DISTANT HYBRIDIZATION AND COMPATIBILITY STUDIES IN WILD ORCHIDS

by

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(2015-21-028)

THESIS

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DEPARTMENT OF PLANT BREEDING AND GENETICS
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2018

DECLARATION

I, hereby declare that this thesis entitled "DISTANT HYBRIDIZATION AND COMPATIBILITY STUDIES IN WILD ORCHIDS" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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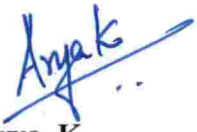
CERTIFICATE

Certified that this thesis entitled "DISTANT HYBRIDIZATION AND COMPATIBILITY STUDIES IN WILD ORCHIDS" is a record of research work done independently by Mrs. Seeja, G. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.



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INTRODUCTION

INTRODUCTION

Orchids, belonging to the most evolved monocotyledonous family Orchidaceae, consist of more than 800 genera and ~30,000 species. It is distributed all over the world except in a few isolated islands and Antarctica. Its' population is rigorous along the riverine and in moist forests since such habitats are suitable for their growth and regeneration. It is considered as an ancient family of angiosperm showing immense ecological and morphological diversity with the highest rate of speciation and species extinction (Gravendeel *et al.*, 2004; Swarts and Dixon, 2009) mainly due to the destruction of natural ecosystem. As part of evolutionary process they showed immense diversity in habits with variously modified vegetative and floral characters, which made each orchid species as unique and distinctive. Based on the growth habit they can be categorized as monopodial and sympodial and based on the growing habitats they can be grouped as terrestrials, epiphytes, lithophytes and saprophytes.

India, one of the 12 mega biodiversity countries in the world, is blessed with varied agro-climatic conditions and has suitable habitats for the luxurious growth of orchids. Although orchids are distributed throughout the country, its diversity and distribution is peak in the Eastern India and the Western Ghats (Jalal, 2012). The other orchid diversity rich regions in the country are North-Eastern and the Western Himalayas and Andaman and Nicobar Islands. In India ~1331 species belonging to 186 genera of orchids were recorded, of these ~ 856 species were from Northeast region. It is also believed that orchids are evolved in the Northeast India (Kumaria and Tandon, 2007). However, more number of endemic species is found from the Western Ghats (Jalal and Jayanthi, 2012). Out of 130 endemic orchid species under 38 genera in peninsular India, the Western Ghats, Deccan Plateau, and Eastern Ghats have 123, 29, 22 species respectively.

Orchids are popular all over the world since they produce the long lasting and most beautiful flowers with amazing unique characteristic features. .The extraordinary beauty of flowers naturally makes them as a multimillion- dollar

industry. In addition to aesthetic value many orchids are used as medicine, cosmetics and spices. They also have pivotal role to balance the forest ecosystem. Orchids are also important biological indicators of environmental degradations because of their niche specificity.

The unprecedented elegancy and diversity of orchid flowers is contributed by the unique floral modification such as morphology of sepals similar to petals which aid in the attraction of pollinators; modification of petal opposite to the sepals into an attractive and beautifully coloured labellum for attracting the pollinators; presence of gynostegium; presence of pollinia-structure formed due to the aggregation of pollen grains to form a consolidated mass and presence of minute dust like seeds without endosperm.

In the global floriculture market orchids rank top most position among the cut flowers and potted plants and it account for 10% of the total floriculture market. The major contributors of orchids in global market are Netherlands (58%) and Columbia (14%). Today >200000 orchid hybrids are available and the top ranked hybrids in the market are *Cymbidium, Dendrobium, Phalaenopsis, Cattleya, Paphiopedilum*, etc. Most of these hybrids have been evolved from wild germplasm. The potential indigenous wild orchids used for hybridization in India are *Aerides multiflorum, Aerides odoratum, Arundina graminifolia, Arachnis, Bulbophyllum, Calanthe masuca, Coelogyne elata, C. flavida C. corymbosa; Cymbidium aloifolium, C. lowianum, C. devonianum, C. hookerianum, C. lancifolium, Dendrobium aphyllum, D. nobile, D. chrysanthum, D. farmeri, D. chrysanthum, D. densiflorum, D. moschatum, D. fimbriatum, D. jenkinsii, Paphiopedilum venustum, P. spicerianum, P. hirsutissimum, P. insigne, Phaiuswallichii, Pleione praecox, Renantheraim schootiana, Rhyncostylis retusa, Vanda cristata, Vanda coerulea, Vanda tessellata, Paphiopedilum druryi*, etc.

Even though India has rich diversity of orchid germplasm, in the commercial hybrid production scenario its contribution is scanty and it is striking to note that many commercial hybrids have been produced abroad by using Indian

wild orchids. One major obstacle in Indian orchid industry is the scarcity of quality planting materials. Considering its economic value, development of elite hybrids using indigenous/wild orchid species and its commercial multiplication through biotechnological intervention has become the need of the hour.

In South India, the Western Ghats harbours richest diversity of orchids and many of the commercially important species are endemic to the Southern Western Ghats. The Queen of Orchids *Paphiopedilum druryi* is the best example. The agro-climatic condition of Kerala is well suited for the commercial cultivation of orchids. About 77 genera and 230 species of orchids including wild types have been reported in Kerala. When compared to other crops orchid cultivation is most economical, gives maximum return per unit area, ensures financial security and employment opportunity.

Distant hybridization is the most effective conventional breeding technique to transfer valuable genes from distantly related individuals. In orchids many interspecific and intergeneric hybrids have evolved under natural conditions. Occurrence of prolific natural hybridization in orchids signifies the rapid evolutionary process in the Orchidaceae family. Lenz and Wimbler (1959) have pointed out that hybridization leads to the transfer of block of genes from one species to another. Therefore, there is no restriction in wide crosses in orchids. Main difficulties encountered in distant hybridization are hybrid sterility due to genetic imbalance, lack of compatibility either between parental genome or between embryo and mother tissue. Orchids can successfully overcome these obstacles by the production of large number of embryos without the presence of endosperm, rapid evolution and speciation through natural hybridization and the production of large number of minute 'dust seeds'.

All the wild orchids are a potent source to create genetic variability and among these some genera/species may or may not be compatible in hybridization.

Hence in a systematic breeding programme, it is necessary to study the compatibility between the parents of hybridization. The orchid seeds are minute,

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non endospermic and germination is erratic. *In vitro* embryo culture is the best option to rescue the embryos obtained after crossing. Producing hybrids of tried and untried combinations and raising the seedlings in flask by the asymbiotic method have become popular all over the world.

A hybrid progeny will take 1-4 years for attaining its reproductive stage. Hence to assess the genetic variability of hybrid progeny rapidly and accurately, molecular marker analysis is essential. Morphological markers are still important as a technique applied to measure a genetic diversity in the plants. However, plant morphology, physical characteristics and plant growth characteristics could be influenced by environmental conditions. Morphological characteristics are also an important method used to distinguish a species or variety in granting plant breeders' right. Morphological characteristics alone are not sufficient and sometimes difficult to distinguish the variability between and within species. Due to this limitation, it has become a problem for taxonomists and breeders to differentiate between two or more species or varieties. However, the problem could be solved and supported through molecular technologies.

In Kerala, R & D focussing on genetic improvement of orchids seldom uses the wild potential genes of the endangered species. Expression of different plant characters is controlled by genetic and environmental factors. It is often difficult to know the proportion of heritable and environmental variation. The progress of breeding is conditioned by the magnitude, nature and interaction of genotypic and environmental variations in the plant characters. So, study of genetic parameters is necessary for a successful breeding program. This will provide valuable information on the mode of inheritance of different characters which would be useful in selecting plants with desirable characters and to estimate correlation of genetic relatedness with the known pedigree to develop strategies for the evolution of new improved varieties with high genetic potential in future. In the light of these, the present investigation entitled "Distant hybridization and compatibility studies in wild orchids " was aimed to carry out

distant hybridization among the selected wild orchid species to determine the cross compatibility by conventional method as well as assessment of genetic variability of seedlings using morphological and molecular markers.

Therefore, the broad objective of this experiment was to study the cross compatibility through distant hybridization in wild orchids and molecular characterization of hybrid seedlings using RAPD/ISSR – markers.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Orchid family of the plant kingdom is unique and represents peak in the evolution of monocots and it is the most successful one among flowering plants rendering wonders of nature's creations with the flower of wealth and nobility. Large number of genera and species exists as wild in the forest ecosystem. These wild orchids are valuable genetic resources having genes determining various qualitative and quantitative traits. But they are experiencing a steady diminution in number with the passage of time. As far as South Indian orchids are concerned, destruction of natural forests is the major threat to their existence. This has been true particularly in Kerala which necessitates the conservation of valuable genetic resources for breeding. Both interspecific and intergeneric hybrids have evolved naturally in orchids. This clearly assures that there is no restriction even in wide crosses in the orchid family.

Considering these favourable opportunities, an attempt was made to investigate the possibility of distant hybridization among wild orchids indigenous to South India. Important aspects of the present investigation hitherto are estimation of components of variance of quantitative traits, heritability, genetic advance, correlation, cross compatibility study, development of hybrid seedlings through *in vitro* seed culture and evaluation of hybrid seedlings through morphological and molecular characterisation.

Literature available pertaining to these particular aspects of the wild types selected for the present investigation was meagre and therefore, relevant information of some other genera and species were collected and reviewed and presented hereunder.

2.1. WILD ORCHIDS AND ITS IMPORTANCE

World average 5.98% of orchid flora and 6.8% of flowering plants is present in India with Himalayas and Western Ghats as their natural habitats (Jain, 1980). Orchids are lovely creatures of nature among flowering plants dwelling in

the forest ecosystem of all continents except Antartica. India is the centre of origin of many orchid species. Wild orchids are puzzling and very peculiar since it possess large showy and fragrant flowers (Vij, 1995). It is estimated that 25,000 species of orchids are present in the world and of these about 1350 species belonging to 186 genera are present in India (Gupta *et.al.*, 1997).

In monocots, it is one of the most important evolutionary lines and still in active state of speciation (Vij, 1995). But species richness is decreasing at an alarming rate day by day due to habitat destruction by over collection, shifting cultivation, deforestation and other development activities. So habitat destruction is the biggest threat to wild orchids present in the forest ecosystem. At present, it is impossible to tell exactly how many species of wild orchids are still alive in their natural habitats. Wild orchids play a good role for balancing the forest ecosystem and hence to be conserved (Mollah, 2001).

Orchids are unique group of flowering plants and really gifts of nature owing to their peculiar floral complexities such as fascinating beautiful flowers, presence of various flower shapes for attracting pollinators and efficiency in holding their flowering phase for a long time unlike any other cut flowers available at present. Besides, orchids have entered in the competition of flowering plants as popular cut flowers, potted plants and hanging baskets. So these flowers of rare beauty have become increasingly important in the 21st century (Dipika *et.al.*, 2017).

Orchids are grown commercially as micro plants, seedlings, cut flowers, pot plants, hanging baskets etc. Apart from these ornamental value, some are found to be medicinal due to the presence of alkaloids, phytoalexins, anthocyanins etc., and have antimicrobial, antimalarial, antituberculosis, antiviral and anticancerous activities and these all reviewed by many authors (Singh, *et al.*, 2012; Pant, 2013). Rare species are used for domestication by botanists and hobbyists worldwide. Wild species without genetic deterioration are used as parents for breeding programme. It is also used as spice, in the preparation of confectionary and

perfumery items and as foods in all continents by many people of different cultures and tribes. Gregarious and distinctive blooming nature was led to describe the orchid species as "living jewels" (Doyle, 1995).

Due to gradual decrease of forest cover, orchid rich countries are taking some steps for germplasm conservation. Many of the precious wild species in pure form are used as parents for breeding programme and are carefully maintaining without alteration in their original genotype by *ex-situ* conservation. Many of the Indian orchids like *Vanda*, *Cymbidium* and *Paphiopedilum* have already proved to be as important parent materials contributed to the production of several outstanding hybrids in the world (Dipika *et al*., 2017).

Important wild orchids with sources of potential genes for different valuable traits available in South India used for the present investigation are briefly described hereunder.

***Vanda tessellata* (Roxb.) Rchb.f. (*Vanda roxburghii*)**

Vanda tessellata is epiphytic in nature and grows up to a height of 30-60 cm possessing leafy stem. The leaves are obtuse, keeled, plicate, recurved and coriaceous type. The inflorescence is an axillary zigzag racemose type with an average of 7-8 flowers. The flowers are greenish yellow with brownish tessellations, mid lobe of lip is mottled brown with purple caruncles. Petals are yellow with brown lines and margins are white coloured. Petals are shorter than sepals. The lip is 16 mm long, bluish and dotted with purple colour. The capsules are long 7.5-9.0 cm with clavate-oblong acute ribs. Flowering period is from March to May and from September to December (Abraham and Vatsala, 1981). It is a therapeutically important orchid used by traditional healers in India. Paste of its leaves is used to treat fevers (Suresh *et al*, 2000) whereas the juice of its leaves is used in the treatment of otitis media. Root is used as antidote against scorpion sting, snakebites, bronchitis, arthritis and rheumatism (Chauhan, 1999; Shrestha, 2000; Baral and Kurmi, 2006). It is popularly used in ayurvedic medicines since

ancient period of Charak and Sushruta. At present it is sold as strong aphrodisiac in ayurvedic medical shops.

***Vanda spathulata* (L.) Spreng.**

It is popularly known as spoon leaf *Vanda*. It is the first *Vanda* species ever described in the 'Hortus Indicus Malabaricus' in 1703 as Ponnampou-maravara and later in 1753, Linnaeus named it as *Epidendrum spathulatumin* and did not get its present name until 1826. *Taprobanea spathulata* (L.) Christenson is commonly known as Svarna-pushpa bandaa or baandaa or mara vazha (means tree-top plantain). It is a perennial herbaceous species endemic to peninsular India including Kerala, Karnataka, Tamil Nadu, Maharashtra and Sri Lanka. It is rare in Madukkarai hills, Coimbatore (Jayanthi *et al.*, 2011) but it is an endangered species (Basha *et al.*, 2012) and its distribution is restricted to narrow pocket due to anthropogenic activity (Miria *et al.*, 2012).

It is epiphytic in nature and blooms during March - September. It possess scandent stem and attains to height of about 120 cm. The leaves are flat, oblong, obtuse or sub-acute apex, entire or emarginated with 10 cm long and 3 cm width. The inflorescence is ~45 cm long raceme with few to many 4-5 cm large golden yellow flowers. Sepals and petals spathulately oblong, flat and lip clawed as long as sepals (Abraham and Vatsala, 1981). It is the only ex-vanda with large golden yellow flowers (Devi *et al.*, 2015).

A therapeutically important orchid used by traditional healers in India (Suresh *et.al.*, 2000) where the crushed leaves and stems are used as ointment for the skin diseases, powdered leaves are used for the treatment of asthma, tuberculosis and epilepsy.

***Rhynchostylis retusa* (L.).Bl.**

It is designated as "Queen of Orchids" by Myanmar flower lovers and cultivators considering the beautiful and attractive flowers. It is popularly known

as 'Fox tail' orchid due to the nature of inflorescence. This is epiphytic and monopodial in growth habit with large stem and thick leaves. The inflorescence is racemose and pendulous in architecture. The flowers are white with purple spots and patches. It is an important parent for developing interspecific and intergeneric hybrids. Whole plant, leaves and root extract are used for treating the rheumatism, emollient, menstrual disorder and epilepsy. Root juice is applied as a wounds healer (Dressler, 1993; Shrestha, 2000; Joshi and Joshi, 2000; Manandhar, 2002; Subedi, 2011; Thammasiri, 2016).

***Aerides ringens* (Lindl.) C.E.C.Fisch.**

It is an epiphytic monopodial orchid, with beautiful small white flushed with pink tinged flowers in axillary raceme. Fleshy mid lobe of the lip possess spur which is almost equal in length of the lip. It grows well in open sunlight and flowering during the months of June-August. This species is popular among the hobbyists and used for breeding programme (Thammasiri, 2016).

***Arundina graminifolia* (D.Don) Hochr.**

Orchid is native to Asia. Found in China, India, Malaysia, Myanmar, the Philippines and Sri Lanka (Arditti, 2008). Popularly known as bamboo orchids and it is a terrestrial type with attractive beautiful flowers mainly sold as cut flowers for decoration and potted flowers. Possess erect long stem able to reach up to a height of 2 m with long and narrow leaves end with a terminal raceme. Flowers are pinkish - white to deep pink, blooms in summer and autumn with many flowers or year round in Malaysia.

It is a valuable plant in medicinal industry also. The root is used to get relief from body pain (Manandhar, 2002) and has detoxification, anti-arthritis activities. It contains phenolic compounds exhibiting potential cytotoxicity and hence used to study the cytotoxicity against the human tumour cell lines. Mainly grows along road sides, rocky sites or open grassy areas.

***Spathoglottis* spp.**

This perennial terrestrial genus is being used as a model for orchid studies since its year round flowering with lateral racemose inflorescence of 25-130 cm long (Kheawwongjun and Thammasiri, 2008). It possesses 4-8 lanceolate leaves each up to 35 cm long and 2-6 cm wide with prominent veins abaxially. Each inflorescence bears many successive and loosely spaced violet, pink, white or yellow flowers.

It is a therapeutically important orchid. Plant decoction is used for treating rheumatism and juice is used for drinking. Seeds are used to prepare ointment and applied to cure skin diseases. This genus is suitable for landscaping. Mainly they are used as parents for developing hybrids with many other colourful orchids (Thammasiri, 2016).

2.2 QUALITATIVE AND QUANTITATIVE TRAITS

2.2.1. Plant Height

Orchid is a crop which expresses a high magnitude of diversity in height and responds very well to the environment (Abraham and Vatsala, 1981). Wide variation was reported in different varieties of *Dendrobium* mainly due to genetic nature, growing conditions and environmental conditions (Roychowdhury *et al.*, 2004; Sugapriya *et al.*, 2012).

Plant height is an inherent genetic character influenced by growing conditions. Height difference was mainly due to differences in internodal length in orchids. Wide variation for this trait was reported among different varieties and hybrids of *Vanda* (Minnu, 2015).

2.2.2. Plant Spread

It is determined as the area required for the growth and density of plants in a particular area. In non-branching types it was largely determined by length,

orientation and arrangement of leaves. Wide variation was reported in different varieties and hybrids of *Vanda* for this trait (Minnu, 2015).

2.2.3. Shoot Girth

It is an indication of strength of the plant and protects the plants from lodging. Wide variability of this trait was reported in different varieties of *Dendrobium* (Sugapriya *et al.*, 2012) and in different varieties and hybrids of *Vanda* (Minnu, 2015).

2.2.4. Number of Leaves Plant^{-1}

Number of leaves plant^{-1} is an indication of leaf yield of a plant. Significant variation was exhibited among different varieties in *Dendrobium* (Sugapriya *et al*, 2012) and among different varieties and hybrids of *Vanda* (Minnu, 2015). This trait directly contributes to the photosynthetic efficiency of plants.

2.2.5. Leaf Length

Wide variation was reported in different cultivars of orchids (Bose *et al.*, 1999; Bhattacharjee *et al.*, 2002; Kaveriamma, 2007). Varieties with more leaf length had less breadth and *vice versa.* Leaves with more length and less breadth drooped and reduced its surface area for photosynthesis in *Dendrobium* (Roychowdhury *et al.*, 2004; Sugapriya *et al.*, 2012) and in *Cymbidium* (Barman *et al.*, 2007). Significant variation was exhibited for leaf length among different varieties and hybrids of *Vanda* (Minnu, 2015) which contributed largely to the leaf area thereby influencing photosynthetic ability.

2.2.6. Leaf Width

Wide variability was reported for width of leaves in different cultivars of orchids (Bose *et al.*, 1999; Bhattacharjee *et al.*, 2002; Kaveriamma, 2007).

Leaves with more breadth and less length exposed more surface area for photosynthesis in *Dendrobium* (Roychowdhury *et al.*, 2004; Sugapriya *et al.*, 2012) and in *Cymbidium* (Barman *et al.*, 2007).

Among different varieties and hybrids of *Vanda* significant variation was exhibited for width of leaves as reported by Minnu (2015). It contributed largely to the leaf area and influenced photosynthetic ability.

2.2.7. Inflorescence

Orchid inflorescence is normally raceme or indeterminate (Goh, 1977). Flowering nature decides how plants are the best grown. Duration of flowering in orchids is a genetically determined trait (Goh and Arditti, 1985).

Plants with single and upright stems are grown in pots and arching stems are ideal for planting towards the edge of a shelf (Squire, 2005).

2.2.8. Season of Flowering

Most hybrid orchids growing in tropical lowlands are probably day neutral plants and uninfluenced by day length (Soon, 1980). In the tropics seasonality in flowering was mainly due to variation in rainfall (Dressler, 1981).

Tropical lowland orchid species are free flowering and flowering peaked from June to October and February to March. May and November received maximum rainfall and showed a steep decline in flowering and it is a genetic trait (Goh, 1984). Flowering season of orchids is a genetically determined trait (Goh and Arditti, 1985).

Among epiphytic orchids flowering peaked during dry season and in early rainy season and decreased with increase in rainfall. Declined flowering from November to January was attributed to shorter day lengths (Gordinez, 1996).

Inflorescence continually flowered for several months in *Spathoglottis plicata* (Freudenstein and Rasmussen, 1996).

Factors determining the flowering in plants with respect to ontogeny and season are juvenility, photoperiodism and vernalisation (Yong and Hew, 2004). Plants respond to changes in photoperiod and temperature and thus flower naturally when the environmental conditions are favourable for flowering and reproduction (Lopez and Runkle, 2004).

Both free and seasonal flowering natures were observed among different varieties of *Dendrobium* (Sobhana, 2000; Nath, 2003; Sugapriya *et al.*, 2012). Free flowering nature is beneficial as far as market demand is considered (Sugapriya *et al.*, 2012).

Different *Vanda* orchids showed considerable variation in flowering nature. Peak flowering was observed in February- June with two blooming periods, three or single blooming period in a year (Minnu, 2015).

2.2.9. Days Taken for First Flowering

Some varieties take one year to show symptom of flower bud initiation due to its genetic constitution (Sanford, 1971).

Significant variation was observed among different varieties of *Dendrobium* for this trait. In some varieties when mature cuttings reached at flowering stage were used for planting, the number of days taken for visible flower bud initiation was less (Das and Bhattacharjee, 2004; Sugapriya *et al.*, 2012).

2.2.10. Number of Spikes Plant^{-1} Annum^{-1}

Vital trait for evaluating orchid plant based on economic feasibility as cut flower and pot plant is number of spikes produced by the plant annually (Garay, 1972).

The number of spikes produced in each plant varies significantly among the different varieties, which directly determines the number of flowers in a plant. The increased flower yield might be due to increased production of more number

of shoots which in turn increased number of spikes plant^{-1} with bigger sized flowers in *Cymbidium* (Barman *et al.*, 2007) and in *Dendrobium* (Sugapriya *et al.*, 2012).

Among different *Vanda* species and hybrids evaluated, hybrid *V. pranermpri* × *V. tessellata* and species *V. pathum* Gold produced maximum number of spikes plant^{-1} year^{-1} and the interval between spike productions was less (Minnu, 2015).

2.2.11. Length of Inflorescence

Environmental condition such as temperature has little or no effect in the expression of this trait in orchids (Robinson, 2002).

Length of inflorescence is very important in the cut flower trade and pot plants. Significant differences were seen for this trait among different *Vanda* species and hybrids evaluated (Minnu, 2015).

2.2.12. Number of Flowers Spike^{-1}

Minnu (2015) reported that significant differences were seen for number of flowers spike^{-1} among different species of *Vanda* and hybrids.

2.2.13. Internodal Length

Internodal length imparts compact appearance in the spike. When different *Vanda* species and hybrids were evaluated (Minnu, 2015) significant differences were exhibited for internodal length between florets.

2.2.14. Flower Size

Among 746 Indian orchids analysed, orchids with large showy flowers and good floret size attracted attention and were vulnerable for commercial exploitation. Floret size was determined by the length and width of petals (Lokesha and Vasudeva, 1994).

2.2.15. Flower Colour and Scent

Flower colour and scent were found to reduce after pollination (Schiestl *et al.*, 1997).

Florets displayed a wide range of colour pattern such as uniform, spotted, tessellated and blotched and shades such as white, yellow, red, purple, violet, blue, pink, magenta, brown, lavender and rose. Among these, blue colour is rare in orchid plants and hence it is highly sought in breeding (Griesbach, 2005).

2.2.16. Days for Inflorescence Emergence to First Flower Opening

Flower bud initiation begins after the spike had grown to a certain length under suitable environmental conditions (Lee and Lin, 1984).

Once the flower bud has initiated blooming time depends upon genotype and environmental conditions (Lopez and Runkle, 2005).

Significant variation was seen among different varieties of *Dendrobium* for this trait. It was mainly determined by the growth rate and length of spike (Rani, 2002; Nath, 2003; Sugapriya *et al.*, 2012).

Significant variation was seen among different varieties and hybrids in *Vanda* for duration from spike emergence to opening of first floret as reported by Minnu (2015).

2.2.17. Days to Last Flower Opening from First Flower Opening

It varied significantly among different varieties of *Dendrobium*. This trait mainly depends on the number of flowers per spike as well as number of days taken for blooming of each flower (Sugapriya *et al.*, 2012).

2.2.18. Spike Longevity

Vanda mostly blooms every month and the flowers retain for two to three weeks. Vital trait for evaluating orchid plant based on economic feasibility as cut

flower and pot plant is single spike longevity (Garay, 1972). It is a major trait for increasing the commercial value of orchids which depends mainly on genetic factors and environmental conditions. After pollination flowers start senescence within 1-5 days. Pollination and pollen removal reduced floral longevity.

2.2.19. Anthesis

Anthesis in some orchids is a genetically controlled trait (Sanford, 1971).

Flower opening time was morning and required 1-2 days for the full blooming of each flower in *Stelis argentia* (Christensen, 1992).

Peak time of anthesis in *Dendrobium* varieties was between 9 and 10 am and also between 3 and 4 pm and the flowers retained its freshness for 45-50 days on the spike (Varghese, 1995).

The flowers at the basal portion of spike matures and opens first and gradually it proceeded to the upper portion of the spike and anthesis occurs in the day time with a peak between 9 and 11am in *Dendrobium* (Sobhana, 2000).

Significant variation was seen among different *Vanda* species and hybrids evaluated for this trait (Minnu, 2015).

2.2.20. Stigma Receptivity

Stigma receptivity remained throughout the day after anthesis and up to five days after anthesis in *Dendrobium amoenum*, up to three days after anthesis in *Spathoglottis plicata,* and up to four days after anthesis in *Aerides odoratum* and up to 11 days after anthesis in *Phaius tankervilleae* although the flowers retained freshness for a longer period. Receptivity period was longer in orchids than other cross pollinated crops. Generally, in orchids it was found maximum between second and fifth day after anthesis but in New Pink (hybrid orchid) it was found from the day of anthesis to the tenth day (Devi and Deka, 1992).

In *Dendrobium* stigma receptivity period was the maximum between 4 and 6 days after anthesis although the stigma remained receptive from the first day to 9th day of anthesis (Varghese, 1995).

Stigma receptivity was maximum one week from flower opening in *Spathoglottis plicata* (Proctor, 1998; Shiau *et al.*, 2002; Lo *et al.*, 2004).

Stigma receptivity remained throughout the day after anthesis and duration of it varied in different hybrids of *Dendrobium* (Sobhana, 2000).

Pollination was effective when selected parents with flowers retained maximum stigma receptivity (1-10 days after blooming) and as the age of stigma receptivity increased, the success of pollination has considerably reduced in *Dendrobium* (Shiau *et al.*, 2002).

In *Rhynchostylis retusa* stigma remained more receptive from the second day of anthesis to 4th day and morning hours. Therefore, pollination was more successful in morning hours (Nitin and Uma, 2014).

2.2.21. Pollen Morphology

In the family orchidaceae the pollen grains are present as agglutinated masses called pollinium.

In *Dendrobium* species pollinium is yellow coloured, ovoid in shape and tightly compressed to form two pairs (Sheehan and Sheehan, 1979; Sobhana, 2000) and pollen grains existed as tetrads (Abraham and Vatsala, 1981; Das and Ghoshal, 1988; Varghese, 1995; Sobhana, 2000) and pollen grains are held together by elastic threads of tapetal origin (Abraham and Vatsala, 1981).

In *Spathoglottis plicata* pollinium was clavate, mature pollen grains were irregular or polygonal in shape and appeared in tetrads and the exine was perforated (Freudenstein and Rasmussen, 1996).

Seeds were dust like and fusiform shaped. Seeds were brown and an aperture was present in the posterior. Seed length was about 100-200μm and width was 50-100μm (Freudenstein and Rasmussen, 1996).

In *Dendrobium* hybrids smaller pollen grains were observed (Varghese *et al.*, 1997).

The wild species of Dendrobium had small sized pollen (Sobhana, 2000).

2.2.22. Pollen Production

Pollen production flower^{-1} anther^{-1} varied from variety to variety within an orchid species (Nair *et al.*, 1964).

The *Dendrobium* hybrids had a low rate of pollen production pollinium^{-1} (Varghese *et al*, 1997).

Pollen production exhibited significant variation ranged from 38282 to 193750 (Varghese, 1995) and 2720 to 13120 (Sobhana, 2000) pollinium^{-1} among different hybrids of *Dendrobium*. The wild species of *Dendrobium* had low pollen count pollinium^{-1} (Sobhana, 2000).

2.2.23. Pollen Fertility

In *Paphiopedilum* the pollinia at anthesis were collected and stained with 1% aceto-carmine solution and pollen with positive staining was taken as viable one (MacFarlane *et al.*, 1989).

In *Dendrobium* high pollen fertility was observed among varieties and low pollen fertility among species with small sized pollens. While in hybrids, a direct relationship between the size and fertility of pollen (Varghese, 1995) and low pollen fertility (Varghese *et al.*, 1997) was reported. Pollen fertility variation was seen in different varieties but the wild species showed low fertility (Sobhana, 2000).

In orchids pollen longevity lasts from few hours to several weeks (Endress, 1994). Environmental conditions such as temperature, light intensity and relative humidity influenced pollen longevity (Pacini *et al.*, 1997) Factors determining pollen longevity are carbohydrate and water content at the time of anthesis, the environment during exposure and dispersal and the site where pollen presentation occurs, i.e., whether the pollen is exposed or protected (Dafni and Firmage, 2000; Nepi *et al.*, 2001). If pollen has high water content (>30%), high sucrose percent and high cytoplasmic polysaccharides then it could resist desiccation (Dafni and Firmage, 2000). Pollen longevity was 8-11 days in *Calypso bulbosa* (Dafni and Firmage, 2000; Luit and Johnson, 2001) and the maximum on 1-8 days after flower opening in *Spathoglottis plicata* (Proctor, 1998; Shiau *et al.*, 2002; Lo *et al.*, 2004).

Pollination was effective when selected parents with flowers retained the maximum pollen viability (1-10 days after blooming) and as the age of pollinium increased, the success of pollination considerably reduced (Shiau *et al.*, 2002). Pollinium of 1-4 day old was more viable for effecting pollination in *Rhynchostylis retusa* (Nitin and Uma, 2014).

2.2.24. Pollen Germination

Sucrose and agar play significant role in pollen germination. The beneficial effect of sucrose on pollen germination might be attributed to nutrition and osmotic phenomenon (Johri and Vasil, 1961) and agar attributed to supply of moisture, carbohydrate and other nutrients to the medium (Stanley and Linskens, 1974). Suitable medium for *Dendrobium* pollen grain germination was 2% sucrose and 1% agar (Varghese, 1995). Pollinia germinated 5-6 hours after incubation and proceeded up to 30-36 hours in *Spathoglottis plicata* and 14 hours after incubation and proceeded up to 12 hours in *Cymbidium ensifolium* in Brewbaker medium with 10% sucrose (Latha and Namboodiri, 1999).

In *Dendrobium* pollen grain germination was the maximum when the pollen grains were treated with 2% sucrose and 1% agar and followed by 2% sucrose and 2% agar, however, variation was observed in different varieties (Sobhana, 2000).

2.3. COMPONENTS OF VARIANCE

Presence of genetic variability followed by subsequent selection of desirable types determines success of any crop improvement programme. Limited attempt has been made for genetic variability and genetic improvement studies in wild orchids.

In crop improvement an important factor guiding the breeder is only the genetic component of variation (Johnson *et al.*, 1955).

Genetic analysis of various traits in orchids grown in the plains of Bengal revealed high amount of genotypic variance for length of inflorescence (506.07) and number of flowers inflorescence^{-1} (178.78) (Rehman *et al.*, 1993).

In the genetic variability evaluation of *Dendrobium*, the phenotypic coefficient of variation was slightly higher than the genotypic coefficient of variation for all the characters. Both phenotypic coefficient of variation and genotypic coefficient of variation were the highest for number of flowers inflorescence^{-1} followed by days for opening of all florets and number of shoots and the lowest for length of leaf (Sobhana, 2000).

Genetic variability evaluation among 20 native *Dendrobium* orchids revealed highly significant variation for all the traits. Wide variation was observed for all the traits and phenotypic coefficient of variation (PCV) was higher than the genotypic coefficient of variation (GCV) for all the characters indicating the influence of environment. The range of variation was high for spike length (2.0-29), flower durability (8.0-31) and plant height (13-71). Difference between phenotypic coefficient of variation and genotypic coefficient of variation was low for spike length, rachis length, plant height, floret number

and flower durability. Environmental influence plays less role in the expression of all these traits in orchids (Moniruzzaman *et al.*, 2012).

Presence of high heritability leads to the improvement of a character. Spike length, rachis length, plant height, floret number and flower durability expressed high heritability out of fifteen traits evaluated. The effectiveness in the selection of genotypes based on the phenotypic appearance is determined by the magnitude of heritability in the hybrids of orchids (Johnson *et al.*, 1955). Heritability is the ratio of genotypic variance to the total variance and it is a good index of the transmission of characters from parents to their offspring (Falconer, 1960).

Heritability estimation of various traits in orchids grown in the plains of Bengal revealed high values for length of inflorescence (78.94%), number of flowers inflorescence^{-1}(95%) and flower size (85.15%) and suggested that consideration should be given for all these three traits for the selection of genotypes or lines for improvement in future (Rehman *et al.*, 1993).

In *Dendrobium* orchids the estimation of heritability exhibited moderate to high values for all the traits. Heritability was high for breadth and length of flower and breadth of leaf and low for number of shoots (Sobhana, 2000).

Estimation of heritability in orchids revealed high values for spike length (94%), rachis length (95.34%) and flower durability indicating the possibility of transfer of traits to the offspring from the parents thereby increasing the possibility of improvement of these traits through selection. If the heritability of a trait is high (80% or more), selection is very effective due to close correspondence between genotypic and phenotypic variances. This is because relatively low contribution of environment to the phenotype. Similarly, if the heritability of a trait is low (40% or less), selection may be ineffective or virtually impractical due to masking effect of environment in the genotypic effects (Faroque, 2003).

Heritability (broad sense) estimation among 20 native *Dendrobium* orchids indicated high values for spike length (94%), flower durability (94%) and floret

number (70.5%). All other floral characters expressed moderate values. High heritability revealed the presence of additive gene action and possibility in the improvement of these three traits through selection (Moniruzzaman *et al.*, 2012).

The effectiveness in the selection of genotypes based on the phenotypic appearance is determined by the magnitude of expected genetic advance also (Johnson *et al.*, 1955).

In gladiolus certain traits expressed moderate heritability with low genetic advance (Negi *et al.*, 1982). In orchids estimation of heritability exhibited high values for plant height (87%) and pod size (74%) and genetic advance exhibited low values for plant height (40.99) and pod size (48.51). The existence of high heritability coupled with low genetic advance indicating the presence of non-additive gene action such as epistasis and dominance (Faroque, 2003).

Estimation of genetic advance in orchids revealed high values for spike length (98.29), rachis length (93.85) and flower durability indicating the possibility of transfer of traits to the offspring from the parents thereby increasing the possibility of improvement of these traits through selection (Faroque, 2003).

Estimation of genetic advance among 20 native *Dendrobium* orchids indicated high values for spike length (98.29), flower durability (89) and floret number (65.47). All other floral characters expressed low values. High genetic advance reveals the presence of additive gene action and possibility in the improvement of these three traits through selection (Moniruzzaman *et al.*, 2012).

2.4. CORRELATION

Genotypic correlation coefficients were higher than phenotypic correlation coefficients for all the traits and it indicating the suppressing effects of environment, which modified phenotypic expression of traits by reducing phenotypic coefficients in gerbera (Anuradha and Gowdha, 1994) and in orchids.

Positive correlation between length of stem and length of leaf, length of leaf and number of leaves, length of leaf and number of flowers, length and breadth of leaf and stem diameter, number of leaves and number of flowers was reported in *Habenaria radiata* (Nagayoshi *et al.*, 1996).

In *Dendrobium* flowers spike^{-1} exhibited significant correlation with height of shoots, number of leaves, length of inflorescence, days for opening of all florets and inter nodal length between first two flowers. Days for opening of all florets registered the highest positive correlation and breadth of leaf registered the lowest positive correlation. Negative correlation was exhibited between vase life up to wilting of first flower and inter nodal length between first two flowers. Highest positive genotypic and phenotypic correlation was exhibited between length and breadth of flowers. Number of shoots had highest positive correlation with breadth of flowers. Days from spike emergence to first flower opening was positively correlated with breadth of leaf and number of shoots. Length of flower was positively correlated with breadth of flower. Breadth of flowers was positively correlated with number of shoots. Length of inflorescence was positively correlated with days for opening of all florets and number of leaves. Vase life up to wilting of first flower was positively correlated with breadth of flower (Sobhana, 2000).

Estimation of correlation between various traits in *Rhynchostylis gigantia* revealed strong positive association between shoot and leaf number and leaf area. Highly significant correlation of physiological parameters existed with plant height, length of inflorescence and number of florets spike^{-1} (Zotz *et al.*, 2001).

Estimation of correlation between various traits among 20 native *Dendrobium* orchids revealed highly significant association for the number of florets spike^{-1} with flower durability and spike length, significant positive correlation for flower durability with flower size and spike length at both genotypic and phenotypic levels indicating that selection of parents should be based on the traits number of florets spike^{-1}, flower durability, spike length and

flower size and it would be useful in a breeding programme (Moniruzzaman *et al.*, 2012).

In gladiolus (Lal *et al.*, 1985; Singh and Singh, 1990) and in orchids reported that flower durability positively correlated with rachis length, flower size and spike length indicating that flower durability increased with increase in the rachis length, flower size and spike length. Therefore, select parents, based on floret number, rachis length and flower durability for improvement.

Length of leaves had positive correlation with number of flowers, flower size, flower durability and rachis length and negative correlation with breadth of leaf, number of pseudobulb and spike length. Breadth of leaf negatively correlated with leaf number and number of spikes and positively correlated with other traits. Number of spikes negatively correlated with number of flowers spike^{-1}indicating that number of flowers will be decreased with increase in the number of spikes plant^{-1}. Number of spikes positively correlated with flower size, flower durability, rachis length and spike length. Number of flowers spike^{-1} positively correlated with flower durability, rachis length and spike length and negatively correlated with flower size in many orchids (Faroque, 2003; Sultana, 2003).

Cane length was correlated with leaf number, cane diameter, spike length, floret number and vase life; cane diameter was correlated with leaf number, cane length, spike length, floret number and vase life; spike length was correlated with leaf number, cane diameter, cane length, floret number and vase life; floret number was correlated with cane diameter, spike length, cane length and vase life and vase life was correlated with cane diameter, spike length, floret number and cane length in orchids (Nair and Tung, 1983; Nolte, 1985; Kamemoto, 1987; Nair and Fong, 1987; Islam *et al.*, 2013).

Significant positive correlation was noticed between plant height and inflorescence size and inflorescence length and inflorescence size in *Dendrobium sambucina*, number of flowers and number of fruits in *Dendrobium sambucina,*

Dendrobium majalis and *Dendrobium bifolia.* Due to fitness of individuals larger ones can produce larger inflorescences (Anna *et al.*, 2015).

2.4. DISTANT HYBRIDIZATION

The first authentic record of a distant hybridization is the production of a hybrid between carnation (*Dianthus caryophyllus*) and sweetwilliam (*Dianthus barbatus*) by Thomas Fairchild in 1717. In agricultural crops most of the interspecific hybrids were of academic interest only and were of no agricultural value but in ornamental crops, served as commercial varieties. At present distant hybridization has a definite role to play in crop improvement programmes: improvement of a few crop species is primarily based on distant hybridization, e.g., sugarcane, brassica, tomato, potato etc.. But in most of the cases it is used for the transfer of a few desirable genes, particularly those for disease resistance. In many crop species, the genes for disease resistance transferred from the wild species were extremely useful. According to Harlan (1976), there are several crop species in which genes from wild relatives stand between man and starvation or economic ruin. A glaring example is the potato famine in Europe.

Distant hybridization is an effective breeding tool to capture hybrid vigour as well as to combine traits that do not occur within a single species (Volker and Orme, 1988).

To introduce valuable economic traits such as disease resistance, flower shape and colour, from wild species into the cultivar assortment, it is essential to overcome interspecific crossing barriers. Several techniques hitherto, as the cut style method, the grafted-style method and the *in vitro* isolated ovule pollination technique, have been developed to overcome pre-fertilisation barriers (Van Tuyl *et al.*, 1991).

Tomar and Singh (1998) reported that hybrid plants will response differently based on the type of alleles that present in the locus of a gene that controlling a particular trait mainly complementary lethal genes and this

phenomena ultimately will leads to the failure of seedling development in distant hybridization.

The environmental conditions such as high temperature, specific protein and exudates (Martin, 1970), hormone treatment (Ascher, 1973), and lipids (Wolters-Arts *et al.*, 1998) also can be applied to overcome these barriers.

In vitro pollination brings pollen grains in direct contact with the ovules is a more effective method (Willemse *et al.*, 1995).

In Lilium various combinations of *in vitro* pollination (cut-style and grafted-style method) and embryo rescue (ovary, ovule and embryo culture, placental pollination) were applied to control the whole fertilisation process (Van Tuyl *et al.*, 1991; Janson, 1993; Chi, 2000).

The introduction of ovule culture and embryo-rescue methods has greatly facilitated wide hybridisation followed by backcrossing in a wide variety of plant species (Hadley and Openshaw, 1980; Van Tuyl *et al.*, 1990; Buitendijk *et al.*, 1995; De Jeu and Jacobsen, 1995; Van Tuyl and De Jeu, 1997). Based on the results of cross combinations, suitable rescue method can be applied (Van Tuyl *et al.*, 2002).

Amphidiploidy has been used to create male and female fertility (Arisumi, 1975; Pasutti, 1977) to overcome sterility problems. Naturally occurring pollen fertility has been found sporadically among interspecific hybrids without the use of amphidiploidy (Weigle and Pasutti, 1976; Pasutti, 1977; Pasutti and Weigle, 1980) in certain crops.

Acetocarmine or Aceto orcein and FDA (Fluoro diacetate) have been used for the staining of fertile pollen in many crops. Especially pollen from interspecific or intergeneric hybrids can be checked for their fertility (Arisumi, 1974; Merlin and Grant, 1986). Methods for estimating pollen fertility include

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counting unstained, normal-looking pollen grains (Arisumi, 1975) or pollen grains stained with acetocarmine solution (Singh, 2007).

There is occurrence of 2n-gametes in hybrids of Orchidaceae through interspecific hybridization.

Intraspecific, intrageneric and intergeneric hybrids are produced in orchids and a large number of crosses involving two genera (bi-generic), three genera (tri-generic), four genera (tetra- generic), five genera (penta- generic) hybrids have been developed and registered. Intergeneric hybrids are very common in orchids. Bigeneric hybrids are Aredovanda (*Aerides* x *Vanda*), Rhynchovanda (*Rhynchostylis* x *Vanda*) etc. and total number of hybrids reported in this group is approximately 6,000 (CITES Identification Manual, 1973).

2.5. CROSS COMPATIBILITY

Cross compatibility is the successful development of hybrid progenies upon cross pollination and fertilization under natural or artificial means between two parent plants.

But in some orchids parthenocarpic development of ovary was brought about by the high auxin content of the pollinia (Laibach and Maschmann, 1933).

