Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid Winter Spot with molecular marker

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled "**Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid Winter Spot with molecular marker"** 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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Certified that the thesis entitled "**Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid Winter Spot with molecular marker"** is a record of research work done independently by **Mrs. Asha Amal Raj** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to her.

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%	Percentage
μg	Microgram
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
BAP	6-Benzylaminopurine
Bp	base pair
cc	Cubic Centimeter
cm	Centimeter
CPBMB	Center for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
CW	Coconut Water
DAI	Days after inoculation
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribo nucleoside Triphosphate
EDTA	Ethylene diamine tetra-acetic acid
$HgCl_2$	Mercuric Chloride
IAA	Indole-3-acetic acid
IBA	lndole-3-butyric acid
Kin	Kinetin
2,4-D	2,4-Dichloro phenenoxyacetic acid
M	Molar

$MgCl_2$	Magnesium chloride
mg	Milligram
μM	Micromolar
mM	mili Molar
MS	Murashige and Skoog (tissue culture medium)
NAA	Naphthalene Acetic Acid
$NaCl_2$	Sodium chloride
NDM	New Dogashima Medium
NTSYS	Numerical Taxonomy and Multivariate Analysis System
PCR	Polymerase Chain Reaction
PLBs	Protocorom-like-bodies
PVP	Polyvinyl pyrrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
SSR	Simple Sequences Repeat
TAE	40 mM Tris-Cl, 20mM sodium acetate, 1 mM EDTA
Taq	*Thermus aquaticus*
TCL	Thin cell layer cross section

TDZ	Thidiazuran
TE	Tris-EDTA buffer
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VW	Vacin and Went (tissue culture medium)

Introduction

1. Introduction

Orchids are one of the most beautiful and bewitching of all flowering ornamental plants. They are often referred to as royals in the plant kingdom. The family orchidaceae represent a peak in the evolution of monocot plants. This is evident from the various adaptation mechanism followed by the flower to attract pollinators. They have a cosmopolitan distribution and are found growing throughout the earth surface except in snow covered areas and in the driest deserts.

The family orchidaceae is the largest among flowering plants in terms of species diversity with 600-800 genera, comprising of 25,000-30,000 species. In India, about 1300 species are distributed all over the north- eastern Himalayas (600 species), north-western Himalayas (300 species), Maharashtra (130 species), Andaman and Nicobar islands (70 species) and western ghats (200 species).

Orchids are marketed both as cut flowers and potted plants. The largest exporters of potted orchids are Taiwan, Thailand, UK, Italy, Japan, New Zealand and Brazil while the largest importer of potted orchids is the United States. Today orchids such as *Cymbidium*, *Dendrobium*, *Oncidium* and *Phalaenopsis* are marketed globally and the orchid industry has contributed substantially to the economy of many ASEAN (Association of the South East Asian Nations) countries (Hew, 1994; Laws, 1995).

Over 75% of all the orchids sold in the U.S. at present are *Phalaenopsis* sp. which had replaced *Cattleya* in the 1980s as the most popular orchid (Griesbach, 2002).

Phalaenopsis is one of the most popular orchids on the Horticulture Market, due to the beauty of its blooms, the wide variety of colors and the availability of a large number of hybrids. The evolution of orchid cultivation in the course of time was a very slow one. In the beginning orchid propagation outside of their natural habitat was impossible, thereby they were very expensive at that point in time; today the orchid culture is being practiced on an industrial level through tissue culture.

Nowadays low production cost and the ease of maintenance made *Phalaenopsi*s the most sought and loved orchid.

The genus name *Phalaenopsis* derives from two Greek terms ("phalaina" = butterfly and "opsisi" = resemblance) refering to the elegant shape of the flowers, that look like the wings of a butterfly. Approximately 60 species of this genus are originally from the thick, moist and warm forests of India, Indonesia and the Philippines. They can be found at altitudes of 200-400m. *Phalaenopsis* are low light orchids; they will grow and flower reliably in natural or artificial light as low as 300-500 foot-candles (fc). However, 1000 to 1500 fc is the perferred level. The ideal annual temperature range is 60 ° F. (16 ° Celsius) to 85 ° F. (24°Celsius). About three weeks of night temperature in the 52 (11.1^{o}c) to 54 ° F (12.2^{o}c) range will insure good bud initiation.

The plant exhibits a monopodial form of growth with a single vertical main stem, which produces a series of thick, fleshy and distichous leaves. One spray of flower develops from each leaf axial. The lower part of the plant consists of root system made out of green fleshy roots that can be found in the growing medium or they can grow outside of the pot up into the air.

Phalaenopsis can be propagated through division and seed culture. These methods can be time consuming and result in only a limited number of plants. The most popular method of propagation for orchids is through *in vitro* propagation as it is said to produce a large number of clones in relatively short duration. Several tissue culture techniques have been developed for *Phalaenopsis* orchids, including the culture of flower stalks with axillary buds, meristems, internodal segments of flower stalks (Arditti and Ernst, 1993; Park *et al*., 1996), leaf segments (Ishii *et al*., 1998 ; Park *et al*., 2002) and root tips (Arditti and Ernst, 1993; Ichihashi, 1997).

Despite its potential to produce numerous new plants from a single leaf segment, it is subjected to unpredictable mutations or somaclonal variation which can occur during the process of multiplication. Somaclonal variation though hailed as a novel source of genetic variation which can result in improved material, is often

undesirable when the objective is micropropagation of elite genotypes. Somaclonal variation can happen because of many reasons such as the type of media used, plant growth regulators and concentration, the type of explant and number of subculture cycles (Reuveni *et al*., 1986).

In the case of *P. bellina,* somaclonal variation may result in the loss of fragrance or change in the color of flowers which appears to be some of the important characteristics of this orchid. Some orchid hybrids are more amenable than others to somaclonal variation. The percentage of the variations can range from 0 - 100% depending on varieties, with an average of 10% among *Phalaenopsis* (Tokuhara and Mii, 1993).

The cause of somaclonal variation in higher plants has been reported during different biochemical and molecular events, including changes in DNA methylation pattern, activation of transposable elements and chromosome remodeling (Hirochika, 1993; Price *et al*., 2002). Molecular markers have been exploited for the detection of somaclonal variation, including random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and microsatellite markers.

The RAPD technique has several advantages such as the ease and rapidity of analysis, a relatively low cost, availability of a large number of primers and the requirement of a very small amount of DNA for analysis (William *et al*., 1990). RAPD analysis using polymerase chain reaction (PCR) in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals.

In this context, "Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid Winter Spot with molecular marker" was taken up at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University.

This study aims to develop an *in vitro* regeneration system and to verify the genetic stability of regenerants in *Phalaenopsis* hybrid Winter Spot using molecular marker.

Review of literature

2. Review of literature

The research programme entitled “Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid Winter Spot with molecular marker” was conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2013- February 2016. Relevant Literature on various aspect of the research is reviewed in this chapter.

2.1 Orchidaceae

Orchidaceae family is one of the largest monocotyledonous families of flowering plants on our planet which has been estimated to include over 70,000 species in the world, and only half of them have been discovered. Orchids are most plentiful in the equatorial regions, but have also been discovered in temperate climates and some even in places close to the tundra regions. Orchids have become the most popularly grown flowers in the world. They are known for great variations in floral morphology, pollinator relationships and diversity of their ecological habitat (terrestrial or epiphytic) (Arditti, 1992; Judd *et al*., 1999).

The family, which includes *Cattleya*, *Dendrobium*, *Epidendrum*, *Paphiopedilum*, *Phalaenopsis*, *Vanda*, *Brassica*, *Cymbidium*, *Laelia*, *Miltonia* and *Oncidium*, is economically important because of the ornamental value (Judd *et al*., 1999).

2. 2 General description

Phalaenopsis is a genus with approximately 60 species of orchid (family Orchidaceae). The generic name originates from the Greek phalaina, "moth" and opsis, "like", descriptive of the inflorescences of some species, which resemble moths in flight. For this reason, the species are sometimes called Moth orchids.

Taxonomically, the genus *Phalaenopsis* belongs to the family *Orchidaceae*, sub-family Epidendroideae, tribe *Vandeae* and subtribe *Aeridinae* which is divided

into five subgenera, namely *Proboscidioides*, *Aphyllae*, *Parishianae*, *Polychilos* and *Phalaenopsis*. It comprises of approximately 66 species according to the latest classification of Christenson (Christenson, 2001).

They are native throughout southeast Asia from the Himalayan mountains to the islands of Polillo and Palawan of the Philippines and northern Australia. Most are epiphytic shade plants; a few are lithophytes. In the wild they are typically found below the canopies of moist and humid lowland forests, protected against direct sunlight. They are typically found of warm temperatures (20 to 35 °C), but are adaptable to conditions more comfortable for human habitation in temperate zones (15 to 30°C); at temperature below 18°C watering should be reduced to avoid the risk of root rot. *Phalaenopsis* requires high humidity (60-70%) and low light of 12,000 to 20,000 lux.

2.3 Propagation

Phalaenopsis can be vegetatively propagated by two methods. The first method is by cutting the flowering stem above a stem internode, the dormant growth "eye" is covered with a triangular sheath. Cut through the inflorescence node, after the last flower has fallen. In most cases, new plants will start from the dormant "eyes ". In the second method, the mother plant is topped. As a monopodial plant, *Phaleanopsis* continue to grow vertically discarding their lower leaves. New roots are produced above the leafless stem, while growing vertically. The stem can be cut below the new roots. The top part, with leaves and roots, can be repotted after proper care of the cut. The remaining stub can be left as is, for a few days/weeks. Soon, new little plants will be found growing out of the old stub (Reuveni *et al*., 1986).

2.4 *In vitro* propagation

Factors influencing *in vitro* propagation are given in the following subtitles.

2.4.1 Explant

The choice of explant is usually the most important initial factor that is considered when establishing a micropropagation protocol for orchids, and may vary depending on the availability of material, seasonality of development or level of infection and/or abundance of tissue / explants (Chugh *et al*., 2009).

Use of flower stalks (Chen and Piluek, 1995) and cytokinin induced nodes (Duan *et al*., 1996) began to be popular for mass propagation of *Phalaenopsis* from 1980's.

2.4.2. Type of explants

Ever since Rotor, (1949) initiated *Phalaenopsis* cultures using flower stalks *in vitro* and Morel, (1960) reported shoot-tip cultures of *Cymbidium*, efficient micropropagation systems using tissue culture techniques have been reported in many genera of the Orchidaceae.

Wimber, (1965) pioneered leaf tissue culture and gave the first well documented report on production of PLBs from *Cymbidium* leaves.

Several tissue culture techniques have been developed for *Phalaenopsis* orchids, including the culture of flower stalks with axillary buds, meristems, flower stalks explants, internodal segments of flower stalks (Arditti and Ernst, 1993; Park *et al*., 1996), leaf segments (Park *et al*., 2002; Ishii *et al*., 1998) and root tips (Arditti and Ernst, 1993; Ichihashi, 1997).

2.4.2.1 Meristem/ shoot tip

The credit for achieving mass clonal propagation of orchids goes to Morel (1960) who was successful in culturing *Cymbidium* shoot apices on nutrient media. It

has been estimated that more than four million plants could be produced in a year from a single explants (Morel, 1964).

Apical meristem of a young shoot is the most commonly used explants, both in monopodial and sympodial orchid. Shoot tip culture was successfully standardized in *Vanda* (kunisaki *et al.*, 1972), *Phalaenopsis* (Intuwong and Sagawa, 1974), *Paphiopedilum* (Stewart and Button, 1976) and *Renantra* (Karim *et al*., 1992).

In *Phalaenopsis*, callus induction was achieved from PLB induced by culturing shoot tips of lateral buds from flower stalks (Ichihashi, 1992) and leaf segments (Ishii *et al*., 1998).

Tokuhara and Mii, (2001) reported that embryogenic calli were induced from *Phalaenopsis* shoot-tip explants excised from flower stalk buds by culturing on New Dogashima Medium (NDM) containing 0.5 mM NAA, 4.4 mM BAP and 29.2 mM sucrose. These calli were subcultured as cell suspension culture in liquid NDM supplemented with 5.4 mM NAA and 58.4 mM sucrose. By reducing the sucrose concentration to 29.2 mM, the cells grew into plantlets.

Shoot tips from *in vitro* grown plants of *Dendrobium primulinum* Lindl. initiated multiple shoot in, MS medium with BAP (1.5 mgl^{-1}) and NAA (0.5 mgl^{-1}) (Pant and Thapa, 2012). Rapid multiplication through PLB formation for shoot tips and leaves of endangered *Vanda coerulea* was achieved through culture of shoot tips of seedlings and mature plants (Seeni and Latha, 2000).

2.4.2.2 Inflorescence stalk

Inflorescence segments have emerged as effective donor organs for micropropagating orchids, ever since Rotor, (1949) initiated *Phalaenopsis* cultures using flower stalks *in vitro*.

Young inflorescence stalk nodal explants is the preferred explants for producing plantlets in *Aranda* (Goh and Wong, 1990*) Dendrobium* (singh and Sagawa, 1972), *Vanda* (Sagawa and Sehal, 1967) and *Cymbidium* (Kim and Kako, 1984).

Kosir, *et al*. (2004) reported high shoot regeneration from inflorescence nodal segment of *Phalaenopsis* using Murashige and Skoog medium supplemented with 2 mgl^{-1} of 6-benzylaminopurine (BAP) and 0.5 mgl^{-1} of α–naphtalenacetic acid (NAA).

Park *et al*. (2002) cultured the leaf segment of *Phalaenopsis* derived *in vitro* from flower stalk on MS medium supplemented with BA 88.8mM and NAA 5.4mM and produced an average of 10-13 protocorm like bodies after 12 week. Optimal number of 13-18 PLBs developed from single PLB section on a modified Hyponex medium.

Chen and Piluek, (1995) observed adventitious bud formation from the vegetative buds of the flower stalks of *Phalaenopsis* on Vacin and went medium with 15% coconut water and 5 to 40μM thidiazuron (TDZ) or 40μM N 6-benzylaminopurine. The highest efficiency of induction was achieved with 5 or 10 μM TDZ. Regerated shoots rooted after about two months of culture on VWC medium with 1% sucrose.

Nodal cuttings taken from senescent flowering stems of *Phalaenopsis* sp. were cultured on ½ strength MS medium containing 2.9mg Ca/litre supplemented with vitamins, adenine, coconut milk, activated charcoal. The best results were generally given by larger stem segments. Vegetative buds rapidly produced leaves and roots whereas flower buds developed into inflorescence with nodal segments and following subculture of these, vegetative growth commenced (Jiménez and Guevara, 1996).

The induction of vegetative shoot from axillary buds of *Phalaenopsi*s flower stalks was studied *in vitro* on the medium of 1/3MS + 6-BA 5mgl^{-1} + NAA 0.2 mgl^{-1}

by the culture of flower-stalk segments each with one bud. About 82.4% of axillary bud sprouting was obtained from the media of 1/3MS with 6-BA 5mgl^{-1} (Li *et al.*, 2006).

Huaiyiu, (1989) also reported clonal propagation of *Phalaenopsis* flower stalk in MS + 0.5-5mgl^{-1} BA + l mgl^{-1} NAA or MS + 0.5-5mgl^{-1} BA medium. When 5mgl^{-1} BA + lmgl^{-1} NAA was added into the medium, 65% of the stem sections formed adventitious buds, and 16.7% of the leaves formed PLB.

2.4.2.3 Leaf

The advantage of using leaves or other organs as explants is that only very little portion of the plant is sacrified in the process.

Many scientists reported successful culturing of younger leaf and leaf tip of *Aranda, Epidendrum, Rhyncostylis, Cattleya* and *Phalaenopsis* (Vij and pathak, 1990).

Khoddamzadeh *et al.,* (2011) established an *in vitro* culture protocol to induce protocorm-like bodies (PLBs) from leaf segments of the *P. bellina* directly from epidermal cells without intervening callus on ½ strength modified Murashige and Skoog (MS) medium supplemented with 1-Naphthaleneacetic acid and Thidiazuron. The best induction percentage for auxin: cytokinin combination was at the combination of NAA and TDZ at 1.0 and 3.0mgl^{-1} respectively which gave 72% induction with nine PLBs per explant.

Callus induction and plant regeneration through somatic embryogenesis were examined in *Phalaenopsis*. Protocorm-like body (PLB) segments formed calli in Vacin and Went medium containing 200 ml (v/v) of coconut water together with 4 % of sucrose (Ishii *et al*., 1998).

Sinha and Jahan, (2011) established a protocol for mass clonal propagation of *Phalaenopsis amabilis* through *in vitro* culture of young leaf segments from mature plant. Explants were cultured on half strength Murashige and Skoog (1/2 MS) medium supplemented with N^6-benzyladenine (2.0 mg l^{-1}), naphthalene acetic acid (0.5 mg l^{-1}), 2% (w/v) sucrose, 10% (v/v) coconut water, 2 g l^{-1} peptone and 1 gl^{-1} activated charcoal. Each section of explant produced 15 protocorm-like bodies (PLBs) after 12 weeks of culture. Leafy shoots rooted on half strength MS medium supplemented with 2gl^{-1} peptone, 2% (w/v) sucrose, 10% (v/v) CW and 1 gl^{-1} activated charcoal. The addition of 2.5 gl^{-1} banana pulp powder enhanced the number and length of root.

In *P. amabilis*, Chen and Chang, (2006) had reported that NAA retards direct embryo formation from leaf explants and reduced the amount of embryos induced by TDZ. In *Phalaenopsis* ‘‘Little Steve’’, 2,4-D exhibited highly inhibitory effect on direct embryo induction from leaf explants even in the presence of TDZ. The result showed that four kinds of auxins (including 2,4-D, IAA, IBA and NAA) highly retarded direct embryogenesis from leaf explants of *P. amabilis* (Kuo *et al.*, 2005).

Adventitious buds of *Phalaenopsis* Blume were induced directly from leaves of plantlets of flower stalk culture without the development of protocorm-like body (PLB). Results showed that the MS + 6-BA 5 mgl^{-1} + NAA 1 mgl^{-1} + CW 100 mgl^{-1} produced an inducing rate of 32.8% adventitious buds and that the adventitious buds produced a rooting rate of 95% when cultured under the Hyponex (7-6-19) 3 g l^{-1} + tryptone 1 gl^{-1} + activated charcoal 1 gl^{-1} + banana homogenate 100 gl^{-1} + IBA 1 mgl^{-1} + NAA 0.5 mgl^{-1} (Xianghua *et al.*, 2004).

Chen and Chang, (2006) demonstrated secondary embryogenesis by culturing leaf explants of *Phalaenopsis amabilis* var. *formosa* segments on 1/2-strength modified Murashige and Skoog medium supplemented with 0.1, 1 and 3 mg l^{-1} TDZ.

Leaf explants of two *Phalaenopsis species*, *P. amabilis* and *P. nebula*, were used to test the effects of auxins (2,4-D, IAA, IBA, NAA), cytokinins (2iP, BA,

kinetin, TDZ, Zeatin), and GA3 on the amount of direct embryo formation of *Phalaenopsis*. The results showed that direct embryo formation was induced by cytokinins. BA at 13.32 μM and 2iP at 4.92 μM found most effective for *P. amabilis* and *P. Nebula*, respectively (Gow *et al*., 2008).

Kuo *et al*. (2005) evaluated the effects of 2,4-D (0.45, 2.26, 4.52 mM), kinetin (2.32, 4.65, 13.95mM), BA (2.22, 4.44, 13.32mM), and TDZ (2.27, 4.54, 13.62mM) on cultured leaf segment of the orchid sp. *Phalaenopsis* 'Little Steve' for the induction of direct somatic embryogenesis. After 20–30d of culture, clusters of somatic embryos formed from leaf surfaces on half-strength Murashige and Skoog medium supplemented with BA and TDZ.

Thin cross sections (TCSs) of actively growing parts such as shoots, and developing protocorm-like bodies (PLBs) have been successfully used by some workers for plantlet regeneration in a few orchids (Begum *et al*., 1994; Lakshmanan *et al*., 1995; Teng *et al*., 1997).

