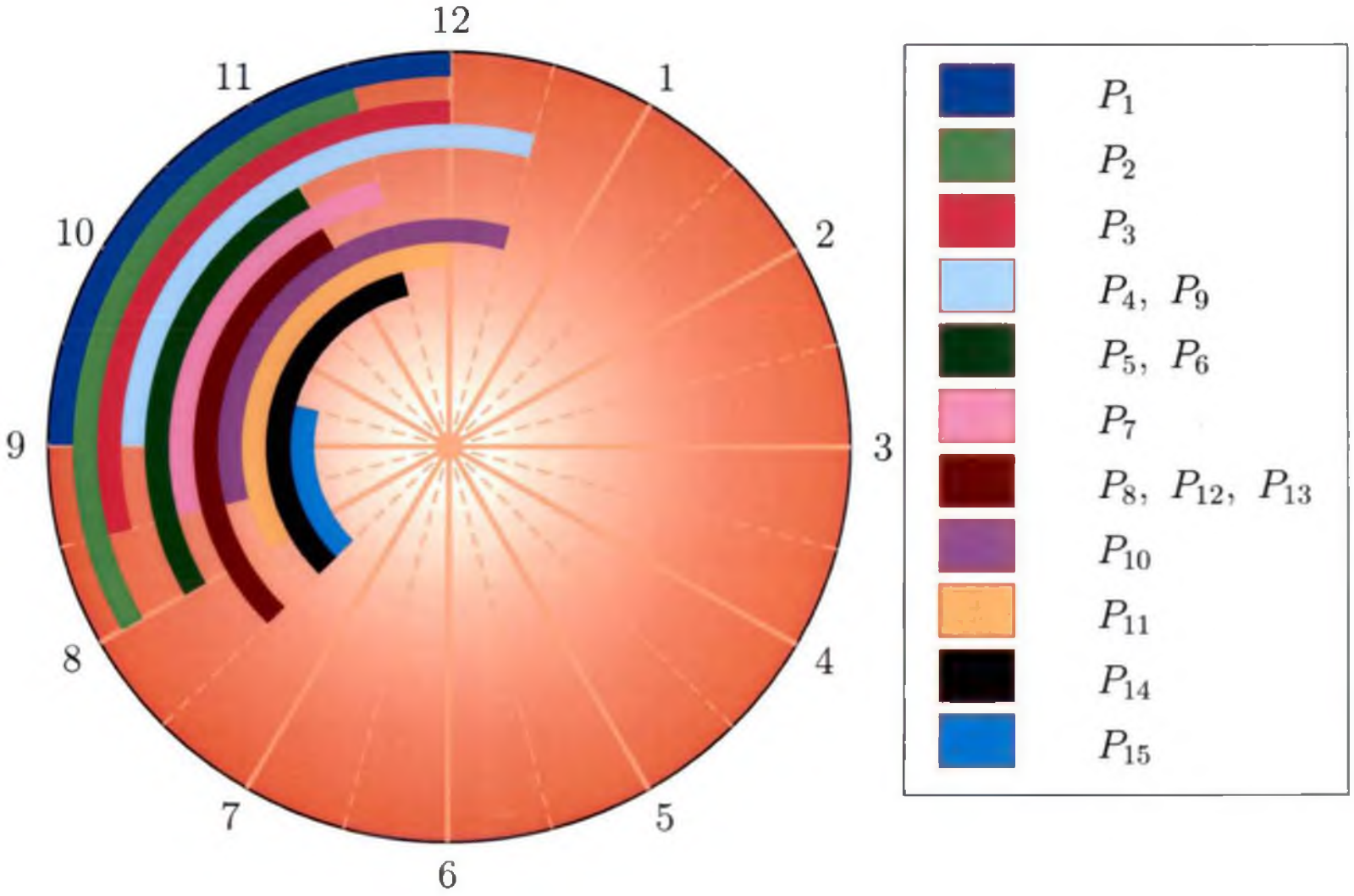


Fig. 8. Flower opening time in 15 parental monopodial orchid genotypes

4.1.3.2.b Anthesis

Anthesis time i.e., the time of maturity of pollen after flower opening was studied, based on the capacity of pollinia to effect capsule set after pollination, in each of the fifteen genotypes studied (Table 10; Fig. 9). Mean anthesis time ranged from 2.0 in P_{15} to 3.8 days in P_{11}. Anthesis time was comparatively early in P_7 (2.4 days), P_1 and P_{12} (for both 2.5 days), P_4 (2.6 days) and P_{13} (2.7 days). This was comparatively late in P_{10} (3.7 days), P_3 and P_9 (for both 3.5 days) and P_2 and P_8 (2.7 days).

4.1.3.2.c. Maximum Stigma Receptivity Period

In all the monopodials analysed, capsule set was obtained irrespective of the time of the day at which pollination was done *viz.*, morning, noon or evening. Maximum stigma receptivity period ranged from second to fourth day in P_{15} to fourth to tenth day in P_3, P_9 and P_{11} (Table 10; Fig. 10). In four genotypes tested *viz.*, P_2, P_7, P_8 and P_{10} stigma receptivity was observed from third to ninth day after flower opening. High stigma receptivity was observed from second to fifth day after flower opening in P_1 where as it was from second to sixth day in four parentals *viz.*, P_5, P_{12}, P_{13} and P_{14}.

4.1.3.3. Pollen Characters

Pollen appeared to be agglutinated in masses known as 'pollinia', as is characteristic of the family Orchidaceae. Each flower possessed a pair of pollinia and each pollinium consisted of a pair of yellow coloured oval-shaped lobes. Pollinia were situated on a conspicuous position at the tip of the column in depressions called 'clinandria', closely covered over by a lid named as 'operculum' (Plate 3). Pollen existed as tetrads, ranging in shape from spherical to rectangular in the various parental genotypes studied (Plate 4). Pollen size, pollen fertility and pollen germination were analysed among the selected fifteen parental genotypes and the results are presented in Table 11 and Fig.11.

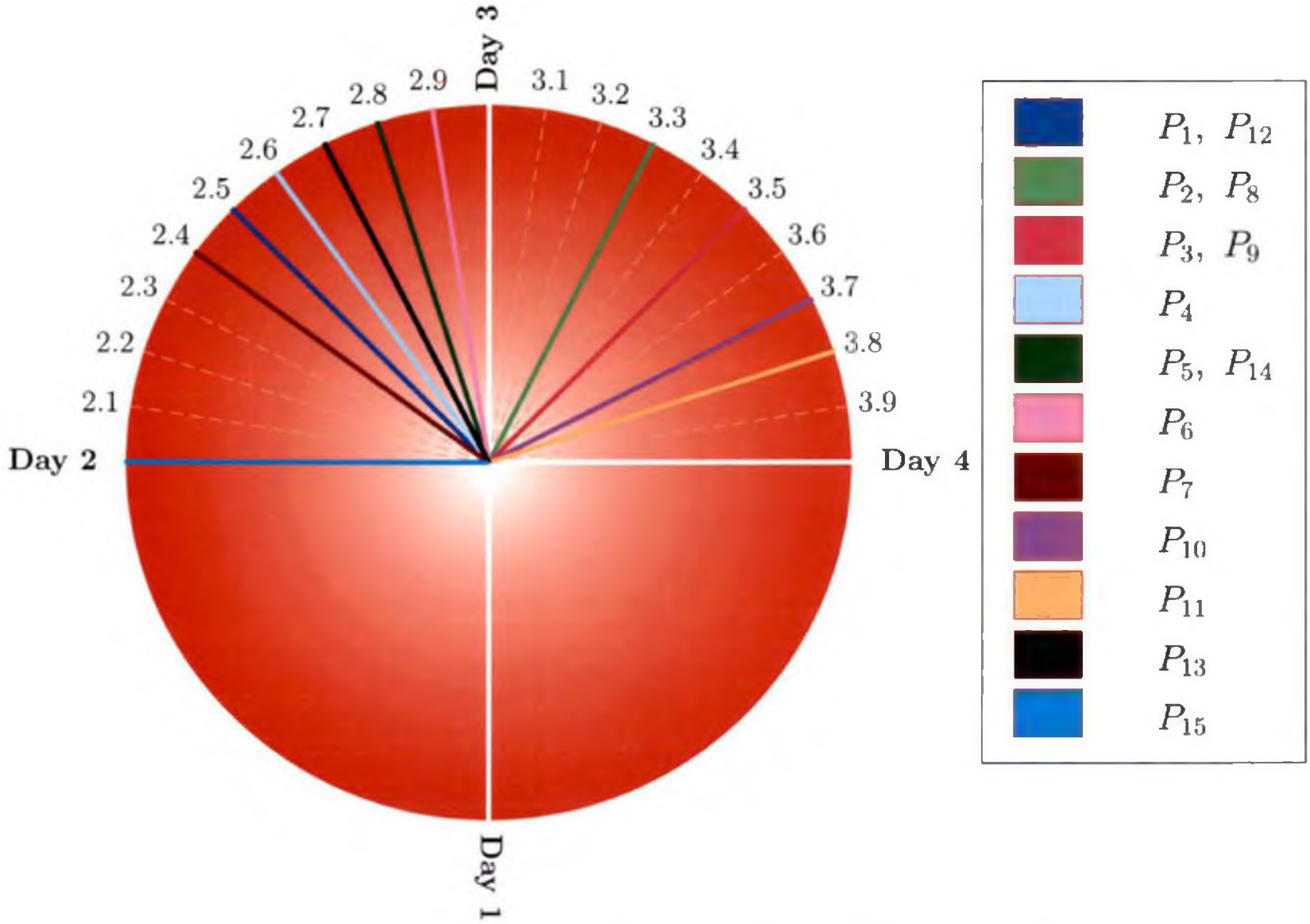


Fig. 9. Anthesis time in 15 parental monopodial orchid genotypes

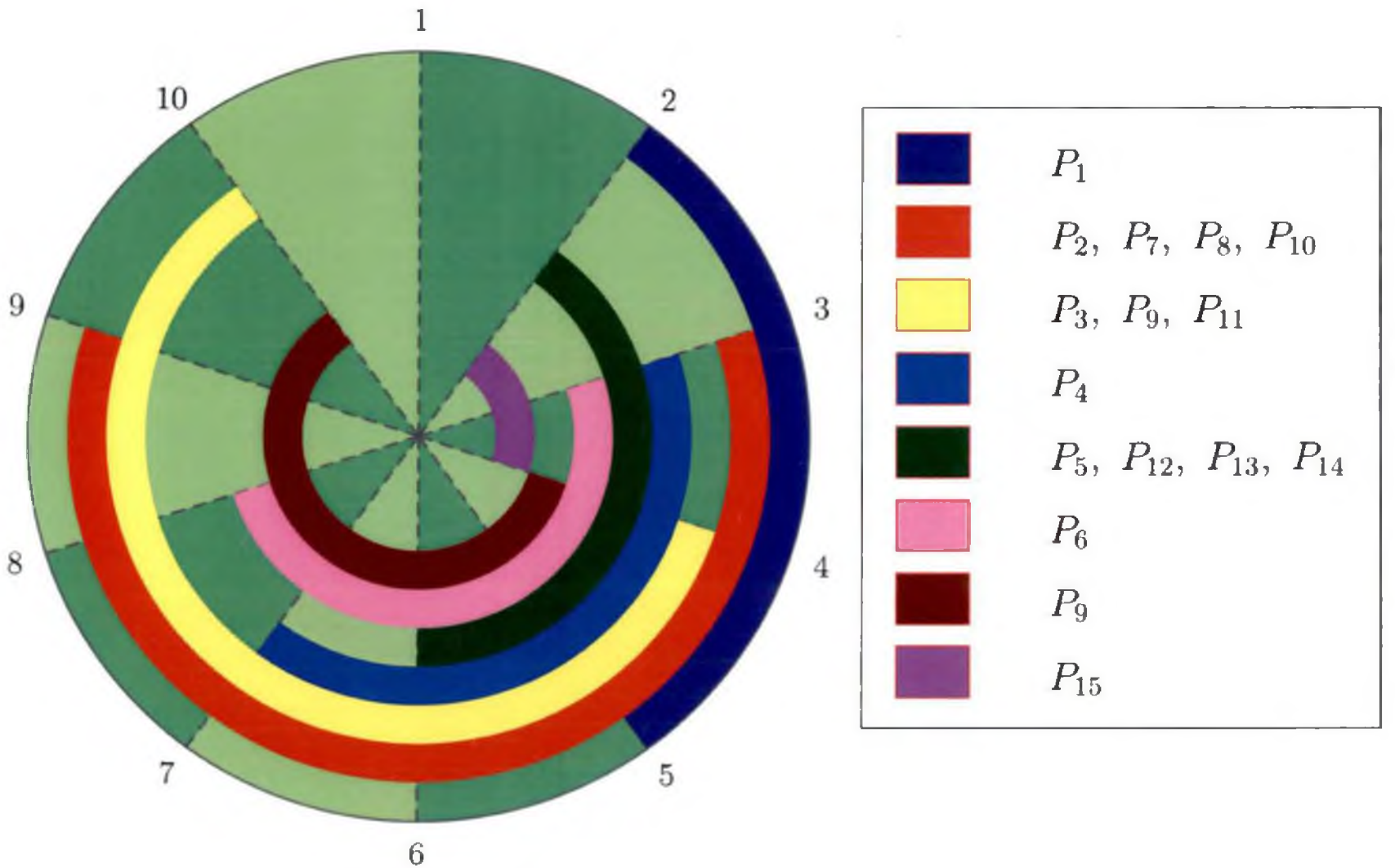


Fig. 10. Stigma receptivity period in 15 parental monopodial orchid genotypes

Table 11. Pollen characters of 15 monopodial orchid parental genotypes

Parents	Pollen size (μ)	Pollen fertility(%)	Pollen germination(%)
P_1	34.06	55.27	42.46
P_2	42.02	74.96	63.24
P_3	43.56	78.47	66.42
P_4	19.79	52.73	15.80
P_5	29.76	50.75	24.30
P_6	37.97	60.98	56.00
P_7	31.74	56.71	43.68
P_8	25.89	53.86	41.28
P_9	31.64	63.37	52.48
P_{10}	23.16	64.97	59.02
P_{11}	22.44	58.67	36.86
P_{12}	37.72	61.33	53.58
P_{13}	36.95	52.91	47.26
P_{14}	34.83	65.55	62.22
P_{15}	22.32	52.82	23.14
CD(0.05)	0.995	0.874	1.138
SE_m	0.352	0.309	0.040

4.1.3.2.a Pollen Size (μ)

Pollen size was comparatively large in the parent P_3 (43.56 μ) followed by P_2 (42.02 μ). The lowest pollen diameter was recorded in the parent P_4 (19.79 μ).

4.1.3.2.b Pollen Fertility (%)

The highest pollen fertility was recorded in P_3 (78.47%) followed by P_2 (74.96 %) while the lowest pollen fertility was recorded in P_5 (50.75 %).

4.1.3.2.c Pollen Germination (%)

Pollen germination varied significantly among the genotypes assessed. The highest pollen germination per cent was observed in P_3 (66.42 %), followed by P_2 (63.24 %), as the trend in pollen fertility. It was the lowest for P_4 (15.80 %) followed by P_{15} (23.14 %) and P_5 (24.30 %).

4.1.3.4. Selection Index

Selection index was calculated based on quantitative floral characters only. The fifteen monopodial orchid parental genotypes included in the present research programme were ranked from 1 to 15 in the descending order of the index. P_2 was ranked first, followed by P_3, P_6 and P_9 (Table 12; Fig. 12). These genotypes are more suitable for large scale commercial cultivation in our environmental conditions.

4.2. HYBRIDIZATION AND COMPATIBILITY / INCOMPATIBILITY STUDIES

Among the selected fifteen monopodial parental genotypes, intercrossing in all possible combinations was done, depending upon the availability of receptive stigma and fertile pollen. This was done with the objective of studying the compatibility / incompatibility relationships between genotypes.

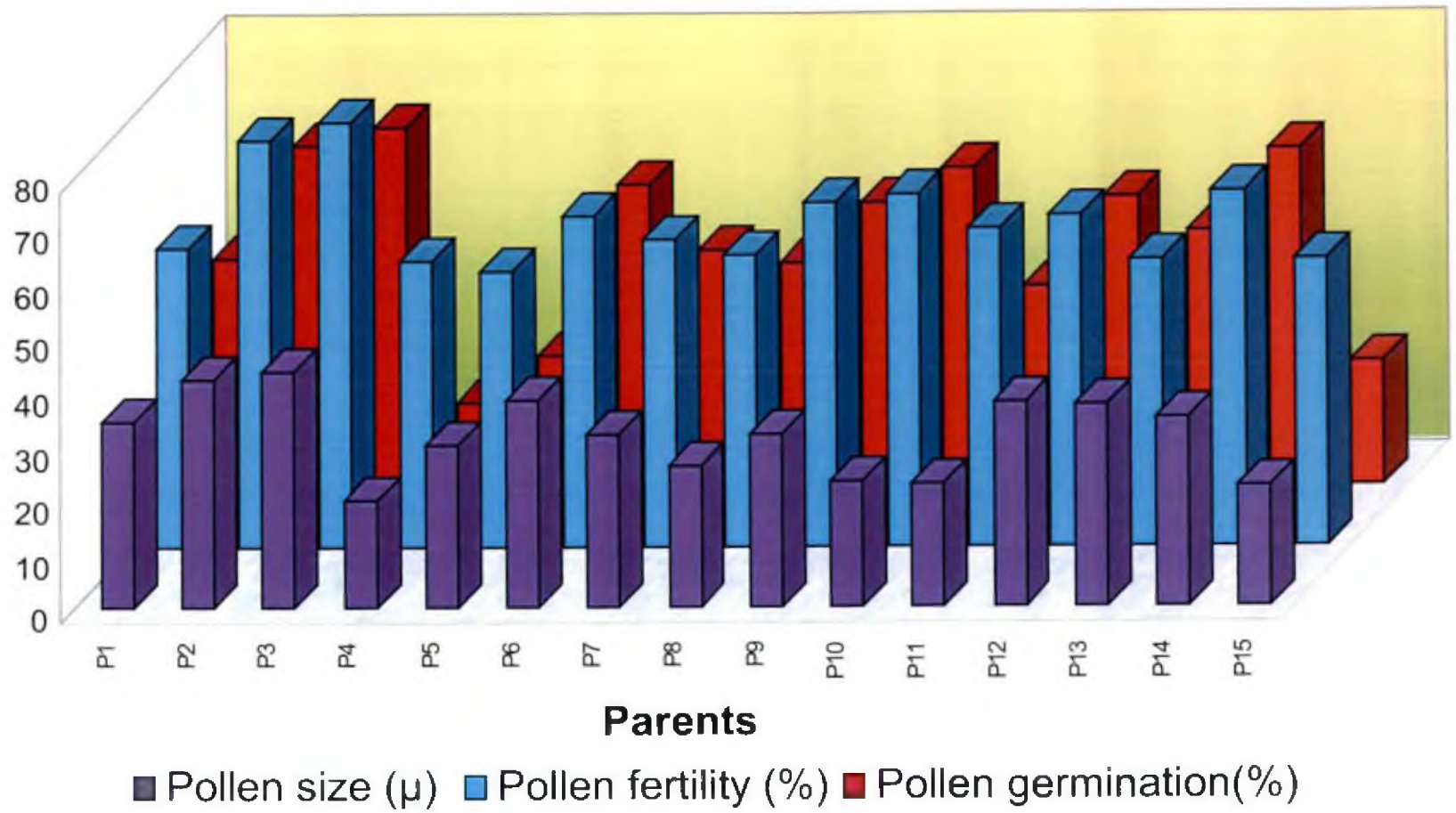
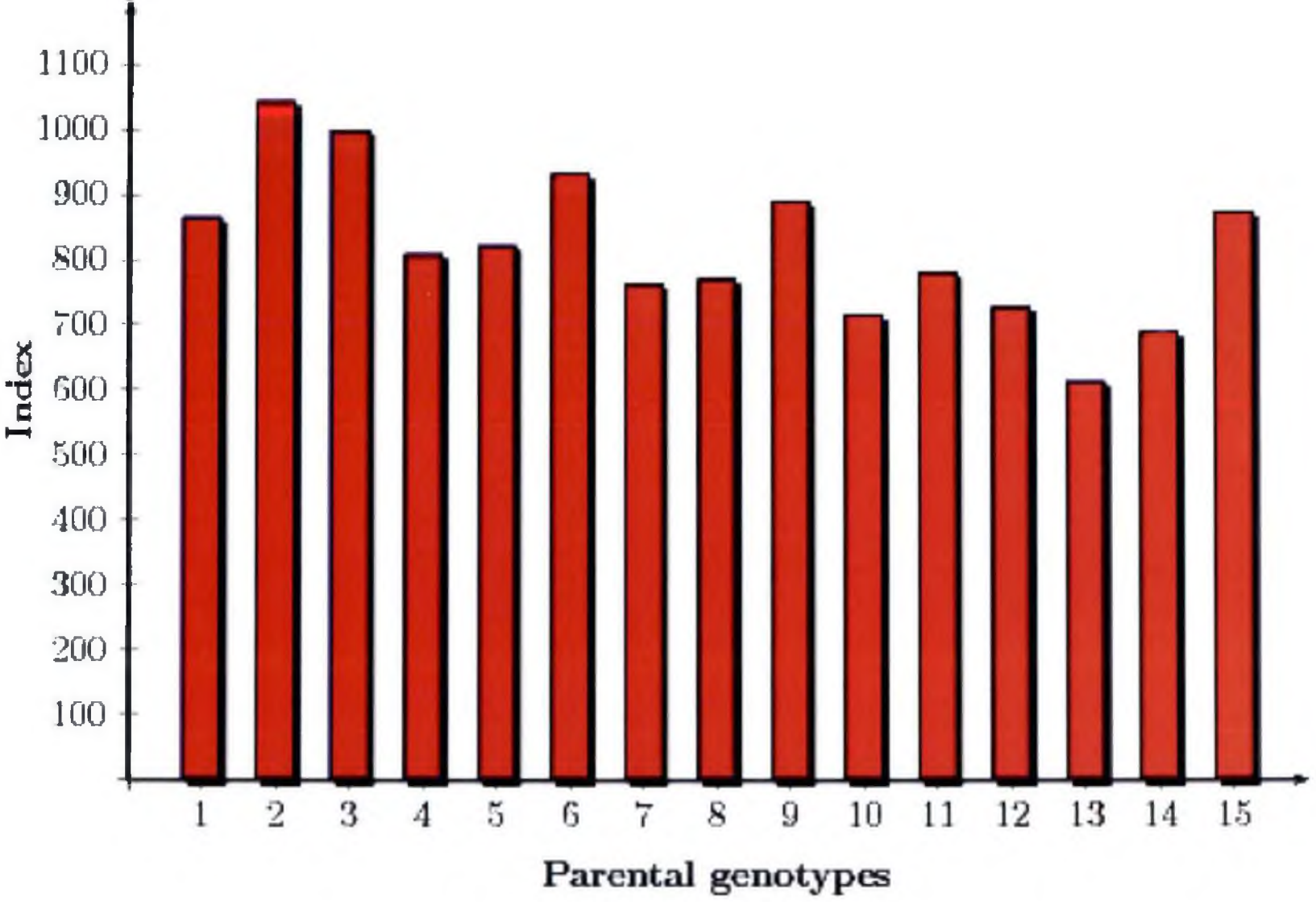


Fig. 11. Pollen characters of 15 monopodial orchid parental genotypes

Table 12. Selection index (based on floral characters only)

Parental genotypes	Index	Rank
P_1	864.84	6
P_2	1043.52	1
P_3	998.99	2
P_4	808.62	8
P_5	821.62	7
P_6	930.67	3
P_7	763.21	11
P_8	770.14	10
P_9	888.94	4
P_{10}	714.55	13
P_{11}	779.63	9
P_{12}	726.77	12
P_{13}	612.58	15
P_{14}	687.45	14
P_{15}	872.77	5


1100
1000
900
800
700
600
500
400
300
200
100
Index
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Parental genotypes

4.2.1. Diallel Crossings Attempted among the Genotypes of Monopodial Orchids

All the 225 (n^2) possible combinations were attempted in diallel pattern. These 225 combinations included 105 crosses, 105 reciprocals and 15 selfs.