Self-incompatible orchids have either homomorphic, gametophytic or polyallelic incompatibility with stigmatic inhibition of pollen germination (Duncan and Curtis, 1943).

The duration between pollination and fertilization is highly variable in the orchidaceae (Swamy, 1943) but it is usually short in terrestrial orchids (Swamy, 1949).

In *Vanda* hybrids within each group exhibited close homology of chromosomes and fertile hybrid produced but between the groups, hybrids exhibited low fertility due to lack of chromosomes homology (Storey, 1955). Seed

set failure in reciprocal crosses was due to either physical barriers, antagonism between maternal cytoplasm and sperm nucleus or sensitivity of one of the plants to its environment so that it would block the development or function of gametes and self-incompatibility and cross sterility encountered in *Vanda* hybrids could be either due to hybrid sterility or polyploidy (Lenz and Wimbler, 1959).

But in intergeneric hybrids, between strap leaved *Vanda* and strap leaved *Ascocentrum* exhibited stronger chromosomes homology than hybrids between strap leaved and terete –leaved *Vanda* (Shindo and Kamemoto, 1962).

Percentage of fruit set in interspecific crosses varied from 0 to 100 and in intergeneric crosses from 0 to 75. By the introduction of foreign pollen effective fertilization leading to embryo formation did not take place and only the ovary was developed parthenocarpically and formed capsules. Out of thirteen hybrid capsules formed, seeds of only three crosses were germinated (Bose and Bhattacharjee, 1980).

Triploidy is common in orchids and it is one of the most frequent causes of sterility. Infertility attributed in polyploids was due to pairing abnormalities during meiosis i.e., an addition of one or more complete or incomplete chromosome sets (Abraham and Vatsala, 1981).

After pollination ovule formed 43 to 45 days in *Cymbidium goeringii* and 58 to 60 days in *Paphiopedilum insigne* var. Sanderae. Embryo maturation was 115 to 120 days in *Cymbidium goeringii* and 195 to 200 days in *Paphiopedilum insigne* var. Sanderae. Seeds germinated in 80 and 165 days in forgoing orchids. Seed germination was the highest when the capsules were harvested with the embryos almost mature (Nagashima, 1982).

Abundant ethylene production after emasculation and pollination led to the senescence of floral organs (Chadwick *et al.*, 1986).

Direct crosses were successful in *Dendrobium primulinum* x *Dendrobium crepidatum* and *Dendrobium transparens* x *Dendrobium pierardii*. Unsuccessful crosses in some *Dendrobium* species was due to unequal chromosome numbers (Das and Ghoshal, 1988).

In hybridization lack of seed setting was due to the absence of anthers and or pollen, pollen sterility, non-viability or genotype specificity (Dadlani *et al.*, 1988).

Sign of capsule maturity was development of brownish and yellowish colour on the surface of the capsule. Post pollination changes include closure of stigma, increase in fresh and dry weight of ovaries and gynostemia, hormone production, synthesis or destruction of pigments, new biochemical pathways, cessation of scent evolution, swelling of the column and cell divisions in the ovary, breaking apart of pollinia due to tetrad dissociation, dehydration of pollen grains and germination of pollens from outside of pollinium to inside. Loss of water from the upper layer of petal cells caused wilting of flowers and led to upward folding of petals (Bose and Yadav, 1989; Slater, 1991).

Auxin content of the pollinia stimulated ethylene production and initiated incompatibility of parents (Johansen, 1990).

Unilateral incompatibility was observed between species of the genus *Dendrobium* and between species of the different genera *viz.*, *Dendrobium, Spathoglottis, Phaius, Coelogyne* and *Rhynchostylis*. Intergeneric and interspecific crosses incompatibility may be due to genetic imbalance. The unsuccessful hybrid pods turned yellow and burst at immature stage or abscised. The ovaries swelled parthenocarpically due to pollination stimulus by the introduction of foreign pollen but fertilization eludes the ovules probably due to incompatibility of parents (Devi and Deka, 1992).

In an attempt to determine compatibility between different species and genera for production of hybrids through embryo culture, 29 interspecific and 47

intergeneric hybridization was made and in many crosses ovary swelled but did not develop pods. Reciprocal crosses were achieved between *Dendrobium crepidatum* and *Dendrobium transparens* and between *Dendrobium crepidatum* x *Dendrobium pierardii* (Devi and Deka, 1994). Emma white was observed as a good female parent since of the different hybrids it had the maximum compatible crosses. Of the six species tried as male parents, *Dendrobium jimbriatum* gave maximum cross compatibility and some reciprocal crosses were not successful. In some crosses capsule development was not seen but the ovaries were swelled due to pollination stimulus and it might have due to post fertilization barriers or the stimulatory parthenocarpy led to the formation of swelled ovaries (Varghese, 1995).

Hybridization between *Dendrobium* species as female parent and wild species as male parent provided varied results. These were the development of small pods, the swelled ovary found to fallen after 30 days before reaching maturity, flower remained intact, retained freshness of flower, faded flowers and abscised flowers 2-3 days after pollination. But in successful crosses ovary remained as part of the spike and gradually enlarged to form the pod with the withered petals and sepals. Time taken for pod maturity varied from 90 to 135 days and more days for culturing ranged from 120 to 135 in crosses involving wild species as male parent. Embryo did not turn green even after 3 months when cultured 60 days old pod. When seeds of different hybrids were cultured, some seeds were not germinated and some germinated seeds were not developed into seedlings (Varghese, 1995).

Seed viability percentage was estimated by staining the seeds extracted from fresh capsules in 1% (w/v) 2,3,5-triphenyl tetrazolium chloride one day in darkness and then observed under light microscope. Presence of red coloured embryo indicated viable seed (Vellupillai *et al.*, 1997).

The duration between pollination and fertilization is highly variable in the orchidaceae. In *Spathoglottis plicata* it was 15 days (Narasak *et al.*, 2015).

2.6. *IN VITRO* CULTURE OF SEEDS

Orchids are the first successful commercially mass micropropagated floriculture crop. With the advancement of germination of the orchid seeds in artificial condition using different culture media, the tissue culture technique has been variously modified and utilized for different species (Knudson, 1922, 1925). Asymbiotic seed culture is germinating seeds and growing seedlings under aseptic conditions in an agar medium supplemented with nutrients required by the plants. It was developed in 1921 by Knudson and now became standard procedure for seed germination.

Commonly used orchid culture media are Knudson C medium (Knudson, 1922, 1925, 1927, 1946), MS medium (Murashige and Skoog, 1962) and Vacin and Went medium (Vacin and Went, 1949).

Asymbiotic seed germination was a major technological advance contributed to modern biotechnology (Johnson *et al.*, 2007). First *in vitro* propagation procedure was asymbiotic seed germination method in orchid developed by Knudson (Yam *et al.*, 2009). Rate of *in vitro* seed germination might be affected by the time of harvesting the capsules (Knudson, 1922; Rajkarnikar, 2014).

In orchids natural seed germination is very poor, only 2-5% (Rao, 1977; Vij, 2002).

In the orchid multiplication and conservation programme, *in vitro* germination of seeds is an important tool since the millions of tiny seeds present in the capsule have the ability to develop into complete seedlings without any fungal aid (Arditti, 1967).

In orchids green pod culture has enhanced the germination and reduced the flowering time. Seeds can be collected before dehiscence of capsules with no seed loss (Tsuchiya, 1954; Rao, 1974; Arditti *et al.*, 1981; Henrich *et al.*, 1981).

Optimum growth and morphogenesis of tissues may vary for different plants as per nutritional requirements and different plant parts have different requirements for good growth (Murashige and Skoog, 1962).

MS medium rich in vitamins and micronutrients gave better results for tropical orchids' seed culture (Reddy, 1992). Half MS medium results rapid protocorm production in orchids, since MS medium contains high ionic concentration of nutrients (Zhang *et al.*, 1992).

Sucrose is the commonly used carbon source supporting initial germination and subsequent growth and its effects depends upon concentration. Organogenesis is promoted at sub optimal concentrations and protocorm proliferation is enhanced at supra optimal concentrations (Arditti, 1979; Singh, 1994). Sucrose acts as morphogenetic trigger in the formation of auxiliary buds and branching of adventitious roots (Vinterhalter and Vinterhalter, 1997).

Vitamins are essential for normal growth and development, act as catalysts in metabolic processes and limiting factors for cell growth and differentiation. Amino acids used for enhancement of cell growth in culture media included glycine, glutamine, asparagine, L-arginine, cysteine and L-tyrosine (Torres, 1989).

Plant growth regulators promote stem elongation, tropism and apical dominance. They are auxins, cytokinins, gibberellins and abscisic acid. Proportion of auxins to cytokinins determines type and extent of organogenesis in plant cell cultures. Cytokinins generally induce shoots and auxins induce roots. Auxins also stimulate callus production and cell elongation (Skoog and Miller, 1957).

Benzyl amino purine (BAP) is a common plant growth regulator enhancing and promoting shoot regeneration, somatic induction, cell division and axillary bud growth. Medium supplemented with appropriate concentration of BAP has increased the rate of shoot proliferation (Talukder *et al.*, 2003) earlier flower induction and protocorm regeneration and plantlet formation (Khatun *et al.*, 2010)

in *Dendrobium*. It is a first-generation synthetic cytokinin applied along with auxin in the medium (Habiba *et al.*, 2014).

BAP has enhanced the asymbiotic seed germination at lower concentrations (0.1, 0.5, 1 mgl^{-1}) in combination with Naphthalein Acetic Acid (NAA) (0.5, 1 mgl^{-1}) in *Spathoglottis plicata* (Uma and Nitin, 2012).

Orchid seeds prefer slightly acidic medium of pH between 4.5-6.5 and it greatly influenced plant growth (Knudson, 1951). MS medium at pH 5.5 is the best for seed germination and protocorm formation in *Vanda tessellata* and suggested that nutritional requirement is species and genera specific (Prakash *et al.*, 1996; Bindiya *et al.*, 2013).

Multiple shoots are produced in medium containing BAP and NAA (Nhat and Dung, 2006; Rahman *et al.*, 2009; Long *et al.*, 2010; Pant and Shrestha, 2011; Bakul and Shahinul, 2014).

NAA induced root formation in *Vanda tessellata* (Rahman *et al.*, 2009).

Coconut water contains cytokinin in a balanced composition which promotes seed germination and differentiation of roots and shoots. Coconut water is a source of sugar, vitamins and inorganic ions required for cell growth (Dhamankar, 1992; Zahed, 2000). Coconut water enhanced seed germination and seedling development in *Vanda*.

Activated charcoal stimulated growth and differentiation in orchids and caused sucrose hydrolysis and acidification in the culture medium (Wang *et al.*, 1976; Druart and Wulf, 1993).

Agar is a polysaccharide obtained from seaweeds and universal gelling agent for preparing semi solid and solid plant tissue culture media. It contains Ca, Mg and trace elements (Pierik, 1997). Hardness of culture medium influenced growth of cultured tissues (Prakash *et al.*, 2002).

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Faster and higher percentage of seed germination was occurred in liquid medium as compared to semi solid Vacin and Went medium (Vacin and Went, 1949).

Contamination can be eliminated by treating the explants in sterilization agents at specific time (Yildiz and Er, 2002). Contamination of media could be controlled by adding antimicrobial agents, acidification or by filtration through microporous filters (Levin and Tanny, 2002).

Seed germination occurred in both Knudson's C (Knudson, 1946) and Vacin and Went (Vacin and Went, 1949) media, with or without coconut water (CW), tomato juice or banana extract on *Arundina bambusifolia* (Kanjilal *et al.*, 1998). *In vitro* raised seedlings exhibited aneuploid variation with 2n=36 and 2n=38 along with the normal one 2n=40.

In vitro seed / embryo germination requirements are influenced by the capsule maturity, genetic potential and specificity of the species. Seeds isolated from undehisced capsules germinate better and earlier than the dehisced one (Arditti *et al.*, 1981; Sharma, 1998).

Germination, protocorm development and seedling growth were better in MS medium in *Coelogyne mossiae*, *Eria reticosa* and *Aerides ringens*. The effect of IAA was low in promoting seedling growth in *Aerides ringens*, *Coelogyne mossiae* and *Eria reticosa*, *Cattleya* (Withner, 1959), *Vanda* (Rao and Avadhani, 1964) and *Spathoglottis plicata*. But early germination and protocorm formation resulted in *Cymbidium macrorhizon* with IAA/NAA (Vij and Pathak, 1988).

A combined treatment of Kinetin, activated charcoal (AC) and coconut water (CW) favoured the development of healthier seedlings in *Aerides ringens*, *Eria reticosa, Spathoglottis, Cymbidium aloifolium* (Bopaiaha and Jorapur, 1986).

Seed maturation stage influenced rate of *in vitro* germination (Linden, 1980). The highest germination was occurred when cultured seeds containing

fully matured embryo from the pods of age 70 days to 150 days (Varghese, 1995). Seeds germinated readily from green pods attained maturity after 8-12 weeks of anthesis in a large number of wild species. Matured seeds were hard to germinate due to dormancy (Mitra, 1986). Seed germination was poor and took longer time when the embryos were at pre-tetrad or intermediary stages in culture (Nagashima, 1993). Seeds isolated and cultured from bursted capsules exhibited poor germination compared to the unbursted capsules in *Vanda* (Sharma, 1998). Embryo culture stage varied with the genus, species, hybrid and local conditions and pod maturity time varied with the habit of the species crossed (Arditti, 1982; Hegde, 1984).

First visible sign of seed germination was the swelling of embryo by imbibing of water followed by greening and emergence out of the seed coat and later developed into protocorm and differentiated into shoot meristem and rhizoids in opposite directions. Leaves are produced from green protocorm and become autotropic in nature. Two leaf stage, protocorm and rhizoid lose the nutritive function and real roots are formed endogenously (Singh, 1993).

Among all the media tried, Knudson medium was found to be the best for seed germination. After inoculation 76-100% germination was found within 35 days in *Cymbidium longifolium* and within 14 days in *Dendrobium aphyllum* and *Rhynchostylis retusa*. Shoot initiation period ranged from 70 - 105 days after seed inoculation. Knudson and Burgeff media combined with coconut water and banana extract was reduced shoot initiation period i.e., 70-98 days after seed inoculation. Root initiation period ranged from 105-126 days after seed inoculation in Knudson and Burgeff basal media. But Knudson and Burgeff media combined with coconut water and banana extract was reduced root initiation period from 98 to119 days. Complete plantlets were obtained 170-180 days after seed inoculation (Das *et al*., 2008).

Protocorm Like Bodies (PLB) are multiplied indefinitely and grow into a complete plantlet. PLB is a spherical tissue mass that resembles the early stage of

embryo developed from initial explants. They can proliferate and regenerate to give large number of individual plantlets within a short period under suitable media and proper growth conditions (Arditti, 2008).

Seed germination was faster in the KC medium (19.5 days) compared to MS medium (24 days) and Vacin and Went medium (50days). A lower concentration of growth regulators enhanced seed germination but higher level of growth regulators promoted subsequent seedling growth in *Spathoglottis plicata* (Sangama and Singh, 1994).

Lipids and proteins stored in the seed are utilized for protocorm development and accumulated starch is utilized for organogenesis in *Spathoglottis plicata* (Krishnan *et al.,* 1993).

Embryos of two intergeneric hybrids namely *Spathoglottis plicata* x *Dendrobium densiflorum* and *Dendrobium muschatum* x *Arundina bambusifolia* germinated with very high frequency to form protocorms in Nitsch medium (Talukdar *et al*., 2002).

Seed germination, protocorm development and seedling growth of *Arundina graminifolia*, *Cymbidium aloifolium*, *Cymbidium pendulum*, *Dendrobium moschatum* x *Arundina graminifolia*, *Dendrobium infundibulum* x *Arundina graminifolia* were the best in modified Nitsch medium but in *Rhynchostylis retusa* and *Spathoglottis plicata* modified Knudson's C medium was the best. Similarly, Seed germination of *Rhynchostylis albiflora*, *Vanda parshii*, *Phalenopsis manii* were better in modified Knudson's C medium while *Calanthe masuca*, *Dendrobium liuiflorum*, *Spathoglottis plicata* x *Dendrobium densiflorum* Nitsch medium was better. The standardised media composition of various orchids seed and tissue culture were well reviewed (Jaime, 2013).

MS medium was the best for culture establishment, multiple shoot production and rooting. Maximum rooting was occurred in ½ MS medium supplemented with IBA and NAA in *Dendrobium osterholt* (Raju *et al*., 2003).

White's and Vacin and Went media were equally effective for seed germination in *Spathoglottis plicata*. Seed germination stimulated in Vacin and Went medium supplemented with banana extract, ripe tomato extract and yogurt water in *Spathoglottis plicata* (Roy and Biswas, 2005).

Seed germination and protocorm development varied with different media and photoperiods among three *Vanda* hybrids (Johnson and Kane, 2007).

Mitra medium supplemented with activated charcoal and Dimethyl sulfoxide (DMSO) enhanced germination and seedling development in *Rhynchostylis retusa* (Vij and Jalota, 1995). Mitra medium supplemented with various organic supplements and plant growth regulators promoted regeneration of plantlets from root segments of *Rhynchostylis retusa* seedlings. Type of regenerants (protocorm or shoot bud) produced from explants depend on growth regulator composition of the medium. Regeneration of protocorm was optimum in the presence of kinetin, peptone and NAA in the medium. Replacement of NAA with IAA promoted shoot bud formation (Sood and Vij, 1986). Seedling growth was the best on MS medium supplemented with BAP, NAA and AC. Regeneration of multiple shoot occurred in medium containing kinetin with or without AC in *Rhynchostylis retusa* (Thomas and Michael, 2007).

Both NAA and IAA were effective in inducing better root system and seedling development and NAA at lower concentration produced seedlings in *Vanda spathulata* (Mitra *et al*., 1976; Chung and Chun, 1983).

Growth of protocorm did not enhance with the application of GA in *Vanda* (Vieitez and Vieitez, 1980). The initial size of explants and media were determined the growth of protocorm in *Vanda* (Mathews and Rao, 1985).

MS medium was efficient for protocorm development and medium supplemented with yeast extract, peptone and NAA enhanced growth and differentiation of protocorms in three interspecific hybrids of *Vanda* (Helena *et al.*, 1980).

In vitro seed culture led to the development of protocorm and seedlings obtained through interspecific hybridization of *Vanda thwaitesii* x *Vanda spathulata* (JNTBGRI, 2014).

MS medium at $1/4^{th}$ strength, Vacin and Went and Knudson-C media supplemented with kinetin, BAP, NAA and IBA enhanced the growth characters like height, leaf length, leaf number, root length and root number of *Dendrobium* New Pink x Emma white hybrid seedlings (Sobhana and Rajeevan, 2002).

MS medium fortified with BAP and NAA significantly increased number of shoot initials explant^{-1}, height and new protocorm explant^{-1} after 60 days of culture and number of leaves shoot^{-1} after 60 and 120 days of subculture. MS medium fortified with kinetin and NAA significantly increased shoot height after 60 and 120 days of subculture, in interspecific hybrid of *Cymbidium aloifolium* x *Cymbidium bicolour* (Krishna, 2015).

Half MS medium fortified with NAA significantly increased the plant height, root number and root length in interspecific hybrid of *Cymbidium aloifolium* x *Cymbidium bicolour* (Krishna, 2015). IAA or IBA oxidation caused by nonspecific peroxidise led to the rooting response.

Seeds of all the selected rare species were germinated after three months of culture, plantlets were vigorous and well acclimatized in green house conditions. Seeds of *Vanda* Motes Primrose x *Ascocenda* Tavivat showed higher germination than V. Paki x (*V.tessellata* x *V.cristata*) and (*V.* Joan Warne x *V.* Paki Paki) x *V.* Loke cross on Knudson C and ½ MS media (Luan *et al.*, 2006).

Under *in vitro* conditions plantlets are heterotrophs and these are gradually turned to autotrophs through the pre-hardening and hardening processes and adapt them to sustain natural conditions (George, 1996).

Success of *in vitro* propagation is mainly depends on the sustainable *ex vitro* establishment under natural conditions (Saxena and Dhawan, 1999; Deb and Imchen, 2010).

For *ex vitro* establishment, vermiculite was effective in *Vanda spathulata* and *Satyrium nepalense* (Mahendran and Narmatha Bai, 2009; Devi *et al.*, 2015).

Hardening medium comprising of brick: charcoal: tree fern: bark pieces: leaf mould: dry sphagnum moss in the 1:1:1:1:1:2 ratio was suitable for survival of *Dendrobium* and *Chrysanthemum* seedlings (Sharma and Chauhan, 1995). Brick pieces: bark pieces: moss: charcoal pieces in the 1:1:1:1 ratio for rooted plantlets of Sonia-17 (Pathania *et al.*, 1998). Charcoal: brick: cocopeat in the 1:1:1 ratio was suitable for *Dendrobium* hybrid Sonia-17 (Indhumati *et al.*, 2003). Brick pieces: charcoal: cocopeat: sphagnum moss: decayed wood in the 1:1:1:1:1ratio for *Dendrobium* hybrid Sonia-17 (Sahoo, 2012).

Rooted plantlets attained 6-7 cm height when planted in hardening medium containing brick pieces, charcoal, coco peat, moss, bark, coco husk and coco chip in the 1:1:1:1:1:1:1 ratio. It significantly increased the height, leaf number, length, width, root number plant^{-1}and percentage of survival after 60 and 120 days of hardening in *Cymbidium aloifolium* x *Cymbidium bicolour* interspecific hybrids (Krishna, 2015).

High humidity of about 90% was essential for hardening of orchid plantlets for two weeks after planting (Puchooa, 2004).

Survival of plantlets at the time of plant out depends on the vigorous growth of the newly produced leaves and number of leaves present initially (Sutter *et al.*, 1985).

Success of acclimatization depends on both pre and post transfer growth conditions. Peatmoss was the best for good stem diameter and height and perlite for more root number (Lim *et al.*, 1993).

The plants were poorly adapted to resist low relative humidity, higher light levels and variable temperature outside and the survival percentage varied in different hybrids (Wainwright, 1988). The medium brick and charcoal pieces selected were successful for good growth. Use of charcoal pieces as a good medium was confirmed in orchids (Seeni and Latha, 1990; Kumar, 1992; Singh, 1993).

Hybrids performed differently for the vegetative traits like height, length of leaf and number of leaves in the field evaluation (Sobhana, 2000).

Vanda hybrids exhibited wide variation in root traits such as number, length and girth of roots (Minnu, 2015). The roots have velamen tissues and it help in easy absorption of water and nutrients through entire length.

Species specific medium composition is required for optimum germination and seedling growth (Olivia and Arditti, 1984).

Twenty four weeks after *in vitro* seed culture protocorm development initiated on seeds cultured on P723 medium and seedling growth initiated 7 weeks after corm formation. Seedling survival was high in all potting media and leaf production and root length were similar in all potting media. Shoot regeneration started 8 weeks after plant out under greenhouse conditions (Philip *el al*., 2006).

In vitro cultures of mature or immature embryo are applied to recover plants that do not produce fertile seeds obtained from intergeneric crosses (Ahmadi *et al*., 2010).

Many native orchids were flowered 2-8 years after seed culture and required 4 or 5 years after seed germination. Standardization of seed culture could be provided efficiency in production of large quantities of faster flowering vigorous plants (Stoutamire, 1974). Therefore, it is necessary to develop appropriate *in vitro* seed culture protocol for every species.

2.7. MOLECULAR CHARACTERIZATION

Morphological markers have limitations due to the influence of developmental stage of the plant and environmental factors on its expression. Therefore, molecular markers based on DNA polymorphism are more informative, independent of environmental conditions and unlimited in number (Agarwal *et al.*, 2008).

In plant species molecular marker based genetic variability analysis is an important tool for breeding and conservation (Nybom and Bartish, 2000).

Genetic analysis by molecular data leads to an invaluable knowledge useful for identification, conservation, sustainable uses, breeding etc. (Thammasiri, 2016).

Generally to establish genetic stability or genetic variability in various plant species RAPD or ISSR or both of the markers have been successfully applied (Arnau *et al.*, 2003).

ISSR is simple quick technique which permits detection of polymorphism in inter-micro satellite loci using a primer designed from dinucleotide or trinucleotide simple sequence repeats (12-13). Based on PCR (Polymerase chain reaction) one of fingerprinting techniques is ISSR and defined as a variant of PCR using sequence primers to amplify simple and repetitive regions between target sequences (Zietkiewicz *et al.*, 1994).

ISSR assess the abundance and random distribution of simple sequence repeats (SSRs) in genomes of different organisms, amplify DNA sequences contained between these SSRs and useful in population analyses (Reddy *et al.*, 2002; Zhang *et al.*, 2013; Pinheiro *et al.*, 2010).

Complementary use of molecular markers along with conventional breeding methods will allow breeders to target more specific characteristic changes and thereby can cut short breeding time. PCR–based techniques used to analyse the

DNA of variant clones found polymorphic fragments and that can be developed as molecular markers (Basiran and Ariffin, 2001).

Evaluation of genetic variability using ISSR molecular markers yielded 40-80% of polymorphism across all the samples in *Rhynchostylis retusa* (Parab and Krishnan, 2008).

ISSR molecular markers are effective in assessing interspecific variation among hybrid populations (Allendorf and Luikart, 2007).

High level of polymorphism was exhibited in molecular analysis using RAPD and ISSR markers in the *Cymbidium labiata* (Pinheiro *et al.*, 2012) and it is an endangered species and suffers from extractivism and habitat loss from deforestation.

Genetic diversity of nine species of *Vanilla* was analysed using eleven ISSR primers and found 108 loci of which 93 were polymorphic (86.11%) (Verma *et al.*, 2009).

Genetic diversity and phylogenetic relationships evaluation among 31 *Dendrobium* species were analysed with 17 ISSR primers and found 278 loci with 100% polymorphism (Wang *et al.*, 2009b).

ISSR markers were sufficient for quantifying genetic diversity and identifying polymorphism between individuals in populations of different orchid species namely *Cymbidium bicolour*, *Cymbidium granulosa*, *Cymbidium labiata* and *Cymbidium schofieldiana*. Also found that to estimate genetic relationships among and within species, 50 -100 bands were sufficient (Colombo *et al.*, 2000).

Measurement of genetic variation and assessment of phylogenetic inferences of five *Laeliinae* species from the Atlantic forest of Brazil was the first use of the ISSR genotyping technique and majority of grouping was with the use of plastid markers and nuclear ITS rDNA (van den Berg *et al.*, 2000, 2009).

Evaluation of natural populations of *Cymbidium labiata* and ten species of the genus was based on RAPD and ISSR markers and high level of polymorphism was observed and it helped to gain knowledge of genetic structure of species used for the breeding programme (Pinheiro *et al.*, 2012).

ISSR are valuable for studying closely related taxa, due to difficulty in finding DNA regions that are variable in different taxa (Archibald *et al.*, 2006).

Dominant ISSR markers are potentially useful for phylogenetic reconstruction of closely related species (Joshi *et al.*, 2000; Hao *et al.*, 2002; Sudupak, 2004).

ISSR technique can generate large number of multi-locus markers and applied to evaluate any organism (Arcade *et al.*, 2000).

ISSR technique used to verify the genetic stability of tissue culture derived micropropagated plantlets in *Vanda stangeana* (Kishor and Devi, 2009). It was used to characterize genetic diversity in several orchids *viz Anoectochilus formosanus* , *Balanophora fungosa*, *Cymbidium goeringii* and *Vanilla species* (Verma *et al.*, 2009; Wang *et al.*, 2009a; Hsiao *et al.*, 2010; Zhang *et al.*, 2010). Useful for informing genetic conservation and sustainable use strategies in *Cymbidium ensifolium* (Wang *et al.*, 2011). ISSR technique used for genetic analysis in endemic South American orchids are rare (Borba *et al.*, 2001; Avila-Diaz and Oyama, 2007).

Analysis of genetic diversity and phylogenetic relationships between *Cattleya granulosa*, an endangered Brazilian orchid and four other native Brazilian species exhibited 23.26% of molecular variance and proved the effectiveness of ISSR genetic markers in detecting genetic differentiation among orchid species (Cristiane *et al.*, 2014).

The efficiency of introgression can be improved by the recently developed highly valuable and potential molecular and cytogenetic tools. A combination of

conventional hybridization and molecular markers are useful for monitoring hybrids, back cross progenies for introgression, elucidating the modes of origin of 2n-gametes, to determine extent of genetic recombination and phylogeny of the species and hybrid cultivars (Tuyl and Lim, 2003).

Availability and increase in ease of development of molecular markers facilitated improvement through hybridization and introgression (Riesberg and Ellstrand, 1993; Martinsen *et al.*, 2001; Moccia *et al.*, 2007; Pinheiro *et al.*, 2010, 2015; Pavarese *et al.*, 2011).

Genetic diversity within and between populations was assessed at molecular level by DNA analysis directly (Bansal *et al.*, 2014).

Genetic variation evaluation using ISSR molecular markers among the *Aerides maculosum* showed 50-100% variation in polymorphism across all the samples (Parab *et al.*, 2008).

Assessment of genetic diversity and differentiation with ISSR molecular markers detected high level of genetic differentiation based on analysis of molecular variance on rare terrestrial orchids *Calanthe tsoongiana* endemic to China (Wang *et al.*, 2013).

Genetic diversity assessment with ISSR molecular markers revealed significant variation among different accessions of *Aerides multiflorum.* ISSR molecular markers are codominant, simple and easy markers to detect by PCR (Omika, 2014).

Molecular markers are used to monitor DNA sequence variation within species, among species and cultivars. It is also useful for the determination of genetic variation and organization of genetic diversity within *Dendrobium* plants. Related knowledge of more species is prerequisite for exploiting *Dendrobium* genetic resources (Wang *et al.*, 2009).

ISSR techniques involve PCR to amplify DNA fragments between two simple sequence repeats with inverse orientations using primers with a single SSR motifs anchored at the 3- or 5-end by a few nucleotides (Zietkiewicz *et al.*, 1994).

ISSR markers are present in both nuclear and organellar genome, highly polymorphic in plant populations, providing a genotyping system with consistency, reliability and codominancy (Peng *et al.*, 2006). In traditional plant systematics a few morphological traits were used as key traits and its variations arising confusion in classification among taxonomists (Tsi *et al.*, 1999; Chen and Tsi, 2000).

ISSR technology is sensitive to low levels of genetic variation, useful tool to study population genetics of different plant species, identifying species, cultivars or population of same species (Zietkiewicz *et al.*, 1994; Raina *et al.*, 2001; Wang *et al.*, 2009).

ISSR analysis is a low-cost and high efficiency technique. It is the best method for identification of morphologically similar species overlapped with geographical distributions and exhibiting minute difference on certain traits (Tsi *et al.*, 1999).

Thirty-one *Dendrobium* species were differentiated by using 17 ISSR primers and two closest species *Dendrobium hancockii* and *Dendrobium hercoglossum* showed 73% similarity and separated 31 species into six clusters (Wang *et al.*, 2009b). But ISSR derived genetic diversity did not match with morphological differences among *Dendrobium* species (Wang *et al.*, 2009).

ISSR analysis is a useful molecular tool for identifying *Cymbidium* cultivars and determining relationship among *Cymbidium goeringii* cultivars (Wang *et al.*, 2009b).

Genetic variability is inherited in a dominant or codominant Mendelian fashion (Gupta *et al.*, 1994). ISSR results a multi-locus marker system useful for

fingerprinting, diversity analysis and genome mapping and a large number of polymorphic bands can be generated (Godwin *et al.*, 1997; Wolfe *et al.*, 1998). It is interpreted as dominant markers and scored diallelically with presence or absence of bands (Wolfe *et al.*, 1998). The absence of band is interpreted as sequence divergence at the site of primer binding and it occurs by loss of a locus through the deletion of site or chromosomal rearrangement (Wolfe and Liston, 1998).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The experiment entitled "Distant hybridization and compatibility studies in wild orchids" was conducted at Saraswathy Thangavelu Extension Centre of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (STEC-JNTBGRI), Puthenthope, Thiruvananthapuram and at College of Agriculture, Vellayani, Thiruvananthapuram during the period of 2015-2018.

3.1. EXPERIMENT 1 - SELECTION OF PARENT MATERIALS AND HYBRIDIZATION TO EVALUATE CROSS COMPATIBILITY

The experiment was carried out in two parts namely evaluation of parents and hybridisation. The materials used for the study included six different genera/species of wild orchids available at the orchid conservatory of STEC-JNTBGRI, Puthenthope, Thiruvananthapuram. These were evaluated for their cross compatibility (Table 1).

Table 1. Details of parent materials used

Sl No	Genus	Species	Remarks
1	*Vanda*	*tessellata*	Epiphytes
1a	*Vanda* *	sp.	Epiphytes
2	*Vanda*	*spathulata*	Epiphytes
3	*Rhynchostylis*	*retusa*	Epiphytes
4	*Aerides*	*ringens*	Epiphytes
5	*Arundina*	*graminifolia*	Terrestrial
6	*Spathoglottis* **		
6a	*Spathoglottis*	*albida*	Terrestrial
6b	*Spathoglottis*	*unguiculata*	Terrestrial
6c	*Spathoglottis*	*plicata*	Terrestrial
6d	*Spathoglottis*	*gracilis*	Terrestrial

* To test the compatibility of wild species of orchids with a hybrid, the first *Vanda* hybrid which was derived from the cross between the wild *Vanda* species *Vanda tessellata* and *Vanda cooperi* was also used as a third parent in the genus *Vanda* for hybridization.

** In the genus *Spathoglottis*, four species were used as parents to test the compatibility.

1. ***Vanda tessellata* (Roxb.) Hook. ex G.Don**

It is an epiphytic valuable medicinal wild orchid exhibiting perennial growth habit. It will flower throughout the year and produces long-lived fragrant flowers. Flowers are with tessellated markings which is a constant distinguishing feature. Exploitation of this orchid for using as traditional medicine has caused significant decline in its population. Currently this orchid is under cultivation in Gurukula Botanical Sanctuary, Wayanad and JNTBGRI, Thiruvananthapuram in South India (Plate1A).

1a. *Vanda* sp.

It is a first *Vanda* hybrid derived from the cross between wild *Vanda* sp. *Vanda tessellata* and *Vanda cooperi* (Plate 1B).

2. ***Vanda spathulata* (L.) Spreng.**

It is popularly known as Spoon-Leaf Vanda. Its synonym is *Taprobanea spathulata* (L.) Christenson. In the red listed category of plants, it is included under the group vulnerable. A large endemic distribution is seen in Southern India. It is a perennial epiphyte with golden yellow flowers, in which flowering and fruiting occurs between September and January. The generation length is fifteen years. It is maintained under *ex situ* cultivation at Gurukula Botanical Sanctuary in Wayanad and JNTBGRI where it has been bred with several Vandaceous orchids (Plate 1C).

3. ***Rhynchostylis retusa* (L.) Blume**

The inflorescence of this wild orchid is pendant consisting of more than 100 pink-spotted white flowers. It is an epiphytic, monopodial orchid and vegetatively resembles closely *with Aerides* except for the shorter stems and thicker leaves. Flowers are fragrant, small and typically white with light to deep magenta, red or lavender-blue spots, spicy aroma is released in the evening. Due to bio-piracy, it is on the verge of extinction in India. It is threatened in its habitat due to excessive harvest (Plate 2A).

Plate 1. Plant with flowers: (A) *Vanda tessellata* (B) *Vanda* sp. (C) *Vanda spathulata*

4. ***Aerides ringens*** **(Lindl.) C.E.C.Fisch.**

It is a monopodial epiphyte with long-lasting, fragrant, waxy flowers, which are often white with purple or pink edges. It possess erect to ascending, rarely branched, densely many flowered inflorescence (Plate 2J). It is popular in horticulture industry as cut flowers and potted plants. Hybrids come in a wide range of colours due to the high degree of genetic diversity.

5. ***Arundina graminifolia*** **(D.Don) Hochr.**

This terrestrial perennial orchid forms into large clumps growing to a height between 70 cm and 2 m. It produces open clusters of showy terminal flowers, ten at the most, rosy lilac and white disk with a purple lip. It is polypodial in growth habit and possesses fragrant flowers and will flower one at a time, which is short-lasting yet successive so the bloom time is extended. It is preferred as a long-blooming orchid for garden areas. This plant attained the importance as a good house plant because of its attractive lilac coloured flower and flower shape (Plate 2I).

6. a. ***Spathoglottis albida*** **Kraenzl**

The garden terrestrial orchid possesses long spike of brightly coloured white flowers that bloom almost continually throughout the growing season (Plate 3D).

b. ***Spathoglottis gracilis*** **Rolfe ex Hook.f.**

It is a tender dwarf perennial producing yellow coloured flowers throughout its life span (Plate 3E).

c. ***Spathoglottis plicata*** **Blume**

Spathoglottis plicata is a fast-growing orchid with a wide environmental tolerance. It has light purple flowers which are self-pollinating; each plant can produce a large number of flowers, fruits and seeds (Plate 3F)

Plate 2. Plant with flowers: (H*) Rhynchostylis retusa* (I) *Arundina graminifolia* (J*) Aerides ringens*

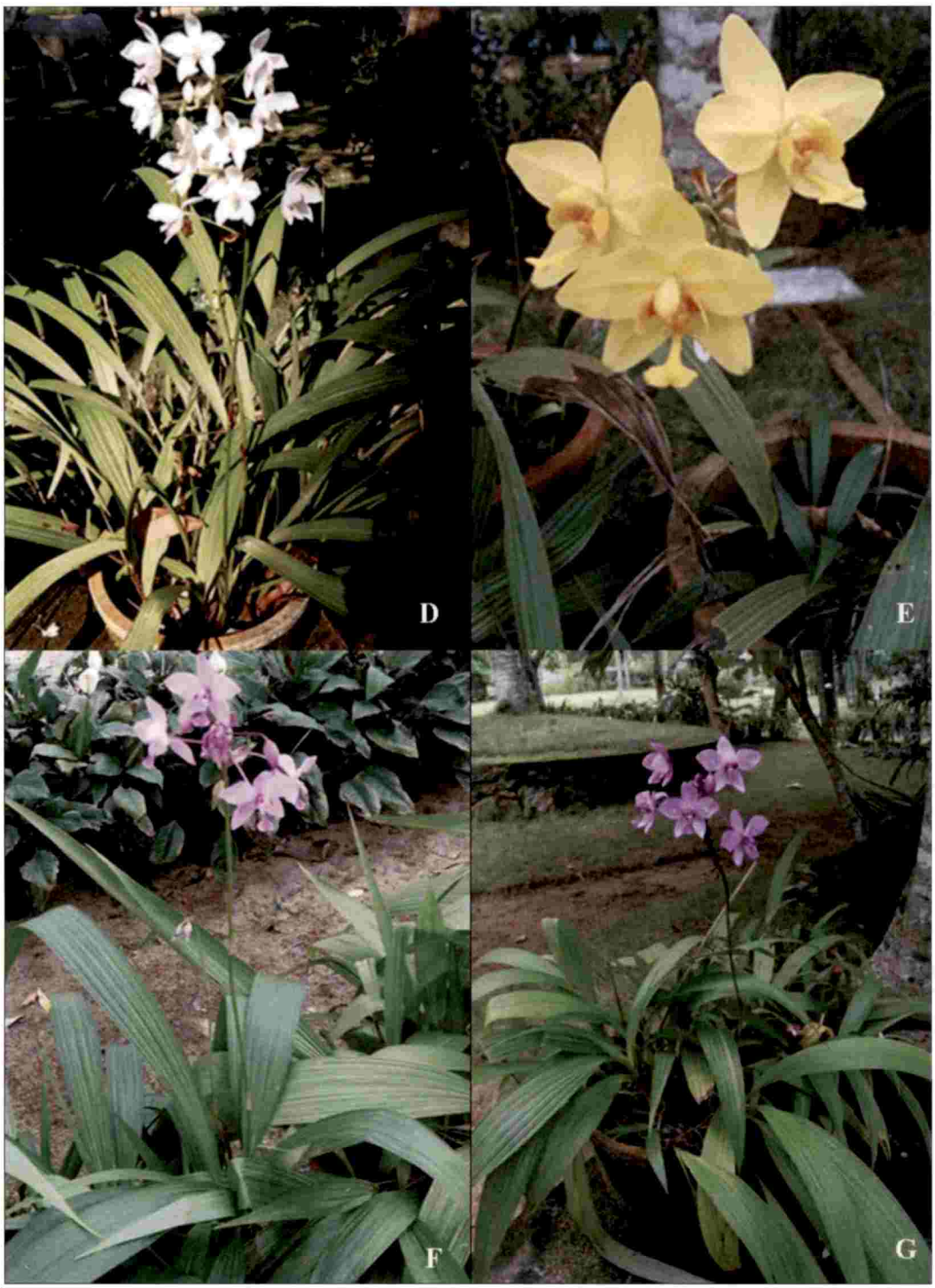

Plate 3. Plant with flowers: (D) *Spathoglottis albida* (E) *Spathoglottis gracilis* (F) *Spathoglottis plicata* (G) *Spathoglottis unguiculata*

d. ***Spathoglottis unguiculata*** **(Labill.) Rchb.f.**

It possess beautiful deep purple flowers with long lasting spikes producing successive flowers over time and have a distinct fragrance of grape juice. They bloom year round and flower stalk reach up to 100 cm, with 10-40 flowers (Plate 3G).

3.1.1. Evaluation of Parents

The parents used for studying compatibility were selected based on variability in floral characteristics and evaluation for various qualitative and quantitative characters. The experimental design used was Completely Randomized Design (CRD) with six treatments each replicated five times with five plants replication^{-1}. Observations with regard to various qualitative and quantitative characters made among different parents were as follows.

A. Plant Characters

3.1.1.1. Plant Height (cm)

Vertical distance from the base of the plant to the tip of the terminal leaf was measured from five plants and worked out mean.

3.1.1.2. Plant Spread (cm)

Spread is the horizontal width of the plant. Horizontal distance between tips of two opposite leaves positioned at the middle portion of main stem was measured from five plants and worked out average.

3.1.1.3. Shoot Girth (cm)

Diameter at collar region of shoot or base of the main stem was measured as girth from five plants and mean was worked out.

3.1.1.4 .Number of Leaves Plant^{-1}

Total number of fully opened leaves from five plants was counted and mean was worked out.

3.1.1.5. Leaf Length (cm)

Length of the middle leaf starting from the base of petiole to apex of leaf blade was measured from five plants and mean was worked out.

3.1.1.6. Leaf Width (cm)

Width of the middle leaf starting from left margin to right margin of leaf blade was measured from five plants and mean was worked out.

B. Floral Characters

3.1.1.7. Nature of Flowering (free / seasonal)

Appearance of inflorescence and flowering time was observed and recorded among different parents in a year.

3.1.1.8. Season of Flowering

Season of flowering among different parents was observed and recorded.

3.1.1.9. Days Taken for First Flowering

Number of days taken from planting to initiation of inflorescence and opening of first flower in a spike was counted and mean was worked out.

3.1.1.10. Number of Spikes Produced Plant^{-1} Year^{-1}

Total number of spikes produced from five plants in a year was counted and average was worked out.

3.1.1.11. Length of Inflorescence (cm)

Length of spike from the base of inflorescence to its tip was measured from five plants and average was worked out.

3.1.1.12. Number of Flowers Inflorescence^{-1}

Total number of flowers present in a spike was counted from five plants and mean was worked out.

3.1.1.13. Internodal Length (cm)

Distance between two adjacent flowering nodes from the base to tip of a spike was measured from five plants and mean was worked out.

3.1.1.14. Flower Size (Flower Length and Flower Width)

Flower length is the distance from the tip of sepal arranged opposite to the labellum to the tip of labellum. It was measured from five plants and mean was worked out.

Flower width is the distance between tip of two side petals of the flower. It was measured from five plants and mean was calculated.

3.1.1.15. Flower Colour

Flower colour was observed and compared with the report in the reference book entitled "Introduction to Orchids" (Abraham and Vatsala, 1981).

3.1.1.16. Days for Inflorescence Emergence to First Flower Opening

Total number of days taken from emergence of inflorescence to opening of first flower in a spike was counted from five plants and mean was worked out.

3.1.1.17. Days to Last Flower Opening from First Flower Opening

Total number of days taken from first flower opening to last flower opening in a spike was counted from five plants and mean was worked out.

