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IN VITRO MICROPROPAGATION PROTOCOL FOR *VANDA* HYBRIDS WITH CLONAL FIDELITY ANALYSIS

By

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(2014-11-108)

THESIS

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2016

DECLARATION

I hereby declare that the thesis entitled **"*In vitro* micropropagation protocol for *Vanda* hybrids with clonal fidelity analysis"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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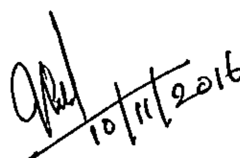

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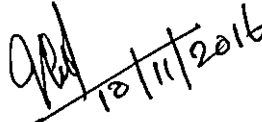

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TABLE OF CONTENTS

CHAPTER	TITLE	PAGE NO.
1	INTRODUCTION	1-3
2	REVIEW OF LITERATURE	4-26
3	MATERIAL AND METHODS	27-48
4	RESULTS	49-89
5	DISCUSSION	90-103
6	SUMMARY	104-105
7	REFERENCES	i-xx
8	ANNEXURES	
9	ABSTRACT	

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
1	Media compositions tested for culture establishment with leaf explants of *Vanda* hybrids	33
2	Media compositions tested for culture establishment with stem segments and apical meristem of *Vanda* hybrids	36
3	Media compositions tested for culture establishment with inflorescence segments of *Vanda* hybrids	38
4	Media compositions tested for the shoot proliferation of established cultures using inflorescence segments of *Vanda* hybrids	39
5	Details of ISSR primers used for screening	46
6	Details of selected ISSR primers	47
7	Composition of the reaction mixture for PCR	47
8	Programme for PCR reaction	48
9	Effect of surface sterilization treatments on leaf explants of *Vanda* 'Dr. Anek'	51
10	Effect of surface sterilization treatments on leaf explants of *Vanda* 'Sansai Blue'	52
11	Response of leaf explants of 'Dr. Anek' for culture establishment in different media combinations	54
12	Response of leaf explants of 'Sansai Blue' for culture establishment in different media combinations	55
13	Effect of surface sterilization treatments on root explants of *Vanda* 'Dr. Anek'	56
14	Effect of surface sterilization treatments on root explants of *Vanda* 'Sansai Blue'	57
15	Response of root explants of 'Dr. Anek' to different media combinations for culture establishment	59
16	Response of root explants of 'Sansai Blue' to different media combinations for culture establishment	60
17	Response of stem segments of 'Dr. Anek' to different media combinations for culture establishment	62
18	Response of stem segments of 'Sansai Blue' to different media combinations for culture establishment	63
19	Effect of surface sterilization treatments on inflorescence segments of *Vanda* 'Dr. Anek'	64

20	Effect of surface sterilization treatments on inflorescence segments of *Vanda* 'Sansai Blue'	65
21	Response of inflorescence segments of 'Dr. Anek' to different media combinations for culture establishment	67
22	Response of inflorescence segments of 'Sansai Blue' to different media combinations for culture establishment	67
23	Response of inflorescence segments of *Vanda* hybrids in culture establishment medium	69
24	Response of cultures of 'Dr. Anek' in shoot proliferation media	71
25	Response of cultures of 'Sansai Blue' in shoot proliferation media	72
26	Response of cultures in elongation medium	74
27	Response of cultures in identified shoot proliferation medium	73
28	Response of cultures in rooting medium	76
29	Biometric observations of plantlets during hardening and acclimatization	77
30	Quality and quantity of genomic DNA of mother plants and clones of 'Dr. Anek'	79
31	Quality and quantity of genomic DNA of mother plants and clones of 'Sansai Blue'	80
32	Details of ISSR primers screened	82
33	DNA Amplification pattern in 'Dr. Anek'	88
34	DNA Amplification pattern in 'Sansai Blue'	89

LIST OF PLATES

PLATE NO.	TITLE	BETWEEN PAGES
1	*Vanda* hybrids selected for the study	27-28
2	Collection and preparation of leaf explants	31-32
3	Collection and preparation of root explants	34-35
4	Collection and preparation of apical meristem and stem segments	35-36
5	Collection and preparation of inflorescence segments	37-38
6	Stages of culture establishment	68-69
7	Cultures in shoot proliferation medium with stalk	72-73
8	Cultures of in shoot proliferation medium without stalk	72-73
9	Cultures in elongation media	76-77
10	Rooted plantlet of *Vanda* hybrid	76-77
11	Regenerated plantlet potted in earthen pots with charcoal, coconut husk and brick pieces	77-78
12	Gel pictures showing the DNA samples of mother plants	78-79
13	Amplification pattern for UBC 808 for different mother plants and their respective clones of 'Dr. Anek'	83-84
14	Amplification pattern for UBC 811 for different mother plants and their respective clones of 'Dr. Anek'	84-85
15	Amplification pattern for UBC 826 for different mother plants and their respective clones of 'Dr. Anek'	84-85
16	Amplification pattern for UBC 835 for different mother plants and their respective clones of 'Dr. Anek'	84-85
17	Amplification pattern for UBC 841 for different mother plants and their respective clones of 'Dr. Anek'	85-86
18	Amplification pattern for UBC 808 for different mother plants and their respective clones of 'Sansai Blue'	86-87
19	Amplification pattern for UBC 811 for different mother plants and their respective clones of 'Sansai Blue'	86-87
20	Amplification pattern for UBC 826 for different mother plants and their respective clones of 'Sansai Blue'	86-87

21	Amplification pattern for UBC 835 for different mother plants and their respective clones of 'Sansai Blue'	87-88
22	Amplification pattern for UBC 841 for different mother plants and their respective clones of 'Sansai Blue'	87-88

LIST OF ANNEXURES

ANNEXURE NO.	TITLE
I	List of laboratory equipments used for the study
II	Chemical composition of Murashige and Skoog (MS) medium
III	Reagents required for DNA isolation
IV	Composition of buffers and dyes used for gel electrophoresis

Introduction

1. INTRODUCTION

Vanda orchids are one of the most sought after genus of orchidaceae family and among the five horticulturally important orchid genera. It is a monopodial orchid with vividly coloured, loosely arranged large beautiful flowers which has a long shelf life. This makes them an important cut flower in the international flower markets. The genus *Vanda* consists of around eighty species. Some species like *Vanda coerulea* and *Vanda teres* are known for its medicinal value while *Vanda cristata* and *Vanda tessellata* are famous for its fragrance. Mostly *Vanda* is an epiphyte. But some terrestrial species have also been reported. Though tropical Asia (India) is considered as its origin, *Vanda* is widely distributed in South East Asia, Philippines, Indonesia, Southern China and northern Australia.

Vanda orchids have evolved greatly adapting to different habitats. The plant morphology varies with its growing conditions. Some *Vanda* orchids have flat and broad leaves known as strap leaved, where as others have cylindrical or terete leaves. Strap leaved *Vandas* are usually lower in plant height and are known as basket vandas. Terete *Vandas* are tall growing and also known as pencil vandas. Semi- terete *Vandas* are also seen which are intermediate to terete and strap leaved types. Those *Vanda* which thrive in the dry areas have fleshy leaves. Long growing roots and thick stem is an important feature of *Vanda* orchids.

Netherlands is the largest exporter of orchids. However, Thailand is the leading country in the export of tropical orchids, and China is the major importer of cut flower orchids from Thailand, followed by Japan, USA, Italy and India. *Vanda* contributes around 8.9 per cent of total orchid trade and in case of cut flower *Vandas,* it is around 0.13 percentage of total orchid export from Thailand (NRC on orchids, 2015). India imported *Vanda* orchids worth 201,183 USD from February 2014- February 2016 from Thailand.

Vanda is a light loving plant but not all species can tolerate direct sunlight. They prefer shade nets or poly houses for better and frequent blooming. They generally bloom twice a year and the blooms last for about three to four weeks.

The climate in India is highly suited for the cultivation of *Vanda* orchids, especially the north eastern states and the humid tropics of Kerala with high rainfall and low temperatures.

Vanda is cross compatible with allied groups of orchid like *Aerides, Ascocentrum, Phalaenopsis* etc. This contributes to the production of elite hybrids which bear magnificent flowers. Availability of skilled labour, demand for cut flower orchid hybrids, both in local and international markets, and growing interest in the protected cultivation emphasize the need for more number of elite planting materials.

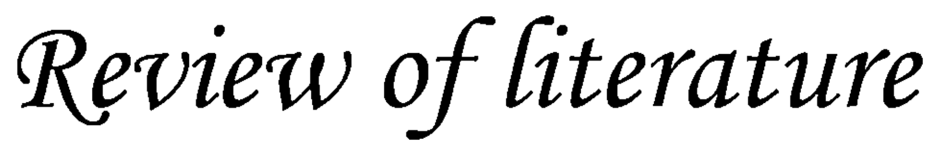
Micropropagation is a highly sought after technique in the commercial production of orchid plants. This has the advantage of providing large number of uniform and true to type plants in a short period of time.

Morel (1960) was the pioneer in reporting that *in vitro* techniques could be used to produce orchids on a large scale using shoot apex cultures of *Cymbidium* species (Begum *et al.*, 2002). This was followed by the development of micropropagation protocols for different economically important orchid species including *Dendrobium, Vanda, Cattleya, Phalaenopsis* etc. using different explants. Kim *et al.* (1972) performed shoot tip culture in *Vanda* and later, Mathews and Rao (1985) used leaf base of *Vanda* hybrid to obtain proliferation. Since then many works had been conducted to study the potential of various explants (Philip and Nainar, 1986; Vij *et al.*, 1986; Seeni and Latha, 2000; Chugh *et al.*, 2009) and the effect of different basal media and hormonal combinations (Seeni and Latha, 2000; Decruse *et al.*, 2003) for the successful regeneration of *Vanda* hybrids.

Recently tissue culture is being extensively used for *Vanda* propagation to meet the increasing market demand as well as for the *ex-situ* conservation of endangered *Vanda* species. Protocols have been standardized at various laboratories using different explants and media composition. But a major constrain in the *in vitro* propagation technique is the somaclonal variation.

Somaclonal variation is the genetic variation observed among progeny of plantlets regenerated from somatic cells cultured *in vitro.* The main reason for this can be attributed to chromosomal rearrangements and spontaneous mutations owing to the culture conditions. This highly limits the practical utility of tissue culture plantlets on commercial scale. Hence it is important to assure the genetic stability and true to type nature of *in vitro* regenerated plantlets. Clonal fidelity analysis can be used to check the genetic similarity and variation produced among the regenerants and the mother plant. ISSR markers serve as an important molecular marker for the clonal fidelity analysis in many of the *in vitro* propagated plants (Alizadeh *et al.,* 2015). ISSR markers have been used in determining the genetic stability in *Vanda* hybrids (Kishor and Devi, 2009).

In this context, "*In vitro* micropropagation protocol for *Vanda* hybrids with clonal fidelity analysis" was taken up at Center for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agriculture University. Two leading *Vanda* hybrids namely Dr. Anek and Sansai Blue were selected for the study. The study was conducted with the objective of developing a viable *in vitro* micropropagation protocol for the selected hybrids and to assure their genetic stability using. ISSR molecular markers. This study can help to identify a protocol suitable for commercial scale entrepreneurship programmes and also for the exploitation of somaclonal variants for crop improvement programmes.


Review of literature

2. REVIEW OF LITERATURE

The research programme entitled "*In vitro* micropropagation protocol for *Vanda* hybrids with clonal fidelity analysis" was conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2014- June 2016 and the literature on various aspects of the research is reviewed in this chapter.

1. Introduction to orchids

Plant taxonomists have classified seeded plants into gymnosperms and angiosperms. Gymnosperms lack flowers whereas angiosperms are flowering plants. Orchidaceae, the family to which orchids belong to, is one of the largest family among angiosperms with an estimated 800 genera and 25,000 species (Chai and Hao, 2007). This family contains the most highly evolved plants with complex flower structures.

Habitat

Orchids can grow in wide range of habitats but are seen abundant in tropical regions which is the homeland for most of the orchid species (Lakshman *et al.,* 2014). However they are also seen in subtropical and alpine regions. Orchids have established themselves in the freezing climates of Alaska, snow covered Himalayas and also in the deserts of Australia and Africa (Mukherjee, 1983). In India, the eastern Himalayas, the western India and the southern hills harbour most of the orchid species.

According to the habitat in which they grow, orchids can be epiphytic, lithophytic or terrestrial (Qiang, 2014). Epiphytic orchids grow on other plants or trees with their roots hanging, but are not parasitic on them. They absorb water and nutrients from the atmosphere using their spongy root epidermis called velamen. Some other orchids grow on the rocks to which their fleshy roots will cling. They take nutrition from the rains which carry dust and other minerals. Such orchids are referred as lithophytes. Terrestrial orchids grow in the ground

and flourish using their roots that spread in the soil just like the normal plants. Their roots may be fleshy or tuberous and are basically of temperate origin.

a. Growth habit

Orchids are herbaceous perennial monocots. The growing pattern observed in orchids is either monopodial or sympodial. Monopodial orchids are those that grow upwards as in case of *Phalaenopsis*, *Paphiopedilum,* and *Vanda*. The development of new leaves in monopodials is to the either sides of a central stem that grows vertically from a single bud. However sympodial orchids develop new growths from the base of the previous growth. Their development is seen side wise or laterally. The common sympodial orchids are *Cattleya, Oncidium, Dendrobium* and *Cymbidium* (Squire, 2005). Sympodials generally have a thickened region at the base of their stem known as pseudobulbs. The main function of pseudobulb is water storage.

b. Roots

Epiphytic and lithophytic orchids have roots that hang from the base of the plant with loose and spongy velamen. According to Pridgeon (1987), velamen is homologous with epidermis, but may have more layers of cells. These are involved in absorbing moisture from the atmosphere and nourishing the plant. Roots in case of terrestrial orchids function as in ordinary plants.

c. Stem

Orchids have typical monocot stem with their vascular tissues scattered in bundles. The stem may be thin and soft as in *Phalaenopsis* or tough as in *Vanda*, whereas *Vanilla* have soft and succulent stem. Stem have nodes on them, from where the leaves arise. In some sympodial orchids, the base of several successive shoots fuse to form the rhizome (Dressler, 1927). In certain others, pseudobulbs are present which is made up of a single thickened internode or several internodes.

d. Leaves

Orchid leaves arise from the nodes on the stem which are typical monocotyledonous. Generally they have simple leaves with parallel venation. However genus *Isotria* and *Pogonia* exhibits reticulate venation (Cammeron and

Dickson, 1998). Leaves are generally arranged in two vertical rows on opposite sides of the central stem known as distichous arrangement. It can be noted that most of the species of genus *Corallorhiza,* commonly known as coralroot, are leafless and rootless. They depend completely on mycorrhizal fungal association for their existence (Taylor *et al.*, 2003). In many cases, basal leaf forms a sheath around the stem whereas in some, leaf base forms a narrow sub-cylindrical petiole.

e. Flowers

The diversity of orchids is much seen in its flower size, shape and colour. The size of the flower varies from 2 mm in diameter as in the genus *Pleurothallis* to 38 cm as in *Brassia* genus. Mariana and Thei-Ben (2008) have explained that a developmental genetic predisposition, which is unique to orchids, is the reason for the morphological diversity of this family.

The shape of orchid flowers are extremely varied that some are commonly known by its appearance. For example, yellow *Oncidium* sp. with large lips is known as 'dancing ladies'. Another species of orchids is known as 'spider orchids' as they resemble spiders in their appearance. *Ophrys apifera* resembles a bee while *Coeloglossum viride* looks like a minute frog.

The variability in colour is also wide, which includes plain as well as variegated colours. This wide diversity in the flower morphology of orchids is closely associated with the mode of pollination of the plant. According to Orlean (1998), "an orchid species and its pollinator are often so closely evolved that the two depend on each other for survival". The flower morphology is such a way evolved that in some species the flowers resemble the shape of the pollinator whereas in some others they produce certain odour that attract the pollinator (Johnson, 2012). The attractiveness of the flowers and its long shelf life even up to few months is also remarkable.

Orchids bloom either as solitary or as inflorescence. It may arise from the base of the plant as in *Cymbidium* or from the axils of leaves as in *Vanda*. In *Catteleya* and few others, the flower appears from the apex of the main stem. Most of the orchid flowers are bilaterally symmetrical. It consists of an outer

whorl of three sepals and an inner whorl of three petals. The medial petal is modified to form a labellum or lip and helps in pollination. This is the most attractive part of orchid flower in most of the species. The androecium and gynoecium in orchid flowers is fused together to form the gynostemium or column. Mostly there is a single anther at the apex and it has the compact mass of pollinia. It contains indistinguishable pollen grains. Some genera have two anthers as in *Cypripedium.* The sticky surfaced area called stigma is the pistillate part of the flower.

2. Economic importance of orchids

Economic importance of the orchid family is very high. They are mostly grown as ornamentals. A large part of global floriculture trade has been shared by orchids both as cut flowers and as potted plants. It is estimated to be around 10 per cent of international fresh cut flower trade (Lakshman *et al.*, 2014).

Though very few, orchids also find applications in the culinary purpose. *Vanilla planifolia*, a member of orchidacea family is grown commercially for its vanilla extract which is used as a food flavor. Hossain (2011) has detailed about 'salep', flour made out of dried tubers of *Orchis mascula, Orchis militaris* and other related species, used for preparing beverages and desserts.

Orchids are also important in certain traditional medicines. Bulpitt *et al.* (2007) have studied the medicinal effects of various orchid species. They have evaluated three orchids namely *Gastrodia elata, Dendrobium* species and *Bletilla striata,* which form an integral component of the traditional Chinese medicines. They were found to contain certain level of alkaloids which is responsible for the treatment of various ailments like low grade fever, migraine, headache, epilepsy etc. According to the studies of Singh and Duggal (2009), orchids have special mention in the Ayurvedic system of medicines also. "Similarly, in America, *Vanilla planifolia* was used as a useful herb for the treatment of hysteria, fevers, impotence, and rheumatism and to increase the energy of muscular systems since 15th century" (Pant, 2013).

3. *Vanda* orchids

These are one among the most demanded orchid genera. It is a monopodial orchid with long hanging roots. They have velamen for water absorption. *Vandas* are mainly of two types: terete leaved (eg. '*Vanda* Miss joaquim' and '*Vanda* Diana') and strap leaved (eg. *Vanda seruli* and *Vanda tessellata*). Terete leaved types require high light conditions for flowering whereas strap leaved ones require less light conditions (Tajuddin and Prakash, 1996).

Magnificent hybrids have been developed within the genus of *Vanda* as well as within species of allied groups. For example, 'Aeridovanda' is a hybrid between *Aerides* and *Vanda* and 'Vandaenopsis' is a hybrid of *Phalaenopsis* and *Vanda* (Arditti, 2009).

Genus *Vanda* is also highly diverse. Typically they have thick, flat and channeled leaves which are arranged in two rows. Flowers are large and long lasting which comes on racemose inflorescence.

Some of the common species of this genus are: *Vanda coerulea, Vanda cristata, Vanda tricolor, Vanda teres wall, Vanda sanderiana, Vanda roxburghii, Vanda parishii* and *Vanda hookeriana*.

Vanda coerulea is the most demanded and charming species of genus *Vanda* which flowers in autumn to winter season. It is seen in the Khasi hills of Assam at 900- 1300 m. *Vanda tricolor* is a fragrant species. *Vanda teres* is of great demand as cut flower. It is widely distributed over the North Eastern states of India. *Vanda sanderiana,* is also known as *Euanthe sanderiana* and was discovered from Mindanao in the Philippines. *Vanda roxburghii* is very common on mango trees in West Bengal and have strong scented flowers. *Vanda parishii* is also a scented species which is dwarf in habit. It is mainly seen in Burma and India-Burma border lines. *Vanda hookeriana* is a Malayan *Vanda* orchid (Mukherjee, 1983).

4. Propagation of orchids:

Orchids can be propagated both sexually as well as asexually. Sexual propagation is brought about by the seeds whereas asexual propagation is done using vegetative methods. Vegetative propagation methods include division, use of back bulbs and keikis (offshoots) (Deyoung *et al.,* 2011).

4.1 Seed propagation

Orchid seeds are unique as they lack endosperm. They are microscopic and contain very less nutrients. Hence they require a symbiotic association with fungi for their germination. As the chance of symbiotic association between orchid seeds and fungi is very limited, only a few seeds can grow into adult plants (Tiwari and Kumar, 2014).

4.2 Division

Orchids are mostly propagated by vegetative methods as it can provide more number of plants which are similar to the mother plants. Dividing a mature plant into two or three parts, each with a new growth can produce a new plant that can come into flowering in the next season. This is one of the simplest methods of orchid propagation. It is common in case of sympodials like *Catelleya.*

4.3 Back bulbs

Another method of vegetative propagation of orchids is by using the older pseudobulbs of the plant known as back bulbs. They lack active growth but contain reserved energy. This energy reserve can be utilized to establish as a new plant. The major problem while propagating orchids using back bulbs is that they may take several years to come to flowering.

4.4 Keikis

'Keiki' is derived from a Hawaiian word 'Kay-key' which means a baby plant. The word is used to represent the new plants produced asexually from certain monopodial genera of orchids like *Vanda, Ascocenda* and *Phalaenopsis.* These are the offshoots that develop from the nodes on the main shoots of the plant. Basal keikis are produced at the base of the mother plant whereas the regular keikis are produced from a node along the flower stem. These can be

separated from the mother plant and can be planted in separate pots to develop as new plants.

5. Micropropagation

The present day demand for orchids is high. Orchid growing has witnessed a change in phase from hobby to small scale and large scale business. This has accelerated the demand for healthy, elite and disease free planting material. The conventional methods of propagation cannot meet the ever increasing demand for orchid plants. Micropropagation can be effectively utilized in the orchid industry to meet the demand.

Micropropagtion is a method of plant tissue culture which is used to produce large number of clones of the mother plant in a short period of time. The conventional method of propagating plants using seeds is not practical in case of orchids as they lack endosperm and require long time for germination. Also, the fungal association is a necessity for orchid seed germination. As orchids are highly heterozygous, seed culture is not a promising option to multiply the required mother plant.

Obtaining large number of selected recombinant types in orchidaceae is possible through vegetative methods. But vegetative methods of orchid propagation are not compatible to meet the demand for orchids as it is a slow method. Plant tissue culture is the technique of growing plant cells, tissues or organs on an artificial media under aseptic conditions to produce large number of identical plantlets. It depends on the property of totipotency. Totipotency is defined as the ability of a plant cell to regenerate into an entire plant.

6. Studies on orchid micropropagation

Micropropagation of orchids has a long history. Initially, seeds were the only source of explants for orchid micropropagation. Later, with the remarkable works of different researchers, other sources such as meristem, shoot tips, inflorescence, nodal buds, leaf, internodes, roots etc. were also used in mass multiplication of orchids (Reddy, 2008).

Knudson (1922) was the pioneer to develop a successful tissue culture technique for asymbiotic germination of orchid seeds on sugar enriched media.

"Rotor in 1949 demonstrated that new orchid plantlets can be developed from the buds on *Phalaenopsis* inflorescence" (Sharma, 1996).

Ito in 1960 used ovaries of *Dendrobium* flowers as explants. Morel (1960) developed a successful clonal propagation technique in orchids using *Cymbidium* shoot apices on Knudson C medium solidified by agar.

Studies by Morel (1960)) showed leaves can produce protocorm- like bodies in cultures of *Cymbidium*. Meristem culture technique had been initiated by Morel (1965). He worked on leaf primodia of *Cymbidium* and *Cattleya*. "The culture of *Arundina* stem sections was first mentioned in 1966 at the Fifth World Orchid Conference at California" (Arditti and Ernst, 1992).

Nodal buds obtained from *Vanda* hybrid 'Miss Joaquim' produced plantlets within three months in culture conditions (Sagawa and Sehgal, 1967). Root segments obtained from *Vanda testacea* successfully produced plantlets (Vij, 1994). Presently almost all parts of orchids are being used as explants for orchid micropropagation.

7. Studies on micropropagation in genus *Vanda*

a. Culture establishment of *Vanda* orchids using different explants

7.1.1 Shoot tip explants

The first experiments in *Vanda* tissue culture were carried at the University of Singapore (Arditti and Ernst, 1992). *Vanda* shoot tip culture was done by Kuniskai *et al.* in 1972 using liquid and solid cultures. Shoot tips of 'Miss Joaquim' inoculated into modified Vacin-Went medium showed cultures proliferating best in liquid medium through intermediate Protocorm like Bodies (PLBs).

Rahman *et al.* (2009) inoculated shoot tip explants in Murashige and Skoog (MS) medium to regenerate *Vanda tessellata.* Around 90 per cent of explants inoculated were found to respond to the basal media enriched with 1.5 mg l^{-1} Naphthalene acetic acid (NAA) and 1.0 mg l^{-1} 6-Benzyladenine (BA).

7.1.2 Stem segments and buds

Sagawa and Seghal in 1967 used sterilized stem segments of 'Miss Joaquim' for rapid clonal multiplication of *Vanda*. Stem segments of 45-50 mm

length were inoculated to Vacin-Went medium solidified using agar. Rooted plantlets were obtained within three months.

In another experiment conducted by Teo *et al.* in 1973, they utilized apical and axillary buds from seedlings of strap leaved *Vanda* to obtain new plantlets.

Vanda pteris was regenerated *in vitro* using axillary bud derived protocorms by Begum *et al.* (2002). Murashige and Skoog (MS) medium without any hormonal supplements could induce 4-6 protocorms from a single axillary bud.

The use of shoot tip and stem segments as explants in monopodials such as *Vanda* results in the arrest of growth and development of the mother plant. Also, the cut ends are susceptible to several infections and ultimately lead to losing of the highly prized mother plants.

7.1.3 Meristems of leaves and roots

Chaturvedi and Sharma (1986) employed meristematic tissues of other organs such as tips of leaf and root from *in vitro* grown seedlings for culture initiation. Bases of leaf tip explants swelled to form PLBs within 10 days in modified Vacin-Went medium. Root tip explants were also found to form PLBs.

Mathews and Rao (1985) concluded that leaf bases were more responsive than the tips and also younger leaves responded better than older leaves. Similar conclusions were drawn by Vij *et al.* (1986) as they worked on the regeneration potential of different leaf segments of *Vanda testacea.* Similarly, leaves of *Vanda coerulea* from mature plants did not form any shoot buds (Seeni and Latha, 2000).