4.2.2. Details of Diallel Crossings

Details of self and cross compatibility among the fifteen monopodial parental genotypes have been analysed (Table13).

Out of the 225 self and cross combinations attempted, 70 combinations succeeded in producing harvestable green capsules which included two selfs and 68 crosses. The relative success of cross was 32.38 per cent (68/210) and self was 13.33 per cent (2/15). The total estimate of success was 31.11 per cent (70/225).

Among the 70 combinations yielding capsules, 15 combinations *viz.*, P_2 x P_8, P_3 x P_5, P_3 x P_7, P_5 x P_3, P_5 x P_{12}, P_5 x P_{13}, P_5 x P_{14}, P_6 x P_{14}, P_7 x P_9, P_7 x P_{12}, P_9 x P_{13}, P_{10} x P_7, P_{11} x P_9, P_{11} x P_{13} and P_{12} x P_6 did not yield any seeds in the capsule while 55 combinations were cultured axenically.

Among the 55 combinations inoculated *in vitro,* no germination was obtained with seeds from 12 combinations *viz.*, P_2 x P_5, P_5 x P_2, P_6 x P_3, P_6 x P_9, P_7 x P_2, P_7 x P_{14}, P_9 x P_3, P_{10} x P_{13}, P_{11} x P_{12}, P_{13} x P_6, P_{14} x P_5. Successful seed germination was obtained in 43 combinations.

Out of the 43 combinations that germinated successfully, 7 combinations showed arrested development of the culture *viz.* P_2 x P_1, P_2 x P_3, P_3 x P_9, P_3 x P_{10}, P_3 x P_{13}, P_{11} x P_{14}, P_{14} x P_7.

Thus out of the total 55 combinations inoculated *in vitro* 36 combinations yielded seedlings which were deflasked successfully.

Table 13. Matrix showing compatibility relationships in diallel crossing among 15 parental genotypes of monopodial orchids

♀ ♂	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}
P_1	0	3	1	1	2	1	2	3	3	3	0	1	0	4	0
P_2	8	7	8	1	7	X	5	6	X	X	X	X	X	X	1
P_3	1	X	2	1	6	5	6	2	8	8	1	X	8	X	0
P_4	1	1	1	0	1	1	1	1	0	1	0	0	1	1	1
P_5	1	7	6	1	2	2	2	3	3	2	4	6	6	6	1
P_6	2	X	7	1	0	2	2	1	7	2	4	5	5	6	1
P_7	2	7	1	1	0	0	5	0	6	5	2	6	2	7	0
P_8	3	1	0	1	2	2	0	2	2	4	1	2	0	0	0
P_9	2	X	7	1	1	2	5	2	1	2	3	2	6	X	1
P_{10}	3	X	2	1	3	2	6	2	2	3	1	0	7	X	1
P_{11}	1	0	4	0	1	0	1	1	6	0	1	7	6	8	0
P_{12}	0	X	2	0	1	6	5	1	2	4	0	1	X	X	1
P_{13}	X	X	X	1	2	7	0	5	2	X	0	X	X	X	2
P_{14}	X	X	X	1	7	X	8	5	X	X	X	X	X	X	X
P_{15}	0	2	1	1	0	1	1	0	1	1	0	0	1	2	1

0- Combinations where pollinated flowers abscised without any change

1- Combinations where pollinated flowers abscised within two weeks

2- Combinations where pollinated flowers abscised during third and fourth weeks

3- Combinations where pollinated flowers abscised during fifth and sixth weeks

4- Combinations where pollinated flowers abscised during seventh and eighth weeks

5- Combinations where pollinated flowers abscised after two months growth

6- Combinations with no seeds in capsule

7- Combinations with no seed germination

8- Combinations lost while in culture

X- Combinations successfully deflasked

Table 14. Matrix of the 36 successful combination that yielded seedlings

♀ \ ♂	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}
P_1															
P_2						X			X	X	X	X	X	X	
P_3		X										X		X	
P_4															
P_5															
P_6		X													
P_7															
P_8															
P_9		X												X	
P_{10}		X												X	
P_{11}															
P_{12}		X											X	X	
P_{13}	X	X	X							X	-	X	X	X	
P_{14}	X	X	X			X			X	X	X	X	X	X	X
P_{15}															

4.2.3. Analysis of Compatibility

Based on compatibility analysis, the monogeneric hybrid P_{14} turned out to be the best female parent generating hardened seedlings in eleven combinations followed by P_2 and P_{13} generating hardened seedlings in seven combinations each (Table 14).

The parental genotypes P_2 and P_{14} turned out to be the best male parents generating successfully hardened seedlings in seven combinations followed by P_{12} and P_{13} yielding four successful combinations. Selfed seedlings obtained from two varieties *viz.*, P_{13} and P_{14} were deflasked successfully. None of the combinations with P_4, P_5, P_7 and P_8 as male parents yielded any viable seedlings for deflasking. Similarly no combination with P_1, P_4, P_5, P_7, P_8, P_{11} and P_{15} as the female parent could generate seedlings which reached deflasking stage.

4.2.4. Analysis of Incompatibility

The levels of incompatibility (Plate 5) were grouped under nine heads as follows:

1. Instances where pollination attempted, but flowers abscised before the onset of any visible post pollination change
2. Instances where pollinated flowers abscised within two weeks, after swelling and greening of ovary
3. Instances where pollinated flowers with swelling ovaries abscised during the third and the fourth weeks
4. Instances where developing capsules abscised during the fifth and the sixth weeks
5. Instances where capsules yellowed and decayed before maturity, abscising during the seventh and the eighth weeks
6. Instances where capsules abscised after two months growth

7. Instances where capsules harvested at normal stage, but did not contain seeds

8. Instances where capsules contained seeds which did not germinate

9. Instances where seeds germinated, but aborted while in culture

1. Instances where pollination attempted, but flowers abscised before the onset of any visible post pollination change

Female parent in cross combination		**No. of combinations**
P_1	*Aranda* Salaya Red	4
P_2	*Aranthera* Annie Black	-
P_3	*Aranthera* James Storei	1
P_4	*Kagawara* Christielow	4
P_5	*Mokara* Calypso	-
P_6	*Mokara* Chak Kuan Pink	1
P_7	*Mokara* Lumsum Sunlight	4
P_8	*Mokara* Singapore Red	5
P_9	*Mokara* Thailand Sunspot	-
P_{10}	*Mokara* Walter Oumae White	1
P_{11}	*Renanthera coccinia*	5
P_{12}	*Vanda* John Clubb	3
P_{13}	*Vanda* Popoe Diana	2
P_{14}	*Vanda* Ruby Prince	-
P_{15}	*Vanda spathulata*	5
Total		35

2. Instances where pollinated flowers abscised within two weeks, after swelling and greening of ovary

Female parent in cross combination		No. of combinations
P_1	*Aranda* Salaya Red	4
P_2	*Aranthera* Annie Black	2
P_3	*Aranthera* James Storei	3
P_4	*Kagawara* Christielow	11
P_5	*Mokara* Calypso	3
P_6	*Mokara* Chak Kuan Pink	3
P_7	*Mokara* Lumsum Sunlight	2
P_8	*Mokara* Singapore Red	3
P_9	*Mokara* Thailand Sunspot	4
P_{10}	*Mokara* Walter Oumae White	3
P_{11}	*Renanthera coccinia*	5
P_{12}	*Vanda* John Clubb	4
P_{13}	*Vanda* Popoe Diana	1
P_{14}	*Vanda* Ruby Prince	1
P_{15}	*Vanda spathulata*	8
Total		57

3. Instances where pollinated flowers with swelling ovaries abscised during the third and the fourth weeks

Female parent in cross combination		No. of combinations
P_1	*Aranda* Salaya Red	2
P_2	*Aranthera* Annie Black	-
P_3	*Aranthera* James Storei	2
P_4	*Kagawara* Christielow	-
P_5	*Mokara* Calypso	4
P_6	*Mokara* Chak Kuan Pink	4
P_7	*Mokara* Lumsum Sunlight	3
P_8	*Mokara* Singapore Red	5
P_9	*Mokara* Thailand Sunspot	5
P_{10}	*Mokara* Walter Oumae White	4
P_{11}	*Renanthera coccinia*	-
P_{12}	*Vanda* John Clubb	2
P_{13}	*Vanda* Popoe Diana	3
P_{14}	*Vanda* Ruby Prince	-
P_{15}	*Vanda spathulata*	2
Total		36

4. Instances where developing capsules abscised during the fifth and the sixth weeks

Combinations (Total = 11)	
1. P_1 x P_2	7. P_8 x P_1
2. P_1 x P_8	8. P_9 x P_{11}
3. P_1 x P_9	9. P_{10} x P_1
4. P_1 x P_{10}	10. P_{10} x P_5
5. P_5 x P_8	11. P_{10} x P_{10}
6. P_5 x P_9	

5. Instances where capsules yellowed and decayed before maturity, abscising during the seventh and the eighth weeks

Combinations (Total = 6)	
1. P_1 x P_{14}	4. P_8 x P_{10}
2. P_5 x P_{11}	5. P_{11} x P_3
3. P_6 x P_{11}	6. P_{12} x P_{10}

6. Instances where capsules abscised after two months growth

Combinations (Total = 10)	
1. P_2 x P_7	6. P_7 x P_{10}
2. P_3 x P_6	7. P_9 x P_7
3. P_6 x P_{12}	8. P_{12} x P_7
4. P_6 x P_{13}	9. P_{13} x P_8
5. P_7 x P_7	10. P_{14} x P_8

7. Instances where capsules harvested at normal stage, but did not contain seeds

Combinations (Total = 15)		
1. P_2 x P_8	6. P_5 x P_{13}	11. P_9 x P_{13}
2. P_3 x P_5	7. P_5 x P_{14}	12. P_{10} x P_7
3. P_3 x P_7	8. P_6 x P_{14}	13. P_{11} x P_9
4. P_5 x P_3	9. P_7 x P_9	14. P_{11} x P_{13}
5. P_5 x P_{12}	10. P_7 x P_{12}	15. P_{12} x P_6

8. Instances where capsules contained seeds which did not germinate

Combinations (Total = 12)	
1. P_2 x P_2	7. P_7 x P_{14}
2. P_2 x P_5	8. P_9 x P_3
3. P_5 x P_2	9. P_{10} x P_{13}
4. P_6 x P_3	10. P_{11} x P_{12}
5. P_6 x P_9	11. P_{13} x P_6
6. P_7 x P_2	12. P_{14} x P_5

9. Instances where seeds germinated, but aborted while in culture

Combinations (Total = 7)	
1. P_2 x P_1	5. P_3 x P_{13}
2. P_2 x P_3	6. P_{11} x P_{14}
3. P_3 x P_9	7. P_{14} x P_7
4. P_3 x P_{10}	

In 35 combinations (15.57 %), flowers abscised without any visible post pollination floral changes. Incompatibility was of the highest degree in these combinations where even the initial swelling of ovary following pollination was not observed.

In 57 combinations (25.33 %), abscission of pollinated flowers occurred within two weeks after pollination. The abscised flowers exhibited initial greening and swelling of ovary into capsules which indicates a reduction in the strength of incompatibility from the first level.

Pollinated flowers abscised during third and fourth weeks of growth, in 36 combinations (16.00 %), showing yellowing and decay.

Growing capsules abscised during fifth and sixth weeks of growth, in 11 combinations (4.89 %), showing yellowing. This indicates a lesser degree of incompatibility reaction.

In six combinations (2.67 %), developing capsules abscised during seventh and eighth weeks of growth, showing yellowing and decay. This again shows a lesser degree of incompatibility reaction.

In ten combinations (4.44 %), capsules abscised showing yellowing, browning and decay before maturity, after two months of capsule growth.

The capsules reached the correct stage of maturity, but when harvested they were found to be empty, without seeds, in 15 combinations (6.67 %).

Green capsules were harvested at the correct stage of maturity, contained seeds; but they failed to germinate when inoculated *in vitro,* in 12 combinations (5.33 %).

In seven combinations (3.11 %) green capsules were harvested at the proper stage, germinated *in vitro,* but later, the protocorms initiated showed declined growth rate and gradually degenerated.

The extent and strength of incompatibility reaction varied among the selected fifteen monopodial orchid parental genotypes (Table 13).

The strength of incompatibility was of the highest degree with P_4 as the male parent. Complete flower drop within two weeks from pollination was observed in all the fifteen combinations, out of which three showed flower abscission without any post pollination change. When P_{15} was used as the male parent, flower abscission before onset of any visible post pollination change, was found in five combinations.

The strength of incompatibility was of the highest degree when P_4 was used as the female parent, recording complete flower drop within two weeks from pollination in all the fifteen unsuccessful combinations out of which four showed flower abscission without any post pollination change.

The extent of incompatibility was of the highest degree when P_4, P_5, P_7 and P_8 were used as male parents, in which all the fifteen combinations failed to produce seedlings. As male parent P_{15} produced seedlings in a single combination (with P_{14}). Seedlings were deflasked and successfully planted out in two combinations each when P_1, P_3, P_6, P_9 and P_{11} were taken as male parents.

The extent of incompatibility was high in seven genotypes i.e., P_1, P_4, P_5, P_7, P_8, P_{11} and P_{15}, when used as the female parent, in which all the combinations failed to develop seedlings.

4.2.5. Details of Post Pollination Developments

The details of post pollination developments are summarized below under seven heads.

1. Post pollination floral changes
2. Stages of capsule development (Plate 5)
3. Duration for green capsule harvest in successful combinations
4. Length of capsule
5. Width of capsule
6. Percentage of capsule yield
7. Percentage of capsules with germinating seeds

1. Post Pollination Floral Changes

Following successful pollination, the sepals, petals. column and ovary underwent a series of changes during the early stages of capsule development (Table 15; Plate 5). Post pollination floral changes include drooping of perianth followed by closure of stigma by overgrowth of column tip. These changes took place rapidly i.e., within five days in P_{12}, P_{13}, P_{14} and P_{15}. Complete covering of the stigma was effected by the wilted sepals and petals. In about two weeks after pollination, complete drying of sepals and petals resulted.

2. Stages of Capsule Development

The developing capsule underwent a series of changes till harvest, following successful pollination. Greening of the ovary with slight swelling was the initial change. This was followed by swelling of the ovary into capsule (Table 16). At 60-75 per cent maturity, the capsules attained maximum length. At this stage, the ribs along the length of the capsule swelled into prominence. The capsules were harvested at 75-90 per cent maturity, which is ideal for green capsule culture. The capsules burst open by separation of capsule wall from ribs,

Table 15. Post pollination floral changes in 15 parental genotypes of monopodial orchids

Parental genotypes	Number of days from pollination for			
	Drooping of sepals and petals	Closure of stigma by over growth of column tip	Covering of stigma by wilted sepals and petals	Complete drying of sepals and petals
P_1	4 – 8	4 – 8	8 – 11	11 – 15
P_2	4 – 7	4 – 7	7 – 10	10 – 15
P_3	4 – 6	4 – 6	7 – 11	12 – 16
P_4	3 – 6	3 – 6	7 – 10	10 – 14
P_5	3 – 7	3 – 7	8 – 11	11 – 16
P_6	4 – 8	4 – 8	9 – 12	12 – 17
P_7	4 – 8	4 – 8	8 – 12	12 – 16
P_8	3 – 7	3 – 7	7 – 11	12 – 16
P_9	4 – 8	4 – 8	8 – 12	13 – 17
P_{10}	3 – 7	3 – 7	8 – 12	12 – 16
P_{11}	4 – 6	4 – 6	7 – 10	10 – 15
P_{12}	2 – 5	2 – 5	5 – 9	10 – 14
P_{13}	2 – 5	2 – 5	5 – 11	12 – 15
P_{14}	2 – 4	2 – 4	4 – 9	9 – 14
P_{15}	2 – 5	2 – 5	5 – 10	10 – 13

Table 16. Stages of capsule development in 15 parental genotypes of monopodial orchids

Parental genotypes	Number of days from pollination for				
	Greening of ovary with slight swelling	Capsule formation	Prominent ribbing of capsule	Slight flattening of capsule rib (green capsule harvest stage)	Bursting of capsule beginning from tip
P_1	5 – 8	–	–	–	–
P_2	5 – 8	85 – 100	94 – 100	108 – 120	126 – 140
P_3	4 – 7	55 – 75	68 – 75	80 – 92	98 – 105
P_4	5 – 7	–	–	–	–
P_5	4 – 6	80 – 92	87 – 92	98 – 102	104 – 109
P_6	5 – 9	85 – 98	93 – 98	104 – 110	114 – 118
P_7	5 – 8	60 – 82	75 – 82	87 – 95	98 – 106
P_8	4 – 7	65 – 80	–	–	–
P_9	5 – 9	85 – 97	92 – 97	105 – 110	113 – 119
P_{10}	4 – 8	82 – 95	90 – 95	102 – 108	120 – 113
P_{11}	5 – 7	80 – 92	87 – 92	97 – 105	109 – 114
P_{12}	5 – 8	70 – 88	83 – 88	94 – 101	106 – 110
P_{13}	4 – 6	63 – 82	75 – 82	88 – 97	105 – 112
P_{14}	5 – 8	75 – 98	92 – 98	104 – 109	115 – 121
P_{15}	6 – 9	–	–	–	–

Plate 5. Stages of capsule development and incompatibility reactions

Stages of capsule development

Drooping of sepals and petals

4 days after pollination

Mokara Thailand Sunspot x *Aranthera* Annie Black

Covering of stigma by wilted sepals and petals

10 days after pollination

Mokara Chak Kuan Pink x *Aranthera* Annie Black

Complete drying of sepals and petals

18 days after pollination

Aranthera Annie Black x *Aranthera* James Storei

Ovary swelling into capsule

35 days after pollination

Aranthera Annie Black x *Mokara* Walter Oumae White

Enlargement of capsule

50 days after pollination

Mokara Thailand Sunspot x *Vanda* Ruby Prince

65 days after pollination

Mokara Walter Oumae White x *Vanda* Ruby Prince

Plate 5

Plate 5. Continued

Maturation of capsule

76 days after pollination

Aranthera Annie Black x *Mokara* Chak Kuan Pink

Prominent ribbing of capsule

90 days after pollination

Mokara Walter Oumae White x *Aranthera* Annie Black

Harvested mature capsule

101 days after pollination

Aranthera Annie Black x *Mokara* Walter Oumae White

Incompatibility – Shrivelling, browning and capsule drop

51 days after pollination

Mokara Chak Kuan Pink x *Renanthera coccinia*

Incompatibility – Yellowing, browning, decay and capsule drop

72 days after pollination

Mokara Lumsum Sunlight x *Mokara* Walter Oumae White

Incompatibility – No seed in mature capsule

100 days after pollination

Vanda John Clubb x *Mokara* Chak Kuan Pink

Plate 5 Continued

beginning near the tip of the capsule and proceeding backwards, if left unharvested.

3. Duration for Green Capsule Harvest in Successful Combinations

Time taken for the harvest of green capsules in compatible combinations (70/225) was analysed (Table 17; Fig.13).

Duration to green capsule harvest ranged from 69.3 days in P_3 x P_5 to 118 days in P_2 x P_{13} in individual crosses.

The average time taken for the harvest of green capsules ranged from 82.3 (P_3) to 107.4 (P_2) days when the genotypes were used as female parents. The variability ranged between 4.2 (P_{14}) and 10.2 (P_3) per cent.

When the same genotypes were used as male parents, the duration ranged from 92.0 (P_5) to 111.3 (P_8) days and the variability ranged from 6.0 (P_1) to 19.5 (P_5) per cent.

4. Length of Capsule

Length of green capsule ranged from 3.4 cm in P_{11} x P_{12} to 8.8 cm in P_{12} x P_{13} in individual crosses (Table 18; Fig.14). This trait was high in P_{12} x P_{14} (8.6 cm), P_{12} x P_2 (8.4 cm) and P_{14} x P_{14} (8.3 cm).

Mean length of green capsules ranged from 4.6 cm in P_2 and P_{11} to 8.1 cm in P_{12} when the genotypes were used as female parents. The variability ranged from 5.9 (P_7) to 22.5 (P_3) per cent.

When the same genotypes were used as male parents, capsule length ranged from 4.1 cm in P_8 to 6.2 cm in P_2 and the variability ranged from 9.6 (P_7) to 33.9 (P_{11}) per cent.