3.1.1.18. Spike Longevity on the Plant

Total number of days taken from emergence of inflorescence to drying of spike tip was counted from five plants and mean was worked out.

3.1.1.19. Time of Anthesis

Time of opening of the flower in an inflorescence from five plants was noticed and mean was worked out.

3.1.1.20. Time of Stigma Receptivity

Total number of days taken for the stigma to remain receptive for effecting pollination in a flower was counted from five plants and mean was worked out.

3.1.1.21. Pollen Morphology

Pollen grains were stained in acetocarmine and observed under microscope for shape, exine and intine ornamentation and nature of pollen and these were recorded.

3.1.1.22. Pollen Production Pollinium^{-1}

The pollen production pollinium^{-1} was estimated using hemocytometer. Pollinium was collected in an Eppendorf tube and crushed well. The prepared pollen suspension was gently filled into the two chambers underneath the coverslip of the hemocytometer and the pollen grains were observed under microscope and counted in four sets of sixteen corner squares and calculated the average number of pollen grains present in pollinium.

3.1.1.23. Pollen Fertility

Pollen fertility was estimated using acetocarmine staining technique. Pollinia were crushed with needle and pollen grains liberated were transferred into acetocarmine stain for one minute. The pollen grains were observed under the microscope. Stained pollen grains were viable and unstained colourless pollen grains were non-viable. The viable and non-viable pollen grains were counted

from five different microscopic fields at random and mean was expressed in percentage.

$$\text{Pollen fertility percentage} = \frac{\text{Number of stained pollen grains}}{\text{Total number of pollen grains}} \times 100$$

3.1.1.24. Pollen Germination

Pollen grains were kept for germination in sucrose solution and observed under microscope to count the total number of germinated and ungerminated pollen grains and pollen germination percentage was calculated using the formula as follows.

$$\text{Pollen germination percentage} = \frac{\text{Number of germinated pollen grains}}{\text{Total number of pollen grains}} \times 100$$

3.1.1.25. Pest/Disease/Physiological Disorder, if any-

Insects and disease appeared in plants were noticed.

3.1.2. Statistical Analysis

The data collected were subjected to statistical analysis. Analysis of variance was carried out in Completely Randomized Design (CRD) to test the significance for various traits among different parents. Further estimation of genetic components of variance such as phenotypic coefficient of variation, genotypic coefficient of variation, environmental coefficient of variation, heritability (in broad sense), genetic advance (percent of mean) and association analysis (correlation coefficient) were worked out.

3.1.2.1. Analysis of Variance

The partitioning of the variation into different sources of variation depends on the arrangement of the various treatments with regard to one another (Table 2).

Table 2. ANOVA

SOURCE	D.F.	S.S.	M.S.	F
Between treatments(Parents)	V-1	VSS	VMS	VMS/EMS
Within the treatments(Error)	n-v	ESS	EMS	
TOTAL	n-1	TSS		

Where V = number of treatment, n = total number of observation

Critical Difference

To compare the means of various treatments, the critical difference (C.D.) was calculated by the following formula:

Critical Difference (C.D.) = S.E. × 't'

Where, S.E. is standard error of the difference of the treatment means to be compared, and

$$S.E = \frac{\sqrt{2MSE}}{r}$$

Where r is number of replication

3.1.2.2. Components of Variance

The mean sum of squares between varieties consisted of the variances

(i) Attributable to varietal differences (i.e., genotypic differences)

(ii) Due to environmental variation among individuals of each genotype.

Thus expected mean sum of squares would be as follows

Expected Mean Sum of Squares for Environment

E (MS_e) = MSe

From this estimated components of variance as suggested by Lush (1940)

Where,

$$GV = \frac{MSv - MSe}{R}$$

GV = Genotypic Variance

Phenotypic Variance (PV) = GV + E (MS_e)

From this estimated Coefficient of Variation as suggested by Burton (1952).

Phenotypic Coefficient of Variation (PCV) = PCV = $\frac{\sqrt{PV}}{\text{Grand mean}} \times 100$

Genotypic coefficient of variation (GCV) = GCV = $\frac{\sqrt{GV}}{\text{Grand mean}} \times 100$

Heritability is the ratio of genotypic variance to the phenotypic variance (Lush, 1940).

Heritability = $\frac{GV}{PV}$

Genetic advance (GA)

As suggested by Johnson *et al.* (1955), genetic advance was estimated as follows.

GA= $\frac{GV}{pSD}$ x K

Where,

GV = Genotypic Variance

pSD = Phenotypic standard deviation

K= selection differential at 5% selection intensity

Genetic advance percent = $\frac{GA}{Mean}$ x 100

3.1.2.3. Components of Covariance

The following factors were estimated under components of covariance (Table 3)

Table 3. ANCOVA

SOURCE	D.F.	S.P.	M.P	F
VARIETIES	V-1	SPv	MSPv	MSPv/MSPe
ENVIRONMENT	R-1	SPr	MSPr	MSPr/MSPe
ERROR	(V-1)(R-1)	SPe	MSPe	
TOTAL	VR-1	TSP		

Expected Mean Sum of Product for Varieties E (MSP_v) = MSPe + R × GC

Expected Mean Sum of Product for Error E (MSP_e) = MSPe

Where,

$$GC = \frac{MSPv - MSPe}{R}$$

GC is the Genotypic Covariance.

Phenotypic Covariance (PC) = GC + MSPe

$$\text{Coheritability} = \frac{GC}{PC}$$

3.1.2.4. Correlation Coefficient

Simple correlation was calculated using the formula

$$r\,(x1x2) = \frac{Cov\,(x1x2)}{\sqrt{Vx\,1 \times Vx2}}$$

Where,

$r\,(x_1x_2)$ is the correlation between x_1 and x_2

$Cov\,(x_1x_2)$ is the covariance between x_1 and x_2

$V(x_1)$ is the variance of x_1

$V(x_2)$ is the variance of x_2

Simple Correlations are of three types.

3.1.2. 4.1. Genotypic Correlations

Estimated from genotypic covariance and variance

3.1.2. 4.2. Phenotypic Correlations

Estimated from phenotypic covariance and variance

3.1.2. 4.3. Environmental Correlations

Estimated from environmental covariance and variance

Based on this variability evaluation, parents were selected

3.1.3. HYBRIDIZATION

Selected parents were crossed in all possible combinations on the basis of synchronisation of flowering among parents.

Total seventy seven cross combinations was made among the parents as indicated in Table 4.

Table 4. Plan of cross compatibility study

♀ \ ♂	Vt	Vs	Vsp	Rr	Ar	Ag	Sa	Sg	Su	Sp
Vt	+	x	-	x	x	x	x	x	x	x
Vs	x	+	x	x	x	x	x	x	x	x
Rr	x	x	-	+	x	x	x	x	x	x
Ar	x	x	x	x	+	x	x	x	x	x
Ag	x	x	-	x	x	+	x	x	x	x
Sa	x	x	x	x	x	x	+	x	x	x
Sg	x	x	-	x	x	x	x	+	x	x
Su	x	x	x	x	x	x	x	x	+	x
Sp	x	x	x	x	x	x	x	x	x	+

Vt-*Vanda tessellata*, Vs - *Vanda spathulata*, Vsp - *Vanda* sp., Rr - *Rhynchostylis retusa*, Ar - *Aerides ringens*, Ag - *Arundina graminifolia*, Sa - *Spathoglottis albida*, Sg - *Spathoglottis gracilis*, Su - *Spathoglottis unguiculata*, Sp - *Spathoglottis plicata*, (+) → selfing, (x) → crossing, (-) → not attempted

The following methods were adopted for the development of hybrids

3.1.3.1. Selection of Ideal Parent Plants for Crossing

Healthy, vigorous, pest and disease free plants were selected as female and male parents for crossing to develop hybrid plants.

3.1.3.2. Crossing

Plants containing inflorescence axis with unopened flower buds were selected as female parent. Similarly plants containing inflorescence axis with unopened flower buds were selected as male parent for collecting pollinium containing pollen grains for pollination. In the female parent plant mature flower buds which would be opened on the next day were selected in the evening and labelled. In male parent also mature flower buds which would be opened on the

next day were selected in the evening and labelled. On the next day anthers (pollinium) were removed from the opened and labelled flower with needle carefully without rupturing anther wall from the female parent plant. Similarly anthers (pollinium) were removed from the opened and labelled flower with needle carefully without rupturing anther wall from the male parent plant. Pollinia taken from the flower of female parent plant were placed over the stigmatic surface of the flower of male parent plant and *vice versa* at the same time. After pollination flowers of both parents were labelled. Pollination was carried out at different time depending on anthesis and also to test stigma receptivity of different species.

3.1.3.3. Harvesting of Mature Green Capsule

After pollination the mature undehisced green capsules or pods developed were harvested.

3.2 EXPERIMENT II - CROSS COMPATIBILITY STUDIES OF SELECTED SPECIES

Cross compatibility between the selected wild orchids was studied based on successful capsule setting after hybridisation. The following observations on capsules were recorded.

3.2.1. Days Taken for Pod Setting

Number of days taken from pollination to greening and swelling of gynoecium present between pedicel and calyx from five plants was recorded and mean was worked out in days.

3.2.2. Days to Green Capsule Harvest

Number of days taken from pollination to harvesting of green undehisced mature capsule from five plants was recorded and mean was calculated.

3.2.3. Length of Capsule (cm)

Distance from the base of pedicel to the base of calyx of the capsule from five plants was measured and mean was calculated.

3.2.4. Width of Capsule (cm)

Diameter of capsule was measured from five plants and mean was worked out.

3.2.5. Percentage of Filled Seeds

Percentage of filled seeds was determined using staining technique. Seeds were scooped out from capsule and transferred into one percent solution of 1,3-5 Triphenyl Tetrazolium Chloride (TTC) and kept for 24 hours in dark at room temperature for staining. After that seeds were washed in distilled water and transferred the seeds into 0.01% solution of malachite green stain for one day to stain the seed coat. Observed the seeds under microscope and counted fully grown seeds with well filled and good shaped green or brown coloured embryo from five different microscopic fields at random and mean was worked out and converted it into percentage (Gayatri and Kavyashree, 2015).

$$\text{Percentage of filled seeds} = \frac{\text{Number of stained seeds}}{\text{Total number of seeds}} \times 100$$

3.3. EXPERIMENT III- *IN VITRO* CULTURE OF RESULTANT DISTANT HYBRIDS AND EVALUATION OF SEEDLINGS

The experiment was carried out in two parts.

3.3.1. *In vitro* Culture of Hybrid Seeds

Hybrid seeds were cultured for rapid germination under *in vitro* condition and evaluated different stages of seed germination and morphology of seedlings at regular intervals.

Mature and green capsules harvested were subjected to surface sterilization using running tap water for one minute followed by rinsing in detergent solution

for ten minutes. The capsules were again washed with running tap water for one minute. Capsules were brought to the laminar air flow chamber and sterilized in 70% ethyl alcohol. After flaming, pods were cut longitudinally with sterilized knife and split opened with sterilized forceps. Seeds were scooped out and inoculated into the culture media. Different types of basal media were prepared for seed germination such as Murashige and Skoog (MS) medium, Mithra medium and half strength of MS medium. The pH of the medium was adjusted to 5.6 to 5.8. Inoculated explants were kept in the culture room for germination which was maintained in 12 hours of photoperiod from cool, dry, light fluorescent tubes and maintained proper light intensity (2000 lux) and temperature (25 ± 2°C). Protocorm developed were sub-cultured into different media for regenerating shoots and roots. The different stages of *in vitro* seed culture are summarized in figure 1.

The following observations were recorded frequently.

3.3.1.1. Days for Initiation of Germination (Greening)

Number of days taken for greening from the day of seed inoculation and average was worked out in days.

3.3.1.2. Days for Initiation of First Leaf

Number of days taken for initiation of first leaf from the day of seed inoculation was observed and average was worked out in days.

3.3.1.3. Days for Initiation of Shoot

Number of days taken for initiation of shoot from the day of seed inoculation was observed and average was worked out in days.

3.3.1.4. Days for Initiation of First Root

Number of days taken for initiation of first root from the day of seed inoculation was observed and average was worked out in days.

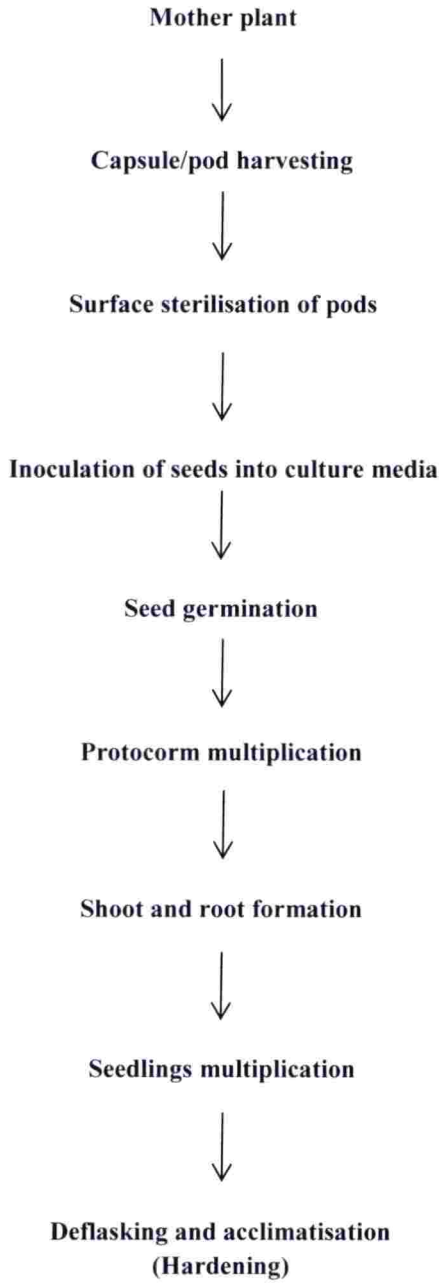

Figure 1. Stages of *in vitro* seed culture

3.3.1.5. Days for Deflasking

Number of days taken for deflasking from the day of seed inoculation was observed and average was worked out in days.

3.3.2. Evaluation of Hybrid Seedlings

Variability for morphological characters of hybrid seedlings was evaluated three times at monthly intervals i.e., one at deflasking, second at one month after deflasking and third at two months after deflasking. The following observations were recorded at monthly intervals.

3.3.2.1. Seedling Height (cm)

Length of seedlings from the base of shoot to tip of terminal leaf was measured and average was calculated.

3.3.2.2. Number of Leaves

Total number of leaves present in seedlings was counted and worked out the average.

3.3.2.3. Leaf Length (cm)

Length of leaf from the base of petiole to tip of largest leaf blade was measured and worked out the average.

3.3.2.4. Leaf Width (cm)

Length of leaf blade from left margin to right margin was measured and worked out average.

3.3.2.5. Number of Roots (at the time of planting out)

Total number of roots present in seedlings at deflasking time was counted and average was worked out.

3.3.2.6. Length of Root (cm) (at the time of planting out)

Length of root from the root emanating part to tip of all the roots was measured and average was calculated.

3.4 EXPERIMENT IV- MOLECULAR CHARACTERIZATION OF HYBRIDS

Seven hybrids evolved through distant hybridization were subjected to molecular characterization using ISSR markers.

3.4.1. Verification of F_1 Hybrids using ISSR Markers

3.4.1.1. DNA Isolation and Primer Selection

ISSR analysis was carried out in immature leaves of parents and three randomly selected F_1 hybrids of each cross. The DNA was extracted as per the procedure of Dellaporta *et al.* (1983). The concentration and purity of DNA were determined using an Eppendorf biophotometer (Eppendorf, Germany) at A260 and A280. Twenty-five ISSR primers homologous to microsatellite repeats and containing other selective anchor nucleotides developed at the University of British Columbia were used. The selected primers were homologous to microsatellite repeats (AC, AG, CA, GA, or TG) anchored at the 3'-end by 1 or 2 nucleotides. After amplification with these twenty-five primers, ten primers showed reproducible results. These ten primers were used for analysis.

3.4.1.2. ISSR Analysis

A total of 10 ISSR primers (UBC primers, University of British Columbia, Vancouver, Canada) were chosen for analyses of genetic diversity based on band reproducibility. The PCR reaction was carried out using a single primer at a time, in 25µL of reaction mixture. Each 25-µL PCR contained 50 ng genomic DNA template, 1X buffer [75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM (NH4)2SO4], 250 µM of each deoxyribonucleotide triphosphate, 3.5 mM $MgCl_2$, 0.4 µM of each ISSR primer (807, 809, 810, 811, 824, 834, 841,842, 849, and 857), and 1 U DNA Polymerase (Finzymes, Thermo USA). The PCRs were subjected to

amplifcation with an initial denaturation at 94°C for 5 min; 35 cycles of denaturing at 94°C for 1 min, annealing at primer specific temperature for 1 min, and extension at 72°C for 2 min, and a final extension at 72°C for 10 min in an Eppendorf Thermal cycler (Eppendorf, Germany). PCR products were electrophoresed on 1.4% agarose gel [stained with ethidium bromide] at 110 V for 65 min. Molecular weights of the DNA bands were estimated with the use of 1 kb Plus DNA Ladder (NEB, UK) as a standard.

3.4.1.3. Verification of F_1 Hybrids

To determine the similarities and differences between male and female parents and between F_1 hybrids and their respective female parents in each cross the pattern of DNA bands of the amplified primers were compared. The experiment was repeated twice and only the DNA bands with consistent results during repeated experiments were considered. The total number of scorable DNA bands for both male and female parents in each cross was documented. Two indices were used to estimate the efficiency of each marker for detecting DNA polymorphism.

I. The percentage of polymorphism between male and female parents of each hybrid, which was used to estimate the ability of each marker to differentiate between male and female parents, was calculated as follows:

$$\frac{\text{No. of polymorphic DNA bands between male and female parent}}{\text{Total number of scorable DNA band}} \times 100$$

II. Similarly, the percentage of polymorphism between the F_1 hybrid and the female parent, which reflected the efficiency of each marker to distinguish the F_1 hybrid from self-pollinated progeny, was calculated as follows:

$$\frac{\text{No. of polymorphic DNA bands between F1 hybrid and female parent}}{\text{Total number of scorable DNA band}} \times 100$$

In vitro grown seedlings were deflasked and washed well in tap water to remove media adhered to the seedlings and planted out in pots filled with potting mixture and kept it in the growth chamber for hardening (*In vitro* acclimatization) for three months. Later seedlings were transferred into large pots filled with potting mixture for the field establishment.

RESULTS

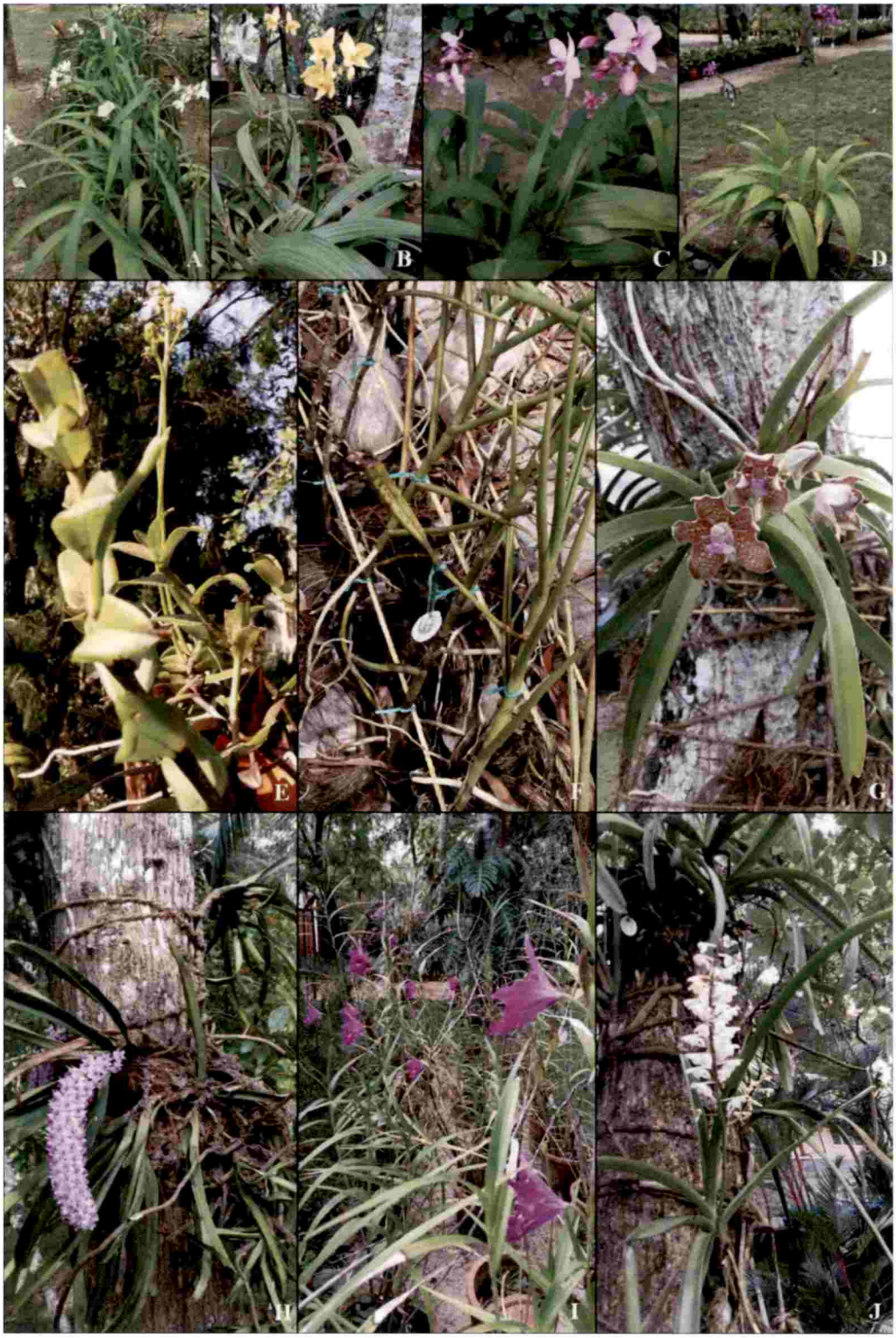

Plate 4. Field view of the experimental plants: (A) *Spathoglottis albida* (B) *Spathoglottis gracilis* (C) *Spathoglottis plicata* (D) *Spathoglottis unguiculata* (E) *Vanda spathulata* (F) *Vanda* sp. (G) *Vanda tessellata* (H) *Rhynchostylis retusa* (I) *Arundina graminifolia* (J) *Aerides ringens*

4. RESULTS

4.1. EXPERIMENT I – SELECTION OF PARENT MATERIALS AND HYBRIDIZATION TO EVALUATE CROSS COMPATIBILITY

4.1.1. Evaluation of Parents

Parents used in the cross compatibility studies were evaluated based on variability in plant and floral characters at the time of flowering and the results obtained were depicted below. The field view of the parents is shown in plate 4.

4.1.1.1. Mean Performance

Mean performance for different traits observed among the parents (Table 5) were as follows.

Plant height was the maximum in *Vanda spathulata* (145.54 cm) and minimum in *Aerides ringens* (26.26 cm) and recorded a significant range of variation among different parents.

Plant spread recorded significant range of variation among parents and was the maximum in *Spathoglottis* spp. (104.46 cm) and minimum in *Vanda spathulata* (14.3 cm).

Significant range of variation recorded for shoot girth among parents was from 2.8 cm to 5.68 cm and the maximum was recorded in *Rhynchostylis retusa* and the minimum in *Vanda spathulata.*

Number of leaves plant^{-1} exhibited significant variation and it ranged from 4.6 to 36.6 in *Spathoglottis* spp. and *Vanda spathulata* respectively.

Among parents, the maximum leaf length was noticed in *Spathoglottis* spp. and the minimum in *Vanda spathulata* and exhibited significant variation from 7.16 cm to 73.16 cm.

Significant variation was noticed for leaf width and it ranged from 1.44 cm to 5.04 cm in *Spathoglottis* spp. and *Vanda spathulata* respectively among parents.

Days taken for first flowering was more in *Vanda spathulata* and less in *Arundina graminifolia* and *Spathoglottis* spp. Significant variation was noticed among different parents, which ranged from 180 to 283 days.

Number of spikes produced plant^{-1} year^{-1} was high (8.6) in *Vanda tessellata* and low (1) in four parents namely *Vanda spathulata, Spathoglottis* spp., *Rhynchostylis retusa* and *Aerides ringens.*

Length of inflorescence was the maximum in *Spathoglottis* spp. (52.6 cm) and the minimum in *Vanda tessellata* (19.02 cm). Significant range of variation was observed among different parents.

Maximum number of flowers inflorescence^{-1} was produced by *Rhynchostylis retusa* (77.2) and minimum by *Vanda tessellata* (6). Significant variation was exhibited by different parents.

The highest internodal length was recorded in *Arundina graminifolia* and the lowest was noticed in *Spathoglottis* spp. Significant range of variation was recorded from 0.62 cm to 2.62 cm among different parents.

Significant variation was noticed for flower length and maximum was recorded in *Vanda tessellata* and minimum in *Aerides ringens.* Flower length varied from 2.28 to 5.54 cm. But the flower width was the maximum in *Vanda spathulata* (5.1cm) and minimum in *Aerides ringens* (1.58cm).

Days for inflorescence emergence to first flower opening showed significant variation (18.8 to 37.2 days) among parents and it was more in *Rhynchostylis retusa* and less in *Arundina graminifolia.*

Days to last flower opening from first flower opening was less in *Vanda tessellata* and more in *Arundina graminifolia* which ranged from 5 to 57.6 days respectively.

Maximum spike longevity was observed in *Aerides ringens* and minimum in *Vanda tessellata*. The values varied significantly from 30 to 103 days.

Table 5. Mean performance of parents

Traits	Vt	Vs	Rr	Ar	Ag	Ss	Vsp
Plant height (cm)	95.84	145.54	65.72	26.26	100.9	80.42	90.22
Plant spread (cm)	37.8	14.3	64.34	54.9	49	104.46	42.6
Shoot girth (cm)	3.4	2.8	5.68	4.58	3.08	3.7	4.2
Number of leaves per plant	29	36.6	17	13	31.4	4.6	26
Leaf length (cm)	22.76	7.16	62.64	31.92	28.82	73.16	26.5
Leaf width (cm)	2.4	1.44	2.78	2.12	1.7	5.04	1.6
Days taken for first flowering	180.6	283	230	247	180	180	240
Number of spikes/plant	8.6	1	1	1	1.2	1	3
Length of inflorescence (cm)	19.02	32.74	25.8	19.02	38.3	52.6	20
Number of flowers per inflorescence	6	20.2	77.2	40.2	15	46	7
Internodal length (cm)	2.02	1.08	0.84	1.04	2.62	0.62	1.5
Flower length (cm)	5.54	5.5	2.3	2.28	4.54	4.364	8.0
Flower width (cm)	5.04	5.1	2.0	1.58	4	4.7	8.3
Days for inflorescence emergence to first flower opening	22.6	25.4	37.2	27.4	18.8	25.8	23
Days to last flower opening from first flower opening	5	47	6.6	9.2	57.6	48.4	28
Spike longevity (days)	30	96.4	60.8	103	61	61	60

Vt - *Vanda tessellata*, Vs - *Vanda spathulata*, Rr- *Rhychostylis retusa*, Ar- *Aerides ringens*, Ag - *Arundina graminifolia*, Ss - *Spathoglotis* spp. , Vsp – *Vanda* sp.

4.1.1.2. Analysis of Variance (ANOVA)

Analysis of variance for different plant and floral biometrical characters was carried out as a basis to find out the test of significance and estimated different genetic components of variance.

The analysis of variance was carried out in Completely Randomised Design (CRD) with five replications. The results pertaining to it are presented below.

Analysis of variance revealed highly significant differences for all polygenic traits included in the evaluation of parents (Table 6). Therefore, proceeded for the estimation of different components of variance such as phenotypic (σ^2p), genotypic (σ^2g) and environmental (σ^2e) variance; phenotypic (PCV), genotypic (GCV) and environmental (ECV) coefficient of variation, broad sense

heritability (H), Co-heritability, genetic advance percent and association analysis namely phenotypic (*r*p), genotypic (*r*g) and environmental correlation (*r*e).

Table 6. ANOVA for biometrical traits among parents

Traits	df	MSE	F	CD
Plant height (cm)	24	511.108	15.4073 **	29.51
Plant spread (cm)	24	58.9693	76.6052 **	10.0242
Shoot girth (cm)	24	0.6176	9.3884**	1.0258
Number of leaves per plant	24	99.4	7.6383**	13.0146
Leaf length (cm)	24	72.6393	43.3313**	11.1256
Leaf width (cm)	24	0.1041	80.7321**	0.4211
Days taken for first flowering	24	1152.71	8.2306**	44.3199
Number of spikes/plant	24	2.25	21.1822**	0.4128
Length of inflorescence (cm)	24	72.0736	11.6074**	11.0822
Number of flowers per inflorescence	24	515.6	6.5633**	29.6412
Internodal length (cm)	24	0.0856	35.2692**	0.3819
Flower length (cm)	24	0.0287	264.7892**	0.2211
Flower width (cm)	24	0.0158	659.2379**	0.1640
Days for inflorescence emergence to first flower opening	24	5	38.192**	2.9189
Days to last flower opening from first flower opening	24	20.4333	146.2372**	5.9007
Spike longevity (days)	24	101	35.8659**	13.1189

4.1.1.2.1. Components of Variance

Phenotypic, genotypic and environmental variances were estimated and the results revealed that phenotypic variance was higher than the genotypic as well as environmental variance for all polygenic traits. Moreover genotypic variance was higher than the environmental variance (Table 7).

4.1.1.2.2. Coefficients of variation

Estimation of phenotypic, genotypic and environmental coefficients of variation from the components of variance was performed for all biometrical characters among different parents and the results obtained are presented as follows (Table 7).

Genotypic coefficient of variation (GCV) values were higher than environmental coefficient of variation (ECV) values but lower than phenotypic coefficient of variation (PCV) values for all traits. GCV was the highest (128.52)

for number of spikes plant $^{-1}$. Out of the sixteen traits, GCV was high for the following fifteen traits *viz*., plant height (45.13), plant spread (55.18) , shoot girth (26.04), number of leaves plant $^{-1}$ (52.49), leaf length (65.73), leaf width (48.84), length of inflorescence (39.35), number of flowers inflorescence^{-1} (70.13), internodal length (54.81), flower length (28.24), flower width (37.73), days for inflorescence emergence to first flower opening (23.29), days to last flower opening from first flower opening (84.10) and spike longevity (37.68). But GCV was moderate for days taken for first flowering (18.93).

ECV was lower than both PCV and GCV. ECV was the highest for number of flowers inflorescence^{-1} (28.83). But moderate for two traits *viz*., number of leaves plant^{-1} (16.51) and number of spikes plant^{-1} (15.41). ECV was low for the remaining thirteen traits *viz*., plant height (5.39), plant spread (1.67), shoot girth (7.91), leaf length (3.64), leaf width (1.42), days taken for first flowering (5.25), length of inflorescence (9.35), internodal length (3.86), flower length (0.3), flower width (0.18), days for inflorescence emergence to first flower opening (1.43), days to last flower opening from first flower opening (1.66) and spike longevity (1.85).

PCV was higher than both GCV and ECV for all traits. The highest and the lowest PCV were for number of spikes plant^{-1} (143.93) and days taken for first flowering (24.18) respectively. PCV was high for all traits *viz*., plant height (50.52), plant spread (56.85), shoot girth (33.95), number of leaves plant^{-1} (69.00), leaf length (69.37), leaf width (50.26), length of inflorescence (48.70), number of flowers inflorescence^{-1} (98.96), internodal length (58.67), flower length (28.54), flower width (37.91), days for inflorescence emergence to first flower opening (24.72), days to last flower opening from first flower opening (85.76) and spike longevity (39.53).

4.1.1.2.3. Heritability (Broad Sense)

Heritability was calculated from genotypic and phenotypic components of variance and the results are presented as follows (Table 7).

Heritability was the highest for flower width (99.06%) and the lowest for number of flowers inflorescence^{-1}(50.21%). It was moderate for three traits *viz.*, shoot girth (58.83%), number of leaves plant^{-1} (57.86%) and number of flowers inflorescence^{-1} (50.21%) and high for the remaining thirteen traits *viz.*, plant height (79.80%), plant spread (94.22%), leaf length (89.76%), leaf width (94.40%), days taken for first flowering (61.30%), number of spikes plant^{-1} (79.72%), length of inflorescence (65.29%), internodal length (87.29%), flower length (97.91%), days for inflorescence emergence to first flower opening (88.77%), days to last flower opening from first flower opening (96.15%) and spike longevity (90.84%).

4.1.1.2.4. Genetic Advance

Genetic advance was estimated as percentage of mean for various traits (Table 7). Results revealed that the highest value was for number of spikes plant^{-1} (239.80) and the lowest value was for days taken for first flowering (30.54). It was high for sixteen traits among parents *viz.*, plant height (83.06), plant spread (110.36), shoot girth (41.18), number of leaves plant^{-1} (82.26), leaf length (128.29), leaf width (98.64), length of inflorescence (65.50), number of flowers inflorescence^{-1} (102.77), internodal length (106.27), flower length (57.75), flower width (112.82), days for inflorescence emergence to first flower opening (45.21), days to last flower opening from first flower opening (170.44) and spike longevity (72.91).

Table 7. Components of variance

Traits	Genotypic variance	Environmental variance	Phenotypic variance	GCV	ECV	PCV	Heritability (%)	Genetic advance (%)
Plant height (cm)	1499.08	379.42	1878.50	45.13	5.39	50.52	79.80	83.06
Plant spread (cm)	892.52	54.75	947.27	55.18	1.67	56.85	94.22	110.36
Shoot girth (cm)	1.02	0.71	1.73	26.04	7.91	33.95	58.83	41.18
Number of leaves plant^{-1}	132.55	96.50	229.05	52.49	16.51	69.00	57.86	82.26
Leaf length (cm)	615.47	70.20	685.67	65.73	3.64	69.37	89.76	128.29
Leaf width (cm)	1.62	0.09	1.71	48.84	1.42	50.26	94.40	98.64
Days taken for first flowering	1684.86	1063.35	2748.20	18.93	5.25	24.18	61.30	30.54
Number of spikes plant^{-1}	8.99	2.29	11.28	128.52	15.41	143.93	79.72	239.80
Length of inflorescence (cm)	151.24	80.39	231.64	39.35	9.35	48.70	65.29	65.50
Number of flowers inflorescence^{-1}	576.42	571.44	1147.86	70.13	28.83	98.96	50.21	102.77
Internodal length (cm)	0.57	0.08	0.66	54.81	3.86	58.67	87.29	106.27
Flower length (cm)	1.48	0.03	1.51	28.24	0.30	28.54	97.91	57.75
Flower width (cm)	2.10	0.02	2.12	37.73	0.18	37.91	99.06	112.82
Days for inflorescence emergence to first flower opening	37.25	4.71	41.96	23.29	1.43	24.72	88.77	45.21
Days to last flower opening from first flower opening	597.56	23.92	621.48	84.10	1.66	85.76	96.15	170.44
Spike longevity (days)	650.90	65.56	716.46	37.68	1.85	39.53	90.84	72.91

4.1.1.2.5. Co-heritability

Co-heritability was estimated from the genotypic and phenotypic components of covariance and the results are presented in Table 8. Co-heritability was the highest (9.13) between number of leaves plant^{-1} and days taken for first flowering and closely followed by two related characters *viz.*, length of inflorescence and number of flowers inflorescence^{-1}(4.18) and days taken for first flowering and length of inflorescence (3.20). It was the lowest between plant height and days taken for first flowering (-11.69) followed by plant height and number of spikes plant^{-1} (0.73).

4.1.1.2.6. Association Analysis

Association analysis was carried out at three levels as phenotypic (*r*p), genotypic (*r*g) and environmental (*r*e) correlation coefficient. The results are presented below.

Genotypic correlation coefficient (*r*g) exhibited highly significant and positive association between five related characters *viz.*, plant spread and leaf length (0.9435), plant spread and leaf width (0.9303), plant height and flower length (0.9345), flower length and flower width (0.9545) and shoot girth and days for inflorescence emergence to first flower opening (0.9359) and highly significant and negative association between two related characters *viz.*, plant spread and number of leaves plant^{-1} (-0.9903) and number of leaves plant^{-1} and leaf length (-0.9506) (Table 9). Genotypic correlation coefficients of flower length with the remaining traits are depicted in figure 2.

Environmental correlation coefficient (*r*e) was positive and highly significant between seven related characters such as plant height and number of leaves plant^{-1} (0.5879), plant spread and leaf length (0.7112), shoot girth and leaf length (0.5660), days taken for first flowering and length of inflorescence (0.5159), flower length and flower width (0.5159), internodal length and days to last flower opening from first flower opening (0.6270) and leaf width and spike longevity (0.5668). But it was negative and highly significant for three other related characters *viz.*, plant height with days taken for first flowering (-0.5109),

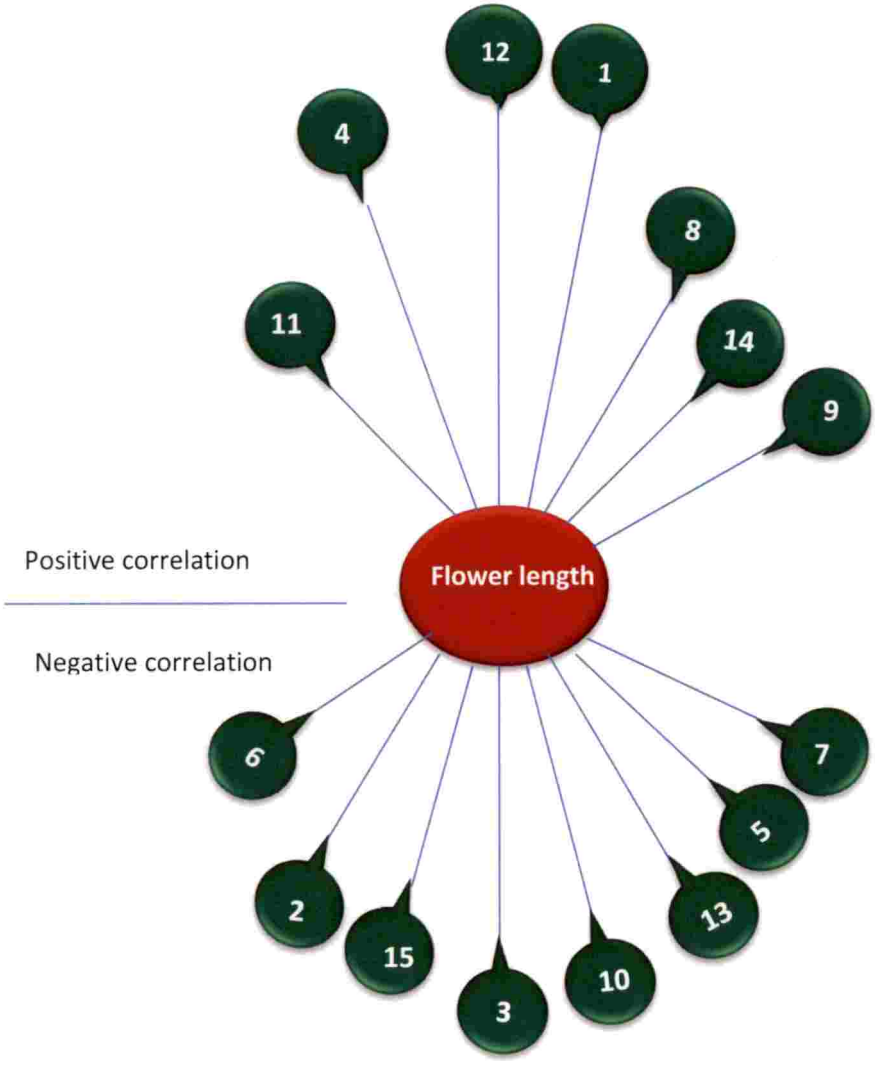

Figure 2. Diagrammatic representation of genotypic correlation of flower length with other traits: 1. Plant height (cm), 2. Plant spread (cm), 3. Shoot girth (cm), 4. Number of leaves plant^{-1}, 5. Leaf length (cm). 6. Leaf width (cm), 7. Days taken for first flowering, 8. Number of spikes/plant, 9. Length of inflorescence (cm), 10. Number of flowers per inflorescence, 11. Internodal length (cm). 12. Flower width (cm), 13. Days for inflorescence emergence to first flower opening, 14. Days to last flower opening from first flower opening, 15. Spike longevity (days).

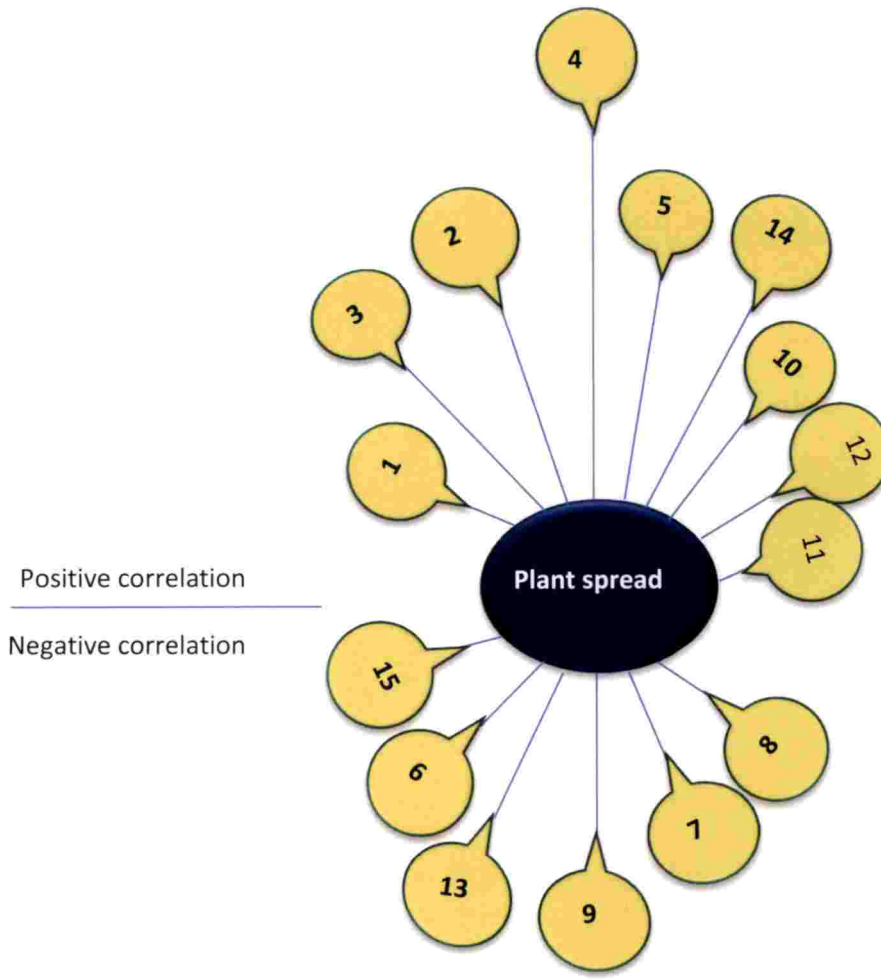

Figure 3. Diagrammatic representation of environmental correlation of plant spread with other traits: 1. Plant height (cm), 2. Shoot girth (cm), 3. Number of leaves plant^{-1}, 4. Leaf length (cm). 5. Leaf width (cm), 6. Days taken for first flowering, 7. Number of spikes/plant, 8. Length of inflorescence (cm), 9. Number of flowers per inflorescence, 10. Internodal length (cm). 11 Flower length (cm), 12. Flower width (cm), 13. Days for inflorescence emergence to first flower opening, 14. Days to last flower opening from first flower opening, 15. Spike longevity (days).