Regeneration potential of *Vanda* Kasem's Delight 'Tom Boykin' (Vij *et al.,* 1994) and *Vanda coerulea* Griff. (Vij and Aggarwal, 2003) was also influenced by the juvenility of the mother plants. The size of the meristematic explants also influenced the regeneration capacity. In the experiments by Mathews and Rao (1985), meristem explants of below 0.3 mm length did not respond.

At Tropical Botanical Garden and Research Institute, Palode, Trivandrum, India, an attempt was made to obtain plantlets of Blue Vanda, *Vanda coerulea*

using leaf base (Anonymous, 1987). Leaf bases of 0.5-0.8 cm of *in vitro* grown seedlings produced callus with PLBs on a medium consisting of 800 mg l^{-1} Gaviota-63, 30 per cent coconut water, 500 mg l^{-1} peptone,. 1mg l^{-1} NAA (Naphthaleneacetic acid), 1 mg l^{-1} BAP (Benzylaminopurine) and 7 g l^{-1} agar. They could obtain 70 plantlets from a single leaf base.

Gantait and Sinniah (2012) induced PLBs from leaf segments of *Vanda* hybrid Aranda Wan Chark Kuan 'Blue' x *Vanda coerulea* Grifft. ex. Lindl. using tissue culture technique. PLBs were obtained at a rate of 94.8% on MS medium supplemented with1.5 mg l^{-1} TDZ (N-phenyl-N-(1,2,3-thidiazol-5-yl)urea).

7.1.4 Nodal explants

Vanda spathulata had been regenerated using tissue culture technique by Decruse *et al.* in 2003. Nodal explants were cultured on Mitra medium supplemented with BA and IAA. Subculturing of the explants lead to multiplication and further rooting of the regenerated shoots. They obtained a moderate establishment rate of 70 per cent.

7.1.5 Inflorescence segments

Inflorescence tips with attached flower buds of *Vanda* were used as explants at University of the Philippines by Valmayor *et al.* in 1986. The bracts covering the buds were removed and the sterilized explants were cultured on liquid Knudson C medium. PLBs formed on this medium differentiated well on Vacin - Went liquid media.

Li *et al.* (2014) could induce new plantlets from the floral stalks of *Vanda* hybrid 'Miss Joaquim' on half strength MS media. They obtained around 65 per cent regeneration on a media supplemented with 0.5 mg l^{-1} NAA and 2.0 mg l^{-1} BA.

7.1.6. *In vitro* explants

In vitro explants are better in culture conditions than *in vivo* explants. Goh (1970) worked on the popular *Vanda* hybrid 'Miss Joaquim' using three different explants namely shoot tips, axillary buds and root tips obtained from *in vitro* grown seedlings. Explants were inoculated on modified Whites medium. He

observed rapid cell division in shoot tip explants and obtained new plantlets from them. However axillary buds and root tip explants failed to produce any new plantlets. Root tip explants elongated in cultures but later turned white. Axillary buds produced side shoots but could not produce any roots.

Root tip cultures of *Vanda* hybrid (*Vanda* TMA X *Vanda* 'Miss Joaquim') also failed to produce any callus (Mathews and Rao, 1985).

Seeni (1988) and Sharma and Chaturvedi (1988) have reported the regeneration of *Vanda* plantlets from *in vitro* grown seedlings. Though Mathews and Rao (1985) observed infrequent proliferation from leaf tips of *Vanda*, Seeni and Latha (2000) could achieve rapid multiplication of endangered *Vanda coerulea* through cultures of shoot tips as well as leaf bases of 8 month old *in vitro* grown seedlings. Mitra medium was supplemented with coconut water and casein hydrolysate along with 8.8 μM BA and 4.1 μM NAA for PLB formation in 8 weeks and direct shoot bud development in 12 weeks. A maximum of 70-80 shoots of varied length were obtained from a single shoot/ leaf tip explants after 30 weeks of culture.

A *Vanda* hybrid between *Ascocentrum ampullaceum* and *Vanda coerulea* was regenerated *in vitro* on half strength MS medium by Kishor *et al.* (2006) using *in vitro* explants. The best response of seedling growth was observed on the media enriched with 2.3 μM Kinetin and 0.5 μM NAA after 150 days of inoculation.

Manners *et al.* (2010) inoculated root tip explants from both *in vivo* and *in vitro* grown plants on MS media containing Indole-3-acetic acid (IAA), 6-Benzylamino purine (BAP), Kinetin and coconut water. Explants from *in vivo* plants failed to respond whereas *in vitro* grown root tips formed PLBs.

In 2014, Sebastinraj *et al.* reported that half strength MS media was better than Knudson C and Vacin-Went medium for rapid plantlet regeneration. *Vanda testacea* showed a high regeneration percentage on half strength MS media supplemented with 0.2 mg l^{-1} TDZ.

In *Vanda tessellata,* Bhattacharjee and Islam (2014) could obtain shoot differentiation after 28 days from *in vitro* grown seedlings on MS medium fortified with 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA.

7.2 Shoot proliferation or multiplication of *Vanda* orchid cultures

The nutritional requirements for proper growth and development of the tissues in culture conditions are different during different stages of its development. In addition to the nutrients present in the basal medium, it requires certain other substances called the plant growth regulators. The relative concentration of different growth regulators in the media determines the route of growth and development of tissues *in vitro.*

Multiplication of the initiated cultures was observed in a strap leaved *Vanda* hybrid (*Vanda insignis* x *Vanda tessellata*) by Teo *et al.* (1973). The culture media was a modification of Vacin- Went media.

Kishi *et al.* (1997) reported a better proliferation of PLBs on one fourth strength MS media containing 10 g l^{-1} sucrose in which Fe- EDTA was replaced by 6.95 mg l^{-1} $FeSO_4.7\ H_2O$. They observed that the fresh weight of the PLBs was greatly influenced by the strength of the basal medium. Both half strength and one eighth strength MS media showed poor response.

Mitra medium enriched with 8.8 μM BA and 1.08 μM NAA showed a good rate of proliferation of PLBs from various explants of *Vanda coerulea,* when fortified with 30 per cent coconut water and 35 g l^{-1} banana pulp (Seeni and Latha, 2000). Another combination of hormones found to be responsive in multiplication of PLBs was observed by Decruse *et al.* (2003). They obtained multiplication of apical buds of *Vanda spathulata* on Mitra medium with 66.6 μM BA and 40 μM IAA whereas 28.5 μM IAA was better for PLB multiplication from leaf explants.

MS media fortified with 2 mg l^{-1} BAP and 1 mg l^{-1} NAA had led to the protocorm multiplication from axillary bud explants of *Vanda pteris* (Begum *et al.,* 2002). In 2006, Lang and Hang produced somatic embryos from callus of various explants of *Vanda coerulea.* They had employed half strength MS media with 0.1mg l^{-1} NAA and 3 mg l^{-1} TDZ for stem internode calli and a lower dose of 0.3 mg l^{-1} TDZ for root tip calli along with 0.1mg l^{-1} NAA.

Gantait and Sinniah (2012) obtained multiplication of PLBs in *Vanda* hybrid by culturing them on MS media supplemented with 1 mg l^{-1} BA and 0.5 mg l^{-1} IBA.

PLB and shoot proliferation is better with respect to *in vitro* explants source than *in vivo*. Sinha and Roy (2004) obtained profuse shooting from *in vitro* explants of *Vanda teres*. They had cultured seeds of *Vanda teres* on Vacin and Went medium and shoots were produced in the same basal media with added hormones. 1mg l^{-1} BAP and 0.5 mg l^{-1} NAA along with 2 g l^{-1} peptone, 10 per cent coconut water, 100 mg l^{-1} casein hydrosylate and 2 g l^{-1} banana pulp were added to the basal Vacin -Went media for shooting.

Kishor *et al.* in 2006 reported that the *in vitro* seedlings cultured on half strength MS enriched with 2.3 µM Kinetin and 0.5 µM NAA attained maximum shoot length and number. In 2009, Kishor and Devi investigated the effect of TDZ on multiple shoot induction. TDZ has a high potential to induce multiple shoots specially in axenic explants. Seedlings of *Vanda* hybrid, *Aerides vandarum* Reichb.f x *Vanda stangeana* Reichb.f developed maximum multiple shoots on half strength MS media supplemented with 2 mg l^{-1} TDZ.

In *Vanda coerulea*, cultures initiated by *in vitro* seed germination were transferred to Vacin-Went media with 1 mg l^{-1} BA and 0.5 mg l^{-1} NAA for multiple shoot induction (Jitsopakul *et al.*, 2013).

In vitro grown seedlings of 4-5 cm height were used for proliferation studies of *Vanda tessellate* by Bhattacharjee and Islam in 2014. Better proliferation was observed on MS media with hormonal combinations of 1 mg l^{-1} each of BAP and NAA.

7.3 Studies on rooting of *Vanda* orchid cultures:

In plant tissue culture, auxins have been used for cell division and root differentiation (Bhojwani and Razdan, 2004). There are many reports substantiating this observation. The most commonly used auxin in plant tissue culture for rooting are IAA and IBA. But others are also being used in varying proportions.

Rooting of plantlets was obtained in *Vanda* orchids when sub-cultured into Mitra medium supplemented with 1.08 μM NAA and 35 per cent banana powder (Seeni and Latha, 2000).

Begum *et al.* (2002) obtained rooting of plantlets from axillary bud explants by culturing them on MS media with 2 mg l^{-1} IBA. In 2003, Decruse *et al.* observed rooting of cultured plantlets in Mitra medium with 5.7 μM IAA and banana powder.

A combination of IBA and NAA could lead to production of roots in plantlets from shoot tip explants (Rahman *et al.*, 2009). MS served as the basal medium with 1 mg l^{-1} IBA and 0.5 mg l^{-1} NAA for an optimum rooting of *Vanda tessellata* tissue cultured plantlets.

In 2004, Sinha and Roy conducted an experiment in *Vanda teres* to obtain tissue culture plantlets. They studied the effects of various hormones on *in vitro* seed germination, shooting and rooting. Profuse rooting of the plantlets were obtained on half strength MS without any hormonal combinations.

In a *Vanda* hybrid, *Vanda coerulea* x *Ascocentrum auranticum,* maximum root length of 1.7 cm was observed on half strength MS media fortified with 2.7 μM NAA (Kishor *et al.,* 2006). But in *Vanda tessellata,* there was optimum root growth and development on half strength MS fortified with 1 mg l^{-1} IAA (Bhattacharjee and Islam, 2014).

In *Vanda coerulea,* Roy *et al.* (2009) could induce rooting on Phytamax media with 3 mg l^{-1} activated charcoal.

In another experiment conducted by Jitsopakul *et al.* (2013), *in vitro* shoot explants of 2-3 mm size of *Vanda coerulea,* showed a better rooting response in Vacin- Went media with 1 mg l^{-1} BA.

Sebastainraj *et al.* (2014) worked with *Vanda testacea* seeds to produce tissue cultured plantlets. The germinated seedlings were found to produce maximum roots when cultured on half strength MS with 0.2mg l^{-1} IBA.

7.4 Hardening and acclimatization of *Vanda* orchids

A large number of micropropagated plantlets do not survive its transfer from *in vitro* to field conditions (Hazarika, 2003). This is because plantlets grown

in vitro are exposed to unique microenvironment conditions to provide minimum stress and maximum plant multiplication. Hence they require a gradual acclimatization to survive in the field conditions (Pospisilova *et al.,* 1999). This is provided by primary and secondary hardening procedures.

Plants from culture rooms are hardened in the hardening unit before planting out in the field. As the plantlets from *in vitro* conditions usually do not have a well developed cuticle and as they had been adjusted to a low light intensity, generally a hardening unit is a mist chamber or a greenhouse. The recommended light intensity is higher in the hardening unit than in the culture rooms to increase growth and reduce mortality of plantlets at acclimatization stage (Chandra *et al.*, 2010). However Seeni and Latha (2000) and Decruse *et al.*, (2003) reported a 95-100 per cent establishment of rooted plants without any hardening.

The rooted plantlets from the culture vessels have to be washed free of agar before they are being transplanted for hardening. Begum *et al.* (2002) had transferred the rooted plantlets into plastic pots containing soil rite: coconut husk: charcoal (1:1:1) and covered them with polythene bags for few days to maintain the humidity and temperature. After 15 days, the polythene covers were removed and were transferred to greenhouse conditions for acclimatization to natural environment.

Sinha and Roy (2004) transferred individual regenerants of *Vanda teres* into earthen pots with equal proportion of charcoal and coconut husk as supporting materials. These pots were kept under indirect sunlight and were given mist thrice a day.

Lang and Hang (2006) potted the regenerants in sphagnum moss in greenhouse conditions and obtained a 90 per cent survival rate. Rahman *et al.* (2009) hardened the rooted plantlets in potting mixture containing coconut husk: perlite: charcoal: brick pieces (2:1:1:1) and eventually established under natural condition.

Roy *et al.* (2009) could record about 80 per cent survival rates for the plantlets potted in a mixture of charcoal, brick pieces and sphagnum moss.

Treating the roots of the regenerated plantlets with fungicide has been found effective in controlling fungal diseases during the acclimatization stage. Gantait and Sinniah (2012) had dipped the basal portion of the root zone in 0.2 per cent carbendazim for 2 minutes. Before transferring to greenhouse conditions, plantlets were hardened in pots with mixture of perlite and sand (2:1) and covered with plastic lids for 15 days. Moisture was maintained by regular water spraying. Bigger community pots were filled with charcoal and coconut fiber (1:1) for greenhouse planting.

In 2012, Paudel and Pant also reported better establishment of regenerants after treating with carbendazim. David *et al.* (2015) treated seedlings of *Vanda helvola* plantlets with 0.5 per cent fungicide for 15 minutes. Later these were planted in pots containing brick pieces and coconut husk (1:2) and mulched with sphagnum moss. Initially plants were covered with a polythene cover for one month and later it was removed for better establishment.

8. Genetic stability analysis

One of the major problems associated with micropropagation is the occurrence of somaclonal variants. Somaclonal variation is defined as genetic variation observed among progenies of regenerated plants from somatic cells cultured *in vitro* (Morrison *et al.,* 1988). Theoretically, clones are expected to be genetically uniform. But there are reports about the occurrence of variants among tissue cultured plantlets in various crops (Bairu *et al.,* 2011).

The frequency of occurrence of somaclonal variants depends on the source of explants, route of regeneration, culture conditions, media composition and the genetic background of the explant (Chen *et al.*, 2008). The cryptic genetic changes in the regenerants, arising due to somaclonal variations seriously limits the utility of micropropagation system.

In commercial micropropagation it is mandatory to regularly check the clonal fidelity or genetic uniformity of the regenerants to confirm their quality (Alizadeh *et al.,* 2015). Several methods are being followed to assess the clonal fidelity of the plantlets including morpho-physiological, biochemical and

cytological approaches (Alizadeh *et al.*, 2015; Kumar and Arya, 2009). Presently, DNA based molecular markers are being used extensively for clonal fidelity testing (Kishor *et al.*, 2008; Teh *et al.*, 2010; Chan *et al.*, 2013). Each tool has its own merits and limitations.

8.1 Methods of assessing clonal fidelity

8.1.1 Morphological markers

Morphological markers refer to the qualitative traits or characters that can be scored visually. Such characters include plant height, disease response, photoperiod sensitivity and other visually scorable traits (Singh, 2004). Morphological markers are present in nature as a result of mutations (Chawla, 2000).

Morphological markers are quick, simple and inexpensive method of assessment. They are usually dominant or recessive. However they have limited applications because morphological mutants occur rarely and often are highly detrimental or even lethal (White *et al.*, 2007). Other limitation is that the expression of the phenotype is highly influenced by the environmental conditions.

Brown (1978) mentioned that the lack of adequate genome coverage is also a major drawback of morphological markers.

Morphologically distinct variants from flower stalk bud cultures were observed by Tokuhara and Mii (1998) in *Phalaenopsis* and *Doritaenopsis.* Variations of less than 10 per cent were observed in the flower and inflorescence axis.

8.1.2 Biochemical markers

Protein markers, particularly, isozymes have been used successfully as biochemical markers (Markert and Moller, 1959). These are protein products of various alleles of one or several genes. They can be isolated and identified by electrophoresis and staining. Isozymes are generally codominant.

Isozymes are phenotypic markers and their genome coverage is low. They also show tissue variability. All these factors limit the reliability of biochemical markers.

Bhattacharyya *et al.* (2014) compared the biochemical parameters such as total phenolic content, total flavonoid content and antioxidant activity of the regenerated plantlets and mother plants of *Dendrobium nobile.*

8.1.3 Molecular markers

Molecular marker is a term used to refer to a specific DNA variation between individuals that has been found to be associated with certain characteristics (Singh *et al.*, 2014). These variations include insertions, deletions, translocations, duplications and point mutations.

The major advantage of molecular markers over other markers is its wide availability. They are widely distributed within the genome and are unaffected by environment.

There are basically two types of DNA markers (White *et al.*, 2007). First based on DNA-DNA hybridization and second based on amplification of DNA using Polymerase Chain Reaction (PCR). Non PCR based molecular marker include Restriction Fragment Length Polymorphism (RFLP) (Botstein *et al.*, 1980) whereas PCR based markers include Random Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1990), Simple Sequence Repeat (SSR) (Hearne *et al.*, 1992), Sequence Tagged Sites (STS) (Fukuoka *et al.*, 1994), Inter Simple Sequence Repeats (ISSR) (Zietkiewicz *et al.*, 1994), Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995).

8.1.3.1 Non PCR based DNA markers

8.1.3.1.1Restriction Fragment Length Polymorphism (RFLP)

RFLP was the first technology utilized to detect the polymorphism at the DNA level. It is a difference in homologous DNA sequences that can be detected as fragments of different lengths after digestion with specific restriction endonucleases. Most RFLP markers are co-dominant.

Though RFLP assay does not require any prior sequence information, it is time consuming and laborious (Powell *et al.*, 1996). It requires large quantity of pure high quality DNA (Kumar *et al.*, 2009) and a constant supply of probes that can reliably detect the variation. The radioactively labeled probes are also a major limitation of RFLPs.

RFLPs are mainly used for linkage mapping of quantitative trait loci and isolation of genes known to have linkage with an RFLP locus. Chang *et al.* (2000) studied the inheritance patterns of chloroplast DNA in intergeneric hybrids of *Phalaenopsis* and *Doritis* using RFLP. They are seldom used for genetic stability studies.

8.1.3.2 PCR based DNA markers

Polymerase Chain Reaction (PCR) is a technique in molecular biology which allows the enzymatic amplification of a given DNA sequence. Since the invention of this technique by Kary Mullis in 1983, it has been widely used in various procedures in molecular biology.

In the process of PCR, a small amount of DNA is amplified to a large extend as it pass through the process of denaturation, annealing and elongation. The major advantage of PCR based techniques is the need of only small quantity of DNA samples. Various PCR based DNA markers used for genetic stability analysis are RAPD, SSR, ISSR and AFLP.

8.1.3.2.1 Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR based technique wherein the given sequence of DNA is amplified using an arbitrary primer. In RAPD, a particular fragment may be generated for one individual but not for the other. This represents the DNA polymorphism and can be used as a molecular marker (Chawla, 2000).

RAPD markers have been successfully used for construction of genetic maps (Xue *et al.*, 2010; Feng *et al.*, 2013), mapping of traits (Grattapaglia *et al.*, 1996; Yano *et al.*, 1997) and genetic diversity analysis (Williams *et al.*, 1990; Heun *et al.*, 1994; Atak *et al.*, 2004).

Antony *et al.* (2012) studied the genetic stability of the PLBs of *Dendrobium* hybrid Bobby Messina using RAPD markers. The uniform RAPD banding profile among the plantlets derived from encapsulated PLBs confirmed the clonal fidelity of the regenerants in *Dendrobium densiflorum* (Mohanty and Das, 2013).

RAPD markers have been reported in many other crops including grape vine protoclones (Schneider *et al.*, 1996), ginger (Rout *et al.*, 1998), *Populus*

deltoids (Rani *et al.*, 1995), date palm (Saker *et al.*, 2000), kiwi fruit (Palombi and Damiano, 2001) etc. for clonal fidelity analysis. It has been used to identify somaclonal variants in moth orchids (Chen *et al.*, 2008), peach (Hashmi *et al.*, 1997) and sugarcane (Taylor *et al.*, 1995).

In some species RAPD markers failed to detect somaclonal variants as in begonia plantlets derived from leaf explants (Bouman and De Klerk, 2001) and garlic mutants (Anastassopoulos and Keil, 1996).

8.1.3.2.2 Amplified Fragment Length Polymorphism (AFLP)

AFLP is a PCR based amplification of selected restriction fragments of total genomic DNA. These markers reveal a restriction site polymorphism and are dominant markers. AFLP technique is reliable since stringent reaction conditions are used for primer annealing (Alizadeh *et al.*, 2015).

Owing to its high reliability, AFLP marker has been utilized extensively for ascertaining the clonal fidelity of micropropagated plantlets (Shenoy and Valil, 1992; Kidwell and Osborn, 1993).

Hornero *et al.* (2001) used AFLP markers to detect the genetic stability of cork oak somatic embryos. Amplification of monomorphic bands with AFLP primers, authenticated the true to type nature of the *in vitro* raised plants of *Dendrocalamus asper* (Singh *et al.*, 2013).

Genetic variations of field established *Bambusa nutans* plants regenerated through tissue cultures were assessed by amplified fragment length polymorphism analysis using 6 primer combinations by Mehta *et al.* (2011). Four hundred and seven scorable fragments were amplified, of which 402 (98.8%) had revealed a high level of genetic stability.

8.1.3.2.3 Simple sequence repeats (SSR) or Microsatellites markers

Simple sequence repeats are also known as microsatellite markers, Short Tandem Repeats (STR) or Simple Sequence Length Polymorphism (SSLP). SSR allelic differences are the results of variable numbers of repeat units within the microsatellite structure. The repeated sequences are usually di-, tri-, and tetra-nucleotide repeats.

The efficiency of microsatellite marker development depends on the abundance of repeats in the target species and the ease with which these repeats can be developed into informative markers (Alizadeh *et al.*, 2015). In *in vitro* cultures, microsatellite markers act as highly sensitive markers for monitoring genetic variations (Lopes *et al.*, 2006).

SSR primers tested for micropropagated plants of olive species *Olea maderensis* and *O. europaea* ssp. *europaea* var. *sylvestris* confirmed its genetic purity with that of the mother plant (Brito *et al.*, 2010).

The genetic fidelity testing of micro-shoots of sugarcane by Pandey *et al.* (2012) based on SSR analysis indicated a strong genetic purity similar to the parent genotype. Lack of variation confirmed the genetic purity of tissue culture plantlets of sugarcane raised through direct organogenesis in young whorl leaf roll explants and also the suitability of overall regeneration protocol.

Nookaraju and Agrawal (2012) could confirm the genetic homogeneity of *in vitro* raised plants of grape vine cultivar Crimson Seedless using microsatellite markers. Allelic composition of 23 *in vitro* raised plants and the mother plant at 5 SSR loci did not show any polymorphism. Their study confirmed the genetic uniformity among the *in vitro* raised plants and demonstrated the reliability of *in vitro* propagation system used for the cultivar.

8.1.3.2.4 Inter Simple Sequence Repeats (ISSR)

Inter-simple sequence repeats (ISSRs) are regions in the genome flanked by microsatellite sequences. ISSR markers are easy to use, low-cost, and methodologically less demanding compared to other dominant markers (Ng and Tan, 2015).

ISSR markers does not require any prior sequence data information for primer construction and the quantity of DNA sample required is very low as it is a PCR based marker (Kumar *et al.*, 2009). Also ISSR markers are randomly distributed over the entire genome.

ISSRs have high reproducibility possibly due to the use of longer primers (16- to 25-mers) as compared to RAPD primers (10-mers) which permits the subsequent use of high annealing temperature (45 to 60^0C) leading to higher stringency (Reddy *et al.*, 2002).

For most genetic variation studies, a good genetic marker is defined by high genetic variability and the ability to generate multilocus data from the genome under study (Anne, 2006). This makes ISSR markers a highly reliable molecular marker for clonal fidelity analysis. Inter simple sequence repeat (ISSRs) have emerged as an efficient and effective tool for clonal fidelity and genome mapping (Alizadeh *et al.*, 2015).

Experimental results obtained by Lakshmanan *et al.* (2007) were a confirmation for the true to type nature of micropropagated plantlets. They investigated the ISSR banding patterns of mother plant as well as the regenerants of banana variety Nanjanagudu Rasabale using 5 ISSR primers. The monomorphic banding pattern obtained confirmed the genetic fidelity of the clones.

Monomorphic banding patterns were observed among the regenerants and the mother plants of monopodial orchid hybrid *Aerides vandarum* X *Vanda*

stangeana when their genetic stability was analyzed by Kishor and Devi (2009) using 12 ISSR primers. Their results confirmed the genetic stability of the regenerated plantlets.

Negi and Saxena (2010) analyzed the bands produced by 15 ISSR primers in *in vitro* raised *Bambusa balcooa* plantlets. Analysis of ISSR patterns revealed that the bands were shared by both the parent clump and the *in vitro* raised plantlets confirming the genetic stability in the regenerants.

Bhatia *et al.* (2011) also obtained monomorphic banding patterns while analyzing the ISSR banding patterns of *in vitro* raised gerbera clones using 10 primers.

Liu and Yang (2012) could confirm the effectiveness of the micropropagation protocol developed by them for *Lilium orientalis* using ISSR markers. Eleven ISSR primers could generate 70 clear and reproducible bands and they could observe a similarity of 92 per cent among the mother plants and the regenerants. Genetic stability of *in vitro* multiplied *Phalaenopsis gigantea* PLBs were assessed by Samarfard *et al.* in 2013. They used 8 ISSR primers which gave monomorphic banding patterns across mother plants and PLBs. Choudhary *et al.* (2015) observed a homogeneous amplification profile for all micropropagated plantlets of commercial banana cultivar Robusta when amplified using 20 ISSR primers.