An efficient and reproducible procedure is outlined for rapid *in vitro* multiplication of commercially important orchids through high frequency shoot proliferation from thin cross sections (TCSs) of protocorm-like bodies (PLBs). Thin cell layer (TCL) system consists of explants of a small size excised from different plant organs *viz*, stem, leaves, floral inflorescences, flower primordia or floral organs, cotyledons, hypo-/epicotyl, apical zone or embryo, either longitudinally (lTCL), or transversally (tTCL) (Nayak *et al*., 2002).

Lakshmanan *et al*., (1995) developed the TCS culture technique by employing sections of 0.6 mm thickness of different plant parts to hasten plant regeneration in a number of orchid genera. According to Rout *et al.*, (2006) the efficiency of thin cell layer culture is very high compared to the conventional technique of tissue culture. The use of TCS explant for *in vitro* plantlet regeneration has also been reported in a few other plant species such as *Panax ginseng* (Ahn *et al*., 1996) *Digitaria sanguinalis*

(Le *et al*., 1998), *Aranda Deborah* (Lakshmanan *et al*., 1995) and *Spathoglottis plicata* (Teng *et al*., 1997) etc.

Development of callus, PLB's and plantlets from TCS depends upon the medium and plant growth regulator used. PLB formation from TCS of stem and leaf was observed in *Dendrobium* (Rangsayatorn, 2009) and *Cymbidium* (Nayak *et al*., 2002). The optimal growth regulator combination for maximal PLB development was 2 mgl^{-1} BA and 1.0 mgl^{-1} NAA, giving rise to average 11 PLBs per explants (Rangsayatorn, 2009). High frequency of shoots regenerated from PLBs were rooted on MS medium containing 9.8mM indole-3-butyric acid (IBA) (Nayak *et al*., 2002).

Thida *et al*. (2009) reported somatic embryogenesis from thin sectioned leaf explants of *Phalaenopsis,* cultured on a half strength MS medium supplement with 9.08 μM thidiazuron 2 weeks in darkness followed by 16hr photoperiod with approximately 60μmol/m/s light intensity.

2.4.2.4 Root

Root tips of orchid species have been considered generally recalcitrant to form PLB or callus *in vitro*, such is the case, with *Epidendrum*, *Oncidium*, and *Cattleya* plants.

Aerial roots have yielded callus and plantlets in *Epidendrum* (Stewart and Button (1978). Tanaka *et al*. (1976) had achieved success in inducing PLBs with the root tips of *Phalaenopsis. amabilis*.

TDZ alone or in combination with auxins has been used to induce embryogenesis in *Cymbidium ensifolium* (Chang and Chang, 1998), *Oncidium* (Chen and Chang, 2000, 2001). Kerbauy (Kerbauy and Colli, 1997; Kerbauy, 1984) and Colli and Kerbauy, (1993) have also shown direct PLB regeneration from root tips of *Catasetum.*

2.4.3 Size of the explants

The size of the explants determines the survival and establishment of culture. In *Phalaenopsis*, the length of flower stalk cuttings has got pronounced influence on shoot development and multiplication (Tanaka *et al*., 1988).Shoot production was enhanced when shorter cuttings were used. Maximum shoot formation (87%) occurred when one cm of flower stalk cuttings were cultured on medium.

2.4.4. Position of explants

Besides the type and size of the explants, its relative position on the plant determine the performance *in vitro*.

Effect of position of the bud on the stalk, temperature and BAP on the node of bud growth of *P. amabilis* hybrid has been extensively investigated (Tanaka and Sakanishi, 1978).While culturing *Phalaenopsis* flower stalks, buds on the upper position showed a tendency to remain dormant while that of the basal section, grow vigorously.

The cut end had a highest competence to form embryos than the other regions of the leaf explants from both *Phalaenopsis amabilis* and *Phalaenopsis* 'Nebula' orchids (Gow, 2010).

The effects of tissue culture conditions and explant characteristics on direct somatic embryogenesis were studied on *Oncidium* 'Gower Ramsey'. Adaxial-side-up orientation significantly promoted embryogenesis in comparison with abaxial-side-up orientation (Chen and Chang, 2002).

2.4.5 Age of explants

Age of the explants is another factor which affects regeneration response of orchid plantlets. Inflorescence explants and explants from younger sources have given better results in *Dendrobium* Miss Hawaii, *Phalaenopsis Capitola*, *Oncidium* Gower

Ramsey, *Ascofinetia and Ponerorchis graminifolia* Rchb. f. (Nuraini and Shaib, 1992; Intuwong and Sagawa, 1973; Mitsukuri *et al*., 2009).

Younger scape nodes (a stalk from the base of the plant bearing flowers, not leaves) proved to be better explants for clonal propagation than older ones as the percentage of shoot formation was higher with younger scape compared to scape from full bloom flower stalks (Nuraini and Shaib, 1992).

The highest frequency of PLB induction occurred on sections inoculated on VW medium supplemented with $1mgl^{-1}$ BAP taken after the flower stalk was formed and before the first flower became visible (Lin, 1986).

2.4.6 Carbon source

Sucrose is a very important component in *in vitro* culture media, serving as a source of carbon and energy and its addition is essential for *in vitro* culture growth and development.

The source of carbon (sucrose, glucose or fructose) is a very important component in *in vitro* culture media. Carbon sources are added to the culture medium because of the light energy deficiency and low CO_2 concentration present in *in vitro* conditions. Sucrose concentrations of 20 and $30g\ l^{-1}$ are the most commonly used in orchid tissue culture studies (Arditti, 1974). A high concentration of sucrose might act as an osmotic stress (George, 1993) or to inhibit chlorophyll formation to induce embryogenic callus formation.

Callus induction and plant regeneration through somatic embryogenesis in *Phalaenopsis* were examined. Protocorm-like body (PLB) segments formed calli in Vacin and Went medium with sucrose. The optimal concentration of sucrose was 40 g l^{-1}. Medium containing 200 mgl^{-1} coconut water together with 40 g l^{-1} sucrose was effective for callus induction (Ishii *et al*.**,** 1998).

Tokuhara and Mii, (2001) obtained embryogenic callus and cell suspensions from shoot tips of flower stalk buds, and later found that carbohydrate sources played a crucial role in somatic embryogenesis in cell suspension cultures. Among the carbohydrates tested on *Doritaenopsis* cultured on gelled medium, glucose at 58.4mM gave the highest efficiency of protocorm-like body (PLB) formation. Maltose and sorbitol only induced PLB formation without callus proliferation. Sucrose induced comparable callus proliferation to glucose but without PLB formation.

The rooting and *in vitro* growth of *Dendrobium nobile* Lindl (Orchidaceae) were studied using different sucrose concentrations (0 g l^{-1}; 5 g l^{-1}; 10 g l^{-1}; 20 g l^{-1}; 30 g l^{-1} and 60 g l^{-1}). Greater increases in plant height and high seedling multiplication were observed in the 60 g l^{-1} sucrose treatment. Sucrose concentration in the culture medium did not influence *in vitro* plant rooting (Faria *et al*., 2004).

2.4.7. Culture Medium

Various culture media, Vacin and Went (VW) medium (Vacin and Went, 1949), Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and Knudson C (KC) medium (Knudson, 1946) have been used for *Phalaenopsis* micropropagation (Tanaka and Sakanishi, 1977, 1980; Griesbach, 1983; Bhattarcharjee, 1999; Chen and Chang, 2004).

The highest frequency of protocorm-like body (PLB) formation in *Phalaenopsis* hybrid was obtained when basal stem explants were cultured on VW medium containing 30 gl^{-1} sucrose, 500 mgl^{-1} activated charcoal, 150 ml/l coconut water, 1 mgl^{-1} NAA, 5 mgl^{-1} 2iP and 2.5 gl^{-1} gelrite (Jo *et al*., 2007).

Young e*t al.*, (2000) found Hyponex medium as best medium for conversion of PLBs into plantlets. PLBs grown in bioreactors were cultured on solid Murashige and Skoog, Vacin and Went, Knudson C, Lindemann and Hyponex media. Hyponex medium was found to be suitable for conversion of 83% of PLBs transformed into plantlets

Ishii *et al*., (1998) examined the effective medium for callus induction from *Phalaenopsi*s hybrid. Medium containing 200ml/l coconut water together with 40gl^{-1} sucrose was effective for callus induction through somatic embryogenesis. Gellan gum was suitable gelling agent for callus induction. The calli easily formed PLBs after being transferred to a medium without sucrose.

Choong *et al.*, (2013) designed a new defined medium containing the micronuturient adjusted with nitrate-nitrogen (NO_3-N) to ammonium-nitrogen (NH_4-N) ratio of 5 and N to S ratio of 15, buffered with 800 mg dm^{-3} KH_2PO_4 and NPK ratio of 2.0:0.8:3.1. Mean fresh weight, dry weight and root to shoot ratio was compared with control medium, this new medium was found to significantly support growth of the seedlings and improved root development of *Phalaenopsis*.

Effect of sterilization methods and culture media on germination of *Phalaenopsis* seeds were studied by Wei *et al.* (2009). Results showed that seeds was sterilized with detergent and 10% $NaCl_2$ were best suitable for seed germination and protocorm formation, when cultured on MS + banana bud 100gl^{-1} + potato 50gl^{-1} and NAA 0.5 mgl^{-1} .

Park *et al*., (2002) tested few media such as MS (Murashige and Skoog, 1962) and VW (Vacin and Went, 1949) for proliferation and differentiation of PLBs in various *Phalaenopsis* hybrids. Survival percentage of the explants and multiplication of PLBs was high on MS followed by VW media. Tanaka and Sakanishi, (1980) used solid and liquid media cultures for proliferation of PLBs and found that solid media cultures had yielded better results than liquid cultures and also found that the type of hormone for induction is dependent of the species studied and rate of proliferation can differ based on the composition of the media.

2.4.8 Vitamin

Al-khayri, (2001) reported that biotin and thiamine incorporated in culture medium enhanced date palm height. Yeast extract, a natural source of B complex vitamin, plays an important role as coenzyme in plant metabolisms, morphogenesis

and development (Arditti and Ernst, 1993). While folic acid, found in leaves and other plant tissues, functions as a B vitamin and demonstrates coenzyme activity in nucleic acid synthesis (Arditti and Harrison, 1977). Biotin, important in fat, protein and carbohydrate metabolism, promoted growth of leaves and stems in some *Cattleya*, *Odontoglossum* and *Paphiopedilum* while it enhanced root growth in *Cymbidium* (Arditti and Harrison, 1977).

2.4. 9 Minerals

Shoots derived from flower stalk node culture of *Phalaenopsis* and *Doritaenopsis* orchids were used to study the effects of some chemical and physical factors on adventitious root formation. Auxin and nitrous oxide releasing agent, sodium nitroperoxide (SNP), added separately or in combination into rooting media, did not increase root formation. Instead, 40 µM SNP inhibited rooting. Addition of 4- 20 μM KCl promoted rooting of *Doritaenopsis* as well as *Phalaenopsis* (Hsuan *et al*., 2009).

Different concentrations of NaH_2PO_4 were supplemented to examine the effects on direct somatic embryogenesis on various leaf regions of *Phalaenopsis* explants. Higher concentrations (85 and 170 mg l^{-l}) of NaH_2PO_4 resulted in a significantly higher embryogenic response in leaf tip regions (Chen and Chang, 2002).

The inorganic form of nitrogen is being used in most plant tissue culture basal media (Dodds and Roberts, 1995). Many organic forms of nitrogen have been used in media for *in vitro* culture of orchids, such as glutamine, peptone, tryptone and casein hydrolysate, etc. (Arditti and Ernst, 1993).

Half strength MS medium supplemented with peptone was found to inhibit growth of considerable number of *Phalaenopsis* species seedlings and this hamper conservation effort. This could be achieved by adjusting the macronutrient concentration. A new defined medium was designed with nitrate-nitrogen (No_3- N) to

ammonium- nitrogen (NH_4-N) ratio of 5 and N to S ratio of 15, buffered with 800mg dm/3 KH_2 Po_4 (Choong *et al*., 2013).

2.4.10 Plant growth regulators

2.4.10.1Auxin

Auxins as a plant morphogen control leaf initiation in the meristem, stimulate root formation, guide tropic responses and organize tissue patterns within the developing embryo.

Indoleacetic acid (IAA) is the most common naturally occurring auxin, and synthetic auxins include compounds such as naphthalene-1-acetic acid (NAA) and 2,4 dichlorophenoxyacetic acid (2,4-D). Acetylamino acids (conjugates of indole acetic acid and amino acids) have also been utilized in the *in vitro* propagation of *Phalaenopsi*s orchids (Griesbach, 1983).

In *Phalaenopsis*, Indole acetyl glycine ($0.5mgl^{-1}$) added to MS medium promoted the formation of a large number of shoots from protocorms derived from inflorescence nodal buds. 2, 4-D, at 0.8 lM has been reported to affect PLBs induction (53%) (Mahmood and Chew, 2008). Bazand *et al.*, (2014) found that NAA at $1.5mgl^{-1}$ enhances the germination percentage (83%) in *Phalaenopsis*.

Jo *et al*. (2007) conducted a study to develop the clonal propagation through *in vitro* culture using basal stem explants in *Phalaenopsis* hybrid grown *in vitro*. The frequency of protocorm- like body (PLB) formation was obtained when basal stem explants were cultured on VW medium containing 30 gl^{-1} sucrose, 500 mgl^{-1} activated charcoal, 150 ml^{-1} coconut water, 1 mgl^{-1} NAA, 5 mgl^{-1} 2iP and 2.5 gl^{-1} gelrite.

Effect of culture media on germination of *Phalaenopsis* seeds were studied by Wei *et al.* (2009). Results showed that seed germination obtained, when cultured on MS with banana bud 100gl^{-1} + potato 50gl^{-1} and NAA 0.5 mgl^{-1}.

In *Dendrobium* culture, the best increase in shoot weight (0.25 g) and shoot number (8.83) were observed from 0.1mgl^{-1} NAA. The highest shoot length (2.60 cm), number of leaves (4.83), number of roots (5.15), and root length (2.67 cm) were obtained in 0.2 mgl^{-1} NAA at 60 DAI (Parvin *et al.*, 2009).

Aktar *et al.* (2007) investigate the effect of different concentrations of IBA (0, 0.5, 1.0, 1.5 and 2.0 mgl^{-1}) with Vacin and Went (VW) medium and charcoal on root formation in *Dendrobium* orchid. The best results were obtained from 1.0 mgl^{-1} IBA treatment in which the number of root was 1.81 plantlet, length of root 0.35cm, fresh weight of root 0.16g at 30 DAI.

2.4.10.2 Cytokinin

Cytokinins generally promote cell division, adventitious bud and axillary bud formation and inhibits root formation. The most commonly used cytokinins are BA, BAP, Kinetin and TDZ.

TDZ was first used for micropropagation of orchids, specifically *Phalaenopsis* (Ernst, 1994). Multiple shoots were formed on media containing 0.23–11.35 mM TDZ, with the higher levels of TDZ (2.27–11.35 mM) producing PLBs also. Effect of TDZ and BAP on shoot regeneration from *Phalaenopsis* hybrid node sections of flower stalks with buds was also reported by Chen and Pileuk, (1995).

In *Phalaenopsis amabilis*, TDZ promoted repetitive embryogenesis from zygotic protocorms (Chen and Chang, 2004), and induced a higher frequency of embryogenesis from leaf explants (Chen and Chang, 2006) than BA and Kinetin (Kuo *et al.*, 2005).

The effects of total nitrogen and 6-benzyladenine (BA) on growth of *Phalaenopsis* orchid were studied. VW medium supplemented with 9 μM nitrogen and 10 mg l^{-1} BA gave the best leaf length, 3.4cm, and width, 1.3cm, with an average of three leaves per plantlet (Kanchanapoom, 2014).

An efficient regeneration system for *in vitro* propagation of butterfly orchid, *Phalaenopsis* was investigated. Thin section of leaves explants cultured on low concentration (4.54- 9.08 μm) of Thidiazuron were observed to be more effective than high concentration of Benzyl adenine (BA: 44.4μm) to induce shoot buds within 2 weeks (Thida *et al.*, 2006).

Nayak, (1997) conducted a study on direct shoot proliferation from shoot segments of three epiphytic orchids, *Cymbidium aloifolium* (L.) SW., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch-Ham) SW. on Murashige and Skoog's medium (MS). Shoots which developed on a TDZ containing medium elongated following transfer to a medium containing 2.2mgl^{-1} BA and 10.8mg l^{-1} naphthalene acetic acid (NAA). Concentrations of TDZ above the optimal level had an inhibitory effect on shoot regeneration.

According to Luo, (2009) conversion of protocorm like bodies (PLBs) to shoots were investigated for the enhancement of micropropagation of *Dendrobium huoshanense*. The best results of shoot development from PLBs occurred on 1/2 MS medium supplemented with 20 μM kinetin and 10 gl^{-1} maltose.

The effect of cytokinins thidiazuron (TDZ), benzyladenine or zeatin on protocorm-like body (PLB) induction from root tips of *Doritaenopsis* grown *in vitro* was studied. Among the cytokinins tested, TDZ (2.3 mM) was found to be more effective cytokinin in the induction of PLBs than benzyladenine (BA) or zeatin (Park *et al*., 2002).

2.4.10.3 Auxin and cytokinin combination

Plant organogenesis is controlled by the ratio of auxin to cytokinin rather than by their absolute levels (Skoog and Miller 1957). Shoot induction is promoted when cytokinin level is higher than the auxin. Root induction can be achieved by increasing auxin level relatively over cytokinin level. Intermediate concentration of these will tend to unorganized tissue.

Tokuhara and Mii (1993) reported that the combination and appropriate concentration of hormones α–naphtalenacetic acid (NAA) and 6-benzylaminopurine (BAP) and the composition of macro- and microelements in the culture medium were of key importance for micropropagation of *Phalaenopsis* and also for callus – induced somatic embryogenesis from the shoot tip of the flower stalk on a commercial scale (Tokuhara and Mii, 2001, 2003). Highest rate of PLB formation occurred with combination of 1 mgl^{-1} NAA and 10 mgl^{-1} BAP in *Phalaenopsis* flower stalk buds and PLB developed into plantlets in a medium without growth regulators (Mariani, 2014).

Khoddamzadeh *et al*., (2011) used NAA and TDZ to induce protocorm-like bodies (PLBs) from leaf segments of the *Phalaenopsis bellina* (Rchb.f.) Christenson directly from epidermal cells. The best induction percentage for auxin: cytokinin combination was at the combination of NAA and TDZ at 1.0 and 3.0 mgl^{-1} on ½ strength MS medium, which gave 72% induction with 9 PLBs per explants.

Talukder *et al*., (2003) reported that in *Dendrobium*, the best shoot proliferation (1.90/explant), root formation (1.93/ explant) leaf number (4.25/plantlet) and the least time requirement for regeneration (8.8 days) was obtained from 2.5 mgl^{-1} BAP + 0.5 mgl^{-1} NAA.

Embryogenic calli were induced from longitudinally bisected segments of protocorm-like bodies (PLBs) of *Cymbidium*, on modified Vacin and Went medium supplemented with 0.1 mgl^{-1} NAA and 0.01 mgl^{-1} TDZ (Huan *et al.*, 2004) and

Mahendran and Bai, (2012) observed best response in protocorms cultured half strength MS medium supplemented with BAP at 1.0 mgl^{-1} and 2,4-D at 2.0 mgl^{-1}.

In *Dendrobium* orchids, Puchooa, (2004) found that maximum number of PLBs was obtained from leaf explants cultured on Murashige and Skoog (MS) liquid medium agitated at 80 rpm and supplemented with 0.1 mgl^{-1} of BA, 1.0 mg l^{-1} of NAA and 15% (v/v) coconut water. Pant and Thapa, (2012) observed that MS medium with BAP (1.5 mg l^{-1}) and NAA (0.5 mg l^{-1}) were most effective for the shoot multiplication and the best rooting response was observed on MS medium with exogenous supply of IAA 0.5 mgl^{-1}.