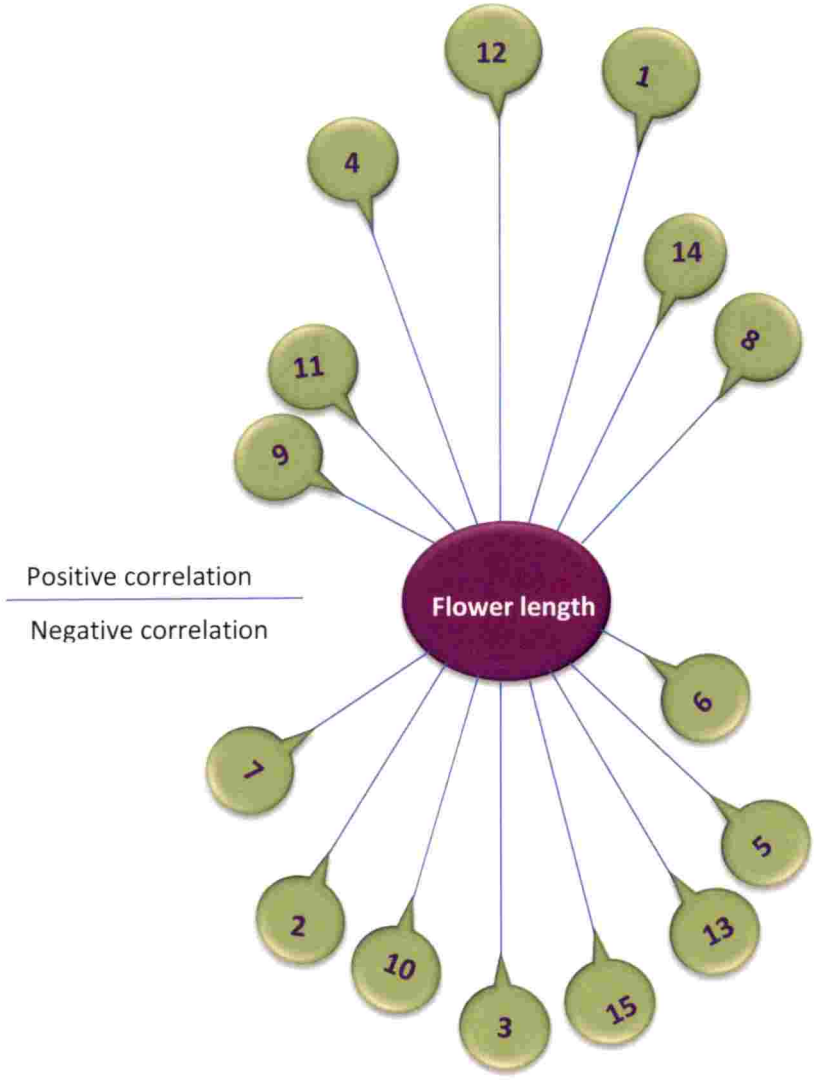

Figure 4. Diagrammatic representation of phenotypic correlation of flower length with other traits: 1. Plant height (cm), 2. Plant spread (cm), 3. Shoot girth (cm), 4. Number of leaves plant^{-1}, 5. Leaf length (cm). 6. Leaf width (cm), 7. Days taken for first flowering, 8. Number of spikes/plant, 9. Length of inflorescence (cm), 10. Number of flowers per inflorescence, 11. Internodal length (cm). 12. Flower width (cm), 13. Days for inflorescence emergence to first flower opening, 14. Days to last flower opening from first flower opening, 15. Spike longevity (days).

number of spikes plant^{-1} and flower width (-0.6075) and days to last flower opening from first flower opening and spike longevity (-0.5530) (Table 10). Environmental correlation coefficients of plant spread with the remaining traits are depicted in figure 3.

Estimated phenotypic correlation coefficient (*r*p) revealed highly significant and positive association between three related characters such as plant spread and leaf length (0.9224), plant spread and leaf width (0.9007) and flower length and flower width (0.9519). Highly significant and negative association was obtained between five other related characters such as plant spread and number of leaves plant^{-1} (- 0.6767), number of leaves plant^{-1} and leaf width (-0.6310), shoot girth and flower width (-0.6274), internodal length and days for inflorescence emergence to first flower opening (-0.6896) and number of spikes plant^{-1} and spike longevity (-0.6555) (Table 11). Phenotypic correlation coefficients of flower length with the remaining traits are depicted in figure 4.

4.1.1.3. Floral Characters (Qualitative Characters) of Parents

Observations related to floral characters which were not estimable quantitatively such as flower colour, nature of flowering, season of flowering, time of anthesis, and time of stigma receptivity among parents are presented below (Table 12).

4.1.1.4. Pollen Characters of Parents

Observations in connection with characters of pollen and pollinia of the parents used in the study are presented below (Table 13). Pollen production pollinium^{-1} was maximum in *Vanda spathulata* (43,75,000) closely followed by *Spathoglottis albida* (11,25,000) whereas minimum in *Rhynchostylis retusa* (2,62,500). Pollen fertility percentage was calculated and the results revealed that *Aerides ringens* produced more number of fertile pollens (93.81) as compared to all other parents. But the genus *Spathoglottis* produced less number of fertile pollen (45.44). High pollen germination percentage was recorded in two parents *viz.*, *Spathoglottis plicata* and *Aerides ringens* (90%) but it was only 24.85% in *Arundina graminifolia* (Plate 6 and Plate 7).

Table 8. Co-heritability

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1		1.0169	0.9113	0.7516	1.0549	1.0604	-11.6986	0.7301	1.6702	1.0760	0.9729	1.0387	1.0250	1.0308	1.0156	1.1008
2			0.8463	1.0806	0.9407	0.9741	0.9431	0.9094	1.0529	1.1477	1.0767	1.0029	1.0162	1.1049	0.8084	0.9826
3				0.9202	0.7953	0.8327	0.7461	0.9672	1.1522	1.3198	0.8814	0.9864	1.0085	0.9330	0.9477	0.8841
4					1.1433	1.0405	9.1314	0.9820	0.7764	1.0207	0.8370	1.0375	1.0288	0.8965	0.8639	1.7247
5						0.9829	0.8947	0.8945	1.1824	0.9805	1.0053	0.9935	1.0279	1.0120	1.1315	1.0068
6							0.8478	1.0005	1.0621	1.1209	0.9821	1.0459	0.9423	1.0142	1.1728	1.1670
7								1.2012	3.2081	0.8942	0.9483	1.0097	0.9738	1.0090	1.3100	1.1734
8									0.8698	0.9299	1.0672	1.0674	1.0724	1.0783	1.0283	0.9768
9										4.1825	1.2786	0.9345	0.9966	0.9861	1.0137	0.7078
10											0.9434	0.9718	1.0100	1.0480	0.9210	0.9541
11												1.0105	1.0200	0.9625	0.8147	0.9918
12													0.9875	0.9517	1.0023	1.0023
13														0.9815	1.0074	1.0091
14															0.9414	0.9005
15																1.3078
16																

1 Plant height (cm)
2 Plant spread (cm)
3 Shoot girth (cm)
4 Number of leaves plant^{-1}
5 Leaf length (cm)
6 Leaf width (cm)
7 Days taken for first flowering
8 Number of spikes plant^{-1}
9 Length of inflorescence (cm)
10 Number of flowers inflorescence^{-1}
11 Internodal length (cm)
12 Flower length (cm)
13 Flower width (cm)
14 Days for inflorescence emergence to first flower opening
15 Days to last flower opening from first flower opening
16 Spike longevity (days)

Table 9. Genotypic correlation matrix

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1		-0.5451**	-0.7950**	0.7635**	-0.5167**	-0.2915**	0.1881**	0.1323*	0.3632**	-0.5913**	0.2965**	**0.9345****	0.8768**	-0.4136**	0.5908**	-0.2167**
2			0.4151**	**-0.9903****	**0.9435****	**0.9303****	-0.5722**	-0.2761**	0.6124**	0.6188**	-0.4207**	-0.4011**	-0.1962**	0.2479**	0.0760	-0.1659**
3				-0.6571**	0.6211**	0.2257**	0.1214*	-0.2570**	-0.3907**	0.0843**	-0.5402**	-0.7543**	-0.8289**	**0.9359****	-0.7106**	0.0941
4					**-0.9506****	-0.8883**	0.3448**	0.3318**	-0.3095**	-0.7705**	0.6650**	0.6978**	0.4837**	-0.4598**	0.2107**	-0.1204*
5						0.8779**	-0.4421**	-0.3084**	0.5184**	0.8346**	-0.5210**	-0.3872**	-0.2559**	0.5258**	-0.0608	-0.2164**
6							-0.5308**	-0.0970	0.6773**	0.4705**	-0.4953**	-0.1049	0.1209*	0.1990**	0.1090	-0.3061**
7								-0.4867**	-0.4341**	0.2231**	-0.5048**	-0.2005**	-0.2794**	0.4365**	-0.1300*	0.8532**
8									-0.4744**	-0.6082**	0.4785**	0.5227**	0.4410**	-0.3361**	-0.4704**	-0.7524**
9										0.1026	-0.1626**	0.2370**	0.4549**	-0.2457**	0.8760**	-0.0527
10											-0.7917**	-0.6527**	-0.6527**	0.0180**	-0.3310**	0.2278**
11												0.3955**	0.2745**	-0.7540**	0.2104**	-0.4369**
12													**0.9545****	-0.4513**	0.3671**	-0.5766**
13														-0.5256**	0.4927**	-0.5209**
14															-0.5531**	0.1712**
15																0.1492*
16																

** Significant at 1% level

1 Plant height (cm)
2 Plant spread (cm)
3 Shoot girth (cm)
4 Number of leaves plant^{-1}
5 Leaf length (cm)
6 Leaf width (cm)
7 Days taken for first flowering
8 Number of spikes plant^{-1}
9 Length of inflorescence (cm)
10 Number of flowers inflorescence^{-1}
11 Internodal length (cm)
12 Flower length (cm)
13 Flower width (cm)
14 Days for inflorescence emergence to first flower opening
15 Days to last flower opening from first flower opening
16 Spike longevity (days)

Table 10. Environmental correlation matrix

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1		0.0727	-0.1837**	**0.5879****	0.1583**	0.1356*	**-0.5109****	0.1928**	-0.3973**	0.0833	0.0430	**-0.4744****	**-0.4367****	0.0691	-0.0901	0.1243*
2			0.3638**	0.3497**	**0.7112****	**0.4097****	-0.1755**	-0.2202**	-0.1702**	-0.3228**	0.3172**	0.0318	0.1296*	-0.2672**	0.3637**	-0.0372
3				-0.0798	**0.5660****	0.2225**	0.0621	-0.0206	0.0846	-0.3154**	-0.2277**	-0.0850	0.0861	0.2260**	-0.2342**	0.0464
4					**0.4135****	0.1664**	**-0.4530****	0.0141	-0.1433*	0.0184	0.3978**	-0.2026**	-0.1632**	-0.1749**	0.1944**	0.1869**
5						0.1853**	-0.1939**	-0.2137**	-0.3247**	0.0494	0.0213	-0.0517	0.2114**	-0.0518	0.1046	0.0136
6							**-0.4927****	0.0003	-0.2229**	-0.2093**	-0.0973	0.1295*	0.3127**	-0.0322	-0.3298**	**0.5668****
7								0.2035**	**0.5159****	0.0333	-0.0907	0.0165	-0.0974	-0.0138	0.1936**	-0.5000**
8									-0.1930**	-0.0912	-0.1565**	**-0.4486****	**-0.6075****	0.1361*	0.1282*	-0.1116
9										-0.1076	0.1274*	0.1560**	0.0218	-0.0133	-0.0813	-0.0940
10											-0.1251*	-0.1304*	0.0668	-0.1316*	-0.1425*	0.0347
11												-0.0740	-0.1454*	-0.2165**	**0.6270****	-0.0299
12													**0.8529****	**-0.4412****	-0.0291	0.0284
13														-0.2871**	-0.1856**	0.1530**
14															**-0.4836****	0.1676**
15																**-0.5530****
16																

** Significant at 1% level

1 Plant height (cm)
2 Plant spread (cm)
3 Shoot girth (cm)
4 Number of leaves plant^{-1}
5 Leaf length (cm)
6 Leaf width (cm)
7 Days taken for first flowering
8 Number of spikes plant^{-1}
9 Length of inflorescence (cm)
10 Number of flowers inflorescence^{-1}
11 Internodal length (cm)
12 Flower length (cm)
13 Flower width (cm)
14 Days for inflorescence emergence to first flower opening
15 Days to last flower opening from first flower opening
16 Spike longevity (days)

Table 11. Phenotypic Correlation matrix

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1		-0.4648**	-0.5977**	0.6903**	-0.4145**	-0.2386**	-0.0112	0.1445*	0.1569**	-0.3479**	0.2544**	0.7952**	0.7606**	-0.3377**	0.5095**	-0.1676**
2			0.3651**	**-0.6767****	**0.9224****	**0.9007****	-0.4611**	-0.2632**	0.4562**	0.3709**	-0.3544**	-0.3841**	-0.1865**	0.2051**	0.0895	-0.1562**
3				-0.4166**	0.5676**	0.2020**	0.0977	-0.1820**	-0.2101**	0.4465**	-0.4392**	-0.5804**	**-0.6274****	0.7250**	-0.5639**	0.0778
4					-0.5992**	**-0.6310****	0.0224	0.2295**	-0.2450**	-0.4069**	0.5647**	0.5062**	0.3560**	-0.3676**	0.1819**	-0.0506
5						0.8221**	-0.3666**	-0.2917**	0.3356**	0.5715**	-0.4588**	-0.3654**	-0.2348**	0.4638**	-0.0499	-0.1941**
6							-0.4763**	-0.0841	0.5007**	0.2890**	-0.4579**	-0.0964	0.1241*	0.1796**	0.0886	-0.2429**
7								-0.2833**	-0.0856	0.1384*	-0.3894**	-0.1539*	-0.2236**	0.3191**	-0.0762	0.5426**
8									-0.3935**	-0.4138**	0.3741**	0.4326**	0.3655**	-0.2623**	-0.4005**	**-0.6555****
9										0.0140	-0.0960	0.2028**	0.3671**	-0.1897**	0.6847**	-0.0573
10											-0.5556**	-0.4710**	-0.4558**	0.6486**	-0.2498**	0.1612**
11												0.3618**	0.2502**	**-0.6896****	0.2366**	-0.3923**
12													**0.9519****	-0.4421**	0.3554**	-0.5426**
13														-0.5022**	0.4774**	-0.4897**
14															-0.5428**	0.1707**
15																0.1066
16																

** Significant at 1% level

1 Plant height (cm)
2 Plant spread (cm)
3 Shoot girth (cm)
4 Number of leaves plant^{-1}
5 Leaf length (cm)
6 Leaf width (cm)
7 Days taken for first flowering
8 Number of spikes plant^{-1}
9 Length of inflorescence (cm)
10 Number of flowers inflorescence^{-1}
11 Internodal length (cm)
12 Flower length (cm)
13 Flower width (cm)
14 Days for inflorescence emergence to first flower opening
15 Days to last flower opening from first flower opening
16 Spike longevity (days)

Table 12. Floral characters of parents

Sl No	Parents	Flower colour	Nature of flowering	Season of flowering	Time of anthesis	Time of stigma receptivity
1	*Vanda tessellata*	Green pedicel, greyish green calyx and corolla tessellated with blue, labellum white speckled with violet tinge inside and mid lobe lilac-blue (Plate 5G)	Free	January-December	6am-6pm	6 days
2	*Vanda spathulata*	Green pedicel, creamy–yellow calyx and corolla, labellum yellow with violet tinge (Plate 5E)	Seasonal	April-December	5-6 am	6 days
1.a	*Vanda* sp.	Cream pedicel, purple calyx and corolla, magenta labellum with red and yellow shades. (Plate 5F)	Free	January-December	5-8 am	3 days
3	*Rhynchostylis retusa*	Purple pedicel, calyx and corolla white with a faint pinkish tinge, labellum deep magenta colour (Plate 5J)	Seasonal	June-August	6 am- 7am	5 days
4	*Aerides ringens*	White pedicel with pink base, calyx and corolla white with pink tinge, labellum white. (Plate 5H)	Seasonal	May-September	6am-10 am	5 days
5	*Arundina graminifolia*	Green pedicel, deep magenta calyx and corolla, labellum deep purple in the throat, lighter towards margin with a triangular white mark in between and the outer surface of the basal half of labellum white (Plate 5I)	Free	January-December-	6am-8am	1 day

(Conti...)

Table 12. Floral characters of parents

Sl No	Parents	Flower colour	Nature of flowering	Season of flowering	Time of anthesis	Time of stigma receptivity
6	*Spathoglottis* spp.					
a	*Spathoglottis albida*	Green pedicel, white calyx , corolla and labellum (Plate 5B)	Free	January-December-	5.30 am - 6 am	6 days
b	*Spathoglottis unguiculata*	Deep violet pedicel, deep purple calyx , corolla and labellum (Plate 5D)	Free	January-December-	6 am-9 am	3 days
c	*Spathoglottis plicata*	Light purple tinged green pedicel, light purple calyx and corolla, base of the labellum white tinge and deep purple and tip of the labellum light purple (Plate 5A)	Free	January-December-	6 am-10 am	4 days
d	*Spathoglottis gracilis*	Greenish brown pedicel, yellow calyx and corolla, labellum yellow with brown spots at the base of the labellum (Plate 5C)	Free	January-December-	6 am-9 am	4days

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Plate 5. Flower colour of the parents: (A) *Spathoglottis plicata* (B) *Spathoglottis albida* (C) *Spathoglottis gracilis* (D) *Spathoglottis unguiculata* (E) *Vanda spathulata* (F) *Vanda* sp. (G) *Vanda tessellata* (H) *Aerides ringens* (I) *Arundina graminifolia* (J) *Rhynchostylis retusa*

Table 13. Pollen characters of the selected parents for hybridisation

Sl No	Parent	Pollen morphology	Pollen production pollinium^{-1}	Pollen fertility %	Pollen germination %
1	*Vanda tessellata*	Ovate to circular, smooth, granular, exine thick and intine thin and inconspicuous, mostly tetrad form.	9,00,000	91.35	78.10
2	*Vanda spathulata*	Ovate to triangular, smooth, granular, exine thick and intine thin and inconspicuous, mostly triad and tetrad form.	43,75,000	73.75	82.83
1. a	*Vanda* sp.	Ovate to circular, smooth, granular, exine thin and inconspicuous and intine thick, mostly tetrad form, diad also present.	8,88,500	92.70	93.70
3	*Rhynchostylis retusa*	Ovate to triangular, smooth, granular, exine thin and inconspicuous and intine thick, mostly tetrad form, triad also present.	2,62,500	86.67	75.53
4	*Aerides ringens*	Ovate to triangular, smooth, granular, exine thick and intine thin, mostly tetrad form, triad also present.	3,00,000	93.81	90.00
5	*Arundina graminifolia*	Ovate to triangular, smooth, granular, exine thick and intine thin and conspicuous, mostly tetrad and triad form.	7,12,500	57.14	24.85
6	*Spathoglottis* spp.				
a	*Spathoglottis albida*	Ovate to circular, smooth, granular, exine thick and intine thin and inconspicuous, mostly tetrad form, diad and triad also present.	11,25,000	45.44	39.44
b	*Spathoglottis unguiculata*	Ovate to circular, smooth, granular, exine thick and intine thin and inconspicuous, mostly tetrad form, diad and triad also present.	4,37,500	86.89	60.13
c	*Spathoglottis plicata*	Ovate to circular, smooth, granular, exine thick and intine thin and inconspicuous, mostly tetrad form, diad and triad also present.	5,00,000	72.49	90.00
d	*Spathoglottis gracilis*	Ovate to circular, smooth, granular, both exine and intine thin and inconspicuous, mostly tetrad form, diad and triad also present.	3,12,500	90.46	83.33

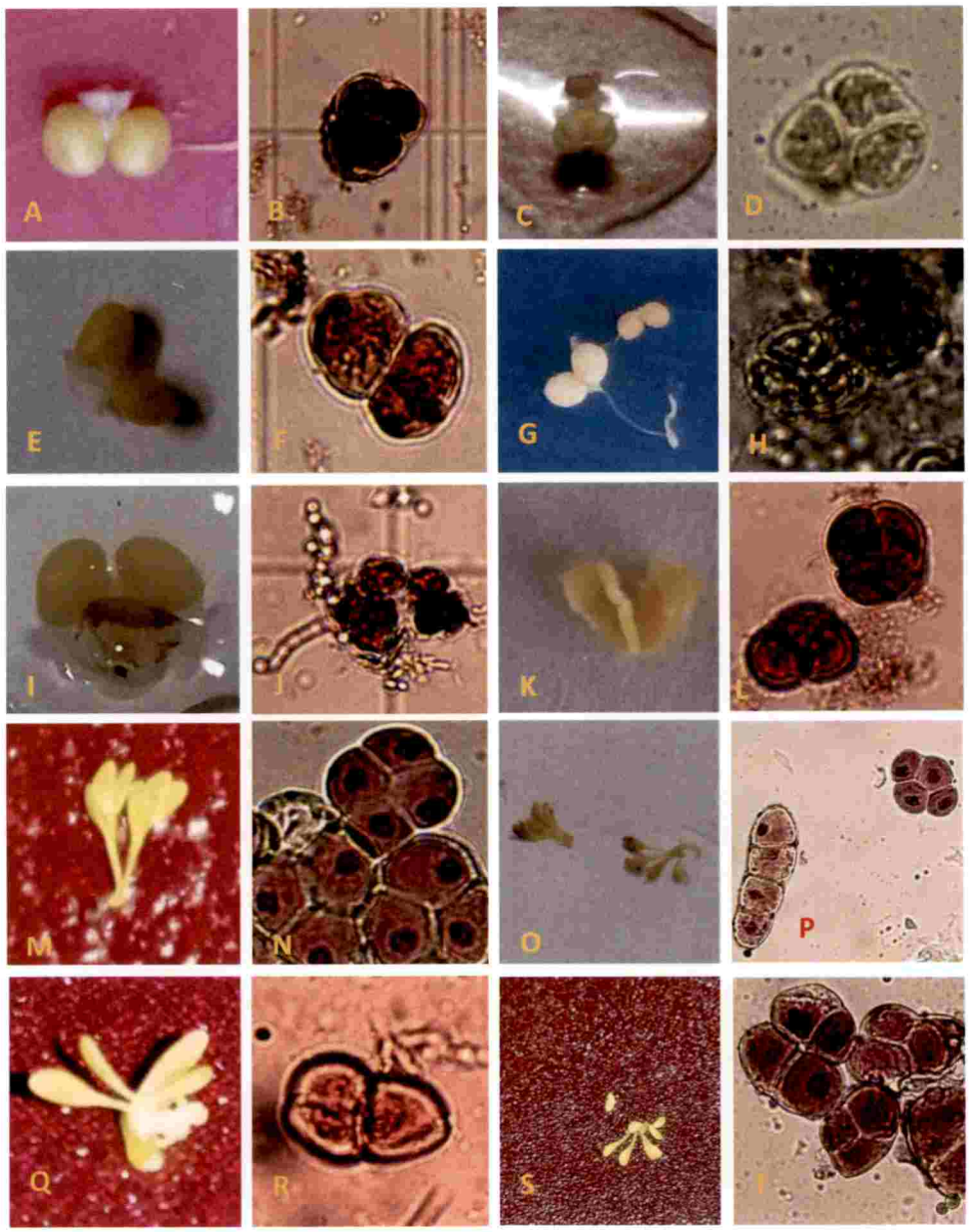


Plate 6. Pollinia and pollen grains of selected parents : (A & B) *Vanda tessellata* (C & D) *Vanda spathulata* (E & F) *Vanda* sp. (G & H) *Rhynchostylis retusa* (I & J) *Aerides ringens* (K & L) *Arundina graminifolia* (M & N) *Spathoglottis albida* (O & P) *Spathoglottis unguiculata* (Q & R) *Spathoglottis plicata* (S & T) *Spathoglottis gracilis*

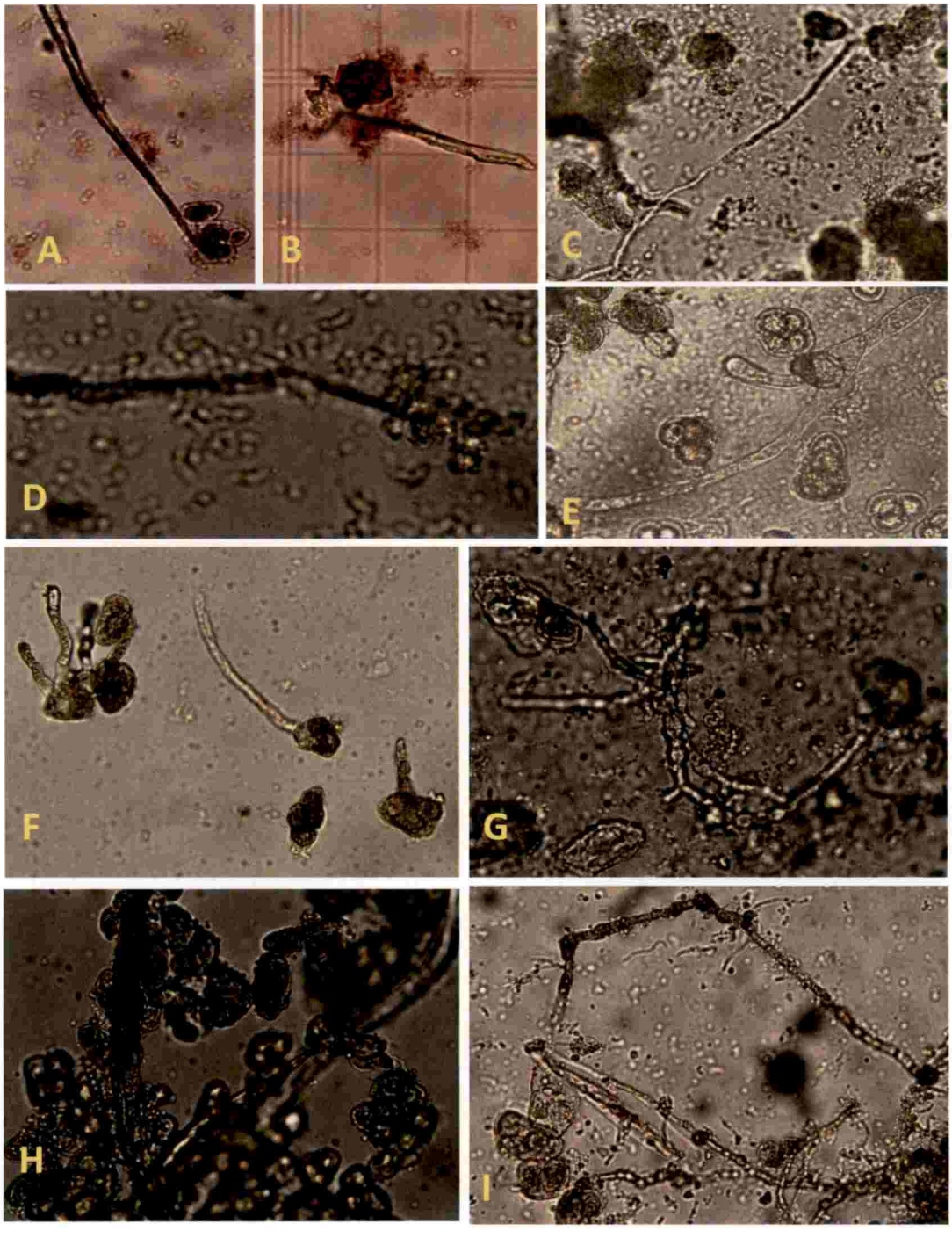


Plate 7. Pollen germination of the parents: (A) *Vanda tessellata* (B) *Vanda spathulata* (C) *Rhynchostylis retusa* (D) *Aerides ringens* (E) *Arundina graminifolia* (F) *Spathoglottis albida* (G) *Spathoglottis unguiculata* (H) *Spathoglottis plicata* (I) *Spathoglottis gracilis*

4.1.1.5. Pest/Disease Incidence

No serious pests and disease incidence were noticed in the plants throughout the course of the experiment.

4.1.2. Hybridization

Parents belonging to the genera *Vanda*, *Rhynchostylis*, *Spathoglottis*, *Aerides* and *Arundina* were selected for hybridization based on evaluation of variability parameters in floral characteristics. Hybridization was carried out in seventy seven combinations.

4.2. EXPERIMENT II- CROSS COMPATIBILITY STUDIES

Results of the cross compatibility revealed that thirty one combinations successfully produced mature capsules whereas in three combinations *viz.*, *Vanda tessellata* x *Vanda spathulata*, *Spathoglottis albida* x *Vanda* sp. and *Spathoglottis plicata* x *Vanda* sp. capsules fell off after 20, 10 and 9 days of maturity respectively (Table 14). Out of these successful combinations, capsules of *Spathoglottis crosses* attained setting four days after pollination whereas capsules of *Rhynchostylis* crosses attained setting ten days after pollination. Similarly capsules attained maturity much earlier (30 days) in all cross combinations when the genus *Spathoglottis* was used as the female parent. But in cross combinations where the species *Vanda spathulata* was used as the female parent more days were required for capsule maturation (150-154 days). Length of capsule was the maximum in the cross of *V. spathulata* x *R. retusa* (8 cm) and the minimum in the combination involved *A. ringens* as female parent (1.7 cm). Width of capsule was the maximum in the cross of *V. spathulata* x *Vanda* sp. (4.1 cm) and the minimum in the combination involved *A. ringens* as female parent (1.4 cm). Percentage of filled seeds was the maximum (100%) in three crosses *viz.*, *V. spathulata* x *R. retusa*, *S. albida* x *S. unguiculata* and *S. albida* x *S. plicata* and the minimum (22.41%) in the cross *A. graminifolia* x *S. unguiculata.* The cross compatibility between parents (plate 8-16) and the plants with pod settings are shown in plate 8-16 and plate 17 respectively.

Table 14.Capsule characteristics of crosses

Sl No	Cross	Days taken for pod setting	Days to green capsule harvest	Length of capsule (cm)	Width of capsule (cm)	Percentage of filled seeds
1	*Vanda tessellata* x *Vanda spathulata*	5.00	20.33	5.77	2.53	0..00
2	*Vanda spathulata* x *Vanda tessellata*	5.00	118.07	5.75	2.71	94.24
3	*Vanda spathulata* x *Aerides ringens*	5.00	151.00	5.00	3.10	95.00
4	*Vanda spathulata* x *Rhynchostylis retusa*	5.00	154.00	8.50	4.00	100.00
5	*Vanda spathulata* x *Vanda* sp.	5.00	108.00	7.10	4.10	80.00
6	*Vanda* sp. x *Vanda tessellata*	7.00				
7	*Rhynchostylis retusa* x *Vanda tessellata*	10.00	120.00	2.73	1.68	95.00
8	*Rhynchostylis retusa* x *Aerides ringens*	10.29	119.00	2.74	1.48	95.00
9	*Aerides ringens* x *Vanda tessellata*	5.00	115.33	2.65	2.07	90.28
10	*Aerides ringens* x *Rhynchostylis retusa*	8.50	120.00	3.25	2.50	31.56
11	*Aerides ringens* x *Vanda* sp.	9.00	73.00	1.70	1.40	82.31
12	*Arundina graminifolia* x *Spathoglottis albida*	5.00	46.09	5.91	3.20	57.45
13	*Arundina graminifolia* x *Spathoglottis unguiculata*	5.00	50.67	5.63	3.17	22.41
14	*Arundina graminifolia* x *Spathoglottis plicata*	4.00	46.00	6.60	2.60	52.22
15	*Arundina graminifolia* x *Vanda tessellata*	4.00	44.00	7.00	3.70	27.00
16	*Spathoglottis albida* x *Spathoglottis unguiculata*	4.00	31.88	6.61	3.14	100.00
17	*Spathoglottis albida* x*Spathoglottis plicata*	4.00	31.00	5.25	3.60	100.00
18	*Spathoglottis albida* x *Spathoglottis gracilis*	4.00	35.33	6.30	3.03	73.33
19	*Spathoglottis albida* x *Arundina graminifolia*	4.00	30.46	5.96	2.84	62.49
20	*Spathoglottis albida* x *Vanda* sp.	4.00	10.00	4.60	0.50	0.00
21	*Spathoglottis unguiculata* x *Spathoglottis albida*	4.00	29.00	6.50	3.75	83.33
22	*Spathoglottis unguiculata* x *Spathoglottis plicata*	4.00	32.50	6.40	3.80	83.33
23	*Spathoglottis unguiculata* x *Spathoglottis gracilis*	4.00	29.00	5.60	2.50	56.43
24	*Spathoglottis unguiculata* x *Arundina graminifolia*	4.00	28.22	5.81	2.50	38.40

(Conti...)

Table 14.Capsule characteristics of crosses

Sl No	Cross	Days taken for pod setting	Days to green capsule harvest	Length of capsule (cm)	Width of capsule (cm)	Percentage of filled seeds
25	*Spathoglottis unguiculata* x *Vanda* sp.	4.00	22.67	5.77	3.07	100.00
26	*Spathoglottis plicata* x *Spathoglottis albida*	4.00	28.50	5.60	3.40	96.30
27	*Spathoglottis plicata* x *Spathoglottis unguiculata*	4.00	30.00	5.50	3.40	89.58
28	*Spathoglottis plicata* x *Spathoglottis gracilis*	4.00	29.00	4.50	1.70	73.33
29	*Spathoglottis plicata* x *Arundina graminifolia*	4.00	30.00	5.00	2.70	26.00
30	*Spathoglottis plicata* x *Vanda* sp.	4.00	9.00	4.60	1.20	0
31	*Spathoglottis gracilis* x *Spathoglottis albida*	7.00	36.00	4.50	2.30	27.00
32	*Spathoglottis gracilis* x *Spathoglottis unguiculata*	7.00	-	-	-	-
33	*Spathoglottisgracilis* x *Spathoglottis plicata*	7.00	31.00	5.00	2.00	68.06
34	*Spathoglottis gracilis* x *Arundina graminifolia*	8.75	28.00	4.62	2.34	32.31

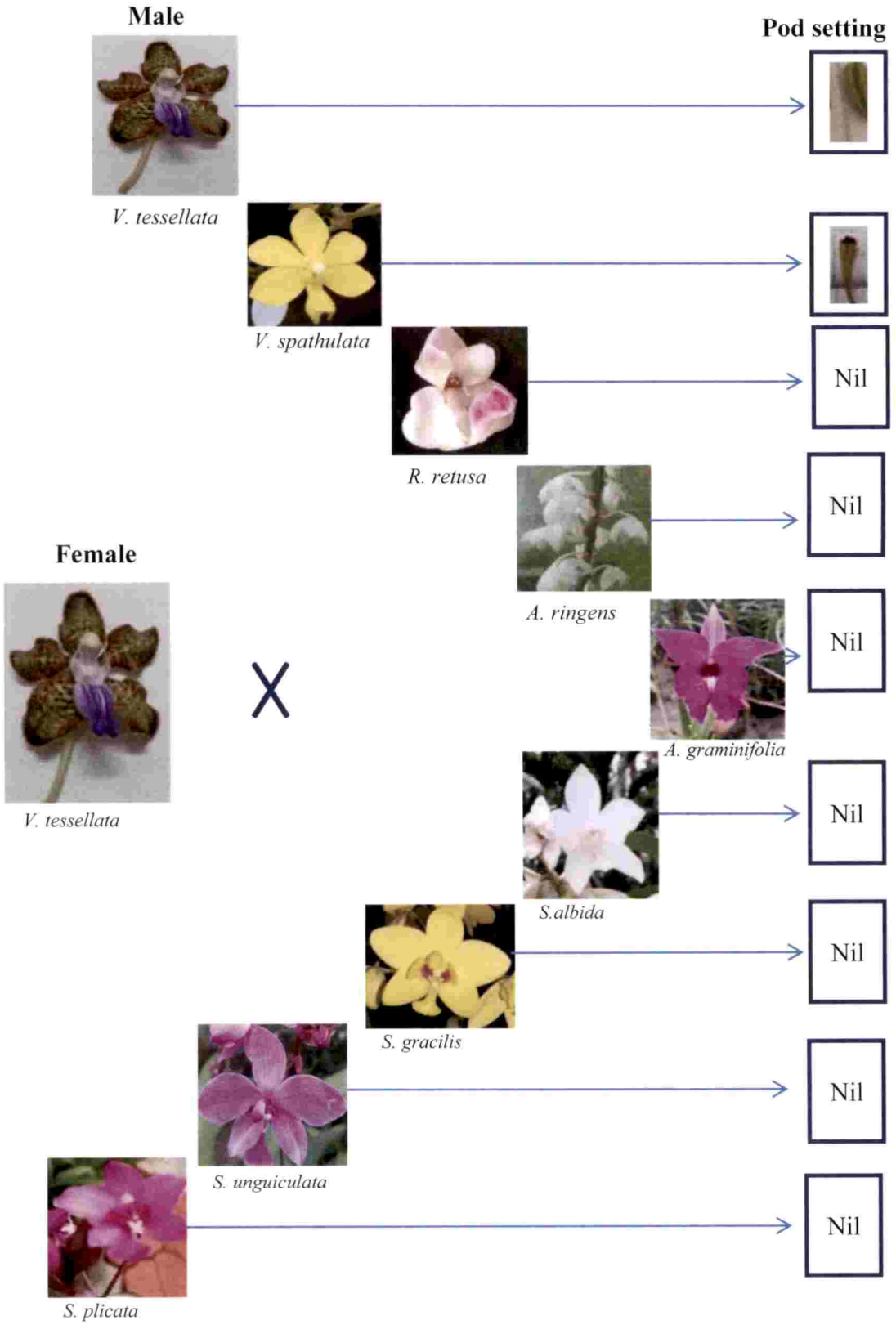


Plate 8. Cross compatibility: *Vanda tessellata* (♀) with other parents (♂)

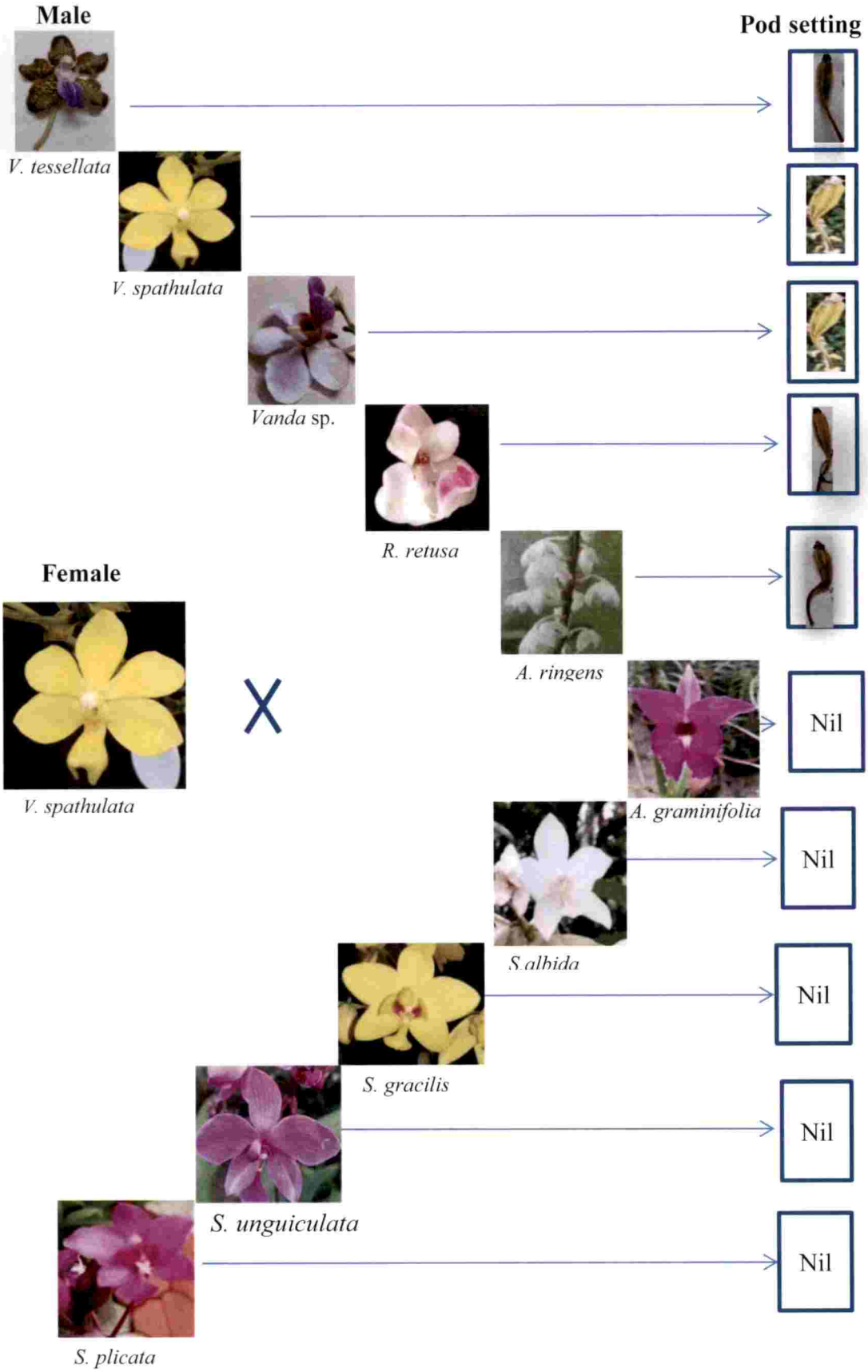


Plate 9. Cross compatibility: *Vanda spathulata* (♀) with other parents (♂)

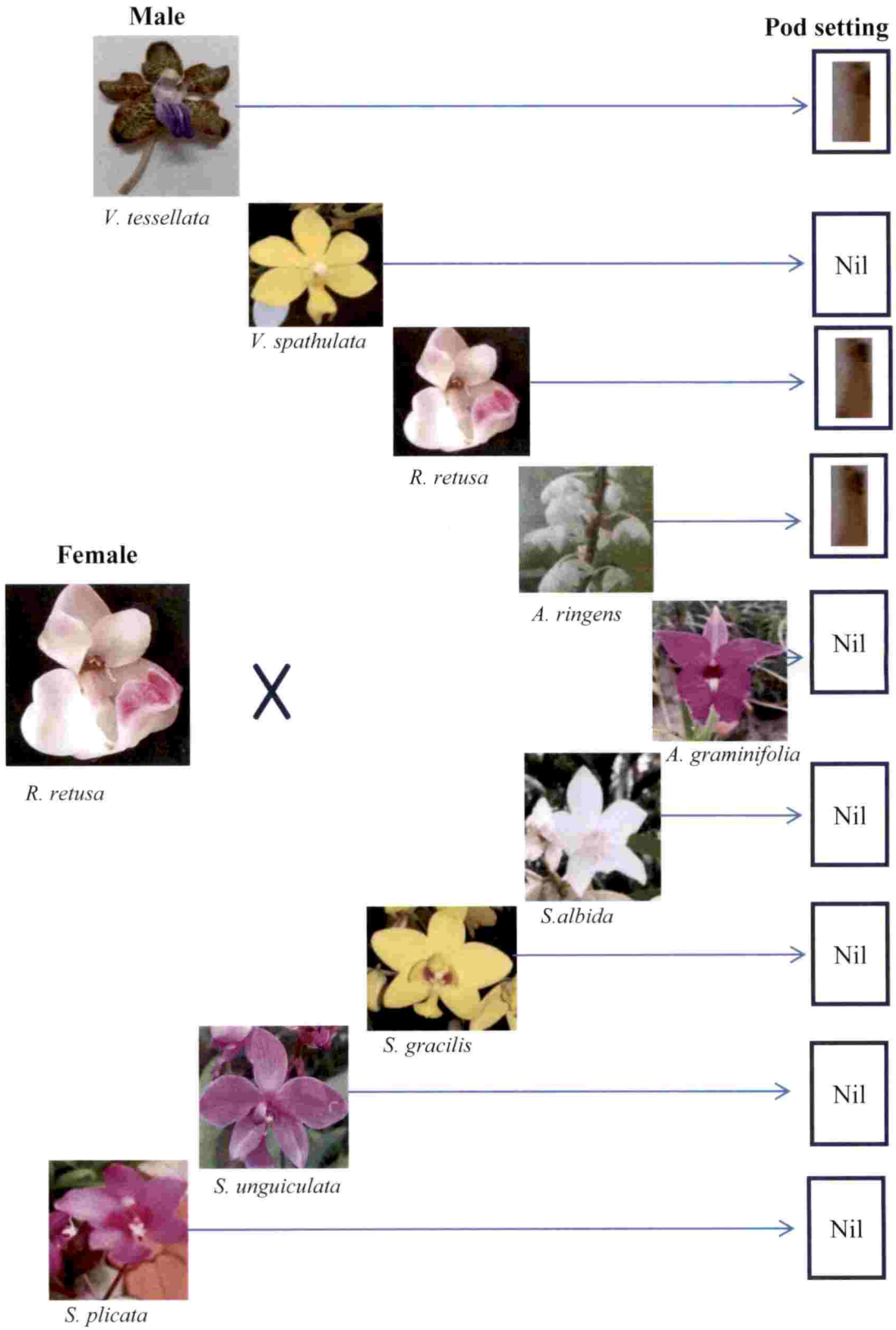


Plate 10. Cross compatibility: *Rhynchostylis retusa* (♀) with other parents (♂)