Table 17. Duration for green capsule harvest (days) in successful combinations

♀ \ ♂	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}	$\bar{x}$	C.V.(%)
P_1																	
P_2	96.3	106.0	120.3		108.7	99.3		111.3	107.3	101.3	116	99.0	118.7	104.0		107.4	7.2
P_3		90.3			69.3		79.3		87.7			72.7	92.3	84.7		82.3	10.2
P_4																	
P_5		97.3	89.7									101.3	91.3	98.3		95.6	4.8
P_6		106.3	98.7						110.7			107.3	98.3	103.0		104.1	4.5
P_7		83.7							74.7			91.7		95.3		86.4	9.7
P_8																	
P_9		109.7	93.3										106.7	100.0		102.4	6.5
P_{10}		95.0					109.3						98.3	102.3		101.2	5.6
P_{11}		100.3							97.3			106.0	93.3	98.7		99.1	4.4
P_{12}		96.3				100.0							85.0	92.3		93.4	6.4
P_{13}	85.0	92.3	98.0			76.7				80.7		88.7	94.3	77.7		86.7	8.8
P_{14}	95.7	107.7	103.7		98.0	109.0	101.3		98.3	106.0	99.3	99.7	106.7	102.7	101.3	102.3	4.2
P_{15}																	
$\bar{x}$	92.3	98.6	100.6		92.0	96.3	96.7	111.3	96.0	96.0	107.7	95.8	98.5	96.3	101.3		
C.V.(%)	6.0	8.1	10.1		19.5	13.0	13.9	0.0	13.0	12.2	8.5	11.3	9.6	8.5	0.0		

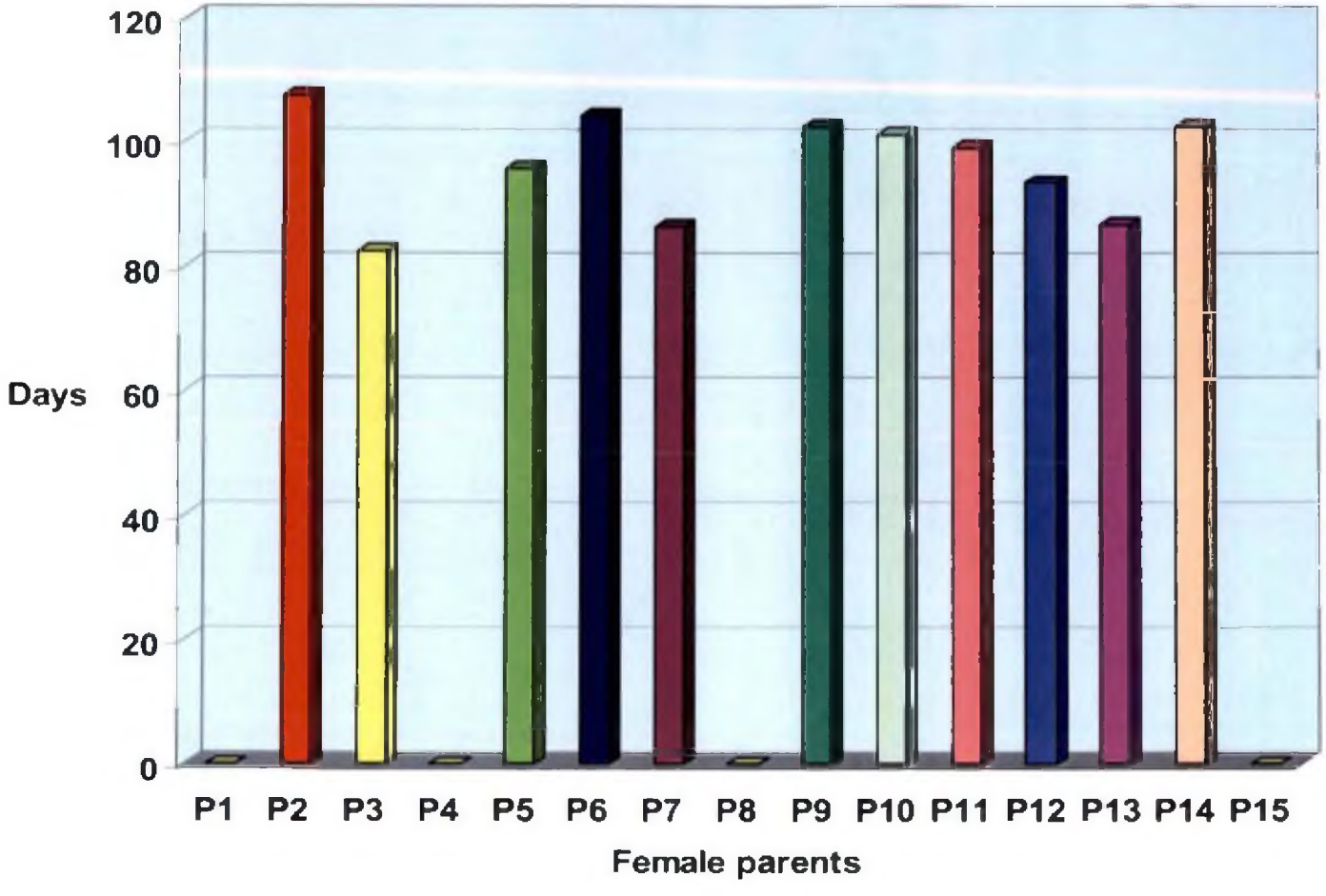


Fig. 13. Duration to green capsule harvest (days) in successful combinations

5. Width of Capsule

Width of green capsule ranged from 0.9 cm in P_3 x P_2, P_{11} x P_9 and P_{11} x P_{12} to 2.2 cm in P_{11} x P_2 (Table 19). This was high in P_3 x P_2 (2.1 cm) also.

Mean width of green capsules ranged from 1.2 cm (P_2) to 1.5 cm (P_9 and P_{12}) when the genotypes were used as female parents. Coefficient of variation was observed to range from 4.4 (P_{12}) to 38.2 (P_{11}) per cent for the character.

When the same genotypes were used as male parents, width of capsule registered a range from 1.1 cm in P_8 to 1.5 cm in P_2 and the variability ranged from 5.5 (P_1) to 23.0 (P_2) per cent.

6. Percentage of Capsule Set

Percentage capsule set from 70 successful combinations were assessed and it ranged from seven in P_2 x P_5 and P_2 x P_6 to 40 in P_{12} x P_{14} and P_{14} x P_{13} (Table 20), in individual combinations.

Mean percentage capsule set ranged from 14.5 in P_{10} to 28.2 in P_{12} with a coefficient of variation ranging from zero in P_4 to 50.1 per cent in P_2 when the genotypes were used as female parents.

When the genotypes were used as male parents, mean percentage capsule set ranged from 10.8 in P_6 to 27.8 in P_{14} and the coefficient of variation ranged from zero in P_8 and P_{15} to 55.3 per cent in P_5.

7. Percentage of Capsules with Germinating Seeds

Green capsules harvested did not contain seeds in 15 combinations (out of 70). When the remaining 55 combinations were cultured *in vitro*, percentage capsules with germinating seeds in individual combinations ranged from 25 in P_{14} x P_9, P_{14} x P_{13} and P_{14} x P_{14} to 100 in P_2 x P_6, P_6 x P_3, P_9 x P_2, P_2 x P_{13}, P_{11} x P_{12}, P_{13} x P_2, P_{13} x P_{13}, P_{14} x P_6 and P_{14} x P_{12} (Table 21).

Table 18. Average length (cm) of green capsules harvested from successful combinations

♀ \ ♂	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}	$\bar{x}$	C.V.(%)
P_1																	
P_2	4.3	5.2	4.5		5.1	4.3		4.1	4.6	4.6	4.2	4.5	4.0	5.2		4.6	8.8
P_3		7.5			4.4		5.3		4.7			4.1	4.1	5.0		5.0	22.5
P_4																	
P_5		4.6	5.2									5.4	4.4	5.7		5.1	10.1
P_6		5.5	6.1						5.2			4.8	4.9	5.2		5.3	8.4
P_7		5.1							4.5			5.1		4.8		4.9	5.9
P_8																	
P_9		6.4	5.6										5.4	6.7		6.0	9.7
P_{10}		6.1					4.9						5.1	5.3		5.4	8.9
P_{11}		6.1							4.2			3.4	4.6	4.5		4.6	19.8
P_{12}		8.4				6.7							8.8	8.6		8.1	10.6
P_{13}	7.3	5.4	7.1			5.3				6.4		7.2	7.1	7.0		6.6	11.9
P_{14}	5.3	8.0	7.3		5.9	6.1	6.1		6.6	5.6	8.0	5.6	6.2	8.3	4.8	6.0	15.6
P_{15}																	
$\bar{x}$	5.6	6.2	6.0		5.1	5.6	5.4	4.1	5.0	5.5	6.1	5.0	5.5	6.0	4.8		
C.V.(%)	23.1	19.6	16.9		12.5	16.9	9.6	0.0	16.5	14.3	33.9	21.5	26.7	22.6	0.0		

Table 19. Average width (cm) of green capsules harvested from successful combinations

♀ \ ♂	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}	$\bar{x}$	C.V.(%)
P_1																	
P_2	1.2	1.2	0.9		1.2	1.2		1.1	1.2	1.3	1.2	1.2	1.3	1.3		1.2	8.7
P_3		2.1			1.3		1.4		1.2			1.2	1.2	1.2		1.4	24.2
P_4																	
P_5		1.4	1.3									1.3	1.5	1.5		1.4	7.6
P_6		1.4	1.5						1.2			1.5	1.3	1.3		1.4	8.9
P_7		1.2							1.1			1.3	1.4	1.4		1.3	9.0
P_8																	
P_9		1.4	1.4										1.6	1.6		1.5	6.8
P_{10}		1.6					1.3						1.3	1.3		1.4	10.6
P_{11}		2.2							0.9			0.9	1.2	1.2		1.3	38.2
P_{12}		1.5				1.5							1.5	1.5		1.5	4.4
P_{13}	1.3	1.3	1.1			1.4				1.4		1.3	1.1	1.1		1.3	11.2
P_{14}	1.3	1.2	1.2		1.5	1.0	1.5		1.2	1.5	1.5	1.1	1.5	1.5	1.3	1.3	13.0
P_{15}																	
$\bar{x}$	1.3	1.5	1.3		1.3	1.3	1.4	1.1	1.2	1.4	1.4	1.2	1.3	1.4	1.3		
C.V.(%)	5.5	23.0	15.1		12.1	16.0	7.3	0.0	10.3	7.3	13.9	13.0	12.0	12.3	0.0		

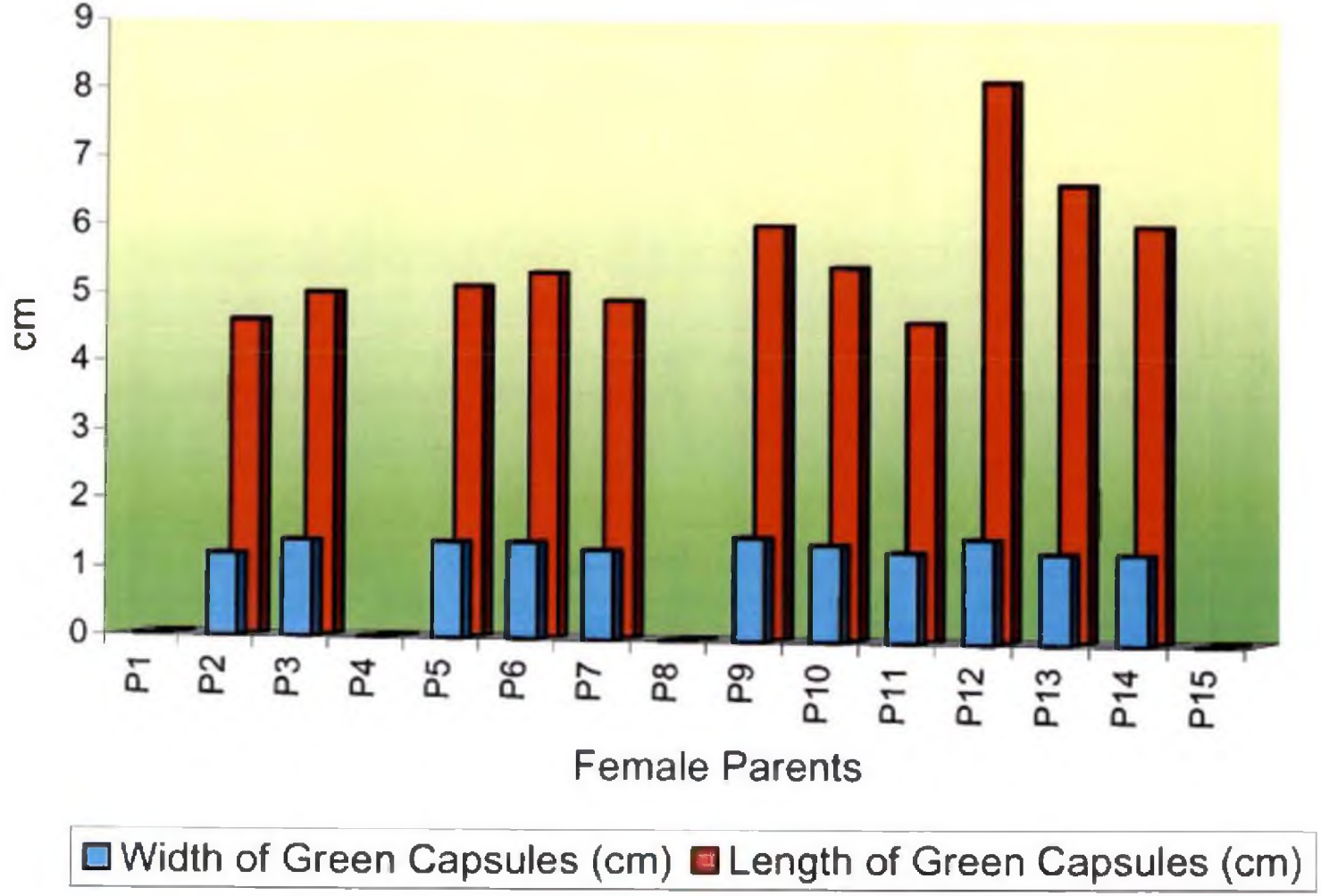

9
8
7
6
5
4
3
2
1
0
cm
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
P14
P15
Female Parents
Width of Green Capsules (cm)
Length of Green Capsules (cm)

Table 20. Capsule set in successful combinations (%)

♀ \ ♂	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}	$\bar{x}$	C.V.(%)
P_1																	
P_2	14	27	20		7	7		13	36	18	17	33	29	38		21.6	50.1
P_3		17			8		14		25			30	25	31		21.4	40.2
P_4			22													22.0	0.0
P_5		21	9									15	12	25		16.4	39.9
P_6		20							13			17	13	30		18.6	37.7
P_7		19	15						18			23		14		17.8	20.0
P_8																	
P_9		17											12	23		17.3	31.8
P_{10}		18					8						13	19		14.5	35.0
P_{11}		22							25			22	25	15		21.8	18.8
P_{12}		13				22							38	40		28.2	45.9
P_{13}	23	38	20			6				13		18	29	33		22.5	47.0
P_{14}	17	36	33		18	8	25		33	23	20	22	40	38	12	25.0	40.8
P_{15}																	
$\bar{x}$	18.0	22.6	19.8		11.0	10.8	15.7	13.0	25.0	18.0	18.5	22.5	23.6	27.8	12.0		
C.V.(%)	25.4	35.3	40.3		55.3	70.1	55.0	0.0	34.8	27.8	11.5	27.8	45.4	33.4	0.0		

Table 21. Capsules with germinating seeds (%)

♀ \ ♂	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}	$\bar{x}$	C.V.(%)
P_1																	
P_2	50	33	33			100			75	50	50	67	100	67		62.5	38.5
P_3		50							67			33	50	25		45.0	36.5
P_4																	
P_5		33														33.0	0.0
P_6		50	100						50							66.7	43.3
P_7		33							33							33.0	0.0
P_8																	
P_9		100												33		66.5	71.3
P_{10}		50												67		58.5	20.6
P_{11}		50										100	50	50		62.5	40.0
P_{12}		100											33	75		69.3	48.8
P_{13}	33	33	50							50		50	100	33		49.9	47.5
P_{14}	50	75	33			100	67		25	67	50	100	25	33	50	56.3	46.6
P_{15}																	
$\bar{x}$	44.3	55.2	54.0			100.0	67.0		50.0	55.7	50.0	70.0	59.7	47.9	50.0		
C.V.(%)	22.1	46.2	58.7			0.0	0.0		42.8	17.6	0.0	42.7	54.8	40.7	0.0		

Mean percentage of capsules with germinating seeds ranged from 33 in P_5 and P_7 to 69.3 in P_{12} with a coefficient of variation ranging from zero in P_5 and P_7 to 71.3 per cent in P_9 when the genotypes were used as female parents.

When the genotypes were used as male parents, mean percentage of capsules with germinating seeds ranged from 44.3 in P_1 to 100 in P_6 and the coefficient of variation ranged from zero in P_6, P_7, P_{11} and P_{15} and to 58.7 per cent in P_3.

4.3. *IN VITRO* CULTURE OF HYBRID SEEDS

4.3.1. Selection of culture media

Effect of media on *in vitro* growth of seedlings in monopodial orchid hybrids were studied using the hybrid combination P_{10} x P_2. Observations were taken at six months after inoculation and the data were analysed statistically. The results are presented in Table 22 and Fig. 15.

1. Height of Seedling (cm)

Significantly high value for height of seedling (3.76 cm) was recorded in MS half strength medium followed by MS quarter strength medium (3.44 cm) which was on par with MS full strength medium (3.12 cm). The value for this trait was lowest in KC medium (2.48 cm).

2. Number of Leaves

The highest number of leaves of 3.6 was recorded in MS half strength medium which was on par with MS quarter strength medium (3.2) and MS full strength medium (2.8). This was the lowest in KC medium.

Table 22. Effect of media on *in vitro* growth of seedlings in monopodial orchid hybrids

Combination : $P_{10} \times P_2$
Period : six months after inoculation

Sl. No.	Medium	Height of seedling (cm)	No. of leaves	Length of leaves (cm)	No. of roots	Length of root (cm)
1.	KC (full strength)	2.48	2.20	1.18	2.00	1.44
2.	MS (1/4 strength)	3.44	3.20	1.78	3.00	1.80
3.	MS (1/2 strength)	3.76	3.60	2.16	3.40	2.08
4.	MS (full strength)	3.12	2.80	1.62	2.80	0.86
5.	VW (full strength)	2.78	2.40	1.36	2.00	1.26
SE_m		0.043	0.219	0.038	0.244	0.032
CD (0.05)		0.128	0.646	0.113	0.723	0.095

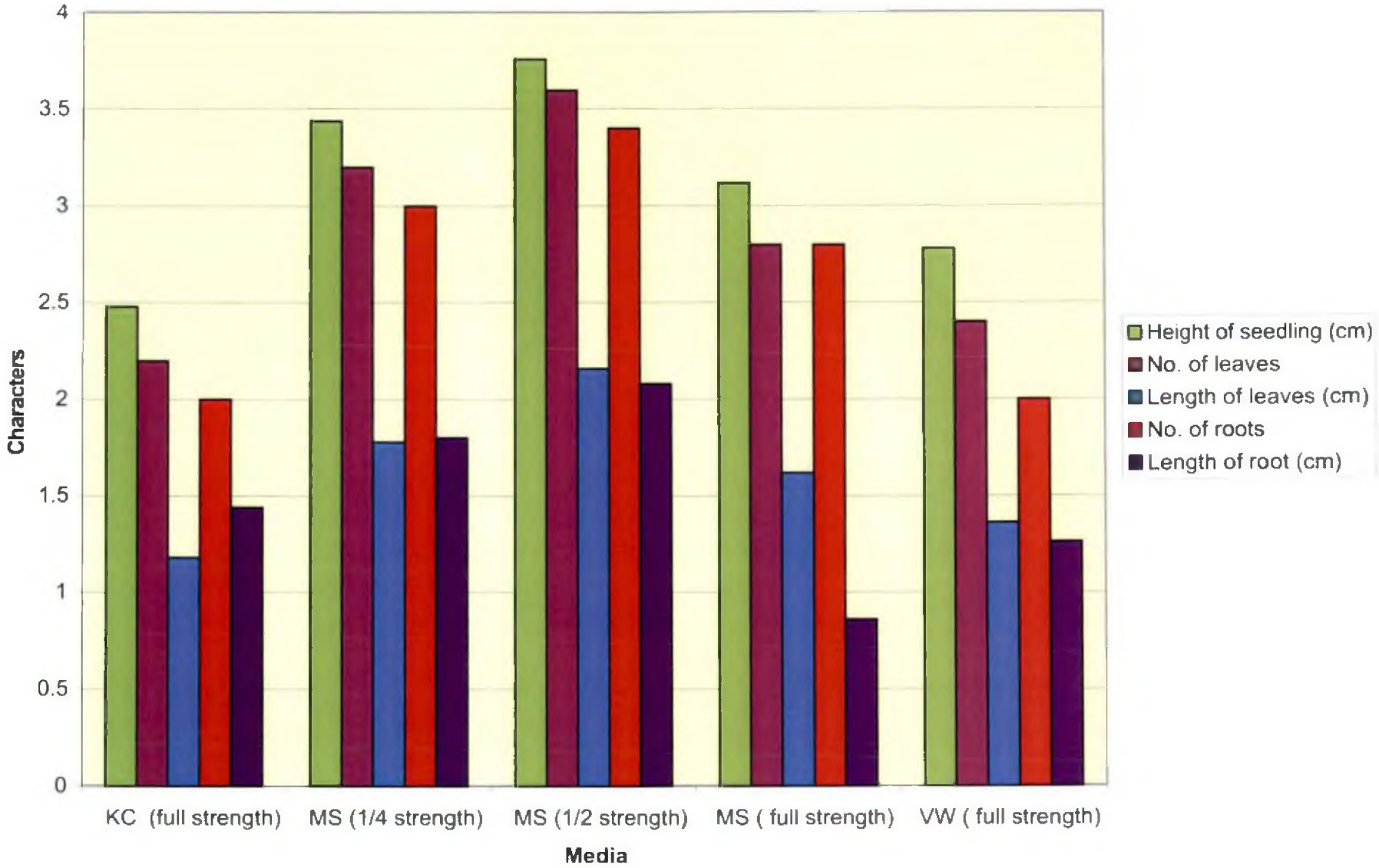


Fig. 15. Effect of media on *in vitro* growth of seedlings in monopodial orchid hybrids

3. Length of Leaves (cm)

Maximum length of leaves was observed in MS half strength medium with a significantly high value of 2.16 cm. This was minimum in KC medium.