Materials and methods

3. MATERIALS AND METHODS

The study entitled "*In vitro* micropropagation protocol for *Vanda* hybrids with clonal fidelity analysis" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2014- June 2016, with the objective to regenerate tissue culture plants of *Vanda* hybrids through a viable protocol and examine suitability of the same for micropropagation with clonal fidelity analysis using ISSR markers. The study mainly focused on the following aspects:

1) Identification of a micropropagation protocol in *Vanda* hybrids using a suitable explant, hardening of the regenerants and its acclimatization.

2) Clonal fidelity analysis of micropropagated plantlets using ISSR assay.

The materials used and methodologies adopted in the study are presented in this chapter.

3.1 Materials

3.1.1 Plant material

Two leading *Vanda* hybrids Dr. Anek (*Vanda* Fuch's Delight X *Vanda* Ponpimol) and Sansai Blue (*Vanda* Crimson Glory X *Vanda coerulea*) were selected for this study (Plate 1). These are two of the most demanded hybrids of *Vanda* and have large showy flowers. Dr. Anek has netted pink coloured flowers whereas Sansai Blue has netted blue flowers.

3.1.2 Laboratory chemicals, glass and plastic wares

Chemicals used for the present study were of good quality (AR grade) from various agencies like Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The *Taq* DNA polymerase, dNTPs, *Taq* buffer and molecular marker (λDNA/*Hind*III+*Eco*RI double digest) were supplied by Bangalore Genei Ltd. All the plastic wares used were obtained from Axygen and Tarson India Ltd. The ISSR primers were obtained from Vision Scientific Pvt. Ltd.

Vanda **'Dr. Anek'**

Vanda **'Sansai Blue'**

Plate 1. *Vanda* hybrids selected for the study

3.1.3 Equipment and machinery

The present research work was carried out using plant tissue culture and molecular biology facilities available at CPBMB, College of Horticulture. All the aseptic manipulations for micropropagation studies were carried out in laminar air flow (LABLIE INDUSTRIES). Media sterilization was done in autoclave (Nat steal equipment Pvt. Ltd.) and centrifugation using high speed refrigerated centrifuge (KUBOTA 6500)/ Dynamical Velocity 14R Refrigerated Centrifuge. NanoDrop^R ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. DNA amplification reaction was carried out in Agilent Technologies thermocycler (SureCycler 8800). Horizontal gel electrophoresis system (BIO-RAD, USA) was used for agarose gel electrophoresis. Gel Doc - BIO-RAD was used for imaging and documenting the agarose gel profile. The details are given in Annexure I.

3.2 Identification of micropropagation protocol for *Vanda* hybrids

Different protocols reported earlier for the culture initiation, multiplication and elongation and rooting of different *Vanda* species were reviewed and three media compositions were selected for identifying a suitable regeneration system for the selected *Vanda* hybrids based on the literature search. The media composition selected for each stage of micropropagation of *Vanda* hybrids are listed below:

1. Culture initiation:
    - MS + 3% sucrose + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) reported by Gantait and Sinniah (2012) for leaf explants
2. Shoot proliferation:
    - MS + 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA (Paudal and Pant, 2012)
3. Elongation and rooting:
    - Rooting: MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} IAA (CPBMB, unpublished)

3.2.1 Source of explants

The source of explants was obtained from a commercial nursery, Pearl Orchids, Moonupeedika, Thrissur. Mother plants of both varieties were obtained in the flowering stage to ensure the procurement of the selected hybrids.

3.2.2 Maintenance of explant source

The mother plants were labelled and maintained in the net house of CPBMB providing 50 per cent shade. The nutrition for the mother plant was provided with both chemical fertilizer complexes and organic supplements in alternate weeks. Mist irrigation was followed to meet the water requirement of plants on daily basis. A systemic fungicide spray of carbendazim (0.1%) was given three days prior to the collection of explants.

3.2.3 Culture media

Full strength MS (Murashige and skoog) and half strength MS fortified with required amount of auxin and cytokinin were used for the identification of suitable micropropagation protocol. Composition of MS medium is given in Appendix II. In case of half strength MS medium, the concentrations of the components were reduced to half expect for the stock V.

3.2.3.1 Preparation of MS medium

Standard procedures were followed for the preparation of MS plant tissue culture media. Five stock (I, II, III, IV and V) solutions of major and minor nutrients were prepared and stored in refrigerated conditions. Stock III was stored in amber coloured bottle. Stock solutions of different growth regulators were also stored under refrigerated conditions.

A clean beaker, rinsed with distilled water was used to prepare the medium. All stock solutions were pipetted in proportionate volume in the beaker. For preparing MS medium of full strength, 20 ml from 50 X stocks and 10 ml from 100 X stocks and required quantities of sucrose (30 g l^{-1}), inositol (0.1 g l^{-1}) and hormones were added and dissolved in it. The desired volume was made up by

adding distilled water. The pH of the medium was adjusted to 5.5 or 5.6 using 0.1 N NaOH.

The volume was finally made up and required quantity of agar (7.5 g l^{-1}) was added to the medium. Agar in the medium was completely melted in a microwave oven and autoclaved. Then, the hot medium was poured into pre-sterilized conical flask with cotton plugs and autoclaved.

3.2.3.2 Autoclaving

The conical flasks with nutrient media were autoclaved at a pressure of 1.06 kg cm^{-2} (121^0C) for 20 minutes. The flasks were then removed from the autoclave and allowed to cool. Next day, the media was melted and sterile cefotaxime (250 mg l^{-1}) was added to the autoclaved culture medium inside the laminar air flow chamber before dispensing into 25 x 150 mm pre-sterilized glass test tubes.

The inoculation was done 4-5 days after media sterilisation ensuring that the tubes were free of microbial contaminations.

3.2.4 Experimental conditions

3.2.4.1 Transfer area and aseptic manipulation

All the aseptic manipulations such as surface sterilisation of the explants and inoculation and subsequent sub-culturing were carried out in the laminar air flow cabinet. The work table of laminar air flow cabinet was sterilized by swabbing with 100 per cent alcohol. The UV light was switched on for half an hour to achieve aseptic environment inside the cabinet and air was allowed to blow off for 10 minutes before working in the lamina air flow cabinet.

3.2.4.2 Culture conditions

The cultures were incubated at 26 ± 2^0C in an air conditioned culture room with 16 h photoperiod (30.4μmoles/m^2/s) from florescent tubes. Humidity in the culture room varied from 60 to 80 per cent according to the prevailing climate.

3.2.5 Culture establishment studies

3.2.5.1 Culture establishment using leaf explants

3.2.5.1.1 Selection and preparation of the explant

The upper new leaves were selected as explant source. Leaves were excised from the mother plants maintained in the net house using sterile blades. Both basal portion and the tips were used as explants. The leaves collected were wiped with 50 per cent ethanol, washed thoroughly under running tap water and further given surface sterilization treatments. After surface sterilization, the explants were allowed to dry by transferring them to sterile tissue paper on a sterile petri dish. All the side ends of the leaf explants were trimmed off. Leaves were further cut into small square pieces of 3-5 mm^2 and allowed for further air drying. The various steps involved in the collection and preparation of leaf explants are given in Plate 2.

3.2.5.1.2 Standardization of surface sterilization of leaf explants

Attempts were made to standardize surface sterilization procedure for both selected hybrids.

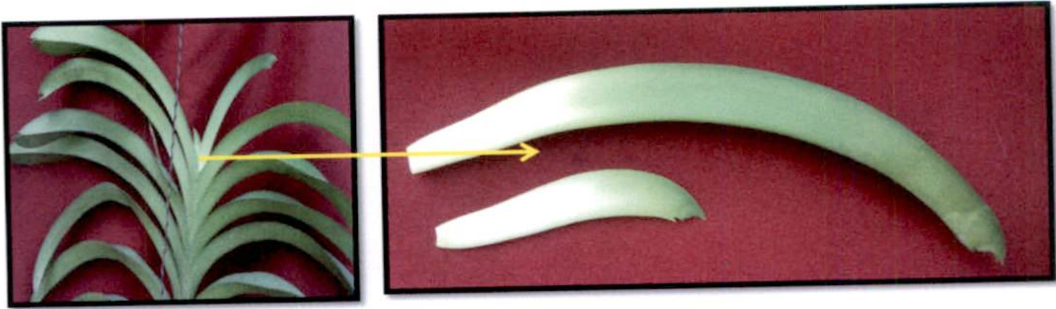
The explants collected from the net house were given a swab with 50 per cent ethanol and washed under running tap water. Then these were given a detergent wash along with 0.1 per cent carbendazime for 20 minutes with thorough shaking. Then it was washed three to four times with distilled water to remove the detergent completely. Further surface sterilization procedures were done in the laminar air flow cabinet with both 70 per cent ethanol and 0.1 per cent mercuric chloride for different time intervals (1, 2, 3, 4, 5, 6, 8 and 10 min each). Thus there were eight treatments as listed below:

T_1: One minute 70 per cent ethanol and one minute 0.1 per cent mercuric chloride.

T_2: Two minutes 70 per cent ethanol and two minutes 0.1per cent mercuric chloride.

T_3: Three minutes 70 per cent ethanol, and three minutes 0.1 per cent mercuric chloride.

T_4: Four minutes 70 per cent ethanol and four minutes 0.1 per cent mercuric chloride.



The young upper leaves of the mother plant

Leaves cut into small pieces after surface sterilization

Leaf base inoculated to medium

Leaf tip inoculated to medium

Plate 2: Collection and preparation of leaf explants

T_5: Five minutes 70 per cent ethanol and five minutes 0.1 per cent mercuric chloride.

T_6: Six minutes 70 per cent ethanol and six minutes 0.1 per cent mercuric chloride.

T_7: Eight minutes 70 per cent ethanol and eight minutes 0.1 per cent mercuric chloride.

T_8: Ten minutes 70 per cent ethanol and ten minutes 0.1 per cent mercuric chloride.

After treating with mercuric chloride, the explants were washed three times in distilled water to remove even the trace amount of sterilants. Then, after cutting into small pieces, the explants were allowed to dry. Once dried completely, the explants were inoculated into the test tubes with MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar (Gantait and Sinniah, 2012). Cefotaxime (250 mg l^{-1}) was also incorporated into the nutrient media. The tubes were kept in dark room for 48 h and were shifted to light room with light intensity of 1000 lux and temperature of 26± 2^0C.

Observations were taken for per cent contamination, per cent survival and the nature of contamination after twenty one days of inoculation.

3.2.5.1.3 Identification of media for culture establishment with leaf explants

Development of an *in vitro* regeneration protocol for micropropagation of plants highly depends on the selection of suitable media composition. The surface sterilized leaf explants of size 3-5 mm^2 were inoculated to different culture establishment media to identify the media composition for establishing cultures. The explants were inoculated such that the abaxial side was in contact with the medium. The different media compositions tried for culture establishment using leaf explants of selected *Vanda* hybrids are given in Table 1. Antibiotic cefotaxime was incorporated in all the media combinations tried.

Observations on the response of inoculated explants in the media were recorded three weeks after inoculation. Sub-culturing was done at an interval of twenty one days.

Table 1. Media compositions tested for culture establishment with leaf explants of *Vanda* hybrids

Treatment No.	Media composition	Reference
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	Gantait and Sinniah, 2012
T2.	$^{1}/_{4}$ MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	Kishi *et al.,* 1997
T3.	Basal MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	Begum *et al.,* 2002
T4.	Mitra + 66.6 µM BA + 28.5 µM IAA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	Decruse *et al.*, 2003
T5.	$^{1}/_{2}$ MS + 10 mg l^{-1} 2,4-D + 1 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	Lang and Hang, 2006
T6.	MS + 1.5 mg l^{-1} NAA + 1 mg l^{-1} BAP + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	Rahman *et al.*, 2009
T7	$^{1}/_{2}$ MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	-
T8.	$^{1}/_{2}$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	CPBMB, unpublished

3.2.5.2 Culture establishment using root explants

3.2.5.2.1 Selection and preparation of root explants

Vanda is a monopodial orchid with long growing roots. For the collection of root segments, fresh, healthy and live roots were selected. They were cut with sterile blades, wiped with 50 per cent ethanol, washed under running tap water and subjected to surface sterilization treatments. After surface sterilization procedure, the explants were allowed to dry by transferring them onto sterile tissue paper on a sterile petri dish. Both the cut ends of the root segments were trimmed off to ensure living cells at the ends which can aid in the better absorption of nutrients from the media. Roots were cut into thin cross sections of size 1-2 mm thick and 0.3 mm diameter and allowed for air dry inside the laminar hood. The steps involved in the collection and preparation of root explants are given in Plate 3.

3.2.5.2.2 Standardization of surface sterilization of root explants

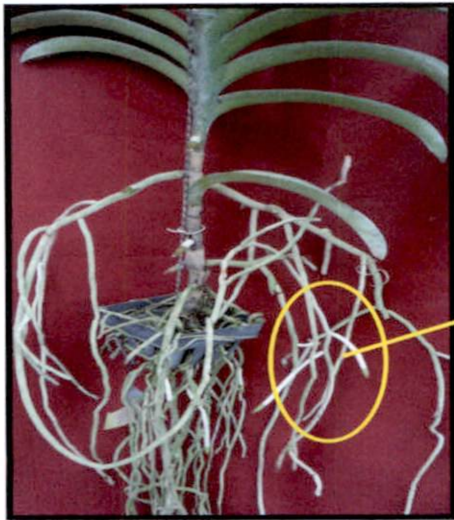
An attempt was made to standardize the surface sterilization treatments for the root explants in both hybrids. The treatments, medium and culture conditions were same as that of the leaf explants.

The explants were prepared in laminar hood and once dried completely, these were inoculated into the test tubes with suitable medium. Observations were taken for the per cent contamination, per cent survival and the nature of contamination after twenty one days of inoculation.

3.2.5.2.3 Identification of media for culture establishment with root explants

Healthy live roots were used as explants to initiate the cultures for developing an efficient micropropagation protocol for selected *Vanda* hybrids using root explants. Different media compositions similar to that tested with leaf explants were also tested for root explants (Table. 1). Ten tubes were inoculated for each media for both the selected hybrids.

Observations on the response of inoculated explants in the media were recorded three weeks after inoculation. Sub-culturing was done at an interval of twenty one days.



Healthy live roots of mother plant

Roots cut into thin cross sections after surface sterilization

Root segments inoculated into medium

Plate 3: Collection and preparation of root explants

3.2.5.3 Culture establishment using stem segments

3.2.5.3.1 Selection and preparation of stem segments

Vanda is a monopodial orchid with a single tough stem. The stem has nodes on them, from where the leaves arise. The upper few leaves were removed carefully to obtain the thick stem at the centre with the nodes. For obtaining the thich stem, mother plant was cut at almost 15 cm from the top of the mother plant. The leaves were removed carefully not to damage any of the nodes and then the explant was washed under running tap water. Apical meristem was collected by removing carefully the upper leaves and exposing the meristematic region on the tip of the stem.

The collected stem segments and the apical meristem were washed well under running tap water and were further given surface sterilization treatments after which it was cut into two noded segments of about 3-4 cm length and inoculated to suitable medium. Plate 4 explains the steps involved in collection and preparation of apical meristem and stem segments.

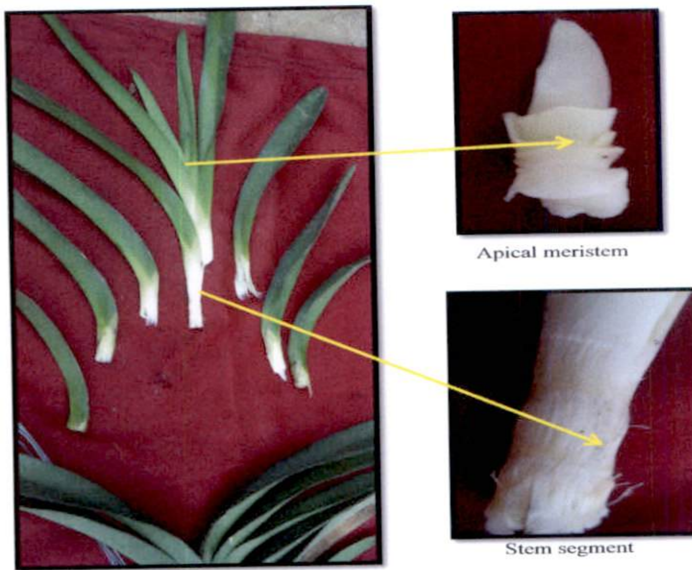
3.2.5.3.2 Surface sterilization of stem segments

Since the number of stem segments that could be obtained was very less, especially because as it destroys the mother plant, the standardization experiment for the surface sterilization procedure was not done for stem segments separately. The treatment that had been standardized for other explants was used for stem segments and apical meristem.

3.2.5.3.3 Identification of media for culture establishment using stem segments

Monopodial growth habit of *Vanda* makes it difficult to obtain large number of stem segments as explants to conduct trials for the development of a micropropagation protocol. Hence in this study only three media compositions were tested for initiating cultures using stem segments and each with only three test tubes for both hybrids. Different media compositions tested for initiating cultures with stem segments and apical meristem are given in Table 2. The inoculated test tubes were kept in dark room for 48h and then shifted to light room of 1000 lux intensity. Three tubes were inoculated for each media tried for both

Mother plant cut at middle



Leaves removed to obtain apical meristem and stem segments

Apical meristem inoculated into medium

Stem segment inoculated into medium

Plate 4: Collection and preparation of apical meristem and stem segments

the selected hybrids in case of stem segments whereas only one test tube could be maintained for apical meristem as the rate of apical meristem collection is only one per mother plant.

Table 2. Media compositions tested for culture establishment with stem segments and apical meristem of *Vanda* hybrids

Treatment No.	Media composition	Reference
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	Gantait and Sinniah, 2012
T2.	½ MS + 10 mg l^{-1} 2,4-D + 1 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	Lang and Hang, 2006
T3.	**For apical meristem** Mitra + 44.4 µM BA + 28.5 µM IAA + 20 g l^{-1} sucrose + 6 g l^{-1} agar	Decruse *et al.*, 2003

Observations on the response of inoculated explants in the media were recorded three weeks after inoculation. Sub-culturing was done at an interval of twenty one days.

3.2.5.4 Culture establishment using inflorescence segments

3.2.5.4.1 Selection and preparation of inflorescence segments

Vanda hybrids maintained for experimental purpose flowered twice in a year and the season being December-February and July-September. The inflorescence stalk was obtained from the mother plant using a sterile forceps at around 10 days from the emergence of the inflorescence on the mother plant. The upper buds were removed from the stalk and the remaining part of stalk was wiped with 50 per cent ethanol and further washed under running tap water. Then it was given surface sterilization treatments inside the laminar hood. After the surface sterilization, the stalk was cut into pieces of 3-4 cm length such that the

segments contained a dormant bud each. Only two such segments could be obtained as explant from one inflorescence stalk. After surface sterilization, the bract covering the node was removed carefully using a sterile forceps. Any amount of moisture near the node was carefully wiped using sterile tissue paper taking care not to damage the bud. The different steps involved in the collection and preparation of inflorescence segments are given in Plate 5.

3.2.5.4.2 Standardization of surface sterilization of inflorescence segments

The inflorescence segments were subjected to surface sterilization treatments to make them free of contamination. The mother plants maintained in the net house were given 0.1 per cent carbendazim spray three days prior to explant collection. The explants collected from the net house were washed under running tap water and then were given a detergent wash. Further surface sterilization procedures were done in the laminar air flow chamber similar to leaf explants as detailed in section 3.2.5.1.2. However owing to the limited number of inflorescence segments, they were given only three different treatments namely T_4, T_5 and T_6 as listed below:

T_4: Four minutes 70 per cent ethanol and four minutes 0.1 per cent mercuric chloride.

T_5: Five minutes 70 per cent ethanol and five minutes 0.1 per cent mercuric chloride.

T_6: Six minutes 70 per cent ethanol and six minutes 0.1 per cent mercuric chloride.

In case of inflorescence segments, the media used for standardization of surface sterilization procedure was half strength MS fortified with 10 mg l^{-1} BA and 2 mg l^{-1} TDZ solidified on 7.5 g l^{-1}agar with 3% sucrose and 250 mg l^{-1} cefotaxime which was the media composition standardized for *Phalaenopsis* tissue culture using inflorescence segments at CPBMB.

Observations on per cent contamination and explant survival were recorded three weeks after inoculation.

Inflorescence on mother plant cut with a sterile blade

Segment with dormant bud selected

Bract covering bud removed after surface sterilization and inoculated into medium

Plate 5: Collection and preparation of inflorescence segments

3.2.5.4.3 Management of phenolic interference

During the standardization of surface sterilization experiments it was observed that the media in which the inflorescence segments were inoculated turned brown during the culture period because of phenolic exudations. Hence the explants were dipped in 0.1 per cent ascorbic acid for five minutes before treating with 70 per cent ethanol during the surface sterilization procedure.

3.2.5.4.4 Identification of media for culture establishment using inflorescence segments

After surface sterilization procedure, once the explants were completely dried, these were inoculated such that the bud is just above the media.Two media compositions were tested to initiate cultures using inflorescence segments as given in Table 3. The inoculated test tubes were kept in dark for 48h and then shifted to light conditions of 1000 lux with a temperature of 26 ±2^{0}C.

Table 3. Media compositions tested for culture establishment with inflorescence segments of *Vanda* hybrids

Treatment No.	Media composition	Reference
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	Gantait and Sinniah, 2012
T2.	½ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1}agar	CPBMB, unpublished

Observations on the response of inoculated explants in the media were recorded three weeks after inoculation. Sub-culturing was done at an interval of twenty one days.

3.2.6 Selection of suitable explant and medium for culture establishment of *Vanda* hybrids

The results of various experiments and trials conducted for the culture establishment of *Vanda* hybrids were analysed to select the suitable explant, medium and surface sterilization treatment to initiate cultures.

Observations on the number of days taken for shoot initiation and per cent culture establishment were recorded three weeks after inoculation. The percentage of culture establishment was calculated by the formula:

$$\% \text{ culture establishment} = \frac{\text{Number of cultures established}}{\text{Number of cultures inoculated}} \times 100$$

3.2.7 Shoot proliferation studies

3.2.7.1 Identification of media composition for shoot proliferation

The inflorescence segments with sprout initials were further sub-cultured into shoot proliferation media after three subcultures in the culture establishment medium. Few of the cultures were inoculated to multiplication media along with the stalk whereas few others were inoculated by separating the sprout from the stalk. The different media compositions tested for shoot proliferations are given in Table 4.

Table 4. Media compositions tested for the shoot proliferation of established cultures using inflorescence segments of *Vanda* hybrids

Treatment No.	Media composition	Reference
T1.	MS + 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA + 30 g l^{-1} sucrose +7.5 g l^{-1}agar	Paudal and Pant, 2012
T2.	½ MS + 1 mg l^{-1} IAA + 1 mg l^{-1} BAP + 30 g l^{-1} sucrose +7.5 g l^{-1}agar	Paul, 2005
T3.	MS + 4.5 ml l^{-1} BA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar	CPBMB, unpublished

The number of multiple shoots produced was recorded after each subculture passages starting from S4 to S7. The sub-culturing was done at every twenty one days interval.

Observations on the mean number of shoots per explant, mean length of the shoots and mean number of leaves per explant were recorded at each subculture passages.

3.2.8 Shoot elongation studies

The *in vitro* micro shoots of size 1.5 cm were taken out from multiple shoot cultures and inoculated in the reported medium for elongation of shoots i.e hormone free basal MS (Bhosle *et al.*, 2005) supplemented with 0.5 g l^{-1} charcoal. The cultures were maintained in this medium for one subculture time period and observations were taken for mean length after 21 days of inoculation. Once the shoots were about 4-5 cm height, they were further sub-cultured into the rooting medium for induction of roots.

3.2.9 Rooting studies

The multiple shoots produced on the cultures along with stalk were directly inoculated to rooting medium while those from without stalk were transferred to rooting medium after elongation stage.

The rooting medium used was MS + 0.5 mg l^{-1} NAA + 1 mg l^{-1} IAA +30 g l^{-1} sucrose + 7.5 g l^{-1} agar. Charcoal at the rate of 0.5 g l^{-1} and 250 mg l^{-1} cefotaxime was also incorporated. This medium had been identified for the elongation and rooting of orchids at CPBMB. Cultures were maintained in rooting medium for two subculture passages.

Observations on the response of cultures in the rooting medium were recorded at twenty one days interval as observed as mean length of shoots, mean number of roots, and number of days taken for rooting. Per cent rooting was calculated using the formula:

$$\% \text{ rooting} = \frac{\text{Number of rooted cultures}}{\text{Number of cultures inoculated in rooting media}} \times 100$$

3.2.10 Hardening and acclimatization

The *in vitro* rooted plantlets were taken out of the culture vessels using clean forceps after soaking the culture in water for five minutes. The solidified medium from plantlets was washed out in running tap water. The plantlets were treated with 0.1 per cent carbendazim for five minutes and were then planted in small earthen pots filled with charcoal, coconut husk and brick pieces. Then plantlets were hardened in hardening unit of 50 per cent shade.

Plantlets were observed for its growth and per cent survival. Biometric observations were made at monthly intervals. Various biometric observations taken for the regenerants included plant height (measured from base of plant to tip of last leaf) and number of leaves.

3.3 Clonal fidelity studies using ISSR molecular markers

For the clonal fidelity analysis of micropropagated plantlets, ISSR assay was carried out. Mother plants and 4-5 regenerated plantlets of each mother plant were subjected to clonal fidelity studies using ISSR assay with five ISSR primers reported for *Vanda* hybrids by Kishor and Devi (2009).

3.3.1 Genomic DNA isolation

The CTAB procedure reported by Rogers and Bendich (1994) was used for the extraction of good quality genomic DNA. The young tender leaves were used for genomic DNA isolation.