2.4.11. Medium supplements

A large number of complex additives like peptone, carrot juice, tomato juice, beef extract, potato extract and especially coconut water (CW), banana extract (BE) etc. are commonly added to orchid media. Beneficial effects of organic additives, such as coconut water and/or banana homogenate and/or potato homogenate added to medium, on seedling growth have been reported in many orchid species like *Aranda Deborah* (Goh and Wong, 1990), *V. coerulea* (Seeni and Latha, 2000), *Dendrobium tosaense* (Lo *et al*., 2004).

2.4.11.1 Banana pulp

Banana pulp is a rich source of natural cytokinins which inhibit culture initiation but promotes differentiation and growth of shoots at later stages (Arditti and Ernst, 1993). Substances of cytokinin nature as well as auxin and gibberellins (Khalifah, 1966a,b) have been found in banana fruit. Promotory effect of banana extract on increase in number and growth of seedling roots was observed in *Cattleya aurantiaca* (Arditti, 1968) and *D. tosaense* (Lo *et al*., 2004) seedling growth.

Gnasekaran *et al*. (2010) demonstrated that shoot tip of *Phalaenopsis violacea* produced maximum growth of PLB, when cultured on Murashige and Skoog (MS) semi-solid medium supplemented with 10% of banana pulp extract.

Vyas *et al*. (2009) have reported more number of roots per shoot as well as increased length of roots of *Dendrobium lituiflorum Lindl*. significantly on Knudson C medium supplemented with banana extract.

Sinha *et al.* (2010) reported that, in P*halaenopsi*s, high frequency regeneration obtained by inoculating the leaf segment on gelrite gelled (1/2) MS medium supplemented with 2% sucrose, 2.0 mgl^{-1} BA, 0.5 mgl^{-1} NAA, 10% coconut water (CW), 2 gl^{-1} peptone and 1 gl^{-1} activated charcoal. For plantlets formation the shoots are cultured on (1/2) MS medium supplemented with 2% sucrose, 10% CW, 2 gl^{-1} peptone, 1 gl^{-1} activated charcoal and 50 gl^{-1} banana pulp.

2.4.11.2. Coconut water

Coconut water (coconut liquid endosperm), is one of the world's most versatile natural product. This refreshing beverage is consumed worldwide as it is nutritious and beneficial for health. There is increasing scientific evidence that supports the role of coconut water in health and medicinal applications. Coconut water is traditionally used as a growth supplement in plant tissue culture/micropropagation. The wide applications of coconut water can be justified by its unique chemical composition of sugars, vitamins, minerals, amino acids and phytohormones (Yong *et al*., 2009)

The liquid endosperm of the coconut promotes growth and differentiation. A number of cell division factors are present in CW including diphenyl urea (Shantz and Steward, 1952). 9-B-ribofuranozyl Zeatin (Letham, 1974) and a compound which co-chromatographs with Zeatin riboside (Van Staden and Drewes, 1975).

Coconut water is a complex additive which contains many nutritional and/or hormonal substances (Dix and Van Staden, 1982). It also contains a large number of free aminoacids including phenylalanine which has the cell division activity in soybean assays (Van Staden and Drewes, 1975).

In *Phalaenopsis,* high frequency regeneration obtained by inoculating the leaf segment on gelrite gelled (1/2) MS medium supplemented with 2% sucrose, 2.0 mgl^{-1} BA, 0.5 mgl^{-1} NAA, 10% coconut water (CW), 2 gl^{-1} peptone and 1 gl^{-1} activated charcoal. (Sinha *et al.*, 2010)

Lakshmanan *et al*. (1995) reported the formation of PLBs from thin sections of shoot tips of *Aranda* Deborah on treatment with CW (5–25%, v/v). In *Dendrobium,* it was observed that the best medium for maximum number of PLB formation was 0.1 mgl^{-1} of BA, 1.0 mgl^{-1} of NAA and 15% (v/v) coconut water in MS liquid medium (Puchooa, 2004).

2.4.11.3 Activated charcoal

Activated charcoal (AC) is composed of carbon arranged in a quasigraphitic form in small particle size. It is a porous and tasteless material and is distinguished from elementary carbon by removal of all non carbon impurities and the oxidation of carbon surface (Budavari, 1996; Mattson and Mark, 1971).

Activated charcoal seems to adsorb the toxic substances that may form in the medium as a result of autoclaving or be released by the explant. It may also stimulate rooting by absorbing the toxins and excluding light from the medium (Paek and Murthy, 1977).

Activated charcoal improves the growth of *Paphiopedilum* (Ernst, 1974) and *Phalaenopsis* (Ernst, 1975) and led to the development of charcoal supplemented media, which gained wide acceptance in a short time. One possible explanation of the

effect of charcoal on orchid seedlings or tissue culture-derived plantlets can be the improvement of aeration. The second possibility is that the charcoal absorbs ethylene and phenolic inhibitors that inhibit growth and development.

Eymar *et al*. (2000) observed that the addition of AC increased and maintained pH levels during culture, increased the nitrogen uptake and improved growth and visual aspects of the explants and reduced the inhibitory effect of exogenous cytokinin on root growth. activated carbon is likely to interfere with other additives as well. Ascorbic acid (vitamin C) has an inhibiting effect on the exudation of phenols as well (Arditti and Ernst, 1993).

Protocorm like bodies (PLB) were induced in *Phalaenopsis* hybrid from leaf segments *in vitro* for bioreactor cultures supplemented with AC. Continuous immersion cultures (air lift column and air lift-balloon bioreactor), and temporary immersion cultures (with or without AC filter attached) were used for the culture of PLB sections. A temporary immersion culture with AC filter attached was most suitable for PLB culture (Young *et al*., 2000).

In *Dendrobium nobile* the asymbiotic seed germination was observed on 1/2 MS medium supplemented with 0.05 gl^{-1} AC. When the cotyledons appeared, the seedlings were transferred to 1/2 MS supplemented with BA (Men *et al*., 2003).

In *Rhynchostylis retusa* the *in vitro* seedling growth was promoted by the addition of AC in the medium. Seedling growth was maximum on MS medium supplemented with BA, NAA and 1 gl^{-1} AC. The multiple shoot induction from seedlings was also promoted by AC and a maximum 8.2 shoots were observed on MS medium fortified with TDZ and 1 gl^{-1} AC (Thomas and Michael, 2007).

2.4.12 PLB development

A method of rapid multiplication in orchids is through PLB formation. When shoot apices of cymbidium was cultured on nutrient media, the explants first turned green and then enlarged slowly as a small bulbllet similar to a protocorm which develop from an embryo. The PLBs further proliferated into clumps of protocorms each in turn developing into a new plantlet. When cut into small pieces and subcultured, these PLBs regenerate more protocorms and it has been estimated that it is possible to obtained more than four millon plants in a year from a single explants (Morel, 1964).

In *Phalaenopsis*, PLB's were indused from shoot tips (Intuwong and Sagawa, 1974; Ichihashi, 1992), stalk tips of flower stalks (Homma and Asahira, 1985), intermodal section of flower stalk (Homma and Asahira, 1985; Lin, 1986; Vij and Pathak, 1989) *in vitro* leaf (Tanaka and Sakanishi, 1977 ; Latha and Seeni, 1991) and *in vitro* root tip (Tanaka *et al*., 1976 ; Latha and Seeni, 1991).

Tanaka and Sakanishi, (1977, 1980) obtained PLB formation from the leaf explants of *Phalaenopsis*, cultured on Murashing and Skoog medium supplement with $1mgl^{-1}$ NAA, $10mgl^{-1}$ Adenine and $1mgl^{-1}$ BA. Culture were Incubated in the dark for 2 week and subsequently transferred to light conditions (16-h photoperiods, 900 lux) for PLB regeneration from leaf explants.

Green protocorm like bodies (PLB) with high multiplication capacity were induced from shoot tips of flower stalk buds using new Dogashima Medium (NDM) containing 0.1 mgl^{-1} NAA and 20 mgl^{-1} BA. These PLB when subcultured on the same medium, more than 10 000 PLBs were obtained from a few buds on a single flower stalk within one year. After transfer onto NDM containing no plant growth regulators, PLBs developed into plantlets. (Tokuhara and Mii, 1993).

For suspension cultures of *Phalaenopsis* Snow Parade and *P*. Wedding Promenade, PLB formation was most efficiently induced by sucrose at 29.2mM for *P*.

Snow Parade and 14.6mM glucose for *P*. Wedding Promenade (Tokuhara and Mii, 2003).

PLBs and plantlets have been reported to form on cut surfaces of root, stems, flower stalk, nodal buds and leaf sections of *Phalaenopsis* flower stalk nodal buds, cultured flower stalk internodes of *Phalaenopsis* and *Doritaenopsis* (Lin, 1986), seeds (Chen and Chang, 2004) and leaf explants (Chen and Chang, 2006).

Rittirat *et al.* (2012) reported development of protocorm–like bodies (PLBs) of *Phalaenopsis. cornu-cervi* in ½ MS medium supplemented with 0.1µm each of NAA and TDZ. They observed plant conversion of PLB's in MS medium in 0.2% activated charcoal.

Niknejad *et al*., (2011) assessed the best medium for the induction of callus and PLBs, from *in vitro* leaf segment cultured on New Dogashima Medium (NDM) supplemented with cytokinins (6 Benzylaminopurine (BAP), Thidiazuron (TDZ), and Kinetin (KIN), each at 0.01, 0.1, 0.5 and 1.0 mgl^{-1}) alone and in combinations with (auxins a-naphthaleneacetic acid (NAA), at 0.01, 0.1, 0.5 and 1.0 mgl^{-1}). TDZ in combination with auxins was found to be the best for the induction of callus and PLBs within 6 weeks of culture.

Rapid multiplication through PLB formation for shoot tips and leaves of endangered *Vanda coerulea* was achieved through culture of shoot tips of seedlings and mature plants (Seeni and Latha, 2000).

Callusing from protocorm-like bodies (PLBs) of *Cymbidium* Twilight Moon 'Day Light' was reported by Huang *et al*. (2004). The medium containing the combination of 0.1 mg l^{-1} NAA and 0.01 mg l^{-1} TDZ was optimal for callus formation and also good for subculture. This callus derived PLBs converted into normal plants with well-developed shoots and roots on the basal medium without hormones after about 4 months.

2.4.13 Culture environment

Light, temperature and humidity condition provided inside the culture room plays a significant role in the success of any tissue culture medium.

2.4.13.1 Light

Light, as a main environmental trigger, plays a central role in regulating plant development.

Tanaka *et al*. (1988) examined various factors affecting the production of shoots from flower stalk cuttings of *Phalaenopsis* hybrids from green house grown plants with an aim to get a large number of leaf explants for culturing. Shoot production, number of leaves per shoot and their length increased when light intensity was decreased from 2.34 to 0.25 W/m^2.

Two *Phalaenopsis* orchids, *Phalaenopsis amabilis* and *Phalaenopsis* 'Nebula', were used to test the effects of induction period on direct somatic embryogenesis of leaf explants from *in vitro* grown seedlings. The suitable culture conditions were 60 days for induction period in darkness and 45 days for subculture period in light (Gow, 2010).

The rate of organ initiation depended on the wavelength of the monochromatic light applied. Red and blue treatments were effective in triggering photo morphogenesis in the evaluated material. The propagation coefficient reached 11.7 under red light, 10.6 under blue, 8.3 under white and 6.2 in darkness. Blue light treatment improved the efficiency of micropropagation and benefitted initiation of rhizogenesis and aerial root elongation, and the resulting plants were true to type (Cybularz-urban *et al*., 2007).

2.4.13.2 Temperature

From the CO_2 uptake pattern, *P. amabilis* showed maximal CO_2 fixation rate in the day temperatures ranging from 28 to 30°C and night temperatures ranging from 20 to 22°C. Kaziwara *et al.*, (1992) found that *Phalaenopsis* grew better under 30/25 than 25/20°C. Kubota and Yoneda, (1990) suggested that the *Phalaenopsis* varieties they tested had the better growth conditions under a constant temperature of 30°C than under 20°C.

Tanaka and Sakanishi, (1978) observed that in *Phalaenopsis* flower stalk culture, sprouting buds placed at 20^oC or 25^oC showed reproductive growth. At 28^oC, all buds developed vegetatively independent of their original position on the stalk.

Blanchard *et al.* (2008) suggested high temperature range of 28 to 32°C for the vegetative stage. These inconsistent results indicated the diverse of the *Phalaenopsis* plants used for research.

Sinha *et al.* (2010) reported high frequency regeneration of *Phalaenopsis* obtained, when the cultures are incubated at 24 +/-2 degrees C under fluorescence light 50 micromol/m(2)/s for 16 h photoperiod per day. The PLBs are induced from gelrite gelled (1/2) MS medium supplemented with 2% sucrose, 2.0 mgl^{-1} BA, 0.5 mgl^{-1} NAA, 10% coconut water (CW), 2 gl^{-1} peptone and 1 gl^{-1} activated charcoal.

2.4.14 Hardening and acclimatization

Acclimatization is an important step in micropropagation. During *in vitro* growth, plants develop under controlled conditions, including enclosed environments, without gaseous exchanges, with high moisture in the air, low light intensity, and the use of sugars from the medium as a source of carbon and energy. After *ex vitro* transfer, these plantlets might easily be impaired by sudden changes in environmental

conditions, and need a period of acclimatization to correct the abnormalities. (Pospišilova *et al*., 1999)

Williams, (1885) suggested rough fibrous peat and live sphagnum moss as a medium for epiphytic orchids. Vacin tested various media for *cymbidiums* and got the best growth medium containing 50 per cent redwood fiber (McLellan, 1956).

Lo *et al.* (2004) reported that tissue culture seedlings after transfer to MS medium with 1.5% sucrose and 8% banana homogenate or potato juice or coconut water and 20 wk of incubation developed into healthy plantlets. Well developed plantlets were transplanted to moss and tree fern or tree fern as substrates in plastic trays and transferred to a greenhouse for hardening. All plants survived, attained maturity, and developed normal flower and capsule after one and a half years.

Growth and flowering of *Phalaenopsis* were greater when commercial medium was mixed with 20% sphagnum moss (Hwang *et al.*, 2004).

Coco peat in combination with tree fern as potting mixture found most suitable for *ex vitro* hardening, which registered maximum per cent increase in shoot length, leaf size and leaf number, recorded 77.2 % survival after a month. This could be mainly due to the retention of optimum moisture, better drainage and good aeration. Perlite as the potting medium registered very high mortality, due to discolouration of plantlets (Nagaraju and Mani, 2005).

Experiments were performed to determine the effects of different media components on rooting and vegetative growth of several clones of *Phalaenopsis.* Plants grown in a medium consisting of 1:1 chunky peat: perlite or 2:1:1 perlite: chunky peat: coconut coir had fewer aerial roots than plants grown in a bark-based or 1:1 perlite: charcoal media. Plants grown in the chunky peat: perlite mix or chunky peat: charcoal mix had the greatest increase in leaf span compared to the other media (Blanchard and Runkle, 2008). An efficient hardening technique for orchid seedling was reported on chips of charcoal, bricks and decayed wood (Deb and Imchen, 2010).

Phalaenopsis plants (*ex vitro*) were planted into containers filled with different media: New Zealand sphagnum moss, mixture of expanded clay pellets and New Zealand sphagnum moss (v:v = 1:1) and expanded clay pellets. Effect of medium on the size of plant, hydration of leaves tissue and flowering was evaluated. Orchids grown in New Zealand sphagnum moss create a significantly greater mass of the above ground plant part and the roots (Trelka *et al.*, 2010).

Indhumathi *et al.* (2003) reported *Dendrobium* Hybrid Sonia - 17 were prehardened in liquid KC medium containing charcoal and brick pieces and get survived better under *in vivo* condition. The root number (18.73) and root length (5.49 cm) of the microshoots were the highest in the medium supplemented with IBA 5.0 mgl^{-1}. Among the five pot mixtures used for hardening, the establishment of plantlets in terms of plant height, leaf size, new growth and root length was good in the medium consisting of Charcoal + Brick pieces + Cocopeat (1:1:1).

Saiprasad and Polisetty, (2003) showed that the orchids *Dendrobium*, *Oncidium* and *Cattleya* had complete survival or establishment in potting media having wood charcoal pieces alone and wood charcoal + brick pieces, which allowed good circulation of air and excellent drainage. Minimum survival (<60 %) was observed when soilrite alone was used as the potting medium, because soil rite retains high moisture, but aeration may be inadequate.

2.5 Genetic stability studies

Ornamental is an important groups of plants in plant kingdom. These groups of plants not only add aesthetic values, it also contributes in forming a billion dollar business. Conventionally plants propagate vegetativly which is a slow process. Since early 1970s, plant cell culture method has been practiced as an alternative method of propagation and over 150 ornamental genera are being propagated through tissue culture worldwide (Rout *et al.*, 2006). The technique has several other advantages including the creation of novel genetic variation.

The occurrence of somaclonal variation during culturing is a frequent and consistent event. The frequency of variation depends on the genotype, culture medium, growth hormones and the way of multiplication. Somaclonal variations not only can be distinguished by their morphological traits (Gamborg *et al.*, 1977) but also by their biochemical, physiological and genetic characteristics. Several reports in the literature indicate that variations can be detected by identifying chromosome numbers (Ogura, 1990), isozyme patterns (Pereira *et al.*, 1996) and random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) in many crops.

Some orchid hybrids are more amenable than others to somaclonal variation. The percentage of the variation can range from 0-100% depending on varieties, with an average of 10% among *Phalaenopsis* (Tokuhara and Mii, 1993). Molecular marker system has been exploited for the detection of somaclonal variation including random amplified polymorphic DNA (RAPD), Methylation sensitive restriction fragment length polymorphism (RFLP) and microsatellite sequence variation.

2.5.1 Methods of assessment of genetic stability

2.5.1.1 Molecular marker

Two type of molecular markers were generally used for detection such as hybridization based Restriction Fragment Length polymorphisms (RFLP) (Botstein *et al.*, 1980)and Polymerace Chain Reaction (PCR) based molecular marker such as Random Amplified polymorphic DNA(RAPD) (Chen *et al.*, 1998), Amplified Fragment Length Polymorphism (AFLP) (Kubis *et al.*, 2003) and microsatellite sequence variation (Alou *et al.*, 2004).

2.5.1.2. Polymerase Chain Reaction (PCR) based detection

Polymerase Chain Reaction (PCR) was invented by Mullis and Co-workers in 1983, and it is based on enzymatic in vitro amplification of DNA (Weising *et al.*,

2005). In PCR, DNA sequence is amplified for primers and thermostable DNA polymerase.

2.5.1.2.1 Random amplified polymorphic DNA (RAPD)

RAPD, which is the simplest version of PCR with arbitrary primers used for detecting DNA variation and is popular due to its convenience, low cost and simplicity (Sun and Wong, 2001), was used to find molecular differences to support physiological differences in tissue culture plantlets.

RAPD analysis is a fingerprinting method using short, random, oligonucleotide primers to search for variation in the entire genomic DNA (Williams *et al*., 1990) and has been widely employed in evaluating genetic distances in many diverse plant genera, e.g., *Acacia* (Casiva *et al*., 2002); *Cicer* (Sudupak *et al*., 2002); *Cupressus* (Rushforth *et al*., 2003); *Linum* (Fu *et al*., 2002) and *Rhizophora* (Lakshmi *et al*., 2002).

In RAPD analysis, sources of DNA polymorphisms may include base changes within the priming site sequence, deletions in the priming site, insertions that render priming sites too distant to support amplifications, and deletions or insertions that change the size of a DNA fragment without preventing its amplification. The RAPD technique has several advantages such as the ease and rapidity of analysis, a relatively low cost, availability of a large number of primers and the requirement of a very small amount of DNA for analysis (Williams *et al*., 1990).

Somaclonal variations in flower and inflorescence axis were investigated among the plants micropropagated through protocorm-like bodies induced by flower stalk bud culture of various cultivars of *Phalaenopsis* and *Doritaenopsis*. Somaclonal variations appeared in flower and inflorescence was classified into 9 categories irrespective of cultivars. The frequencies of these somaclonal variations in each

genotype ranged from 0 to 100%, but most of the cultivars showed variations less than 10% (Tokuhara and Mii, 1993).