4. Number of Roots

The highest number of roots were produced when the hybrid seedlings were inoculated in MS half strength medium with a significantly high value of 3.4, followed by MS quarter strength medium (3.0).

5. Length of Root (cm)

Mean length of roots were significantly high (2.08 cm) in MS half strength medium. Shortest root was observed in MS full strength medium with a value of 0.86 cm.

4.3.2. Refinement of Culture Media

Effect of BA, IAA and NAA on *in vitro* growth of seedlings in monopodial orchid hybrids was evaluated in detail using the hybrid combination P_{10} x P_2. Observations on various morphological characters were recorded six months after inoculation and statistical analysis was carried out. The results of this media refinement study are presented below (Table 23; Fig. 16).

1. Height of Seedling (cm)

Significantly high value (4.24 cm) of seedling height was recorded in T_5 followed by T_2. Shortest seedlings (2.80 cm) were developed by T_3.

Table 23. Effect of BA, IAA and NAA on *in vitro* growth of seedlings in monopodial orchid hybrids

Medium : MS half strength

Combination : $P_{10} \times P_2$
Period : six months after inoculation

Treatments	Concentration (mg l^{-1}) of			Height of seedling (cm)	No. of leaves	Length of leaves (cm)	No. of roots	Length of root (cm)
	BA	IAA	NAA					
T_1	0	0	0	3.20	2.80	1.58	2.60	1.50
T_2	8	2	0	4.04	3.80	2.30	4.80	2.32
T_3	8	0	2	2.80	2.00	1.26	2.20	1.32
T_4	0	2	8	3.86	3.60	2.04	3.80	2.04
T_5	0	8	2	4.24	4.60	2.44	4.20	2.20
T_6	0	10	0	3.56	3.00	1.80	3.00	1.78
T_7	0	0	10	3.10	2.40	1.46	2.60	1.08
SE_m				0.033	0.227	0.031	0.262	0.032
CD (0.05)				0.094	0.657	0.090	0.758	0.095

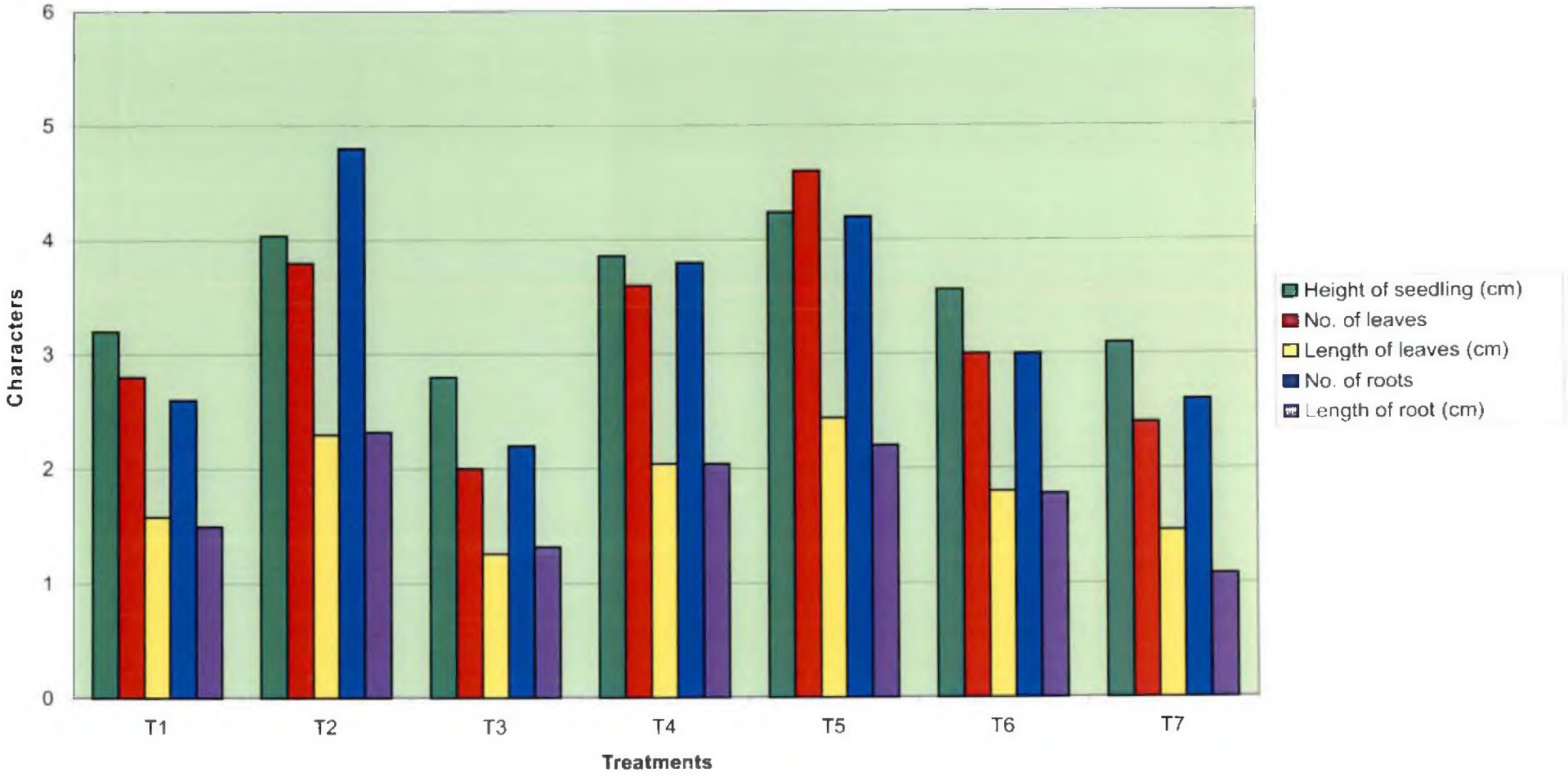


Fig. 16. Effect of BA, IAA and NAA on *in vitro* growth of seedlings in monopodial orchid hybrids

2. Number of Leaves

Maximum number of leaves i.e., 4.6 was produced in T_5 followed by T_2 (3.8) which was on par with T_4 with a value of 3.6. Number of leaves (2.0) was minimum in T_2.

3. Length of Leaves (cm)

The longest leaves were formed when hybrid seeds were cultured in T_5 with a length of 2.44 cm which was observed to be statistically on par with T_2 (2.30 cm).

4. Number of Roots

The highest number of roots were found in T_2 (4.8). This was followed by T_5 (4.2) and T_4 (3.8). Number of roots was the lowest in T_3 (2.2).

5. Length of Root (cm)

Significantly high values for root length were registered in T_2 (2.32 cm) followed by T_5 (2.20 cm) and T_4 (2.04 cm). This was lowest in T_7 (1.08 cm).

4.3.3. Rate of Attainment of Different *In vitro* Developmental Stages in Successful Combinations

The details of embryo culture of hybrid seeds obtained from 43 successful combinations were analysed (Table 24; Plate 6). Significant differences could be observed between the hybrid combinations with respect to the time taken to attain each of the different *in vitro* developmental stages.

1. Days for Initiation of Germination

The number of days taken for germination initiation was found to be significantly low in $P_{13} \times P_{13}$ (20.20 days). This was low for $P_{14} \times P_{14}$ (22.20) also

Table 24. Details of *in vitro* culture of hybrid seeds obtained from 43 successful combinations

Sl. No	Cross combination	Number of days to germination initiation	Number of days taken for the development of					No.of days for planting out
			Protocorm	Chlorophyll	First leaf primordium	First shoot primordium	First root primordium	
1	$P_2 \times P_1$	26.00	43.20	-	-	-	-	-
2	$P_2 \times P_3$	28.60	43.00	65.40	-	-	-	-
3	$P_2 \times P_6$	27.20	44.20	61.80	76.80	118.00	131.60	219.60
4	$P_2 \times P_9$	25.80	42.80	56.60	81.20	137.80	156.60	247.00
5	$P_2 \times P_{10}$	31.60	60.00	73.80	83.60	136.80	160.60	254.00
6	$P_2 \times P_{11}$	28.20	53.00	69.20	81.00	136.40	156.20	254.00
7	$P_2 \times P_{12}$	28.20	50.80	69.20	85.20	142.80	166.20	258.40
8	$P_2 \times P_{13}$	32.00	54.00	73.20	92.60	147.00	171.20	265.20
9	$P_2 \times P_{14}$	29.20	40.20	54.00	73.60	108.80	134.00	196.40
10	$P_3 \times P_2$	33.80	52.40	70.20	87.00	123.20	143.40	216.80
11	$P_3 \times P_9$	37.00	55.20	78.40	-	-	-	-
12	$P_3 \times P_{10}$	39.00	58.60	-	-	-	-	-
13	$P_3 \times P_{12}$	34.00	61.20	75.00	91.80	130.40	154.40	235.40

Table 24. Continued

Sl. No	Cross combination	Number of days to germination initiation	Number of days taken for the development of					No.of days for planting out
			Protocorm	Chlorophyll	First leaf primordium	First shoot primordium	First root primordium	
14	$P_3 \times P_{13}$	34.80	62.20	-	-	-	-	-
15	$P_3 \times P_{14}$	31.00	44.80	60.80	76.60	105.20	130.00	197.00
16	$P_6 \times P_2$	41.00	60.80	82.00	99.20	148.40	171.60	256.40
17	$P_9 \times P_2$	41.00	64.60	84.00	100.60	152.00	174.80	262.20
18	$P_9 \times P_{14}$	38.20	63.20	80.20	100.00	149.00	172.00	275.40
19	$P_{10} \times P_2$	42.80	65.20	80.00	99.80	148.60	173.60	279.20
20	$P_{10} \times P_{14}$	37.80	61.40	80.60	100.60	149.00	177.80	283.80
21	$P_{11} \times P_{14}$	27.40	51.00	-	-	-	-	-
22	$P_{12} \times P_2$	29.80	51.60	67.80	81.60	131.00	157.20	261.20
23	$P_{12} \times P_{13}$	30.40	56.20	72.60	90.80	138.00	164.00	266.40
24	$P_{12} \times P_{14}$	28.80	54.00	71.00	81.60	129.20	154.00	259.20
25	$P_{13} \times P_1$	32.80	53.40	64.80	90.80	142.60	170.60	271.40
26	$P_{13} \times P_2$	31.60	50.80	67.40	84.80	126.20	144.60	224.80
27	$P_{13} \times P_3$	27.20	47.20	62.80	82.00	120.80	144.60	230.80
28	$P_{13} \times P_9$	35.60	62.00	78.00	98.20	149.40	168.20	269.60
29	$P_{13} \times P_{10}$	37.60	64.40	83.20	98.80	145.40	167.80	270.40

Table 24. Continued

Sl. No	Cross combination	Number of days to germination initiation	Number of days taken for the development of					No.of days for planting out
			Protocorm	Chlorophyll	First leaf primordium	First shoot primordium	First root primordium	
30	$P_{13} \times P_{12}$	25.20	50.60	64.60	88.40	134.80	156.00	249.20
31	$P_{13} \times P_{13}$	20.20	40.60	62.60	87.40	131.00	151.80	242.20
32	$P_{13} \times P_{14}$	35.00	56.60	72.20	96.20	128.80	146.00	220.80
33	$P_{14} \times P_{1}$	28.00	47.40	60.80	88.00	117.80	140.60	217.80
34	$P_{14} \times P_{2}$	29.80	51.20	69.80	87.60	120.20	141.20	225.80
35	$P_{14} \times P_{3}$	29.60	48.00	61.80	80.60	114.40	139.80	213.60
36	$P_{14} \times P_{6}$	32.80	63.40	78.60	96.60	146.60	168.80	264.60
37	$P_{14} \times P_{7}$	31.60	52.80	72.00	-	-	-	-
38	$P_{14} \times P_{9}$	34.20	61.20	75.20	96.20	146.20	168.20	274.20
39	$P_{14} \times P_{10}$	31.40	57.60	73.80	96.20	144.80	162.40	272.20
40	$P_{14} \times P_{12}$	33.00	55.00	70.00	93.00	141.40	157.60	263.60
41	$P_{14} \times P_{13}$	23.00	42.20	60.00	73.40	106.60	129.60	204.80
42	$P_{14} \times P_{14}$	22.20	39.20	53.80	68.80	105.00	127.00	202.80
43	$P_{14} \times P_{15}$	27.20	44.00	60.80	76.20	117.60	131.40	214.00
	SE_m	0.486	0.489	0.495	1.302	0.492	0.562	0.587
	CD(0.05)	1.346	1.355	1.371	3.608	1.365	1.558	1.627

which was observed to be on par with the combination P_{14} x P_{13} (23.00 days). It was found to be significantly high in P_{10} x P_2 (42.80 days).

2. Days for Protocorm Development

The time taken for the development of protocorm was recorded the lowest in P_{14} x P_{14} (39.20 days). This was on par with P_2 x P_{14} (40.20 days) and P_{13} x P_{13} (40.60 days). The number of days taken for development of protocorm in P_{10} x P_2 (65.20 days) was found to be significantly high. This was found to be on par with P_9 x P_2 (64.60 days) and P_{13} x P_{10} (64.40 days).

3. Days for Development of Chlorophyll

The duration taken for development of chlorophyll was minimum in P_{14} x P_{14} (53.80 days) which was on par with P_2 x P_{14} (54.00 days). This period was observed to be the longest i.e., 84 days in P_9 x P_2 which was on par with P_{13} x P_{10} (83.20 days).

4. Days for First Leaf Initiation

The time taken for formation of first leaf primordium was recorded significantly low in P_{14} x P_{14} (68.80 days). This was observed to be low in P_{14} x P_{13} (73.40 days) which was on par with P_2 x P_{14} (73.60 days). The combinations P_9 x P_2 and P_{10} x P_{14} recorded the longest duration of 100.60 days for development of first leaf primordium which was found to be on par with P_9 x P_{14} (100 days), P_{10} x P_2 (99.80 days), P_6 x P_2 (99.20 days), P_{13} x P_{10} (98.80 days) and P_{13} x P_9 (98.20 days).

5. Days for Shoot Initiation

The time taken for the development of first shoot primordium was found to be significantly low in P_{14} x P_{14} (105 days) which was observed to be on par with P_3 x P_{14}(105.20 days). This was found to be the highest in P_9 x P_2 (152 days).

6. Days for First Root Initiation

Number of days taken for the initiation of first root was observed to be significantly low in P_{14} x P_{14} (127 days). It was also recorded to be low in P_{14} x P_{13} (129.60 days) which was on par with P_3 x P_{14} (130 days). The longest duration taken for development of root primordia was observed in P_{10} x P_{14} (177.80 days) which was found to be on par with P_9 x P_4 (172 days).

7. Days for Deflasking

Deflasking required 197 days in P_3 x P_{14} which was found to be on par with P_2 x P_{14} (196.40 days) whereas the seedlings in P_{10} x P_{14} recorded the longest duration taken for deflasking (283.80 days).

4.4. *EX VITRO* ESTABLISHMENT OF HYBRID SEEDLINGS

4.4.1. Observations on Seedling Morphology at Deflasking

Important morphological characters of 36 successfully deflasked seedlings were studied and analysed statistically (Table 25; Plate 7).

1. Height of Seedling

Height of seedling at deflasking was significantly low in P_{14} x P_1 (2.08 cm). This was observed to be low in P_2 x P_{11} (2.14 cm) which was found to be on par with P_{13} x P_{12} (2.16 cm), P_{12} x P_2 and P_{13} x P_{13} (both recorded 2.20 cm). The hybrid combination P_{12} x P_{13} recorded a height of 4.02 cm which was significantly high followed by P_{13} x P_{13} (3.68 cm).

2. Number of Leaves

Mean number of leaves per seedling was observed to range from 2.20 in P_2 x P_{11}, P_{13} x P_2, P_{13} x P_{12}, P_{14} x P_2 and P_{14} x P_{12} to 3.80 in the hybrids P_{12} x P_{13}

Plate 6. ***In vitro*** **embryo culture of hybrid seeds**

Inoculated hybrid seeds

10 days after inoculation

Mokara Walter Oumae White x *Aranthera* Annie Black

Protocorm development

65 days after inoculation

Mokara Walter Oumae White x *Aranthera* Annie Black

Leaf primordia initiation

90 days after inoculation

Mokara Walter Oumae White x *Aranthera* Annie Black

Leaf regeneration

93 days after inoculation

Vanda Ruby Prince *x Vanda* John Clubb

Leaf development

100 days after inoculation

Mokara Walter Oumae White x *Aranthera* Annie Black

Root development

157 days after inoculation

Vanda Ruby Prince *x Vanda* John Clubb

Plate 6

Plate 6. Continued

Seedlings ready for deflasking

255 days after inoculation

Mokara Chak Kuan Pink x *Aranthera* Annie Black

275 days after inoculation

Mokara Thailand Sunspot x *Vanda* Ruby Prince

279 days after inoculation

Mokara Walter Oumae White x *Aranthera* Annie Black

Subculturing in laminar air flow

Master flasks ready for subculturing

Incubation of subcultured flasks






Plate 6 Continued

Table 25. Seedling morphology of 36 successfully planted out hybrid combinations at deflasking and planting out

Sl.No	Cross combination	Height of seedling	No. of leaves	Length of longest leaf	Breadth of longest leaf	Number of roots	Length of longest root	Thickness of longest root
1	$P_2 \times P_6$	2.46	3.20	1.72	0.50	2.40	1.26	0.14
2	$P_2 \times P_9$	2.24	3.00	1.66	0.42	2.60	1.32	0.22
3	$P_2 \times P_{10}$	3.52	3.20	1.84	0.46	2.80	1.22	0.22
4	$P_2 \times P_{11}$	2.14	2.20	1.54	0.32	2.00	0.56	0.10
5	$P_2 \times P_{12}$	3.52	3.20	2.08	0.38	2.80	1.36	0.12
6	$P_2 \times P_{13}$	3.34	3.60	2.20	0.52	3.20	1.34	0.16
7	$P_2 \times P_{14}$	3.14	3.00	2.32	0.40	2.20	1.24	0.14
8	$P_3 \times P_2$	3.08	2.80	2.08	0.38	2.80	1.36	0.14
9	$P_3 \times P_{12}$	2.66	3.00	1.78	0.16	3.20	1.34	0.16
10	$P_3 \times P_{14}$	3.24	3.00	2.32	0.40	2.20	1.24	0.14
11	$P_6 \times P_2$	3.16	3.40	2.20	0.46	2.60	1.20	0.16
12	$P_9 \times P_2$	3.06	3.20	2.10	0.48	2.80	1.24	0.18
13	$P_9 \times P_{14}$	3.16	3.00	2.04	0.38	2.80	1.36	0.14

Table 25. Continued

Sl.No	Cross combination	Height of seedling	No. of leaves	Length of longest leaf	Breadth of longest leaf	Number of roots	Length of longest root	Thickness of longest root
14	$P_{10} \times P_2$	3.18	3.60	2.20	0.54	3.20	1.34	0.16
15	$P_{10} \times P_{14}$	3.40	3.60	2.32	0.42	2.60	1.30	0.16
16	$P_{12} \times P_2$	2.20	2.40	1.24	0.32	2.20	0.72	0.12
17	$P_{12} \times P_{13}$	4.02	3.80	3.40	0.22	3.00	1.36	0.14
18	$P_{12} \times P_{14}$	3.68	3.00	3.08	0.20	2.20	1.14	0.12
19	$P_{13} \times P_1$	2.36	2.60	2.08	0.32	2.60	1.24	0.14
20	$P_{13} \times P_2$	2.24	2.20	1.44	0.32	2.00	0.64	0.12
21	$P_{13} \times P_3$	2.36	2.40	1.32	0.26	2.20	0.68	0.14
22	$P_{13} \times P_9$	3.16	3.60	2.92	0.38	3.20	1.20	0.16
23	$P_{13} \times P_{10}$	3.38	3.80	3.04	0.44	3.00	1.28	0.16
24	$P_{13} \times P_{12}$	2.16	2.20	1.44	0.22	2.00	0.64	0.12
25	$P_{13} \times P_{13}$	2.20	2.60	1.32	0.22	2.20	0.68	0.14
26	$P_{13} \times P_{14}$	2.50	2.40	1.36	0.22	2.60	0.68	0.14

Table 25. Continued

Sl. No	Cross combination	Height of seedling	No. of leaves	Length of longest leaf	Breadth of longest leaf	Number of roots	Length of longest root	Thickness of longest root
27	$P_{14} \times P_1$	2.08	2.80	2.08	0.30	2.40	1.24	0.14
28	$P_{14} \times P_2$	3.24	2.20	1.44	0.32	2.00	0.64	0.12
29	$P_{14} \times P_3$	2.24	2.40	1.34	0.24	2.20	0.68	0.14
30	$P_{14} \times P_6$	2.46	3.20	1.72	0.50	2.40	1.26	0.14
31	$P_{14} \times P_9$	2.42	3.00	1.66	0.42	2.60	1.32	0.22
32	$P_{14} \times P_{10}$	3.52	3.20	1.86	0.46	2.80	1.22	0.22
33	$P_{14} \times P_{12}$	2.24	2.20	1.44	0.32	2.00	0.64	0.12
34	$P_{14} \times P_{13}$	2.36	2.40	1.32	0.26	2.20	0.68	0.14
35	$P_{14} \times P_{14}$	2.36	2.40	1.32	0.26	2.20	0.68	0.14
36	$P_{14} \times P_{15}$	3.12	3.20	1.82	0.34	2.40	1.20	0.16
	SE_m	0.045	0.27	0.032	0.025	0.267	0.032	0.023
	CD(0.05)	0.124	0.748	0.09	0.069	0.739	0.089	0.063

Plate 7. Deflasking and *ex vitro* establishment of hybrid seedlings

Hybrid seedlings after deflasking

Seedlings kept on graph paper for taking measurements

Seedling singled out for planting

Fungicide treatment before planting out

Seedlings planted out

Repotted seedlings – four months after deflasking

Humidity chamber for acclimatization

Seedlings kept with in humidity chamber


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Plate 7

and P_{13} x P_{10}. Three more hybrids recorded values on par with the highest number of leaves. These were P_2 x P_{13}, P_{10} x P_2 and P_{10} x P_{14} registering 3.60 leaves each.