Reagents (Details of composition of reagents are provided in the Annexure III)

1. 2 X CTAB extraction buffer
    - 2 per cent CTAB (w/v)
    - 100mM Tris (pH 8.0)
    - 20mM EDTA (pH 8.0)
    - 1.4M NaCl
    - 1 per cent PVP
2. CTAB (10 per cent)
    - 10 per cent CTAB (w/v)
    - 0.7M NaCl

3. TE buffer

   -10mM Tris (pH 8.0)

   -1mM EDTA (reagent 1 and 3 autoclaved and stored at room temperature)

4. β- mercaptoethanol
5. Chloroform : isoamyl alcohol (24:1)
6. Isopropanol (chilled)
7. Ethanol (70 %)
8. Distilled water

3.3.1.1 Procedure for DNA isolation

Procedure

Young and tender leaf tissue (0.5g) was weighed and ground in a pre-chilled mortar and pestle in the presence of liquid nitrogen and a pinch of PVP. One ml of extraction buffer (2X) and 50μl of β-mercaptoethanol were added to it. The homogenized sample was transferred to an autoclaved 2ml centrifuge tube and added 1ml of pre-warmed extraction buffer. After mixing thoroughly, the mixture was incubated at 65°C for 30 minutes with occasional mixing by gentle inversion. Equal volume (1ml) of chloroform: isoamyl alcohol (24:1v/v) was added and the mixture was mixed by inversion to emulsify. Then, it was centrifuged at 12,000 rpm for 15 minutes at 4°C. After the centrifugation, the top aqueous layer was transferred to another clean centrifuge tube and again equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion and centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a clean centrifuge tube and added 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till the DNA was precipitated. The mixture was kept for overnight incubation in -20^{0}C. After the incubation, the mixture was again centrifuged at 10,000 rpm for 15 minutes at 4°C and the supernatant was gently poured off. The DNA pellet was washed with 70 per cent ethanol and the ethanol was decanted. The DNA pellet was air dried for 20 minutes and the pellet was dissolved in 50μl of TE buffer or sterilized water and stored at - 20°C.

3.3.1.2 Purification of DNA

The DNA isolated would had RNA contamination and hence was purified by RNase treatment and precipitation (Sambrook *et al.*, 1989).

Reagents

1. Chilled isopropanol
2. 70 % ethanol
3. TE buffer
4. Chloroform: Isoamyl alcohol (24:1, v/v)
5. RNase (1%)

The RNase-A from Sigma, USA was used to prepare RNase. One per cent solution was prepared by dissolving RNase-A in autoclaved distilled water. The solution was dispensed into aliquots and stored at -20°C.

Procedure

RNase solution (2µl) was added to 100µl DNA sample and incubated at 37°C in dry bath (GeNei, Thermocon) for one hour. The volume was made up to 250µl with distilled water and equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently. This was then centrifuged at 10,000 rpm for 15 minutes at 4°C. The aqueous phase was collected into a fresh micro centrifuge tube and equal volume of chloroform: isoamyl alcohol (24:1) was added. Again it was centrifuged at 10,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a fresh micro centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by gentle inversion till the DNA was precipitated. The mixture was incubated at -20°C for half an hour for complete precipitation and centrifuged at 10,000 rpm for 15 minutes at 4°C. The pellet of DNA obtained was washed with 70 per cent ethanol. The pellet was air dried and dissolved in 50µl of autoclaved distilled water.

3.3.2 Assessing the quality of DNA by electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al.*, 1989).

Materials for agarose gel electrophoresis

1. Agarose
   - 0.8 per cent (for genomic DNA)
   - 1.8 per cent for ISSR
2. 50X TAE buffer (pH 8.0)
   - Tris buffer
   - Acetic acid
   - 0.5mM EDTA
3. 6X Loading /Tracking dye
   - 0.03% bromophenol blue
   - 0.03% xylene cyanol
   - 60% glycerol
4. Ethidium bromide (0.5μg/ml)
5. Electrophoresis unit, power pack, gel casting tray, comb
6. UV transilluminator
7. Gel documentation and analysis system

Chemical composition of buffers and dye are given in Annexure IV. The procedure followed for agarose gel electrophoresis was as follows:

1 X TAE buffer was prepared from the 50 X stock solution. Agarose (0.8%) was weighed and dissolved in a solution of TAE buffer (1X) and distilled water by boiling, and then ethidium bromide was added at a concentration of 0.5μg/ml and mixed well. The open end of the gel casting tray was sealed with cello tape and kept on a horizontal surface. The comb was placed suitably and the dissolved agarose was poured in to the tray. The gel was allowed to solidify for 30 minutes after which the comb was removed carefully. The tray was kept in the electrophoresis unit with the wells directed towards the cathode. 1X TAE buffer was added to the tank. Then, DNA sample (5μl) along with tracking dye (1μl) was loaded into the wells using a micropipette carefully. λDNA/*Eco*RI+*Hind*III double digest was used as the ladder. After closing the tank, the anode and cathode ends

were connected to the power pack and the gel was run at the voltage (80V). The power was turned off when the tracking dye reached 80% length of the gel.

Then, the gel was taken from the electrophoresis unit and observed in Gel doc XR+ for presence of DNA. The DNA fluoresces under UV light due to ethidium bromide dye. The image was documented in gel documentation system. The gel profile was examined for intactness and clarity of DNA band.

3.3.2.1 Assessing the quality and quantity of genomic DNA

The quality and quantity of genomic DNA from both parents and clones was estimated using NanoDrop[R] ND-1000 spectrophotometer. Before taking sample readings, the instrument was set zero by taking 1µl autoclaved distilled water as blank. One micro litre of nucleic acid sample was measured at a wavelength of 260 nm and 280 nm and OD_{260}/ OD_{280} ratios were recorded to assess the purity of DNA.

A ratio of 1.8 to 2.0 for OD_{260}/OD_{280} indicated good quality DNA. The quantity of DNA in the pure sample was calculated using the formula $OD_{260}= 1$ is equivalent to 50 µg double stranded DNA/ µl sample.

1OD at 260 nm = 50 µg DNA/ml

Therefore $OD_{260} \times 50$ gives the quantity of DNA in µg/ml.

3.3.3 Inter Simple Sequence Repeats (ISSR) marker analysis

ISSR assay was performed to detect the polymorphism in amplification patterns in the region between two SSR's. This was carried out by amplifying the DNA by using specific primers relating to the SSR region flanking the ISSR.

Good quality genomic DNA (50 to 100ng/µl) isolated from the regenerated plantlets was subjected to ISSR assay. An initial primer screening was done with eight primers reported for *Vanda* hybrids by Kishor and Devi (2009). Based on the resolving power of primers and number of bands, five were selected for further analysis. ISSR primers were supplied by 'Vision Scientific Pvt. Ltd.

3.3.3.1 Primer screening for ISSR assay

Primer screening was done with eight primers and the details are furnished in Table 5.

Table 5. Details of ISSR primers used for screening

Sl. No.	Primers	Nucleotide sequence (5'-3')	Bands scored	Size range (bp)
1	UBC 808	5'AGAGAGAGAGAGAGAGC 3'	9	183-1534
2	UBC 811	5'GAGAGAGAGAGAGAGAC 3'	10	231-1484
3	UBC 818	5' CACACACACACACACAG 3'	9	274-1693
4	UBC 826	5'ACACACACACACACACC 3'	9	411-1100
5	UBC 835	5'AGAGAGAGAGAGAGAGYC 3'	11	121-1500
6	UBC 841	5' GAGAGAGAGAGAGAGAYC 3'	8	372-2323
7	UBC 847	5' CACACACACACACACARC 3'	8	441-2203
8	UBC 850	5' GTGTGTGTGTGTGTGTYC 3'	12	200-2300

R= (A+G), Y= (C+T)

3.3.3.2 ISSR assay

ISSR assay was performed with DNA samples isolated from mother plants and *in vitro* plantlets. Five selected primers as shown in Table 6 were used for the study.

Table 6: Details of selected ISSR primers

Sl. No.	Primers	Nucleotide sequence (5'-3')	Bands scored	Size range (bp)
1	UBC 808	5'AGAGAGAGAGAGAGAGC 3'	9	183-1534
2	UBC 811	5'GAGAGAGAGAGAGAGAC 3'	10	231-1484
3	UBC 826	5'ACACACACACACACACC 3'	9	411-1100
4	UBC 835	5'AGAGAGAGAGAGAGAGYC 3'	11	121-1500
5	UBC 841	5' GAGAGAGAGAGAGAGAYC 3'	8	372-2323

DNA amplification was done in Agilent thermocycler as per the PCR conditions cited in Table 7. Amplification was performed in a 20 µl reaction mixture.

Table 7. Composition of the reaction mixture for PCR

Reagents	Quantity (µl)
Genomic DNA (50 ng/ µl)	2.0
10X *Taq* assay buffer	2.0
$MgCl_2$	2.0
dNTP mix (10Mm each)	1.5
Taq DNA polymerase (3 U)	0.4
Primer	1.5
Autoclaved distilled water	10.6
Total volume	20.0

The thermocycler was programmed as given in Table 8:

Table 8. Programme for PCR reaction

Sl. No.	Step	Temperature (^0C)	No. of cycles
1.	Initial denaturation	94	1
2.	Denaturation	94	
3.	Primer annealing	48-58	35
4.	Primer extension	72	
5.	Final extension	72	1
6.	Final hold	4	

PCR product was electrophoresed and gel profiles data were documented. Amplification profile of mother plants and micropropagated plantlets generated by each selected primers was examined for maximum number of amplicons, polymorphism and molecular weight of amplicons. Percentage of polymorphism generated by each primer was worked out as given below:

$$\text{Percentage of polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Results

4. RESULTS

The results of the study on "*In vitro* micropropagation protocol for *Vanda* hybrids with clonal fidelity analysis" conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2014 to June 2016 are presented in this chapter under different sub headings.

4.1 Studies on micropropagation

Two leading *Vanda* hybrids namely Dr. Anek and Sansai Blue obtained from a commercial nursery were used for the present study. Flower colour of Dr.Anek is dark netted pink and that of Sansai Blue is netted blue.

Different explants were used for the present micropropagation study namely basal leaf segments, leaf tip segments, root segments, stem segments, and inflorescence segments. All explants were collected from the mother plants maintained in the net house at CPBMB, College of Horticulture.

4.2 Culture establishment studies with leaf explants

4.2.1 Standardization of surface sterilization of leaf explants

Separate experiments were conducted to standardize surface sterilization procedure for both the hybrids namely 'Dr. Anek' and 'Sansai Blue' and the results obtained are given in Table 9 and Table 10. The medium used for standardization experiment was MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime and the culture conditions given were initial 48 h dark and further light of intensity 1000 lux.

From the results obtained, it was observed that in 'Dr. Anek', microbial contamination was least in T_7 and T_8 (0%) but had a lower survival percentage (10% and 0%). However in T_5 the survival per cent was highest (80%) and hence

T_5 was considered to be the best treatment for surface sterilization of leaf explants of Dr. Anek. The fungal contamination in all treatments ranged from 0-100 per cent while bacterial contamination was in the range of 0-20 percentage.

As the time period was increased from 6-10 minutes, drying of the explants were observed and the drying percentage increased from 50-100 percentage.

Similarly, in 'Sansai Blue', microbial contamination was least in T_7 and T_8 (0%) but had a lower survival percentage (20% and 0%). However in T_5 the survival per cent was highest (90%) and hence T_5 was considered to be the best treatment for surface sterilization of leaf explants of 'Sansai Blue'. The fungal contamination in all treatments ranged from 0-100 per cent while bacterial contamination was in the range of 0-20 percentage.

As the time period was increased from 6-10 minutes, drying of the explants were observed and the drying percentage increased from 40-100 percentage in 'Sansai Blue'.

The results of the experiments conducted for the standardization of surface sterilization of leaf explants showed that T_5 i.e. treating the explants with 0.1 per cent carbendazim for 20 minutes, followed by 70 per cent ethanol for 5 minutes and 0.1 per cent mercuric chloride for 5 minutes can eliminate maximum microbial contamination with highest per cent survival of the tissues.

4.2.2 Identification of media for culture establishment using leaf explants

The leaf explants collected from the maintained mother plants were prepared for culture establishment as described in section 3.2.5.1.1. Response of the leaf explants of 'Dr. Anek' and 'Sansai Blue' in various culture establishment media tested are given in Table 11 and 12.

Table 9. Effect of surface sterilization treatments on leaf explants of *Vanda* 'Dr. Anek'

Treatment no.	Surface sterilization treatment	Per cent contamination (%)	Per cent survival (%)	Nature of contamination	Percentage fungal contamination (%)	Percentage bacterial contamination (%)	Percentage of cultures dried (%)
T1.	70% ethanol 1 min + 0.1% $HgCl_2$ 1 min	100	0	Fungus	100	0	0
T2.	70% ethanol 2 min+ 0.1% $HgCl_2$ 2 min	100	0	Fungus	100	0	0
T3.	70% ethanol 3 min+ 0.1% $HgCl_2$ 3 min	80	20	Fungus Bacteria	60	20	0
T4.	70% ethanol 4 min+ 0.1% $HgCl_2$ 4 min	40	60	Fungus Bacteria	30	10	0
T5.	70% ethanol 5 min+ 0.1% $HgCl_2$ 5 min	20	80	Fungus	20	0	0
T6.	70% ethanol 6 min+ 0.1% $HgCl_2$ 6 min	20	30	Fungus Drying	20	0	50
T7.	70% ethanol 8 min+ 0.1% $HgCl_2$ 8 min	0	10	Drying	0	0	90
T8.	70% ethanol 10 min+ 0.1% $HgCl_2$ 10 min	0	0	Drying	0	0	100

No. of cultures inoculated: 10 per treatment
Medium: MS + 1.5 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime
Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Table 10. Effect of surface sterilization treatments on leaf explants of *Vanda* 'Sansai Blue'

Treatment no.	Surface sterilization treatment	Per cent contamination (%)	Per cent survival (%)	Nature of contamination	Percentage fungal contamination (%)	Percentage bacterial contamination (%)	Percentage of cultures dried (%)
T1.	70% ethanol 1 min+ 0.1% $HgCl_2$ 1 min	100	0	Fungus	100	0	0
T2.	70% ethanol 2 min+ 0.1% $HgCl_2$ 2 min	100	0	Fungus	100	0	0
T3.	70% ethanol 3 min+ 0.1% $HgCl_2$ 3 min	60	40	Fungus Bacteria	50	10	0
T4.	70% ethanol 4 min+ 0.1% $HgCl_2$ 4 min	40	60	Fungus Bacteria	20	20	0
T5	70% ethanol 5 min+ 0.1% $HgCl_2$ 5 min	10	90	Fungus	10	0	0
T6.	70% ethanol 6 min+ 0.1% $HgCl_2$ 6 min	20	40	Fungus Drying	20	0	40
T7.	70% ethanol 8 min+ 0.1% $HgCl_2$ 8 min	0	20	Drying	0	0	80
T8.	70% ethanol 10 min+ 0.1% $HgCl_2$ 10 min	0	0	Fungus	0	0	100

No. of cultures inoculated: 10 per treatment
Medium: MS + 1.5 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime
Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Sub-culturing was done after three weeks of inoculation. The leaf explants did not respond to any of the media compositions tested under the present study even after first subculture. Drying of explants was observed after three weeks of inoculation. It was observed that the basal segments remained green for longer period of about two months and leaf tip segments started to dry after two weeks of inoculation. However, none of the leaf explants could produce any culture initiation.

4.3 Culture establishment with root explants

4.3.1 Standardization of surface sterilization of root explants

Healthy live roots were used as explants to initiate the cultures for developing an efficient micropropagation protocol for selected *Vanda* hybrids. Surface sterilization treatments were standardized for root explants of both hybrids. The media used for standardization experiment was MS + 3% sucrose + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) and culture condition was initial 48 h dark and further light of 1000 lux intensity.

Observations were recorded after three weeks of inoculation. The results of the experiment performed to find the best treatment for surface sterilization of root explants of 'Dr. Anek' and 'Sansai Blue' are presented in Table 13 and Table 14 respectively.

Table 11. Response of leaf explants of 'Dr. Anek' for culture establishment in different media combinations

Treatment No.	Media composition	Culture establishment after 21 days of inoculation
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250mg l^{-1} cefotaxime	Nil
T2.	$^1/_4$ MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T3.	Basal MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T4.	Mitra + 66.6 µM BA + 28.5 µM IAA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T5.	$^1/_2$ MS + 10 mg l^{-1} 2,4-D + 1 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T6.	MS + 1.5 mg l^{-1} NAA + 1 mg l^{-1} BAP + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T7.	$^1/_2$ MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T8.	$^1/_2$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime	Nil

No.cultures inoculated: 10 per medium

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Table 12. Response of leaf explants of 'Sansai Blue' for culture establishment in different media combinations

Treatment No.	Media composition	Culture establishment after 21 days of inoculation
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250mg l^{-1} cefotaxime	Nil
T2.	¼ MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T3.	Basal MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T4.	Mitra + 66.6 µM BA + 28.5 µM IAA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T5.	½ MS + 10 mg l^{-1} 2,4-D + 1 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T6.	MS + 1.5 mg l^{-1} NAA + 1 mg l^{-1} BAP + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T7.	½ MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T8.	½ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime	Nil

No. of cultures inoculated: 10 per medium

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Table 13. Effect of surface sterilization treatments on root explants of *Vanda* 'Dr. Anek'

Treatment no.	Surface sterilization treatment	Per cent contamination (%)	Per cent survival (%)	Nature of contamination	Percentage fungal contamination (%)	Percentage bacterial contamination (%)	Percentage of cultures dried (%)
T1.	70% ethanol 1 min+ 0.1% $HgCl_2$ 1 min	100	0	Fungus	100	0	0
T2.	70% ethanol 2 min+ 0.1% $HgCl_2$ 2 min	100	0	Fungus	100	0	0
T3.	70% ethanol 3 min+ 0.1% $HgCl_2$ 3 min	90	10	Fungus, Bacteria	80	10	0
T4.	70% ethanol 4 min+ 0.1% $HgCl_2$ 4 min	50	50	Fungus, Bacteria	30	20	0
T5.	70% ethanol 5 min+ 0.1% $HgCl_2$ 5 min	30	70	Fungus	20	10	0
T6.	70% ethanol 6 min+ 0.1% $HgCl_2$ 6 min	20	20	Fungus, Bacteria, Drying	10	10	60
T7.	70% ethanol 8 min+ 0.1% $HgCl_2$ 8 min	10	20	Drying, Fungus	10	0	70
T8.	70% ethanol 10 min+ 0.1% $HgCl_2$ 10 min	0	0	Drying	0	0	100

No. of cultures inoculated: 10 per treatment

Medium: MS + 1.5 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Table 14. Effect of surface sterilization treatments on root explants of *Vanda* 'Sansai Blue'

Treatment no.	Surface sterilization treatment	Per cent contamination (%)	Per cent survival (%)	Nature of contamination	Percentage fungal contamination (%)	Percentage bacterial contamination (%)	Percentage of cultures dried (%)
T1.	70% ethanol 1 min+ 0.1% $HgCl_2$ 1 min	100	0	Fungus	100	0	0
T2.	70% ethanol 2 min+ 0.1% $HgCl_2$ 2 min	100	0	Fungus	100	0	0
T3.	70% ethanol 3 min+ 0.1% $HgCl_2$ 3 min	80	20	Fungus, Bacteria	50	30	0
T4.	70% ethanol 4 min+ 0.1% $HgCl_2$ 4 min	50	50	Fungus, Bacteria	40	10	0
T5.	70% ethanol5 min+ 0.1% $HgCl_2$ 5 min	20	80	Fungus	10	0	0
T6.	70% ethanol 6 min+ 0.1% $HgCl_2$ 6 min	10	30	Fungus, Drying,	10	0	60
T7.	70% ethanol 8 min+ 0.1% $HgCl_2$ 8 min	10	10	Drying, Fungus	10	0	80
T8.	70% ethanol 10 min+ 0.1% $HgCl_2$ 10 min	0	0	Drying	0	0	100

No. of cultures inoculated: 10 per treatment

Medium: MS + 1.5 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Results showed that for root explants, as observed for leaf explants, microbial contamination was least in T_8 for both hybrids but survival percentage was zero. The survival was maximum in T_5 for both hybrids i.e. 70 per cent for Dr. Anek and 80 per cent for Sansai Blue. T_5 consisted of treating the explants with 70 per cent ethanol for 5 minutes followed by treating with 0.1 per cent mercuric chloride for 5 minutes. In this treatment, contamination percentage for both hybrids ranged from 20-30 percentage.. In Dr. Anek the percentage of fungal contamination in different treatments ranged from 0-100 percentage but for bacterial contamination only 0-20 percentage. In Sansai Blue, fungal contamination was in the range of 0-100 per cent and bacterial contamination was 0-30 percentage.

The results of the experiments conducted for the standardization of surface sterilization of root explants showed that T_5 was the best treatment for surface sterilization of root explants in both hybrids.

4.3.2 Culture establishment using root explants

For establishing cultures for *Vanda* hybrids with root explants, different media compositions were tested and the response was recorded after twenty one days as detailed in Table 15 and Table 16 for Dr. Anek and Sansai Blue respectively. The culture conditions given was initial 48 h dark followed by light of 1000 lux intensity with 26 ± 2^0C.

The root explants did not respond to any of the media compositions tested under the present study. Drying was more fast and severe in root explants than in the leaf explants. Even before the first subculture all the inoculated explants were dried completely.

Table 15. Response of root explants of 'Dr. Anek' to different media combinations for culture establishment

Treatment No.	Media composition	Culture establishment after 21 days of inoculation
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250mg l^{-1} cefotaxime	Nil
T2.	$^{1}/_{4}$ MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T3.	Basal MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T4.	Mitra + 66.6 μM BA + 28.5 μM IAA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T5.	$^{1}/_{2}$ MS + 10 mg l^{-1} 2,4-D + 1 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T6.	MS + 1.5 mg l^{-1} NAA + 1 mg l^{-1} BAP + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T7.	$^{1}/_{2}$ MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T8.	$^{1}/_{2}$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime	Nil

No. of cultures inoculated: 10 per medium

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Table 16. Response of root explants of 'Sansai Blue' to different media combinations for culture establishment

Treatment No.	Media composition	Culture establishment after 21 days of inoculation
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250mg l^{-1} cefotaxime	Nil
T2.	¼ MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T3.	Basal MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T4.	Mitra + 66.6 µM BA + 28.5 µM IAA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T5.	½ MS + 10 mg l^{-1} 2,4-D + 1 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T6.	MS + 1.5 mg l^{-1} NAA + 1 mg l^{-1} BAP + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T7.	½ MS + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T8.	½ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime	Nil

No. of cultures inoculated: 10 per medium

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2^{0}C

4.4 Culture establishment using stem segments

Stem segments with two nodes and the upper meristematic region on the stem were inoculated for culture establishment. The surface sterilization procedure that was standardized for leaf and root segments were followed for the stem segments. However both the stem segments and meristems also failed to respond in the media under trial. The observations recorded after twenty one days of inoculation are presented in Table 17 for 'Dr. Anek' and Table 18 for 'Sansai Blue'.

Stem segments also did not respond positively to the different media tested. The stem segments remained as such without any change for two weeks. Further it started to dry from the upper part of inoculated explant. The apical meristem inoculated dried in one week.

4.5 Culture establishment using inflorescence segments

4.5.1 Standardization of surface sterilization of inflorescence segments

Inflorescence segment was another explant used for the culture establishment in the present experiment.

Surface sterilization treatments were standardized for inflorescence segments for both hybrids. The medium used for standardization experiment was half strength MS fortified with 10 mg l^{-1} BA and 2 mg l^{-1} TDZ solidified on 7.5 g l^{-1}agar with 3 per cent sucrose which was the medium standardized for *Phalaenopsis* tissue culture using inflorescence at CPBMB. The medium was incorporated with 250 mg l^{-1} cefotaxime.

The results of the experiment performed to find the best treatment for surface sterilization of inflorescence segments of *Vanda* hybrids are presented in Table 19 and Table 20 for 'Dr. Anek' and 'Sansai Blue' respectively.

Table 17. Response of stem segments of 'Dr. Anek' to different media combinations for culture establishment

Treatment no.	Media composition	Culture establishment after 21 days of inoculation
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T2.	½ MS + 10 mg l^{-1} 2,4-D + 1 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T3.	**For apical meristem** Mitra + 44.4 μM BA + 28.5 μM IAA + 20 g l^{-1} sucrose + 6 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil

No. of cultures inoculated: 3 per medium
Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Table 18. Response of stem segments of 'Sansai Blue' to different media combinations for culture establishment

Treatment no.	Media composition	Culture establishment after 21 days of inoculation
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T2.	½ MS + 10 mg l^{-1} 2,4-D + 1 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T3.	**For apical meristem** Mitra + 44.4 µM BA + 28.5 µM IAA + 20 g l^{-1} sucrose + 6 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil

No. of cultures inoculated: 3 per medium

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Table 19. Effect of surface sterilization treatments on inflorescence segments of *Vanda* 'Dr. Anek'

Treatment no.	Surface sterilization treatment	Per cent contamination (%)	Per cent survival (%)	Nature of contamination (%)	Percentage fungal contamination (%)	Percentage bacterial contamination (%)	Percentage of cultures dried (%)
T4.	70% ethanol 4 min+ 0.1% $HgCl_2$ 4 min	60	40	Fungus Bacteria	50	10	0
T5.	70% ethanol 5 min+ 0.1% $HgCl_2$ 5 min	20	80	Fungus	20	0	0
T6.	70% ethanol 6 min+ 0.1% $HgCl_2$ 6 min	20	40	Fungus Drying	20	0	40

No. of cultures inoculated: 5 per treatment
Medium: ½ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime
Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Table 20. Effect of surface sterilization treatments on inflorescence segments of *Vanda* 'Sansai Blue'

Treatment no.	Surface sterilization treatment	Per cent contamination (%)	Per cent survival (%)	Nature of contamination	Percentage fungal contamination (%)	Percentage bacterial contamination (%)	Percentage of cultures dried (%)
T4.	70% ethanol 4 min+ 0.1% $HgCl_2$ 4 min	40	60	Fungus	40	0	0
T5.	70% ethanol 5 min+ 0.1% $HgCl_2$ 5 min	0	100	Fungus	0	0	0
T6.	70% ethanol 6 min+ 0.1% $HgCl_2$ 6 min	40	20	Fungus Drying	10	10	40

No. of cultures inoculated: 5 per treatment
Medium: $^1/_2$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime
Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

From the results obtained, it was observed that T_5 recorded least microbial contamination with highest explant survival in both the hybrids. In 'Dr. Anek' one explant, out of the five inoculated showed fungal contamination whereas in 'Sansai Blue' there was no contamination in T_5. The percentage of contamination was observed to be 20 and 0 per cent respectively for 'Dr. Anek' and 'Sansai Blue' in T_5. The fungal contamination ranged from 20-10 per cent in 'Dr. Anek' and bacterial contamination was in the range of 0-10 percentage. However 0-10 per cent drying was observed in various treatments in 'Dr. Anek'.