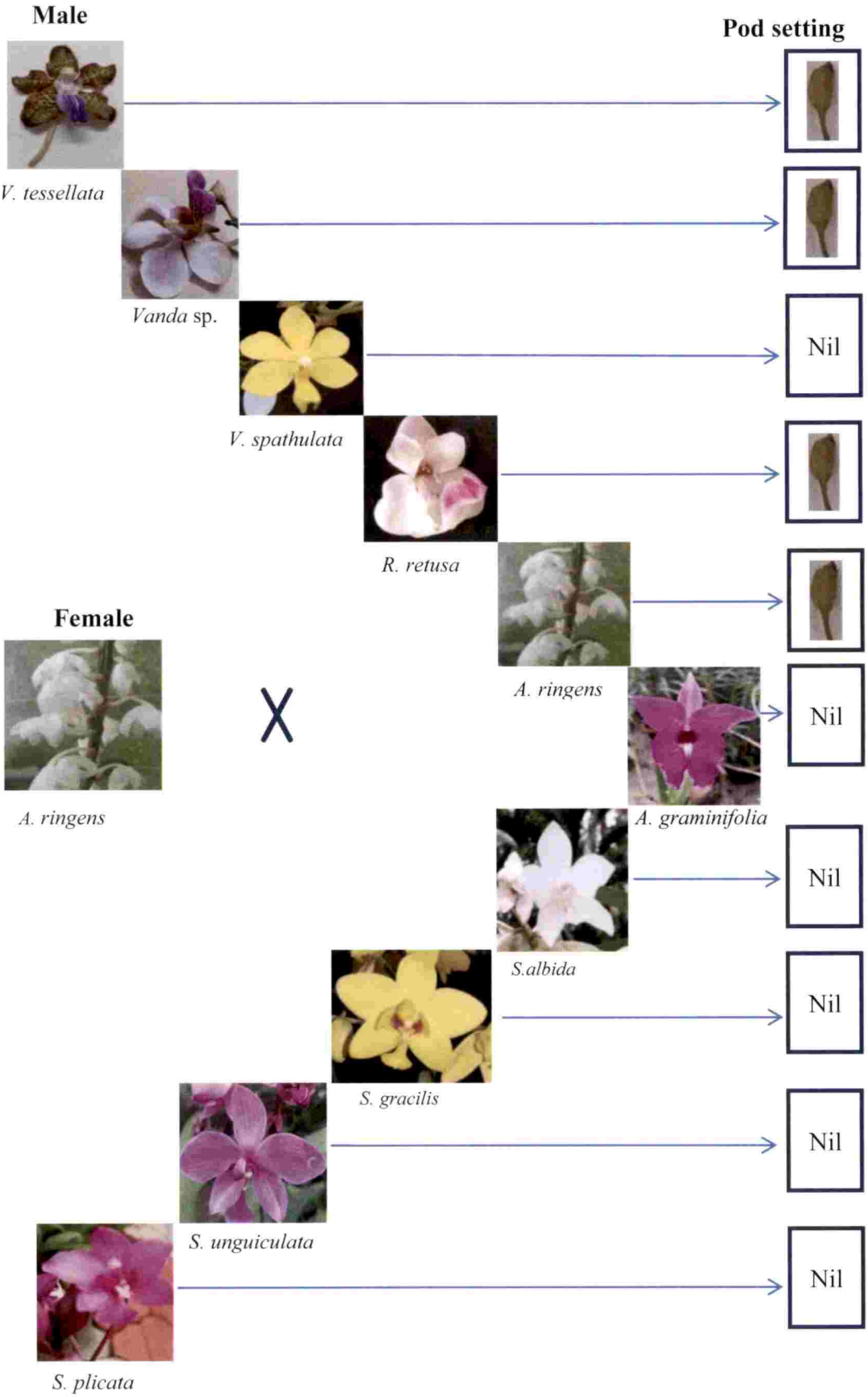


Plate 11. Cross compatibility: *Aerides ringens* (♀) with other parents (♂)

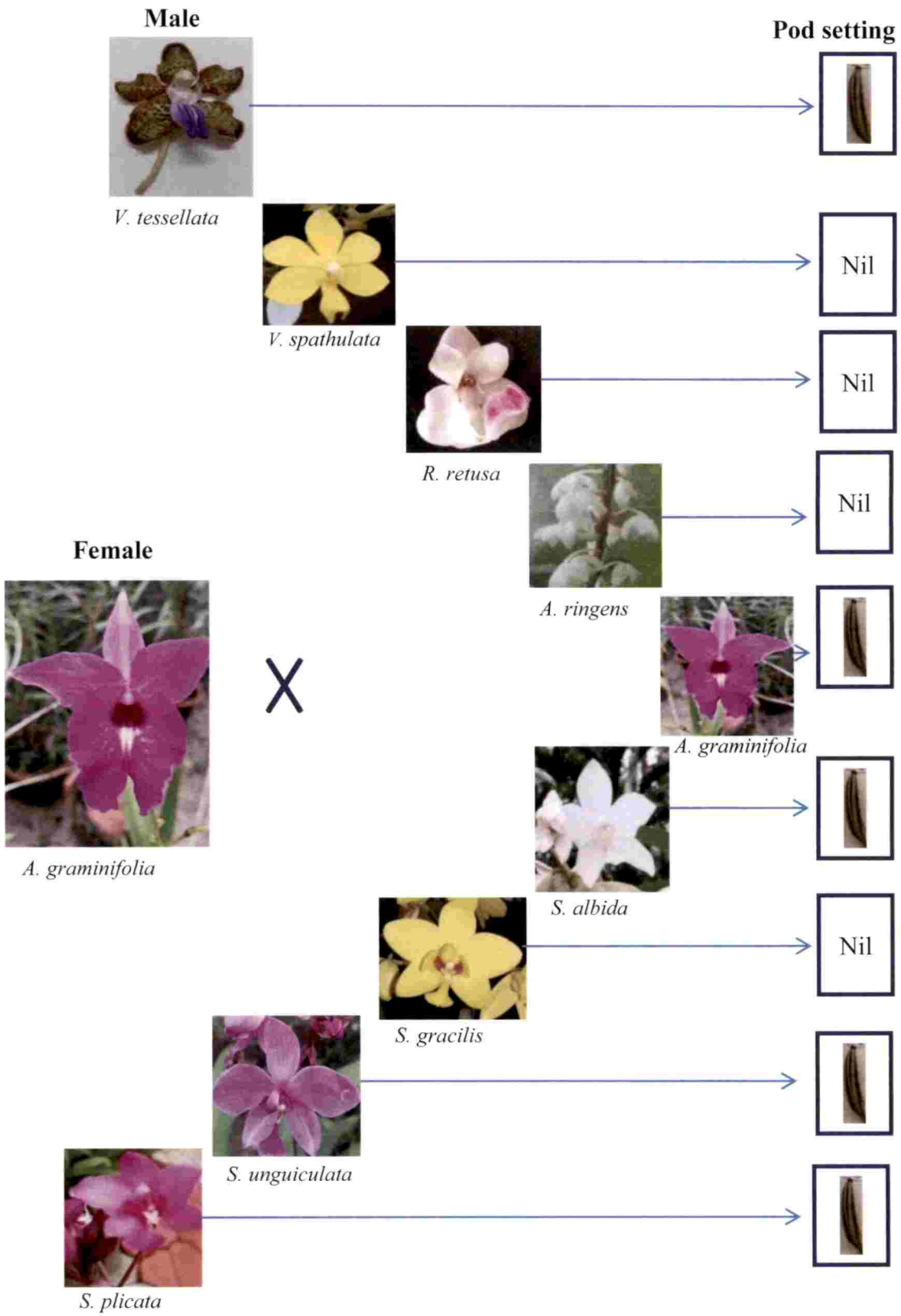


Plate 12. Cross compatibility: *Arundina graminifolia* (♀) with other parents (♂)

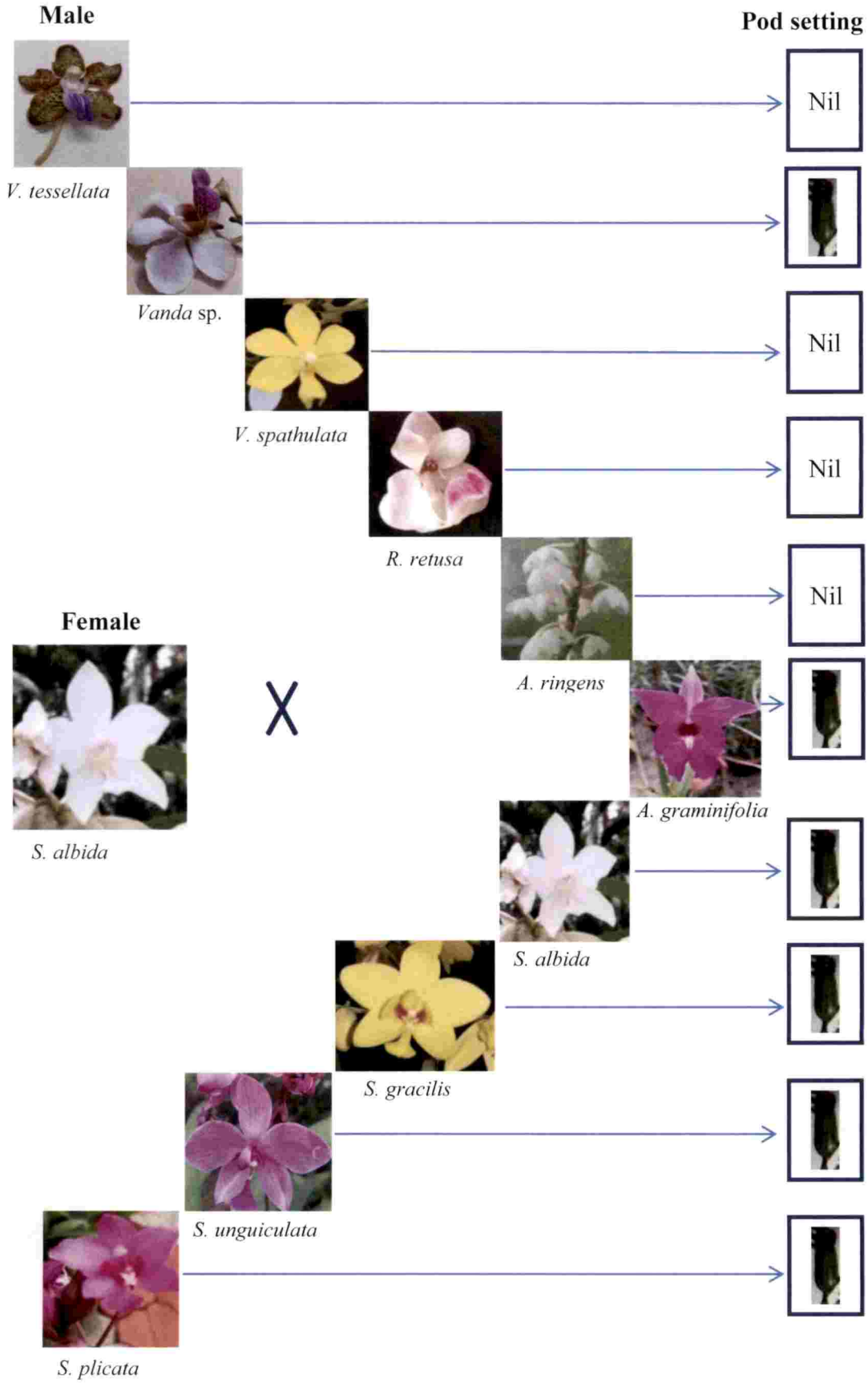


Plate 13. Cross compatibility: *Spathoglottis albida* (♀) with other parents (♂)

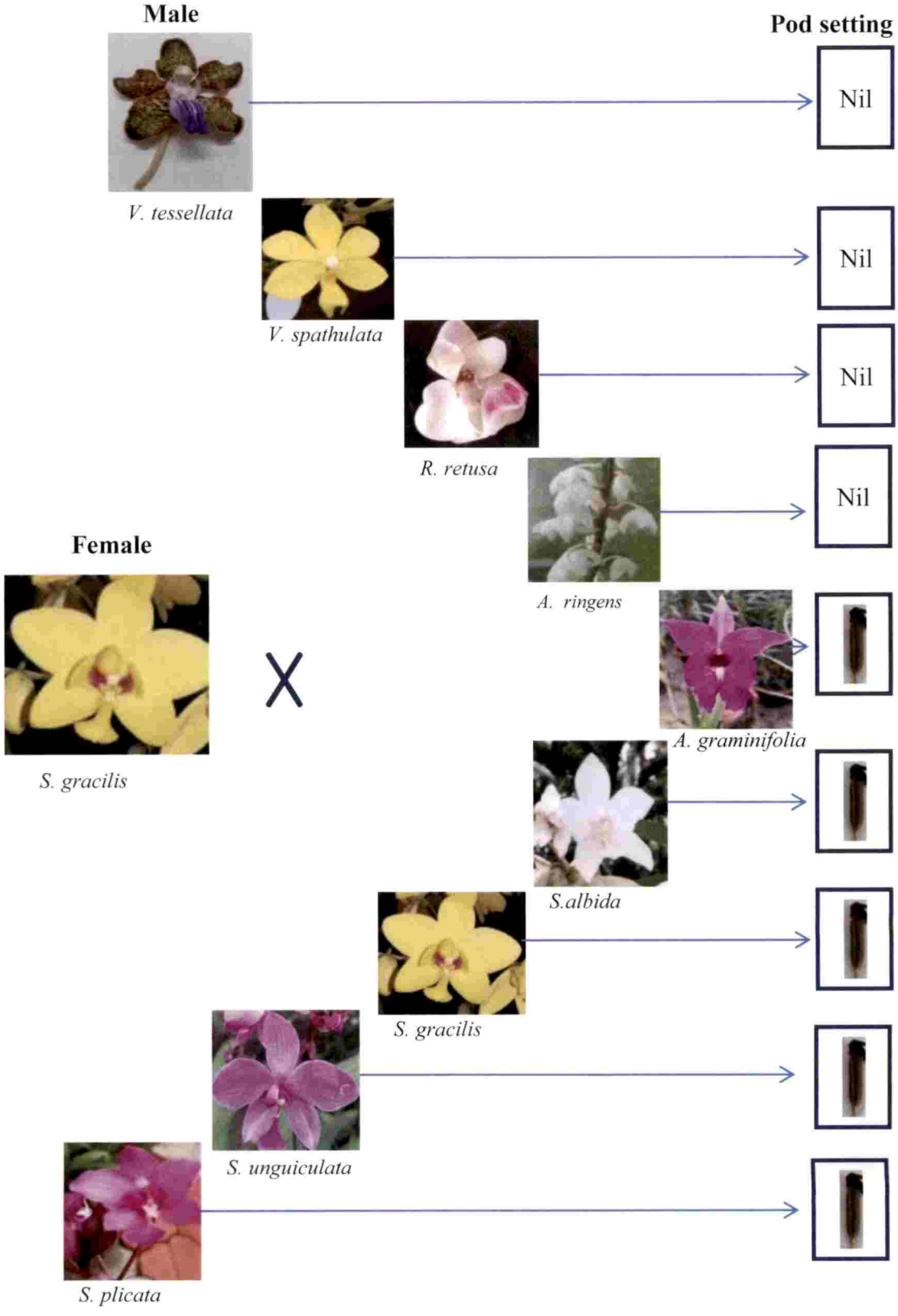


Plate 14. Cross compatibility: *Spathoglottis gracilis* (♀) with other parents (♂)

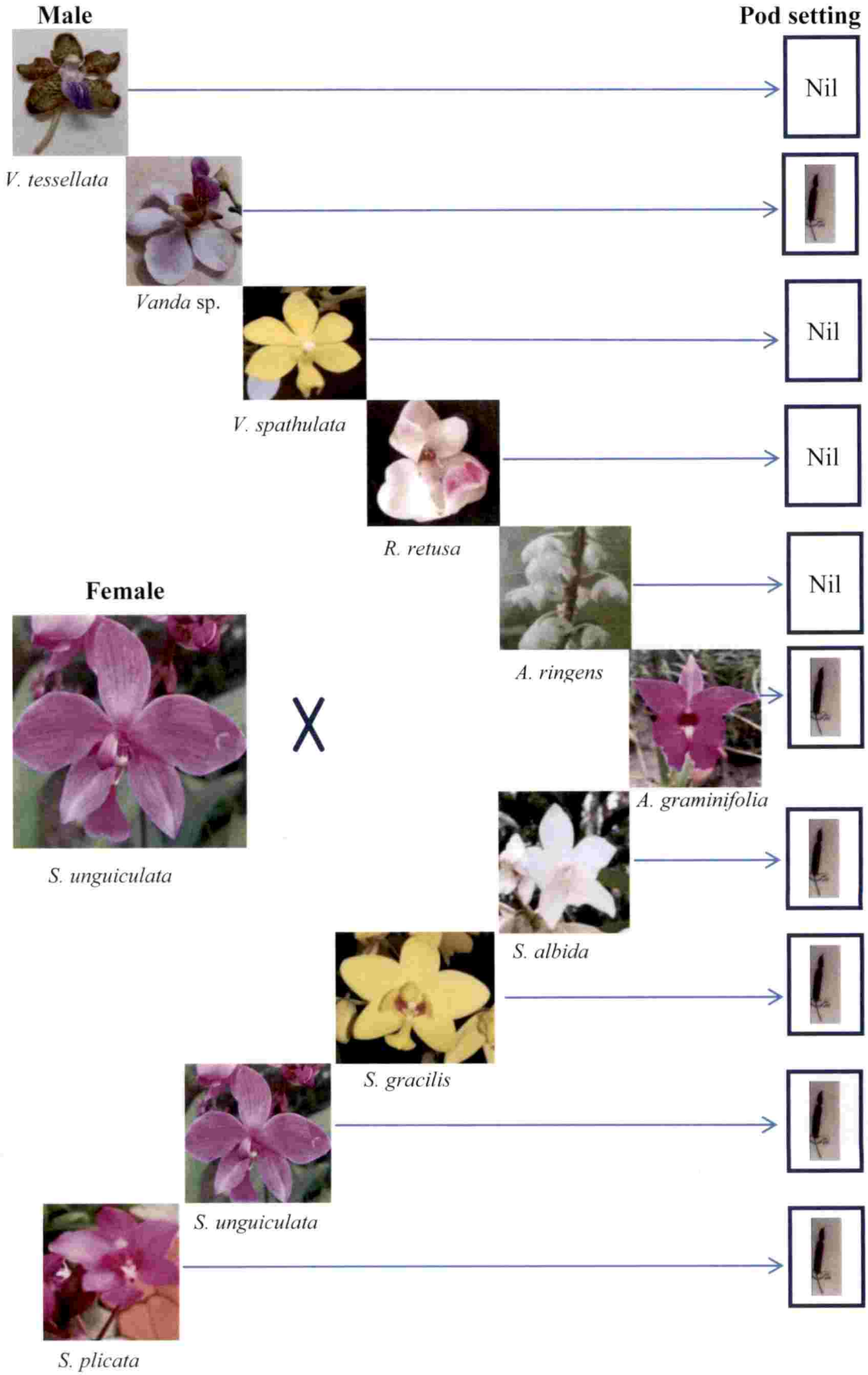


Plate 15. Cross compatibility: *Spathoglottis unguiculata* (♀) with other parents (♂)

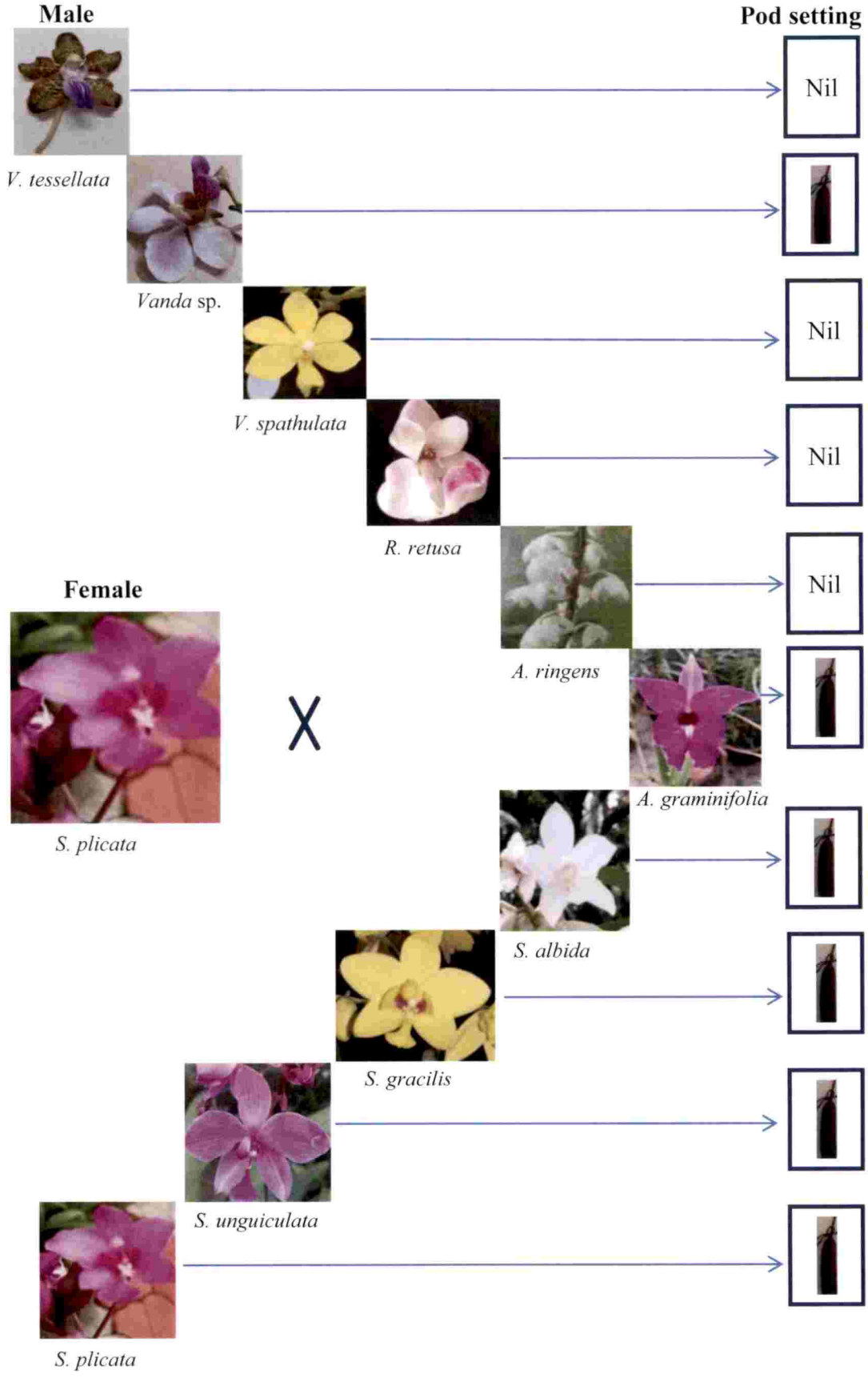


Plate 16. Cross compatibility: *Spathoglottis plicata* (♀) with other parents (♂)

Plate 17. Pod setting among the parents through hybridization : (A) *Spathoglottis albida* (B) *Spathoglottis gracilis* (C) *Spathoglottis unguiculata* (D) *Spathoglottis plicata* (E) *Vanda spathulata* (F) *Vanda tessellata* (G) *Vanda* sp. (H) *Aerides ringens* (I) *Arundina graminifolia* (J) *Rhynchostylis retusa*

4.3. EXPERIMENT III- *IN VITRO* CULTURE OF RESULTANT DISTANT HYBRIDS AND EVALUATION OF SEEDLINGS

The experiment was conducted in two parts and the results are presented below

4.3.1. *In Vitro* Culture of Hybrid Seeds

The mature capsules of hybrids were harvested from mother plants and used for *in vitro* seeds culture. The results of the experiment are presented in table 15 and plate 18.

Capsules of thirty four crosses were used for seed culture under *in vitro* condition. The seeds of distant hybrids obtained were extracted from the capsules, cultured under *in vitro* condition for germination and seedling development and observations were recorded.

Out of the thirty four crosses, seeds of sixteen crosses exhibited signs of greening. The cross *Spathoglottis unguiculata* x *Spathoglottis albida* attained seed germination stage seventeen days after inoculation and the cross *Arundina graminifolia* x *Spathoglottis plicata* attained seed germination 320 days after inoculation.

Seeds of six crosses germinated and developed into protocorm and seedlings. The cross *Spathoglottis unguiculata* x *Spathoglottis albida* produced first leaf 38 days after inoculation but in *Spathoglottis unguiculata* x *Vanda* sp. the first leaf was produced 125 days after inoculation. The cross *Spathoglottis unguiculata* x *Spathoglottis albida* attained shoot initiation after 68 days of inoculation whereas *Spathoglottis unguiculata* x *Vanda* sp. attained shoot initiation after 153 days of inoculation. *Spathoglottis unguiculata* x *Spathoglottis albida* attained initiation of first root 89 days after inoculation but in the cross *Spathoglottis unguiculata* x *Vanda* sp. root initiation stage was attained 213 days after inoculation. Of these *Spathoglottis unguiculata* x *Spathoglottis plicata* cross was ready for deflasking 192 days after inoculation whereas the cross

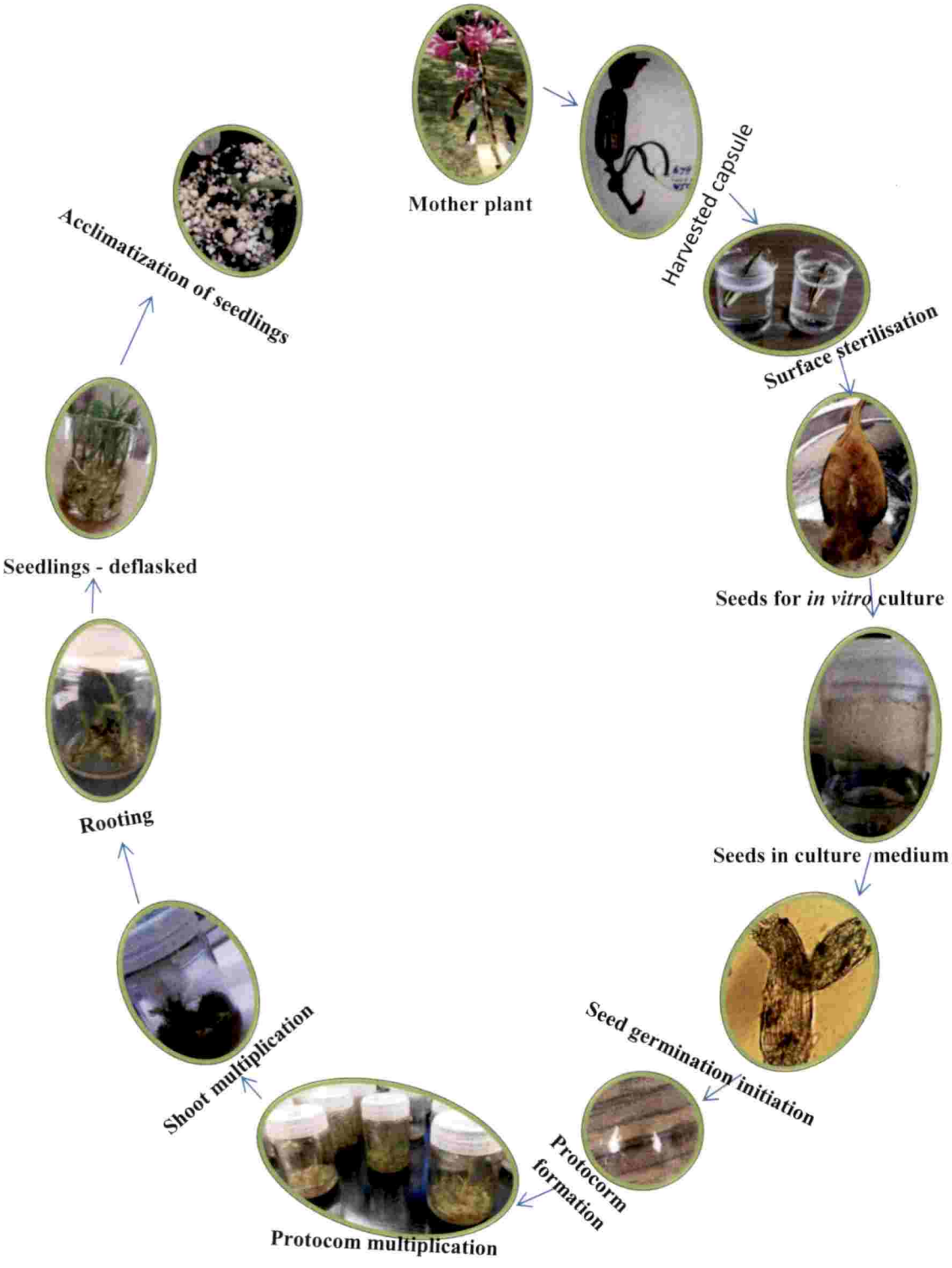


Plate 18. Different stages of *in vitro* seed culture of orchid hybrids

Spathoglottis albida x *Spathoglottis unguiculata* attained deflasking stage at 363 days after inoculation.

4.3.2. Evaluation of hybrid seedlings

4.3.2.1. Morphological characters (at monthly interval)

Seedlings of seven crosses (Plate 21-27) were planted out for hardening (Plate 28). Capsules and seeds of seven crosses are shown in plate 19 and 20 respectively. Morphological characters were recorded at monthly intervals as depicted in table 16 and figure 5A-G. In the cross *Spathoglottis albida* x *Spathoglottis unguiculata* (culture 778 and 1368), seedling height increased gradually but number of leaves remained same at deflasking, one month after deflasking and two months after deflasking. Leaf length and width also increased at monthly intervals. Similar growth performance was exhibited by the remaining all five crosses *viz.*, *Spathoglottis unguiculata* x *Spathoglottis albida* (culture 622), *Spathoglottis unguiculata* x *Vanda* sp. (culture 679), *Spathoglottis plicata* x *Spathoglottis unguiculata* (culture 1386), *Spathoglottis unguiculata* x *Spathoglottis plicata* (culture 1388) and *Spathoglottis gracilis* x *Spathoglottis unguiculata* (culture 652).

4.4. EXPERIMENT IV- Molecular characterization

4.4.1. ISSR Assay

Hybrids from seven crosses were verified using ISSR markers. A total of 60 ISSR loci were clearly amplified from all hybrids using ten ISSR primers. Among these amplified fragments, approximately 39 DNA bands (66.6%) were found to be polymorphic between male and female parents. Total polymorphic DNA bands between hybrids and their respective female parents to distinguish the true hybrids from the self-pollinated progeny of female parents were approximately 14 (22, 36%).

Total number of scorable DNA bands amplified by each primer ranged from four (ISSR 824, 834,849) to ten (ISSR 807) with an average of 5 bands primer^{-1}. The size of amplified products was in the range of 220-2000 bp (Table 17). All

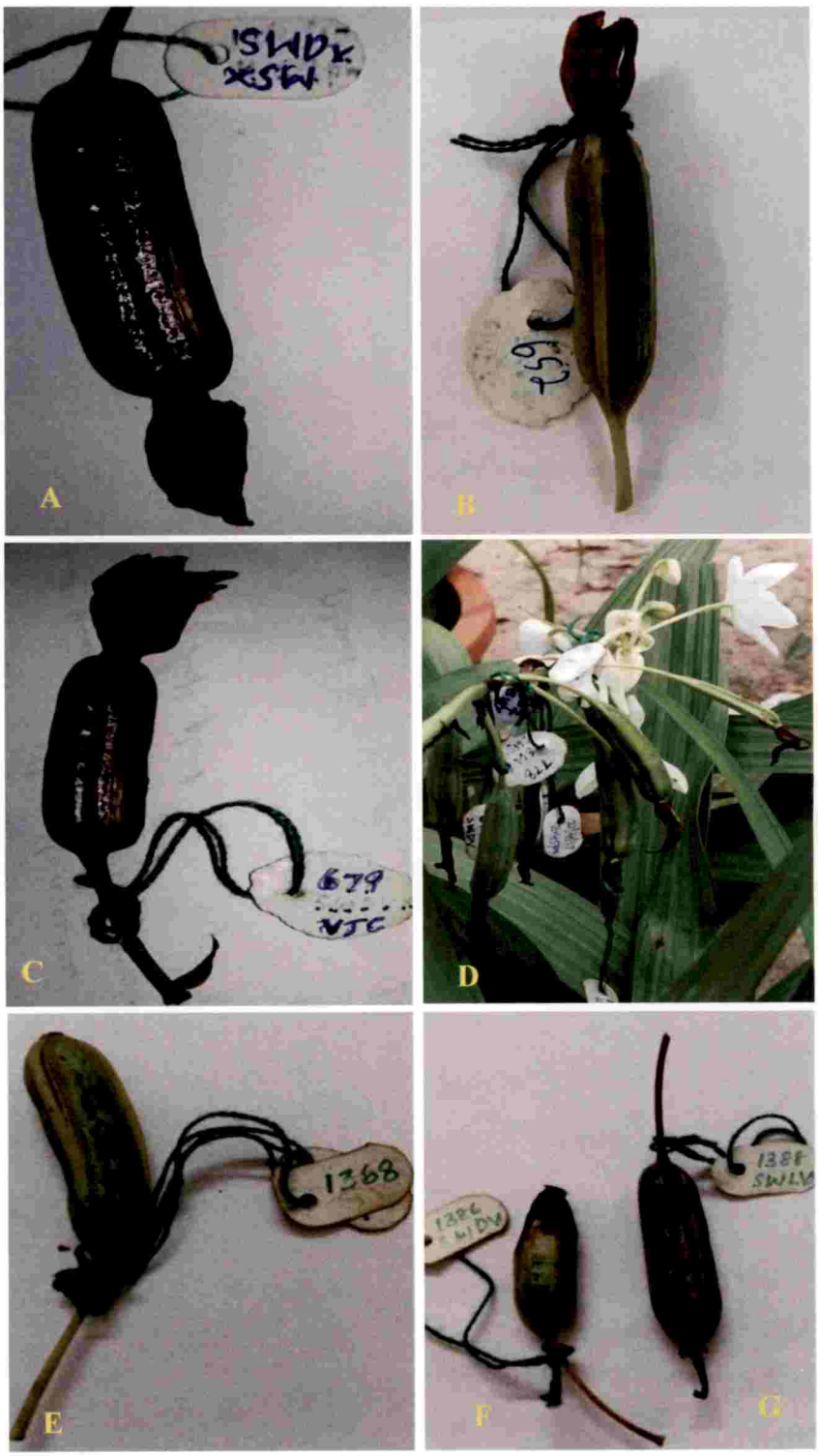
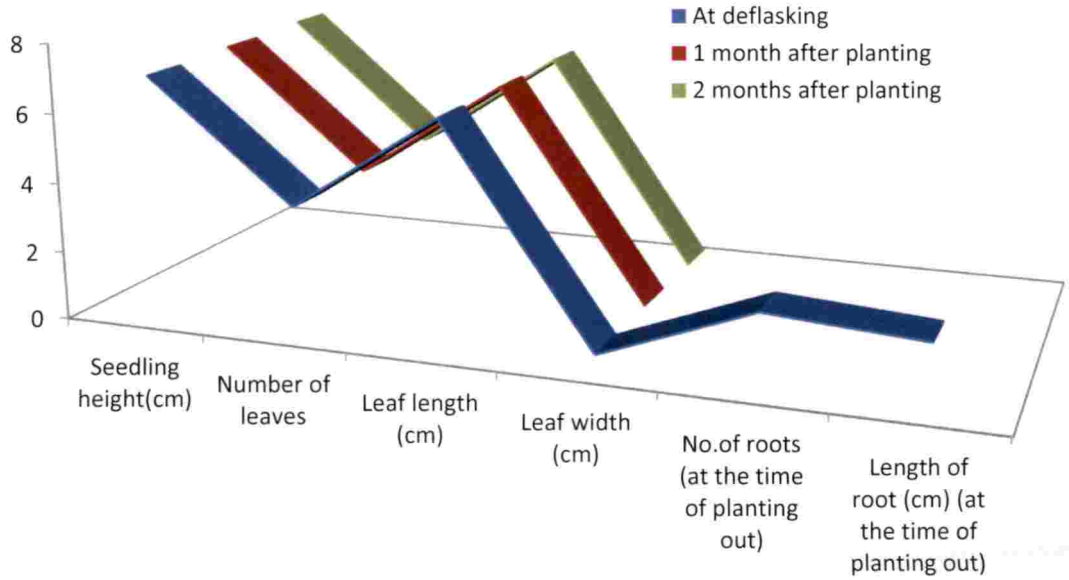


Plate 19. Hybrids capsules : (A) *Spathoglottis unguiculata* x *Spathoglottis albida* (Culture 622) (B) *Spathoglottis gracilis* x *Spathoglottis unguiculata* (Culture 652) (C) *Spathoglottis unguiculata* x *Vanda* sp. (Culture 679) (D) *Spathoglottis albida* x *Spathoglottis unguiculata* (Culture 778) (E) *Spathoglottis albida* x *Spathoglottis unguiculata* (Culture 1368) (F) *Spathoglottis plicata* x *Spathoglottis unguiculata* (Culture 1386) (G) *Spathoglottis unguiculata* x *Spathoglottis plicata* (Culture 1388)

Plate 20. **Seeds of crosses** – (A) *Spathoglottis unguiculata* x *Spathoglottis albida* (Culture 622) (B) *Spathoglottis gracilis* x *Spathoglottis unguiculata* (Culture 652) (C) *Spathoglottis unguiculata* x *Vanda* sp. (Culture 679) (D) *Spathoglottis albida* x *Spathoglottis unguiculata* (Culture 778) (E) *Spathoglottis albida* x *Spathoglottis unguiculata* (Culture 1368) (F) *Spathoglottis plicata* x *Spathoglottis unguiculata* (Culture 1386) (G) *Spathoglottis unguiculata* x *Spathoglottis plicata* (Culture 1388)

(a) *S. albida* x *S. unguiculata*



(b) *S. unguiculata x S. albida*

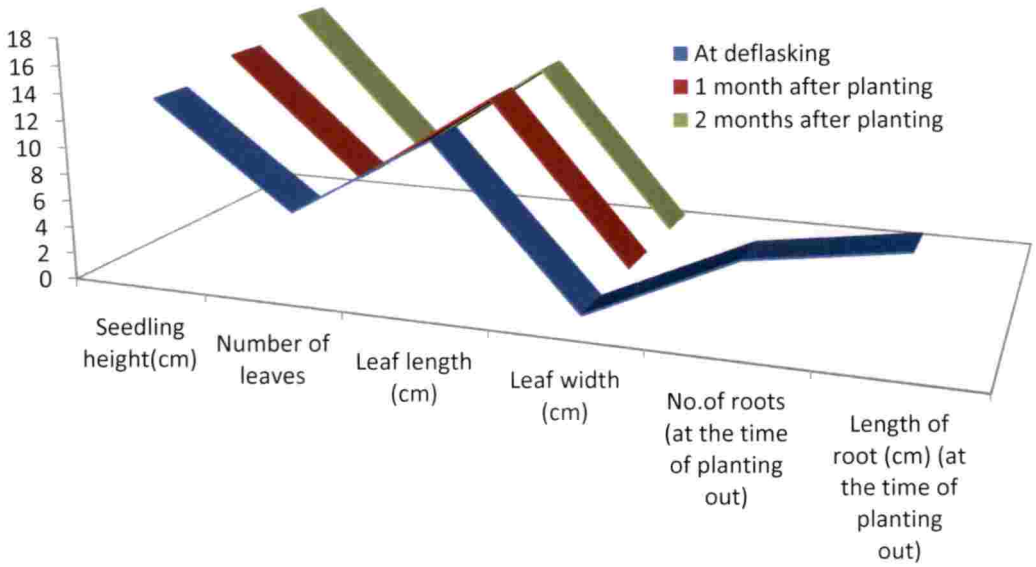
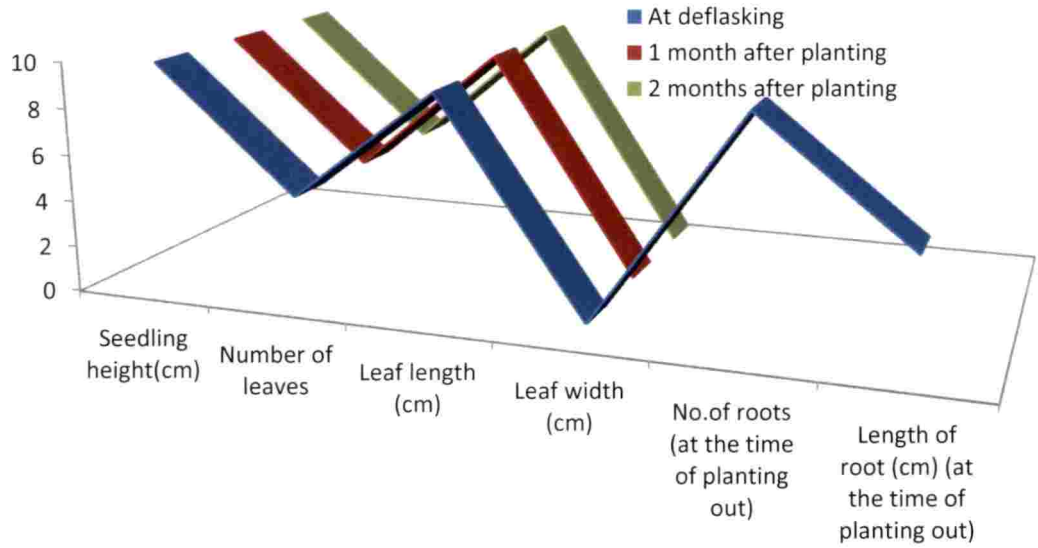


Figure 5. Morphological characters of hybrid seedlings at monthly interval

(c) *S. unguiculata* x *Vanda* sp.