3. Length of the Longest Leaf

Length of the longest leaf was recorded maximum in the hybrid combination P_{12} x P_{13} (3.40 cm), followed by P_{12} x P_{14} (3.08 cm) and P_{13} x P_{10} (3.04 cm). The hybrid combination P_{12} x P_2 produced the shortest leaf (1.24 cm) among the different combinations.

4. Breadth of the Longest leaf

The breadth of longest leaf was recorded highest in P_{10} x P_2 (0.54 cm) which was on par with P_2 x P_{13} (0.52 cm) and P_2 x P_6 (0.50 cm). It was found to be the lowest in P_3 x P_{12} (0.16 cm).

5. Number of Roots

Number of roots was found to be highest in the seedlings of P_2 x P_{13}, P_3 x P_{13} and P_{13} x P_9 (3.20). This was found to be on par with P_{12} x P_{13} and P_{13} x P_{10} (3.00). P_{13} x P_{12} and P_{14} x P_{12} recorded the lowest number of roots in the seedling (2.00).

6. Length of the Longest Root

Length of root in P_2 x P_{12}, P_3 x P_2, P_9 x P_{14} and P_{12} x P_{13}, recorded the maximum value with 1.36 cm whereas the root length was found to be the lowest in P_2 x P_{11} (0.56 cm).

7. Diameter of the Longest Root

The diameter of the longest root was found to be maximum in the hybrids P_2 x P_9, P_2 x P_{10}, P_{14} x P_9 and P_{14} x P_{10} (0.22 cm). The hybrid combination P_2 x P_{11} recorded the lowest root diameter with 0.10 cm.

4.4.2. Observations on seedling morphology four months after planting out

In vitro raised seedlings were acclimatized in humidity chamber for one month and transferred to net house for hardening. Seedling morphology of these 36 successful hybrid combinations was studied after four months of planting out also (Table 26).

1. Height of Seedling

Height of seedling four months after deflasking was observed to range from 2.52 cm in $P_{13} \times P_2$, $P_{13} \times P_{12}$ and $P_{14} \times P_{12}$ to 4.68 cm in $P_{12} \times P_{13}$ which was significantly high. The hybrid combination $P_{10} \times P_{14}$ with a value of 4.44 cm was found to be the second highest.

2. Number of Leaves

Mean number of leaves per seedling was observed to range from 3.20 in five hybrid combinations such as $P_2 \times P_{11}$, $P_{13} \times P_2$, $P_{13} \times P_{12}$, $P_{14} \times P_2$ and $P_{14} \times P_{12}$ to 5.00 in the hybrid $P_{12} \times P_{13}$. The hybrids $P_{10} \times P_{14}$, $P_{13} \times P_9$ (4.80 for both), $P_{10} \times P_2$ and $P_{13} \times P_{10}$ (4.60 for both) recorded values on par with the highest for number of leaves.

3. Length of the Longest Leaf

Length of the longest leaf was recorded maximum in the hybrid combination $P_{12} \times P_{13}$ with a significantly high value of 3.84 cm. The hybrid combination $P_{12} \times P_2$ produced the shortest leaf (1.62 cm) among the different combinations.

4. Breadth of the Longest Leaf

The breadth was recorded high in $P_9 \times P_2$ and $P_{10} \times P_2$ (0.74 cm for both) whereas it was found to be the lowest in $P_{12} \times P_{14}$ and $P_{13} \times P_{12}$ (0.26 cm).

Table 26. Seedling morphology of 36 successfully planted out hybrid combinations after four months of hardening

Sl.No	Cross combination	Height of seedling	No.of leaves	Length of longest leaf	Breadth of longest leaf	Number of roots	Length of longest root	Thickness of longest root
1	$P_2 \times P_6$	3.28	4.20	2.18	0.70	3.20	1.70	0.18
2	$P_2 \times P_9$	3.08	4.20	2.16	0.66	3.80	1.82	0.24
3	$P_2 \times P_{10}$	4.10	4.20	2.42	0.72	4.00	1.72	0.24
4	$P_2 \times P_{11}$	2.74	3.20	1.82	0.46	2.60	1.24	0.14
5	$P_2 \times P_{12}$	4.32	3.80	2.66	0.60	4.00	2.14	0.24
6	$P_2 \times P_{13}$	4.02	4.20	2.58	0.68	4.00	2.30	0.24
7	$P_2 \times P_{14}$	4.12	4.00	2.78	0.56	3.40	2.14	0.20
8	$P_3 \times P_2$	3.90	3.80	2.66	0.60	3.80	2.14	0.20
9	$P_3 \times P_{12}$	3.54	4.00	2.10	0.46	4.00	2.10	0.18
10	$P_3 \times P_{14}$	4.16	4.00	2.78	0.56	3.40	2.14	0.20
11	$P_6 \times P_2$	3.78	3.80	2.68	0.68	3.60	2.10	0.24
12	$P_9 \times P_2$	3.82	4.20	2.68	0.74	4.00	2.18	0.28
13	$P_9 \times P_{14}$	3.94	4.20	2.60	0.60	3.80	2.14	0.24

Table 26. Continued

Sl.No	Cross combination	Height of seedling	No.of leaves	Length of longest leaf	Breadth of longest leaf	Number of roots	Length of longest root	Thickness of longest root
14	$P_{10} \times P_2$	4.00	4.60	2.58	0.74	4.00	2.30	0.24
15	$P_{10} \times P_{14}$	4.44	4.80	2.78	0.58	4.00	2.28	0.24
16	$P_{12} \times P_2$	2.60	3.20	1.62	0.44	3.00	1.18	0.16
17	$P_{12} \times P_{13}$	4.68	5.00	3.84	0.34	4.20	1.86	0.22
18	$P_{12} \times P_{14}$	4.24	4.00	3.44	0.26	3.40	1.56	0.18
19	$P_{13} \times P_1$	3.22	3.60	2.34	0.36	3.40	1.56	0.16
20	$P_{13} \times P_2$	2.52	3.20	1.72	0.42	3.40	1.66	0.16
21	$P_{13} \times P_3$	2.76	3.40	1.66	0.34	3.40	1.56	0.18
22	$P_{13} \times P_9$	3.92	4.80	3.36	0.54	4.40	1.88	0.30
23	$P_{13} \times P_{10}$	4.18	4.60	3.54	0.50	3.80	1.90	0.24
24	$P_{13} \times P_{12}$	2.52	3.20	1.72	0.26	3.40	1.66	0.16
25	$P_{13} \times P_{13}$	2.76	3.60	1.66	0.28	3.40	1.56	0.18

Table 26. Continued

Sl.No	Cross combination	Height of seedling	No.of leaves	Length of longest leaf	Breadth of longest leaf	Number of roots	Length of longest root	Thickness of longest root
26	$P_{13} \times P_{14}$	2.76	3.40	1.66	0.32	3.40	1.56	0.18
27	$P_{14} \times P_1$	3.04	3.60	2.34	0.36	3.40	1.56	0.14
28	$P_{14} \times P_2$	3.52	3.20	1.72	0.42	3.40	1.62	0.14
29	$P_{14} \times P_3$	2.74	3.60	1.66	0.34	3.40	1.56	0.16
30	$P_{14} \times P_6$	3.28	4.20	2.18	0.70	3.20	1.70	0.18
31	$P_{14} \times P_9$	3.08	4.20	2.16	0.68	3.80	1.82	0.24
32	$P_{14} \times P_{10}$	4.20	4.20	2.42	0.70	4.00	1.70	0.22
33	$P_{14} \times P_{12}$	2.52	3.20	1.72	0.42	3.40	1.66	0.16
34	$P_{14} \times P_{13}$	2.76	3.40	1.66	0.34	3.40	1.56	0.18
35	$P_{14} \times P_{14}$	2.76	3.40	1.66	0.34	3.40	1.56	0.18
36	$P_{14} \times P_{15}$	3.74	4.40	2.30	0.52	3.60	1.62	0.28
	SE_m	0.046	0.296	0.035	0.03	0.31	0.035	0.025
	CD(0.05)	0.128	0.821	0.098	0.082	0.859	0.098	0.069

5. Number of Roots

Number of roots was found to be significantly high in the seedlings of P_{12} x P_{13} (4.40), followed by P_{13} x P_9 (4.20). P_2 x P_{11} recorded the lowest number of roots in the seedling (2.60).

6. Length of the Longest Root

Root length in P_2 x P_3 and P_{10} x P_2 recorded the maximum value with 2.30 cm. This was found to be on par with P_{10} x P_{14} (2.28). The root length in P_{12} x P_2 (1.18 cm) was found to be the lowest and was found to be on par with P_2 x P_{11} (1.24 cm).

7. Thickness of the Longest Root

The thickness of the longest root was found to be maximum in the hybrid P_9 x P_2 and P_{14} x P_{15} (0.28 cm). The hybrid combinations P_2 x P_{11}, P_{14} x P_1 and P_{14} x P_2 recorded the lowest root thickness with 0.14 cm .

4. 5. MOLECULAR CHARACTERIZATION

In the present research work, RAPD (Random Amplified Polymorphic DNA) analysis was used for molecular characterization of 20 monopodial orchid hybrids, making use of arbitrary primers to amplify random DNA sequence in the genome. The results are presented below.

4.5.1. Isolation of Genomic DNA

Genomic DNA was extracted from leaves of hybrids based on the method of Mondal et al., (2000) with slight modifications. When fresh and tender leaves were used, the purity and yield of DNA were good. The DNA yield of 20 monopodial orchid hybrids ranged from 660 to 990 ng μl^{-1} and its purity ranged from 1.60 to 1.83 $\mu g\ \mu l^{-1}$ (Table 27). Rahana et al., (2007) also recorded similar

Table 27. Quality and yield of DNA from 20 monopodial orchid hybrids

Sl. No.	Hybrid No.	A_{260}	A_{280}	A_{260}/A_{280}	DNA yield (ng μl^{-1})
1	H_1	0.028	0.016	1.75	840
2	H_2	0.029	0.018	1.61	870
3	H_3	0.033	0.019	1.74	990
4	H_4	0.027	0.016	1.69	810
5	H_5	0.021	0.012	1.75	630
6	H_6	0.032	0.019	1.68	960
7	H_7	0.023	0.013	1.77	690
8	H_8	0.024	0.015	1.60	720
9	H_9	0.026	0.016	1.63	780
10	H_{10}	0.022	0.012	1.83	660
11	H_{11}	0.028	0.017	1.65	840
12	H_{12}	0.031	0.018	1.72	930
13	H_{13}	0.025	0.014	1.79	750
14	H_{14}	0.023	0.013	1.77	690
15	H_{15}	0.029	0.017	1.71	870
16	H_{16}	0.026	0.015	1.73	780
17	H_{17}	0.024	0.014	1.72	720
18	H_{18}	0.029	0.017	1.71	870
19	H_{19}	0.028	0.016	1.75	840
20	H_{20}	0.029	0.016	1.81	870

Table 28. Primers, their sequence and total number of amplified bands in 20 monopodial orchid hybrids

Sl. No.	Primer	Sequence	Total no. of bands amplified
1	OPB - 07	GGTGACGCAG	6
2	OPB - 15	GGAGGGTGTT	10
3	OPB - 17	AGGGAACGAG	7
4	OPC - 04	CCGCATCTAC	5
5	OPC - 05	GATGACCGCC	6
6	OPC - 08	TGGACCGGTG	6
7	OPC - 15	GACGGATCAG	9
8	OPD - 02	GGACCCAACC	8

findings in *Dendrobium* hybrids where the DNA yield ranged from 630 to 960 ng μl^{-1} and its purity was observed to vary from 1.60 to 1.85 $\mu g\ \mu l^{-1}$.

Quality of DNA was assessed, for which all the samples were run on 0.8 per cent agarose gel. The gel was stained with ethidium bromide and bands appearing in the gel were visualized, using ultraviolet trans-illuminator. The DNA should be free of RNA and protein, for RAPD analysis. Also, it requires sufficient quantity of intact, unsheared DNA.

4.5.2. Polymerase Chain Reaction

The procedure for amplification of DNA, standardized by Lim et al. (1999) for *Vanda* was adapted, with slight modification. To identify the promising primers for RAPD analysis, 70 decamer primers of series A, B, C and D were screened using the DNA of hybrid H_2. Among these, based on the performance in DNA amplification, eight decamer primers were identified for RAPD analysis. Primers that produced highest number of intense, polymorphic and reproducible bands were selected. They were OPB-07, OPB-15, OPB-17, OPC-04, OPC-05, OPC-08, OPC-15 and OPD-02 (Table 28).

The amplification profiles generated by these selected primers are shown in Plates 8 to 15. A total of 57 scorable bands (average of 7.125 bands per primer) were generated by the selected eight primers of which six were monomorphic (10.53 %) and the remaining 51 were polymorphic (89.47 %). The number of amplification products ranged from five to ten with an average of two per primer.

The highest number of scorable bands (10 bands) was given by OPB-15 followed by OPC-15 (9 bands), OPD-02 (8 bands), OPB-17 (7 bands), OPC-05 (6 bands), OPC-08 (6 bands), OPB-07 (6 bands) and OPC-04 (5 bands). All the ten bands obtained from OPB-15 and the nine bands produced by primer OPC-15 were polymorphic. Among the six scorable bands produced by primer OPC-05 two bands were monomorphic. Out of the six bands produced by OPB-07, one

was monomorphic and among the five scorable bands from OPC-04 one was monomorphic. The primers, OPC-08 and OPD-02 also produced one monomorphic band each.

Statistical analysis was carried out using the data obtained from the eight primers that produced scorable bands. The genetic similarity matrix constructed using Jaccard's coefficient method is presented in Table 29.

4.5.3. Cluster analysis

The clustering of 20 monopodial orchid hybrids using UPGMA is depicted in Fig.17. The overall similarity coefficients ranged from 0.40 to 0.84 as revealed in the dendrogram. The genetic distance indicated the genetic variability that exists between the hybrids.

Cluster analysis revealed that at 0.69 similarity coefficient, the 20 monopodial orchid hybrids got divided into six groups. Cluster I A contained four hybrids H-1, H-10, H-3 and H-9. H-1 showed 75 per cent similarity with H-10, 71.74 per cent similarity with H-3 and 67.39 per cent similarity with H-9.

The three hybrids H-5, H-6 and H-8 fell in cluster I B. H-5 showed 78.57 per cent similarity with H-6 and 62.79 per cent with H-8. At a similarity coefficient of 0.75, cluster I C could be divided into two groups *viz.*, I C_1 and I C_2. H-2, H-12 and H-11 belonged to the first subgroup

Among the 20 hybrids, H-18, H-7, H-13, H-17, H-19 and H-20 stand separately in clusters II, III A, III B, IV, V and cluster VI respectively. H-17 exhibited comparatively low similarity per cent (<50 %) with H-18, H-19 and H-20.

H-7 and H-13 could be included in a single group, cluster III, at similarity coefficient of 0.66. They showed 67.65 per cent similarity with one another. Other groups remain the same as clustering at similarity coefficient of 0.69.

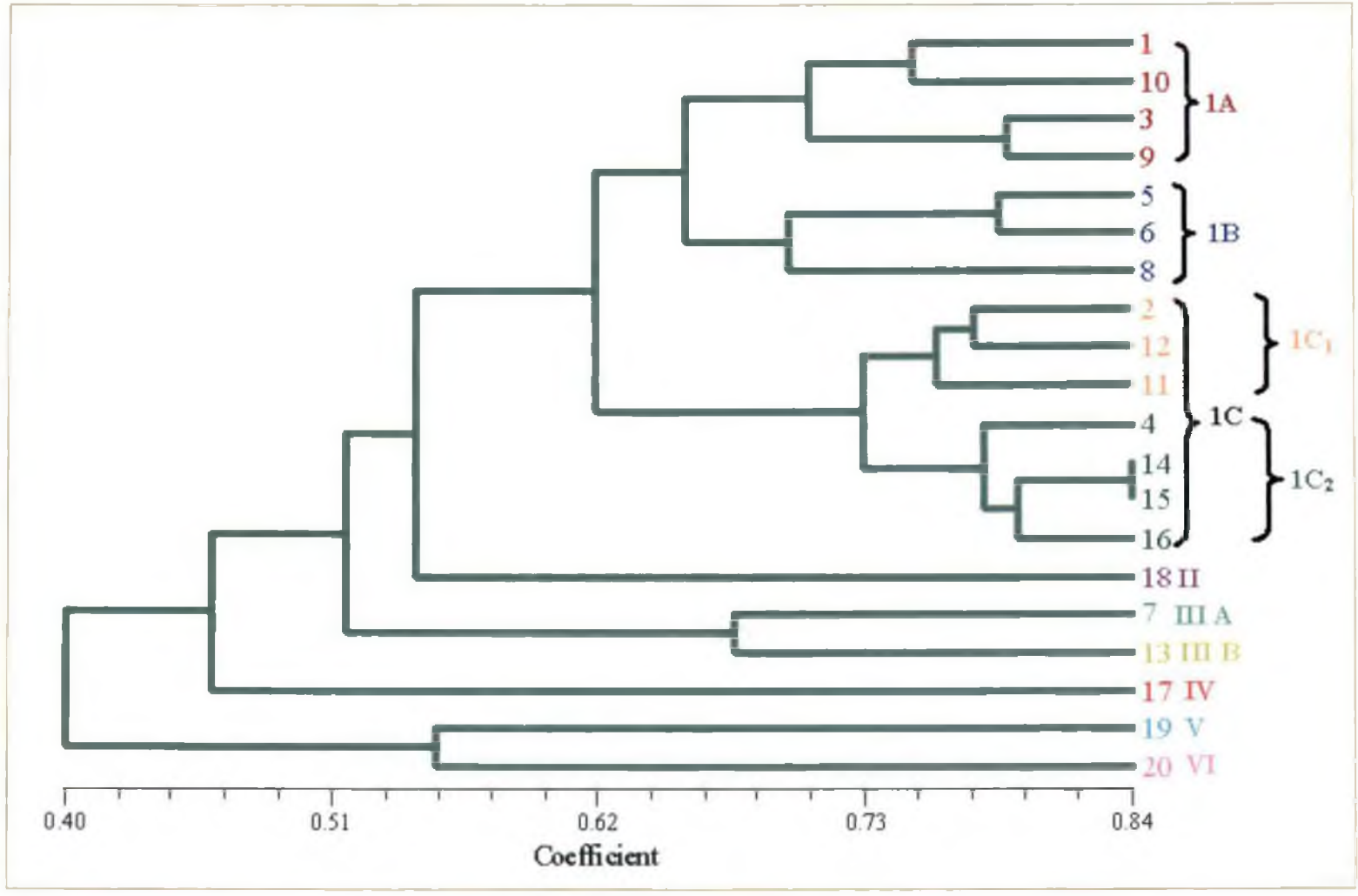

Fig. 17. Dendrogram for 20 selected monopodial orchid hybrids based on RAPD analysis

Table 29. Jaccard's similarity coefficient

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1.0000																			
2	0.6735	1.0000																		
3	0.7174	0.5870	1.0000																	
4	0.5962	0.7111	0.5745	1.0000																
5	0.6400	0.6522	0.6977	0.7500	1.0000															
6	0.6531	0.5625	0.7561	0.6889	0.7857	1.0000														
7	0.5745	0.5111	0.6667	0.4375	0.6190	0.6341	1.0000													
8	0.5833	0.5217	0.6341	0.5778	0.6279	0.7692	0.5897	1.0000												
9	0.6739	0.5778	0.7895	0.5652	0.6512	0.7073	0.7027	0.7568	1.0000											
10	0.7500	0.6512	0.7000	0.6000	0.6136	0.5909	0.6579	0.5854	0.7368	1.0000										
11	0.5294	0.7561	0.5000	0.6977	0.6364	0.5106	0.4545	0.4667	0.5227	0.5581	1.0000									
12	0.6042	0.7750	0.5455	0.7143	0.5778	0.5909	0.4651	0.5854	0.6098	0.6098	0.7632	1.0000								
13	0.4490	0.5116	0.5500	0.4348	0.4773	0.4884	0.6765	0.4750	0.5789	0.5385	0.4878	0.5000	1.0000							
14	0.6667	0.7174	0.6170	0.7391	0.6458	0.6250	0.4490	0.5532	0.6087	0.6087	0.7442	0.7619	0.4468	1.0000						
15	0.6800	0.7333	0.6667	0.8372	0.7333	0.7111	0.5217	0.6744	0.6977	0.7381	0.7209	0.7805	0.4565	0.8409	1.0000					
16	0.7200	0.7391	0.6383	0.7609	0.7021	0.6458	0.5319	0.5745	0.6304	0.7045	0.6889	0.7442	0.5000	0.7660	0.8222	1.0000				
17	0.4348	0.4286	0.5000	0.4186	0.5000	0.4390	0.4444	0.4595	0.5278	0.4865	0.4359	0.4474	0.5313	0.4318	0.4419	0.4884	1.0000			
18	0.4808	0.5435	0.5814	0.5319	0.5778	0.4894	0.3696	0.4773	0.5349	0.4667	0.5581	0.5349	0.3636	0.6444	0.5870	0.5957	0.3750	1.0000		
19	0.2500	0.2857	0.3784	0.2791	0.3500	0.3250	0.2778	0.3333	0.3611	0.2895	0.2500	0.2564	0.3030	0.2955	0.3023	0.2609	0.4074	0.4412	1.0000	
20	0.4600	0.5227	0.4884	0.5111	0.4565	0.4667	0.3409	0.4186	0.4762	0.4762	0.5000	0.5122	0.3659	0.5909	0.5682	0.5106	0.4571	0.5500	0.5517	1.0000

Plate 8. Amplification profiles of the DNA of 20 monopodial orchid hybrids using the primer OPB-07