The genetic distance and relationships of 149 accessions representing 46 species in the genus *Phalaenopsis* and four species in *Paraphalaenopsis* were studied using random amplified polymorphic DNA (RAPD) markers. The genus *Paraphalaenopsis* was used as an out group. A total of 20 random primers were screened and out of these, six random primers provided 123 polymorphic bands and zero monomorphic bands. Pairwise genetic distances between accessions were estimated according to Nei and Li, (1979). Cluster analysis of data using the UPGMA algorithm placed the species in seven groups based on morphological characters (Goh *et al*., 2005).

According to Khoddamzadeh *et al.* (2010) twelve decamer random amplified polymorphic DNA (RAPD) primers were used to study somaclonal variation among the mother plant, the initially induced PLBs and proliferated PLBs after 3 and 6 months in culture. Protocorm-like bodies (PLBs) of *P. bellina* were induced from leaf segments using ½ strength Murashige and Skoog (MS) media. Eight out of twelve primers produced 172 bands with 18 polymorphic bands in all the treatments. The amplified products varied between 125 to 8000 bp. Among the primers used, P 16 produced the highest number of bands (29), while primer OPU 10 produced the lowest number (15). The range of similarity coefficient was from 0.83 to 1.0 among the different sub-cultures and mother plant (MP). It was reported that no change occurred between the mother plant and the PLBs produced after 3 months of culture, but 17% dissimilarity reported after 6 months of subculture.

Random amplified polymorphic DNA (RAPD) analysis was conducted to determine their genetic distances and relationships. Among 20 different primers used for RAPD analysis, 10 primers showed polymorphism and 26 to 54 DNA fragments were amplified. A total of 414 polymorphic fragments were generated by 10 primers and used for correlation group analysis. The highest value of Similarity index was 0.28

between *P. violacea malaysia* and *P. violacea witte*. The dendrogram resulting from UPGMA (Unweighted Pair Group Method using Arithmetic average) hierarchical cluster analysis separated the 20 *Phalaenopsis* species into three groups (Atienzar *et al.*, 2002)

RAPD markers can thus be successfully applied in this economically important group of orchids for the study of molecular characterization and relationships. The data acquired from this study could be used for identification and classification of other orchid genera and oriental *Phalaenopsis* (Niknejad *et al.*, 2009).

2.5.1.2.2 Amplified Fragment Length Polymorphism (AFLP)

AFLP is a PCR-based tool used in genetics research, DNA fingerprinting, and in the practice of genetic engineering. Developed in the early 1990s by Keygene, AFLP uses restriction enzymes to digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments is then selected to be amplified. This selection is achieved by using primers complementary to the adaptor sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments.

Sixteen primer pairs were used in the AFLP analyses of different *Phalaenopsis* cultivars grouped by flower colour, i.e. red, white and white with red lips. AFLP patterns generated by E1M6 and E3M4 primer pairs successfully distinguished all mother plants and regenerants of 'W1-3' and 'WR1-3' into the three flower colour groups by similarity analysis. The mother plant of 'WR3-21' and its tissue-cultured derivatives, classified as normal plants and somaclonal variants by their phenotypes, were used for genetic fidelity test. Clear polymorphic banding patterns from E4M5, E6M8 and E7M5 primer pairs were observed on samples of somaclonal variants but not on normal regenerants. These polymorphic bands were considered as potential molecular markers for the detection of somaclonal variation (TsuHwie *et al.*, 2003).

Chang and Veilleux, (2009) Studied the genetic variability of 16 *Phalaenopsis* species hybrids using using amplified fragment length polymorphism (AFLP) markers. Ten AFLP primer combinations amplified 1353 DNA fragments ranging in size from 100 to 350 bp and 1285 (95%) of them were polymorphic. The genetic similarity among *Phalaenopsis* species and hybrids ranged from 0.298 to 0.774 based on Dice coefficient. A significant linear relationship ($r = 0.724$, $P < 0.0001$) was observed between known pedigrees and AFLP-derived genetic similarity for 136 pairwise comparisons of *Phalaenopsi*s species and hybrids.

2.5.1.2.3 Microsatellite marker or Simple Sequence Repeat

Simple Sequence Repeats (SSR) is tandem repeats of DNA sequence only a few base pair (1-6bp) in length. The term microsatellite was introduced to characterized the simple sequence stretches amplified by PCR (Hearne *et al.*, 1992) The most abundant being the dinucleotide repeat (McCouch *et al*., 2002)

Huang *et al.* (2009) were used a set of 13 expressed sequence tag (EST)-derived simple sequence repeat (SSR) to analyze 103 cultivars of six species of Chinese orchid (*Cymbidium* spp.). The 13 SSR primer pairs generated a total of 168 polymorphic bands, with an average of 12.92 bands per primer and a range of 6–24 bands which clearly revealed the difference between cultivars inter- or intra-species of Chinese orchid. Cluster analysis based on UPGMA method showed a dendrogram with three basic clusters.

Moe *et al.* (2010) studied polymorphic analysis using 14 newly developed SSRs, a total of 201 alleles across 96 *Cymbidium* accessions were detected with an average of 14.4 per locus. The average gene diversity and polymorphism information content values were 0.394 and 0.639, respectively. The mean genetic similarity coefficient was 0.4297, indicating a wide genetic variation among the *Cymbidium* accessions. These SSRs will be used for genotype identification, germplasm conservation, molecular breeding, and assessments of genetic diversity and population structure in *Cymbidium*.

Materials and Methods

3. Material and Methods

The investigations on "Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid Winter Spot with molecular marker" were carried out at the Center for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2013- February 2016, with the objective to develop an *in vitro* regeneration system and to verify the genetic stability of regenerants in *Phalaenopsis* hybrid Winter Spot using molecular marker. The study mainly focused on the following aspects:

1. To develop an *in vitro* regeneration system
2. To verify the genetic stability of regenerants in *Phalaenopsis* hybrid Winter Spot using molecular marker

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

3.1 Source of explants

Ten flowering plants of *Phalaenopsis* hybrid Winter Spot maintained in the net house of PPNMU, KAU, Vellanikkara were used as explants source (plate no. 1). This is a monopodial orchid having single vertical main stem producing fleshy and prominent leaves. This hybrid produces beautiful white flowers with purple patch at the center.

Mother plants selected were serially marked from 1 to 10. Mother plants were sprayed with fungicide at weekly interval. Observation of mother plants selected are provided in Table 1.

Plate no. 1 *Phalaenopsis* hybrid Winter Spot used for the study

Table 1: Observation of mother plants selected

Plant No.	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
No. of leaf	4	6	4	4	2	5	6	5	6	4
Inflorescence	2	1	2	2	2	2	1	1	1	1
No. of flowers	10	8	10	13	12	12	9	6	8	8

3.2 Culture medium

3.2.1 Laboratory chemicals, glassware and plastic wares

Chemicals used for the present study were of good quality from various agencies like Merck india Ltd., Himedia and Sisco Research Laboratories. The Taq DNA polymerase, dNTPs, Taq buffer and molecular marker were supplied by Banglore Genei Ltd. All the plastic wares were obtained from Axygen and Tarson India Ltd. The RAPD primers obtained from Vision Scientific chemicals.

3.2.2 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 asceptic manipulations for micropropagation studies were carried out in laminar air flow (LABLINE INDUSTRIES). Media sterilization was done in autoclave (Natsteal equipment Pvt. Ltd.). Centrifugation was done in high speed refrigerated centrifuge KUBOTA 6500/ Dynamica Velocity 14R refrigerated centrifuge. Nanodrop^{R} ND-1000spectrophotometer 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 electropharosis. Gel Doc-BIO-

RAD was used for imaging and documenting the agarose gel profile. The details are given in Annexure I.

3.2.3 Composition of media

Basal MS medium (Murashige and Skoog, 1962) supplemented with different growth regulators was used for plant tissue culture in the present study. The composition of MS medium is given in Appendix-1.

3.2.4 Preparation of MS media

Standard procedures were followed for the preparation of MS plant tissue culture media. Five stock (1, 11, 111, 1V and V) solutions for major and minor nutrients were prepared and stored in pre cleaned glass bottles in refrigerated conditions. Stock 111 was stored in amber coloured bottle. Stock solutions of different growth regulators were 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 to the beaker. For preparing MS medium of full strength, 20ml taken from 50x stocks and 10ml from 100x stock. Required quantities of sucrose, Inositol 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 using 0.1N NaOH.

The volume was finally made up and required quantity of agar was added to the medium. Agar in the medium was completely melted in a microwave oven. Then 15-20 ml of hot medium was poured into 25 X 150 mm pre-sterilized glass culture tubes and plugged with non-absorbent cotton and autoclaved. Sterile Cefotaxime ($250mgl^{-1}$) was added in autoclaved culture establishment medium before dispensing into test tubes.

3.2.5 Autoclaving

The test tubes with nutrient media were autoclaved at a pressure of 1.06 kg cm^{-2} ($121^{o}c$) for 20 minutes. The tubes were then removed from the autoclave and allowed to cool. Inoculation was done 4-5 days after media sterilization ensuring that the tubes were free of microbial contaminations.

3.3 Transfer area and aseptic manipulation

All the aseptic manipulation such as surface sterilization 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 70 per cent alcohol. The UV light was switched on for 20 minutes to achieve aseptic environment inside the cabinet and air was allowed to blow for 20 minutes before working in the laminar air flow cabinet.

The sterilized explants were inoculated to culture establishment medium. The cut ends of explants were kept in such a way that they had maximum contact with the medium.

3.4 Culture condition

The cultures were maintained at $26\pm2^{0}C$ in an air conditioned culture room with 16 hr photoperiod. White fluorescent tubes light were provided to meet the light requirements (2000 lux) of the explants. Humidity in the culture room varied from 60 to 80 per cent according to the prevailing climate. Sub culturing was carried out in 3 weeks interval.

3.5 Collection and preparation of explants

Explants were collected from the source plants maintained in the net house. Three types of explants were used *viz*, inflorescence node, leaf segments and root segments. The method of preparation of explants for inoculation varied with the type of explants.

3.5.1 Inflorescence node

Inflorescence stalks were collected from mother plants individually by cutting with a sharp knife at the base retaining the basal node on the stalk for the development of new inflorescence (plate no. 2a). The stalks were wiped with cotton dipped in 70 per cent alcohol, then cut into nodal section and washed with distilled water. The bracts covering the dormant buds were removed and the sections were subjected to surface sterilization.

3.5.2 Leaf

Leaf explants were collected from young emerging leaves of length 3cm (Plate no. 2b). After collection leaves were washed in tap water, blotted dry, and wiped with 70% alcohol, followed by surface sterilization. Transverse thin cell layer of leaves were taken and inoculated in the media.

3.5.3 Root

Root piece of 5cm length (Plate no. 2c) including the root tip were collected from mother plants, washed in tap water and subjected to surface sterilization.

3.6 Standardization of surface sterilization

Surface sterilization of explants was done in order to make the explants free of microbial contamination. Explants were subjected to the following pre treatments prior to surface sterilization. Explants were washed thoroughly in running tap water. Then they were immersed in a solution of bavistin (0.1%) with two drops of prill for 30 minutes and washed thoroughly with distilled water.

In order to formulate an effective surface sterilization technique, mercuric chloride (0.1%) at different time periods (5min, 6min, 7min and 8min) was used with leaf and inflorescence nodal segment.

After mercuric chloride treatment explants were washed with sterile double glass distilled water for three to four times in Laminar air flow cabinet to remove any traces of $HgCl_2$. The end portion from both sides of the inflorescence

a) Immature inflorescence node

b) Transverse thin cell layer of leaf

c) Root tip

Plate no 2. Explants used for *in vitro* propagation of *Phalaenopsis* hybrid Winter Spot

segments were removed and made to 2 cm long piece. The leaf pieces were trimmed on the four sides and size reduced to 1cm. Using sharp sterile blade thin transverse section also taken from leaf segment. Root pieces were also trimmed to a size of 0.5cm thickness. After washing the explants were blotted dry on sterile tissue paper on sterile petridish.

3.7 Effect of basal media for culture establishment

To identify the best mineral salt composition favoring shoot production, a trial was conducted using two different mineral salt compositions. They were

MS- MS medium containing full strength of both organic and inorganic compounds.

1/2MS- MS medium containing half the concentration of inorganic salts and full strength of organic compounds.

Twenty number each of the three explants were inoculated for each treatment. TDZ at 1-2 mgl^{-1} incorporated in the medium as reported by Thida *et al*. (2006). Sterile Cefotaxime was added in autoclaved culture establishment medium before dispensing into test tubes. Observations were recorded on the percentage of culture establishment, number of shoots per culture after 4 weeks of culturing. The composition of media and explants used are detailed in Table 2.

Table 2: Different media used for identifying the best mineral strength

Sl. No	Media composition	Explants used
1.	MS + 1.0 mgl^{-1} TDZ	- Inflorescence node - Root segment - TCL
2.	½ MS + 1.0 mgl^{-1} TDZ	- Inflorescence node - Root segment - TCL
3.	MS +2.0 mg l^{-1} TDZ	- Inflorescence node - Root segment - TCL
4.	½ MS +2.0 mgl^{-1} TDZ	- Inflorescence node - Root segment - TCL

3.7.1 Observations recorded

1. Percent of culture establishment
2. Explants response
3. Days taken for culture establishment

3.8 Effect of growth regulators on culture establishment

Growth regulators have a profound influence on the growth of cultures. Treatments formulated to identify the best growth regulator and their combinations supporting *in vitro* culture of inflorescence node, leaf and roots explants based on the micropropagation protocol reported by Thida *et al*., 2006 and Rittirat *et al*., 2012 on *Phalaenopsis*.

3.8.1 Treatment combinations

Trials were conducted with different growth regulators and their combination in MS medium containing sucrose at 30 per cent level. The details of treatment combinations of growth regulators tried are given in Table 3.

Table 3: Different growth regulators and their combinations used for culture establishment with different explants of *Phalaenopsis*

SL No	Media composition
1.	MS +1.0 mgl^{-1} BA +0.1 mgl^{-1} NAA
2.	MS + 2.0 mgl^{-1} BA + 0.1mgl^{-1} NAA
3.	MS + 5.0 mgl^{-1} BA + 0.1mgl^{-1} NAA
4.	MS+ 4.5 mgl^{-1} BA
5.	MS +1.0 mgl^{-1} BA +0.2 mgl^{-1} IAA
6.	MS +2.0 mgl^{-1} BA +0.2 mgl^{-1} IAA
7.	MS +5.0 mgl^{-1} BA +0.2 mgl^{-1} IAA
8.	MS +1.0 mgl^{-1} BA + 1.0 mgl^{-1} Kinetin
9.	MS +1.0 mgl^{-1} BA + 2.0 mgl^{-1} Kinetin
10.	MS +1.0 mgl^{-1} BA + 5.0 mgl^{-1} Kinetin
11.	MS +1.0 mgl^{-1} TDZ
12.	MS +2.0 mgl^{-1} TDZ

3.8.2 Observations recorded

1. Number of culture inoculated

2. Percentage of culture establishment

3. Period for shoot induction

3.9 Effect of media additives

Trials were conducted using different additives like, coconut water, banana pulp, activated charcoal and adenine sulphate for enhancing the growth of the cultures. TDZ at 2mgl^{-1} incorporated in the medium as reported by Thida *et al*.

(2006). Additives were added to MS basal medium supplement with 2mgl^{-1} of TDZ. The details of treatment combination are presented below.

Table 4: Different additives tried for *in vitro* culture of *Phalaenopsis* inflorescence node explants

Sl. No.	Media composition
1	MS + 2.0 mgl^{-1} TDZ
2	MS + 2.0 mg^{-1} TDZ +10% Coconut water
3	MS + 2.0 mgl^{-1} TDZ +15% Coconut water
4	MS + 2.0 mgl^{-1} TDZ +20% Coconut water
5	MS + 2.0 mgl^{-1} TDZ + 50 mg l^{-1} Banana pulp
6	MS + 2.0mgl^{-1} TDZ + 75 mg l^{-1} Banana pulp
7	MS + 2.0 mgl^{-1} TDZ +0 .1 % AC
8	MS + 2.0 mgl^{-1} TDZ + 1% AC
9	MS + 2.0 mgl^{-1} TDZ + 2% AC
10	MS + 2.0 mgl^{-1} TDZ + 10 mgl^{-1} Adenine sulphate
11	MS + 2.0 mgl^{-1} TDZ + 50 mgl^{-1} Adenine sulphate

3.9.1 Observations recorded

1. No of explants inoculated

2. Period for shoot induction

3. Percentage of shoot regenerated

3.10 Multiple shoots induction

Shoots formed from inflorescence node were taken out after four weeks of inoculation from the media of MS + 2mgl^{-1} TDZ. The single shoots were detopped and inoculated to multiplication media for induction of multiple shoot. Observations regarding the response of inoculated culture to different media compositions were recorded. The growth regulator combination used for shoot multiplication detailed in Table 5.

Table 5: Different growth regulator combinations tested for multiple shoots induction in *Phalaenopsis*

SL. No.	Treatment combination
1	MS + 2.0 mgl^{-1} BA
2	MS+ 2.0 mgl^{-1} BA +0.1 mgl^{-1} NAA
3	MS + 4.5 mgl^{-1} BA
4	MS+ 4.5mgl^{-1} BA +0.1 mgl^{-1} NAA
5	MS+ 4.5 mgl^{-1} BA+ 0 .1mgl^{-1} IAA

Observations recorded

1. No of shoot inoculated

2. Period for multiple shoot induction

3. Percentage of culture responded

4. Average no of multiple shoot

3.11 Elongation and Rooting

The healthy shoots were transferred to elongation and rooting medium for elongation of shoots. The shoots of three to four cm in length were taken out from multiple shoot cultures and kept for induction of rooting. Observations regarding

the number of shoots, length of shoots, percentage of rooting were recorded. Different media combinations tried are detailed in Table 6.

Table 6: Different media combination used for elongation and rooting of *in vitro* culture of *Phalaenopsis*

SL. No.	media
1.	MS + 0.1 mgl^{-1} Kinetin + 1.0 mgl^{-1} NAA
2.	MS + 4.5 mgl^{-1} BA +1.0 mgl^{-1} IAA
3.	MS+5.0 mgl^{-1} BA+ 0.01 mgl^{-1} NAA

Observations recorded

1. No of explants inoculated
2. Days taken for root initiation
3. Mean length of shoots(cm)
4. Percentage of rooting
5. Mean no. of roots

3.12 Hardening and acclimatization

The *in vitro* rooted plantlets were taken out of the culture vessels using forceps after soaking the culture in water for five minutes. The solidified medium from plantlets was washed out in running water. The plantlets were treated with 0.1 percent bavistin for five min and then planted in small earthen pots filled with potting media and hardened in hardening unit.

Observation regarding the percentage of plant established was documented.

3.13 Genetic stability analysis using RAPD marker

To study the genetic stability of micropropagated plants, RAPD molecular assay was carried out. The mother plants and multiple shoots from inflorescence node of the mother plant were subjected to genetic stability studies using RAPD assay with 12 RAPD markers reported for *Phalaenopsis.* RAPD markers are very simple, fast, cost-effective, highly discriminative and reliable. They do not need

any prior sequence information to design the primer. They are largely distributed throughout the genome. Thus, they are suitable for the assessment of the genetic fidelity of *in vitro* regenerated plants.

3.13.1 Genomic DNA isolation

Isolation of good quality genomic DNA is one of the most important prerequisite for RAPD analysis. The CTAB procedure reported by Doyle & Doyle, (1990) was used for the extraction of good quality genomic DNA. The young tender leaves were used for genomic DNA isolation.