(d) *S. unguiculata* x *S. plicata*

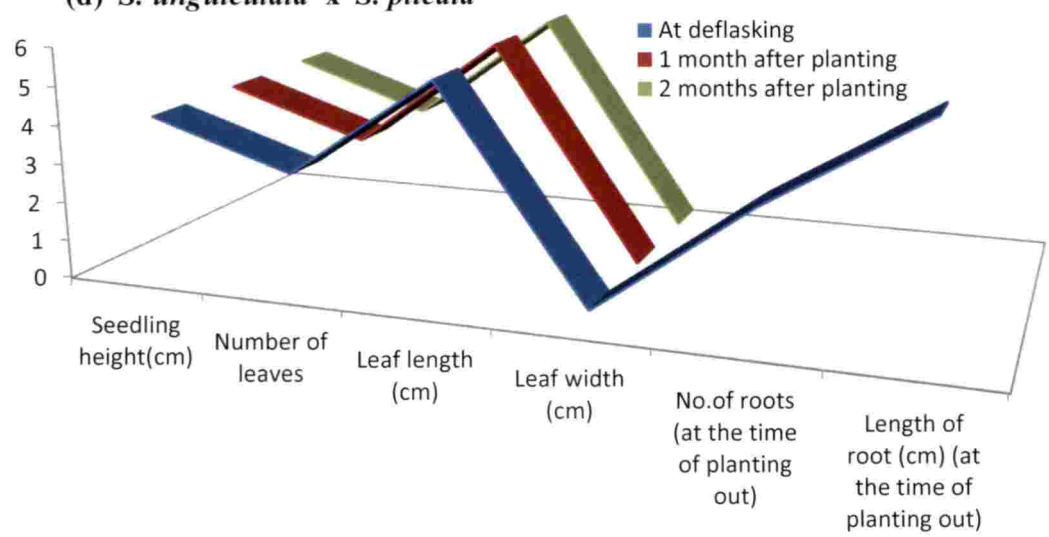
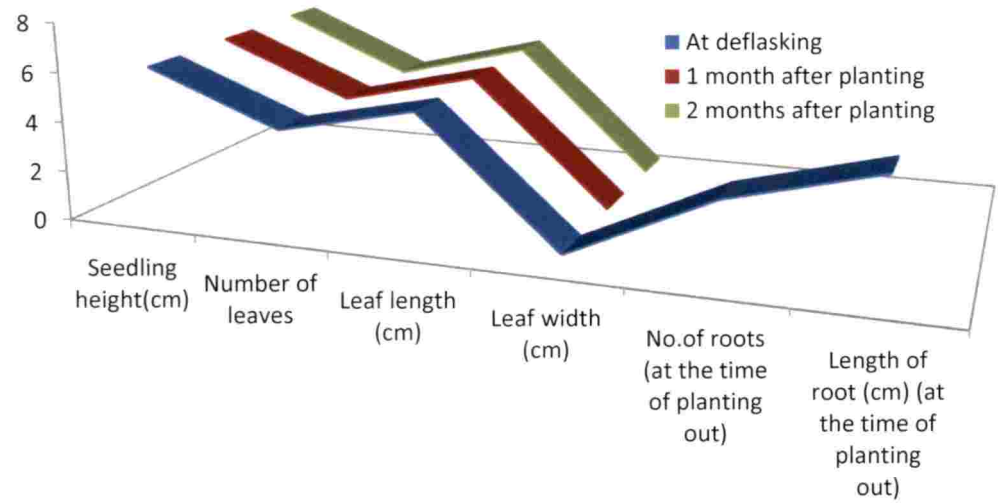


Figure 5. Morphological characters of hybrid seedlings at monthly intervals

(e) *S. plicata* x *S. unguiculata*



(f) *S. gracilis* x *S. unguiculata*

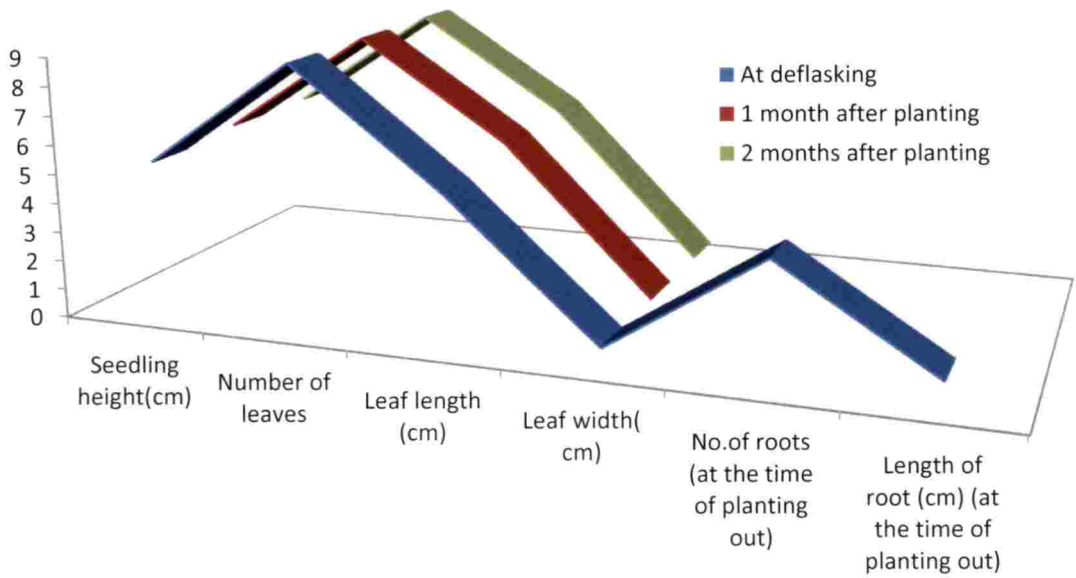


Figure 5. Morphological characters of hybrid seedlings at monthly intervals

(g) *S. albida* x *S. unguiculata*

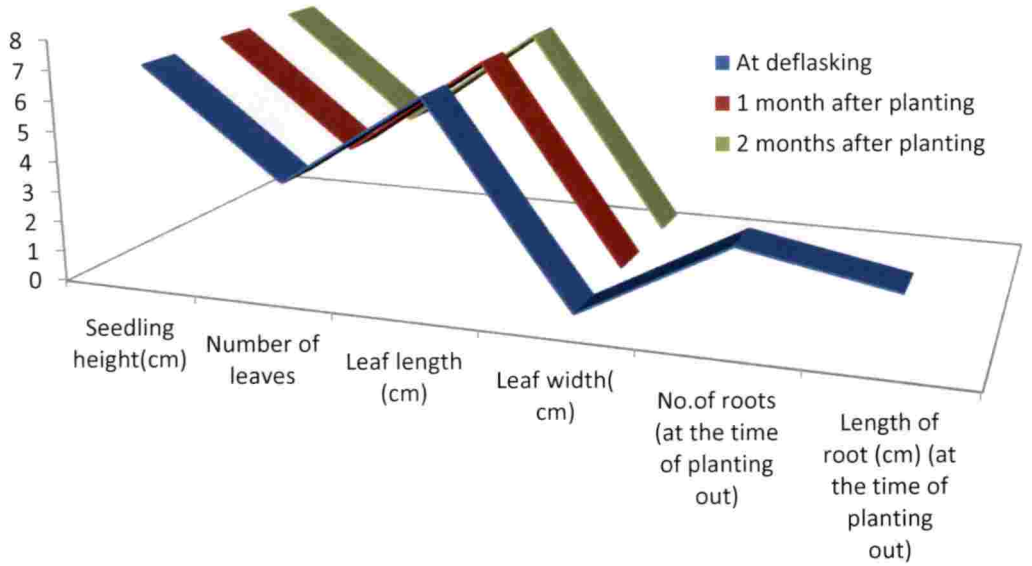


Figure 5. Morphological characters of hybrid seedlings at monthly intervals

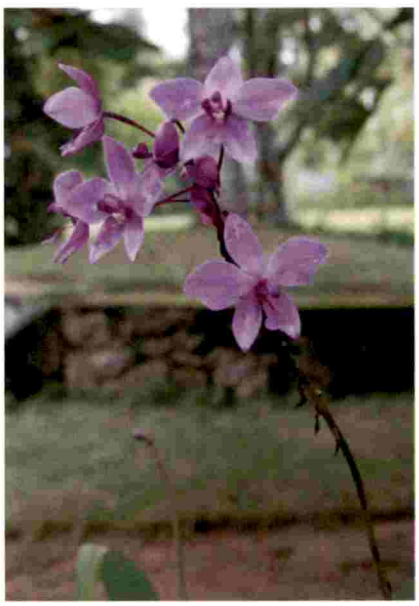
Spathoglottis unguiculata
♀

***Vanda* sp.**
♂

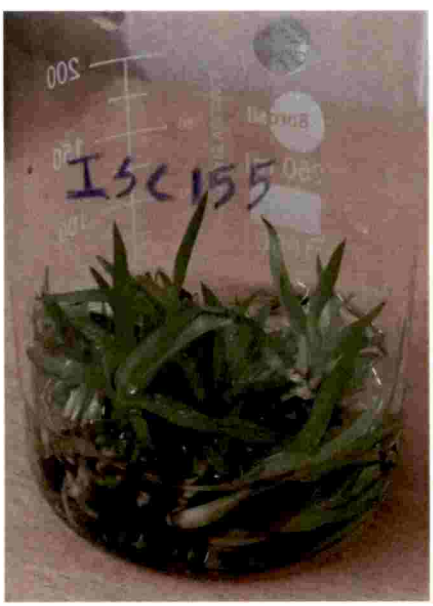
F1

Plate 21. F_1 intergeneric hybrid between *Spathoglottis unguiculata* and *Vanda* sp.

Spathoglottis gracilis
♀



Spathoglottis unguiculata
♂

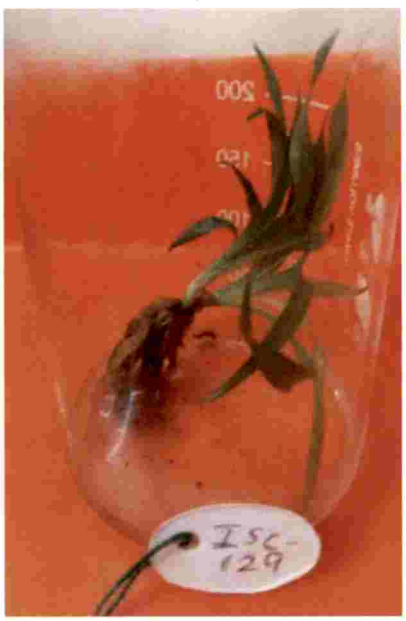


F1

Plate 22. F_1 interspecific hybrid between *Spathoglottis gracilis* and *Spathoglottis unguiculata*

Spathoglottis unguiculata
♀

Spathoglottis albida
♂



F1

Plate 23. F_1 interspecific hybrid between *Spathoglottis unguiculata* and *Spathoglottis albida*

Spathoglottis albida
♀

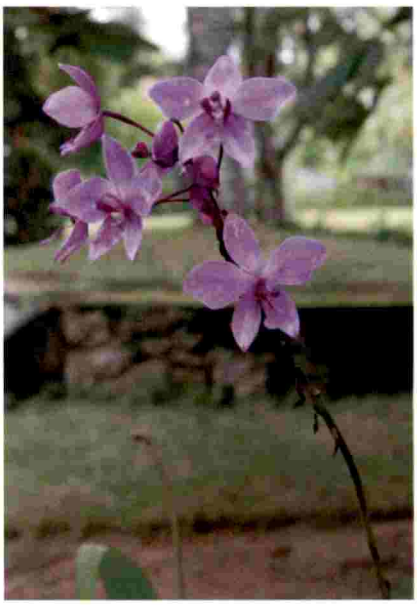
Spathoglottis unguiculata
♂

F1

Plate 24. F_1 interspecific hybrid between *Spathoglottis albida* and *Spathoglottis unguiculata*

Spathoglottis albida
♀

×

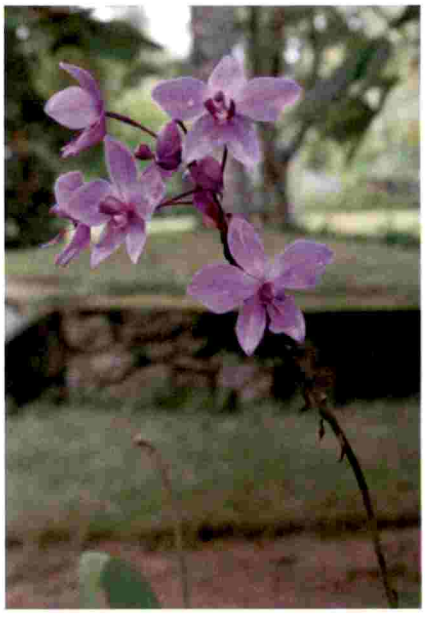


Spathoglottis unguiculata
♂

F1

Plate: 25. F_1 interspecific hybrid between *Spathoglottis albida* and *Spathoglottis unguiculata*

Spathoglottis plicata
♀



Spathoglottis unguiculata
♂

F1

Plate 26. F_1 interspecific hybrid between *Spathoglottis plicata* and *Spathoglottis unguiculata*

Spathoglottis unguiculata
♀

Spathoglottis plicata
♂

F1

Plate 27. F_1 interspecific hybrid between *Spathoglottis unguiculata* and *Spathoglottis plicata*

Plate 28. Hybrid seedlings under hardening and acclimatization:
(1) *Spathoglottis plicata* x *Spathoglottis unguiculata* (2) *Spathoglottis gracilis* x *Spathoglottis unguiculata* (3) *Spathoglottis unguiculata* x *Vanda* sp. (4) *Spathoglottis unguiculata* x *Spathoglottis plicata* (5) *Spathoglottis plicata* x *Spathoglottis unguiculata* (6) *Spathoglottis albida* x *Spathoglottis unguiculata* (7) *Spathoglottis unguiculata* x *Spathoglottis albida*.

ten primers could effectively differentiate between male and female parents in all seven crosses and nine primers were successful in distinguishing true hybrids from female parents. The highest percentage of polymorphism between male and female parents (95.83%) was achieved using ISSR 841 primer followed by ISSR 849 (84.37%) and ISSR 810 (70.83%). Similarly primers displayed the highest percentages of polymorphism between the hybrid and female parents were ISSR 841 (33.33%), ISSR 849 (34.37%) and ISSR 810 (33.33%) (Table 17).

Table 15. Seed germination and seedling development period

Sl No	Cross	Days for initiation of germination (greening)	Days for initiation of first leaf	Days for initiation of shoot	Days for initiation of first root	Days for deflasking
1	*V. tessellata* x *V. spathulata*	NC	NC	NC	NC	NC
2	*V. spathulata* x *V. tessellata*	104	NC	NC	NC	NC
3	*V. spathulata* x *A. ringens*	NC	NC	NC	NC	NC
4	*V. spathulata* x *R. retusa*	85	NC	NC	NC	NC
5	*V. spathulata* x *Vanda* sp.	NC	NC	NC	NC	NC
6	*Vanda* sp. x *V. tessellata*	NC	NC	NC	NC	NC
7	*R. retusa* x *V. tessellata*	NC	NC	NC	NC	NC
8	*R.retusa* x *A. ringens*	NC	NC	NC	NC	NC
9	*A. ringens* x *V. tessellata*	122	NC	NC	NC	NC
10	*A. ringens* x *R. retusa*	139	NC	NC	NC	NC
11	*A. ringens* x *Vanda* sp.	NC	NC	NC	NC	NC
12	*A. graminifolia* x *S. albida*	42	NC	NC	NC	NC
13	*A. graminifolia* x *S. unguiculata*	NC	NC	NC	NC	NC
14	*A. graminifolia* x *S. plicata*	320	NC	NC	NC	NC
15	*A. graminifolia* x *V. tessellata*	269	NC	NC	NC	NC
16	*S. albida* x *S. unguiculata*	20	52	91	138	363
17	*S. albida* x *S. plicata*	NC	NC	NC	NC	NC
18	*S. albida* x *S. gracilis*	NC	NC	NC	NC	NC
19	*S. albida* x *A. graminifolia*	235	NC	NC	NC	NC
20	*S. albida* x *Vanda* sp.	NC	NC	NC	NC	NC
21	*S. unguiculata* x *S. albida*	17	38	68	89	276
22	*S. unguiculata* x *S. plicata*	24	83	114	175	192
23	*S. unguiculata* x *S. gracilis*	NC	NC	NC	NC	NC
24	*S. unguiculata* x *A. graminifolia*	91	NC	NC	NC	NC
25	*S. unguiculata* x *Vanda* sp.	93	125	153	213	326
26	*S. plicata* x *S. albida*	NC	NC	NC	NC	NC
27	*S. plicata* x *S. unguiculata*	43	73	101	162	200
28	*S. plicata* x *S. gracilis*	NC	NC	NC	NC	NC
29	*S. plicata* x *A. graminifolia*	184	NC	NC	NC	NC
30	*S. plicata* x *Vanda* sp.	NC	NC	NC	NC	NC
31	*S. gracilis* x *S. albida*	NC	NC	NC	NC	NC
32	*S. gracilis* x *S. unguiculata*	33	65	96	164	349
33	*S. gracilis* x *S. plicata*	NC	NC	NC	NC	NC
34	*S. gracilis* x *A.graminifolia*	NC	NC	NC	NC	NC

NC-No change in seed culture

Table 16. Morphological characters of hybrid seedlings at monthly intervals

Sl No	Cross	Culture No	Time of planting	Seedling height (cm)	Number of leaves	Leaf length (cm)	Leaf width (cm)	No. of roots (at the time of planting out)	Length of root (cm) (at the time of planting out)
1	*S. albida* x *S.unguiculata*	778	At deflasking	7.0	3.6	6.42	0.3	2	1.7
			1 MAD	7.1	3.6	6.44	0.31	-	
			2 MAD	7.2	3.6	6.47	0.31	-	
2	*S. albida* x *S. unguiculata*	1368	At deflasking	7.1	3.6	6.7	0.32	3	2.1
			1 MAD	7.2	3.6	6.9	0.33	-	-
			2 MAD	7.3	3.6	7.0	0.34	-	-
3	*S. unguiculata* x *S. albida*	622	At deflasking	13.39	5.7	12	0.59	5.7	7.4
			1 MAD	14.69	5.7	12.7	0.59	-	-
			2 MAD	16.19	5.7	12.9	0.61	-	-
4	*S. unguiculata* x *S. plicata*	1388	At deflasking	4.16	3.0	5.6	0.3	3.2	5.6
			1 MAD	4.2	3.0	5.8	0.31	-	-
			2 MAD	4.25	3.0	5.8	0.32	-	-
5	*S. unguiculata* x *Vanda* sp.	679	At deflasking	9.88	4.6	9.42	0.48	9.6	4.68
			1 MAD	9.91	4.6	9.81	0.49	-	-
			2 MAD	9.94	4.6	9.91	0.50	-	-
6	*S. plicata* x *S. unguiculata*	1386	At deflasking	6.1	4	5.2	0.3	3	4.5
			1 MAD	6.2	4	5.3	0.31	-	-
			2 MAD	6.3	4	5.4	0.32	-	-
7	*S. gracilis* x *S. unguiculata*	652	At deflasking	5.3	9	5.1	0.5	4	0.7
			1 MAD	5.5	9	5.6	0.51	-	-
			2 MAD	5.5	9	5.7	0.53	-	-

Table 17. Primer sequences, DNA size, percentages of polymorphism between male and female parents, percentages of polymorphism between hybrid and female parent for each ISSR primers in all 7 crosses of Orchids.

Sl. No	Primers	Primer sequence	DNA size	Number of scorable bands	Percentage polymorphism (male and female parents)	Percentage polymorphism (Hybrid and female parents)
1	807	$(AG)_8T$	250- 1500bp	10	38.75	12.50
2	809	$(AG)_8G$	200- 1200bp	6	62.49	16.66
3	810	$(GA)_8T$	200-1800bp	6	70.83	33.33
4	811	$(GA)_8C$	200-1800bp	9	64.03	15.27
5	824	$(TC)_8G$	200-1300bp	4	62.50	34.37
6	834	$(AG)_8Y*T$	200-1200bp	4	65.62	28.12
7	841	$(GA)_8Y*C$	200-1000bp	3	95.83	33.33
8	842	$(GA)_8Y*G$	400-2000bp	6	56.25	6.25
9	849	(GT)8Y*A	500-1400bp	4	84.37	34.37
10	857	(AC)8Y*G	220-1200bp	8	65.62	9.37

*Y= pyrimidines (C, T)

4.4.2. Confirmation of Hybridity

To confirm the hybrid purity, only the primers which amplified bands specific to the female parent of each hybrid were considered. Analysis of the inheritance of the single locus for each cross combination was performed. The bands that were common to the F_1 hybrids and to only one of the parents were defined as discriminative ISSR loci. The percentages of discriminative ISSR loci were calculated for both the male and the female parents (Table 19). The highest total values were found for two hybrids *viz.*, *Spathoglottis unguiculata* x *Spathoglottis plicata* [SU1388 X SP1388 (52.72%)] and *Spathoglottis albida* x *Spathoglottis unguiculata* [SA1368 X SU1368 (42.11%)]. For all cross combinations, the percentage of discriminative loci for the female parent was higher than for the male parent; the percentage of discriminative ISSR loci for the female parent was 17%, while for the male parent the discriminative loci ranged from 0-15% (Table 19). Inconsistent percentage of bands was evident in the hybrids but not in the parents. These recombinant bands were obtained majorly with the primers, 841, 849 and 810 (Table 17). The percentage of polymorphism

between male and female parents could reveal the genetic difference between male and female parents in each cross combination. When considering all ten primers, the highest percentage of polymorphism between male and female parents (73.83%) was found in the cross *Spathoglottis albida* x *Spathoglottis unguiculata* [SA778 X SU778]; followed in the cross *Spathoglottis albida* x *Spathoglottis unguiculata* [SA1368 X SU1368 (71.97%)] (Table 18 and Plate 29).

Table 18. Number of scorable DNA bands, percentages of polymorphism between male and female parents, percentages of polymorphism between hybrid and female parent for all 10 ISSR primers in each cross of Orchids.

Sl. No	Crosses	N	% Polymorphism (Female- Male parents)	% Polymorphism (Hybrid - Female parents)
1	SU622 X SA622	60	65.97	30.47
2	SG652 X SU652	60	65.72	30.47
3	SU679 X VA679	60	61.14	32.97
4	SA778 X SU778	60	73.83	26.42
5	SA1368 X SU1368	60	71.97	19.61
6	SP1386 X SU1386	60	56.64	11.99
7	SU1388 X SP1388	60	68.00	26.94

Table 19. Analysis of the discriminative ISSR loci revealed by 10 primers in 7 orchid crosses.

Primer	N	(D) Discriminative Loci			% D
		F1-♀	F1-♂	T	
SU622 X SA622					
807	10	2	0	2	20.00
809	6	1	0	1	16.67
810	6	3	0	3	50.00
811	9	2	0	2	22.22
824	4	3	0	3	75.00
834	4	2	0	2	50.00
841	3	1	1	2	66.67
842	6	0	0	0	0.00
849	4	2	1	3	75.00
857	8	1	0	1	12.50
Total	60	17	2	19	36.30

Conti..

Primer	N	(D) Discriminative Loci	% D	Primer	N
SG652 X SU652					
807	10	2	0	2	20.00
809	6	1	0	1	16.67
810	6	3	0	3	50.00
811	9	2	0	2	22.22
824	4	3	0	3	75.00
834	4	2	0	2	50.00
841	3	1	0	1	33.33
842	6	0	0	0	0.00
849	4	1	0	1	25.00
857	8	1	0	1	12.50
Total	60	17	0	17	30.48
SU679 X VA679					
807	10	2	0	2	20.00
809	6	1	0	1	16.67
810	6	3	0	3	50.00
811	9	2	0	2	22.22
824	4	3	0	3	75.00
834	4	2	0	2	50.00
841	3	1	0	1	33.33
842	6	0	0	0	0.00
849	4	2	0	2	50.00
857	8	1	0	1	12.50
Total	60	17	0	17	32.97
SA778 X SU778					
807	10	1	0	1	10.00
809	6	3	3	6	100.00
810	6	2	0	2	33.33
811	9	0	0	0	0.00
824	4	1	0	1	25.00
834	4	2	0	2	50.00
841	3	1	1	2	66.67
842	6	0	0	0	0.00
849	4	2	0	2	50.00
857	8	1	0	1	12.50
Total	60	13	4	17	34.75

Conti..

Primer	N	(D) Discriminative Loci			% D
SA1368 X SU1368					
807	10	1	0	1	10.00
809	6	0	2	2	33.33
810	6	4	2	6	100.00
811	9	1	0	1	11.11
824	4	0	2	2	50.00
834	4	1	1	2	50.00
841	3	1	1	2	66.67
842	6	0	3	3	50.00
849	4	1	0	1	25.00
857	8	2	0	2	25.00
Total	60	11	11	22	42.11
SP1386 X SU1386					
807	10	2	2	4	40.00
809	6	0	1	1	16.67
810	6	0	2	2	33.33
811	9	0	0	0	0.00
824	4	1	0	1	25.00
834	4	0	0	0	0.00
841	3	1	2	3	100.00
842	6	1	0	1	16.67
849	4	1	0	1	25.00
857	8	0	0	0	0.00
Total	60	6	7	13	25.66
SU1388 X SP1388					
807	10	0	3	3	30.00
809	6	2	1	3	50.00
810	6	1	2	3	50.00
811	9	4	1	5	55.56
824	4	0	3	3	75.00
834	4	1	1	2	50.00
841	3	2	1	3	100.00
842	6	2	2	4	66.67
849	4	2	0	2	50.00
857	8	0	0	0	0.00
Total	60	14	14	28	52.72

[Where N= Total number of loci amplified; D= number od discriminative ISSR loci; F1-♀ = loci common to hybrid and female parent; F1-♂ = loci common to hybrid and male parent; % D = discriminative power of primer to differentiate between hybrid and parents.]

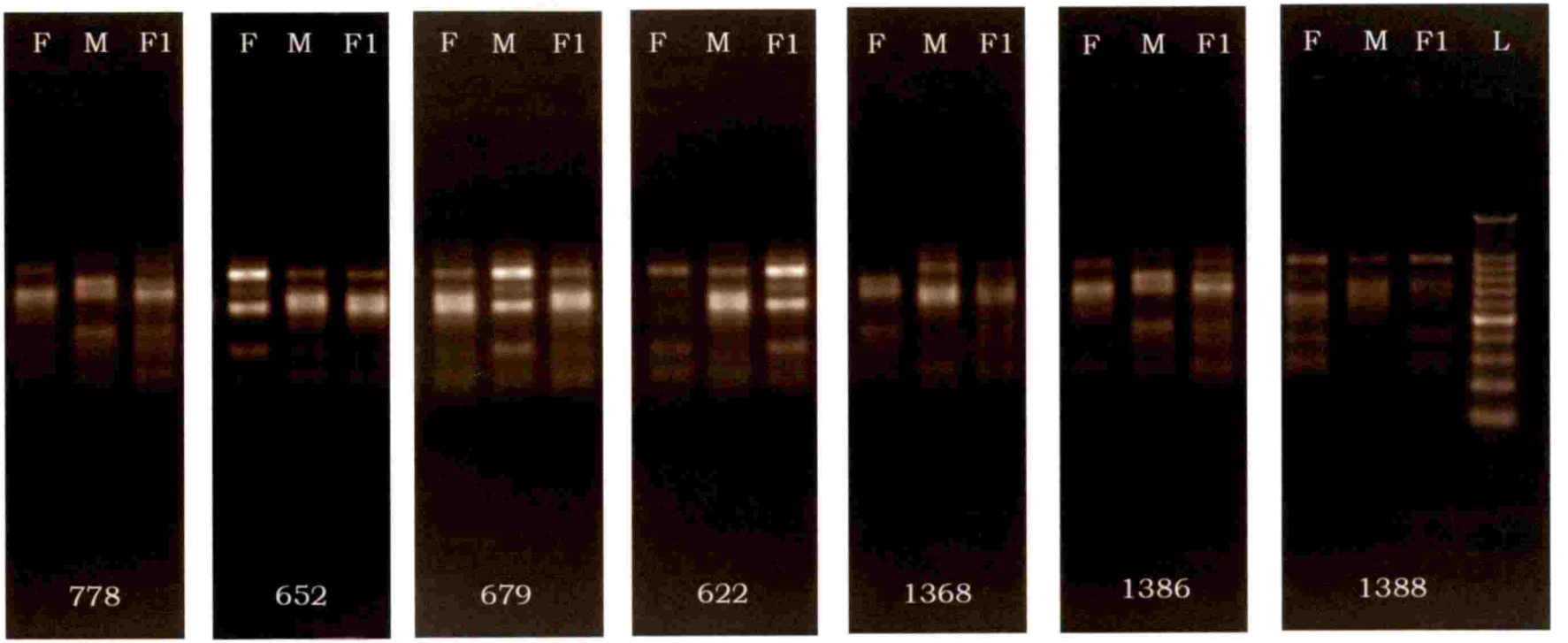


Plate 29. Representative gel showing genetic variation among parents : (F= Female; M= Male) and F1 progeny in different crosses of orchids. [L= 1Kb DNA Ladder; The numbers below the bands denote the different crosses in the experiment]
(778) *Spathoglottis albida* x *Spathoglottis unguiculata* (652) *Spathoglottis gracilis* x *Spathoglottis unguiculata* (679) *Spathoglottis unguiculata* x *Vanda* sp. (622) *Spathoglottis unguiculata* x *Spathoglottis albida* (1368) *Spathoglottis albida* x *Spathoglottis unguiculata* (1386) *Spathoglottis plicata* x *Spathoglottis unguiculata* (1388) *Spathoglottis unguiculata* x *Spathoglottis plicata*

DISCUSSION

5. DISCUSSION

Natural flora present in the forest ecosystem is enriched with the existence of wild orchids contributing enormous variety of eye lashing flowers with unique articulate. Orchids are growing in land as well as as epiphytes and a few of them are saprophytes. It belongs to the second largest family among angiosperms. Due to the fascinating and long-lasting flowers it became the top ranked cut flowers in the global market. In India more than 1330 orchid species are reported, of these, a major share is endemic to India. These valuable genetic resources are catching the attention for genetic improvement. Moreover, evolution of large number of beautiful and attractive flower producing species and varieties is generating enthusiasm and pay attention of the breeders to the tempo for developing new and varied combinations through distant hybridization. But utilization of these genetic resources for genetic improvement through hybridization is seldom practiced in India, particularly in Kerala.

Distant hybridization is an effective breeding tool to capture hybrid vigour as well as to combine traits that do not occur within a single species. Meanwhile, three broad classes of difficulties encountered in the production of wide crosses are 1) failure of zygote formation 2) failure of zygote development and 3) failure of F_1 seedling development. These difficulties make it essential to determine how easily two species or genera involved in a cross will actually hybridize.

As a pre breeding, this investigation was aimed to the evaluation of the cross compatibility among different wild genera and species indigenous to South India, particularly in Kerala before launching into a successful genetic improvement programme through distant hybridization. Therefore, prior to hybridization essential steps to be taken hitherto are evaluation of parents and choice of elite parents.

The present investigation was focused on evaluation of parents with the estimation of extent of genetic variability in various quantitative traits in terms of components of variance, heritability, genetic advance and correlation; evaluation

of cross compatibility of different parental species; *in vitro* culture of hybrid seeds and development of hybrid seedlings and its evaluation through morphological and molecular characterization.

The results pertaining to the investigation are discussed hereunder.

5.1. EXPERIMENT I

5.1.1. Evaluation of Parents

If the performance of parents in the area where breeding is to be done is not known, evaluation is essential. The parents or genotypes are evaluated based on the expression of different metric characters. The quantitative trait is measured and expressed as phenotypic value of an individual. The phenotypic value of an individual for a particular trait is assessed by simple measures of variability such as mean, range, variances etc. Selection of parents on the basis of phenotypic performance is the foremost step in a breeding programme.

5.1.1.1. Mean

The phenotypic values of parents for different traits measured using the variability parameter mean is discussed as follows.

Among different parents, significant range of variation was recorded for plant height, plant spread, shoot girth, number of leaves plant^{-1}, leaf length and width, days taken for first flowering, number of spikes plant^{-1} year^{-1}, length of inflorescence, number of flowers inflorescence^{-1}, internodal length, flower length and width, days for inflorescence emergence to first flower opening, days to last flower opening from first flower opening and spike longevity.

Wide variation for plant height was reported in different varieties of *Dendrobium* and it is mainly due to genetic nature, growing conditions and environmental conditions (Roychowdhury *et al.*, 2004; Sugapriya *et al.*, 2012). Wide variation was reported for plant height, plant spread, shoot girth, number of leaves plant^{-1}, leaf length and width, number of spikes plant^{-1} annum^{-1}, length of

inflorescence, number of flowers spike^{-1}, internodal length, days for inflorescence emergence to first flower opening and spike longevity among different varieties and hybrids of *Vanda* (Minnu, 2015).

Of the different parents studied, the species *Vanda spathulata* recorded maximum plant height, number of leaves plant^{-1} and days taken for first flowering and minimum plant spread, shoot girth, leaf length and width and number of spikes plant^{-1} year^{-1}.

Spathoglottis spp. recorded the maximum plant spread, leaf length and width and length of inflorescence and the minimum number of leaves plant^{-1}, days taken for first flowering, number of spikes plant^{-1} year^{-1} and internodal length.

In consonance with the earlier report in *Vanda* (Minnu, 2015) in the present investigation plant spread was largely determined by length of leaves in *Vanda spathulata* and *Spathoglottis* spp.

Varieties with more leaf length had less breadth and *vice versa* (Sugapriya *et al.*, 2012). It is contradictory with the present findings in the species *Vanda spathulata* and *Spathoglottis* spp. *Rhynchostylis retusa* recorded the maximum shoot girth, number of flowers inflorescence^{-1} and days for inflorescence emergence to first flower opening and the minimum number of spikes plant^{-1} year^{-1}.

Vanda tessellata recorded the maximum number of spikes plant^{-1} year^{-1} and the minimum length of inflorescence, number of flowers inflorescence^{-1}, days to last flower opening from first flower opening and spike longevity.

Arundina graminifolia recorded the maximum internodal length and days to last flower opening from first flower opening and the minimum days taken for first flowering and days for inflorescence emergence to first flower opening.

Aerides ringens recorded the maximum spike longevity and the minimum plant height, number of spikes plant^{-1} year^{-1}, flower length and width.

Vanda sp. recorded maximum flower length and width.

Floret size was determined by the length and width of petals (Lokesha and Vasudeva,1994). It is in agreement with the present results in *Vanda* sp. and *Aerides ringens.*

5.1.1.2. Floral Characters (Qualitative Characters) of Parents

Floral characters which were not estimable quantitatively are flower colour, nature of flowering, season of flowering, time of anthesis and time of stigma receptivity among parents.

5.1.1.2.1. Flower Colour

All the parents exhibited dissimilarity with each other with respect to flower colour. This oligogenic trait is solely genotype specific in nature. This is in agreement with the report made in orchids (Griesbach, 2005).

5.1.1.2.2. Nature of Flowering

Among different parents, *Vanda tessellata*, *Vanda* sp., *Arundina graminifolia*, *Spathoglottis albida*, *Spathoglottis unguiculata*, *Spathoglottis plicata* and *Spathoglottis gracilis* were in free flowering nature whereas *Vanda spathulata*, *Rhynchostylis retusa* and *Aerides ringens* were in seasonal flowering nature. In the tropics, seasonality in flowering was mainly due to variation in rainfall (Dressler, 1981). This flowering nature is a genotype specific oligogenic trait in orchids. This is in agreement with the report of Goh (1984).

Both free and seasonal flowering nature was observed among different varieties of *Dendrobium* (Sobhana, 2000; Nath, 2003; Sugapriya *et al.*, 2012).

Inflorescences continually flowered for several months in *Spathoglottis plicata* (Freudenstein and Rasmussen, 1996). This is in close correspondence with the present results in *Spathoglottis plicata.*

5.1.1.2.3. Season of Flowering

Variation observed in the season of flowering among different species is genotype specific and environment sensitive and a physiologically determined trait.

Plants respond to changes in photoperiod and temperature and thus flowering naturally when the environmental conditions are favourable for reproduction (Lopez and Runkle, 2004).

5.1.1.2.4. Time of Anthesis

In almost all the species, anthesis time was observed from early morning to noon except *Vanda tessellata*. The same observation was reported in *Dendrobium* (Sobhana, 2000) while in *Vanda tessellata* anthesis was observed from morning to evening in a day. It is a genetically controlled trait (Sanford, 1971).

5.1.1.2.5. Time of Stigma Receptivity

In all the species of orchids studied, stigma receptivity period lasted for one week only. Similar results were reported in *Spathoglottis plicata* (Proctor, 1998; Shiau *et al*., 2002; Lo *et al*., 2004) and in *Rhynchostylis retusa* (Nitin and Uma, 2014). But time of stigma receptivity varied among different species. In *Arundina graminifolia* stigma receptivity was retained for only one day from morning to evening.

5.1.1.2.6. Pollen morphology

In the family orchidaceae, the pollen grains are present as agglutinated masses called pollinium.

Colour, size and shape of pollinia and size, shape and exine ornamentation of pollen grains varied among different species.

In the present investigation the pollen grains existed as tetrads. This is in agreement with the reports of following researchers (Abraham and Vatsala, 1981; Das and Ghoshal, 1988; Varghese, 1995; Sobhana, 2000).

In all the orchids studied, pollen grains were held together by elastic threads of tapetal origin. It is in line with the contribution of Abraham and Vatsala (1981).

The microscopic observations revealed the presence of perforated exine in *Spathoglottis plicata*. This is in agreement with the report of Freudenstein and Rasmussen (1996). These are genotype dependent traits.

5.1.1.2.7. Pollen Production Pollinium^{-1}

Pollen production varied among different species, in the present study which is a genotype and environment dependent trait. This is in conformation with the report of Nair *et al.* (1964).

5.1.1.2.8. Pollen Fertility Percentage

In general, this trait showed variation among different species studied. The similar result was reported earlier in *Dendrobium* varieties and wild species (Sobhana, 2000). It was the minimum in *Spathoglottis albida* and the maximum in *Aerides ringens* .

5.1.1.2.9. Pollen germination percentage

In accordance with the earlier reports in *Dendrobium* (Sobhana, 2000), in the present study this trait showed variation among different species. It was minimum in *Arundina graminifolia* and maximum in *Vanda* species.

Based on the evaluation of mean performance for different traits, all the species can be selected as elite parents for hybridization.

5.1.2. ANALYSIS OF VARIANCE (ANOVA)

Analysis of variance splits the total variation into different components. It is useful in estimating the different components of variance. The total variation is

divided into two parts such as variation between genotypes and within genotypes. It helps in partitioning of phenotypic variation into genotypic and environmental components. It also permits estimation of coefficients of variation.

Assessment of existing variability for any character present in a crop species is of utmost importance for starting a judicious plant breeding programme. The efficiency of selection largely depends on the extent of genetic variability present in the population.

Components of variability include phenotypic, genotypic and environmental variation, heritability and genetic advance under selection.

Analysis of variance for different biometrical, vegetative and floral characters was carried out as a basis to find out the test of significance and to estimate different components of variance.

Analysis of variance revealed highly significant differences for all polygenic traits included in the evaluation of parents and therefore, different components of variance were estimated.

5.1.2.1. Components of Variance

Phenotypic, genotypic and environmental variances were estimated and the results revealed that phenotypic variance was higher than the genotypic as well as environmental variance for all polygenic traits. Moreover genotypic variance was higher than the environmental variance. These results indicated that the reflecting phenotypic value is mainly relying on the genotypic value and the contribution of non-heritable component is practically little or nil. In this situation, selection of elite parents based on the phenotypic expression alone will lead to yield fruitful results through hybridization in future.

High amount of genotypic variance was reported earlier in orchids (Rehman *et al.*, 1993).

5.1.2.2. Coefficients of Variation

Estimation of phenotypic, genotypic and environmental coefficients of variation from the components of variance was performed for all biometrical characters among different parents.

5.1.2.2.1. Genotypic Coefficient of Variation (GCV)

GCV values were higher than ECV values but lower than PCV values for all traits. Similar results were reported earlier in *Dendrobium* (Sobhana, 2000; Moniruzzaman *et al*., 2012).

GCV was the highest (128.52) for number of spikes plant^{-1}. Out of the sixteen traits, GCV was high for fifteen traits *viz*., plant height, plant spread, shoot girth, number of leaves plant^{-1}, leaf length, leaf width, length of inflorescence, number of flowers inflorescence^{-1}, internodal length, flower length, flower width, days for inflorescence emergence to first flower opening, days to last flower opening from first flower opening and spike longevity including number of spikes plant^{-1} since all the values were more than 20%. But GCV was moderate for days taken for first flowering (18.93) since the value was between 10% and 20% according to the classification of coefficient of variation as suggested by Sivasubramanian and Madhavamenon (1973). In crop improvement only the genetic component of variation is important since only this component will be transmitted to the next generation. Environmental influence plays less role in the expression of the traits. Similar result was reported earlier in orchids by Moniruzzaman *et al*. (2012).

5.1.2.2.2. Environmental Coefficient of Variation (ECV)

ECV was lower than both PCV and GCV. Similar results were reported earlier in *Dendrobium* (Sobhana, 2000; Moniruzzaman *et al*., 2012).

ECV was the highest for number of flowers inflorescence^{-1} (28.83) since it was more than 20%. It was moderate for two traits *viz*., number of leaves plant^{-1} and number of spikes plant^{-1} since the values were between 10 and 20%. But ECV

was low for the remaining thirteen traits *viz*., plant height, plant spread, shoot girth, leaf length, leaf width, days taken for first flowering, length of inflorescence, internodal length, flower length, flower width, days for inflorescence emergence to first flower opening, days to last flower opening from first flower opening and spike longevity because all these values are less than 10% (Sivasubramanian and Madhavamenon, 1973). It indicates that in majority of traits, the contribution of ECV was negligible. Therefore, all these traits are amenable for selection for further improvement.

5.1.2.2.3. Phenotypic Coefficient of Variation (PCV)

PCV was higher than both GCV and ECV for all traits. Similar results were reported earlier in *Dendrobium* (Sobhana, 2000; Moniruzzaman *et al*., 2012).

The highest and the lowest PCV were for number of spikes plant^{-1} (143.93) and days taken for first flowering (24.18) respectively. PCV was high for all traits *viz*., plant height, plant spread, shoot girth, number of leaves plant^{-1}, leaf length, leaf width, length of inflorescence, number of flowers inflorescence^{-1}, internodal length, flower length, flower width, days for inflorescence emergence to first flower opening, days to last flower opening from first flower opening and spike longevity because all these values were more than 20% (Sivasubramanian and Madhavamenon, 1973). It indicates that selection of elite parents on the basis of phenotypic performance alone is a sound procedure for breeding programme since the phenotypic value is contributed by the influence of genotypic value and the contribution of environmental effect is negligible or nil. Environmental influence plays less role in the expression of all these traits in orchids (Moniruzzaman *et al*., 2012).

5.1.2.3. Heritability (Broad Sense)

The extent of contribution of genotype to the phenotypic variation for a trait in a population is expressed as the ratio of genetic variance to the total variance (phenotypic variance) and this ratio is known as heritability. The efficiency of selection largely depends on the heritability of the concerned character.

Heritability was the highest for flower width (99.06 %) and the lowest for number of flowers inflorescence^{-1}(50.21). It was moderate for three traits *viz.*, shoot girth, number of leaves plant^{-1} and number of flowers inflorescence^{-1} since the values were between 30 and 60%. But it was high for the remaining thirteen traits *viz.*, plant height, plant spread, leaf length, leaf width, days taken for first flowering, number of spikes plant^{-1}, length of inflorescence, internodal length, flower length, days for inflorescence emergence to first flower opening, days to last flower opening from first flower opening and spike longevity since the values were more than 60% according to the categorization of heritability values as suggested by Johnson *et al.* (1955). So selection for all these traits is more effective because there would be a close correspondence between the genotype and the phenotype due to a relatively smaller contribution of environment to the phenotype.

Similarly high heritability was reported for various traits in orchid hybrids (Johnson *et al.*, 1955; Rehman *et al.*, 1993; Faroque, 2003) and in *Dendrobium* (Sobhana, 2000; Moniruzzaman *et al.*, 2012).

It is a good index of the transmission of traits from parents to their offspring (Falconer, 1960).

5.1.2.4. Genetic Advance

Improvement in the mean genotypic value of the selected individual over the base population before selection is genetic advance under selection. It mainly depends on the phenotypic variability in the population, heritability of the trait under selection and intensity of selection. Genetic advance was estimated as percentage of mean for various traits.

It was the highest for number of spikes plant^{-1} (239.80) and the lowest for days taken for first flowering (30.54). It was high for all the sixteen traits among parents *viz.*, plant height, plant spread, shoot girth, number of leaves plant^{-1}, leaf length, leaf width, length of inflorescence, number of flowers inflorescence^{-1}, internodal length, flower length, flower width, days for inflorescence emergence

to first flower opening, days to last flower opening from first flower opening and spike longevity because the values were more than 20% according to the classification range of genetic advance as suggested by Johnson *et al.* (1955). It indicates that selection was more rewarding for improvement of all these traits.

High genetic advance selection was reported for various traits in orchid hybrids (Faroque, 2003) and in *Dendrobium* (Moniruzzaman *et al.*, 2012). The effectiveness in the selection of genotypes based on the phenotypic appearance is determined by the magnitude of expected genetic advance also (Johnson *et al.*, 1955).