In 'Sansai Blue' fungal contamination ranged between 10-40 per cent and bacterial contamination between 0-10 per cent. Drying in 'Sansai Blue' ranged between 0-40 per cent.

During the standardization of surface sterilization of inflorescence segments it was observed that the colour of the media turned brown due to the exudation of phenolic compounds. Hence the inflorescence segments were dipped in 0.1 per cent ascorbic acid for five minutes before treating with 70 per cent ethanol during the surface sterilization procedure to manage media browning.

4.5.2 Culture establishment using inflorescence segments

An attempt was made to initiate cultures using inflorescence segments to develop an efficient micropropagation protocol for *Vanda* hybrids. The response of inflorescence segments of 'Dr. Anek' in different culture establishment media tested is detailed in Table 21 and of 'Sansai Blue' in Table 22.

Table21. Response of inflorescence segments of 'Dr. Anek' to different media combinations for culture establishment

Treatment no.	Media composition	Culture establishment after 21 days of inoculation
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T2.	$^{1}/_{2}$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime	Yes

No. of cultures inoculated: 5 per medium
Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

Table22. Response of inflorescence segments of 'Sansai Blue' to different media combinations for culture establishment

Treatment no.	Media composition	Culture initiation after 21 days of inoculation
T1.	MS + 1.5 mg l^{-1} N phenyl-N'-(1, 2, 3-thidiazol-5yl) urea (TDZ) + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	Nil
T2.	$^{1}/_{2}$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime	Yes

No. of cultures inoculated: 5 per medium
Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 ^{0}C

The inflorescence segments inoculated into the media reported by Gantait and Sinniah (2012) did not respond positively. The bud on the segments turned yellow after one week of inoculation and dried in three weeks. However in $^1/_2$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime, the explants showed positive response. The buds on the segments showed sprout initiation in two weeks. Further development of the bud was observed during the following subcultures at an interval of twenty one days.

4.6 Identification of suitable explant and medium for culture establishment of *Vanda* hybrids

From the various explants tested under the study namely leaf explants, root segments, stem segments and inflorescence segments, a response in the cultures was recorded only in the case of inflorescence segments. The medium in which the response was recorded was $^1/_2$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime. Hence the study further concentrated in developing the *in vitro* regeneration protocol for *Vanda* hybrids using inflorescence segments in the identified medium.

4.6.1 Response of inflorescence segments in culture establishment medium

There was gradual increase in the size of the bud after fifteen days of inoculation and the buds showed prominent enlargement after twenty one days. The dormant buds present on the young immature inflorescence on the *Vanda* hybrids produced visible shoot initials in the identified culture establishment medium by sixty days of inoculation (Plate 6). Response of the cultures in the medium is detailed in Table 23 for both hybrids. It was observed that average time taken for sprout initiation for 'Dr. Anek' was 14.20 days whereas for 'Sansai Blue' it was 17.40 days. On an average, direct shoot initials were obtained in 9 weeks for both the hybrids. The percentage of culture establishment was 80 per cent for 'Dr. Anek' whereas it was only 60 per cent for 'Sansai Blue'.

On the day of inoculation

At S1 passage

At S3 passage

At S2 passage

Plate 6. Stages of culture establishment

Table 23. Response of inflorescence segments of *Vanda* hybrids in culture establishment medium

Sl. No.	Hybrid	No. of explants inoculated	Mean days for sprout initiation	Response			Percentage of culture establishment (%)
				At S1 passage (21 days)	At S2 passage (42 days)	At S3 passage (63 days)	
1.	Dr. Anek	20	14.20	Bud enlargement	Bud elongation	Shoot initiation	80
2.	Sansai Blue	20	17.40	,,	,,	,,	60

Medium: $^1/_2$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ

Culture condition: Initial 48 h dark followed by light conditions of 1000 lux with 26 ± 2 ^{0} C

4.7 Shoot proliferation studies

The cultures initiated from the inflorescence segments were transferred to three different media for shoot proliferation. Response of cultures of 'Dr. Anek' in different shoot proliferation media tested is given in the Table 24. Table 25 gives the response of cultures of 'Sansai Blue' in various shoot proliferation media tested.

The explants of both Dr. Anek and Sansai Blue inoculated into MS + 4.5 ml l^{-1} BA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime both with and without stalk produced multiples. Number of multiples was less in cultures with stalk compared to cultures without stalk. No shoot proliferation was observed in T1 i.e. $^1/_2$ MS + 1 mg l^{-1} IAA + 1 mg l^{-1} BAP + 30 g l^{-1} sucrose and T2 i.e. MS + 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA + 30 g l^{-1} sucrose.

4.7.1 Response of cultures in shoot proliferation medium

The cultures from both hybrids were repeatedly subcultured in the shoot proliferation medium of MS + 4.5 ml l^{-1} BA at 21 days interval for a period of 84 days upto S7 passages and the details of the response of cultures are given in Table 26. It was observed that the inflorescence segments inoculated in shoot proliferation medium along with the stalk produced elongated shoots whereas those inoculated without stalk had smaller shoots (Plate 7 and 8).

In Dr. Anek, for cultures inoculated with stalk, the mean length of multiple shoots increased from 0.85cm to 4.20 cm from the day of inoculation to 84 days after inoculation in shoot proliferation medium. While for the cultures inoculated without stalk, the increase in mean shoot length was from 0.25 cm to 1.53 cm during the same period of culturing. The mean number of multiple shoots produced was less in cultures inoculated with stalk and was observed to be 4.50 per culture for Dr. Anek at S7 passage. Whereas the mean number of multiples produced in cultures without stalk was 6.67 per culture for Dr. Anek. The mean number of leaves per shoot was almost same for cultures inoculated with and without stalk i.e. 3.03 for cultures with stalk and 3.06 for cultures without stalk.

In Sansai Blue, the mean length of shoots increased from 0.98 cm on the day of inoculation to shoot proliferation medium to 4.07 cm after 84 days for those cultures inoculated with stalk. While for those cultures inoculated without stalk, the mean length increased from 0.20 cm to 1.55 cm. The mean number of shoots per culture was 4.00 for cultures with stalk at S7 passage while for cultures without stalk, the mean number of shoots was observed to be 7.50. Mean number of leaves produced was 3.46 for cultures with stalk and 3.14 for cultures without stalk.

So it can be concluded that multiple shoot production is more in cultures without stalk for both hybrids and shoot elongation was more in cultures with stalk.

Table 24: Response of cultures of 'Dr. Anek' in shoot proliferation media

Treatment no.	Media	No. of cultures inoculated		Response after 21 days of inoculation	Multiple shoot initiation
		With stalk	Without stalk		
T1.	½ MS + 1 mg l^{-1} IAA + 1 mg l^{-1} BAP + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	2		Dried	-
			3	Dried	-
T2.	MS + 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	2		Dried	-
			3	Dried	-
T3.	MS + 4.5 ml l^{-1} BA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	3		Multiple initiation	Less
			3	Multiple initiation	Numerous

Table 25: Response of cultures of 'Sansai Blue' in shoot proliferation media

Treatment no.	Media	No. of cultures inoculated		Response after 21 days of inoculation	Multiple shoot initiation
		With stalk	Without stalk		
T1.	½ MS + 1 mg l^{-1} IAA + 1 mg l^{-1} BAP + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	1		Dried	-
			2		
T2.	MS + 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	1		Dried	-
			2		
T3.	MS + 4.5 ml l^{-1} BA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime	3		Multiple initiation	Less
			3	Multiple initiation	Numerous

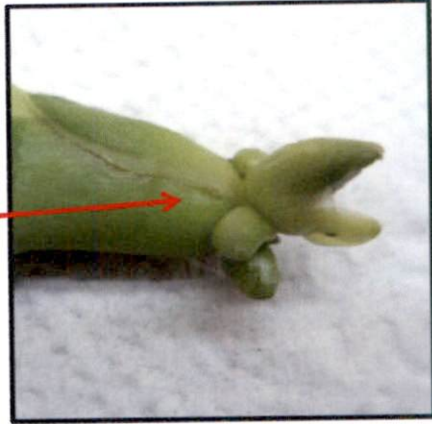
At subculture 4 passage

At subculture 5 passage

At subculture 6 passage

At subculture 7 passage

Plate 7. Cultures in shoot proliferation medium with stalk



At subculture 4 passage

At subculture 5 passage

At subculture 6 passage

At subculture 7 passage

Plate 8. Cultures of in shoot proliferation medium without stalk

The average number of multiples produced was limited to 3.5 per explants in 'Dr. Anek' and 3.67 in 'Sansai Blue' for those explants inoculated with stalk and the leaves produced were thick. For those explants inoculated without stalk, the mean number of multiples produced per explants was higher to up to 6.67 in 'Dr. Anek' and 7.5 in 'Sansai Blue', but the multiples were formed as micro-shoots.

4.8 Elongation studies

One cycle of subculture in hormone free basal MS media with charcoal led to elongation of the micro-shoots produced from those cultures without stalk (Plate 9). Table 27 give the details of cultures of both hybrids in shoot elongation medium cultured under light conditions of 1000 lux at 26 ± 2^0C.S

Table 27. Response of cultures in elongation medium

Sl. No.	Hybrid	No. of cultures inoculated	Mean no. of shoots per culture	Mean length at inoculation (cm)	Mean length after 21 days of inoculation (cm)
1.	Dr. Anek	3	6.66	1.53	4.07
2	Sansai Blue	2	7.50	1.55	4.10

Medium: Hormone free basal MS

Culture condition: Light conditions of 1000 lux with $26 \pm 2\ ^0$C

The cultures in elongation media showed positive results with respect to the mean length of the shoots. It was observed that the mean length of the micro-shoots increased from 1.53 cm to 4.07 cm in 'Dr. Anek' and 1.55 cm to 4.10 cm in 'Sansai Blue' after one subculture passage.

Table26. Response of cultures in identified shoot proliferation medium

Sl. No.	Hybrids	Cultures	At inoculation to SPM*		At S4 passage (After 21 days in SPM)		At S5 passage (After 42 days in SPM)		At S6 passage (After 63 days in SPM)			At S7 passage (After 84 days in SPM)		
			Mean no. of shoot initials per culture	Mean length of shoots (cm)	Mean no. of shoot initials per culture	Mean length of shoots (cm)	Mean no. of shoot initials per culture	Mean length of shoots (cm)	Mean no. of shoots per culture	Mean length of shoots (cm)	Mean no. of leaves per shoot	No. of shoots per culture	Mean length of shoots (cm)	Mean no. of leaves per shoot
1.	Dr. Anek	With stalk	1	0.85	2.00	1.69	3.00	2.18	3.50	2.88	2.30	4.50	4.20	3.03
2.		Without stalk	1	0.25	3.33	0.61	4.00	0.85	5.00	1.34	2.22	6.67	1.53	3.06
3.	Sansai Blue	With stalk	1	0.98	2.00	1.54	3.00	2.29	3.67	2.91	2.39	4.00	4.07	3.46
4.		Without stalk	1	0.20	3.50	0.48	4.50	0.76	6.00	0.92	2.46	7.50	1.55	3.14

Medium: MS + 4.5 ml l^{-1} BA

Culture condition: Light conditions of 1000 lux with 26 ± 2 ^{0}C

***SPM**: Shoot Proliferation Medium

4.9 Rooting studies

The elongated shoots were further subcultured into the identified rooting medium by separating them into individual shoots. The details of the response of the shoots in elongation and rooting media are furnished in Table 28. The cultures were maintained in rooting medium for two subculture passages. The rooted plantlets of *Vanda* hybrid is shown in Plate 10.

Rooting percentage in Dr. Anek was observed to be 72.41 per cent whereas in Sansai Blue it was 70.37 per cent.

The mean number of roots for Dr. Anek was 6.63 with a mean length of 5.93 cm. In Sansai Blue, the mean number of roots per shoot was observed to be 5.83 with a mean root length of 5.61 cm.

Table 28: Response of cultures in rooting medium

Sl. No.	Hybrid	No. of cultures	Response at S9 passage	Mean no. of roots per plant at S9	Mean root length at S9 (cm)	Mean no. of roots per plant at S10	Mean root length at S10 (cm)	No. of cultures lost due to contamination and drying	Rooting percentage (%)
1.	Dr. Anek	29	Root initiation	4.46	3.30	6.63	5.93	8	72.41
2.	Sansai Blue	27	Root initiation	4.11	3.50	5.83	5.61	8	70.37

Medium: MS + + 0.5 mg l^{-1} NAA + 1 mg l^{-1} IAA

Culture condition: Light conditions of 1000 lux with 26 ± 2 ^{0}C

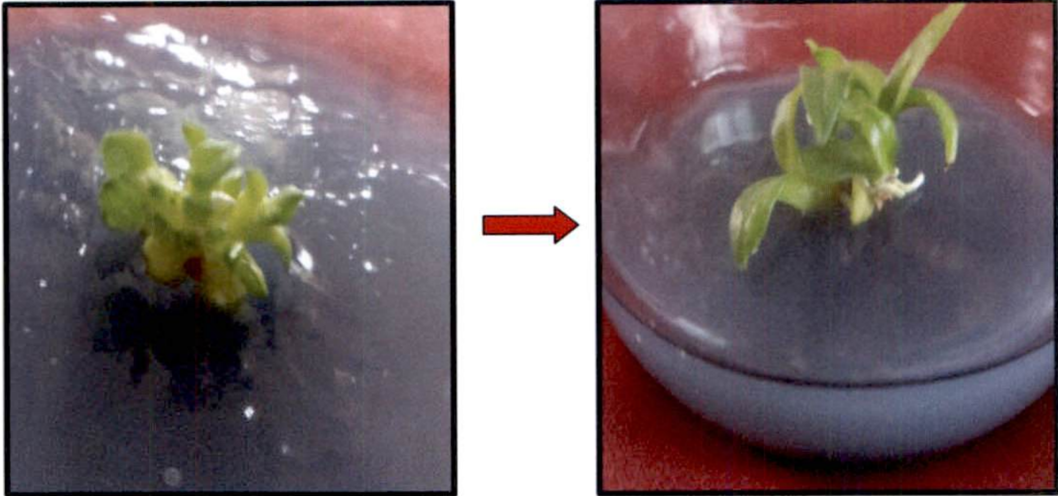


Plate 9: Cultures in elongation media

Plate 10. Rooted plantlet of *Vanda* hybrid

4.9 Hardening and acclimatization

All the rooted plantlets were planted in small earthen pots filled with charcoal, coconut husk and brick pieces (Plate 11). Further these were hardened in hardening unit of 50 per cent shade.

All the transferred plantlets could acclimatize well and 100 per cent survival was observed. Results of the biometric observations taken are furnished in Table 29.

Table 29: Biometric observations of plantlets during hardening and acclimatization

Sl. No.	Hybrid	Mean plant height (cm)		Mean no. of leaves	
		At plant out	30 days after hardening	At plant out	30 days after hardening
1.	Dr. Anek	4.80	7.10	3.64	4.63
2.	Sansai Blue	4.85	7.14	3.86	4.18

The mean plant height for Dr .Anek increased from 4.80 cm to 7.10 cm and in Sansai Blue the mean plant height increased from 4.85 cm to 7.14 after one month of hardening. The mean number of leaves was 3.64 and 3.86 in Dr. Anek and Sansai Blue before hardening and it was found to be 4.63 and 4.18 respectively one month after hardening.

Plate 11. Regenerated plantlet potted in earthen pots with charcoal, coconut husk and brick pieces

4.10 Clonal fidelity analysis studies using ISSR molecular marker

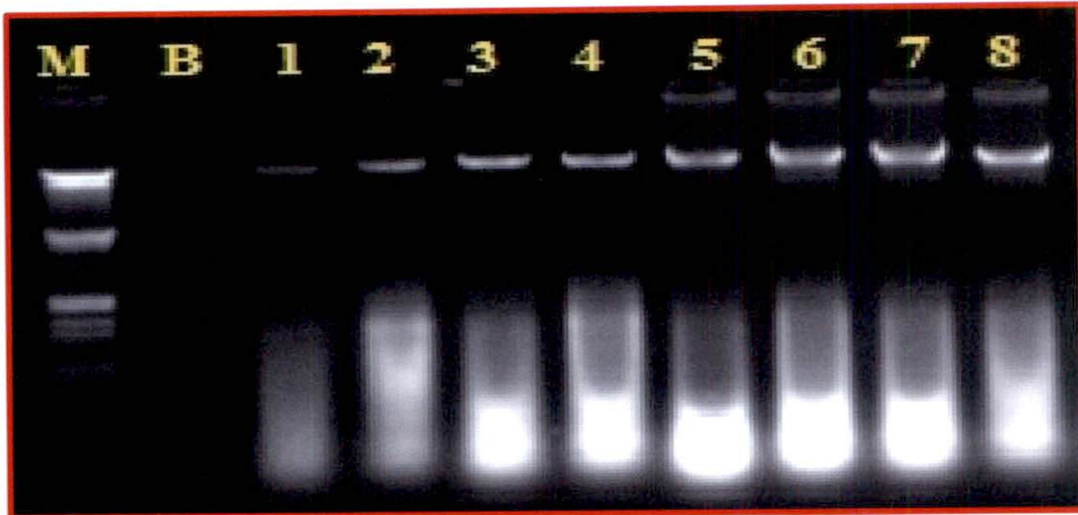
4.10.1 Genomic DNA isolation and purification

For isolation of genomic DNA, tender leaf samples were collected from mother plants maintained at net house as well as from the regenerated clones using CTAB method reported by Roger and Bendich (1994). As the isolated DNA had RNA contamination, RNase treatment was given which provided with good quality DNA (Plate12).

4.10.2 Quality and Quantity of genomic DNA

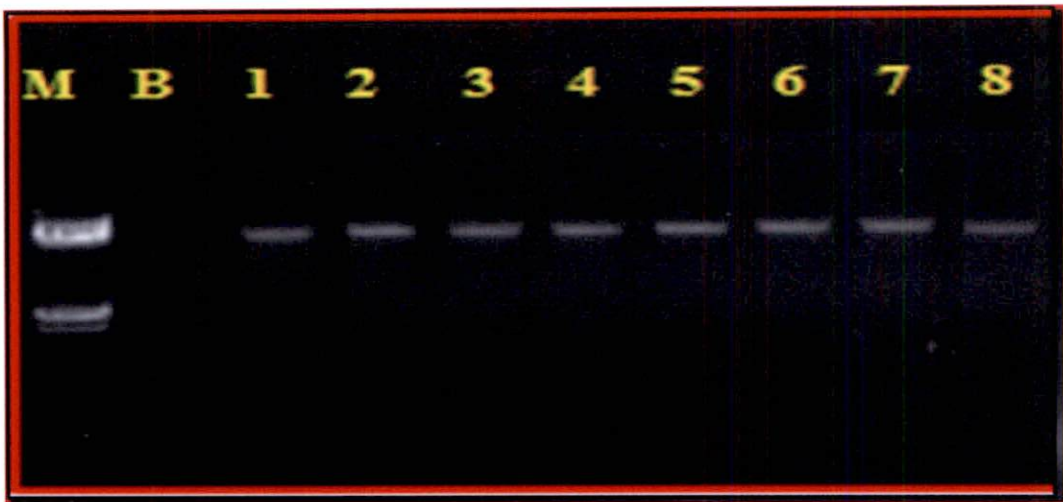
The quality and quantity of isolated DNA was analysed using both electrophoresis and NanoDrop spectrophotometer. Intact clear bands indicated that DNA extracted was non-degraded and was of good quality. The DNA from both parents and regenerated clones after appropriate dilution was used as template for ISSR analysis.

The Nanodrop spectrophotometer results obtained from source mother plant along with the clones are presented in Table 30 and Table 31. Spectrophotometric analysis gave ratio of UV absorbance ($A_{260/280}$) between 1.77 – 1.96 in Dr. Anek and 1.79-2.00 in Sansai Blue.



M: Marker Lambda DNA(EcoRI/ Hind III digest 1000bp), B: Blank, 1-4: Mother plants of Dr. Anek, 5-8: Mother plants of Sansai Blue

a. Isolated DNA samples of mother plants before RNAse treatment



M: Marker Lambda DNA(EcoRI/ Hind III digest 1000bp), B: Blank, 1-4: Mother plants of Dr. Anek, 5-8: Mother plants of Sansai Blue

b. Isolated DNA samples of mother plants after RNAse treatment

Plate 12 (a and b). Gel pictures showing the DNA samples of mother plants

Table 30: Quality and quantity of genomic DNA of mother plants and clones of 'Dr. Anek'

Sl. No.	Sample ID of mother plants	Sample ID of progenies	UV absorbance at 260/280 ($A_{260/280}$)	Quantity (ng/ul)
1.	M1		1.85	551.3
		C1	1.87	478.2
		C2	1.91	964.1
		C3	1.96	1004.1
		C4	1.86	689.7
		C5	1.80	702.3
2.	M4		1.88	498.2
		C1	1.80	459.9
		C2	1.94	682.4
		C3	1.83	341.5
		C4	1.77	852.3
3.	M5		1.87	367.2
		C1	1.98	884.7
		C2	1.78	966.8
		C3	1.85	781.9
		C4	1.87	668.1
		C5	1.89	493.4
4.	M8		1.81	1001.0
		C1	1.96	799.5
		C2	1.86	784.2
		C3	1.84	946.3
		C4	1.88	563.2
		C5	1.87	456.3

Table 31: Quality and quantity of genomic DNA of mother plants and clones of 'Sansai Blue'

Sl. No.	Sample ID of mother plants	Sample ID of progenies	UV absorbance at 260/280 ($A_{260/280}$)	Quantity (ng/ul)
1.	M1		1.88	881.3
		C1	1.79	895.1
		C2	1.80	925.6
		C3	1.88	743.2
		C4	1.80	636.3
2.	M5		1.95	600.4
		C1	1.81	701.8
		C2	1.87	653.4
		C3	1.90	489.2
		C4	1.83	946.5
		C5	1.83	746.1
3.	M6		1.86	1003.9
		C1	1.90	980.3
		C2	1.84	881.5
		C3	1.79	765.5
		C4	1.93	745.8
4.	M8		2.00	966.3
		C1	1.87	879.4
		C2	1.90	963.4
		C3	1.82	899.2
		C4	1.88	513.5

4.10.3 Primer screening for ISSR assay

A total of 8 primers were screened with good quality DNA isolated from one mother plant of two selected hybrids. The isolated DNA was amplified in gradient PCR-Agilent at different temperatures ranging from 46°C to 58°C. Amplification was performed in a 20 μl reaction mixture as shown below in Table 7 and the thermocycler was programmed as given in Table 8.

The amplified products were run on 1.8 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA/*Eco*RI+*Hind*III double digest). The profile was visualized and documented. The documented ISSR profiles were carefully examined for total number amplicons generated by each primer.

Primers giving DNA amplification were selected for further analysis. The optimum temperatures for DNA amplification by each primer were also identified. The results of primer screening are presented in Table 32.

Table 32. Details of ISSR primers screened

Sl. No.	Primers	Nucleotide Sequence (5'-3')	Dr. Anek		Sansai Blue		Annealing temperature (°C)	Comments
			Size range	Total number of bands	Size range	Total number of bands		
1	UBC 808	5'AGAGAGAGAGAGAGAGC 3'	250-1200	8	250-900	7	54	Selected
2	UBC 811	5'GAGAGAGAGAGAGAGAC 3'	150-1200	9	250-1000	10	58	Selected
3	UBC 818	5' CACACACACACACACAG 3'	-	-	-	-	-	Not selected
4	UBC 826	5'ACACACACACACACACC 3'	150-1200	9	150-1200	9	53	Selected
5	UBC 835	5'AGAGAGAGAGAGAGAGYC 3'	300-2000	11	500-2500	9	49	Selected
6	UBC 841	5' GAGAGAGAGAGAGAGAYC 3'	300-800	8	230-1000	8	46	Selected
7	UBC 847	5' CACACACACACACACARC 3'	-	-	-	-	-	Not selected
8	UBC 850	5' GTGTGTGTGTGTGTGTYC 3'	-	-	-	-	-	Not selected

4.10.4 ISSR marker analysis

In the present study, ISSR primers were used for checking the fidelity of *in vitro* generated clones. Out of eight ISSR primers reported for *Vanda* hybrids by Kishor and Devi (2009), five were selected based on resolving power. The details of selected ISSR primers are given in Table 6 under section 3.3.3.2.Genomic DNA was isolated from both mother plants and the regenerants and were subjected to ISSR analysis.

4.10.4.1Clonal fidelity analysis

Four mother plants with five clones each regenerated from the inflorescence segments of *Vanda* hybrids Dr. Anek and Sansai Blue at subculture passage eight were compared for their DNA amplification pattern using five selected ISSR primers.

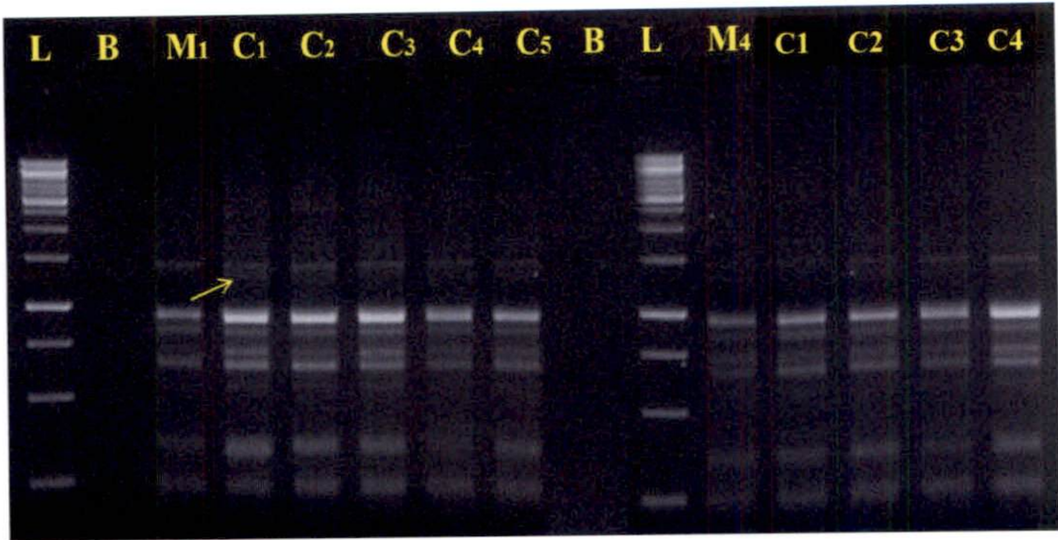
The gel profiles were examined for total number of amplicons and its molecular weight, number of polymorphic and monomorphic bands and percent polymorphism was worked out.