Reagents

1. 2x CTAB extraction buffer
   -2 per cent CTAB (w/v)
   - 100mM Tris (pH8.0)
   - 20mMEDTA (pH 8.o)
   - 1.4M NaCI
   - 1 per cent PVP

2. CTAB (10 per cent)
   - 10 per cent CTAB (w/v)
   - 0.7M NaCI

3. TE buffer

4. ß- mercaptoethanol

5. Chloroform: Isoamyl alcohol (24:1)

6. Isopropanol chilled

7. Ethanol (70%)

8. Distilled water

3.13.1.2 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. Extraction buffer (2x) 1ml and 50μl of ß- mercaptoethanol were added to it. The homogenized sample was transferred to an autoclaved 2ml centrifuge tube. After mixing thoroughly, the mixture was incubated at 65^{o}C for 20-30 minutes with occasional mixing by gentle inversion. Equal volume of chloroform: isoamyl alcohol 24: 1 (v/v) was added and the mixture was mixed by inversion to emulsify. Then it was spinned at 10,000 rpm for 15minutes at 4^{o}C. After the centrifugation, the top aqueous layer was transferred to a clean centrifuge tube and 1/10th volume of ten per cent CTAB was added. Equal volume of chloroform: isoamylalcohol (24:1) was added and mixed by inversion and centrifuged at 10, 000 rpm for 15 minutes at 4^{o}C. The aqueous phase was transferred to a clean centrifuge tube and added 2/3 volume of chilled isopropanol and mixed by gentle inversion till the DNA was precipitated. The mixture was again centrifuged at 10,000 rpm for 15 minutes at 4^{o}C and supernatant was gently poured off. The DNA pellet was washed with 70 per cent ethanol followed by 100 per cent ethanol, spun for 5 minutes at 10,000 rpm and decanted the ethanol. The DNA pellet was air dried for 30 minutes and the pellet was dissolved in 50μl of TE buffer or sterilized water and stored at – 20^{o}C.

3.13.2 Purification of DNA

The DNA isolated contained RNA as contaminant and hence was purified by treatment with RNase and further precipitated.

3.13.2.1 Assessing the quality of DNA by Electrophoresis

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

Materials for agarose gel electrophoresis

1. Agarose
   - 0.8 per cent for genomic DNA
   - 2 per cent for RAPD
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

TAE buffer was prepared from the 50x stock solution. Agarose (0.8%) was weighed and dissolved in TAE buffer (1x) by boiling, 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 desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 30 minutes after which the comb was removed carefully. The tray was kept in the electrophoresis unit with well side directed towards the cathode. 1X TAE buffer was loaded into the wells using a micropipette carefully. After closing the tank , the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (80V) and current (50 A). The power was turned off when the tracking dye reached 2/3rd length of the gel.

Then the gel was taken from the electrophoresis unit and viewed under UV transilluminator for the presence of DNA. DNA fluorescence under UV light due

to 2 drops ethidium bromide dye. The image was documented in gel documentation system. The gel profile was examined for intactness and clarity of DNA band.

3.13.2.2 Assessing the quality and quantity of DNA by NanoDrop method

The quality and quantity of genomic DNA was estimated using NanoDrop 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 260nm 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 50μg double stranded DNA / μl sample.

1OD at 260 nm = 50 µg DNA/ml

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

3.13.3 Molecular marker assays

RAPD primers were used for assessing genetic stability of micropropagated plants based on earlier reports on RAPD assays in *Phalaenopsis* by Khoddamzadeh *et al.*, 2010. Details RAPD primers used are given in Table 7.

Table 7. Details of RAPD primers used for screening

No	Primer	Nucleotide Sequence (5'-3')
1	OPU 08	GGCGAAGGTT
2	OPU 09	CCACATCGGT
3	OPU 10	ACCTCGGCAC
4	OPU11	AGACCCAGAG
5	OPU12	TCACCAGCCA
6	OPU13	GGCTGGTTCC
7	OPU14	TGGGTCCCTC
8	OPU15	ACGGGCCAGT
9	OPU16	CTGCGCTGGA
10	P 12	CCAAGCTTGC
11	P 14	AGGATACGTG
12	P 16	GGATCTGAAC

3.13.3.1 RAPD analysis

RAPD assays was performed to detect the polymorphism in amplification patterns in the region b/w 2 SSR's. This was carried out by amplifying the DNA by using specific primers relating to the SSR region flanking the RAPD.

Good quality genomic DNA (30 to 50ng/μl) isolated from the leaf sample of plantlets regenerated from inflorescence node were subjected to RAPD assay. RAPD primers with good resolving power were selected for amplifying DNA.

3.13.3.2 RAPD primers selected for the study

Twelve RAPD primes were selected and screened for the study. Details of selected RAPD primers are presented in Table 8. The selected primers were checked for amplification using bulked DNA from different groups of plants in the present study.

Table 8: Details of RAPD primers selected for the study

SL .No	Primer No	Nucleotide Sequence (5'-3')
1	OPU 10	ACCTCGGCAC
2	OPU13	GGCTGGTTCC
3	OPU15	ACGGGCCAGT
4	OPU16	CTGCGCTGGA
5	P 12	CCAAGCTTGC
6	P 16	GGATCTGAAC

The reaction was carried out in an Agilent thermocycler. Amplification was performed in a 20μl reaction mixture as shown below:

3.13.3.3 Composition of the reaction mixture for PCR

Materials	Quantity (μl)
a) Genomic DNA(30ng/μl)	-2.0
b) 10X Taq assays buffer B	-2.0
c) $MgCl_2$	-2.0
d) dNTP mix (10mM each)	-1.5
e) Taq DNA polymerase (3U)	-0.4
f) Decamer primer	-1.5
g) Autoclaved distilled water	-10.6
Total volume	- 20.0

The thermo cycler was programmed as follows:

- Initial denaturation – 94^0c 5mts
- Denaturation - 94^0c 45 sec
- Primer annealing - 30^0c -34^0c 1mts
- Primer elongation -72^0c 2mts
- Final extension -72^0c 5mts

35 cycles

- 4^oC for infinity to hold the sample

The amplified sample were run on 2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (1 kb plus Ladder). The profile was visualized under UV transillumitor and carefully examined for polymorphism.

3.13.3.4 RAPD assay

RAPD assay was performed with DNA samples isolated from four different mother plant plants and *in vitro* regenerated shoots from source plants using 12 primers.

3.13.3.5 RAPD data analysis

Amplification profile of source plants and micropropagated inflorescence node derived plants were compared with selected primers. Bands of DNA fragments were scored manually as (1) for presence and (0) for absence. The DNA amplification patterns with selected primers in different groups of mother plants were analyzed and variability exhibited at DNA level was calculated.

Percentage of polymorphism generated by each primer was worked out as given below:

$$\text{Percentage of polymorphism} = \frac{\text{No of polymorphic band}}{\text{Total no of band}} \times 100$$

3.13.3.6 Comparison of mother plant with clones using NTSYS software

The amplification patterns generated by primer for mother plant and regenerated clones were subjected to NTSYS software. The similarity matrix were subjected to the unweighted pair group method with arithematic averages (UPGMA) and a dendrogram was generated by using NTSYS pc- software (Rohlf, 2000). Similarity coefficient between clone and mother plants was worked out for the study.

Results

4. RESULTS

The results of the study on "Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid Winter Spot with molecular marker" conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2013 to February 2016 are presented in this chapter under different sub headings.

4.1 Studies on micropropagation

The micropropagation protocols reported by Thida *et al*., 2006 ; Rittirat *et al*., 2012 were used as basic for regeneration of plantlets from three explants of Inflorescence node, transverse thin cell layer of leaf and root tip. Flowering mother plants of *Phalaenopsis* hybrid Winter Spot maintained at PPNMU were used as explant source.

4.1.1 Standardization of surface sterilization of inflorescence node of *Phalaenopsis*

The explants were prepared as given in chapter three section 3.5. The explants were subjected to surface sterilization treatment as given in chapter three section 3.6. Observations on percentage of explant survival recorded two weeks after inoculation are shown in table 9.

Microbial contamination was recorded least in the treatment T4 ($HgCl_2$ 0.1% 8min) which was 20 per cent closely followed by T3 ($HgCl_2$ 0.1% 7min) with 45 per cent. Maximum percent of contamination was observed in the treatment T1 ($HgCl_2$ 0.1% 5min) with 80%. Treatment T3 ($HgCl_2$ 0.1% 7min) recorded maximum per cent (55%) of explant survival followed by treatment of T4 ($HgCl_2$ 0.1% 8 min) with 40 per cent.

Based on the observations, treatment T3 ($HgCl_2$0.1% 7 min) was followed for further surface sterilization of inflorescence node. Cefotaxime 250mgl^{-1} was added into the media after autoclaving for reducing the bacterial contamination.

Table 9: Standardization of surface sterilization treatments for inflorescence node

Treatment	Percentage of contamination	Percentage of explant browning	Percentage of explant survival	Type of contamination
T1 - 5 minutes 0.1 per cent $HgCl_2$	80	0	20	Fungi & bacteria
T2 - 6 minutes 0.1 per cent $HgCl_2$	65	0	35	Fungi & bacteria
T3 - 7 minutes 0.1 per cent $HgCl_2$	45	0	55	Fungi & bacteria
T4 - 8 minutes 0.1 per cent HgCl2	20	40	40	Fungi & bacteria

Pre treatment : 20 minutes 0.1% bavistin + 2 drops prill , wiped with 70 per cent ethanol

Medium : MS+ 2mgl^{-1} TDZ

No of explants inoculated: 20

4.1.1.2 Standardization of surface sterilization of leaf explants of *Phalaenopsis*

Observations on surface sterilization experiments of leaf explants are given in Table 10.

The per cent of contamination was observed maximum (75%) in treatment T1 (5 minutes 0.1 per cent $HgCl_2$) closely followed by T2 (6 minutes 0.1 per cent $HgCl_2$) with 55 per cent.

The treatment T4 (8 minutes 0.1 per cent $HgCl_2$) recorded least contamination (35 per cent) with maximum per cent of explants survival (65 %) followed by T3 with 50% contamination and 50 per cent explants survival. Based on the observations treatment T4 (8 minutes 0.1 per cent $HgCl_2$) was followed for further surface sterilization of leaf explants. Cefotaxime 250mgl^{-1} was added into the media before autoclaving for reducing the bacterial contamination.

Table 10: Standardization of surface sterilization treatments for leaf explants of *Phalaenopsis*

Surface sterilization treatment	Percentage contamination	Percentage of explants survival	Type of contamination
T1- 5 minutes 0.1 per cent $HgCl_2$	75	25	Fungi & bacteria
T2- 6 minutes 0.1 per cent $HgCl_2$	55	45	Fungi & bacteria
T3- 7 minutes 0.1 per cent $HgCl_2$	50	50	Fungi & bacteria
T4 -8 minutes 0.1 per cent $HgCl_2$	35	65	Fungi & bacteria

Pre treatment : 20 minutes 0.1% bavistin + 2 drops prill and wiped with 70 per cent ethanol

Medium : MS+ 2mgl^{-1} TDZ

No of explants inoculated: 20

4.1.2 Effect of basal media for culture establishment of *Phalaenopsis* explants

Response of different explants like inflorescence node, leaf segment and root segment to different basal media are given in Table 11. TDZ at different concentration (1-2mgl^{-1}) was used for study. From different media tried, response was observed only in the medium of Full MS with 2mgl^{-1} of TDZ, for the explants of inflorescence node (Plate.3). From inoculated explants, 55 per cent established and sprouted within the period of 4 weeks.

Six weeks after inoculation

Eight weeks after inoculation

Plate no.3. Shoot regeneration from the inflorescence node of *Phalaenopsis* hybrid Winter Spot

In other media, inflorescence node exhibited bulging of bud and later turned to brown. Leaf explants remained green up to 2 weeks, thereafter started drying. Root segment remained as such without any change. based on the observation full strength of MS medium was used for further tissue culture works.

Table 11: Effect of basal media on culture establishment of *Phalaenopsis*

SL. No.	Media composition	Explants used	Percent of culture establishment	Explants response	Days taken for culture establishment
1	MS +1.0 mgl^{-1} TDZ	Inflorescence node Root segment TCL	35 0 0	Bulging of bud NC NC	28 - -
2	1/2MS +1.0mgl^{-1} TDZ	Inflorescence node Root segment TCL	25 0 0	Bulging of bud NC NC	28 - -
3	MS + 2.0mgl^{-1} TDZ	Inflorescence node Root segment TCL	55 0 0	Sprouting NC NC	30 - -
4	½ MS +2.0mgl^{-1} TDZ	Inflorescence node Root segment TCL	35 0 0	Bulging of bud NC NC	29 - -

No of culture inoculated : 20

NC : No change

TCL= Thin cell layer cross section

4.1.3 Response of different explants to various growth regulators combination

Response of different explants to various growth regulators is given in Table 12. BA at (1-5mgl^{-1}), IAA (0.2mgl^{-1}),NAA (0.2mg l^{-1}), TDZ (1-2mgl^{-1}) and kinetin (1-5mgl^{-1}) were used for the study.

MS medium supplemented with BA and TDZ was found to give good shoot regeneration from inflorescence node explants.

In the medium MS +2 mgl^{-1} TDZ recorded highest percentage (80%) of culture establishment, followed by MS + 4.5 mg l^{-1} of BA with 55 per cent of sprouting. In MS medium supplemented with various concentrations of auxin *viz*, IAA (0.2 mg l^{-1}) and NAA (0.1mg l^{-1}) along with various concentrations of cytokinin BA (1-5mg l^{-1}) observed the bulging of dormant bud of the inflorescence node.

Among the explants tried, inflorescence node sprouted and established. Root segment remained as such without any change, whereas leaves explants remained green up to 2 weeks, thereafter started drying in all the growth regulators combination.

Table 12: Response of different explants to various growth regulators combination

SL No	Media composition	Explants used	Explants response	Percentage of culture establishment	Days taken for sprouting
1.	MS +1.0 mg l^{-1} BA +0.1 mg l^{-1} NAA	Inflorescence node Root segment TCL	Bulging of bud NC NC	25 Nil Nil	28 0 0
2.	MS + 2.0 mg l^{-1} BA + 0.1mg l^{-1} NAA	Inflorescence node Root segment TCL	Bulging of bud NC NC	45 Nil Nil	30 0 0
3.	MS + 5.0 mg l^{-1} BA + 0.1 mg l^{-1} NAA	Inflorescence node Root segment TCL	Bulging of bud NC NC	30 Nil Nil	28 0 0
4.	MS+ 4.5 mg l^{-1} BA	Inflorescence node Root segment TCL	Sprouting NC NC	55 Nil Nil	28 0 0
5.	MS +1.0mg l^{-1} BA + 0.2 mg l^{-1} IAA	Inflorescence node Root segment TCL	Bulging of bud NC NC	40 Nil Nil	30 0 0
6.	MS +2.0 mg l^{-1} BA + 0.2 mg l^{-1} IAA	Inflorescence node Root segment TCL	Bulging of bud NC NC	30 Nil Nil	30 0 0
7.	MS +5.0 mg l^{-1} BA + 0.2 mg l^{-1} IAA	Inflorescence node Root segment TCL	Bulging of bud NC NC	25 Nil Nil	30 0 0
8.	MS +1.0 mg l^{-1} BA + 1.0 mg l^{-1} Kinetin	Inflorescence node Root segment TCL	NC NC NC	Nil Nil Nil	0 0 0
9.	MS +1.0 mg l^{-1} BA + 2.0 mg l^{-1} Kinetin	Inflorescence node Root segment TCL	NC NC NC	Nil Nil Nil	0 0 0
10.	MS +1.0 mg l^{-1} BA + 5.0 mg l^{-1} Kinetin	Inflorescence node Root segment TCL	NC NC NC	Nil Nil Nil	0 0 0
11	MS +1.0 mg l^{-1} TDZ	Inflorescence node Root segment TCL	Bulging of bud NC NC	30 Nil Nil	28 0 0
12	MS +2.0 mg l^{-1} TDZ	Inflorescence node Root segment TCL	Sprouting NC NC	80 Nil Nil	28 0 0
13	MS +1.0 mg l^{-1} BA + 0.1 mg l^{-1} NAA	Inflorescence node Root segment TCL	Bulging of bud NC NC	25 Nil Nil	28 0 0
14	MS + 2.0 mg l^{-1} BA + 0.1mg l^{-1} NAA	Inflorescence node Root segment TCL	Bulging of bud NC NC	45 Nil Nil	30 0 0

No of culture inoculated: 20

NC: No Change

4.1.4 Effect of different additives for shoot regeneration from inflorescence node

Experiment was conducted using coconut water, banana pulp, activated charcoal and adenine sulphate for enhancing the growth of the cultures. The inflorescence node with dormant bud was cultured on MS medium with TDZ along with additives. Response of inflorescence node to different additives for shoot regeneration was given in table 13.

With coconut water at 10%, shoot regeneration was observed in15 days after inoculation. With 15% and 20% of coconut water, the sprouting was observed within 20 days of inoculation. Percentage of inflorescence node explants established and sprouted was 100 per cent in all the media containing coconut water was additive.

Addition of banana pulp in the medium did not induce any sprouting of inflorescence node. On addition of activated charcoal ($1\text{-}5gl^{-1}$), bulging of dormant bud of the inflorescence node was observed after 28 days of inoculation. With the addition of adenine sulphate in the medium, there was no remarkable growth promotion for inflorescence node.

Table 13: Effect of different additives for shoot regeneration from inflorescence node

Sl. No.	Media composition	No of inflorescence node inoculated	Days taken for culture establishment	Percentage of shoot regenerated	Response
1	MS + 2.0 mg l^{-1} TDZ	20	28 days	100	Sprouting
2	MS + 2.0 mg l^{-1} TDZ +10% Coconut water	20	15 days	100	Sprouting
3	MS + 2.0 mg l^{-1} TDZ +15% Coconut water	20	20 days	100	Sprouting
4	MS + 2.0 mg l^{-1} TDZ +20% Coconut water	20	20 days	100	Sprouting
5	MS + 2.0 mg l^{-1} TDZ + 50 mg l^{-1} banana pulp	20	-	-	NC
6	MS + 2.0 mg l^{-1} TDZ + 75 mg l^{-1} Banana pulp	20	-	-	NC
7	MS + 2.0 mg l^{-1} TDZ + 1g l^{-1} AC	20	28	100	Bulging
8	MS + 2.0mg l^{-1} TDZ +2g l^{-1} AC	20	28	100	Bulging
9	MS +2.0mg l^{-1} TDZ +5g l^{-1} AC	20	28	100	Bulging
10	MS +2.0mg l^{-1} TDZ +10 mg l^{-1} Adenine sulphate	20	-	-	NC

NC : No change

4.1.5 Effect of different growth regulators for multiple shoot induction from inflorescence node

Response of different growth regulators for multiple shoot induction was given in Table14. For multiple shoot induction, the sprouted shoots were transferred to media with different growth regulator combinations for assessing the response of multiple shoot induction. Growth regulators like BA at 1- 4.5 mgl^{-1}, IAA and NAA at 0.1mgl^{-1} were used for the study.

From the different growth regulators tried, BA (2.0 and 4.5mgl^{-1}) and IAA (0.1mgl^{-1}) and their combinations resulted in multiple shoot induction(Plate no.4). The percentage of multiple shoot induction was observed maximum (86.6 %) in MS + 4.5 mg^{-1} BA followed by MS+ 4.5 mgl^{-1} BA+ 0.1mgl^{-1} IAA with 80 per cent.

The media MS + 4.5 mgl^{-1} BA resulted in the highest average number of multiple shoot (4.1) followed by MS + 2.0 mgl^{-1} BA and MS+ 4.5 mgl^{-1} BA+ 0.1mgl^{-1} IAA (4.0). The number of days taken for multiple shoot induction ranged from 40 to 45.

Medium: MS+ 4.5 mgl^{-1} BA

40 days after subculturing

Medium: MS+ 2mgl^{-1} BA

42 days after subculturing

Plate no.4. Multiple shoot induction from inflorescence node of *Phalaenopsis* hybrid Winter Spot

Table 14: Effect of different growth regulators for multiple shoot induction from inflorescence node

SL. No.	Media composition	No of shoot inoculated	Multiple shoot induction	Percentage of culture responded	Average no of multiple shoots/ culture	Days taken for multiple shoot induction
1	MS +2.0mg l^{-1} BA	15	+	33.3	4.0	42
2	MS+ 2.0mg l^{-1} BA +0.1 mg l^{-1} NAA	15	-	Nil	Nil	-
3	MS + 4.5 mg l^{-1} BA	15	+	86.6	4.15	40
4	MS+ 4.5mg l^{-1} BA +0.1mg l^{-1} NAA	15	-	Nil	Nil	-
5	MS+ 4.5 mg l^{-1} BA+ 0.1mg l^{-1} IAA	15	+	80	4.0	45

+ = Multiple shoot induction

- = No multiple shoot induction

4.1.6 Effect of different growth regulators for shoot elongation and rooting

Response of different growth regulators for shoot elongation and rooting is given in Table 15. The proliferated shoots were kept for elongation and root induction. On an average, shoot elongation of 3cm was observed in the elongation medium (plate.5).