5.1.2.5. Co-heritability

Co-heritability was estimated from the genotypic and phenotypic components of covariance. Co-heritability was the highest (9.13) between number of leaves plant^{-1} and days taken for first flowering. So selection on number of leaves plant^{-1} will results more increase in the days taken for first flowering.

5.1.2.6. Association Analysis

Direct selection is not effective for polygenic traits with low heritability and indirect selection is worthful in this situation. Association (correlation) analysis provides information about the relative contribution of component traits which in turn help in the selection of superior genotypes from the breeding population.

Association analysis was carried out at three levels as phenotypic, genotypic and environmental correlation coefficient.

5.1.2.6.1. Genotypic Correlation Coefficient (rg)

Genotypic correlation coefficient exhibited highly significant and positive association between five related characters *viz.*, plant spread and leaf length, plant spread and leaf width, plant height and flower length, flower length and flower width and shoot girth and days for inflorescence emergence to first flower opening. Highly significant and negative association was observed between two

related characters *viz.*, plant spread and number of leaves plant^{-1} and number of leaves plant^{-1} and leaf length. It indicates that at genetic level this positive association is due to coupling phase of linkage and negative association is due to repulsion phase of linkage. Contradictory results reported in *Habenaria radiata* (Nagayoshi *et al.*, 1996). Length of flower was positively correlated with breadth of flower as reported in *Dendrobium* (Sobhana, 2000) which is in agreement with the present results. Length of leaves had positive correlation with number of flowers reported in orchids (Faroque, 2003; Sultana, 2003) is in agreement with the present results. Plant height was correlated with leaf number and spike length as reported in orchids (Nair and Tung, 1983; Nolte, 1985; Nair and Fong, 1987; Kamemoto, 1987; Islam *et al.*, 2013; Anna *et al.*, 2015) is also in agreement with the present results.

5.1.2.6.2. Environmental Correlation Coefficient (re)

Environmental correlation coefficient was positive and highly significant between seven related characters such as plant height and number of leaves plant^{-1}, plant spread and leaf length, shoot girth and leaf length, days taken for first flowering and length of inflorescence, flower length and flower width, internodal length and days to last flower opening from first flower opening and leaf width and spike longevity. But it was negative and highly significant for three related characters *viz.*, plant height with days taken for first flowering, number of spikes plant^{-1} and flower width and days to last flower opening from first flower opening and spike longevity. It indicates that at genetic level this positive association is due to coupling phase of linkage and negative association is due to repulsion phase of linkage.

5.1.2.6.3. Phenotypic Correlation Coefficient (rp)

Estimated phenotypic correlation coefficient revealed highly significant and positive association between three related characters such as plant spread and leaf length, plant spread and leaf width and flower length and flower width. Whereas highly significant and negative association was obtained between five related

characters such as plant spread and number of leaves plant^{-1}, number of leaves plant^{-1} and leaf width, shoot girth and flower width, internodal length and days for inflorescence emergence to first flower opening and number of spikes plant^{-1} and spike longevity. It indicates that at genetic level this positive association is due to coupling phase of linkage and negative association is due to repulsion phase of linkage. Length of flower was positively correlated with breadth of flower reported in *Dendrobium* (Sobhana, 2000) is in agreement with the present results. Length of leaves had positive correlation with number of flowers reported in orchids (Faroque, 2003; Sultana, 2003) is in corroboration with the present results. In accordance with the earlier reports in orchids (Nair and Tung, 1983; Nolte, 1985; Nair and Fong, 1987; Kamemoto, 1987; Islam *et al.*, 2013; Anna *et al.*, 2015) plant height was correlated with leaf number and spike length.

5.1.3. HYBRIDIZATION

Hybridization is the most potent conventional technique for evolving elite varieties. The selection of suitable parents is one of the most important steps in hybridization. Parents should be chosen on the basis of genetic value. After selection of parents the breeding procedure to be followed for successful results mostly depends on the genetics of quantitative traits. In plant breeding, hybrids between genotypes of diverse origin or distantly related, generally display a greater heterosis and throw more recombinants than those between closely related parents. Occurrence of natural hybridization among wild members was noticed from the earliest days of orchid collection and cultivation. In distant hybridization genetically unrelated parents are chosen and the hybrids are evaluated in terms of heterosis and superior hybrids are selected for further breeding programme. But in distant hybridization the main difficulties encountered are the lack of compatibility between the parental genomes or between the embryos of two parents. Therefore, it is an important problem to look into distant hybridization of orchids.

Parents belonging to the genera *viz*., *Vanda*, *Rhynchostylis*, *Spathoglottis*, *Aerides* and *Arundina* were selected based on variability in floral characteristics and evaluation to study cross compatibility through hybridization. Hybridization was carried out in seventy seven combinations.

5.2. EXPERIMENT II - CROSS COMPATIBILITY STUDIES OF SELECTED SPECIES

Thirty one combinations successfully produced mature capsules but in three combinations *viz*., *Vanda tessellata* x *Vanda spathulata*, *Spathoglottis albida* x *Vanda* sp. and *Spathoglottis plicata* x *Vanda* sp. capsules fell off after 20, 10 and 9 days of maturity respectively. This result is in agreement with the following report. The ovaries swelled due to pollination stimulus and it might have due to post fertilization barriers or the stimulatory parthenocarpy led to the formation of swollen ovaries (Varghese, 1995).

In the *Vanda tessellata* x *Vanda spathulata* combination capsule development occurred, but embryo tested were nonviable and it indicates that it is a partially compatible combination. But its reciprocal cross was fully compatible and exhibited >90% seed viability. Similar result was reported by Devi and Deka (1992) as follows. Unilateral incompatibility was observed between species of the genus *Dendrobium* and between species of the different genera *viz*., *Dendrobium*, *Spathoglottis*, *Phaius*, *Coelogyne* and *Rhynchostylis*. Intergeneric and interspecific crosses incompatibility may be due to genetic imbalance.

In the *Spathoglottis albida* x *Vanda* sp. and *Spathoglottis plicata* x *Vanda* sp. combinations also capsule development occurred, but embryo tested were nonviable. This also indicates that these two are partially compatible combinations. The stimulatory parthenocarpy led to the formation of swelled ovaries. This is in confirmation with the result reported by Varghese (1995). Seed set failure in reciprocal crosses was due to either physical barriers, antagonism between maternal cytoplasm and sperm nucleus or sensitivity of one of the plants to its environment so that it would block the development or function of gametes

and self-incompatibility and cross sterility encountered in hybrids could be either due to hybrid sterility or polyploidy. The present result is in confirmation with the earlier report (Lenz and Wimber, 1959).

Capsules of *Spathoglottis* crosses attained setting four days after pollination whereas capsules of *Rhynchostylis* crosses attained setting only ten days after pollination. Similarly capsules attained maturity much earlier in all cross combinations where the genus *Spathoglottis was* used as the female parent. But cross combinations where the species *Vanda spathulata* was used as the female parent required more days for capsule maturation. Normally capsule maturity in *Spathoglottis* is one month and in *Vanda spathulata* 150-180 days, depending on the genotype specificity. The reason for this was explained as time taken for pod maturity varied from 90 to 135 days and more days for culturing ranged from 120 to 135 in crosses involving wild species as male parent. Embryo did not turn green even after 3 months when 60 days old pod was cultured. When seeds of different hybrids were cultured, some seeds did not germinated and some germinated seeds did not develop into seedlings (Varghese, 1995).

Length of capsule was the maximum in the cross of *Vanda spathulata* x *Rhynchostylis retusa* and the minimum in the combination involved *Aerides ringens* as female parent. Width of capsule was the maximum in the cross of *Vanda spathulata* x *Vanda* sp. and the minimum in the combination involved *Aerides ringens* as female parent. Normally the pods of *Aerides ringens* are small in size.

Percentage of filled seeds was the maximum in three crosses *viz*., *Vanda spathulata* x *Rhynchostylis retusa*, *Spathoglottis albida* x *Spathoglottis unguiculata* and *Spathoglottis albida* x *Spathoglottis plicata* and the minimum in the cross *Arundina graminifolia* x *Spathoglottis unguiculata.* It indicates that the combination *Arundina graminifolia* x *Spathoglottis unguiculata* is exhibiting hybrid sterility due to incompatibility between two parental species. Parthenocarpic development of ovary was brought about by the high auxin

content of the pollinia (Laibach and Maschmann,1933). Auxin content of the pollinia stimulated ethylene production and initiated incompatibility of parents (Johansen, 1990).

5.3. EXPERIMENT III - *IN VITRO* CULTURE OF HYBRID SEEDS

Orchid seeds are difficult to germinate naturally because the seeds lack stored food and the embryo is virtually naked. In distant hybridization incomplete embryo development may occur at the time of seeds maturity. To overcome all these problems, *in vitro* seed culture is inevitable and attains *prima facie* importance in orchid hybridization. Young or mature embryos or seeds are excised from sterilized pods and placed on suitable nutrient medium aseptically. The embryos or seeds develop into seedlings directly or through callus formation. This method is desirable to recover hybrid progenies from distant hybridization.

First visible sign of seed germination was the swelling of embryo followed by greening and emergence out of the seed coat and later developed into protocorm and differentiated into shoot meristem and rhizoids in opposite directions. Leaves were produced from green protocorm. Real roots were formed later. This has explained in detail by Singh (1993).

Mature undehisced green capsules of hybrids developed were used for culturing seeds under *in vitro* condition. Out of thirty four crosses, seeds of sixteen crosses exhibited signs of greening. The cross *Spathoglottis unguiculata* x *Spathoglottis albida* attained seed germination stage seventeen days after inoculation and the cross *Arundina graminifolia* x *Spathoglottis plicata* attained seed germination 320 days after inoculation. *In vitro* seed / embryo germination requirements are influenced by capsule maturity, genetic potential and specificity of the species. Seeds isolated from undehisced capsules germinated better and earlier than the dehisced one. This result is in agreement with the earlier reports (Arditti *et al.*, 1981; Sharma, 1998). The highest germination was occurred when cultured seeds containing fully matured embryo from the pods of age 30 days to 150 days. This is also in line with the report of Varghese (1995). Seeds isolated

and cultured from burst capsules exhibited poor germination compared to the unburst capsules in *Spathoglottis* and *Arundina* species. This result is in agreement with report of Sharma, 1998 in *Vanda*. Seed culture stage varied with the genus, species and hybrid and pod maturity time varied with the genus and species crossed. This is in confirmation with the report made by Arditti (1982) and Hegde (1984) in *Dendrobium*.

Seeds of six crosses germinated and developed into protocorm and seedlings in MS medium supplemented with growth regulators, BAP and IBA at lower concentrations.

Contradictory to the forgoing results, embryos of two intergeneric hybrids namely *Spathoglottis plicata* x *Dendrobium densiflorum* and *Dendrobium muschatum* x *Arundina bambusifolia* germinated with very high frequency to form protocorms in Nitsch medium (Talukdar *et al*., 2002).

The cross *Spathoglottis unguiculata* x *Spathoglottis albida* produced first leaf 38 days after inoculation into the medium but *Spathoglottis unguiculata* x *Vanda* sp. produced first leaf 125 days after inoculation. *Spathoglottis unguiculata* x *Spathoglottis albida* cross attained shoot initiation 68 days after inoculation whereas *Spathoglottis unguiculata* x *Vanda* sp. attained shoot initiation 153 days after inoculation. *Spathoglottis unguiculata* x *Spathoglottis albida* attained initiation of first root 89 days after inoculation but the cross *Spathoglottis unguiculata* x *Vanda* sp. attained root initiation stage 213 days after inoculation. Of these, *Spathoglottis unguiculata* x *Spathoglottis plicata* cross was ready for deflasking 192 days after inoculation. But the cross *Spathoglottis albida* x *Spathoglottis unguiculata* attained deflasking stage 363 days after inoculation. This indicated that crosses in which *Spathoglottis* spp. was used as female parent exhibited early seed germination, protocorm and seedling development as compared to other crosses.

Days required for attaining different seed germination stages has been explained in detail by Das *et al.* (2008) in *Cymbidium longifolium*, *Dendrobium aphyllum* and *Rhynchostylis retusa*.

A lower concentration of growth regulators enhanced seed germination in liquid media but higher level of growth regulators promoted subsequent seedling growth in *Spathoglottis plicata*.

Many authors have observed similar results in various wild species and interspecific and intergeneric hybrids *viz.* *Spathoglottis plicata Arundina graminifolia*, *Cymbidium aloifolium*, *Cymbidium pendulum*, *Dendrobium moschatum* x *Arundina graminifolia*, *Dendrobium infundibulum* x *Arundina graminifolia*, *Rhynchostylis retusa* and *Spathoglottis plicata* (Sangama and Singh, 1994).

5.3.1. Evaluation of hybrid seedlings

Hybridization programme will be successful only if the hybrid progenies are well established in the field. In orchids even though pods could be successfully developed through hybridization, the seedlings planted out in the field may not live beyond a few days. Therefore, frequent evaluation would be helpful in the selection of superior cultures for further improvement.

5.3.1.1. Morphological characters (at monthly interval)

Seedlings of seven crosses were planted out for hardening. Morphological characters were recorded at monthly intervals. In the cross *Spathoglottis albida* x *Spathoglottis unguiculata* (culture 778 and 1368), seedling height increased gradually but number of leaves remained same as at deflasking, one month after deflasking and two months after deflasking. Leaf length and width also increased at monthly intervals. Similar growth performance was exhibited by the remaining five crosses *viz.*, *Spathoglottis unguiculata* x *Spathoglottis albida* (culture 622), *Spathoglottis unguiculata* x *Vanda* sp. (culture 679), *Spathoglottis plicata* x *Spathoglottis unguiculata* (culture 1386), *Spathoglottis unguiculata* x

Spathoglottis plicata (culture 1388) and *Spathoglottis gracilis* x *Spathoglottis unguiculata* (culture 652).

Increased plant height, leaf length and width was reported earlier in *Cymbidium aloifolium* x *Cymbidium bicolour* interspecific hybrids in green house condition (Krishna, 2015).

5.4. EXPERIMENT IV - MOLECULAR CHARACTERIZATION

Analysis of genetic variation in plants has been traditionally assessed by analysis of morphological or biochemical traits. The analysis of plant DNA allows the direct assessment of variation in plants. PCR based marker systems are applied to assess genetic diversity in populations in order to supplement and refine the morphological based classification. Molecular markers based on DNA polymorphism are more informative, independent of environmental conditions and unlimited in number (Agarwal *et al.*, 2008).

Generally to establish genetic stability or genetic variability in various plant species RAPD or ISSR or both of the markers have been successfully applied (Arnau *et al.*, 2003). ISSR molecular markers are effective in assessing interspecific variation among hybrid populations (Allendorf and Luikart, 2007).

5.4.1. ISSR assay

The highest percentage of polymorphism between male and female parents (95.83%) was achieved using ISSR 841, ISSR 849 and ISSR 810 primers. Similarly primers displayed the highest percentages of polymorphism between the hybrid and female parents were ISSR 841, ISSR 849 and ISSR 810.

5.4.2. Confirmation of hybridity

ISSR molecular markers are effective in assessing interspecific variation among hybrid populations (Allendorf and Luikart, 2007). To confirm the hybrid purity, only the primers which amplified bands specific to the female parent of each hybrid were considered. The highest total values were found for two hybrids

viz., *Spathoglottis unguiculata* x *Spathoglottis plicata* [SU1388 X SP1388 (52.72%)] and *Spathoglottis albida* x *Spathoglottis unguiculata* [SA1368 X SU1368 (42.11%)]. The percentage of discriminative ISSR loci for the female parent was 17%, while for the female parent the discriminative loci ranged from 0- 15%. Inconsistent percentage of bands was evident in the hybrids but not in the parents. These recombinant bands were obtained majorly with the primers, 841, 849 and 810. The percentage of polymorphism between male and female parents could reveal the genetic difference between male and female parents in each cross combination. When considering all ten primers, the highest percentage of polymorphism between male and female parents (73.83%) was found in the *Spathoglottis albida* x *Spathoglottis unguiculata* [SA778 X SU778] cross; the next highest was the *Spathoglottis albida* x *Spathoglottis unguiculata* [SA1368 X SU1368 (71.97%)] .

Similarly 23.26% of molecular variance was exhibited in *Cattleya granulosa*, an endangered Brazilian orchid and four other native Brazilian species (Cristiane, 2014), 50-100% variation in polymorphism among the *Aerides maculosum* (Parab *et al.*, 2008) and 73% similarity and separated thirty-one *Dendrobium* species into six clusters (Wang *et al.*, 2009b).

ISSR primers were able to differentiate the hybrids from the parents and male parents from the female parents. Primers were successful in identifying hybrids in seven crosses. Primers 810, 841and 849 can be further utilized to identify hybrids from a mass cultivation experiment using these parental crosses.

The above study revealed that through distant hybridization in wild orchids, one intergeneric hybrid *Spathoglottis unguiculata* x *Vanda* sp. and six interspecific hybrids *viz.*, *Spathoglottis unguiculata* x *Spathoglottis albida*, *Spathoglottis gracilis* x *Spathoglottis unguiculata*, *Spathoglottis albida* x *Spathoglottis unguiculata*, *Spathoglottis albida* x *Spathoglottis unguiculata*, *Spathoglottis plicata* x *Spathoglottis unguiculata*, *Spathoglottis unguiculata* x

Spathoglottis plicata could be obtained and molecular characterization confirmed the hybridity of the intergeneric and interspecific hybrids.

Therefore, the intergeneric and six interspecific F_1 hybrids evolved through distant hybridization can carry forward for the development of varieties or can be utilised for genetic improvement programme in future.

SUMMARY

6. SUMMARY

India is blessed with rich repository of wild orchids' germplasm in the forest ecosystem. Wild species may contribute valuable "yield genes" to the cultivated species. But utilization of these valuable genetic resources for developing the commercial varieties through distant hybridization is seldom attempted in Kerala. To achieve this goal it is essential to look into the possibility of cross compatibility of different genus and species. In this backdrop, the present investigation entitled "Distant hybridization and compatibility studies in wild orchids" was carried out with the objective of evaluating the cross compatibility through distant hybridization among wild orchids and molecular characterization of hybrid seedlings using RAPD/ISSR – markers.

The experiment was conducted at Saraswathy Thangavelu Extension Centre of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (STEC-JNTBGRI), Puthenthope, Thiruvananthapuram and at College of Agriculture, Vellayani, Thiruvananthapuram during 2015-2018. The wild orchid genera /species available at the orchid conservatory of STEC-JNTBGRI *viz.*, *Vanda tessellata*, *Vanda* sp., *Vanda spathulata*, *Rhynchostylis retusa*, *Aerides ringens*, *Arundina graminifolia*, *Spathoglottis albida*, *Spathoglottis unguiculata* , *Spathoglottis plicata* and *Spathoglottis gracilis* were selected for evaluating its cross compatibility. To test the compatibility of wild species with a hybrid, first *Vanda* hybrid, *Vanda* Miss Joaquim which was derived from the cross between *Vanda tessellata* and *Vanda cooperi* was used as a third parent under the genus *Vanda*.

The present investigation included four experiments *viz.*, (i) evaluation and selection of parents based on the study of genetics of polygenic traits in terms of components of variance, heritability, genetic advance and correlation; (ii) cross compatibility study of different parental species; (iii) culturing of hybrid seeds for developing seedlings and (iv) evaluation of seedlings based on morphological and molecular characterization.

The experimental design used was Completely Randomized Design (CRD) with six treatments, each replicated five times with five plants replication^{-1}. Parents were evaluated based on variability in plant (vegetative) and floral characters at the time of flowering.

Significant range of variation was recorded among different parents for different vegetative and floral characters *viz*., plant height, plant spread, shoot girth, number of leaves plant^{-1}, leaf length, leaf width, days taken for first flowering, number of spikes produced plant^{-1} year^{-1}, length of inflorescence, number of flowers inflorescence^{-1}, internodal length, flower length, flower width, days for inflorescence emergence to first flower opening and spike longevity.

Analysis of variance revealed highly significant differences for all polygenic traits included in the evaluation of parents. Phenotypic, genotypic and environmental variances revealed that phenotypic variance was higher than the genotypic as well as environmental variance for all polygenic traits. Moreover genotypic variance was higher than the environmental variance. Phenotypic (PCV), genotypic (GCV) and environmental coefficients of variation (ECV) showed that GCV values were higher than ECV values but lower than PCV values for all traits. GCV was the highest for number of spikes plant^{-1}. PCV was higher than both GCV and ECV for all traits. The highest and the lowest PCV were for number of spikes plant^{-1} and days taken for first flowering respectively. Heritability was the highest for flower width and the lowest for number of flowers inflorescence^{-1}. Genetic advance was the highest for number of spikes plant^{-1} and the lowest for days taken for first flowering. Co-heritability was the highest between number of leaves plant^{-1} and days taken for first flowering. Among association analysis, genotypic correlation coefficient (*r*g) exhibited highly significant and positive association between five related characters *viz*., plant spread and leaf length, plant spread and leaf width, plant height and flower length, flower length and flower width and shoot girth and days for inflorescence emergence to first flower opening. Environmental correlation coefficient was positive and highly significant between seven related characters. Phenotypic

correlation coefficient revealed highly significant and positive association between three related characters such as plant spread and leaf length, plant spread and leaf width and flower length and flower width.

Floral characters (Qualitative characters) of parents which were not estimable quantitatively such as flower colour, nature of flowering, season of flowering, time of anthesis, and time of stigma receptivity exhibited variation. Pollen production pollinium^{-1} was maximum in *Vanda spathulata* and minimum in *Rhynchostylis retusa*. Pollen fertility percentage was more in *Aerides ringens* and less in *Spathoglottis* spp.. High pollen germination percentage was recorded in two parents *viz*., *Spathoglottis plicata* and *Aerides ringens* and low in *Arundina graminifolia*.

Parents belonging to the genera *Vanda*, *Rhynchostylis*, *Spathoglottis*, *Aerides* and *Arundina* were selected based on the evaluation of variability in vegetative and floral characteristics. Hybridization was carried out in seventy seven combinations. Of these, thirty one combinations successfully produced mature capsules but in three combinations viz., *Vanda tessellata* x *Vanda spathulata* , *Spathoglottis albida* x *Vanda* sp. and *Spathoglottis plicata* x *Vanda* sp. capsules fell off after 20, 10 and 9 days of maturity respectively. Out of these successful combinations, capsule settings was observed four days after pollination in all *Spathoglottis* crosses whereas in *Rhynchostylis* crosses capsule setting occurred only ten days after pollination. Similarly, capsules maturity period was 30 days in all cross combinations when the genus *Spathoglottis* was used as the female parent. But when *Vanda spathulata* was used as the female parent the capsule maturity period was 150-154 days. Length of the capsule was the maximum in the cross of *Vanda spathulata* x *Rhynchostylis retusa* and the minimum in the combination involved *Aerides ringens* as female parent. Width of capsule was the maximum in the cross of *Vanda spathulata* x *Vanda* sp. and the minimum in the combination involved *Aerides ringens* as female parent. Percentage of filled seeds was the maximum in three crosses viz., *Vanda spathulata* x *Rhynchostylis retusa*, *Spathoglottis albida* x *Spathoglottis*

unguiculata and *Spathoglottis albida* x *Spathoglottis plicata* and the minimum in the cross *Arundina graminifolia* x *Spathoglottis unguiculata.*

Capsules of thirty four crosses were used to culture seeds under *in vitro* condition for germination and seedling development. Seeds of sixteen crosses exhibited signs of greening. The cross *Spathoglottis unguiculata* x *Spathoglottis albida* the sign of seed germination started after seventeen days of inoculation and the cross *Arundina graminifolia* x *Spathoglottis plicata* seed germination was noticed only after 320 days of inoculation. Seeds of six crosses germinated and developed into protocorm and seedlings. The cross *Spathoglottis unguiculata* x *Spathoglottis albida* produced first leaf 38 days after inoculation but *Spathoglottis unguiculata* x *Vanda* sp. produced first leaf 125 days after inoculation. *Spathoglottis unguiculata* x *Spathoglottis albida* cross showed shoot initiation 68 days after inoculation whereas *Spathoglottis unguiculata* x *Vanda* sp. shoot initiation noticed only after 153 days of inoculation. *Spathoglottis unguiculata* x *Spathoglottis albida* root initiation was noted after 89 days of inoculation but the cross *Spathoglottis unguiculata* x *Vanda* sp. root initiation stage observed after 213 days of inoculation. Of these, *Spathoglottis unguiculata* x *Spathoglottis plicata* cross was ready for deflasking after 192 days of inoculation. But the cross *Spathoglottis albida* x *Spathoglottis unguiculata* attained deflasking stage after 363 days of inoculation.

Seedlings of seven crosses were planted out for hardening. Morphological characters were recorded at monthly intervals. In the cross *Spathoglottis albida* x *Spathoglottis unguiculata* (culture 778 and 1368), seedling height increased gradually but number of leaves remained same even two months after planting of the seedlings into green-house for hardening. Leaf length and width also increased at monthly intervals. Similar growth performance was exhibited by the remaining all five crosses *viz.*, *Spathoglottis unguiculata* x *Spathoglottis albida* (culture 622), *Spathoglottis unguiculata* x *Vanda* sp. (culture 679), *Spathoglottis plicata* x *Spathoglottis unguiculata* (culture 1386),

Spathoglottis unguiculata x *Spathoglottis plicata* (culture 1388) and *Spathoglottis gracilis* x *Spathoglottis unguiculata* (culture 652).

Hybrids from seven crosses were verified using ISSR markers. In the confirmation of hybrid purity the highest percentage of polymorphism was found for two hybrids *viz.*, *Spathoglottis unguiculata* x *Spathoglottis plicata* [SU1388 X SP1388 (52.72%)] and *Spathoglottis albida* x *Spathoglottis unguiculata* [SA1368 X SU1368 (42.11%)]. For all cross-combinations, the percentage of discriminative loci for the female parent was higher than for the male parent; the percentage of discriminative ISSR loci for the female parent was 17%, while for the male parent the discriminative loci ranged from 0-15%. Inconsistent percentage of bands was evident in the hybrids but not in the parents. These recombinant bands were obtained mainly with the primers, 841, 849 and 810. When considering all ten primers, the highest percentage of polymorphism between male and female parents was found in the *Spathoglottis albida* x *Spathoglottis unguiculata* [SA778 X SU778] followed by *Spathoglottis albida* x *Spathoglottis unguiculata* [SA1368 X SU1368].

The above study revealed that through distant hybridization in wild orchids, one intergeneric hybrid *Spathoglottis unguiculata* x *Vanda* sp. and six interspecific hybrids *viz.*, *Spathoglottis unguiculata* x *Spathoglottis albida*, *Spathoglottis gracilis* x *Spathoglottis unguiculata*, *Spathoglottis albida* x *Spathoglottis unguiculata*, *Spathoglottis albida* x *Spathoglottis unguiculata*, *Spathoglottis plicata* x *Spathoglottis unguiculata*, *Spathoglottis unguiculata* x *Spathoglottis plicata* could be obtained and molecular characterization confirmed the hybridity of the intergeneric and interspecific hybrids.

Therefore, the intergeneric and six interspecific F_1 hybrids evolved through distant hybridization can be recommended for the development of varieties or can be utilised for genetic improvement programme in future.

REFERENCES

7. REFERENCES

Abraham, A. and Vatsala, P. 1981. *Introduction to Orchids with Illustrations and Descriptions of 150 South Indian Orchids*. Tropical Botanic Garden and Research Institute, Thiruvananthapuram, India, Kerala, 533p.

Agarwal, M., Shrivastava, N., and Padh, H. 2008. Advances in molecular techniques and their applications in plant sciences. *Plant Cell Rep.* 27:617-631.

Ahmadi, A., Azadfar, D., and Mofidabadi, A.J. 2010. Study of inter-generic hybridization possibility between *Salix aegyptica* and *Populus caspicato* achieve new hybrids. *Int. J. Plant Prod.* 4(2): 143-147.

Allendorf, F.W. and Luikart, G. 2007. *Conservation and the Genetics of Populations*. Blackwell, London, 642 p.

Anna, E., Vojtkó, J.S., Balázs, A. L., and Attila, M.V. 2015. Factors affecting reproductive success in three entomophilous orchid species in Hungary, *Acta Biol. Hung.* 66(2): 231-241.

Anuradha, S. and Gowdha, J.V. 1994. Correlation studies in *Gladiolus*. *In: Floriculture -Technology, Trades and Trends*. (eds.) J. Prakash and K. R. Bhandry, Oxford and IBH Publishing Co. Pvt. Ltd. Calcutta, pp. 285-287.

Arcade, A., Anselin, F., Rampant, F.P., Lesage, M.C., Paques, L.F., and Prat, D. 2000. Application of AFLP, RAPD and ISSR markers to genetic mapping of European and Japanese larch. *Theor. Appl. Genet.* 100:299-307.

Archibald, J.K., Mort, M.E., Crawford, D.J., and Santos-Guerra, A. 2006. Evolutionary relationships within recently radiated taxa: Comments on methodology and analysis of inter-simple sequence repeat data and other hypervariable dominant markers. *Taxon.* 55 (3):747-756.

Arditti, J. 1967. Factors affecting the germination of orchid seeds. *Bot. Rev.* 33:1-97.

Arditti, J. 1979. Aspect of the physiological of Orchids. *Adv. Bot. Rev.*33: 1-97.

Arditti, J. 1982. *Orchid Biology - Reviews and Perspectives II.* Comstock Publishing Association, Cornell University Press, London, p. 273-274.

Arditti, J. 2008. *Micropropagation of Orchids*, 2nd edn. Blackwell, Cambridge.

Arditti, J., Michaud, I.D., and Oliva, A.P. 1981. Seed germination of North American Orchids. *Bot. Gaz.* 142:442-453.

Arisumi, T. 1974. Chromosome numbers and breeding behavior of hybrids among Celebes, Java, and New Guinea species of *Impatiens* L. *Hortic. Sci.* 9:478-479.

Arisumi, T. 1975. Phenotypic analysis of artificial and natural amphidiploid cultivars of New Guinea and Indonesian species of *Impatiens* L. *J. Am. Soc. Hortic. Sci.* 100: 381–383.

Arnau, G., Lallemand, J., and Bourgoin, M. 2003. Fast and reliable strawberry cultivar identification using inter simple sequence repeat (ISSR) amplification. *Euphytica*, 129, 69-79.

Ascher, P.D. 1973. The effect of pre-pollination stylar flush on pollen tube growth in heat treated styles of *Lilium longiflorum*Thunb. incompatibility. *Newsl.* 3:4-6.

Avila-Diaz, I. and Oyama, K. 2007. Coservation genetics of an endemic and endangered epiphytic *Laelia speciosa* (Orchidaceae). *Am.J. Bot.* 94(2):184-193.

Bakul, B. and Shahinul Islam, S.M. 2014. Effects of plant growth regulators on multiple shoot induction in *Vanda tessellata* (Roxb.) Hook. ex G. Don an endangered medicinal orchid. *Int. J. Sci. Nat.* 5 (4): 707-712.

Bansal M., Kumar, A., and Reddy, M.S. 2014. Diversity among wild accessions of *Bacopa monnieri* (L.) Wettst. and their morphogenetic potential. *Acta Physiol. Plant,* 36:1177-1186.

Baral, S.R. and Kurmi, P.P. 2006. *A Compendium of Medicinal Plants of Nepal*. Publisher Rachana Baral, Printed in Nepal by Mass Printing Press, Kathmandu. 55p.

Barman, D., Basak, J., Rai, B., Devadas, R., Nagarase, V., and Medhi, R.D. 2007. Performance of *Cymbidium* hybrids in Mid hill situation of Sikkim. *J. Orn. Hortic.* 10 (1):30-33.

Basha, S.K.M., Umamaheswari, P., Rajyalakshmi, E., Rambabu, M., and Pullaiah, T. 2012. Medicinal flora of Penusila Narasimha Sacred Grove, Eastern Ghats, SPSR Nellore District, Andhra Pradesh, India. *Indian J. Fundamental Appl. Life Sci.* 2(2):334-344.

Basiran, M.N. and Ariffin, S. 2001. Molecular techniques as complementary in orchid mutagenesis.Proceedings of the FNCA workshop on plant mutation breeding. *Molecular Biological Technique-August, 20*-27, Bangkok, Thailand, pp.90-102.

Bhattacharjee, S.K., Pushkar, S., and Kumar, P.N. 2002. Passport data of flower crops germplasm. *AICRP on Floriculture*. Technical Bulletin No. 23, Indian Council of Agricultural Research, New Delhi. 210p.

Bindiya, P.I., Ritu, T.B., Prathibha, S., and Shagufta, K. 2013. Effect of different pH on *in vitro* seed germination of *Vanda tessellata* (Roxb.) Hook. ex G. an endangered medicinal orchid. *Adv. Life Sci. Technol.* (On line) 8.

Bopaiah, A.K. and Jorapur, S.M. 1986. Studies on growth and development of *Cymbidium aloifolium* Sw. seedlings *in vitro*. *Biology, Conservation and Culture of Orchids* (ed. Vij, S.P.). The Orchid Society of India, Chandigarh, pp. 423-427.

Borba, E.L., Felix, J.M., Solferini,V.N., and Semir, J. 2001. Fly-pollinated *Pleurothallis* (Orchidaceae) species have high genetic variability: Evidence from isozyme markers. *Am. J. Bot.*88:419-428.

Bose, T.K. and Bhattacharjee, S.K. 1980. *Orchids in India*. Naya Prakash Publishers, Calcutta, West Bengal,India, 538p.

Bose, T.K. and Yadav, L.P. 1989. *Commercial Flowers*. Nayaprokash, Calcutta, India, pp.151-195.

Bose, T.K., Bhattacharjee, S.K., Das, P., and Basak, U. C. 1999. *Orchids of India*. Naya Prokash, Kolkatta, 487p.

Buitendijk, J.H., Pinsonneaux, N., Van Donk, A. C., Ramanna, M. S., and Van Lammeren, A.A.M. 1995. Embryo rescue by half-ovule culture for the production of interspecific hybrids in *Alstroemeria*. *Sci. Hortic.* 64:65-75.

Burton, G.W. 1952. Quantitative inheritance in grasses. *Proc.6th Int. Grassland Cong.* 1:227-83.

Chadwick, A.V., Nyman, L. P., and Arditti, 1. 1986. Sites of ethylene evolution in orchid flowers. *Lindleyana*, 1:164-168.

Chauhan, N.S. 1999. *Medicinal and Aromatic Plants of Himachal Pradesh*. Indus Publishing Company, New Dehli.

Chen, S.C. and Tsi, Z. H. 2000. *The Orchards of China, 2nd edition*. The Chinese Forestry Press, Beijing.

Chi, H.S. 2000. Interspecific crosses of lily by *in vitro* pollinated ovules. *Bot. Bull. Acad. Sci.* 41:143-149.

Christensen, D.E. 1992. Notes on the reproductive biology of *Stelis argentata* Lindl. (Orchidaceae: *Pluerothallidinae*) in Eastern Ecuador. *Lindleyana,* 7(1):28-33.

Chung, J.D. and Chun, C.K. 1983. Asymbiotic germination of *Cymbidium ensifolium*. I - Effect of basal media and growth regulators on germination

of seeds and shoot emergence from rhizomes. *J. Korean Soc. Hortic. Sci.* 24: 236-242.

CITES. 1973. *The Convention on International Trade in Endangered species of Wild Fauna and Flora.* Identification manual.

Colombo, C., Second, G. and Charrier, A. 2000. Diversity within American cassava germplasm based on RAPD markers. *Genet. Mol. Biol.* 23(1):189-199.

Cristiane, G., Fa'bio de, A.V., and Wagner, F. M. 2014. Interspecific genetic analysis of orchids in Brazil using molecular Markers. *Plant Syst. Evol.* 300:1825–1832.

Dadlani, N.K., More, T.A., and Singh, B. 1988. Studies on pollen fertility and viability in *Jasminum*. *Ann. Agric. Res.* 9(2): 247-251.

Dafni, A. and Firmage, D. 2000. Pollen viability and longevity: Practical ecological and evolutionary implications. *Plant Syst. Evol.* 222(1-4):113–132.

Das, A. and Ghoshal, K.K. 1988. Breeding behaviour of some *Dendrobium* species in plains of West Bengal. *J. Orchid Soc. India*, 2(1-2):33-37.

Das, A. K., Das, J., Gogoi, H. K., and Srivastava, R.B. 2008. Mass propagation of orchids through *in vitro* seed culture technology. *J. Cell Tiss. Res.* 8(2):1585-1588.

Das, S.P. and Bhattacharjee, S.K. 2004. *Orchids: In Advances in Ornamental Horticulture* (Ed. Bhattacharjee, S. K.), 2: pp 1-65.

De Jeu, M.J. and Jacobsen, E. 1995. Early post-fertilisation ovule culture in *Alstroemeria* L and barriers to interspecific hybridisation. *Euphytica*, 86:15-23.

Deb, C.R. and Imchen. 2010. An efficient *in vitro* hardening technique of tissue culture raised plants. *Biotechnol.* 9(1): 79-83.

Dellaporta, S.L., Wood, J., and Hicks, J.B. 1983. A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter*, 1(4). pp. 19-21.

Devi, J. and Deka, P.C. 1992. Pollen viability, stigma receptivity and cross-compatibility of some Indian orchids. *J. Orchid Soc. India*, 6(1-2):79-84.

Devi, J. and Deka, P.C. 1994. Embryo culture of orchid hybrids. *Advances in Plant Tissue Culture in India.* (ed. Pramod-Tandon). Pragathi Prakashan, Meerut, pp.51-59.

Devi, N.P., Lisipriya, B., and Narmatha, B. 2015. Asymbiotic seed germination and mass multiplication of *Taprobanea spathulata* (L.) Christenson (Asparagales: Orchidaceae): a medicinally important epiphytic orchid. *Brazilian J. Biol.Sci.* 2(4): 271-286.

Dhamankar, V.S. 1992. Molasses, a source of nutrients for *in vitro* sugar cane culture. *Sugar Cane*, 4:14- 15.

Dipika, S., Swathi, K., Monoj, S., Brijesh, K., Tapas, M., and Nirmal, M. 2017. A Review on: *In vitro* Cloning of Orchids. *Int. J. Curr. Microbiol. App. Sci.* 6(9): 1909-1927.

Doyle, J. 1995. Black Market Orchids / A global underground smuggling network may drive some rare species into extinction. Available online https://www.sfgate.com/crime/article/Black-Market-Orchids-A-global-underground-3048840.php (27 Sept., 2018).

Dressler, R.L. 1981. *The Orchids, Natural History and Classification.* Harvard University Press, Cambridge, USA, 171p.

Dressler, R.L. 1993. *Phylogeny and Classification of the Orchid Family.* Cambridge University Press, London. pp 59-82.

Druart, P.H. and Wulf, O. 1993. Activated charcoal catalyses sucrose hydrolysis during autoclaving. *Plant Cell Tiss. Organ Cult.* 32: 97- 99.

Duncan, R.E. and Curtis, I.T. 1943. Growth of fruits in *Cattleya* and allied genera in the Orchidaceae. *Bull. Torrey Bot. Club* 70: 104-119.

Endress, P. K. 1994. *Diversity and Evolutionary Biology of Tropical Flowers.* Cambridge University Press.

Falconer, D.S. 1960. *Introduction to Quantitative Genetics.*Longman, New York.

Faroque, A.A. 2003. Study on the variabilities, correlation and morphological characteristics of different local orchids. M.Sc. thesis , BAU, Mymensingh, Bangladesh, pp 4-90.

Freudenstein, J.V. and Rasmussen, F.N. 1996. Pollinium development and number in the Orchidaceae. *Am. J. Bot.* 83: 813-824.

Garay, L. 1972. On the systematics of the monopodial orchids, *Bot. Mus. Leafl.* Harvard University, 23(4):149-212.

Gayatri, M.C. and Kavyashree, R. 2015. *Plant Tissue culture Protocols in Plant Biotechnology.* Narosa Publishing House, New Delhi. pp.12.1-12.8.

George, E.F. 1996. Plant propagation by tissue culture Part 2. Exegetics Ltd., Edington, England.

Godwin, I.D., Aitken, E.A., and Smith, L.W. 1997. Application of inter-simple sequence repeats (ISSR) markers to plant genetics. *Electrophoresis*, 18:1524-1528.

Goh, C.J. 1977. Regulation of floral initiation and development in orchid hybrid *Aranda* Deborah. *Ann. Bot.* 41:763-769.

Goh, C.J. and Arditti, J. 1985. *Orchidaceae, Handbook of Flowering:* 1.CRC Press, pp.309-336.

Goh, C.J. 1984. Physiology of flowering in orchids. In: Tan, K.N. (ed.), *Proceedings of the Eleventh World Orchid Conference, Florida*, pp.166-173.

Gordinez, E.S. 1996. Trends in the phenology of flowering in the Orchidaceae of Western Mexico. *Biotropica,* 28(1):130-136.

Gravendeel, B., Smithson, A., Slik, F.J.W., and Schuiteman, A. 2004. Epiphytism and pollinator specialization: drivers for orchid diversity?. *Philos. Trans. Royal Soc. London Ser. B, Biol. Sci.* 359: 1523–1535.

Griesbach, R.J. 2005. A Scientific approach to breeding blue orchids: exploring new frontiers in search of elusive flower colours. *Orchids.* 74(5): 378-379.

Gupta, P.D., Forkan, M.A., and Bhadra, S.K. 1997. Micropropagation through seeds and shoot segments in *Dendrobium crepida. Plant Tiss. Cult.* 8(1):1-10.

Gupta, M., Chyi,Y.S., Romero-Severson, J., and Owen, J.L. 1994. Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple sequence repeats. *Theor. Appl. Genet.* 89: 998-1006.

Habiba, S.U., Shimasaki, K., Ahasan, M., and Alam, M. 2014. Effect of different cytokinins on *in vitro* organogenesis in protocorm-like bodies (PLBs) of *Epidendrum* 'Rouge Star No. 8'. Middle-East *J. Sci. Res.* 21:1843-1847.

Hadley, H.H. and Openshaw, S.J. 1980. Interspecific and intergeneric hybridisation. In: W.F.R. Fehr and H.H. Hadley. W.I. Madison (eds.), *Hybridisation of Crop Plants.* 696 p.

Hao, G., Lee, D.H., Lee, J.S., and Lee, N.S. 2002. A study of taxonomical relationships among species of Korean *Allium* sect. Sacculiferum (Alliaceae) and related species using inter-simple sequence repeat (ISSR) markers. *Bot. Bull. Acad. Sci.* 43:63-68.

Harlan, J.R. 1976. Genetic resources in wild relatives of crops. *Crops. Sci.*16:329-333.

Hegde, S.N. 1984. *Orchids of Arunachal Pradesh.* Forest Department, Itanagar, Arunachal Pradesh, India. 85p.

Helena, V., Mathews, and Rao, P.S. 1980. *In vitro* multiplication of *Vanda* hybrids through tissue culture technique. *Plant Sci. Lett.* 17(3): 383-389.

Henrich, I.E., Stimart, D.P., and Ascher, P.D. 1981. Terrestrial orchid seed germination *in vitro* on a defined medium. *Am. Soc. Hortic. Sci.*106:193-196.

Hsiao, S.C., Huang, W.T., and Lin, M.S. 2010. Genetic diversity of *Balanophora fungosa* and its conservation in Taiwan. *Bot. Stud.* 51: 217-222.

Indhumati, K., Kannan, M., Jawaharlal, M., and Amarnath, V. 2003. Standardization of prehardening and hardening techniques for *in vitro* derived plantlets of *Dendrobium* Orchid Hybrid Sonia-17. *J. Ornamental Hortic.* 6(3): 212-216.

Islam, M.S., Mehraj, H., Roni, M.Z.K., Shimasaki, K., and Jamaluddin, A.F.M. 2013. Correlation between cane growth and flowering behaviour of *Dendrobium* orchid cultivars . *J. Bangladesh Acad. Sci.* 37(2):205-209.

Jaime, A.T.S. 2013. Orchids: Advances in Tissue Culture, Genetics, Phytochemistry and Transgenic Biotechnology. *Floriculture and Ornamental Biotechnology*, 7 (1):1-52.

Jain, S.K. 1980. Orchid and mountain flora of India. *67th session Indian Sci. Congr. Assn.* Calcutta.

Jalal, J.S. 2012. Distribution pattern of orchids in Uttarakhand Western Himalayas, India. *Int. J. Plant Biol.* 3(e5):24-26.