4.10.4.1.1 Clonal fidelity analysis for *Vanda* hybrid Dr.Anek

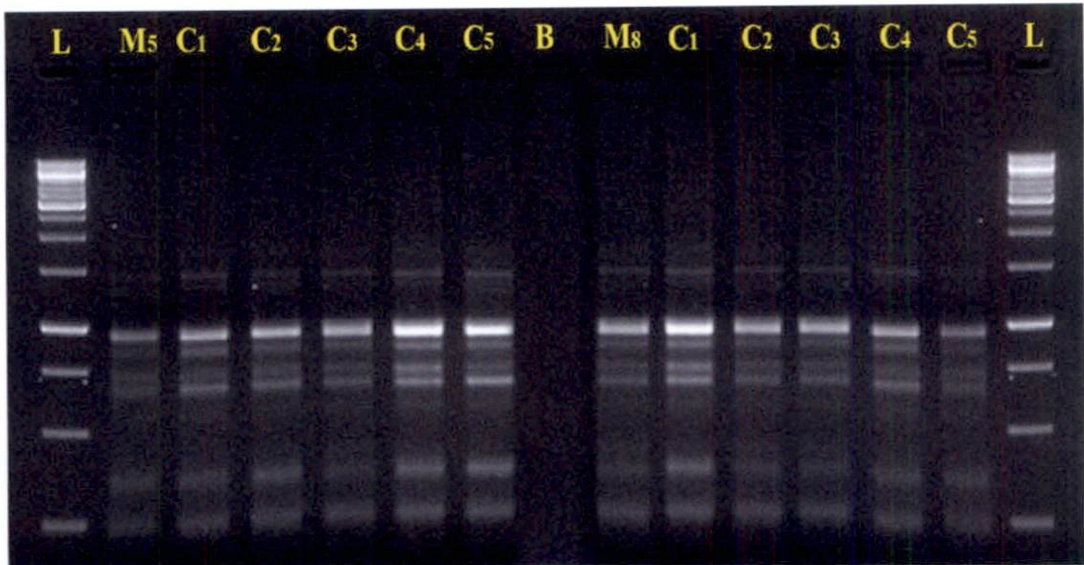
The amplification patterns obtained for each of the primers are detailed below:

UBC 808

Amplification with the primer UBC 808 generated eight clear amplicons in 4 clones out of 5 tested in mother plant 1 of Dr. Anek. The bands obtained were in the size range of 250-1200 bp. The amplified DNA profile showed one polymorphic band of 1000bp size in one of the clones (C_1) among five for one mother plant (M1) which was absent for other clones (Plate 13). In all other mother plants of Dr. Anek (M4, M5 and M8) all clones as well as mother plants showed monomorphic bands which showed that the clones are exactly similar to



(a). L: Marker (12 kb); B: Blank; M_1: Mother plant 1 of Dr.Anek; C_1-C_5: Clones of M_1; M_4: Mother plant 4 of Dr.Anek; C_1- C_4: Clones of M_4



(b). L: Marker (12 kb); B: Blank; M_5: Mother plant 5 of Dr.Anek; C_1-C_5: Clones of M_5; M_8: Mother plant 8 of Dr.Anek; C_1- C_5: Clones of M_8

Plate 13 (a and b). Amplification pattern for UBC 808 for different mother plants and their respective clones of 'Dr. Anek'

the mother plants. The amplification pattern for primer UBC 808 for the four mother plants and their respective clones detailed in Plate 13.

UBC 811

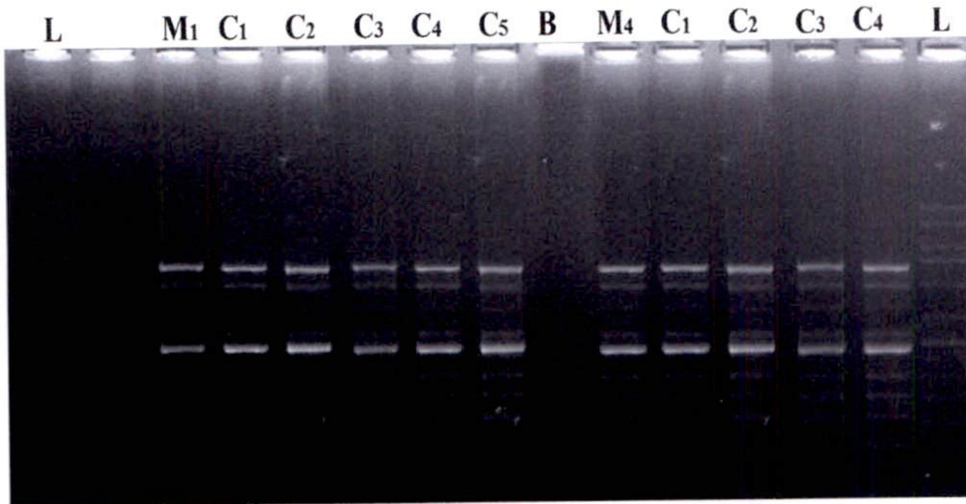
Amplification with the primer UBC 811 generated nine clear amplicons in all clones of all four tested mother plants of Dr. Anek. The bands obtained were all monomorphic bands in the size range of 150-1200 bp. Monomorphic banding pattern was observed for all the amplified band classes across the mother plant and its regenerants. The amplification pattern for primer UBC 811 for the four mother plants and their respective clones detailed in Plate 14.

UBC 826

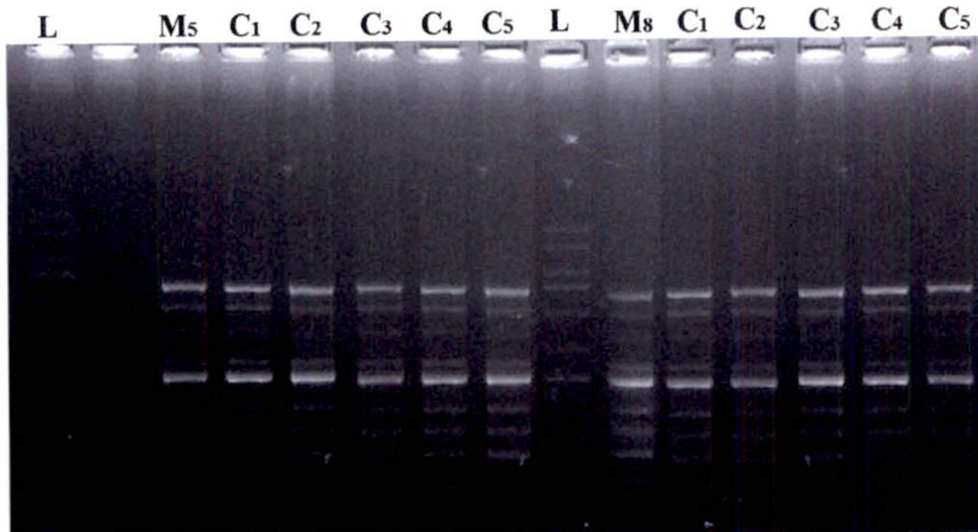
Amplification with the primer UBC 826 generated nine clear amplicons in all clones of all four tested mother plants of Dr. Anek. The bands obtained were all monomorphic in the size range of 150-1200 bp. The monomorphic bands obtained in the ISSR analysis with UBC 826 primer showed that the clones regenerated through the identified *in vitro* propagation protocol are exactly similar to the mother plants. The amplification pattern for primer UBC 826 for the four mother plants and their respective clones are detailed in Plate 15.

UBC 835

Amplification with the primer UBC 835 generated eleven clear amplicons in all clones of all four tested mother plants of Dr. Anek and bands obtained were in the size range of 300- 2000 bp. In one clone of mother plant 1(C_3 of M1), there was the absence of a band of 850bp size which showed variation in genetic content of clone 3 (Plate 16). The other monomorphic bands obtained in the ISSR analysis with UBC 835 primers showed that the clones regenerated through the identified *in vitro* propagation protocol are exactly similar to the mother plants whereas the polymorphic band showed that the particular clone may have difference in some traits. The amplification pattern for primer UBC 835 for the four mother plants and their respective clones are provided in Plate 16.

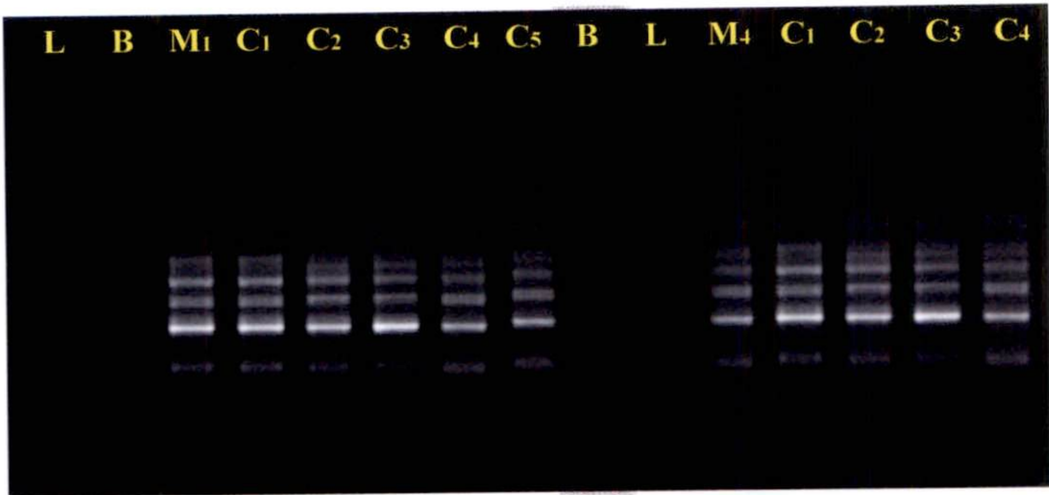


(a). L: Marker (3kb); B: Blank; M_1: Mother plant 1 of Dr.Anek; C_1-C_5: Clones of M_1; M_4: Mother plant 4 of Dr.Anek; C_1- C_4: Clones of M_4

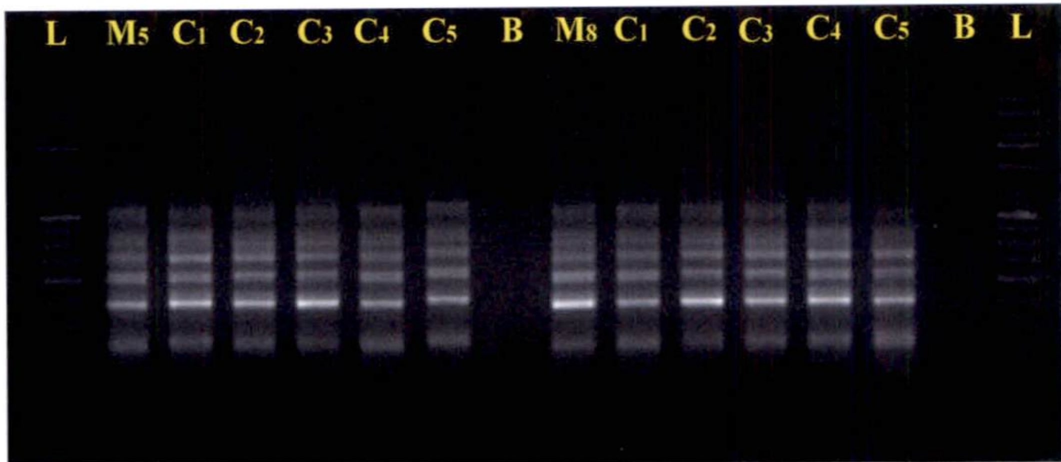


(b). L: Marker (3 kb); B: Blank; M_5: Mother plant 5 of Dr.Anek; C_1-C_5: Clones of M_5; M_8: Mother plant 8 of Dr.Anek; C_1- C_5: Clones of M_8

Plate 14 (a and b). Amplification pattern for UBC 811 for different mother plants and their respective clones of 'Dr. Anek'

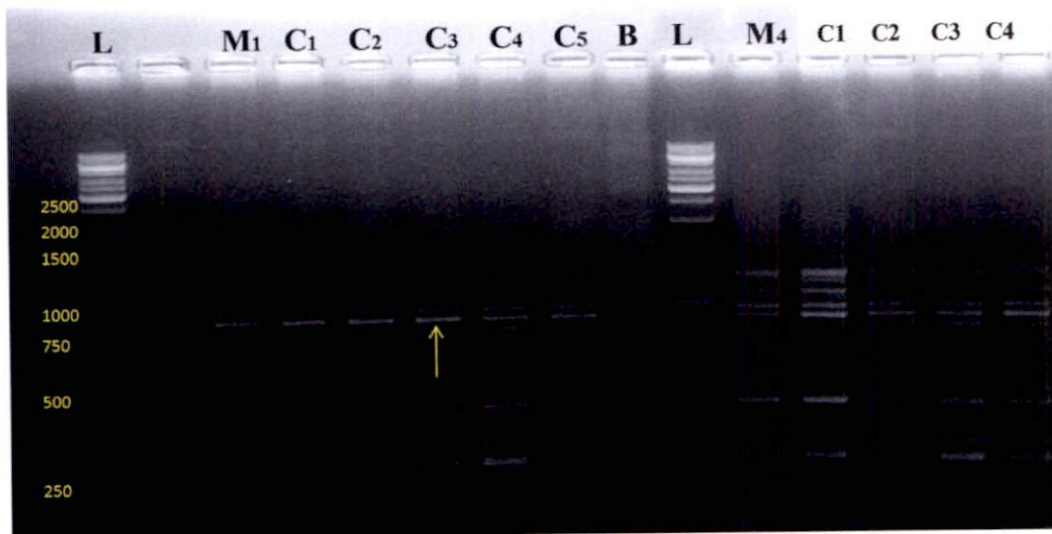


(a). L: Marker (3 kb); B: Blank; M_1: Mother plant 1 of Dr.Anek; C_1-C_5: Clones of M_1; M_4: Mother plant 4 of Dr.Anek; C_1- C_4: Clones of M_4

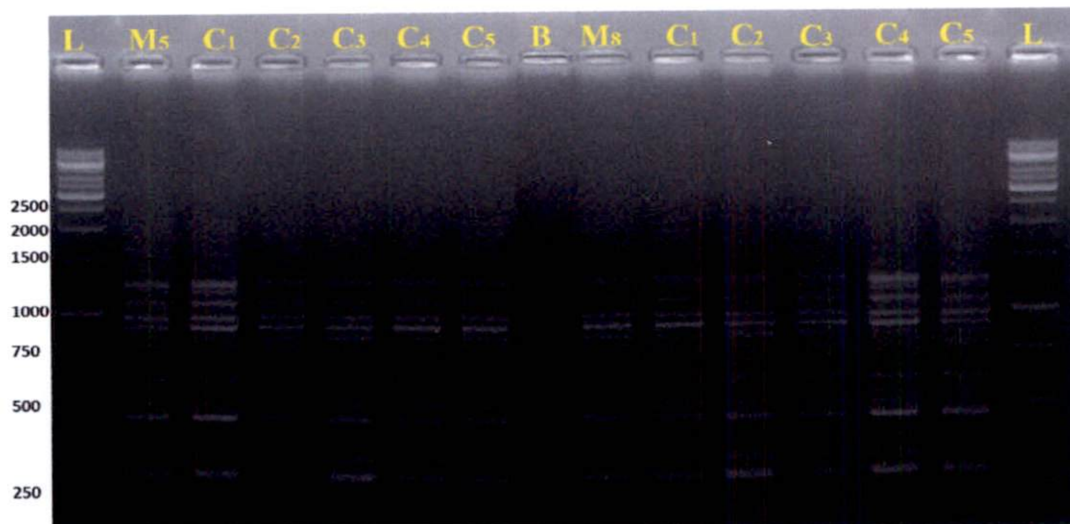


(b). L: Marker (3 kb); B: Blank; M_5: Mother plant 5 of Dr.Anek; C_1-C_5: Clones of M_5; M_8: Mother plant 8 of Dr.Anek; C_1- C_5: Clones of M_8

Plate 15 (a and b). Amplification pattern for UBC 826 for different mother plants and their respective clones of 'Dr. Anek'



(a). L: Marker (12 kb); B: Blank; M_1: Mother plant 1 of Dr.Anek; C_1-C_5: Clones of M_1; M_4: Mother plant 4 of Dr. Anek; C_1- C_4: Clones of M_4

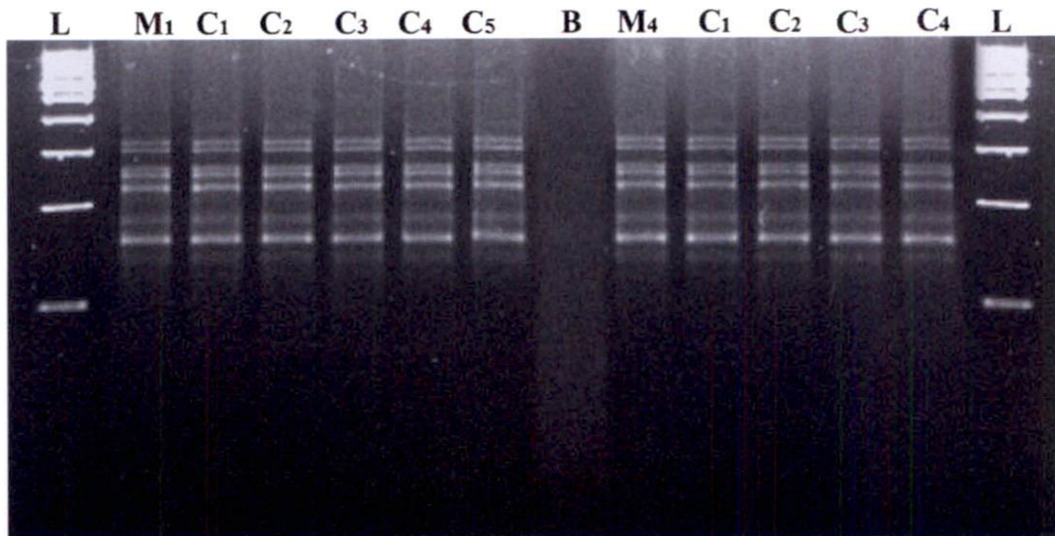


(b). L: Marker (12 kb); B: Blank; M_5: Mother plant 5 of Dr.Anek; C_1-C_5: Clones of M_5; M_8: Mother plant 8 of Dr.Anek; C_1- C_5: Clones of M_8

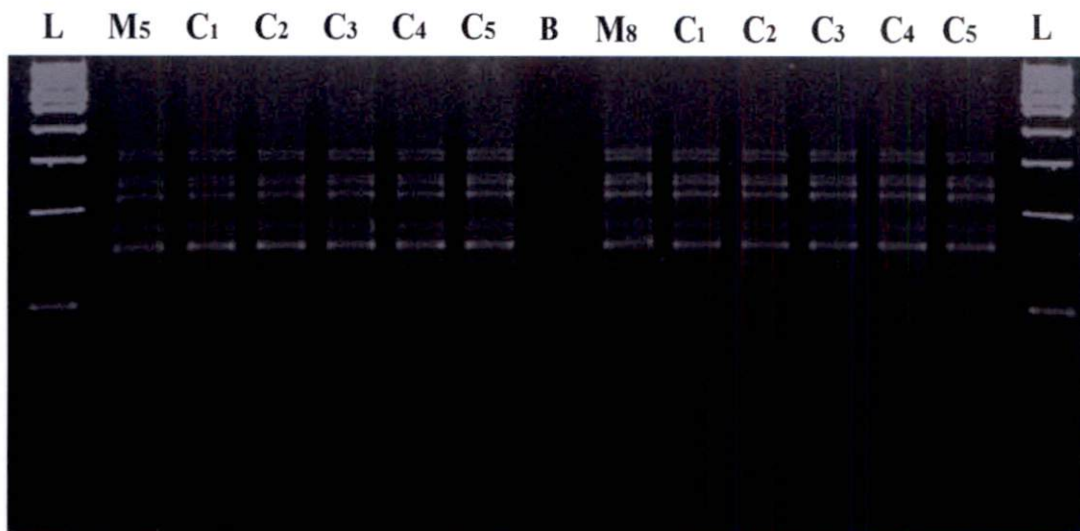
Plate 16 (a and b). Amplification pattern for UBC 835 for different mother plants and their respective clones of 'Dr. Anek'

UBC 841

Amplification with the primer UBC 841 generated eight clear amplicons in all clones of all four tested mother plants of Dr. Anek. All the bands obtained were monomorphic in all four mother plants in the size range of 300-800 bp. The monomorphic bands obtained in the ISSR analysis with UBC 841 primers showed that the clones regenerated through the identified *in vitro* propagation protocol are identical to the mother plants. The amplification pattern for primer UBC 841 for the four mother plants and their respective clones are given in Plate 17.



(a). L: Marker (12 kb); B: Blank; M_1: Mother plant 1 of Dr.Anek; C_1-C_5: Clones of M_1; M_4: Mother plant 4 of Dr.Anek; C_1- C_4: Clones of M_4



(b). L: Marker (12 kb); B: Blank; M_5: Mother plant 5 of Dr.Anek; C_1-C_5: Clones of M_5; M_8: Mother plant 8 of Dr.Anek; C_1- C_5: Clones of M_8

Plate 17 (a and b). Amplification pattern for UBC 841 for different mother plants and their respective clones of 'Dr. Anek'

4.10.4.1.2 Clonal fidelity analysis for *Vanda* 'Sansai Blue'

The amplification patterns obtained for each of the primers are detailed below:

UBC 808

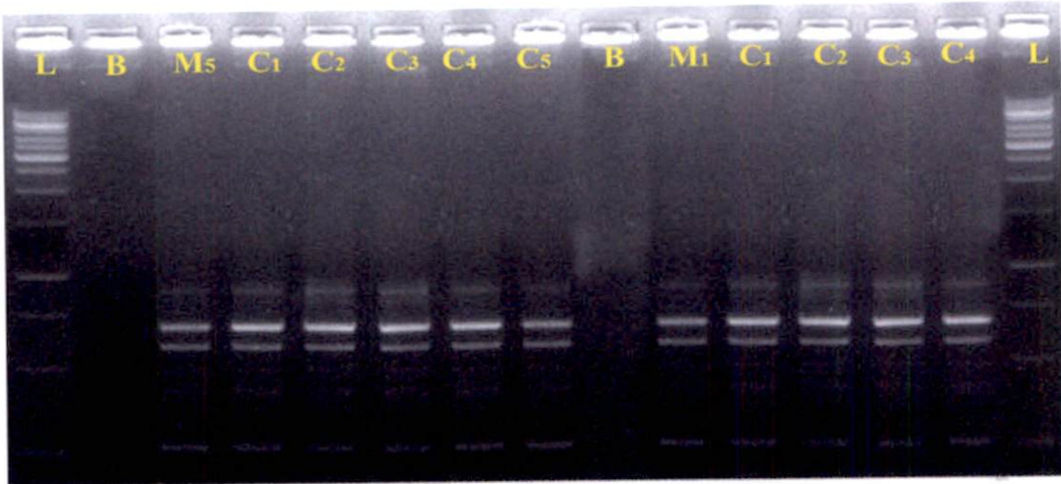
Amplification with the primer UBC 808 generated seven clear amplicons in all mother plants and their respective clones for 'Sansai Blue' with size range from 250 to 900 bp. No polymorphic bands were observed in the amplification pattern which showed that the mother plants and their clones were true-to-type. The amplification profile for primer UBC 808 for the four mother plants and their respective clones in 'Sansai Blue' are given in Plate 18.

UBC 811

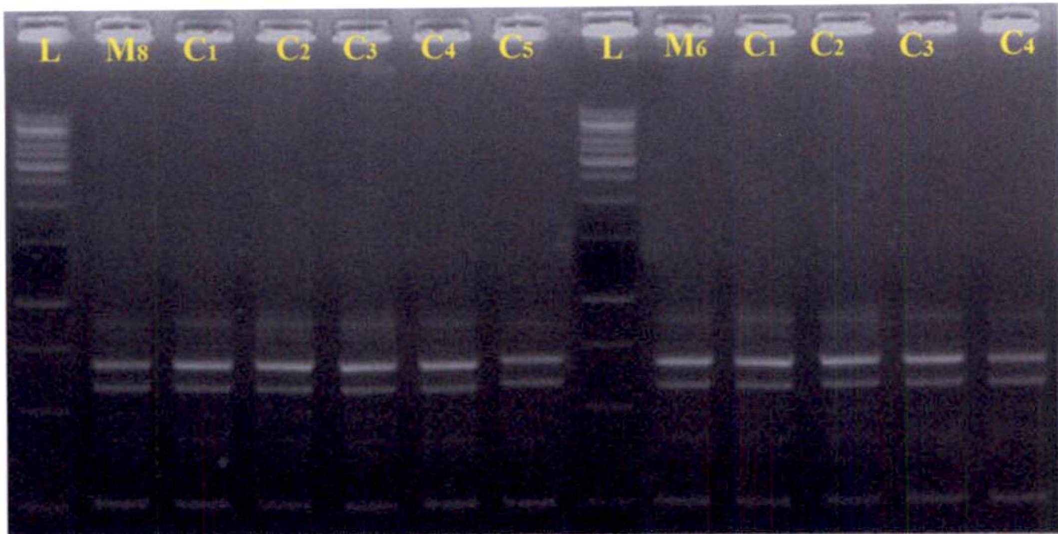
Amplification with the primer UBC 811 generated ten clear amplicons in all clones of all four tested mother plants of 'Sansai Blue'. The bands obtained were all monomorphic bands in the size range of 250- 1000 bp. The monomorphic bands obtained in the ISSR analysis with UBC 811 revealed that the clones regenerated through the identified *in vitro* propagation protocol are genetically identical to their mother plants. The amplification profile for primer UBC 811 for the four mother plants and their respective clones with monomorphic banding pattern are given in Plate 19.