In MS medium supplemented with BA 4.5mgl^{-1} +IAA 1mgl^{-1}, 80% rooting was observed followed by MS +Kinetin 0.1mgl^{-1} +1mgl^{-1} NAA with 65% rooting. The media MS + 1mg l^{-1} IAA +4.5 mgl^{-1} BA recorded good root formation with the highest number of roots (3).

From different growth regulators tried, auxin like IAA was found to induce the maximum shoot length as evidenced by treatment MS + 1mgl^{-1} IAA + 4.5mgl^{-1} BA, where the mean length of shoots was 2.98cm (Plate.6).

The number of days taken for *in vitro* rooting initiation ranged from 50-56 days. Among different growth regulators tried, IAA with BA was found to cause early root induction in 50 days after inoculation.

Table 15: Effect of different growth regulators for shoot elongation and rooting

SL. No.	media	Days taken for root initiation	Mean length of shoots (cm)	Mean no. of roots	% of rooting
1.	MS +Kinetin 0.1 mgl^{-1} + 1mgl^{-1} NAA	54	2.35	2.7	65
2.	MS + 1mgl^{-1} IAA +4.5 mgl^{-1} BA	50	2.98	3.0	80
3.	MS+5 mgl^{-1} BA+ 0.01 mgl^{-1} NAA	56	2.12	2.6	50

Plate no. 5. Shoot elongation of *Phalaenopsis* hybrid Winter Spot

Plate no.6. *In vitro* rooting of *Phalaenopsis* hybrid Winter Spot

4.1.7 Hardening of *in vitro* plantlets of *Phalaenopsis*

The potting media of charcoal, brick pieces and sphagnum moss (1:1:1) was used for hardening micropropagated plantlets of *Phalaenopsis*. Observations recorded after 2 months of planting out revealed a survival rate of 100% with mean plant height of 5 cm and mean number of leaves 3 (Plate.7).

4.1.8 Genetic stability analysis using RAPD marker

4.1.8.1 Genomic DNA isolation

For isolation of genomic DNA, tender leaf sample was collected from mother plants and plantlets regenerated from inflorescence node explants. Three flower bud regenerants each from four mother plants were used for RAPD marker analysis.

4.1.8.2 Isolation and purification of genomic DNA

Isolation of genomic DNA was carried out using CTAB method reported by Doyle & Doyle, 1990. RNA contamination was observed after DNA isolation hence, RNase treatment was given, that resulted in good quality DNA. Addition of RNase at the rate of 2 μl/100μl DNA sample and incubation at 37^{o}c in dry bath for one hour removed RNA contaminations.

4.1.8.3 Quality and Quantity of genomic DNA

The quality and quantity of isolated DNA was analyzed by electrophoresis and NanoDrop Spectrophotometer. Intact clear band indicated that DNA extracted was non-degraded and was of good quality. Spectrophotometer analysis gave ratio of UV absorbance (A260/280) between 1.8-2.0 .The DNA from both parents and regenerated clones after appropriate dilution was used as template for RAPD analysis.

Plate no.7 *Ex vitro* establishment of *Phalaenopsi*s hybrid winter spot

Table 16: Quality and quantity of genomic DNA of M1 mother plant and regenerants

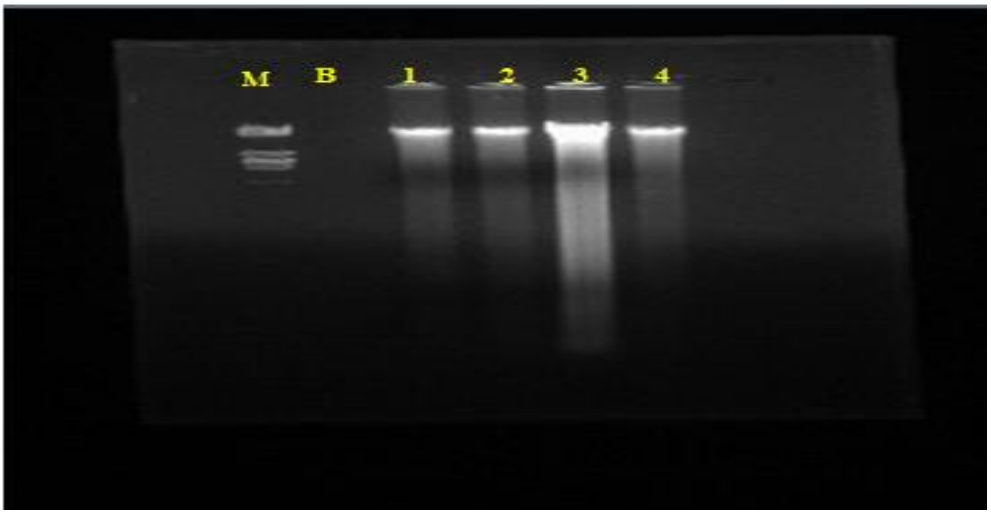
SI. No.	Sample ID of progenies and mother plant	Quantity(ng/μl)	UV absorbance at 260/280 (A260/280)
1	M1(Mother plant)	384.80	2.04
2	C1(Regenerants)	227.53	1.99
3	C2(Regenerants)	136.80	2.00
4	C3(Regenerants)	1036.30	1.86

Table 17: Quality and quantity of genomic DNA of M2 mother plant and regenerants

SI. No.	Sample ID of progenies and mother plant	Quantity(ng/μl)	UV absorbance at 260/280 (A260/280)
1	M2 (Mother plant)	355.40	1.98
2	C1 (Regenerants)	473.46	1.90
3	C2 (Regenerants)	830.57	1.86
4	C3 (Regenerants)	523.14	2.02

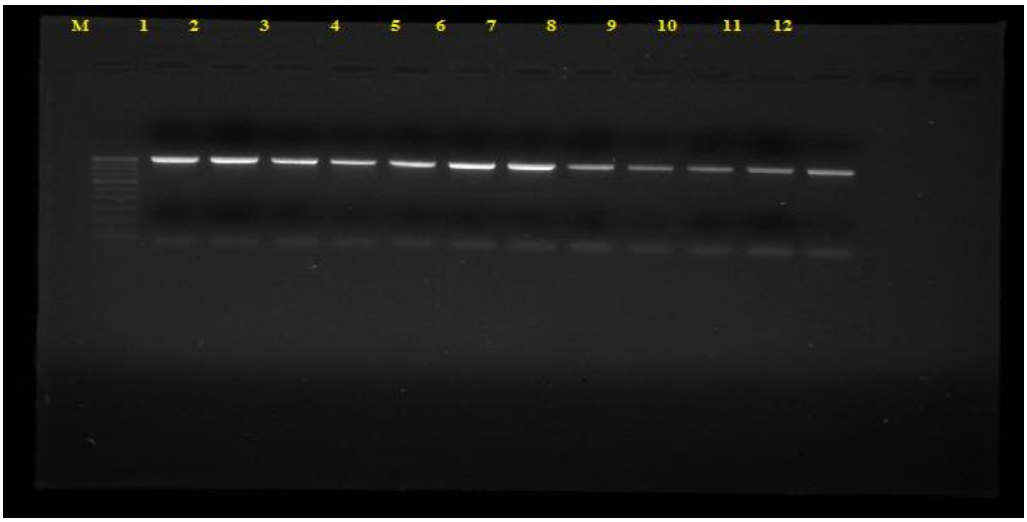
Table 18: Quality and quantity of genomic DNA of M3 mother plant and regenerants

SI. No.	Sample ID of progenies and mother plant	Quantity(ng/μl)	UV absorbance at 260/280 (A260/280)
1	M3 (Mother plant)	486.81	1.97
2	C1 (Regenerants)	194.57	2.06
3	C2 (Regenerants)	369.81	1.91
4	C3 (Regenerants)	802.04	1.80



M- 1kb plus Ladder 1, 2, 3, 4 - M1, M2, M3 and M4 Mother plants

a. Isolated DNA from four mother plant of *Phalaenopsis* hybrid Winter Spot



M- 1 kb plus ladder 1,2,3- Regenerants from M1 mother plant 4,5,6- Regenerants from M2 mother plants 7,8,9- Regenerants from M3 mother plant 10,11,12- regerants from M4 mother plant

b. Isolated DNA from regenerated clones of *Phalaenopsis* hybrid Winter Spot

Plate no.8 Isolated DNA from mother plant and regenerated clones of *Phalaenopsis* hybrid Winter Spot

Table 19: Quality and quantity of genomic DNA of M4 mother plant and regenerants

SI. No.	Sample ID of progenies and mother plant	Quantity(ng/μl)	UV absorbance at 260/280 (A260/280)
1	M4 (Mother plant)	262.74	1.94
2	C1 (Regenerants)	258.2	2.06
3	C2 (Regenerants)	872.0	1.83
4	C3 (Regenerants)	455.4	2.05

4.1.8.4 Primer screening

Twelve RAPD primers reported by Khoddamzadeh *et al.* (2010) was used for RAPD analysis. The details of selected primers are given in Table 7.

Twelve RAPD primers were screened with good quality DNA isolated from mother plants. The isolated DNA was amplified in PCR-Agillent at five different gradient temperatures ranging from 30-34^{o}C for fixing the optimum temperature. Amplification was performed in a 20μl reaction mixture as stated in Section 3 (3.13.3.3)

The amplified product were run on two percent agarose gel using 1X TAE buffer stained with ethidium bromide using marker (λDNA/*Eco*RI+*Hind*III). The profile was visualized under UV transilluminator and documented in gel doc XR+. The gel profiles are carefully examined for total number of amplicons generated by each primer.

Primers giving DNA amplification were selected for further RAPD analysis. The optimum temperatures for DNA amplification by each primer are presented in Table 20.

4.1.8.5 RAPD marker analysis

Out of twelve RAPD primes screened, six were selected based on good DNA amplification pattern. The detail of selected RAPD primers are given in table7 under section 3 (3.13.3.2).

The DNA isolated from mother plant and regenerants were subjected to RAPD analysis.

Table 20: Details of amplification generated using selected primers

No	Primer Name	Nucleotide Sequence	Size range	Total number of bands	Annealing temperature	Comments
1.	OPU 10	ACCTCGGCAC	200-4000	5	30^{o}c	selected
2.	OPU13	GGCTGGTTCC	200-7000	6	31.8^{o}c	Selected
3.	OPU15	ACGGGCCAGT	100-8000	6	30.1^{o}c	Selected
4.	OPU16	CTGCGCTGGA	200-4000	6	32^{o}c	Selected
5.	P 12	CCAAGCTTGC	100-4000	5	32^{o}c	Selected
6.	P 16	GGATCTGAAC	400-8000	8	34^{0}c	Selected

4.1.8.5.1 Clonal fidelity analysis of M1 mother plant

The amplification pattern obtained for plants derived from inflorescence node with six selected RAPD primer is provided in plates. Primer specific amplification details are given in Table 21.

4.1.8.5.2 DNA amplification with selected RAPD primers

OPU 10

Amplification with the primer OPU10 generated six clear amplicons. No polymorphic bands. All were monomorphic amplicons (plate no. 9a). The molecular weight of amplicons ranged from 200 to 3000 bp

OPU 13

A total of four clear amplicons were obtained with the primer OPU13. Both monomorphic and polymorphic bands were produced. The amplicon of size 650bp was absent in clone C3 (plate no.9b). The molecular weight of amplicons ranged from 100 to 2000 bp.

OPU 15

Amplification with primer OPU 15 generated six clear amplicons. Two amplicons of size 6000bp to 7000bp were absent in mother plant, clone C1, C2 and C3. The molecular weight of amplicons ranged from 300 to 8000 bp (Plate no.10a).

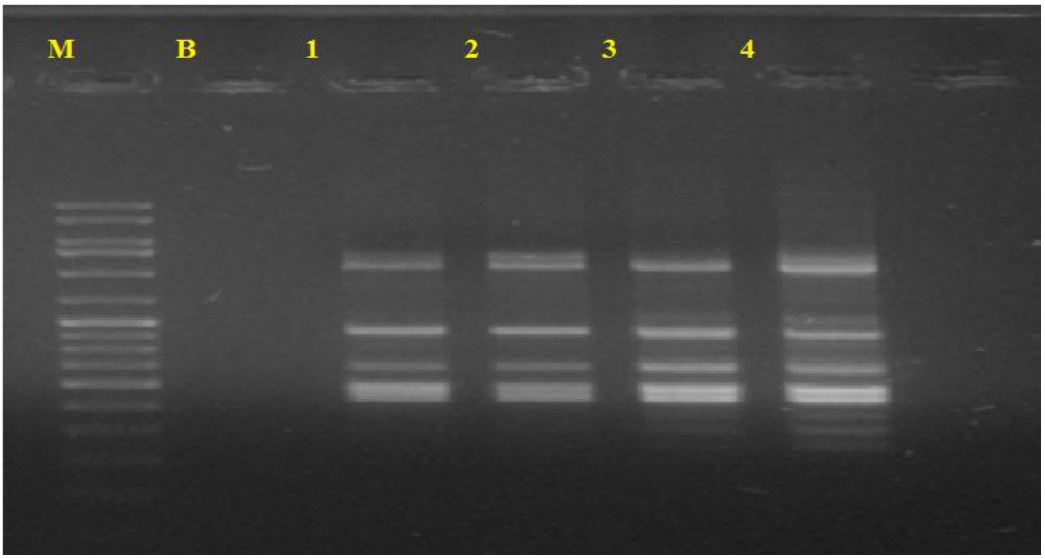
OPU 16

Amplification with the primer OPU16 generated five clear amplicons which are monomorphic. The amplicon of size 6000bp was absent in mother plant and clone C2 (Plate no.10b). The molecular weight of amplicons ranged from 400 to 7000 bp.

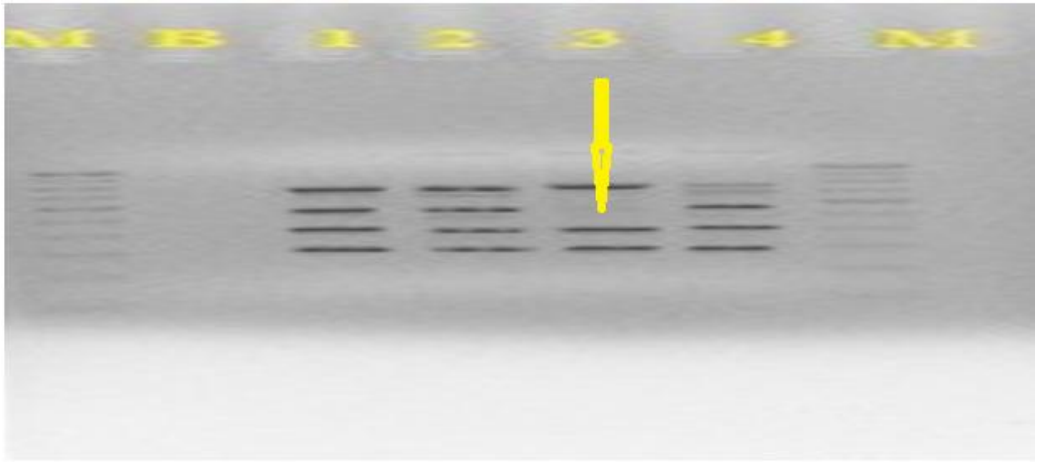
P12

Amplification with primer P12 generated five clear amplicons. The amplicon of size 400bp was absent in clone C1. The molecular weight of the product ranged from 200 to 8000 kb (Plate no. 11a).

a. Amplification with OPU10



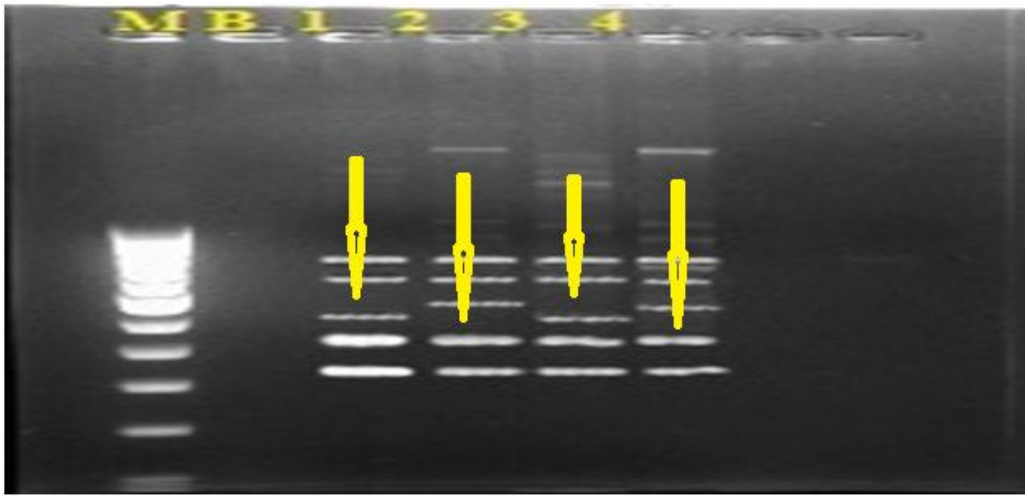
b. Amplification with OPU13



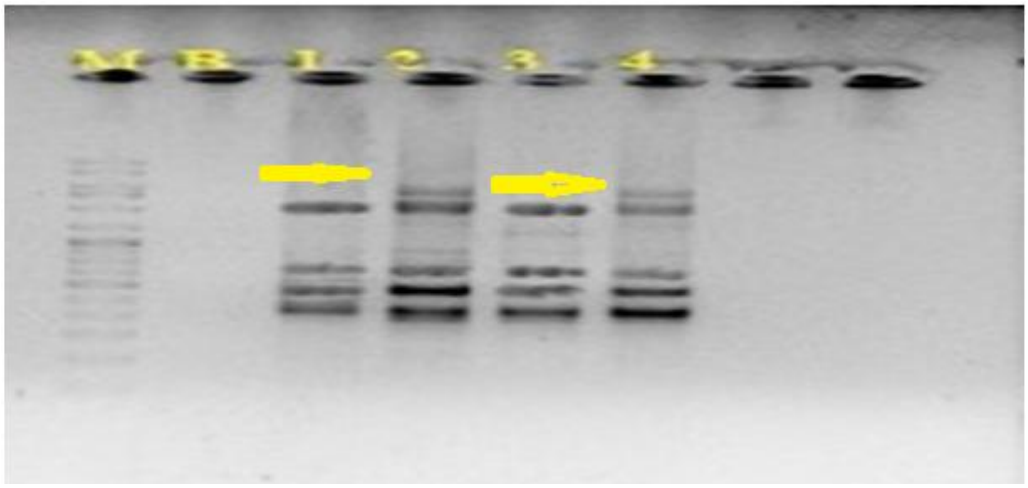
M- Marker (1Kb plus Ladder), B- Blank, 1- Mother plant, 2, 3, 4 - Regenerant