Jalal, J.S. and Jayanthi, J. 2012. Endemic orchids of peninsular India: a review. *J. Threat.Taxa.* 4(15): 3415–342.

Janson, J. 1993. Placental pollination in *Lilium longiflorum*Thunb. *Plant Sci.* 99:105-115.

Jayanthi, P., Rajendran, A., Binu Thomas, Aravindhan, V., and Sivalingam, R. 2011. Biodiversity of lithophytes in Madukkarai Hills of Southern Western Ghats of Coimbatore District, Tamil Nadu, India. *Int. J. Biol. Sci. Technol.* 2(2) :76-82.

JNTBGRI, 2014. *Annual report*, www.jntbgri.res.in

Johansen, B. 1990. Incompatibility in *Dendrobium* (Orchidaceae). *Bot. J. Linn. Soc.* 103(2):165-196.

Johnson, T.R. , Stewart, S.L. , Dutra, D. , Kane, M.E., and Richardson, L. 2007. Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)-preliminary evidence for the symbiotic culture advantage. *Plant Cell Tiss. Org. Cult.* 90:313-323.

Johnson, H.W., Robinson, H.F., and Comstock, R.E. 1955. Estimates of genetic and environmental variability in soybean. *Agron. J.* 47:314-18.

Johnson, T.R. and Kane, M.E. 2007. Asymbiotic germination of ornamental *Vanda*: *in vitro* germination and development of three hybrids. *Plant Cell Tiss. Org. Cult.* 91:251-26.

Johri, B.M. and Vasil, L.K. 1961. Physiology of pollen. *Bot. Rev.* 27(3):325-381.

Joshi, K.K. and Joshi, S.D. 2000. *Genetic Heritage of Medicinal and Aromatic Plants of Nepal Himalayas*. Buddha Academy Publisher and Distributors, Pvt. Kathmandu, Nepal. 320p.

Joshi, S.P., Gupta,V.S., Aggarwal, R.K., Ranjekar, P.K., and Brar, D.S. 2000. Genetic diversity and phylogenetic relationship as revealed by inter-simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. *Theor. Appl. Genet.*100:1311-1320.

Kamemoto, H. 1987. Genome breeding in *Dendrobium* orchids. In: C.H. Huang (cd.). *The Breeding of Horticultural Crops. Food and Agriculture Technology*. Taipei, Taiwan. pp.182-188.

Kanjilal, B., Dutta, K.B., and Sarker, D.D. 1998. Aneuploid lines in *Arundina graminifolia*. (Don) Hocher. (Orchidaceae) obtained through *in vitro* micropopagation, *Pers. in Cytology and Genetics*, 9:417-421.

Kaveriamma, M.M. 2007. Evaluation of monopodial orchids for cut flower. M.Sc. thesis. Kerala Agricultural University, Thrissur, 120p.

Khatun, M.M., Khatun, H., Khanam, D., and Al-amin, M.D. 2010. *In vitro* root formation and plantlet development in *Dendrobium* orchid. *Bangladesh J. Agric. Res.* 35(2) : 257-265.

Kheawwongjun, J. and Thammasiri, K. 2008. Breeding *Spathoglottis* spp. for commercial potted orchids. *Acta Hortic.* 788:47-52.

Kishor, R. and Devi, 2009. Induction of multiple shoots in a monopodial orchid hybrid (*Aerides vandarum* Reichb.f x *Vanda stangeana* Reichb.f) using thidiazuron and analysis of their genetic stability. *Plant Cell Tiss. Organ Cult.* 97:121-129.

Knudson, L. 1922. Nonsymbiotic germination of orchid seeds. *Bot. Gaz.* pp.1-25.

Knudson, L. 1925. Physiological study of the symbiotic germination of orchid seeds. *Bot. Gaz.* 79:345-379.

Knudson, L. 1927. Symbiosis and asymbiosis relative to orchids. *New Phytol.* 26:328-336.

Knudson, L. 1946. A nutrient for germination of orchid seeds. *Am. Orchid Soc. Bull.*15:214-217.

Knudson, L. 1951. Nutrient solutions for orchids. *Bot. Gaz.* 112: 528-532.

Krishna, D.K. 2015. *In vitro* seed culture of interspecific hybrid in *Cymbidium* spp. M.Sc. thesis, Orissa University of Agriculture and technology, Bhubaneswar, Odisha.

Krishnan, P.N., Latha, P.G., and Seeni, S. 1993. Biochemical changes during protocorm formation from in *vitro* grown embryos of *Spathoglottis plicata* Blume. *J. Orchid Soc. India,* 7(1-2):87-91.

Kumar, P.K.S. 1992. Potting media and post-transplantation growth of *Dendrobium* hybrid seedlings. *J. Orchid Soc. India,* 6(1-2):131-133.

Kumaria, S. and Tandon, P. 2007. Biotechnological approaches to conservation of orchids, the wondrous and mystic plants of North-East India. Man and Society, *J. N. E. Stud., Spr.* 4:57-71.

Laibach, F. and Maschmann, E. 1933. Uber den wuchstoff der orchideen-pollinien. *J. Wiss. Bot.* 78:399-430.

Lal, S.D., Shah, A., and Seth, J. N. 1985. Genetic variability in gladiolus. *Prog. Hortic.*17(1): 31-34.

Latha, P.G. and Namboodiri, A.N. 1999. Unusual pattern of *in vitro* pollen tube growth in orchids: possible role of pollinium borne auxin induced ethylene synthesis in control of tube growth. *J. Orchid Soc. India,* 13(1-2):37-40.

Lee, N. and Lin, G.M. 1984. Effect of temperature on growth and flowering of *Phalaenopsis* white hybrid. *J. Chinese Soc. Hortic. Sci.* 30: 223-231.

Lenz, L.W. and Wimbler, D.E. 1959. Hybridsation and inheritance of orchids. *The Orchids -A Scientific Survey* (ed. Withner, C.L.). The Ronald Press Co., New York, pp. 261-313.

Levin,R. and Tanny, G. 2002. Bioreactors as a low cost option for tissue culture. In: International Atomic Energy Agency (ed.): Low cost options for tissue culture technology in developing countries. *Proceedings of a technical meeting, 26-30 August*, Vienna, Austria.

Lim, H.C., Park, N.B., Choi, D.C., Jin, S.G., Park, K.H., and Choi, B.J. 1993. Studies on micropropagation *in vitro* and hardening culture in *Dendrobium moniliforme*. *J. Agric. Sci. Biotech.* 35(2):221-225.

Linden, B. 1980. Aseptic germination of seeds of Northern terrestrial orchids. *Ann. Bot. Fennici.* 17:174-182.

Lo, S.F., Nalawade, S.M., Kuo, C.L., Chen, C.L., and Tsay, H.S. 2004. Asymbiotic germination of immature seeds, plantlet development and ex-*vitro* establishment of plants of *Dendrobium tosaense* Makino, a medicinal orchid. *In vitro Cell Dev. Biol.* 40:528-35.

Lokesha, R. and Vasudeva, R. 1994. Do floral features determine the endangered status of Indian Orchids. *J. Orchid Soc. India.* 8(1): 53-54.

Long, B., Niemiera, A.X., Cheng, Z., and Long, C. 2010. *In vitro* propagation of four threatened *Paphiopedilum species* (Orchidaceae), *Plant Cell Tiss. Organ Cult.*101: 151-162.

Lopez, R.G. and Runkle, E.S. 2005. Environmental physiology of growth and flowering of orchids. *Hortic. Sci.* 40(7):1969-1973.

Lopez, R.G. and Runkle, E.S. 2004. The flowering of orchids [on-line]. Available: https://staugorchidsociety.org/PDF/FloweringofOrchids-LopezRunkle.pdf [27 Sept. 2018].

Luan, V.Q., Thien, N.Q., Khiem, D.V., and Nhut, D.T. 2006. *In vitro* germination capacity and plant recovery of some native and rare orchids. *Proceedings of International Workshop on Biotechnology in Agriculture.* Nong Lam University, Ho Chi Minh City, 111p.

Luit, R. and Johnson, S.D. 2001. Hawkmoth pollination of the African epiphytic orchid *Mystacidium venosum*, with special reference to flower and pollen longevity, *Plant Syst. Evol.* 228:49–62.

Lush, J.L. 1940. Intra-sire correlation and regression of offspring in rams as a method of estimating heritability of characters. *Proc. Am. Soc. Anim. Prod.* 33:292-301.

MacFarlane, S.W.H., Jones, J.K. , and Sebastiam pillai, A.R. 1989. Pollen storage of *Fragaria* and *Potentilla*, *Euphytica*, 41:65–69.

Mahendran, G. and Narmatha Bai, V. 2009. Mass propagation of *Satyrium nepalense* D. Don: a medicinal orchid via seed culture. *Sci. Hortic.* 119:203-207.

Manandhar , N.P. 2002. *Plants and People of Nepal.* Timber Press, Portland, Oregon, USA .

Martin, F.W. 1970. Compounds of the stigmatic surface of *Zea mays* L. *Ann. Bot.* 34:835–842.

Martinsen, G.D., Whitam, T.G., Turek, R.J., and Kaim, P. 2001. Hybrid populations selectively filter gene introgression between species. *Evol.* 55: 1325–1335.

18

Mathews, V.H. and Rao, P.S. 1985. *In vitro* culture of *Vanda* hybrid (*Vanda* TMA x *Vanda* Miss. Joaquim) I. Studies on protocorm explants. *Proc. Indian Nat. Sci. Acad.* 51(1):pp. 96-103.

Merlin, C.M. and Grant, W.F. 1986. Hybridisation studies in the genus *Impatiens*. *Can. J.Bot.* 64:1069–1074.

Minnu, S. 2015. Evaluation of *Vanda* orchids for commercial traits. M.Sc. thesis, Kerala Agricultural University, Thrissur-Kerala, India.

Miria, A., Khan, A.B., and Rao, B.R.P. 2012. Orchids of Talakona Sacred Grove, Andhra Pradesh, India. *Am. Eur. J. Agric. Environ. Sci.* 12(4):469-471.

Mitra, G. 1986. *In vitro* culture of orchid seeds for obtaining seedlings. *Biol. Conserv. Cult.Orchids*. 15:210-212.

Mitra, G.C., Prasad, R.N., and Roychowdhury, A. 1976. Inorganic salts and differentiation of protocorms in seed callus of orchid and correlative changes in its free amino acid content. *Indian J. Exp. Biol.* 14:350-351.

Moccia, M.D., Widmer, A., and Cozzolino, S. 2007. The strength of reproductive isolation in two hybridizing food- deceptive orchid species. *Mol.Ecol.* 16:2855–2866.

Mollah, S. 2001. *Orchid.* Floriculture Division, HRC, BARI, pp 1-30.

Moniruzzaman, M., Zaman, M.A., Earshad Hossain, M., Bhuiyan, M.M.H., and Rahman, M.Z. 2012. Genetic Variability and character association in some native orchid species (*Dendrobium* spp.). *Agric.*10 (1):1-9.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.

Nagashima, T. 1982. Studies on embryogenesis and seed germination in *Cymbidium goeringii* and *Paphiopedilum insignae* var. Sanderae. *J. Jap. Soc. Hortic. Sci.* 51(1):82-93.

Nagashima, T. 1993. Studies on relationship between embryogenesis and germination in orchidaceae. *J. Jap. Soc. Hortic. Sci.* 62:581-594.

Nagayoshi, T., Hatanaka, T., and Suzuki, T. 1996. Seed propagation of *Habenaria radiata* - morphological and physiological characteristics of plants derived from *in vitro* cultured seedlings. *Nat. Hum. Act.*1:67-81.

Nair, H. and Fong, T.H. 1987. Ethylene production and 1-aminocyclopropane-1-carboxylic acid levels in detached orchid flowers of *Dendrobium* 'Pompadour'. *Sci. Hortic.* 32:145-151.

Nair, H. and Tung, H.F. 1983. Some post harvest studies on detached flowers of *Dendrobium pompadour*. *Malay. Orchid Rev.* 17:22-30.

Nair, P.C.S., Balasubramanyan, V.R., and Khan, H.H. 1964. Palynological investigations of some guava varieties. *Indian J. Hortic.* 21 :79-84.

Narasak,S., Achra, T., and Piyada, T. 2015. Embryology of *Spathoglottis plicata* Blume: A reinvestigation and additional data. *Trop. Nat. Hist.*15(2):97-115.

Nath, N.C. 2003. Compatibility studies in monopodial orchids. M.Sc. thesis, Kerala Agricultural University, Thrissur, Kerala, India.

Negi, S.S., Sharma, T.V.R.S., Raghava, S.P.S., and Srinivasan, V.R. 1982. Variability studies in gladiolus. *Indian J. Hortic.* 39: 269 -272.

Nepi, M., Franchi, G.G., and Pacini, E. 2001. Pollen hydration status at dispersal: cytophysiological features and strategies. *Protoplasma*, 216:171–180.

Nhat, N.T.H. and Dung, T.T. 2006. *In vitro* propagation of *Dendrobium* orchid through thin stem section culture, In: *Proceedings of International Workshop on Biotechnology in Agriculture*, Ho Chi Minh City, Vietnam, pp.154-155.

Nitin, D. and Uma, T. 2014. Artificial pollination and its role in orchid conservation, https://www. researchgate. net/publication.

Nolte, F. 1985. Longevity of cut *Dendrobium* from Thailand. *Deutscher Gartenbau* 39:2178.

Nybom, H. and Bartish, I. 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. *Perspect Plant Ecol. Evol. Syst.* 3(2):93-114.

Olivia, A.P. and Arditti, J. 1984. Seed germination of North American Orchids. II. Native California and related species of *Aplectrum, Cypripedium and Spiranthes*. *Bot. Gaz.* 145: 495-501.

Omika, T. 2014. Diversity among the wild population of *Aerides multiflorum* from Kangra valley. M.Sc. dessertation, Thapar University, Patiala, India.

Pacini, E., Franchi, G.G., Lisci, M., and Nepi, M. 1997. Pollen viability related to type of pollination in six angiosperm species. *An. Bot.* 80: 83–87.

Pant, B. 2013. Medicinal orchids and their uses: Tissue culture a potential alternative for conservation. *Afr. J. Plant Sci.* 7(10):448-467.

Pant, B. and Shrestha, S. 2011. *In vitro* mass propagation of a ground orchid-*Phaius tancarvilleae* (L'Her.) Blume through shoot tip culture, *Plant Tiss. Cult. Bioteh.* 21: 181-188.

Parab, G.V. and Krishnan, S. 2008. Assessment of genetic variation among populations of *Rhynchostylis retusa* , an epiphytic orchid from Goa, India using ISSR and RAPD markers. *Indian J. Biotechnol.*7: 313-319.

Parab, G.V., Krishnan,S., Janarthanam,M.K., Sivaprakash,K.R., and Parida,A. 2008. ISSR and RAPD markers assessed genetic variations of *Aerides maculosum* – an epiphytic orchid from Goa, India. *J. Plant Biochem. Biotechnol.* 17: 107-109.

Pasutti, D.W. 1977. Cytological study of some New Guinea, Java, and Celebes *Impatiens* interspecific hybrids. M.Sc. thesis, Iowa State University, Ames, IA.

Pasutti, D.W. and Weigle, J.L. 1980. Pollen fertility in Java - New Guinea *Impatiens* inter-specific hybrids. *Can. J. Bot.* 58: 384–387.

Pathania, N.S., Shegal, O.P., Debojit, P., Dutta, B.S., and Paul, D. 1998. Studies on micropropagation in *Dendrobium* cv Sonia. *J. Orchid Soc.* 12 (1-2): 35-38.

Pavarese, G., Tranchida-Lombardo, V., Cogoni, A., Cristaudo, A., and Cozzolino, S. 2011. Where do Sardinian orchids come from: a putative African origin for the insular population of *Platanthera bifolia* var. kuenkelei ? *Bot. J. Linnean Soc.* 167: 466–475.

Peng, X., Liu, J.J., Xiang, Y., and Huang, S. 2006. *A Practical Handbook of Plant Molecular Biotechnology*.Chemical Industry Press, Beijing.

Philip, J.K., Wagner, A.V., and Michael, E.K. 2006. *In vitro* seed culture and seedling development of *Calopogon tuberosus*. *Plant Cell Tiss. Organ Cult.* 85:91–102.

Pierik, R.L.M. 1997. *In vitro Culture of Higher Plants*. Dordrecht: Klower Acad. Publ.

Pinheiro, F., Cardoso-Gustavson, P., Suzuki, R.M., Abrão, M.C.R., Guimarães, L.R.S., Draper, D., and Moraes, A.P. 2015. Strong post zygotic isolation prevents introgression between two hybridizing Neotropical orchids *Epidendrum denticulatum* and *E. fulgens*. *Evol. Ecol.* 29: 229–248.

Pinheiro, F., de Barros, F., Palma-Silva, C., Meyer, D., Fay, M.F., Suzuki, R.M., Lexer, C., and Cozzolino, S. 2010. Hybridization and introgression across different ploidy levels in the Neotropical orchids *Epidendrum fulgens* and *E.puniceoluteum* (Orchidaceae). *Mol. Ecol.* 19: 3981-3994.

Pinheiro, L.R., Silva, A.V.C., and Ledo, A.S. 2012. Genetic diversity and population structure in Brazilian *Cattleya labiata* (Orchidaceae) using RAPD and ISSR markers. *Plant Syst. Evol.* 13: 366-370.

Prakash, L., Lee, C.L., Loh, C.S., and Goh, C. J. 1996. *In vitro Propagation of Commercial Orchids:* An assessment of current methodologies and development of a novel approach – thin cross-section culture. In: Islam, A. S. (ed.), Oxford and IBH Publishing Company, New Delhi. pp. 42-49.

Prakash, S., Hoque, M.I., and Brinks, T. 2002. Culture media and containers. In: International Atomic Energy Agency (ed.): Low cost options for tissue culture technology in developing countries. *Proceedings of a technical meeting on 26-30* , Vienna, Austria.

Proctor, H.C. 1998. Effect of pollen age on fruit set, fruit weight, and seed set in three orchid species. *Can. J. Bot.* 76:420–427.

Puchooa, D. 2004. Comparison of different culture media for the *in vitro* culture of *Dendrobium* (Orchidaceae). *Int. J. Agric. Biol.* 6: 884-888.

Rahman, M.S., Hasan, M.F., Das, R., Hossain, M.S., and Rahman, M. 2009. *In vitro* micropropagation of orchid (*Vanda tessellata* L.) from shoot tip explants. *J. Biol. Sci.* 17:139-144.

Raina, S.N., Rani, V., Kojima,T., Ogihara,Y., Singh, K.P., and Devarumath, R.M. 2001. RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity varietals identification and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. *Genome*, 44:763-772.

Rajkarnikar, K.M. 2014. *In vitro* propagation of *Dendrobium amoenum* Wall. ex Lindl. from shoot-tip culture. *Bull. Dept. Plant Res.* 36: 52-55.

Raju, B., George, A., Jaganath, J., Jayaprasad, K.V., and Honabyraiah. 2003. Micropropagation of *Dendrobium*. *National Symposium on Recent Advances in Indian Floriculture*.12-14.

Rani, L.C. 2002. Intra and interspecific hybridization in *Dendrobium* spp., Ph.D. thesis, Kerala Agricultural University, Thrissur, (India).

Rao, A. 1977. Tissue culture in the orchid industry. *Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture*. J. Reinert and YPS Bajaj (eds.). McGraw-Hill, New York, pp.44-69.

Rao, A.N. 1974. Tissue culture in the orchid industry. *The Orchids - Scientific Studies* (ed. Withner, C.L.). John Wiley and Sons, New York, pp. 44-69.

Rao, A.N. and Avadhani, P.N. 1964. Some aspects of *in vitro* culture of *Vanda* seeds. *Proc. 4th World Orchid Conf.*, Singapore, pp.194-202.

Reddy, M.P., Sarla, N., and Siddiq, E.A. 2002. Inter-simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica,* 128:9-17.

Reddy, P.V. 1992. Study of the effect of culture media on seed germination of *Dendrobium* Orchid. *J. Orchid Soc.India,* 6:75-78.

Rehman, M., Jena, S.C., Biswas, M.R., and Chattopadhyay, T.K. 1993. Genetic analysis of some characters of orchids grown in the plains of West Bengal. *J. Orchid Soc. India,* 7(1-2):17-19.

Rieseberg, L.H. and Ellstrand, N.C. 1993. What can molecular and morphological markers tell us about plant hybridization? *Critical Rev. Plant Sci.* 12: 213–241.

Robinson, K. A. 2002. Effects of temperature on the flower development rate and morphology of *Phalaenopsis* orchid. M.Sc.(Ag) thesis, Mich. State University, East lansing, 120p.

Roy, S. and Biswas, A.K. 2005. Isolation of a white flowered mutant through seed culture in *Spathoglottis plicata* Blume, *Cytologia* .70(1):1–6, 2005.

Roychowdhury, N., Mandal, T., and Munsi, P. S. 2004. Evaluation of different *Dendrobium* spp. under polyhouse in North-East Indian hills. *Acta Hortic.* 658:315.

Sahoo, S. 2012. High frequency multiplication of orchid through *in vitro* culture. M.Sc. (Ag.) thesis, OUAT, Bhubaneswar.pp.77.

Sanford, W.W. 1971. The flowering time of West African orchids. *Bot. J. Linn. Soc.* 64: 163-181.

Sangama and Singh, F. 1994. Effect of growth regulators on *in vitro* seed germination of *Spathoglottis plicata*. J. Prakash and K.R. Bhundary (Eds). *Floriculture Technology, Trades and Trends,* Oxford & IBH publishing co pvt. Ltd. pp.389-391.

Saxena, S. and Dhawan, B. 1999. Regeneration and largescale production of bamboo *Dendrocalamus strictus* Nees through somatic embryogenesis. *Plant Cell Rep.*18:438-443.

Schiestl, F.P., Ayasse, M., Hannes, F.P., Erdman, D., and Francke, W. 1997. *J. Chem. Ecol.* 23:2881–2895.

Seeni, S. and Latha, P.G. 1990. Post transplantation growth of *Phalaenopsis* hybrid seedlings in community pots. *J. Orchid Soc. India,* 4:127-135.

Sharma, J. and Chauhan, Y.S. 1995. Establishment of *in vitro* raised seedlings of *Dendrobium* and *Paphiopedilum*. *J. Orchid Soc.India*, 9(1-2):37-41.

Sharma, J. 1998. Studies on *Vanda*. Effect of age of capsules (pods) on *in vitro* seed germination. *J. Orchid Soc. India* , 12(1-2):43-45.

Sheehan, T. and Sheehan, M. 1979. *Orchid Genera illustrated.* Van Nostrand Reinhold Company, New York, pp 12-81.

Shiau, Y.J., Sagare, A.P., Chen, U.C., Yang, S.R., and Tsay, H.S. 2002. Conservation of *Anoectochilus formosanus* Hayata by artificial cross-pollination and *in vitro* culture of seeds. *Bot. Bull. Acad. Sci.* 43:123–130.

Shindo, K. and Kamemoto, H. 1962 . Genome relationships of *Neofinetia* and some allied genera of Orchidaceae. *Cytol.* 27:402-409.

Shrestha, R. 2000 . Some medicinal orchids of Nepal. In: The Himalayan plants, can they save us? *Proceeding of Nepal-Japan joint symposium on conservation and utilization of Himalayan medicinal resources* (Eds. T. Watanabe, A. Takano, M.S. Bista and H.K. Saiju), Society for the Conservation and Development of Himalayan Medicinal Resources (SCDHMR), pp.153-156.

Singh, B. and Singh, M. 1990. Studies on variability and genetic advance parameters in gladiolus. *Prog. Hortic.* 19(3-4):271-275.

Singh, B.D. 2007. *Plant Breeding: Principles and Methods*. Kalyani publishers, New Delhi, pp. 762-788.

Singh, F. 1993. *In vitro* orchid seed germination and cloning of orchids - a success story. *Plant Biotechnology - Commercial Prospects and Problems*. (eds. Prakash,1. and Pierik, RL.M.). Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, pp.85-96.

Singh, F. 1994. Advances in production technology for tropical Orchids in: J. Prakash and K.R. Bhandary (Eds) *Floriculture – Technology Trades and Trends*, Oxford and IBH publishing co pvt. Ltd: pp.378-384.

Singh, S., Singh, A.K., Kumar, S., Kumar, M., Pandey, P.K., and Singh, M.C.K. 2012. Medicinal properties and uses of orchids: A concise review, *Elixir Appl. Bot.* 52:11627-11634.

Sivasubramanian, V. and Madhavamenon, P. 1973. Path analysis for yield and yield components of rice. Madras Agric.J., 60:1217-1221.

Skoog, F. and Miller, R.A. 1957. Chemical regulations of growth and organ formation in plant tissue culture *in vitro*. *Sym. Soc. Exp. Biol.* 11:118-131.

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Slater, A.T. 1991. Interaction of the stigma with the pollinium of *Dendrobium speciosum*. *Aust. J. Bot.* 39(3):273-282.

Sobhana, A. and Rajeevan, P.K. 2002. Refinement of embryo culture medium in *Dendrobium*. *Proceedings of the Abstract National Symposium on Indian Floriculture in the New Millenium*. p.65.

Sobhana, A. 2000. Improvement of *Dendrobium* through hybridization and *In vitro* mutagenesis. Ph.D. thesis, Kerala Agricultural University, Vellanikkara, Thrissur -680 656, Kerala, India.

Sood, A. and Vij, S.P. 1986. *In vitro* root segment culture of *Rhyncostylis retusa* Bl. In: S.P. Vij (Ed), *Biology, Conservation and Culture of Orchids*. Orchid Society of India, Affiliated East West Press, New Delhi, pp.463 468.

Soon, T.E. 1980. *Asian Orchids*. Times Books International, Singapore, 287p.

Squire, D. 2005. *The Orchid Specialist*. New Holland Publishers, 80p.

Stanley, R.G. and Linskens, H.F. 1974. *Pollen Biology, Biochemistry and Management*. Springer-Verlang, Berlin Heidelberg, New York, pp.39-85.

Storey , W.B. 1955. Fertility and sterility in *Vanda. Na Pua Okika o Hawaii Nei.* 5:160-168.

Stoutamire, W.P. 1974. Australian terrestrial orchids, thynnid wasps and pseudocopulation. *Am. Orchid. Soc. Bull.* 43: 13-18.

Subedi, A. 2011. New species, pollinator interactions and pharmaceutical potential of Himalayan orchids.Ph.D. thesis, Leiden University, The Netherlands.

Sudupak, M.A. 2004. Inter and intra- species inter-simple sequence repeat (ISSR) variations in the genus *Cicer*. *Euphytica*, 135:229-238.

Sugapriya, S., Mathad, J.C., Patil, A.A., Hegde, R.V., Lingaraju, S., and Biradar, M.S. 2012. Evaluation of *Dendrobium* orchids for growth and yield grown under greenhouse. *Karnataka J. Agric. Sci.* 25 (1): 104-107.

Sultana, K.S. 2003. Study on variabilities and morphological characteristics of some hybrid orchids. M.Sc. thesis, BAU, Mymensingh, Bangladesh, pp.10-80.

Suresh, P.K., Subramoniam, A., and Pushpangadan, P. 2000. Aphrodisiac activity of *Vanda tessellata*. *Indian J. Pharmacol.* 32:300-304.

Sutter, E.G., Fabbri, A., and Dunston, S. 1985. Morphological adaptation of leaves of strawberry plant grown *in vitro* after removal from culture. *Tissue*

culture in Forestry and Agriculture. (eds. Henke, R.R., Hughesh, K.W., Constantin, MJ. and Hollaendar, A.) Plenum Press, New York, pp. 358-359.

Swamy, B.G.L. 1943. Embryology of Orchidaceae. *Curr. Sci.* 12: 13-17.

Swamy, B.G.L. 1949. Embryological Studies in the Orchidaceae. II. Embryogeny. *Am. Midland Nat.* 41: 202-232.

Swarts, N.D. and Dixon, K.W. 2009. Terrestrial orchid conservation in the age of extinction. *Ann. Bot.* 104: 543–556.

Talukdar, M.C., Ahmed, H., and Deka, H. 2002. Embryo culture and isozyme analysis in two intergeneric hybrids of Orchid. *National Symposium on Indian Floriculture in the New Millenium*. 25-27.

Talukder, S.K., Nasiruddin, K.M., Yasmin, S., Hassan, L., and Begum, R. 2003. Shoot proliferation of *Dendrobium* Orchid with BAP and NAA. *J. Biol. Sci.* 3(11):1058-1062.

Thammasiri, K. 2016. Thai orchid genetic resources and their improvement, *Hortic. J.* 1-13.

Thomas, T.D. and Michael. A. 2007. High-frequency plantlet regeneration and multiple shoot induction from cultured immature seeds of *Rhynchostylis retusa* Blume., an exquisite orchid. *Plant Biotechnol. Rep.* 1:243-249.

Tomar,S. M.S. and Singh, B. 1998 . Hybrid chlorosis in wheat x rye crosses. *Euphytica*. 99:1-4.

Torres, K.C. 1989. *Tissue Culture Techniques for Horticultural Crops*. New York, London: Chapman and Hall.

Tsi, Z.H., Luo, Y.B., Cribb, P.J., McGough, N., and Siu, G. 1999. A preliminary report on the population size, ecology and conservation status of some *Paphiopedilum* species (Orchidaceae) in Southwest China, *Lindleyana*, 14:12-23.

Tsuchiya, I. 1954. Germination of orchid seeds from premature pods. *Na. Pua. Oxiha. O. Hawaii Nei.* 4:11-16.

Tuyl, J.M.V. and Lim, K. 2003. Interspecific hybridization and polyploidisation as tools in ornamental plant breeding, Proc.21st IS on Classical/Molecular Breeding, pp.13-22.

Uma, T. and Nitin, D. 2012. Artificial pollination and *in vitro* asymbiotic seed germination in garden orchid *Spathoglottis plicata* Blume (Orchidaceae). *Rec. Res. Sci. Technol.* 4(2):13-18.

Vacin, E. and Went, F. 1949. Some pH changes in nutrient solution. *Bot. Gaz.*110:605-13.

Van den Berg, C., Higgins, W.E., Dressler, R.L., Whitten, W.M., Soto Arenas, M.A., Culham, A., and Chase, M.W. 2000. A phylogenetic analysis of *Laeliinae* (Orchidaceae) based on sequence data from internal transcribed spacers (ITS) of nuclear ribosomal DNA. *Lindleyana* . 15(2):96-114.

van den Berg, C., Higgins, W.E., Dressler,R.L., Whitten,W.M., Soto Arenas, M.A., and Chase, M.W. 2009. A phylogenetic study of *Laeliinae* (Orchidaceae) based on combined nuclear and plastid DNA sequences. *Ann. Bot.* 104: 417-430.

Van Tuyl, J.M. and De Jeu, M.J. 1997. Methods for overcoming interspecific crossing barriers. Chapter13 In: Sawhney and Shivanna (eds.), *Pollen Biotechnology for Crop Production and Improvement*, Cambridge University Press, pp. 273-293.

Van Tuyl, J.M., Bino, R.J., and Custers, J.B.M. 1990. Application of *in vitro* pollination, ovary culture, ovule culture and embryo rescue techniques in breeding of *Lilium*, *Tulipa* and *Nerine*. In: J. de Jong (ed), *Integration of in vitro Techniques in Ornamental Plant Breeding*, pp. 86-97.

Van Tuyl, J.M., Maas, I.W.G.M., and Lim, K.B. 2002. Introgression in interspecific hybrids of lily. *Acta Hortic.* 570: 213-218.

Van Tuyl, J.M., Van Dien, M.P., Van Creij, M.G.M., Van Kleinwee, T.C.M., Franken, J., and Bino, R.J. 1991. Application of *in vitro* pollination, ovary culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. *Plant Sci.* 74: 115–126.

Varghese, S. 1995. Floral Biology and compatibility studies in *Dendrobium.* M.Sc.(Hort.) thesis, Kerala Agricultural University, Thrissur.

Varghese, S., Sadhankumar, P.G., and Rajeevan, P.K. 1997. Pollen Biology in *Dendrobium* hybrids. *J. Orchid Soc. India*,11(1-2):13-17.

Vellupillai, M., Swaru, P.S., and Goh, C.J. 1997. Histological and protein changes during early stage of seed germination in the orchid *Dendrobium acurmenatum*. *J. Hortic. Sci.* 76: 94-98.

Verma, P.C., Chakrabarty, D., Jena, S.N., Mishra, D.K.K, Singh, P., Sawant,S.V., and Rakesh, T. 2009. The extent of genetic diversity among *Vanilla* species: comparative results for RAPD and ISSR. *Ind. Crops Prod.* 29:581-589.

Vieitez, A.M. and Vieitez, M.L. 1980 . Culture of chestnut shoots from buds *in vitro*. *J. Hortic. Sci.* 55: 83-84.

Vij , S. 2002 . Orchids and tissue culture: current status. *Role of Plant Tissue Culture. In Biodiversity Conservation and Economic Development.* Gyanodaya Prakashan, Nainital India. 491p.

Vij, S.P. 1995. Genetic Resources of Orchids. In: *Advances in Horticulture,* 12-*Ornamental Plant* Ed. K.L. Chadha and S.K. Bhattacharjee. Malhotra Publishing House, New Delhi. pp.153-181.

Vij, S.P. and Jalota, R.K. 1995. Green pod culture of *Rhyncostylis retusa. Am. Orch. News.* 11(1-2):17-18.

Vij, S.P. and Pathak, P. 1988. Asymbiotic germination of the saprophytic orchid, *Cymbidium macrorhizon*: A study *in vitro*, *J. Orchid Soc. India.* 2: 25-32.

Vinterhalter, D. and Vinterhalter, B.S. 1997. Micropropagation of *Dracaena* sp. In: Bajaj YPS (ed.) *Biotechnology in Agriculture and Forestry* 40, *High-tech. and Micropropagation VI.* Berlin, Heidelberg: *Springer*; pp.131- 146.

Volker, P.W. and Orme, R.K. 1988. Provenance trials of *Eucalyptus globulus* and related species in Tasmania. *Aus.For.* 51(4): 257-265.

Wainwright, H. 1988. Overcoming problems in establishing micropropagules guidelines for growers. *Prog. Hort.* 2(3):67-72.

Wang, C.X., Qian, X., and Tian, M. 2013. Genetic diversity and population differentiation of *Calanthe tsoongiana:* a rare and endemic orchid in China. *Int. J. Mol. Sci.* 14: 20399-20413.

Wang, H.Z., Feng, S.G., Lu, J.J., Shi, N.N., and Liu,J.J.2009a. Phylogenetic study and molecular identification of 31 *Dendrobium* species using inter-simple sequence repeat (ISSR) markers. *Sci. Hortic.* 122: 440-447.

Wang, H.Z., Lu,J.J., Hu, X., and Liu, J.J. 2011. Genetic variation and cultivar identification in *Cymbidium ensifolium. Plant Syst. Evol.* 293(1):101-110.

Wang, H.Z.,Wu, Z.X., Lu, J.J., Shi, N.N., Zhao,Y., Zhang, Z.T., and Liu, J.J. 2009b. Molecular diversity and relationships among *Cymbidium goeringii* cultivars based on inter-simple sequence repeat (ISSR) markers. *Genetica,* 136: 391-399.

Wang, H.Z.,Wu, ZX., Lu, J.J., Shi, N.N., Zhao,Y., Zhang, ZT., and Liu, J.J. 2009. Molecular diversity and relationships among *Cymbidium goeringii* cultivars based on inter-simple sequence repeat (ISSR) markers. *Genetica,* 136:391-399.

Wang, W.C. , Yung, Y.L. , Lacis, T.M., and Hansen, J.E. 1976. Greenhouse effects due to man-made perturbation of trace gases. *Sci.* 194: 685- 690.

Weigle, J.L. and Pasutti, P.W. 1976. Approaches to transfer of characteristics between ploidy levels in New Guinea *Impatiens*. *Acta Hortic.* 63:109–112.

Willemse, M.T.M., Plyushch, T.A., and Reinders, M.C. 1995. *In vitro* micropylar penetration of the pollen tube in the ovule of *Gasteria verrucosa* (Mill) H. Duval and *Lilium longiflorum* Thunb.: conditions, attraction and application. *Plant Sci.* 108: 201-208.

Withner, C.L. 1959. Orchid Physiology. *The Orchids - A Scientific Survey.* (ed.Withner, C.L.) The Ronald Press Co., New York, pp. 315-360.

Wolfe, A.D. and Liston, A. 1998. Contributions of PCR-based methods to plant systematics and evolutionary biology. In Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.).*Plant Molecular Systematics II.*, Newyork. Chapman Hall.

Wolfe, A.D., Xiang, Q.Y., and Kephart, S.R. 1998. Assessing hybridization in natural populations of *Penstemon* (Scrophulareaceae) using hypervariable inter-simple sequence repeat (ISSR) bands. *Mol. Ecol.* 7:1107-1125.

Wolters-Arts, M., Mary-Lush, W., and Mariani, C. 1998. Lipids are required for directional pollen-tube growth. *Nat.* 392: 818–821.

Yam, T.W., Arditti, J. , and Cameron, K.M. 2009. "The orchids have been a splendid sport"-an alternative look at Charles Darwin's contribution to orchid biology. *Am. J. Bot.* 96: 2128-2154.

Yildiz, M. and Er, C. 2002. The effect of sodium hypochlorite solutions on *in vitro* seedling growth and shoot regeneration of flax (*Linum usitatissimum*). *Nat.* 89: 259-261.

Yong, J.W.H. and Hew, C.S. 2004. *The Physiology of Tropical Orchids in Relation to the Industry.* World Scientific Publishing Co Pte. Singapore. 370p.

Zahed, M.A.M. 2000. Studies on morphogenesis of three elite species of orchids. M.Sc. thesis, University, Dhaka, Bangladesh.

Zhang, Z.G., Liu, Y., Wang, L., Cai, Z.G., Han, X.Z., Zhao, L.H., and Luo, Z.J. 1992. Study on factors of protocorm proliferation of *Dendrobium candicum*. *Chinese Tradit. Herb. Drugs*, 23 (8):431-433.

Zhang, C.S., Xing, F.G., Selvaraj, J.N., Yamg, Q.L., Zhao,Y.J., and Liu,Y.2013. The effectiveness of ISSR profiling for studying genetic diversity of *Aspergillus flavus* from peanut-cropped soils in China. *Biochem. Syst. Ecol.* 50:147-153.

Zhang, F., Lv, Y., Dong, H., and Guo, S. 2010. Analysis of genetic stability through inter-simple sequence repeats molecular markers in

micropropogated plantlets of *Anoectochilus formosanus* Hayata, a medicinal plant. *Biol. Pharm. Bull.* 33(3): 384-388.

Zietkiewicz, E. Rafalski, A., and Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR) anchored polymerase chain reaction amplification. *Genome*. 20:176-183.

Zotz, G., Hietz, P., and Schmidt, G. 2001. Small plant, large plants: the importance of plant size for the physiological ecology of vascular epiphytes. *J. Exp. Bot*. 52:2051-2056.

DISTANT HYBRIDIZATION AND COMPATIBILITY STUDIES IN WILD ORCHIDS

by

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ABSTRACT

The present investigation entitled "Distant hybridization and compatibility studies in wild orchids" was carried out with the objective of studying the cross compatibility through distant hybridization in wild orchids and molecular characterization of hybrid seedlings using RAPD/ISSR- markers. The study was carried out at Saraswathy Thangavelu Extension Centre of KSCSTE- JNTBGRI, Puthenthope, Thiruvananthapuram and at College of Agriculture, Vellayan, Thiruvananthapuram.

The research work included experiment I, II, III and IV. Experiment I was carried out in two parts as evaluation of parents and hybridization, experiment II was cross compatibility studies of selected species, experiment III was *in vitro* culture of resultant hybrids and evaluation of seedlings and experiment IV was molecular characterization of hybrids.

In experiment I, wild types belonging to different genera/species namely *Vanda* (*Vanda tessellata*, *Vanda spathulata*, *Vanda* sp.), *Rhynchostylis retusa*, *Spathoglottis* (*Spathiglottis albida*, *Spathoglottis unguiculata*, *Spathoglottis gracilis*, *Spathoglottis plicata*), *Aerides ringens* and *Arundina graminifolia* available at the orchid conservatory of Saraswathy Thangavelu Extension Centre of JNTBGRI, Puthenthope, Thiruvananthapuram were selected as parents based on variability in floral characteristics and these were evaluated for various quantitative and qualitative characters.

Statistical analysis was carried out in Completely Randomized Design and analysis of variance revealed highly significant differences for all the biometrical characters included in the evaluation. Estimation of components of variance revealed that Phenotypic Coefficient of Variation (PCV) was higher than both Genotypic Coefficient of Variation (GCV) and Environmental Coefficient of Variation (ECV) for all the traits studied. PCV (143.93) and GCV (128.52) were the highest for number of spikes plant^{-1}. Heritability was the highest for flower

width (99.06 %) and it was high for all thirteen traits. Genetic advance as percent of mean was high for all the traits studied. It was the highest for number of spikes plant^{-1} (239.80). Estimated phenotypic correlation coefficient revealed highly significant and positive association between flower length and flower width (0.9519). The highly significant and negative association was obtained between internodal length and days for inflorescence emergence to first flower opening (-0.6896). Similarly genotypic correlation coefficient exhibited highly significant and positive association between flower length and flower width (0.9545) and highly significant and negative association between plant spread and number of leaves plant^{-1} (-0.9903).

Based on this, parents were selected and were crossed in all possible combinations. In *Vanda* a hybrid evolved from the wild species was available at the centre and it was also crossed with the wild parents to study the cross compatibility of wild species with hybrids.

In experiment II, cross compatibility of 152 cross combinations made was studied based on successful capsule setting. Capsule characteristics were recorded. The genus *Spathoglottis* took only four days for attaining pod setting whereas for the genus *Rhynchostylis* it was ten days for pod setting. Capsule attained maturity much earlier (30 days) in the genus *Spathoglottis* but the species *Vanda spathulata* took more days for attaining capsule maturity i.e., harvesting stage (150-154 days).

In experiment III, capsules developed among thirty one cross combinations were subjected to seed culture for rapid germination under *in vitro* condition and seedling development. Observations regarding seed germination were recorded and seeds of ten crosses exhibited seed greening and initiation of protocorm development. Seeds of seven cross namely one intergeneric hybrid and six interspecific hybrids were developed into seedlings. Evaluation of hybrid seedlings was made based on variability in morphological characters at monthly intervals. The six interspecific hybrids were *Spathoglottis unguiculata* x

Spathoglottis albida (Culture 622), *Spathoglottis gracilis* x *Spathoglottis unguiculata* (Culture 652), *Spathoglottis albida* x *Spathoglottis unguiculata* (Culture 778), *Spathoglottis albida* x *Spathoglottis unguiculata* (Culture 1368), *Spathoglottis plicata* x *Spathoglottis unguiculata* (Culture 1386), *Spathoglottis unguiculata* x *Spathoglottis plicata* (Culture 1388) and intergeneric hybrid was *Spathoglottis unguiculata* x *Vanda* sp. (Culture 679).

In experiment IV, molecular characterisation of seedlings evolved through the crossing was done based on ISSR analysis to distinguish F_1 hybrid seedlings from selfed seedlings and their parents. Percentage of polymorphism between hybrids and parents ranged from 11.99 to 32.97 and these results confirmed that all the seven crossed seedlings tested were F_1 hybrids and these were distinct from their relevant parents.

The above study revealed that through distant hybridization in wild orchids, one intergeneric hybrid *Spathoglottis unguiculata* x *Vanda* sp. and six interspecific hybrids *viz.*, *Spathoglottis unguiculata* x *Spathoglottis albida*, *Spathoglottis gracilis* x *Spathoglottis unguiculata*, *Spathoglottis albida* x *Spathoglottis unguiculata*, *Spathoglottis albida* x *Spathoglottis unguiculata*, *Spathoglottis plicata* x *Spathoglottis unguiculata*, *Spathoglottis unguiculata* x *Spathoglottis plicata* could be obtained and molecular characterization confirmed the hybridity of the intergeneric and interspecific hybrids. Therefore, the intergeneric and six interspecific F_1 hybrids evolved through distant hybridization can be carried forward for the development of varieties or can be utilised for genetic improvement programme in future.