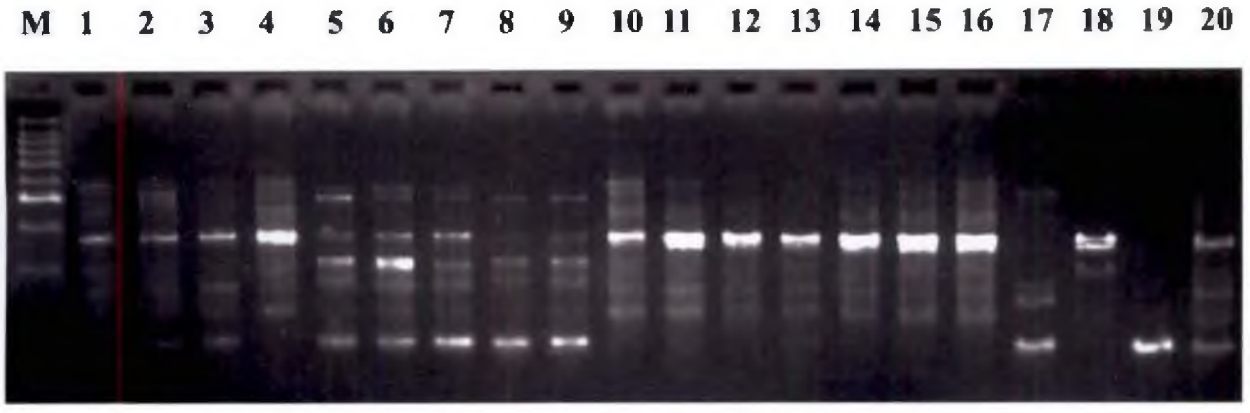

Plate 9. Amplification profiles of the DNA of 20 monopodial orchid hybrids using the primer OPB-15

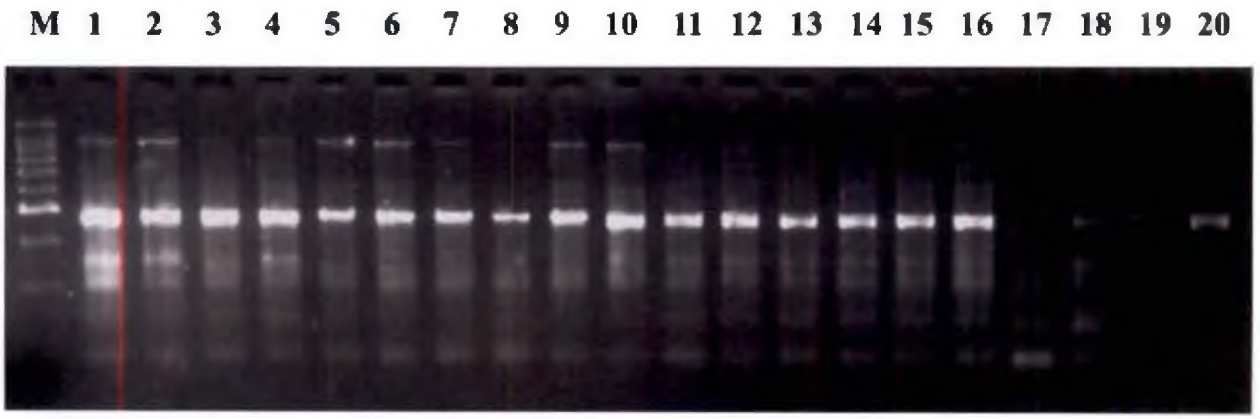

Plate 10. Amplification profiles of the DNA of 20 monopodial orchid hybrids using the primer OPB-17

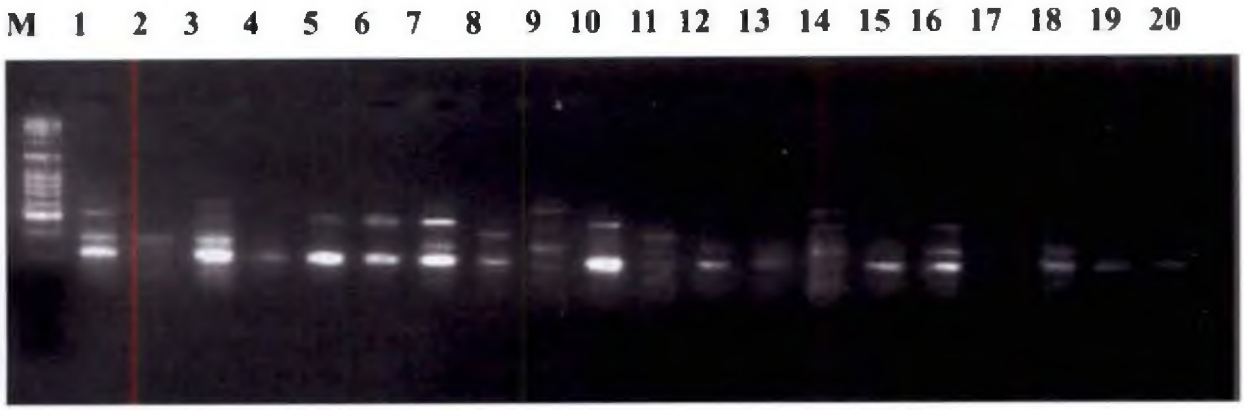

Plate 11. Amplification profiles of the DNA of 20 monopodial orchid hybrids using the primer OPC-04

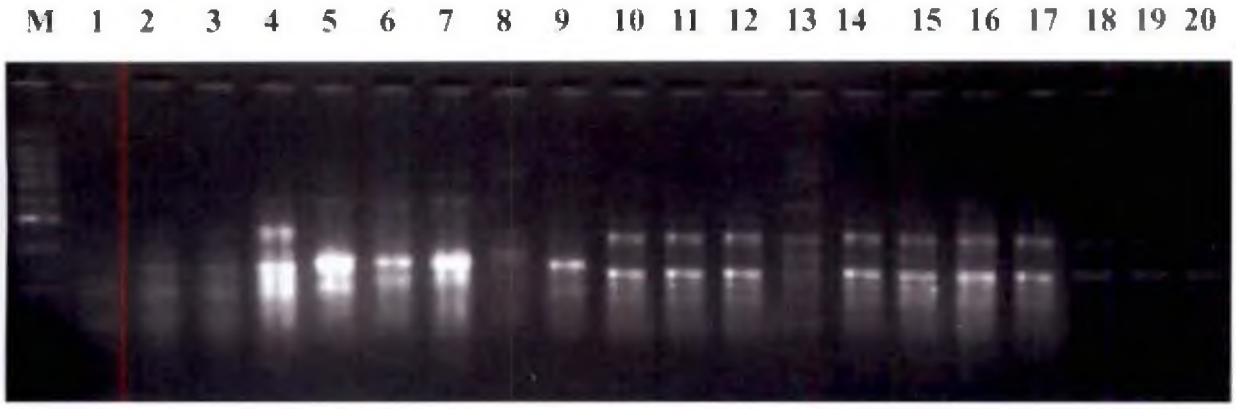

Plate 12. Amplification profiles of the DNA of 20 monopodial orchid hybrids using the primer OPC-05

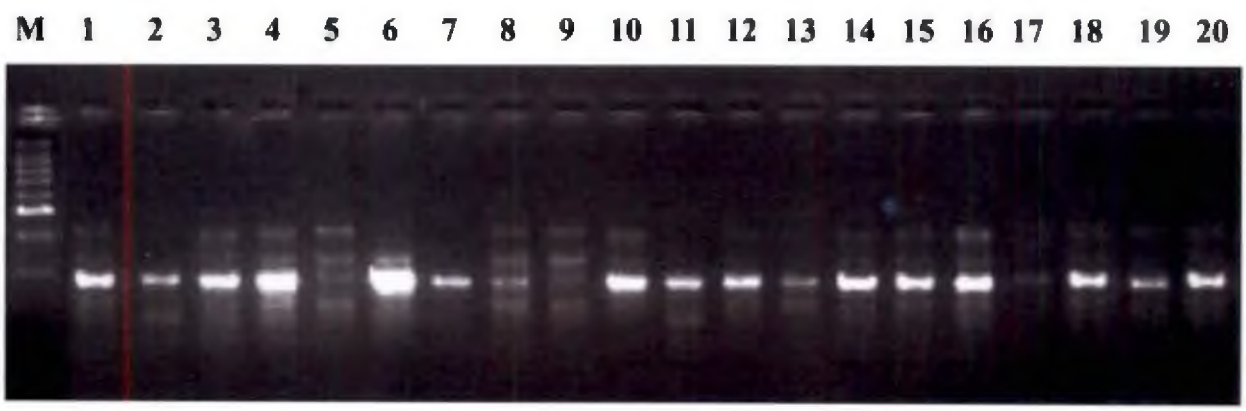

Plate 13. Amplification profiles of the DNA of 20 monopodial orchid hybrids using the primer OPC-08

Plate 14. Amplification profiles of the DNA of 20 monopodial orchid hybrids using the primer OPC-15

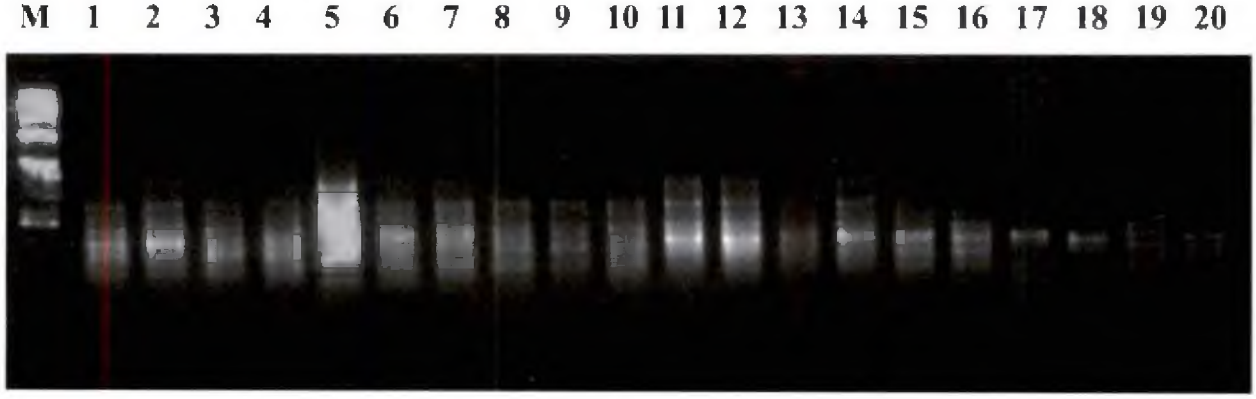

Plate 15. Amplification profiles of the DNA of 20 monopodial orchid hybrids using the primer OPD-02

When clustering was done at a similarity coefficient of 0.58, 14 hybrids *viz.*, H-1, H-10, H-3, H-9, H-5, H-6, H-8, H-2, H-12, H-11, H-4, H-14, H-15 and H-16, stood together in cluster I. The dissimilarity between H-14 and H-15 found to be negligible showing 84.09 per cent similarity with each other. High similarity was observed between H-15 and H-16 with a value of 82.22 per cent . H-18 remained separately as cluster II. H-7 and H-13 belonged to cluster III. The hybrids, H-17, H-19 and H-20, were singled out as clusters IV, V and VI respectively.

DISCUSSION

5. DISCUSSION

Orchids are considered as the most fascinating plants because of their diversity and specialization in floral characteristics and morphological features. They are famous all over the world as plants of ornamentation and are referred to as gems in the field of horticulture. Among them, monopodial orchids have comparative advantages over sympodials such as easier maintenance, longer vase-life etc. They are highly priced in the international florist trade due to their spectacular flowers having wonderful beauty with numerous colour combinations and patterns. Monogeneric and bigeneric hybrids as well as improved multigeneric grexes have been developed in them. The production of multigeneric orchid hybrids which have more than two genera in their parentage, has vastly broadened the environmental range of orchids. They also usually have improved flower size, colour and pattern, as well as habit of inflorescence when compared to the individual species or hybrids of the parental genera (Mercy and Dale, 1997). The present research programme was conducted for developing novel hybrids in monopodial orchids which have demand in the national and international markets and to characterise them using molecular markers. Monogenerics *viz., Vanda* and *Renanthera* and bigenerics such as *Aranda* and *Aranthera* were included in the study. Multigeneric grexes *viz., Kagawara* and *Mokara* which are gaining more popularity in the national and international markets were also utilized. The study resulted in the development of 36 hybrid combinations. Salient research findings during their development are discussed below.

5.1. COMPARISON BETWEEN PARENTS BASED ON VEGETATIVE CHARACTERS

A detailed study of vegetative as well as floral traits is important for understanding the diversity of monopodial orchid genotypes and selecting them as parents for a successful hybridization programme. Although the ultimate success of an orchid hybrid is decided by the beauty of its bloom, vegetative vigour is also

important (McDonald, 1991). He emphasised the importance of vegetative vigour, stating that vigorous hybrids result in bigger, better blooms and more floriferous nature with greater flower substance. Also while selecting parents for any hybridization programme, the general health and superior vegetative qualities of the plants are of great importance. Hence a comparative analysis of the vegetative characters of the genotypes utilized in the present study is presented below.

The 15 genotypes used in the present study were evaluated with respect to growth characters one year after planting. A wide range of variation for vegetative biometric characters was observed among them. The range was prominent for most of the vegetative characters like length of shoot (46.92 to 75.54 cm), number of leaves per cane (18.6 to 33.6), number of aerial roots (3.4 to 14.8), length of aerial root (13.66 to 39.02 cm), length of leaf (6.14 to 21.1 cm), width of leaf (0.36 to 3.36 cm) and leaf area (2.96 to 58.82 cm). This wide range of variations may be due to the fact that the majority of the genotypes employed in the present study are themselves higher order monogeneric, bigeneric and multigeneric hybrids. This finding is in conformity with the reports of Hurst (1898) that in orchids higher order multigeneric hybrids showed a far wider range of character variation as compared to lower order primary hybrids. Similar results have been observed in orchids by McConnel and Kamemoto (1983). They found that even reciprocal crossings in multigeneric hybrids yielded offsprings differing in cane height, pseudobulb production and flower yield, bearing evidence to their highly heterozygous nature. In the present study characters like thickness of stem (0.79 to 1.618 cm) and length of internode (1.788 to 3.764 cm) also showed a high range of variation. This wide variation in external vegetative morphology bears evidence to their diverse genetic makeup. When hybridized, these will give rise to a wide array of variants from which more desirable hybrids can be selected.

5.2. COMPARISON BETWEEN PARENTS BASED ON FLORAL CHARACTERS

A clear background knowledge on floral biology is of special significance in the breeding of orchids due to the structural and functional peculiarities of their flowers. The important biometric characters deciding the size and nature of inflorescence are length of inflorescence, length of scape, number of flowers per inflorescence, length of internode of inflorescence and thickness of inflorescence axis. Days to first flower opening from inflorescence emergence is primarily decided by the length of inflorescence and its rate of growth (Lekha Rani, 2002). Ninitha Nath (2003) observed that this character was following the same trend as the length of inflorescence in all varieties, while the rate of inflorescence growth did not vary much between varieties. Flowering time is decided by the number of flowers per inflorescence and the rate of flower opening. Correlation was not observed between flowering time and number of flowers per inflorescence in the parental varieties studied denoting that the rate of flower opening varied with the variety.

Length of inflorescence has been pointed out as a character of prime importance in any orchid breeding programme (McDonald, 1991). Proper balancing between the length of inflorescence and length of scape is important in deciding the elegance and grace of an inflorescence. In the present study, significant positive correlation was observed between these two characters. Diameter of inflorescence axis also has a major say in deciding the shape and nature of the inflorescence axis. Length of internode of inflorescence should be optimum for proper display of flowers; ample clearance between successive flowers is essential to prevent overcrowding of flowers whereas more clearance leads to the ungainly, prominent exposure of inflorescence axis.

Number of flowers per inflorescence is a character of prime importance in orchid breeding, as has been pointed out by Kamemoto (1983), McConnel and Kamemoto (1983), Singh (1986) and McDonald (1991). As all the genotypes used

in the present study are higher order hybrids, the increased average flower number exhibited by the majority of them is in accordance with the observations of Singh (1982) that in orchids, higher order hybrids show increased number of flowers per spike. Bobisud and Kamemoto (1982) arrived at the same conclusion that flower production in *Dendrobium* hybrids was primarily influenced by parental genotypes. The inheritance of the character number of blooms per inflorescence has been reported by several scientists. Tippit (1997) was of the opinion that in a new hybrid, the number of flowers per inflorescence was the geometric mean of the two genetically dissimilar parents involved in the cross. Among the fifteen genotypes, flower length was found to be greater than flower width in all the varieties excluding P_4, P_5, P_{12}, P_{13} and P_{15}. Atwood (1989) and Porter (1989) encountered a similar situation where flowers in *Paphiopedilum* hybrids were found to be generally longer and wider when parents having different length : breadth ratios were crossed. Oakeley (1991) in *Lycaste* species has described in detail the advantages of breeding for reduced size by selecting parents having reduced flower length and width.

In monopodials, flowering is either throughout the year or seasonal. In the study conducted by Ninitha Nath (2003), out of the 12 monopodial genotypes, five were free-flowering and seven were seasonal, flowering from May to October and June to December. Sobhana (2000) and Lekha Rani (2002) conducted similar evaluation in two sets of *Dendrobium* prior to hybridization. They also observed both free-flowering and seasonal flowering nature. In the present investigation, ten out of 15 varieties studied exhibited free-flowering nature, while seasonal flowering was shown by five varieties. Seasonal flowering is not as advantageous as free-flowering because of the restriction of flowering to certain months or seasons. This limits the availability of flowers of varieties of *Renanthera* and *Vanda* to certain months of the year, and reduces their market share. The flowers of *Aranda, Aranthera, Kagawara* and *Mokara* which are free-flowering are available in the market throughout the year. Ninitha Nath (2003) reported seasonal flowering in seven monopodial orchids and free-flowering nature in five

genotypes. The general trend noticed on demand of flowers is related to their constant availability in the market which is seen in free-flowering commercial hybrid varieties. Nature of inflorescence axis in monopodials may be erect or arching. The mode of display of flowers is alternate and facing opposite directions or whorled. In the present study, the genotypes exhibited erect as well as arching inflorescence axes. The mode of display was whorled, presenting a bunched appearance or alternate, with flowers facing opposite directions. Both these modes of display are advantageous as the inflorescence presents the same appearance when viewed from either sides. The inflorescence axes were erect with the flowers alternate and facing opposite directions in *Dendrobium* (Lekha Rani, 2002). Davidson (1994) reported that while selecting parents for hybridization programmes in orchids, the distinctive shape of the inflorescence axis and attractive mode of display were important.

Fullness value gives a clue about the degree of fullness or the perfection in shape and arrangement of sepals and petals. Leonhardt (1977), while breeding with *Cymbidium* and related genera, made use of fullness value to obtain an estimate of the degree of fullness. Fullness value in diploid *Cymbidium* was 4.7 and that in tetraploid was 4.1, indicating that the tetraploid was comparatively fuller. Ninitha Nath (2003) observed that *Vanda* Miss Joaquim produced remarkably full flowers with a fullness value of 2.15. Also flowers were observed to be full in parents like *Vanda* Popoe Diana (2.22) and *Vanda* TMA Mandai (2.27). In the present study, fullness values ranged from 2.60 in *Vanda* John Clubb to 5.33 in *Aranthera* Annie Black. Here also monogeneric *Vanda* varieties were found to be fuller as compared to bigeneric *Aranthera* types. Oakeley (1991) observed that slight reflexing and overlapping of sepals and petals are desirable attributes in orchids.

A basic understanding of flower opening, anthesis and stigma receptivity is of special importance in the breeding of orchids due to the structural and functional peculiarities of their flowers. These peculiarities leading to a lack of knowledge of the method of pollination was the main reason for starting artificial

hybridization much later in orchidaceae than in other angiosperm families. In all the monopodial orchid genotypes studied, flowers opened during day-time in acropetal succession. Flower opening commenced from twenty seven to thirty eight days after inflorescence emergence, lasting for eight to eighteen days. These findings are in conformity with the reports of Varghese (1995), Sobhana (2000) and Lekha Rani et al. (2006) in three different sets of *Dendrobium* varieties. In the present investigation, flower opening commenced from 7.30 am on sunny days; but was delayed till 12.30 pm on rainy days. Christenson (1992) and Lekha Rani et al. (2006) have also reported such a weather dependent variation in the time of opening of flowers in orchids. A concise study of the maximum stigma receptivity period revealed that any monopodial genotype assessed could be pollinated with maximum success between 4 and 6 days after anthesis. This is in accordance with the findings of Varghese (1995) and Lekha Rani et al. (2006) in two different sets of *Dendrobium* varieties.

Pollen in Orchidaceae is agglutinated to form masses called 'pollinia' (Sheehan and Sheehan, 1979). This minimizes the wastage of pollen. Abraham and Vatsala (1981) observed that pollen in orchids exists as tetrads, held together by elastic bands of tapetal origin. In the present study pollen diameter of the fifteen monopodial parental genotypes ranged from 19.798 µ in P_4 to 43.566 µ in P_3 and pollen fertility ranged from 50.754 per cent in P_5 to 78.472 per cent in P_3. Similar results were obtained in a study conducted among twelve genotypes of monopodial orchids by Ninitha Nath (2003) in which pollen size ranged from 34.86 µ in *Vanda* TMA Mandai to 44.56 µ in *Arachnis* Kapama. She also observed that pollen fertility ranged from 50.80 per cent in *Vanda* Popoe Diana to 78.00 per cent in *Aranthera* James Storei.

Highest pollen germination per cent was observed in P_3 (66.42 %) followed by P_2 (63.24 %) following the same trend as in pollen fertility. This supports the fact that if fertile pollen is more, germination per cent will also be more. Lekha Rani et al. (2006) reported that pollen germination varied significantly among the *Dendrobium* varieties tested with the commercial hybrid,

Dendrobium Candy Stripe x *Dendrobium* Tomie Drake recording highest germination of 77.1 per cent. Percentage pollen germination was the lowest in the species, *Dendrobium philippica* and *Dendrobium barbatulum*. In the present study, it was lowest for P_4, P_{15} and P_5, which may be attributed to their low pollen fertility. Low pollen fertility and germination were found to reflect to some extent on percentage of hybrid seed set, in the present research work. This is in agreement with the findings of Lekha Rani (2002).

From the preliminary evaluation it may be concluded that wide range of variability exists among the fifteen monopodial orchid genotypes studied, for most of the biological traits analysed. Hence, these can be selected and utilized as parents in combination breeding programmes.