Plate no.9 RAPD amplification pattern in M1 mother plant and regenerated clones from inflorescence node in variety Winter Spot of *Phalaenopsis* with primer OPU10 and OPU13

a. Amplification with OPU15



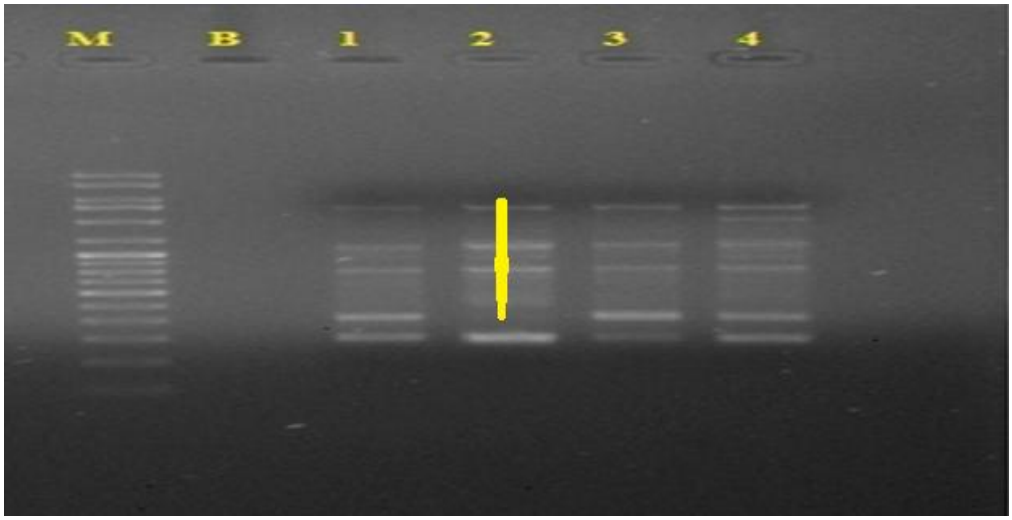
b. Amplification with OPU16



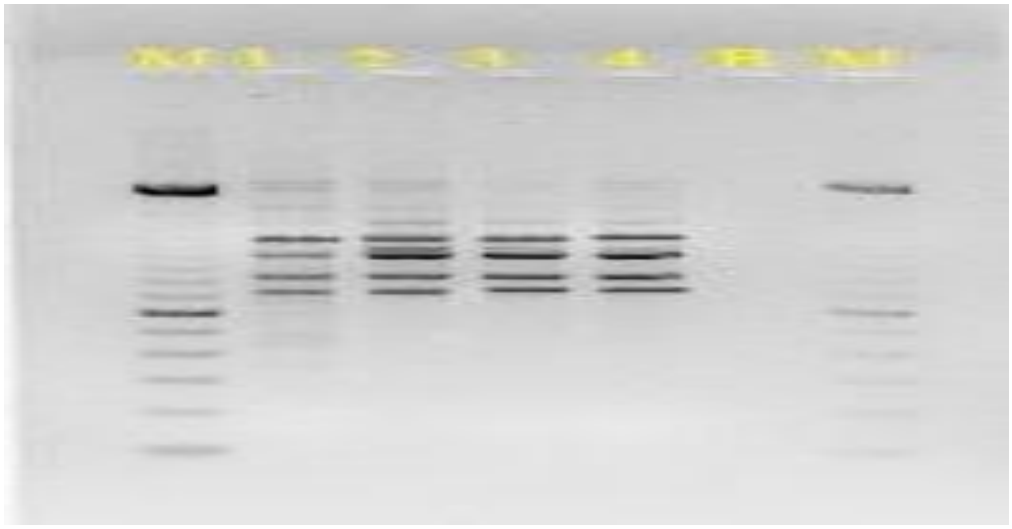
M- Marker (1Kb plus Ladder), B- Blank, 1- Mother plant, 2, 3, 4 - Regenerants

Plate no.10: RAPD amplification pattern in M1 mother plant and regenerants from inflorescence node in hybrid Winter Spot of *Phalaenopsis* with primer OPU15 and OPU16

a. **Amplification with P12**



a. **Amplification with P16**



M- Marker (1Kb plus Ladder), B- Blank, 1- Mother plant, 2, 3, 4 - Regenerants

Plate no.11: RAPD amplification pattern in M1 mother plant and regenerants from inflorescence node from hybrid Winter Spot of *Phalaenopsis* with primer P12 and P16

P16

Amplification with the primer P16 generated five clear amplicons. The amplicons obtained with the primers were monomorphic (Plate no. 11b). The molecular weight of amplicons ranged from 650 to 5000 bp.

Table 21: DNA Amplification pattern of M1 mother plant and regenerated clones with RAPD primers

Sl. no.	Primer	Total number of amplicons	Number of polymorphic amplicons	Number of monomorphic amplicons	Polymorphism (%)
1	OPU 10	**6**	**0**	**6**	**00.00**
2	OPU 13	**4**	**1**	**3**	**25.00**
3	OPU 15	**6**	**2**	**4**	**33.30**
4	OPU16	**5**	**2**	**3**	**40.00**
5	P12	**5**	**1**	**4**	**20.00**
6	P16	**5**	**0**	**4**	**00.00**
	Total	**31**	**6**	**24**	**118.3**
	Average	**5.1**	**1**	**4**	**19.70**

4.1.8.5.3 Clonal fidelity analysis of M2 mother plant

OPU 10

A total of five clear amplicons were obtained with the primer OPU 10. The molecular weight of amplicons ranged between 200 to 3000 bp. One amplicons of size range 300-400 bp absent in clone C2 (Plate no. 12a).

OPU13

A total of six clear amplicons were obtained when DNA was amplified with primer OPU13. The amplicons produced were monomorphic. The molecular weight of amplicons ranged between 100 and 3000 bp (Plate no. 12b).

OPU15

A total of seven clear amplicons were obtained after DNA amplification with the primer OPU15. One amplicons were found polymorphic in clone C2 and C3. The molecular weight of amplicons ranged from 100 and 8000 bp. The amplicons of size 400 bp was absent in clone C2 and C3 (Plate no. 13a).

OPU16

Two clear amplicons were observed in agarose gel for DNA amplified with the primer OPU16**.** All the amplicons are monomorphic in both the parent and clones. The molecular weight of the amplicon ranged from 300 to 650 bp (Plate no. 13b).

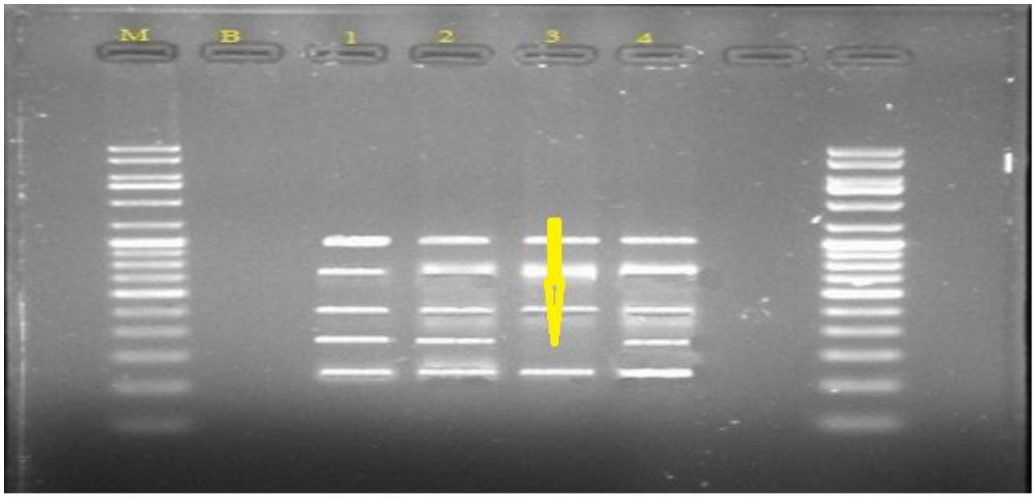
P12

Five amplicons were observed on the agarose gel for the DNA amplified with the primer P12**.** Amplicons showed monomorphic and polymorphic amplicon. One polymorphic amplicon at 200bp found in mother plant. The molecular weight of the product ranged between 200 to 3000 bp (Plate no. 14a).

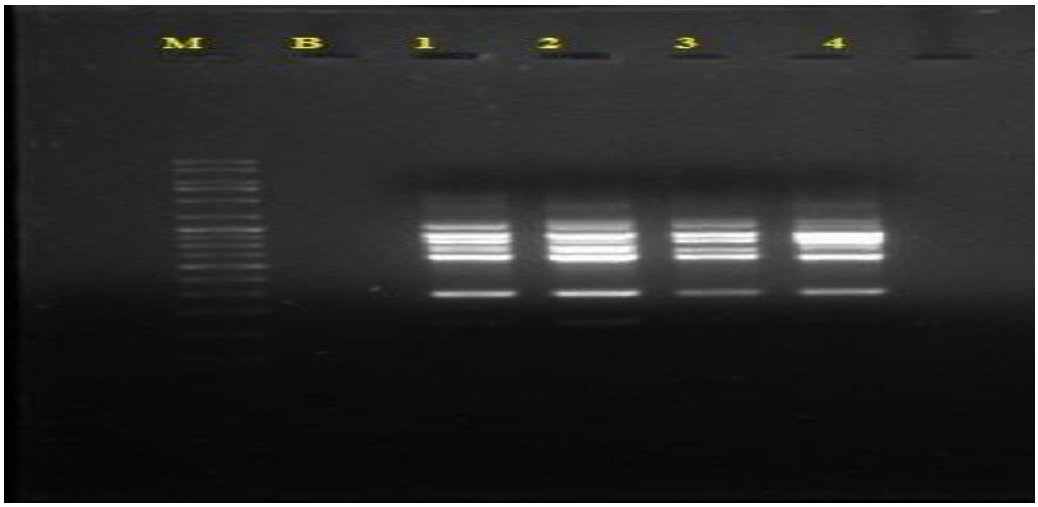
P16

A total of four clear amplicons were obtained with the primer P16. The molecular weight of amplicons ranged from 400 to 3000 bp. Amplicons show monomorphic in mother plant and in most of the clones (Plate no. 14b).

a. Amplification with OPU10



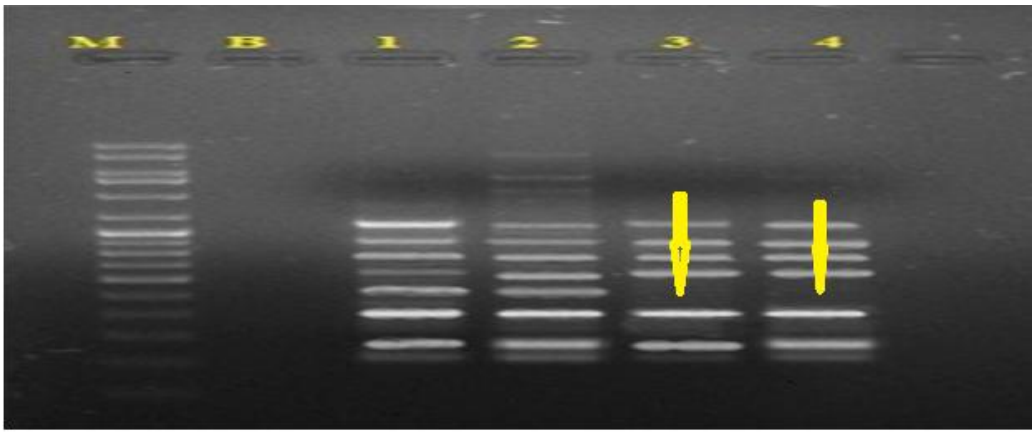
b. Amplification with OPU10



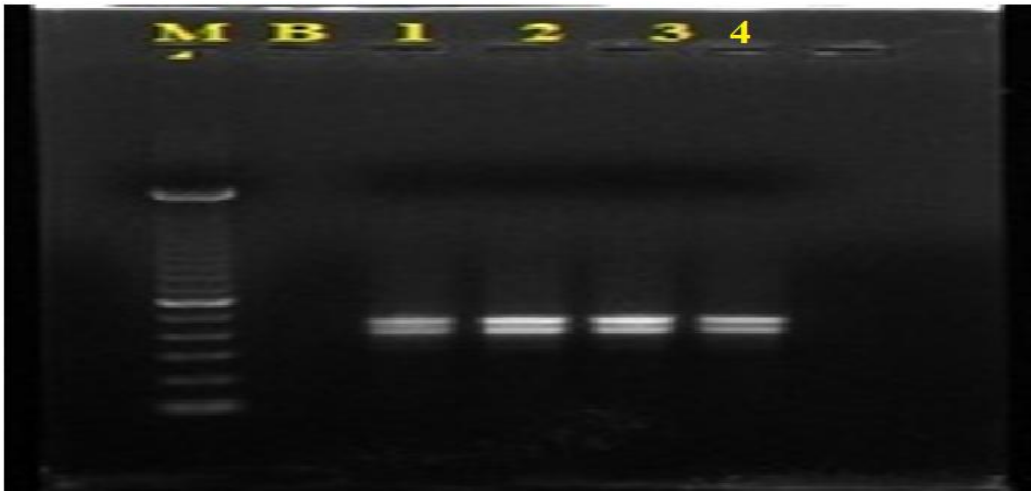
M- Marker (1Kb plus Ladder) , B- Blank, 1- Mother plant, 2, 3, 4 - Regenerants

Plate no.12: RAPD amplification pattern in M2 mother plant and regenerants from inflorescence node of *Phalaenopsis* with primer OPU10 and OPU13

a. Amplification with OPU15



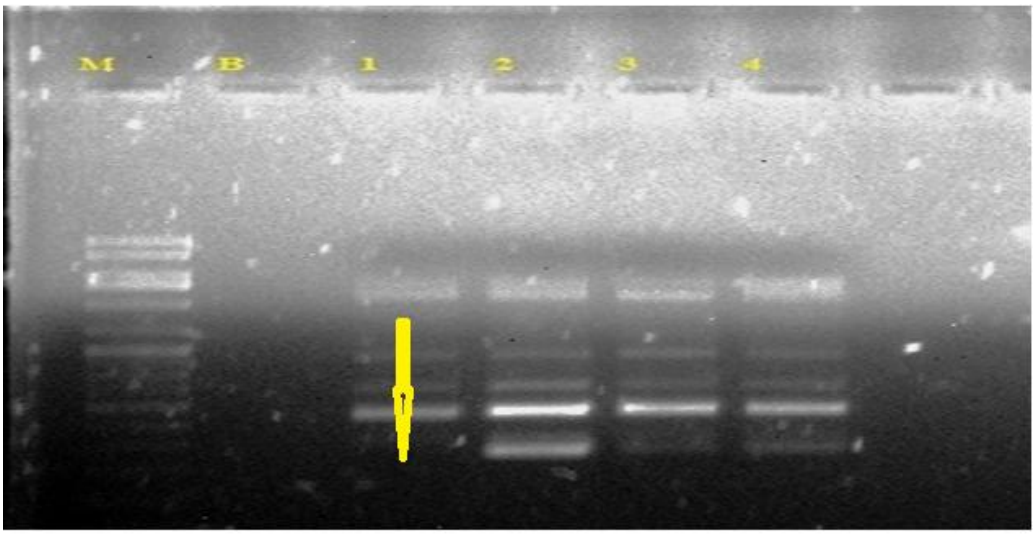
b. Amplification with OPU16



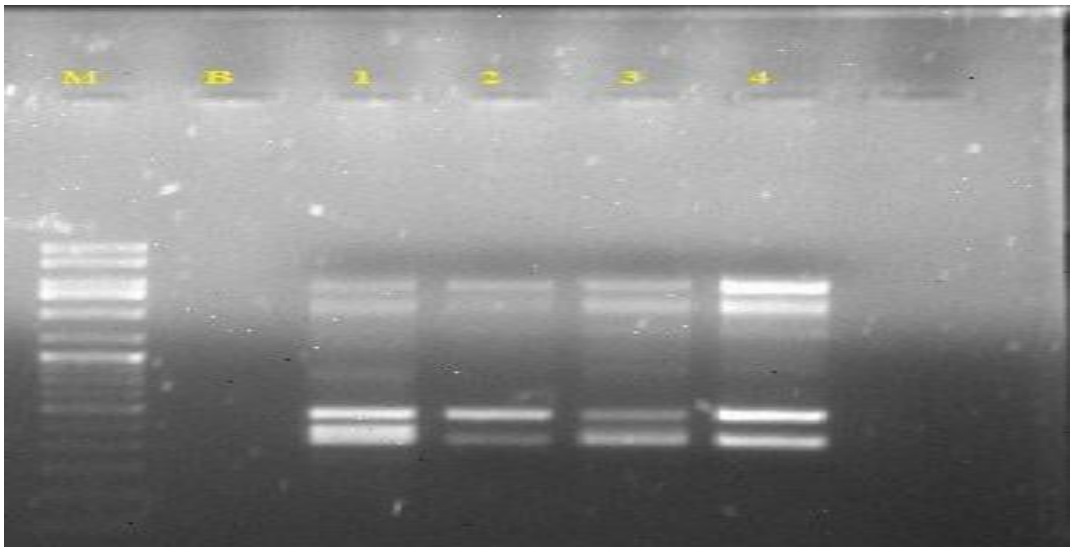
M- Marker (1Kb plus Ladder), B- Blank, 1- Mother plant, 2, 3, 4 - Regenerants

Plate no.13: RAPD amplification pattern in M2 mother plant and regenerants from inflorescence node of *Phalaenopsis* with primer OPU15 and OPU16

a. Amplification with P12



b. Amplification with P16



M- Marker (1Kb plus Ladder), B- Blank, 1- Mother plant, 2, 3, 4 - Regenerants

Plate no.14: RAPD amplification pattern in M2 mother plant and regenerants from inflorescence node of *Phalaenopsis* with primer P12 and P16

Table 22: DNA Amplification pattern of M2 mother plant and regenerated clones with RAPD primers

Sl no.	Primer	Total number of amplicons	Number of polymorphic amplicons	Number of monomorphic amplicon	Polymorphism (%)
1	OPU 10	**5**	**1**	**4**	**20.00**
2	OPU 13	**6**	**0**	**6**	**00.00**
3	OPU 15	**7**	**1**	**6**	**14.28**
4	OPU16	**2**	**0**	**2**	**00.00**
5	P12	**5**	**1**	**4**	**20.00**
6	P16	**2**	**0**	**4**	**00.00**
	Total	**27**	**4**	**25**	**54.28**
	Average	**4.5**	**0.6**	**4.7**	**9.04**

4.1.8.5.4 Clonal fidelity analysis of M3 mother plant

OPU10

A total of five clear amplicons were obtained when DNA was amplified with OPU 10. The amplicons produced monomorphic bands. No polymorphic bands. The molecular weight of amplicons ranged from 200 to 3000 bp (Plate no. 15a).

OPU13

A total of five clear amplicons were obtained when DNA was amplified with primer OPU13. Amplicons produced were monomorphic and polymorphic (Plate no.15b). One amplicon found polymorphic in mother plant and clone C1and C3.The molecular weight of amplicons ranged between 100 and 1000 bp.

OPU15

A total of five clear amplicons were obtained after DNA amplification with the primer OPU15. No amplicons were found polymorphism. The molecular weight of amplicons ranged from 200 and 8000 bp (Plate no. 16a).

OPU16

Six clear amplicons were observed in agarose gel for DNA amplified with the primer OPU 16**.** One amplicons were polymorphic at 6000bp. All other amplicons were monomorphic in both the parent and the clones (Plate no. 16b). The molecular weight of the amplicon ranged from 400 to 7000 bp.

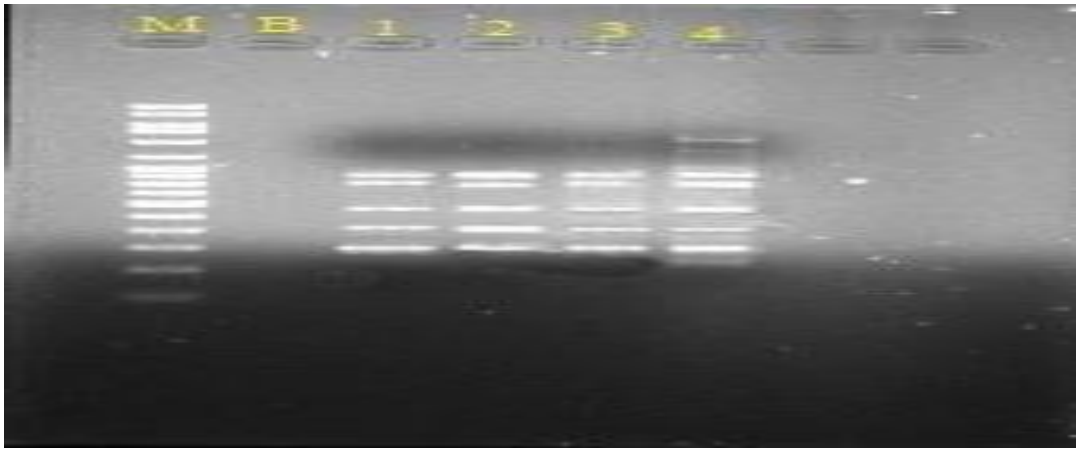
P12

A total of five clear amplicons were obtained with the primer P12. The molecular weight of amplicons ranged from 100 to 850 bp. No amplicons were found polymorphic. All amplicons were monomorphic (Plate no. 17a).