UBC 826

Amplification with the primer UBC 826 generated nine clear amplicons in all clones of all four tested mother plants and their clones of 'Sansai Blue'. The bands obtained were all monomorphic bands in the size range of 150- 1200 bp. Monomorphic banding pattern was observed for all the amplified band classes across the mother plant and its regenerants. The amplification profile for primer UBC 826 for the four mother plants and their respective clones with monomorphic banding pattern is given in Plate 20.



(a). L: Marker (12 kb); B: Blank; M_1: Mother plant 1 of Sansai Blue; C_1-C_4: Clones of M_1; M_5: Mother plant 5 of Sansai Blue; C_1- C_5: Clones of M_5



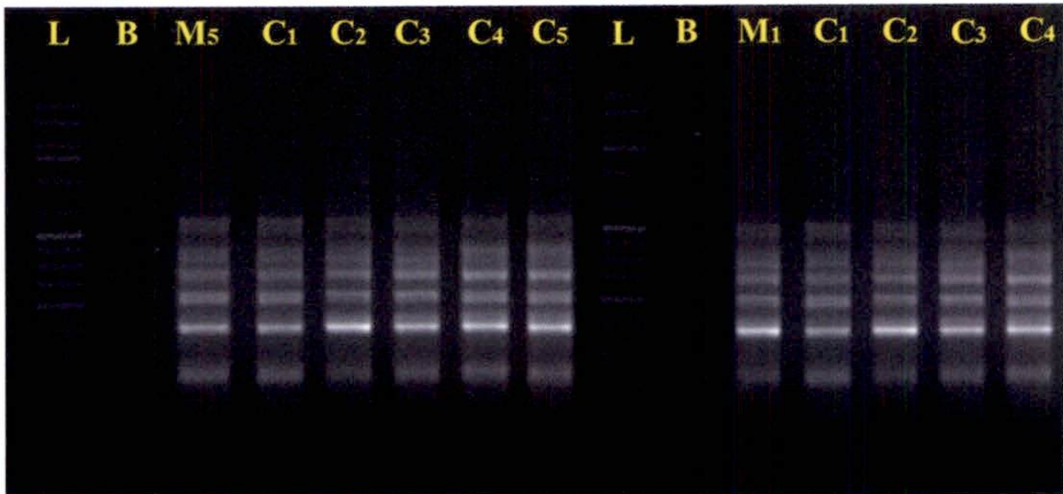
(b). L: Marker (12 kb); M_6: Mother plant 6 of Sansai Blue; C_1-C_4: Clones of M_6; M_8: Mother plant 8 of Sansai Blue; C_1- C_5: Clones of M_8

Plate 18 (a and b). Amplification pattern for UBC 808 for different mother plants and their respective clones of 'Sansai Blue'

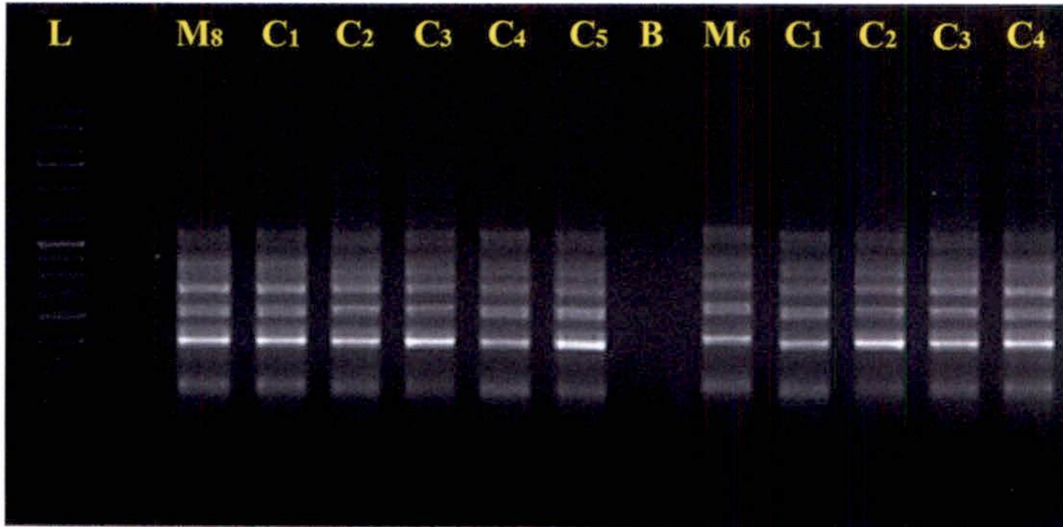
(a). L: Ladder (12 kb); B: Blank; M_1: Mother plant 1 of Sansai Blue; C_1-C_4: Clones of M_1; M_5: Mother plant 5 of Sansai Blue; C_1- C_5: Clones of M_5

(b). L: Marker (12 kb); B: Blank; M_6: Mother plant 6 of Sansai Blue; C_1-C_4: Clones of M_6; M_8: Mother plant 8 of Sansai Blue; C_1- C_5: Clones of M_8

Plate 19 (a and b). Amplification pattern for UBC 811 for different mother plants and their respective clones of 'Sansai Blue'



(a). L: Marker (12 kb); B: Blank; M_1: Mother plant 1 of Sansai Blue; C_1-C_4: Clones of M_1; M_5: Mother plant 5 of Sansai Blue; C_1- C_5: Clones of M_5



(b). L: Marker (12 kb); M_6: Mother plant 6 of Sansai Blue; C_1-C_4: Clones of M_6; M_8: Mother plant 8 of Sansai Blue; C_1- C_5: Clones of M_8

Plate 20 (a and b): Amplification pattern for UBC 826 for different mother plants and their respective clones of 'Sansai Blue'

UBC 835

Amplification with the primer UBC 835 generated nine clear amplicons in all clones of all four tested mother plants of 'Sansai Blue'. The monomorphic bands were in the size range of 500-2500 bp. The monomorphic bands obtained in the ISSR analysis shows that the clones regenerated through the identified *in vitro* propagation protocol are exactly similar to the mother plants. The amplification pattern for primer UBC 835 for the four mother plants and their respective clones are detailed in Plate 21.

UBC 841

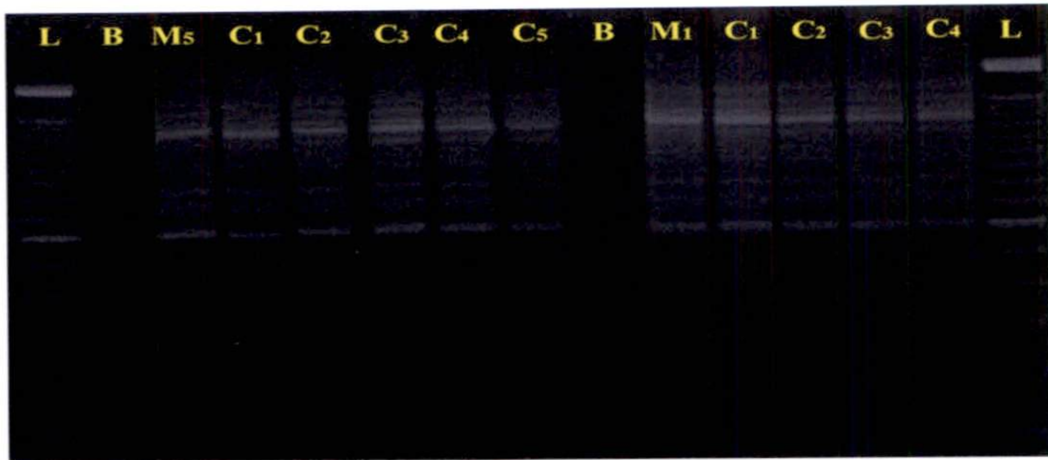
Amplification with the primer UBC 841 generated eight clear amplicons in all clones of all four tested mother plants of 'Sansai Blue'. All the bands obtained were monomorphic in all four mother plants in the size range of 230-1000 bp. The monomorphic bands obtained in the ISSR analysis revealed that the clones are genetically identical to their mother plants. The amplification profile for primer UBC 841 for the four mother plants and their respective clones are given in Plate 22.

4.10.4.2 Amplification pattern in regenerated clones from 'Dr. Anek' with ISSR primers

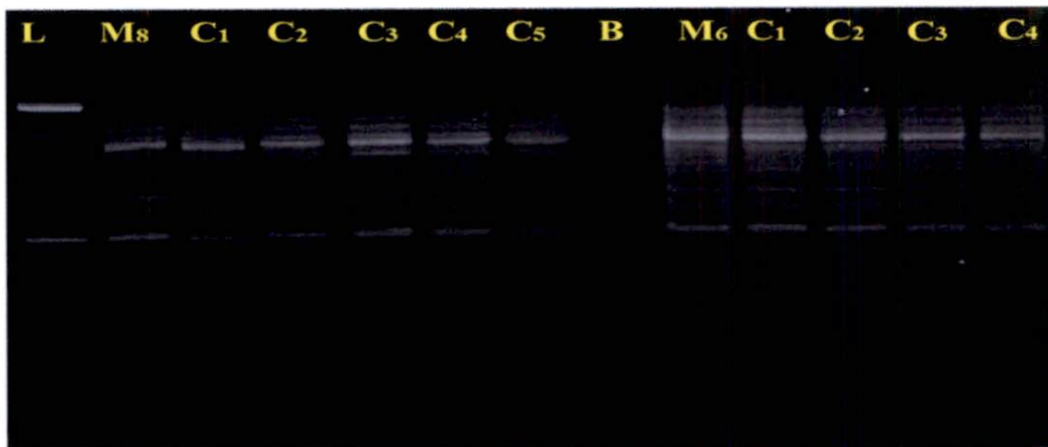
In 'Dr. Anek', four mother plants and their respective regenerants were analysed for the clonal fidelity using five ISSR primers out of which two primers namely UBC 808 and UBC 835 showed polymorphism. The average polymorphism was observed to be 1.11 per cent (Table 33).

4.10.4.3 Amplification pattern in regenerated clones from 'Sansai Blue' with ISSR primers

In Sansai Blue, four mother plants and their respective regenerants were analysed for the clonal fidelity using five ISSR primers. All the primers exhibited monomorphism showing no variation among the mother plant and regenerants (Table 34).

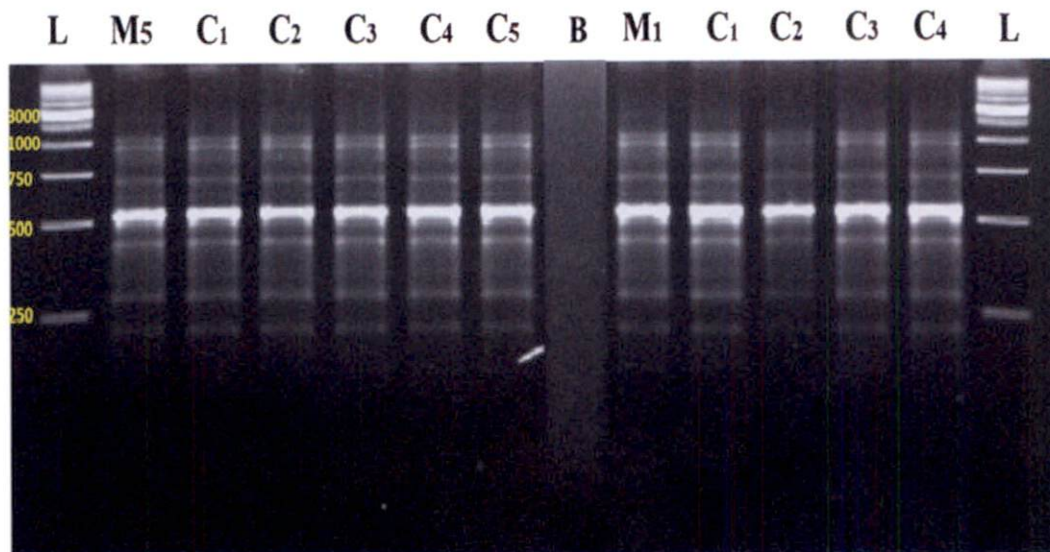


(a). L: Marker (12 kb); B: Blank; M_1: Mother plant 1 of Sansai Blue; C_1-C_4: Clones of M_1; M_5: Mother plant 5 of Sansai Blue; C_1- C_5: Clones of M_5

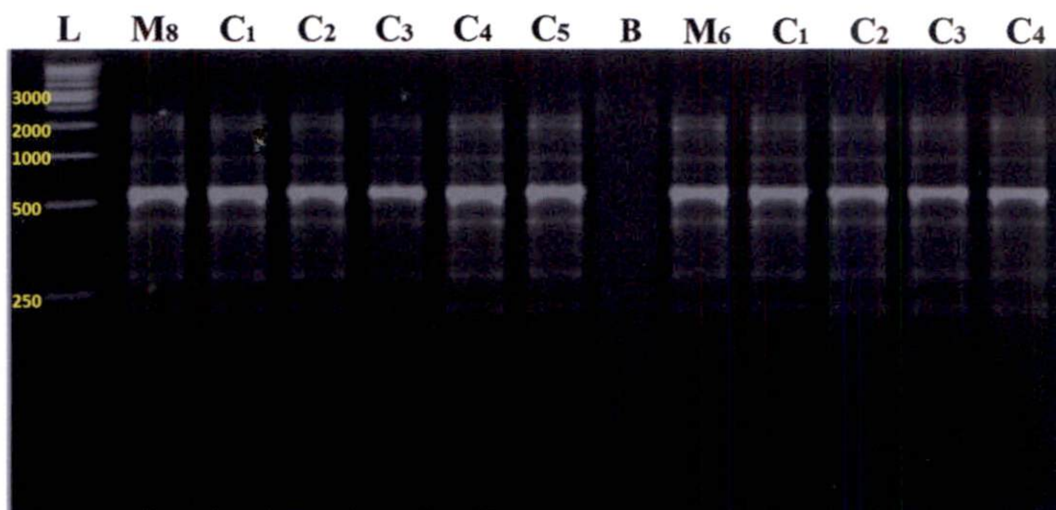


(b). L: Marker (12 kb); M_6: Mother plant 6 of Sansai Blue; C_1-C_4: Clones of M_6; M_8: Mother plant 8 of Sansai Blue; C_1- C_5: Clones of M_8

Plate 21(a and b). Amplification pattern for UBC 835 for different mother plants and their respective clones of 'Sansai Blue'



(a). L: Marker (12 kb); B: Blank; M_1: Mother plant 1 of Sansai Blue; C_1-C_4: Clones of M_1; M_5: Mother plant 5 of Sansai Blue; C_1- C_5: Clones of M_5



(b). L: Marker (12 kb); M_6: Mother plant 6 of Sansai Blue; C_1-C_4: Clones of M_6; M_8: Mother plant 8 of Sansai Blue; C_1- C_5: Clones of M_8

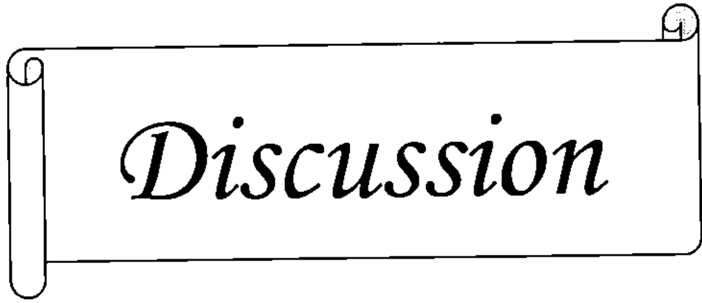
Plate 22(a and b). Amplification pattern for UBC 841 for different mother plants and their respective clones of 'Sansai Blue'

Table 33. DNA Amplification pattern in 'Dr. Anek'

Sl. No.	Primer name		Total No. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	Polymorphism (%)
1	UBC 808	M1	8	0	8	3.13
		M4	8	0	8	
		M5	8	1	7	
		M8	8	0	8	
2	UBC 811	M1	9	0	9	0
		M4	9	0	9	
		M5	9	0	9	
		M8	9	0	9	
3	UBC 826	M1	9	0	9	0
		M4	9	0	9	
		M5	9	0	9	
		M8	9	0	9	
4	UBC 835	M1	11	1	10	2.27
		M4	11	0	11	
		M5	11	0	11	
		M8	11	0	11	
5	UBC 841	M1	8	0	8	0
		M4	8	0	8	
		M5	8	0	8	
		M8	8	0	8	
	Total		**180**	**2**	**178**	**1.11**
	Average		**9.0**	**0.10**	**8.9**	

Table 34. DNA Amplification pattern in 'Sansai Blue'

Sl. No.	Primer name		Total No. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	Polymorphism (%)
1	UBC 808	M1	7	0	7	0
		M5	7	0	7	
		M6	7	0	7	
		M8	7	0	7	
2	UBC 811	M1	10	0	10	0
		M5	10	0	10	
		M6	10	0	10	
		M8	10	0	10	
3	UBC 826	M1	9	0	9	0
		M5	9	0	9	
		M6	9	0	9	
		M8	9	0	9	
4	UBC 835	M1	9	0	9	0
		M5	9	0	9	
		M6	9	0	9	
		M8	9	0	9	
5	UBC 841	M1	8	0	8	0
		M5	8	0	8	
		M6	8	0	8	
		M8	8	0	8	
	Total		**172**	**0**	**172**	**0**
	Average		**8.6**	**0**	**8.6**	


Discussion

5. DISCUSSION

Vanda hybrids are becoming prominent both in the domestic and international flower markets. Presently, the demand for these hybrids is met mainly by importing them from Thailand, Singapore and Malaysia. As the demand is more for the true to type plants, micropropagation is mostly recommended for orchid propagation. This scenario throws light on the need for developing an efficient propagation method for the highly demanded *Vanda* hybrids. The present study "*In vitro* micropropagation protocol for *Vanda* hybrids with clonal fidelity analysis" was carried out in order to generate tissue culture plantlets of selected *Vanda* hybrids using a viable protocol and to examine its suitability for micropropagation with the help of clonal fidelity analysis using ISSR markers. The results of the experiments are discussed in this chapter.

5.1 Studies on micropropagation

Two leading *Vanda* hybrids namely Dr. Anek and Sansai Blue were used for the present study. Different explants were collected from the mother plants maintained in the net house at CPBMB to develop an efficient micropropagation protocol for *Vanda* hybrids.

5.2 Culture establishment studies

5.2.1 Standardization of surface sterilization of explants

In the present study, four different explants were tested for culture initiation and regeneration of plantlets. They were leaf, root, stem and inflorescence segments. Since the explants are collected from the external environment, they carry numerous microbes on them. These are the potential contaminants in any tissue culture procedures. Hence it is necessary to give surface sterilization for the explants using chemical sterilants before they are inoculated.

In the present study, eight different surface sterilization treatments were tested for both hybrids for the different explants and the results for leaf explants is given in Table 9 and 10, root explants in Table 13 and 14, and inflorescence

segments in Table 19 and 20. Owing to limited number of explants, the surface sterilization experiment was not conducted for stem segments. The culture medium in which the sterilization experiments conducted was MS + 1.5 mg l^{-1} TDZ + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime for leaf and root segments as reported by Gantait and Sinniah, 2012 while for inflorescence segments the medium used was ½ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime which was standardized at CPBMB. The culture condition given was initial 48 h dark and further light conditions of 1000 lux with 26 ± 2^0C.

In the present study, the best treatment identified for all the explants was treating the explants with 20 minutes of 0.1per cent carbendazim followed by 5 minutes of 70 per cent ethanol and 5 minutes of 0.1per cent mercuric chloride as it recorded maximum per cent survival and minimum contamination. Endress (1994) reported that the main objective of any sterilization procedure is to obtain minimum contamination with maximum tissue survival.

Considerable drying was observed in the tissues as the duration of exposure to both ethanol and mercuric chloride was increased from 5 minutes to ten minutes. Increase in time of exposure to sterilants proved to be lethal for the explants. Bhadane and Patil (2016) reported that the duration of exposure to the sterilants is an important factor for successful regeneration of *in vitro* plant cultures. Chawla (2000) stated that it is commonly observed that the over use of chemical sterilants is lethal to plant tissues.

Standardization of the surface sterilization procedure is important as it varies with chemicals used as well as the crops. In orchids, Chen and Chang (2000) reported successful regeneration of tissues after surface sterilizing the explants with 0.25 per cent mercuric chloride for 5 minutes whereas Seeni and Latha (2000) reported 95-100 per cent explants survival when the explants were surface sterilized using 70 per cent ethanol for 20 seconds and 0.1per cent mercuric chloride for 3 minutes. Begum *et al.* (2002) used 0.1per cent mercuric

chloride for 5 minutes to surface sterilize *Vanda pteris* axillary bud explants to obtain an efficient micropropagation protocol.

In all the treatments, the per cent bacterial contamination was found to be lower than the fungal contamination. The possible reason for this would the effect of antibiotic cefotaxime (250 mg l^{-1}) which was used in the nutrient media. Cefotaxime have been used in the tissue culture protocols for many crops for effective control of bacterial contamination (Silva and Fukai, 2001; Yu *et al.*, 2001; Donzo, 2015).

Browning of the media was observed in inflorescence segments during the culture period. This would be probably because of the exudation of phenolic compounds. Treating of the explants with 0.1per cent ascorbic acid for five minutes before treating with 70 per cent ethanol during the surface sterilization procedure could efficiently manage the problem of media browning in the present study. It has been reported by Seeni and Latha (2000) that addition of ascorbic acid to the nutrient media can help to overcome the inhibitory effect of phenolics in *Vanda coerulea.* Ascorbic acid can even inhibit the exudation of phenols (Arditti and Ernst, 1992).

5.2.2Identification of suitable explant and medium for culture establishment

In the present study, four different explants were tested on different reported media compositions and out of which only inflorescence segments showed a positive response on the medium of $^{1}/_{2}$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ. Though Chugh *et al.* (2009) reported various potential explants in orchid micropropagation, among the different explants tried in this study (leaf, root, stem and inflorescence segments), only inflorescence segments could initiate the culture response. In contrary to the present study, shoot tips (Seeni and Latha, 2000), leaf segments (Mathews and Rao, 1985; Vij *et al.,* 1986), axillary buds and *in vitro* derived explants of various species of *Vanda* orchids showed positive results to different media compositions.

An observation similar to the present study was found when Korah and Shylaraj (2011) tried to establish cultures using leaf explant, shoot tips, shoot tips of *in vitro* raised plantlets, root tips and inflorescence segments in 15 hybrids of *Vanda* including Dr. Anek. Only the shoot tips of *in vitro* raised plantlets responded positively to the different media under trial.

Reports have revealed that the age of the explant is an important factor that decides its response in the culture media. Chung *et al.* (2009) stated that young leaves respond better than the older leaves. The age of the plant from which the explants is obtained may also influence the response of the tissues. In the experiment conducted by Gaintait and Sinniah (2012), the explants were obtained from two year old mother plants.

The time and cost involved in the standardization of various factors such as nutrient media composition, growth hormones, source of leaf, part of leaf taken and importantly the age of the leaf makes leaf-explant mediated tissue culture protocol development restricted (Chugh *et al.,* 2009) even though there are many reports on orchid micropropagation using leaf explants.

Dr. Anek is a hybrid between *Vanda* Fuch's Delight and *Vanda* Ponpimol. Fuch's Delight is a hybrid between Kaseem Delight which is recalcitrant to tissue culture and *Vanda* Gordon Dillon. The influence of this recalcitrant nature of the parentage may also influence the response of Dr. Anek in the culture media.

The capacity of orchid roots to form shoots is very low (Kerbauy, 1984). Vij (1994) reported root explants from mature plants failed to respond in the culture conditions. Arditti (2009) reported that the orchid roots are one of the most recalcitrant orchid explant both with respect to its inability to survive under *in vitro* conditions as well as for the production of callus or PLBs.

The study also showed that the drying was more fast and severe in root explants than in the leaf explants. The possible reason would be the higher amount of phenolics present in the roots of *Vanda* hybrids than in the leaves. Oxidation of

these phenolic exudates from the explants has been reported to be a major hurdle in the success of orchid tissue culture (Chugh *et al.*, 2009).

With regard to the stem segments, one of the major limitations of the study was the limited number of explants. Since the explants were limited, it was possible to try only two media compositions to initiate the cultures.

The *in vitro* propagation procedure using inflorescence segments involve exposing the dormant bud either to high auxin levels, high cytokinin levels or high anti-auxin levels (Chugh *et al.*, 2009). In this study, inflorescence segments with the dormant bud responded positively to the media composition with high level of cytokinins ($^1/_2$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose +7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime).

The shoot initials were obtained from the buds on inflorescence segment on half strength MS medium with hormones. This might be due to the optimum dose of nitrogen in the media. TDZ is a plant growth hormone. It is a non purine cytokinin which is a phenylurea derivative and is not catabolised by cytokinin oxidase (Kishor and Devi, 2009). TDZ have a stronger effect than BA on *in vitro* morphogenesis in many crops (Malik and Saxena, 1992; Park *et al.*, 2003). The combined effect of TDZ and BA would be a possible reason for the bud sprout and development in the culture establishment of *Vanda* hybrids. The effect of TDZ was also prominent on direct somatic embryogenesis from epidermal cells in *Phalaenopsis* (Chen and Cheng, 2006).

It was observed that average time taken for sprout initiation for Dr. Anek hybrid was 14.20 days whereas for Sansai Blue it was 17.40 days. The percentage of culture establishment was 80 per cent for Dr. Anek whereas only 60 per cent cultures were established in Sansai Blue. The quality of the explants can influence the response of the cultures *in vitro*. It was observed that the inflorescence stalks of Sansai Blue were thin and the buds present inside the bract were smaller when compared to Dr. Anek. This may be a possible reason for a longer time for sprout initiation and a lower culture establishment percentage.

5.3 Shoot proliferation studies

5.3.1 Identification of shoot proliferation media for *Vanda* hybrids

The cultures produced multiple shoots on the medium of MS + 4.5 ml l^{-1} BA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime. The cultures were maintained in the shoot proliferation medium for four subculture passages (S4, S5, S6 and S7). The response of cultures was different for those inoculated with and without the stalk. Those cultures which were inoculated with the stalk produced less number of multiples per explant but were elongated whereas those cultures inoculated without stalk produced numerous multiples but were micro-shoots. The details of the response of explants in shoot proliferation medium are given in Table 26.