5.3. COMPATIBILITY ANALYSIS

Several cases of incompatibility have been reported in orchids, particularly in monopodials, although uninhibited intermingling of genomes has been pointed out as a characteristic feature of orchidaceae. Leonhardt (1977) found that incompatibility systems in orchids are of two types :

1) exogenous barriers such as geographical isolation, pollinator specificity and seasonal flowering habit.

2) endogenous barriers of genic or chromosomal nature.

Exogenous barriers can be easily overcome by hybridization under controlled conditions whereas endogenous systems may act as permanent barriers to hybridization. Endogenous incompatibilities of genic origin may be associated with an inability of pollen to germinate on a given stigmatic surface or an inability of the pollen tubes to grow down the column and reach the ovules. In a comparatively milder form of incompatibility, inhibition may occur in the ovary, preventing fertilization. All these forms of incompatibility are manifested externally as initial flower drop following pollination.

In the present investigation, abscission of pollinated flowers without any visible change occurred in 35 combinations. After initial swelling and greening of ovary 57 combinations abscised within two weeks. In 36 combinations, abscission took place during third and fourth weeks. During fifth and sixth weeks also developing capsules dropped off. Growing capsules abscised during seventh and eighth weeks also. This large scale initial ovary drop can be attributed to the above mentioned genic incompatibility. The monopodial trigeneric hybrids included in the experiment *viz.*, *Kagawara* and *Mokara* orchids are having highly complex genetic make up as they contain genes from three different genera. *Kagawara* is a multi generic comprising of *Ascocentrum*, *Renanthera* and *Vanda*. *Mokara* is derived from the artificial hybridization of *Arachnis* , *Ascocentrum* and *Vanda*. This complex genetic constitution of these grexes certainly led to the high percentage of incompatibility (189/225 = 84 %) which was categorized into nine groups. Only 36 combinations were planted out successfully among the 225 combinations attempted i.e., 16 %. While analysing the successful combinations, it is obvious that when these trigenerics were used as female parent, only five hybrids were obtained for planting out.

Ninitha Nath (2003) also opinioned that high ovary drop (20.70 %) after initial swelling followed by pollination in the pre and post-zygotic phases may be due to genic incompatibility. Devi and Deka (1994), Varghese (1995), Sobhana (2000) and Lekha Rani (2002) have also reported very high initial ovary drop in orchids like *Dendrobium*. Changes following pollination and the control exerted by the rostellar stigmatic region in bringing about these changes have been studied at length by Harrison and Arditti (1972), Arditti (1979) and Slater (1991). In the present study, post pollination floral changes comprising of the changes happening to the perianth, column and ovary have been analysed in detail. The changes consisted of drooping of sepals and petals and closure of stigma by over growth of column tip which were completed within two to eight days, covering of stigma by wilted sepals and petals which was over within four to twelve days and complete drying of sepals and petals which took nine to seventeen days in the

different varieties studied. A female parent dependent variation was observed for the onset of these changes in the varieties studied. Ninitha Nath (2003) conducted similar studies in twelve monopodial orchid genotypes. In *Cymbidium*, Leornhadt (1977) encountered 89.30 per cent initial ovary drop. He further observed that in analysing inter and intra group compatibilities, the following four parameters were the most useful :

1) Percentage capsule yield
2) Percentage capsules with filled / apparently normal seeds
3) Percentage filled seeds per capsule
4) Percentage capsules with germinating seeds

Leonhardt (1977) in *Cymbidium* reported that a total of 2466 pollinations were made with 265 (10.75 %) fruits harvested of which 182 (68.68 %) contained an average of 31.30 per cent seeds with apparently viable embryos. Of these, seeds from 142 fruits (53.58 %) germinated, producing seedlings.

In the present study, out of the 225 combinations attempted, 70 (31.11 %) succeeded in producing harvestable green capsules. Out of these, 55 (24.44 %) combinations which contained filled seeds were inoculated *in vitro*. Seeds from 43 (19.11 %) germinated *in vitro*. Ninitha Nath (2003) conducted similar studies in monopodials. Out of the 116 cross combinations attempted, 58 (50.00 %) succeeded in producing harvestable green capsules. Among these, 46 combinations cultured *in vitro* and capsules from 36 (78.26 %) combinations contained germinating seeds. Similar findings were reported by Lekha Rani (2002) in *Dendrobium*. She carried out 1696 pollinations in 84 combinations with 218 (12.58 %) green capsules harvested, of which 211 (96.78 %) in 81 combinations contained an average of 33.54 per cent seeds with apparently viable embryos. Out of these, seeds from 197 green capsules (93.36 %) belonging to 76 combinations germinated *in vitro*.

Parthenocarpic fruit development without seed set has been reported in orchids by several workers (Devi and Deka, 1992; Varghese, 1995; Sobhana, 2000; Lekha Rani, 2002). Some pre or post-fertilization barriers may be operational in such cases. This type of parthenocarpic fruit development was observed in fifteen cross combinations, in the present study. Similar results were obtained by Ninitha Nath (2003) in twelve combinations, in monopodial orchids. The production of non-germinating, yet apparently viable seeds is another incompatibility system which was found operational in twelve cross combinations. Ninitha Nath (2003) also observed a similar situation, in ten monopodial cross combinations. Such embryos may be dormant or non-functional due to various reasons such as structural hybridity, genic and chrormosomal imbalances, chemical inhibitors etc.

In the present study, incompatibility was found to strike at later stages also. Out of the 43 germinating combinations, 36 combinations provided mature seedlings for deflasking. In seven combinations incompatibility was found to strike at different stages in the post zygotic phase. In these combinations, growth rate slowed down considerably prior to degeneration *in vitro,* suggesting perhaps the involvement of genic and chromosomal incompatibility mechanisms. Similar observations were also reported by Ninitha Nath (2003) were out of the 36 germinating combinations, 24 combinations provided mature seedlings for deflasking and in 12 combinations incompatibility was found after germination.

Failure of fruit development has been observed in many crosses especially when particular varieties were used as male parents (Ninitha Nath, 2003). In *Dendrobium,* the same problem had been reported by Devi and Deka (1992), Varghese (1995) and Lekha Rani (2002). This bears evidence to the functioning of an unidirectional incompatibility system within the family. Leonhardt (1977) has reported a similar incompatibility system in hybridization between *Cymbidium* and *Ansellia* whereby seedlings were easily produced when *Cymbidium* was used as the female parent and rarely produced when *Ansellia* was used as the female parent.

From the analysis of compatibility relationships of the present research programme, it can be understood that strong incompatibility systems exist in monopodial orchid species such as *Renanthera coccinea* and *Vanda spathulata*. When used as female parents, they did not produce any seedlings. When large flowers were pollinated using pollen from these small flowers, the incompatibility noted may be purely physical rather than genetic. The pollen tubes may not have had the physical capacity to grow down the length of the column to reach the unfertilized ovules. This may be the reason for strong incompatibility when the above mentioned two species were taken as male parents.

Incompatibility reaction is strongest when it strikes at an early stage i.e., flower drop after pollination before the onset of any post pollination floral change. So when a particular parent as male or female presents this symptom after pollination in several cross combinations, the strength of its incompatibility reaction can be considered to be high. In the present investigation strongest incompatibility was observed in P_4 as pollinated flowers in all the combinations attempted, abscised without any changes or within two weeks after pollination.

Extent of incompatibility reaction denotes the number of cross combinations in which incompatibility reaction is presented (at any stage) when a particular parent is used as male or female. The more the number of crosses presenting incompatible reaction, the greater the extent of incompatibility. Extent of incompatibility was the highest in P_4, P_5, P_7 and P_8 denoting that the highest number of crosses expressed incompatibility reaction at various stages from pollination to deflasking in these parents. Incompatibility reaction was presented by all the fifteen combinations when these were used as either the female or the male parent.

Ranking of the fifteen parental genotypes was done based on compatibility, i.e., the number of successful combinations that were deflasked (Table 30). The parent P_{14} was ranked first, providing a total of eighteen successful combinations – seven and eleven as male and female parents

Table 30. Ranking of 15 monopodial orchid parental genotypes based on compatible crosses

Parental genotypes	No. of combinations providing hardened seedlings			Rank assigned
	As female parent	As male parent	Total	
P_1	0	2	2	8
P_2	7	7	14	2
P_3	3	2	5	5
P_4	0	0	0	10
P_5	0	0	0	10
P_6	1	2	3	7
P_7	0	0	0	10
P_8	0	0	0	10
P_9	2	2	4	6
P_{10}	2	3	5	5
P_{11}	0	2	2	8
P_{12}	3	4	7	4
P_{13}	7	4	11	3
P_{14}	11	7	18	1
P_{15}	0	1	1	9

respectively. Second position was for P_2 with fourteen successful combinations to its credit which included seven combinations each, as male and female parents. P_{13} ranked third with seven and four successful combinations as the male and female parents, respectively, making a total of eleven. The multigenerics P_4, P_5, P_7 and P_8 turned out to be the poorest combiners, ranking last. No combination turned out to be successful when these were used as either male or female parent. Self compatibility was noticed in two genotypes *viz.*, P_{13} and P_{14}, while the remaining thirteen were self incompatible.

5.4. *IN VITRO* EMBRYO CULTURE

As orchid seeds lack nutritive tissues in them , they are to be cultured *in vitro*. Green capsule culture was a major advancement in increasing the germination of orchid seeds in culture media. Withner (1959) was of opinion that very young as well as fully mature ovules did not form good explants *in vitro* due to dormancy, pH, inhibitory and other metabolic factors. Sauleda (1976) found that the pistillate parent was mainly responsible for determining the correct capsule maturity stage. Sobhana (2000) harvested green capsules of *Dendrobium* at 75-90 per cent maturity, *viz.*, 90-140 days after pollination. Lekha Rani (2002) too confirmed these findings in *Dendrobium* and the capsules were harvested at 62-130 days after pollination *viz.*, between 75-90 per cent maturity. In the present study also, capsules were harvested at 74-135 days after pollination with very high success in terms of *in vitro* germination.

The inherent genetic and physiological features were found to play a direct role in *in vitro* seed germination and differentiation of organs. Hazarika and Sarma (1995) in *Dendrobium transparens* observed seed germination 16-18 days after inoculation and Krishnan et al. (1993) observed the same in *Spathoglottis plicata* around two weeks of culture.

Hybrid seeds were *in vitro* cultured, after selecting suitable medium for monopodials i.e., MS half strength, in the present research work. Refinement of

culture medium to promote seedling growth was done, identifying 8 mg l^{-1} IAA + 2 mg l^{-1} NAA as the best treatment. Coconut water 200 ml l^{-1} was also added to improve the *in vitro* growth of seedlings in all situations. This was proved to be the best organic additive for *Dendrobium* hybrid seedlings by Lekha Rani (2002) and Sivamani (2004). Activated charcoal was added at a concentration of 1g l^{-1} to all the media studied due to its beneficial effect of adsorption of inhibitory phenolic and carboxylic compounds produced by the tissues in culture.

The present investigation exhibited a wide range in duration for *in vitro* germination from 20.20 days (P_{13} x P_{13}) to 42.80 days (P_{10} x P_2). Generally, when complex bigenerics and trigenerics are included in the parentage the hybrid combinations exhibited more number of days for all the *in vitro* developmental stages, till deflasking. Ninitha Nath (2003) revealed similar findings in monopodials. A wide range in duration from 15.50 to 40.50 days was found for *in vitro* germination. The highly bred bigeneric hybrid varieties included in the parentage required more number of days. The same general trend of slow growth was followed by the complex hybrids throughout *in vitro* growth, till deflasking.

5.5. SEEDLING MORPHOLOGY AT PLANTING OUT AND HARDENING

Post deflasking survival of plantlets depended greatly on the number of leaves present at the time of deflasking and the rate of leaf growth. Sutter *et al.* (1985) expressed the view point that the stage at which deflasking is done is important in determining further survival. The results of the present study were also in full conformity with his findings that survival was the highest with seedlings that had developed a minimum 2-3 leaves and 2-4 roots. The lesser the time taken to reach this stage, the more vigorous the hybrid combination was considered to be. Thus the duration to deflasking is important in that it is an indication of the vegetative vigour or heterotic potential of the hybrid.

In *Dendrobium*, Lekha Rani (2002) reported that fast growing cultures yielding vigorous seedlings continued the rapid rate of growth in the greenhouse also, reaching flowering stage much earlier than slow growing cultures. Hence a detailed analysis of days taken for deflasking and seedling morphology at deflasking are important because they reflect further growth and development in the greenhouse.

In the present research programme, seedling morphology at deflasking and four months after planting out were analysed with respect to height of seedling, number of leaves per seedling, length and breadth of the longest leaf, number of roots per seedling and length and thickness of the longest root. Seedling height at deflasking showed a highly significant wide range from 2.08 cm in $P_{14} \times P_1$ to 4.02 cm in $P_{12} \times P_{13}$ with variation between combinations, indicating the effect of genotype in character expression. Length of the longest leaf ranged from 1.24 in $P_{12} \times P_2$ to 2.32 cm in $P_2 \times P_{14}$ and $P_3 \times P_{14}$ and length of the longest root ranged from 0.56 cm in $P_{14} \times P_1$ to 1.36 cm in $P_2 \times P_{12}$, $P_3 \times P_2$, $P_9 \times P_{14}$ and $P_{12} \times P_{13}$. In general, an enhanced seedling height at deflasking stage indicates higher root length and leaf length as has been reported by Lekha Rani (2002).

5. 6. MOLECULAR CHARACTERIZATION

In orchids, molecular characterization is possible using a wide range of molecular markers. These are utilized to evaluate DNA polymorphism and among them, the most important is polymerase chain reaction (PCR) based markers. PCR based molecular markers have been developed into powerful tools to analyse genetic relationships and genetic diversity. Among them RAPD is the most extensively used technique. This has significantly contributed to our understanding of the species at the genetic level. RAPD techniques have been used for the identification of hybrids and their parentage determination.

Wang et al. (2003) proposed RAPD fingerprinting as a convenient tool for the identification, protection and parentage determination of plant hybrids. Bhat

and Jarret (1995) suggested that the number of polymorphisms might be more important than the number of primers for the generation of stable phenogram and it would vary with plant material under investigation and the sequences that are amplified.

In the present study, RAPD was employed for studying the genetic diversity and for the fingerprinting of the 20 monopodial orchid hybrids, making use of arbitrary primers to amplify random DNA sequences in the genome. The results are discussed below.

Isolation of genomic DNA of monopodial orchid hybrids was done using Mondal et al. (2000) method with slight modification. Tissues from young tender leaves were found to yield good quality DNA. The DNA yield for 20 monopodial orchid hybrids ranged from 660 to 990 ng μl^{-1}. The purity of DNA (A_{260}/A_{280} ratio) ranged from 1.60 to 1.83 $\mu g\ \mu l^{-1}$.

To identify the promising primers for RAPD analysis, 70 decamer primers of kit A, B, C and D were screened using the DNA of hybrid H-2. The procedure standardized by Lim et al. (1999) for *Vanda* was adapted for amplification.

Based on the performance in DNA amplification, eight decamer primers were identified for RAPD analysis. Primers that produced highest number of polymorphic bands which were intense and reproducible were selected. They were OPB-07, OPB-15, OPB-17, OPC-04, OPC-05, OPC-08, OPC-15 and OPD-02. Pillai (2003) concluded that four primers OPA-10, OPB-02, OPB-04 and OPB-10 were yielding good resolution bands when used for the molecular characterization of fifteen *Dendrobium* varieties using RAPD. Three primers OPB-11, OPB-12 and OPB-17 were used for RAPD analysis to characterize genetic variability and relationships among 12 cultivars of *Dendrobium* at molecular level (Krishnapriya, 2005).

Rahana (2006) conducted similar RAPD analysis for the molecular characterization of 40 selected *Dendrobium* hybrids, making use of arbitrary

primers to amplify random DNA sequence in the genome. Eight decamer primers were identified for RAPD analysis based on the performance in DNA amplification and production of highest number of polymorphic bands. They were OPA-03, OPA-04, OPA-10, OPA-16, OPA-18, OPB-02, OPB-06 and OPB-10.

Rahana et al. (2007) reported that a total of 69 scorable bands (average of 8.63 bands per primer) were generated by the selected eight primers of which three were monomorphic and the remaining 66 were polymorphic (95.65 per cent). The number of amplification products ranged from four to twelve with an average of two per primer. The highest number of scorable bands (12 bands) was given by OPA-18 followed by OPA-10 (11 bands), OPA-16 (11 bands), OPB-06 (9 bands), OPA-04 (8 bands), OPB-02 (7 bands), OPB-10 (7 bands) and OPA-03 (4 bands). Out of the four scorable bands produced by the primer OPA-03, two bands were monomorphic while one band was monomorphic among the eight scorable bands produced by primer OPA-04.

In the present study a total of 57 scorable bands (average of 7.125 bands per primer) were generated by the selected eight primers of which six were monomorphic and the remaining 51 were polymorphic (89.47 %). The number of amplification products ranged from five to ten with an average of two per primer. In similar studies conducted in fifteen *Dendrobium* varieties, Pillai (2003) obtained 44 amplified RAPD markers of which 39 were polymorphic (88.6 %) and five were monomorphic. Chakrabarti (2005) obtained a total of 227 distinct major RAPD bands of which 97 per cent were polymorphic whereas out of the 27 bands amplified, 24 were found to be polymorphic (88.9 %) by Krishnapriya (2005) in *Dendrobium.*

The highest number of scorable bands (10 bands) was given by OPB-15 followed by OPC-15 (9 bands), OPD-02 (8 bands), OPB-17 (7 bands), OPC-05 (6 bands), OPC-08 (6 bands), OPB-07 (6 bands) and OPC-04 (5 bands). All the 10 bands obtained from OPB-15 were polymorphic. The nine bands produced by primer OPC-15 were also polymorphic. Out of the six bands produced by

OPB-07, one was monomorphic and among the five scorable bands from OPC-04 one was monomorphic. The primers, OPC-08 and OPD-02 also produced one monomorphic band each. Among the six scorable bands produced by primer OPC-05 two bands were monomorphic.

The estimation of Jaccard's coefficients and construction of dendrogram by using UPGMA revealed the presence and extent of genetic similarities among the 20 monopodial orchid hybrids. The overall similarity coefficients ranged from 0.40 to 0.84. The genetic distance expressed the genetic variability that exists between the hybrids. Rahana et al. (2007) undertook similar research work and reported that Jaccard's coefficients and dendrogram using UPGMA revealed the presence and extent of genetic similarities among the 40 selected *Dendrobium* hybrids. The overall similarity coefficients ranged from 0.29 to 1.00. In agreement with the present results, the genetic distance revealed the genetic variability existing between the hybrids.

Cluster analysis revealed that at 0.69 similarity coefficient, the 20 monopodial orchid hybrids got divided into six groups. Among the 20 hybrids, H-18, H-7, H-13, H-17, H-19 and H-20 stood separately in clusters II, III A, III B, IV, V and cluster VI respectively. This substantiates the moderately broad distribution of genetic variability, which can be attributed to the broad genetic base in their ancestry. Out of the 20 hybrids included in the present study, 16 (H-1 to H-16) are higher order hybrids with three to four distinct species in varying dosages involved in their parentage. The parents involved in the development of these hybrids and their ancestry are presented in Appendix II. The four remaining hybrids (H-17 to H-20) were obtained by selfing *Arachnis* Maggie Oei Red Ribbon, which inturn was produced artificially by crossing *Arachnis hookeriana* with *Arachnis flos-aeris*. In a highly heterogenous family like orchidaceae, even selfing creates variability. The presence of two parents in the ancestry of these hybrids supports their grouping into separate clusters.

Cluster I A contained four hybrids H-1, H-10, H-3 and H-9. Their grouping in the same cluster indicates the presence of a common parent i.e., *Arachnis* Maggie Oei Red Ribbon in their parentage. This reveals the involvement of two common species *viz.*, *Arachnis hookeriana* and *Arachnis flos-aeris* in their ancestry, supporting their belonging to the same cluster.

The three hybrids H-5, H-6 and H-8 fell in cluster I B. H-5 and H-6 were developed from the same grex combination (*Aranthera* James Storei x *Arachnis* Maggie Oei Red Ribbon). H-8 is having *Arachnis* Maggie Oei Red Ribbon as one of the parents. Similarity among these hybrids can be substantiated by their pedigree relationship which reveals two parental species *viz.*, *Arachnis hookeriana* and *Arachnis flos-aeris* , in common for them (Appendix II).

At a similarity coefficient of 0.75, cluster I C can be bifurcated into two groups *viz.*, I C_1 and I C_2. H-2, H-12 and H-11 belonged to the first subgroup which can be supported by the presence of *Arachnis hookeriana* in their ancestry. Second subgroup included H-4, H-14, H-15 and H-16. This was supported by the fact that they have a common parent *viz.*, *Aranthera* James Storei , which was developed by hybridization between two species *i.e., Arachnis hookeriana* and *Renanthera storiei*

H-7 and H-13 can be included in a single group, cluster III, at similarity coefficient of 0.66. This can be justified by the fact that these two hybrids have two species common in their pedigree chart *viz., Arachnis hookeriana* and *Arachnis flos-aeris*. They showed 67.65 per cent similarity with one another. Other groups remain the same as at similarity coefficient of 0.69.

When clustering was done at a similarity coefficient of 0.58, 14 hybrids *viz.*, H-1, H-10, H-3, H-9, H-5, H-6, H-8, H-2, H-12, H-11, H-4, H-14, H-15 and H-16, stood together in cluster I. H-18 remained separately in cluster II. H-7 and H-13 belonged to cluster III. The hybrids, H-17, H-19 and H-20, were singled out into clusters IV, V and VI respectively, eventhough they were developed by

selfing as discussed earlier. Similar cluster analysis was done by Rahana et al. (2007), grouping 40 selected *Dendrobium* hybrids into two main clusters, cluster I and II, at a similarity coefficient of 0.46. Among the hybrids, H-376 fell in the first group showing high degree of variability from other hybrids.

In the present study, the cluster based on RAPD analysis using eight primers clearly demonstrates the existence of genetic variation within the 20 monopodial orchid hybrids. It reveals the expression of ancestral characters and thereby the occurrence of recombination in these hybrids. Polymorphism revealed here will be useful, in the fingerprinting of these hybrids. The results prove that RAPD technique being relatively simpler, quicker, less expensive and non-radioactive, can detect sufficient polymorphisms in genetic distance studies. It also suggests that these hybrids can be utilized in further orchid improvement programmes for producing novel grexes as they are genetically dissimilar.

The objectives of the present study were to undertake intra and intergeneric hybridization, *in vitro* embryoculture and molecular characterization in monopodial orchids as a preliminary step to develop novel hybrids. With these objectives in focus, hybridization in all possible combinations was carried out among the selected parental genotypes. Hybrid seedlings were deflasked at the 2 to 3 leaves and 2 to 3 roots stage, seedling morphology was studied. They were planted using mixture of broken tiles + charcoal + coconut husk (each item as 2 x 2 x 2 cm pieces) in 2 : 2 : 1 ratio, in plastic cups with holes for drainage. Detailed analysis of compatibility incompatibility relations was done at the different stages starting from immediately after pollination till deflasking. Among the 225 cross combinations, seedlings from 36 combinations were successfully deflasked, for *ex vitro* establishment and they were acclimatized in the humidity chamber for one month. After acclimatization, they were transferred to the net house (50 % shade), for hardening. Four months after planting out they were repotted in small clay pots with the same initial potting medium and were kept in the net house for further development.

The following future lines of work have been suggested based on the outcome of the present investigation :

a) Periodical evaluation of the new hybrids.
b) Morphological and molecular characterisation of the newly developed hybrids.
c) Chromosomal, cytological, genetic and pollen studies of the parents and hybrids.
d) Estimation of heterosis exhibited by the novel hybrids with respect to important biometric characters.
e) Selection and micropropagation of promising hybrids.
f) Adaptability trials for variety release.
g) Detailed analysis of the pre and post zygotic incompatibility systems, so as to select compatible parents in further breeding programmes.

SUMMARY

6. SUMMARY

A research programme on "Intra and inter generic hybridization and molecular characterisation in monopodial orchids" was conducted in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2005-2008 with the objectives of undertaking intra and intergeneric hybridization, *in vitro* embryo culture and molecular characterization in monopodial orchids, as a preliminary step to develop novel hybrids.

- Fifteen genotypes of monopodial orchids belonging to six genera *Aranda, Aranthera, Kagawara, Mokara, Renanthera* and *Vanda*, with good cut flower qualities and high demand in the market were evaluated adopting completely randomized design with ten replications.

- Observations were collected for vegetative characters and floral characters from the selected parental genotypes.

- Analysis of variance revealed significant differences among the parental genotypes with respect to all the 22 (ten vegetative + twelve floral) biometric characters studied.

- High phenotypic and genotypic coefficients of variation were observed for thickness of leaf, leaf area and number of aerial roots indicating high variability for these characters and scope for improvement through selection.

- Genetic advance (% mean) was high (>70) for six characters considered. It was exhibited in the range of 30-70 per cent by sixteen characters analysed.

- High heritability (>70 %) combined with 30-70 per cent genetic advance was observed for length of shoot, number of leaves per shoot, length of aerial roots, thickness of shoot, length of internode, length of leaf, days to last flower opening from first flower opening, length of inflorescence,

length of scape, thickness of inflorescence axis, length of internode of inflorescence, length of flower, width of flower, pollen size, vase life and fullness value.

- High heritability (>70 %) combined with genetic advance greater than 70 per cent was observed for number of aerial roots, width of leaf, thickness of leaf, leaf area, number of spikes per shoot and number of flowers per inflorescence, indicating additive gene action for these characters. This suggests that permanent improvement could be attained by practising selection on the above traits.

- The genotypic, phenotypic and environmental correlations of the fifteen parents were studied for ten biometric characters. High positive correlation at genotypic and phenotypic levels was observed between most of the vegetative and floral characters studied.

- Significant positive inter-correlation at genotypic and phenotypic levels was observed for length of flower and width of flower with number of spikes per shoot. The character number of spikes per shoot recorded significant positive correlation with leaf area. Number of flowers per inflorescence was positively correlated with number of leaves per shoot and length of inflorescence.

- Vase life showed highest positive correlation with length of inflorescence followed by number of flowers per inflorescence and number of leaves per shoot. This trait showed highly significant negative correlation with width of flower and number of aerial roots.

- Environmental correlation was observed to be low in comparison with genotypic and phenotypic correlations for all pair-wise character combinations.

- In the case of length of inflorescence, its environmental correlation with length of scape was positive and significant which was observed to be higher than the genotypic and phenotypic correlations.

- Flowering and floral quality of the parental genotypes were analysed with respect to flowering nature, nature of inflorescence axis, mode of display of flowers and shape of flower.

- Out of the six genera studied, four *viz., Aranda, Aranthera, Kagawara* and *Mokara*, exhibited free flowering nature. Seasonal flowering was observed mainly from June to December in *Vanda*, whereas it was confined to two seasons, from February to March and August to October in *Renanthera.* Inflorescence axis was found to be arching in *Aranthera* and *Renanthera* while the rest of the genotypes produced erect inflorescence axis.

- The flowers were full and flat with broad sepals and petals in almost all the parental genotypes except P_2, P_3 and P_{11} where the flowers presented a flat appearance with narrow spatulate sepals and petals with the tips slightly incurved.

- In all the monopodial genotypes studied, flowers opened during the day-time, in acropetal succession. Each flower in an inflorescence opened almost during the same time of the day at a uniform time interval. Flower opening commenced from 7.30 am on sunny days and was delayed till 12.30 pm on rainy days.

- Anthesis time was studied based on the capacity of pollinia to effect capsule set after pollination. Mean anthesis time ranged from 2.0 in P_{15} to 3.8 days in P_{11}. Anthesis time was comparatively early in P_7, P_1, P_{12}, P_4 and P_{13}. This was comparatively late in P_{10}, P_3 and P_9.

- Maximum stigma receptivity period ranged from second to fourth day in P_{15} to fourth to tenth day in P_3, P_9 and P_{11}. In four genotypes tested *viz.*, P_2, P_7, P_8 and P_{10} stigma receptivity was observed from third to ninth day after flower opening. High stigma receptivity was observed from second to fifth day after flower opening in P_1 where as it was from second to sixth day in four parentals *viz.*, P_5, P_{12}, P_{13} and P_{14}.

- Pollen was found to exist as tetrads which were spherical to rectangular in shape and agglutinated in masses called 'pollinia'. Although similar in shape, pollen size ranged from 19.79 µ in P_4 to 43.56 µ in P_3.

- Pollen fertility percentage showed much variation among the parental genotypes. The highest pollen fertility was recorded by P_3 (78.47 %) and lowest by P_5 (50.75 %).

- Pollen germination varied significantly among the genotypes assessed. Highest pollen germination per cent was observed in P_3 (66.42 %), followed by P_2 (63.24 %), as the trend in pollen fertility. It was lowest for P_4 (15.80 %).

- Intercrossing in all possible combinations involving the fifteen parental genotypes of monopodial orchids was attempted, depending on the availability of receptive stigma and fresh pollen. These 225 combinations include 105 crosses, 105 reciprocals and 15 selfs.

- Mature green capsules were harvested from 70 combinations at 70 to 90 per cent maturity, out of which no seeds were obtained from the capsules of 15 combinations. Seeds from the remaining 55 combinations were cultured axenically.

- Percentage capsule yield ranged from seven in $P_2 \times P_5$ and $P_2 \times P_6$ to 40 in $P_{12} \times P_{14}$ and $P_{14} \times P_{13}$ in individual combinations.

- Mean percentage capsule yield ranged from 14.50 in P_{10} to 28.25 in P_{12} when the genotypes were used as female parents and from 11.00 in P_5 to 27.82 in P_{14} when they were used as male parents.

- Mean percentage of capsules with germinating seeds ranged from 33 in P_5 and P_7 to 69.33 in P_{12} when the genotypes were used as female parents. The range was from 44.33 in P_1 to 100 in P_6 when the genotypes were used as male parents.

- Effect of media on *in vitro* growth of seedlings in monopodial orchid hybrids were studied using the hybrid combination P_{10} x P_2. MS half strength was selected as the best basal medium. Coconut water 200ml l^{-1} was used as additive in all the cases.

- Effect of BA, IAA and NAA on *in vitro* growth of seedlings in monopodial orchid hybrids was evaluated in detail using the hybrid combination P_{10} x P_2. For improving the *in vitro* growth of hybrid monopodial orchid seedlings refinement of medium by supplementing with IAA (8 mg l^{-1}) and NAA (2 mg l^{-1}) was beneficial.

- No germination was obtained with seeds from 12 out of the 55 combinations inoculated *in vitro*. Successful seed germination was observed in 43 combinations.

- Further development was found to be arrested in seven combinations at various stages of *in vitro* development. Mature seedlings were obtained from 36 combinations.

- The levels of incompatibility reactions were grouped under nine heads ranging from flower abscission before the onset of any visible post pollination change to instances where seeds germinated but aborted in culture. A total of 189 combinations attempted succumbed to incompatibility at these different stages from pollination to deflasking.

- The successful cross combinations were cultured and taken through three to four subculture passages. Seedlings having 2-3 leaves and 2-3 roots were deflasked. Time taken for attaining this stage varied from 196.40 days to 283.80 days. Significant differences among the combinations were observed with respect to number of days taken for germination initiation, number of days for development of protocorms, chlorophyll, first leaf and first root primoridia and for deflasking.

- Significant differences in seedling morphology were observed among the 36 combinations at deflasking and four months after planting out, with respect to various vegetative characters studied.

- The hybrid seedlings were kept in humidity chamber for acclimatization for one month, transferred to net house for hardening and maintained there for further growth.

- In the present investigation, RAPD was employed for studying the genetic diversity and for the fingerprinting of 20 monopodial orchid hybrids, making use of arbitrary primers to amplify random DNA sequence in the genome.

- The DNA yield of 20 monopodial orchid hybrids ranged from 660 to 990 ng μl^{-1} and its purity ranged from 1.60 to 1.83 $\mu g\ \mu l^{-1}$.

- To identify the promising primers for RAPD analysis, 70 decamer primers of kit A, B, C and D were screened using the DNA of hybrid H-2.

- Based on the performance in DNA amplification, eight decamer primers were identified for RAPD analysis. Primers that produced highest number of polymorphic bands which were intense and reproducible were selected. They were OPB-07, OPB-15, OPB-17, OPC-04, OPC-05, OPC-08, OPC-15 and OPD-02.

- A total of 57 scorable bands (average of 7.125 bands per primer) were generated by the selected eight primers of which six were monomorphic and the remaining 51 were polymorphic (89.47 %).

- The number of amplification products ranged from five to ten with an average of two per primer.

- The estimation of Jaccard's coefficients and construction of dendrogram by using UPGMA revealed the presence and extent of genetic similarities among the 20 monopodial orchid hybrids. The overall similarity coefficients ranged from 0.40 to 0.84.

- Cluster analysis revealed that at 0.69 similarity coefficient, the 20 monopodial orchid hybrids got divided into six groups. Among the 20 hybrids, H-18, H-7, H-13, H-17, H-19 and H-20 stood separately in clusters II, III A, III B, IV, V and cluster VI respectively. This substantiates the moderately broad distribution of genetic variability, which can be attributed to the broad genetic base in their ancestry.

- Cluster I A contained four hybrids *viz.*, H-1, H-10, H-3 and H-9. This grouping is justified by the presence of a common parent i.e., *Arachnis* Maggie Oei Red Ribbon in their parentage. Moreover, two common species *viz.*, *Arachnis hookeriana* and *Arachnis flos-aeris* are involved in their ancestry. All these support their belonging to the same cluster.

- The three hybrids *viz.*, H-5, H-6 and H-8 fell in cluster I B where H-5 showed 78.57 % similarity with H-6 and 62.79 % with H-8.

- At a similarity coefficient of 0.75, cluster I C could be divided into two groups *viz.*, I C_1 and I C_2. The hybrids such as H-2, H-12 and H-11 belonged to the first subgroup.

- RAPD technique is relatively simpler, quicker, less expensive and non-radioactive than other molecular characterization techniques. The results of present research proved that it can detect sufficient polymorphisms in genetic distance studies in monopodial orchids. These hybrids can be utilized in further orchid improvement programmes for producing novel grexes as they are genetically dissimilar.

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* originals not seen

APPENDICES

APPENDIX I

Basic chemical composition of the media employed for *in vitro* hybrid seed culture

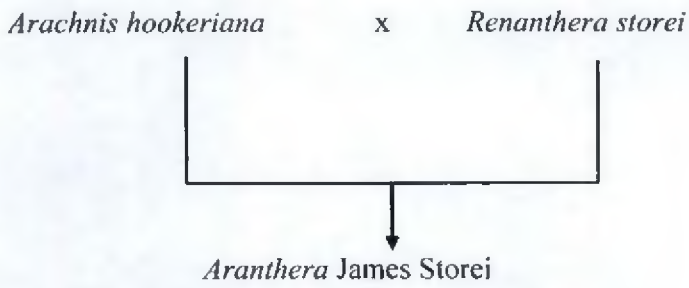
Chemical	Quantity (mg l^{-1})		
	KC	MS	VW
Major Elements			
$Ca(PO_4)_2$	-	-	200.0
$CaCl_2\ 2H_2O$	-	440.00	-
$Ca(NO_3)_2\ 4H_2O$	1000.0	-	-
Ferric citrate	-	-	28.0
$FeSO_4\ H_2O$	25.0	27.800	-
KNO_3	-	1900.000	525.0
KH_2PO_4	250.0	170.000	250.00
$MgSO_4\ 7H_2O$	250.0	370.000	250.00
$(NH_4)_2\ SO_4$	250.0	-	500.0
NH_4NO_3	-	1650.000	-
Na_2 EDTA	-	37.300	-
Minor Elements			
$CoCl_2\ 6\ H_2O$	-	0.025	-
$CuSO_4\ 5H_2O$	-	0.025	-
H_3Bo_3	-	6.200	-
KCl	-	0.830	-
$MnSO_4$	7.5	22.300	7.0
Na_2MoO_4	-	0.250	-
$ZnSO_4$	-	8.600	-
Organic Constituents			
Glycine	-	2.000	-
Myo-inositol	-	100.000	-
Nicotinic acid	-	0.500	-
Pyridoxine HCl	-	0.100	-
Thiamine HCl	-	0.100	-

KC Knudson C medium
MS Murashige and Skoog medium
VW Vacin and Went medium

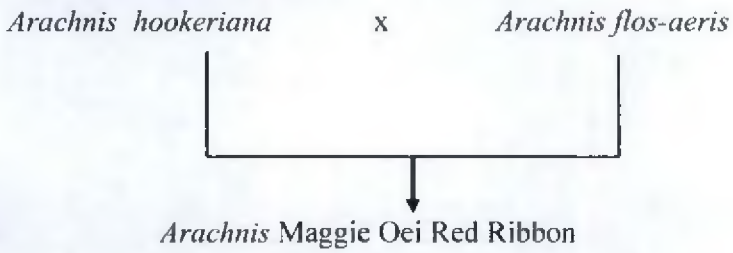
APPENDIX –II

Pedigree chart showing the ancestry of parental varieties of hybrids H_1 to H_{20}

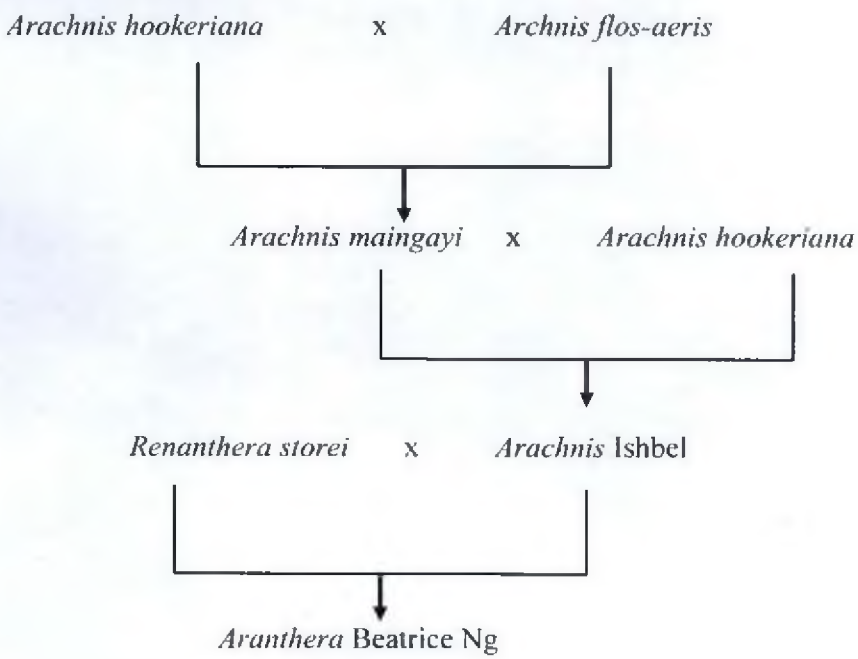
(1) *Aranthera* James Storei



(2) *Arachnis* Maggie Oei Red Ribbon



(3) *Aranthera* Beatrice Ng



ABSTRACT

INTRA AND INTER GENERIC HYBRIDIZATION AND MOLECULAR CHARACTERIZATION IN MONOPODIAL ORCHIDS

BEENA THOMAS

Abstract of Thesis

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Department of Plant Breeding and Genetics,
COLLEGE OF AGRICULTURE,
VELLAYANI, THIRUVANANTHAPURAM 695 522

ABSTRACT

A research programme entitled "Intra and inter generic hybridization and molecular characterization in monopodial orchids" was carried out in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2005-2008 with the objective of undertaking intra and intergeneric hybridization, *in vitro* embryo culture and molecular characterization in monopodial orchids, as a preliminary step to develop novel hybrids.

Fifteen monopodial orchid genotypes comprising of six genera *viz.*, *Aranda, Aranthera, Kagawara, Mokara, Renanthera* and *Vanda*, with good cut flower qualities and high demand in the market were selected as parents after initial evaluation. They were evaluated adopting completely randomized design with five replications.

Analysis of variance revealed significant differences for almost all the characters studied. Genotypic and phenotypic coefficients of variation were high for thickness of leaf, leaf area and number of aerial roots. High heritability (>70 %) combined with high genetic advance (>70 %) was observed for number of aerial roots, width of leaf, thickness of leaf, leaf area, number of spikes per shoot and number of flowers per inflorescence.

Significant positive inter-correlation at genotypic and phenotypic levels was observed for length of flower and width of flower with number of spikes per shoot. The character number of spikes per shoot recorded significant positive correlation with leaf area. Number of flowers per inflorescence was positively correlated with number of leaves per shoot and length of inflorescence.

Out of the six genera studied, four *viz.*, *Aranda, Aranthera, Kagawara* and *Mokara*, exhibited free-flowering nature. Seasonal flowering was observed mainly from June to December in *Vanda*, whereas it was confined to two seasons, from

February to March and August to October in *Renanthera.* Inflorescence axis was found to be arching in *Aranthera* and *Renanthera* while the rest of the genotypes produced erect inflorescence axis.

The 15 parental genotypes were crossed in all possible combinations after preliminary studies on floral biology. A total of 225 cross combinations were attempted including 105 crosses, 105 reciprocals and 15 selfs. Incompatibility reactions were noticed at different stages ranging from flower abscission before the onset of any visible post pollination change to instances where seeds germinated but aborted in culture. Mature green capsules were harvested from 70 combinations at 70 to 90 per cent maturity. Among them 15 combinations did not yield any seeds in the capsule while the remaining 55 combinations were cultured axenically. Among the 55 combinations inoculated *in vitro,* no germination was obtained from seeds of 12 combinations. Out of the 43 combinations that germinated successfully, seven combinations showed arrested development.

Thus out of the total 55 combinations inoculated *in vitro* 36 combinations developed successfully. These were subcultured three to four times. Seedlings having 2-3 leaves and 2-3 roots were deflasked and planted out. MS half strength was selected as the best basal medium. For improving the *in vitro* growth of hybrid monopodial orchid seedlings refinement of this medium by supplementing with IAA (8 mg l^{-1}) and NAA (2 mg l^{-1}) was beneficial.

Significant differences among the combinations were observed with respect to number of days taken for germination initiation, number of days taken for development of protocorms, chlorophyll, first leaf, first shoot and first root primordia and for deflasking.

Significant differences in seedling morphology were observed among the 36 hybrid combinations at deflasking. These were kept in humidity chamber for acclimatization for one month, transferred to net house for hardening and maintained there for further growth.

In the present study, RAPD was employed for studying the genetic diversity and for the fingerprinting of 20 monopodial orchid hybrids, making use of arbitrary primers to amplify random DNA sequences in the genome. To identify the promising primers for RAPD analysis, 70 decamer primers of kit A, B, C and D were screened using the DNA of hybrid H-2.

Based on the performance in DNA amplification, eight decamer primers were identified for RAPD analysis. Primers that produced highest number of polymorphic bands which were intense and reproducible were selected. They were OPB-07, OPB-15, OPB-17, OPC-04, OPC-05, OPC-08, OPC-15 and OPD-02. a total of 57 scorable bands (average of 7.125 bands per primer) were generated by the selected eight primers of which six were monomorphic and the remaining 51 were polymorphic (89.47%).

The estimation of Jaccard's coefficients and construction of dendrogram by using UPGMA revealed the presence and extent of genetic similarities among the 20 monopodial orchid hybrids. The overall similarity coefficients ranged from 0.40 to 0.84.

Cluster analysis revealed that at 0.69 similarity coefficient, the 20 monopodial orchid hybrids got divided into six groups. Among the 20 hybrids, H-18, H-7, H-13, H-17, H-19 and H-20 stood separately in clusters II, III A, III B, IV, V and cluster VI respectively. This substantiates the moderately broad distribution of genetic variability, which can be attributed to the broad genetic base in their ancestry.

Cluster I A contained four hybrids *viz.*, H-1, H-10, H-3 and H-9. This grouping is justified by the presence of a common parent *i.e., Arachnis* Maggie Oei Red Ribbon in their parentage. Moreover, two common species *viz., Arachnis hookeriana* and *Arachnis flos-aeris* are involved in their ancestry. All these

support their belonging to the same cluster. The three hybrids such as H-5, H-6 and H-8 fell in cluster I B.

RAPD technique is relatively simpler, quicker, less expensive and non-radioactive than other molecular characterization techniques. The results of present investigation proved that it can detect sufficient polymorphisms in genetic distance studies in monopodial orchids.