The prominent effect of TDZ in inducing shoot bud differentiation in comparision with other cytokinins has been proved in various crops by different experiments (Ernst, 1994). But one of the major disadvantages of using TDZ in the regeneration protocols is the difficulty observed in the elongation of the regenerated shoots (Chugh *et al.*, 2009). The lower mean length of the shoots may be because of the incorporation of TDZ in the establishment media which leads to high cytokinin activity and persistence of TDZ in the tissues.

Lin (1986) reported the influence of developmental stage and age of flower stalk on the frequency of multiples formed during *in vitro* culture of flower stalk internodes in *Phalaenopsis* and *Doritaenopsis*. Highest number of multiples was obtained when the explants were taken from the flower stalk before the first flower became visible. When the explants were obtained after the appearance of first flower on the stalk, the explants from the base of the stalk did not produce any multiples.

Proliferation of regenerated shoots were observed on MS media fortified with 4.5 mg l^{-1} BA. Similar observations have been reported by many researchers. Latip *et al.* (2010) obtained 28 per cent shoot proliferation in *Phalaenopsis* after 40 days of culture in a medium containing 0.5-3.5 mg l^{-1} BA. At a concentration

of 2 mg l^{-1} of BAP, Begum *et al.* (2002) obtained an average of 13.2 multiples per protocorm in combination with 1 mg l^{-1} NAA in *Vanda pteris*. Influence of BA on the cellular division and shoot growth induction in the axillary buds may be the underlining factor for shoot multiplication of plant *in vitro.*

5.4 Elongation and rooting studies

In the present study the cultures were proliferated both with and without stalk. The cultures with stalk were of about 4.0 cm in length but those without stalk were shorter and were about 1.5 cm (Table 26). Since the cultures without stalk did not elongate as compared to the cultures with stalk, these cultures were transferred to basal MS medium without any hormones for its elongation. It was observed that the regenerated shoots elongated to an average length of 4.08 cm after one subculture passage (Table 27). This was now on par with the length of those shoots regenerated from the cultures with stalk (4.20 cm for Dr. Anek and 4.09 cm for Sansai Blue).

Bhosle *et al.* (2005) reported a similar observation as they could achieve about 3 to 3.5 cm elongation of shoots on basal MS medium without any hormones in *Alysicarpus rugosus.* Thanh *et al.* (2012) transferred the adventitious shoots produced from the explants of *Anoectochilus setaceus,* a medicinal orchid into hormone free MS medium for shoot elongation before transferring to the rooting medium. Hormone free MS medium was also reported to produce healthy roots in *Vanda teres* (Sinha and Roy, 2004). In the present study, in few culture bottles, rooting was observed by the end of one subculture passage in hormone free MS medium.

Healthy shoots of about 4 cm were transferred into rooting medium with a composition of MS + 0.5 mg l^{-1} NAA + 1 mg l^{-1} IAA +30 g l^{-1} sucrose solidified on 7.5 g l^{-1} agar and fortified with 250 mg l^{-1}cefotaxime which was standardized at CPBMB for micropropagation of orchids. Charcoal (0.5 g l^{-1}) was incorporated in the medium. The rooting percentage was calculated to be 72.41per cent in Dr. Anek and 70.37 per cent in Sansai Blue. Plantlets recorded good root characters

with an average 6.63roots for Dr. Anek and 5.82for Sansai Blue having a mean root length of 5.93 cm and 5.61 cm respectively.

Paudal and Pant (2012) reported 75 per cent rooting of rare orchid *Esmeralda clarkei* regenerants with about 3 roots per plant with a length of about 2 cm when inoculated on MS medium supplemented with 0.5 mg l^{-1} NAA. The present study used a combination of both NAA and IAA. Rahman *et al.* (2009) obtained a maximum root induction on MS medium fortified with 0.5 mg l^{-1} NAA and 1 mg l^{-1} IBA. Findings by Dutta *et al.* (2011) in *Dendrobium* also support the effect of IAA on rooting when supplemented with MS media. The enhanced level of auxins in the medium can improve rooting of the shoots as they have a strong absorption affinity for many of the inhibitory compounds (Paudal and Pant, 2012).

Incorporation of charcoal and its positive effect on the rooting of *in vitro* plantlets have been well established (Roy *et al.,* 2009). The use of activated charcoal in the present study also had a positive influence on rooting of the cultures. The root induction promoting effect of charcoal is due to its ability to absorb the phenolic exudates from the plantlets into the nutrient media. A similar result showing positive influence of activated charcoal on *Phalaenopsis* rooting was observed by Bhaskar (1996). Jitsopakul *et al.* (2013) reported that activated charcoal induced root formation in *Vanda coerulea* on Vacin and Went medium without adding any plant growth regulators. Arditti (2008) explained that the possible reason behind the effect of activated charcoal on orchid seedling growth *in vitro* is that it improves the aeration and also absorbs ethylene which is an inhibitor of plant growth and differentiation.

5.5 Hardening and acclimatization

Well rooted plantlets of the regenerated *Vanda* hybrids were transferred to the hardening unit for better field establishment of the *in vitro* grown plantlets. The plantlets were given 0.1 per cent carbendazim treatment for five minutes before planting them into small earthen pots filled with charcoal, coconut husk and brick pieces. All the plantlets transferred to the hardening unit acclimatized

well with a 100 per cent survival rate. The regenerated seedlings showed good growth and development in the hardening unit of 50 per cent shade. One month after hardening, the seedlings showed a mean plant height of 7.10 cm in Dr. Anek and 7.14 cm in Sansai Blue (Table 29).

Different potting mixtures have been reported for orchids for the hardening procedures. Paudal and Pant (2012) used sand, soil, sawdust (1:1:1) for the hardening and establishment of rooted seedlings of rare orchid *Esmeralda clarkei* and obtained 85 per cent survival. Gantait and Sinniah (2012) used perlite and sand (2:1; v/v) for the acclimatization of hybrid *Aranda* Wan Chark Kuan 'Blue' x *Vanda coerulea* and obtained more than 95 per cent seedling survival. However the potting mixture used in this study was charcoal, coconut husk and brick pieces (1:1:1) which has been standardized at CPBMB for orchids and showed an excellent survival percentage for the regenerated seedlings of *Vanda* hybrids.

5.6 Clonal fidelity analysis using ISSR molecular marker

5.6.1 Genomic DNA isolation and purification

DNA was isolated from young leaves of mother plants and regenerated plantlets. Young leaves from mother plants were collected early morning hour. The extraction method reported by Rogers and Bendich, (1994) with 2X CTAB extraction buffer yielded good quality DNA.

Liquid nitrogen helped in maintaining the frozen tissue, preventing nucleic acid degradation and effect of secondary metabolites and a better mechanical disruption of tissues (Hernandez and Oyarzun, 2006). The problem of polyphenols was overcome by the addition of β- mercaptoethanol and poly vinyl pyrrolidone (PVP) along with the extraction buffer. β- Mercaptoethanol disrupted the protein disulphide bond and was thereby capable of initiating protein degradation. Advantageous effect of CTAB along with PVP on the quality of DNA has been reported by Gallego and Martinez (1996).

The detergent used in the extraction buffer, CTAB helped in the release of nucleic acids into buffer by disrupting the cell membrane. The released DNA was protected from the action of DNase enzyme by EDTA present in the extraction buffer. It is a chelating agent, which efficiently blocks Mg^{2+}, the major cofactor of DNase enzyme. The DNA isolated by CTAB method was made free of chlorophyll by using the chloroform: isoamyl alcohol which aided in the separation of organic mixture and aqueous phase of the DNA isolation. EDTA was also a major component of TE buffer in which the DNA was dissolved and stored as reported by Sambrook *et al.* (1989).

A DNA sample is analysed as good quality if it appears as a band of high molecular weight with a low amount of RNA (Wettasinghe and Peffley, 1998). The use of RNase for removal of RNA contamination from the isolated DNA was reported by several workers (Wettasinghe and Peffley, 1998; Raval *et al.*, 1998; Gallego and Martinez, 1996). In the present study, RNase treatment was given and it yield good quality DNA.

5.6.2 Quality and Quantity analysis of genomic DNA

Good quality DNA isolated from mother plants and regenerants were used for ISSR marker analysis. The quality and quantity of DNA was calculated using the absorbance ratio as OD at $^{260}/_{280}$ for the various samples using Nanodrop spectrophotometer (Table 30 and Table 31). Those samples with ratio between1.8-2.0 were considered to be of high quality DNA. If the value goes beyond 2.0, it indicates RNA contamination and if less than 1.8, it indicates protein contamination. The yield of DNA in the present study ranged from 459.9 ng/µl to1004.1 ng/µl in Dr. Anek and 489.2 ng/µl to 1003.9 ng/µl and the purity (A_{260}/ A_{280}) of DNA ranged from 1.77 to 1.98 in Dr Anek and 1.79 to 2 in Sansai Blue.

5.2.2 ISSR marker analysis

ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent

regions (16-18 bp). ISSR primers are now proved to be much more efficient in assessing the genetic integrity among clonally propagated plants as reported by many workers in different species (Zietekiewicz *et al.,* 1994; Bhatia *et al.*, 2011; Vanijajiva, 2012). High reproducibility with ISSR technique is attributable to the use of longer primers allowing for higher annealing temperatures than those of RAPDs (Pradeep *et al.*, 2002).

Genomic DNA isolated from mother plants and regenerants were subjected to ISSR analysis. A total of 8 primers were screened with good quality DNA isolated from 4 mother plants in both hybrids. Five ISSR primers reported by Kishor and Devi (2009) were used (Table 32).

5.2.3 Clonal fidelity analysis

In the present study, ISSR marker assay was employed to validate the clonal fidelity of *in vitro* raised *Vanda* plantlets multiplied using inflorescence segments. Martins *et al.* (2004) reported the use of combination of two types of markers which amplify different regions of the genome for better analysis of genetic stability of plantlets. ISSR markers require only little amount of DNA sample, does not involve any radioactivity tests and are simple as well as faster. Hence ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various plants (Carvalho *et al.,* 2004).

5.2.3.1Clonal fidelity analysis for clones of hybrid Dr. Anek

Mother plants and regenerants of four mother plants of Dr. Anek were subjected to ISSR assay. With five primers tested, UBC 808 and UBC 835 exhibited polymorphism while all the other primers gave monomorphic amplicons in the size range of 150 bp to 2000 bp. The number of scorable bands for each primer varied from 8 to 11. A total of 180 bands were generated from mother plant and regenerants, out of which 178 were monomorphic and only 2 clones showed polymorphic bands. Primer UBC 808 showed 3.13 per cent polymorphism whereas UBC 835 showed only 2.27 per cent polymorphism

173897

between clones and their mother (Table 33). The polymorphism may be due to somoclonal variation or by addition of antibiotics in the cultureing medium. Monomorphic bands reveal the genetic stability of progenies inherited from their parents indicating the true-to-type nature of plantlets.

5.2.3.2 Clonal fidelity analysis for hybrid Sansai Blue

In Sansai Blue, four mother plants and their respective regenerants were subjected to ISSR assay. All the five primers tested gave monomorphic bands for the mother plant as well as the clones. The five ISSR primers produced 172 distinct and scorable bands in the size range of 150 bp to 2500 bp. The number of scorable bands for each primer varied from 7 to 10. A total of 172 bands were generated from mother plant and regenerants, out of which all 172 bands were monomorphic (Plate 34).

Rahman and Rajora (2001) reported that the presence of variability among the mother plant and the clonal regenerants is a major limiting factor in the commercial utilization of *in vitro* propagation. Hence it has to be assured that the regenerants produced through any *in vitro* regeneration protocol must be true to type. Use of molecular markers can be utilized for this and it has been reported that ISSR markers are extremely reliable marker system that can give reproducible and authentic variability information. It has been used to access the clonal fidelity of the micropropagated plantlets of many crops (Chavan *et al.*, 2014; Devi *et al.*, 2014).

Kishor and Devi (2009) analysed clonal fidelity for orchid hybrid *Aerides vandarum* × *Vanda stangeana* using ISSR primers and found 100 per cent monophorphism in clones with respect their parents.

Joshi and Dhawan (2007) have employed ISSR marker assay to validate the genetic fidelity of *Swertia chirayita* plantlets multiplied *in vitro* by axillary multiplication upto forty-two passages. Gantait *et al.* (2010) reported the clonal fidelity of micropropagated and sustained cultured clones of *Allium ampeloprasum* L. using ISSR primers.

The polymorphism in mother plant and regenerated plants when amplified with selected primers could result from change in either the sequence of primer binding site or change which alter the size and prevent successful amplification of target DNA. The variations in regenerated plants might be due to gene amplification, chromosomal irregularities, point mutation and alteration in DNA methylation during *in vitro* culture (Saker *et al.*, 2000). The direct formation of plant structures without any intermediate callus phase minimises the possibility of variation (Karp, 1994).

In the present study per cent polymorphism was calculated only to 1.11 per cent in Dr. Anek and zero per cent for Sansai Blue. This results show that the protocol identified for *in vitro* regeneration of *Vanda* hybrids can maintain the genetic stability of the mother plants and is suitable to obtain true to type plantlets.

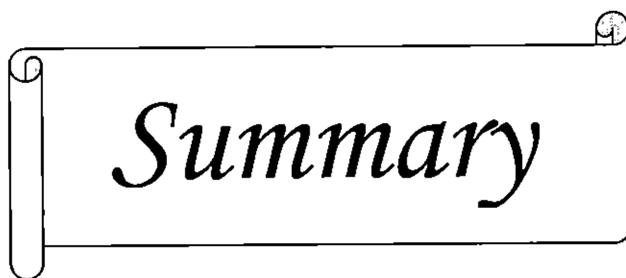
Similar observations were reported by Kishor and Devi (2009) in *Vanda* hybrid *Aerides vandarum*x *Vanda stangeana.* Across the randomly selected mother plants and nine of its regenerants, they could obtain monomorphic banding profiles. The molecular analysis of the mother plants and clones did not show any genomic alterations for the shoots regenerated on 2 mg l^{-1}TDZ which was in accordance with the present study. Marco *et al.* (2015) reported 71.66 per cent polymorphism in vanilla by the use of indirect morphogenic routes to establish vanilla cultures.

Some authors have indicated that various treatments used in tissue culture, with high growth rate, may increase the variant numbers (Bairu *et al.,* 2006). The regeneration systems from organized meristems, such as shoot tip and axillary buds, are considered to be the most efficient methods to guarantee genetic integrity of the micropropagated material. The regeneration methods from leaf explants (Kawiak and Lojkowska, 2004) and callus (Skirvin*et al.,* 1994) are considered to be less stable permitting the occurrence of genetic variation.

Goto *et al.* (1998) reported that the presence or absence of variations during *in vitro* propagation depends upon the source of explants and the method

of regeneration. Martins *et al.* (2004) reported that sub-optimal levels of plant growth substances, especially synthetic plant growth hormones, have also been associated with somaclonal variation. Even at optimal levels, long term multiplication and high chromosome number of the plant may often lead to somaclonal or epigenetic variations in micropropagated plants, consequently, questioning fidelity of their clonal nature. In present study, regenerated plantlets were obtained with very little variation.

The study can be further carried forward by attempting to increase the multiplication rate during the shoot proliferation stage. The identified protocol may be used for the micropropagation of other *Vanda* hybrids. Hybridization programmes can be done to obtain variation in the existing germplasm and the media composition can be used for its commercial propagation.


Summary

6. SUMMARY

The study entitled "*In vitro* micropropagation protocol for *Vanda* hybrids with clonal fidelity analysis" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2014- June 2016, with the objective of regenerating tissue culture plantlets of two selected *Vanda* hybrids through a viable protocol and to examine its suitability for micropropagation by clonal fidelity analysis using ISSR markers.

The main findings of the study are as follows:

- Treating the explants with 0.1 per cent carbendazim for 20 minutes, followed by 70 per cent ethanol for 5 minutes and 0.1 per cent mercuric chloride for 5 minutes can eliminate maximum microbial contamination with minimum destruction to the plant tissues and was found to be the best surface sterilization procedure in *Vanda* tissue culture protocol development.

- Among the various explants selected for initiating *in vitro* regeneration of two selected *Vanda* hybrids namely Dr. Anek and Sansai Blue, inflorescence segments responded positively whereas the leaf, root and stem segments failed to respond under the various media combinations tested.

- The media compositions for initiating the cultures using inflorescence segments, multiplication of shoots and elongation and rooting of the regenerants were identified for the hybrids.

- Culture initiation in *Vanda* hybrids using inflorescence segments were brought about on $^{1}/_{2}$ MS + 10 mg l^{-1} BA + 2 mg l^{-1} TDZ + 30 g l^{-1} sucrose

173897

+7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime with an establishment percentage of 80 per cent in Dr. Anek and 60 per cent in Sansai Blue.

- The medium for multiplication of the shoots was identified as MS + 4.5 mg l^{-1} BA + 30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime.

- The micro-shoots produced on the proliferation medium were successfully elongated on hormone free basal MS medium supplemented with 0.5 g l^{-1} charcoal.

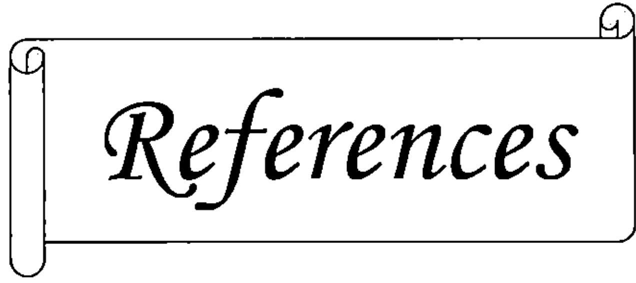
- Rooting of healthy and elongated shoots was about 70 per cent in both hybrids of *Vanda* under the present study on MS + 0.5 mg l^{-1} NAA + 1 mg l^{-1} IAA + 30 g l^{-1} sucrose solidified on 7.5 g l^{-1} agar and fortified with 250 mg l^{-1} cefotaxime.

- Acclimatization of *in vitro* regenerated *Vanda* plantlets was observed to be 100 per cent in earthen pots with charcoal, coconut husk and brick pieces in 50 per cent shade house.

- Genetic stability of the clones was confirmed using ISSR molecular markers.

- The ISSR primers UBC 808, UBC 811, UBC 826, UBC 835 and UBC 841 could be used for genetic stability analysis of *Vanda* hybrids in micropropagation.

- In Dr. Anek the polymorphism percentage was calculated to be 1.11 per cent which is negligible whereas in Sansai Blue there was no polymorphism.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IN VITRO MICROPROPAGATION PROTOCOL FOR *VANDA* HYBRIDS WITH CLONAL FIDELITY ANALYSIS

By

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ABSTRACT OF THESIS

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ABSTRACT

Vanda orchids are one of the most sought after orchids in the international as well as domestic flower markets both as cut flower and potted plants. It is a monopodial orchid with vividly coloured, loosely arranged large beautiful flowers which has a long shelf life. Presently, many *Vanda* hybrids are becoming prominent even in the home gardens. However the present scenario of importing these hybrids from Thailand, Singapore and Malaysia to meet the Indian demands throws light on the need for developing an efficient propagation method for *Vanda* orchids. One of the major limiting factors for its spread and large scale cultivation in India is the non-availability of good quality and true to type planting material at a reasonable price.

As the demand is more for the true to type plants, micropropagation is mostly recommended for orchid propagation. Hence this study was undertaken to develop an efficient micropropagation protocol for two *Vanda* hybrids namely Dr. Anek and Sansai Blue and to check the variability between the parents and regenerated plantlets.

The different explants tested to initiate the cultures were leaf, root, stem and inflorescence segments. Initially the surface sterilization procedure was standardized for the explants. The results of the experiment showed that treating the explants with 0.1 per cent carbendazim for 20 minutes, followed by 70 per cent ethanol for 5 minutes and 0.1 per cent mercuric chloride for 5 min effectively reduced the microbial contamination with highest percentage of explant survival. Trial was made to initiate cultures using eight reported media compositions. The study showed positive results for inflorescence segments inoculated on to $^1/_2$ MS + 10 mg l^{-1} BA+ 2 mg l^{-1} TDZ+30 g l^{-1} sucrose+7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime as observed as direct shooting of the dormant buds. About 80 per cent and 60 per cent culture establishment was brought about in Dr. Anek and Sansai Blue respectively in 9 weeks.

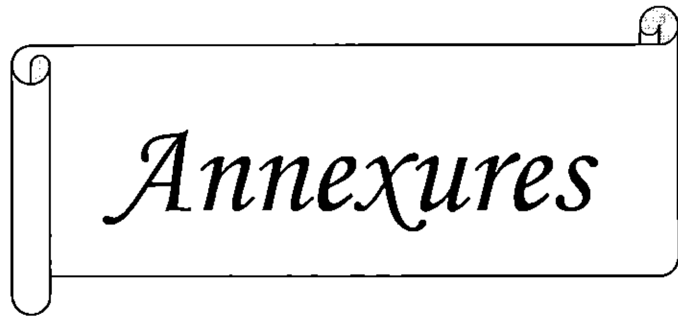
The established cultures successfully produced multiple shoots on MS+4.5 ml l^{-1}BA +30 g l^{-1} sucrose + 7.5 g l^{-1} agar + 250 mg l^{-1} cefotaxime both when

inoculated with and without the stalk in about 100 days of inoculation of explant. The micro-shoots from cultures without stalk were further transferred to hormone free basal MS media for elongation. Elongated shoots of about 4 cm were transferred to rooting media with a composition of MS + 0.5 mg l^{-1} NAA + 1 mg l^{-1} IAA +30 g l^{-1} sucrose +7.5 g l^{-1}agar + 250 mg l^{-1} cefotaxime for better rooting of the regenerants. The percentage of rooting was observed to be 72.41 per cent for Dr. Anek and 70.37 per cent in Sansai Blue.

The rooted plantlets with ample number of healthy roots were planted out in small earthen pots with charcoal, coconut husk and brick pieces. These were successfully hardened in net house of 50 per cent shade and showed a hundred percent plantlet survival.

Good quality DNA isolated from the mother plants and their respective clones using Rogers and Bendich procedure were analyzed for the clonal fidelity. ISSR analysis was done using 5 UBC (University of British Columbia) primers such as UBC 808, UBC 811, UBC 826, UBC 835 and UBC 841. An average of 8 to 9 bands was obtained from all primers in Dr. Anek and Sansai Blue. Out of 5 primers, UBC 808 and UBC 835 generated polymorphic bands in two clones of Dr. Anek. For Sansai Blue, all five primers generated monomorphic bands for all the mother plants and their respective clones analyzed. The per cent polymorphism in Dr. Anek was calculated to be 1.11 per cent whereas for Sansai Blue, there was no polymorphism detected revealing the true to type nature of the clones.

The results showed that the identified protocol for *in vitro* regeneration of selected *Vanda* hybrids is a viable protocol since there were no changes in the banding pattern observed in tissue culture plants as compared with that of mother plant. Hence it can be concluded that the developed micropropagation protocol can be used for commercial production of *Vanda* hybrids without much risk of genetic instability. ISSR markers were effective to evaluate the genetic stability of the clones regenerated from the mother plants by the identified protocol.


Annexures

ANNEXURE I

List of laboratory equipments used for the study

Refrigerated centrifuge	:	Kubota, Japan
Horizontal electrophoresis System	:	Biorad, USA
Thermal cycler	:	Veriti Thermal Cycler (Applied Biosystem, USA)
Gel documentation system	:	Biorad, USA
Nanodrop® ND-1000 spectrophotometer	:	Nanodrop®Technologies Inc. USA

ANNEXURE II

Chemical composition of Murashige and Skoog (MS) medium

Stock	Chemical	mg/litre	Stock concentration	Stock
I	$(NH_4)NO_3$	1,650	50 X	82.5g/l
	KNO_3	1,900		95.0g/l
	KH_2PO_4	170		8.5g/l
	$MgSO_4.7H_2O$	370		18.5g/l
II	$CaCl_2.2H_2O$	440	50 X	22.0g/l
	(Prepare the stock separately or it may precipitate)			
III	Na_2EDTA	37.3	100 X	3.7g/l
	$FeSO_4.7H_2O$	27.8		2.8g/l
(Remember to prepare this as described under stock solution preparation)				
IV	$MnSO_4.7H_2O$	22.3	100 X	2.23g/l
	$ZnSO_4.7H_2O$	8.6		0.86g/l
	H_3BO_3	6.2		0.62g/l
	Kl	0.83		0.083g/l
	$Na_2MoO_4.\ 2H_2O$	0.250		0.025g/l

	$CuSO_4.5H_2O$	0.025		0.002g/l
	$CoCl_2.2H_2O$	0.025		0.002g/l
V	Vitamins			
	Glycine	2.0	100X	200mg/l
	Nicotinic acid	0.5		50 mg/l
	Pyridoxine acid – HCL	0.5		50 mg/l
	Thiamine – HCL	0.1		10 mg/l

100 mg/l myo – inositol

30 g/l sucrose

2 g/ l CleriGel

pH 5 – 5.8

ANNEXURE III

Reagents required for DNA isolation

Reagents:

1. **2x CTAB extraction buffer (100 ml)**

CTAB	:	2g
(Cetyl trimethyl ammonium bromide)		
Tris HCl	:	1.21 g
EDTA	:	0.745 g
NaCl	:	8.18 g
PVP	:	1.0 g

Adjusted the pH to 8 and made up final volume up to 100 ml.

2. **CTAB (10 %, 100 ml)**

CTAB	:	10 g
NaCl	:	4.09 g

3. **Chloroform- Isoamyl alcohol (24:1 v/v)**

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

4. **Chilled isopropanol**

Isopropanol was stored in refrigerator at 0°C and was used for the study.

5. **Ethanol (70 %)**

To the 70 parts of absolute ethanol (100 %), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. TE buffer (pH 8, 100 ml)

Tris HCl (10 mM)	:	0.1576 g
EDTA (1 mM)	:	0.0372 g

The solution was prepared, autoclaved and stored at room temperature.

ANNEXURE IV

Composition of buffers and dyes used for gel electrophoresis

1. **TAE Buffer 50X**

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5M EDTA (pH 8.0)	:	100 ml

2. **Loading Dye (6X)**

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. **Ethidium bromide**

The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in a dark bottle.