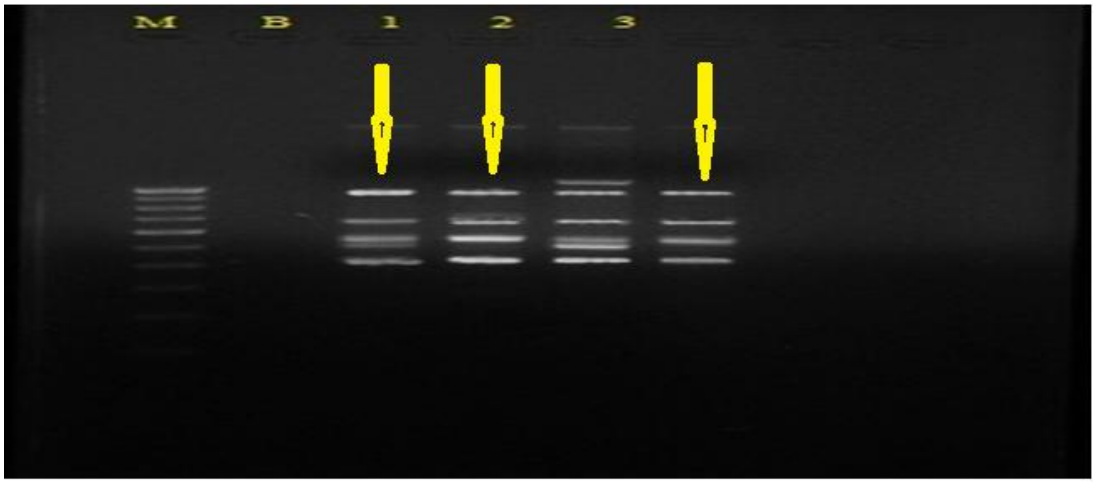
P16

Eight amplicons were observed on the agarose gel for the DNA amplified with the primer P16**.** Amplicons show polymorphism in clone 1 at 2000bp (Plate no. 17b). All others were monomorphic. The molecular weight of the product ranged between 400 to 8000 bp**.**

a. Amplification with OPU10



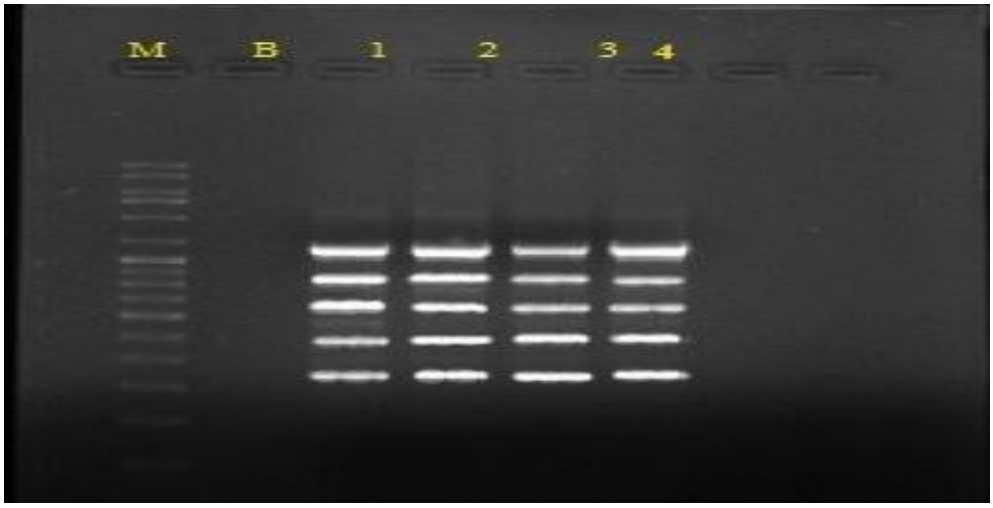
b. Amplification with OPU13



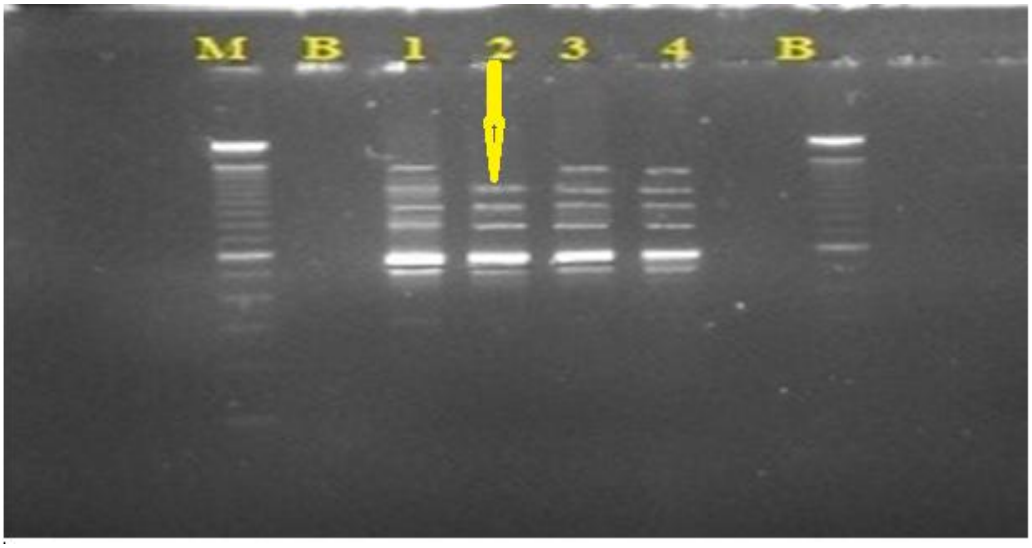
M- Marker (1Kb plus Ladder), B- Blank, 1- Mother plant, 2, 3, 4 - Regenerants

Plate no.15: RAPD amplification pattern in M3 mother plant and regenerants from inflorescence of *Phalaenopsis* with primer OPU10 and OPU13

a. **Amplification with OPU15**



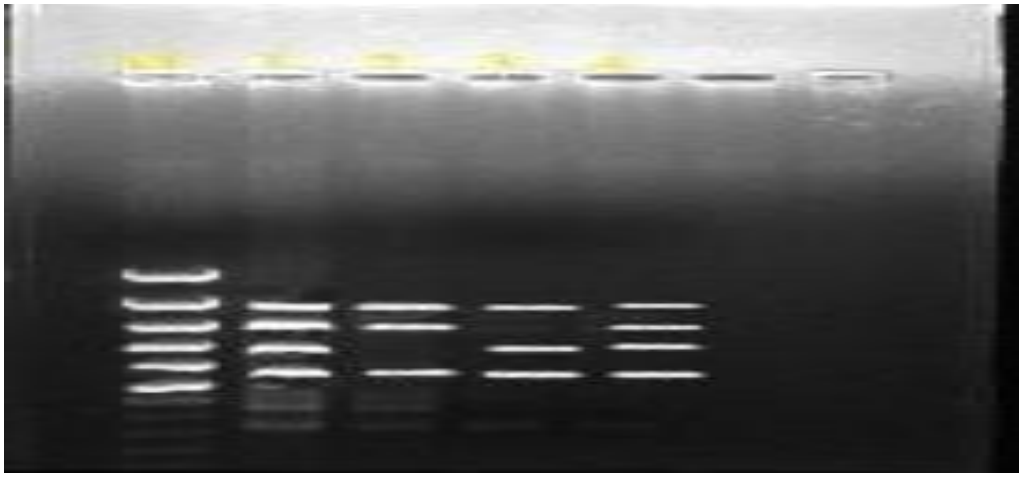
b. **Amplification with OPU16**



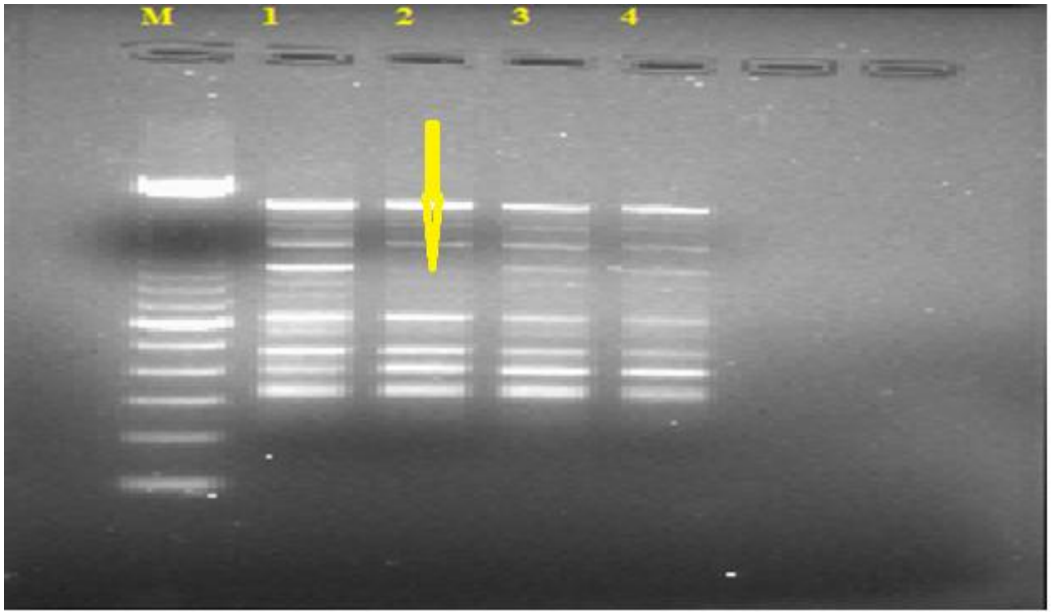
M- Marker (1Kb plus Ladder), B- Blank, 1- Mother plant, 2, 3, 4 - Regenerants

Plate no.16: RAPD amplification pattern in M3 mother plant and regenerants from inflorescence node of *Phalaenopsis* with primer OPU15 and OPU16

a. Amplification with P12



b. **Amplification with P16**



M- Marker (1Kb plus Ladder), 1- Mother plant, 2, 3, 4 - Regenerants

Plate no.17: RAPD amplification pattern in M3 mother plant and regenerants from inflorescence node of *Phalaenopsis* with primer P12 and P16

Table 23: DNA Amplification pattern of M3 mother plant and regenerated clones with RAPD primers

Sl no.	Primer	Total number of amplicons	Number of polymorphic amplicons	Number of monomorphic amplicons	Polymorphism (%)
1	OPU 10	**5**	**0**	**5**	**00.0**
2	OPU 13	**5**	**1**	**4**	**20.0**
3	OPU 15	**5**	**0**	**5**	**00.0**
4	OPU16	**6**	**1**	**5**	**16.6**
5	P12	**5**	**0**	**5**	**00.0**
6	P16	**8**	**1**	**7**	**12.5**
	Total	**34**	**3**	**31**	**49.1**
	Average	**5.6**	**0.5**	**5.1**	**8.15**

4.1.8.5. 5 Clonal fidelity analysis of M4 mother plant

OPU10

Five amplicons were observed on the agarose gel for the DNA amplified with the primer OPU10**.** One amplicons of size 850bp was absent in clone C3. The molecular weight of the product ranged between 200 to 4000 bp (Plate no. 18a).

OPU13

Amplification with the primer OPU13 generated six clear amplicons which are monomorphic. The molecular weight of amplicons ranged from 150 bp and 6000 bp (Plate no. 18b).

OPU15

A total of four clear amplicons were obtained with the primer OPU15. The amplicons of size 150 bp was absent in clone C2**.** The molecular weight of amplicons ranged from 100 to 850 bp (Plate no. 19a).

OPU16

Amplification with the primer OPU16 generated four clear amplicons which were monomorphic (Plate no. 19b). The molecular weight of amplicons ranged from 200 bp and 850 bp. All amplicon are monomorphic.

P12

A total of four clear amplicons were obtained with the primer P12. The molecular weight of amplicons ranged from 650 to 4000 kb. Two amplicon found polymorphic. All others were monomorphic (Plate no. 20b).

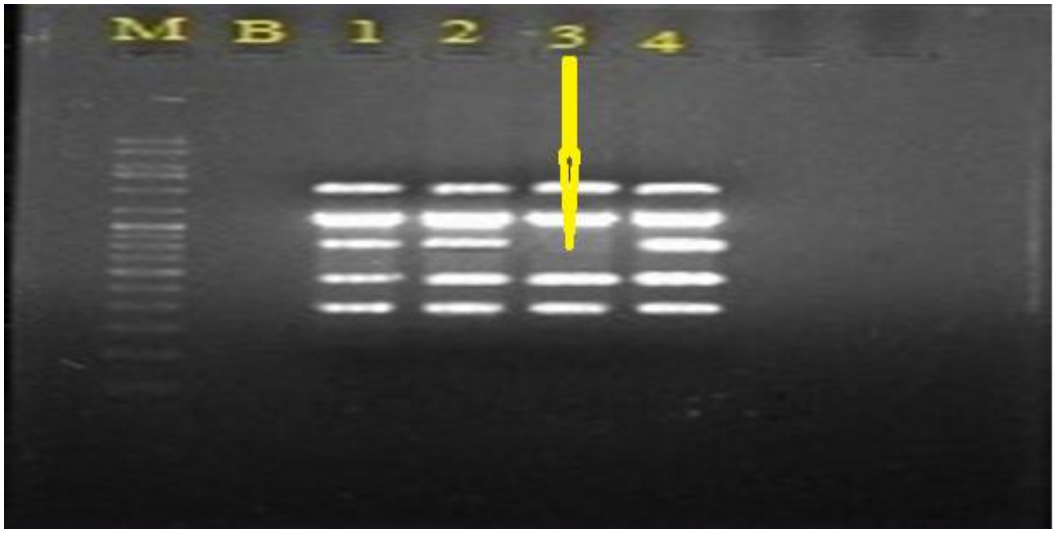
P16

Amplification pattern of P16 shows six clear amplicons. No amplicons were found polymorphic. All amplicons were monomorphic. The molecular weight of amplicons ranged from 100 to 500 bp (Plate no. 20b).

Table 24: DNA Amplification pattern of M4 mother plant and regenerated clones with RAPD primers

Sl no.	Primer	Total number of amplicons	Number of polymorphic amplicons	Number of monomorphic amplicon	Polymorphism (%)
1	OPU 10	**5**	**1**	**4**	**20**
2	OPU 13	**6**	**0**	**6**	**0**
3	OPU 15	**4**	**1**	**3**	**25**
4	OPU16	**4**	**0**	**4**	**0**
5	P12	**4**	**2**	**4**	**50**
6	P16	**6**	**0**	**6**	**0**
	Total	**29**	**2**	**27**	**95**
	Average	**4.83**	**0.33**	**4.5**	**15.8**

a. Amplification with OPU10

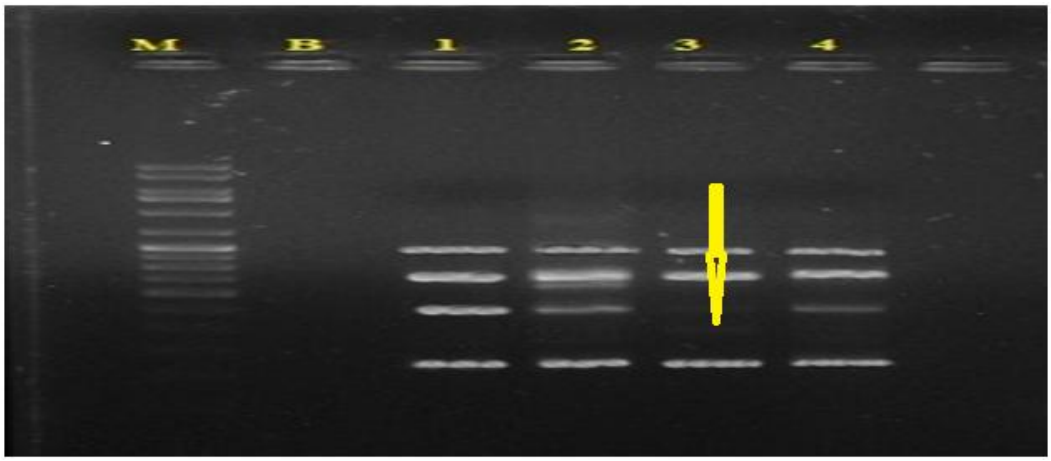


b. **Amplification with OPU13**

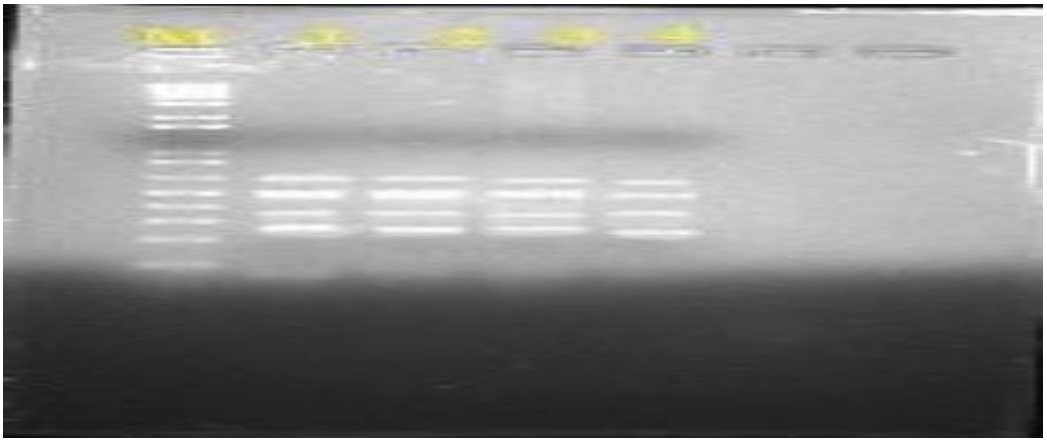
M- Marker (1Kb plus Ladder), B- Blank, 1- Mother plant, 2,3, 4 - Regenerants

Plate no.18: RAPD amplification pattern in M4 mother plant and regenerants from inflorescence node of *Phalaenopsis* with primer OPU10 and OPU13

a. Amplification with OPU15



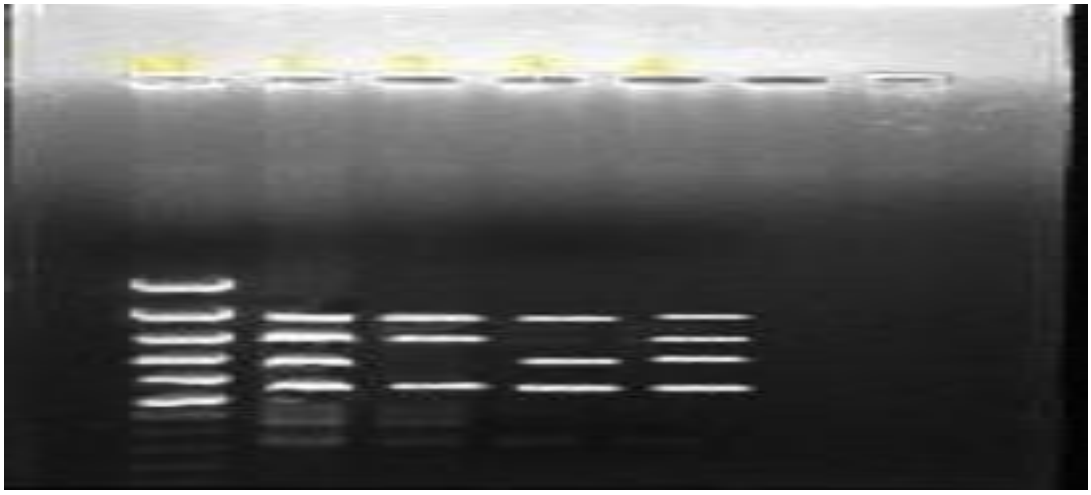
a. Amplification with OPU16



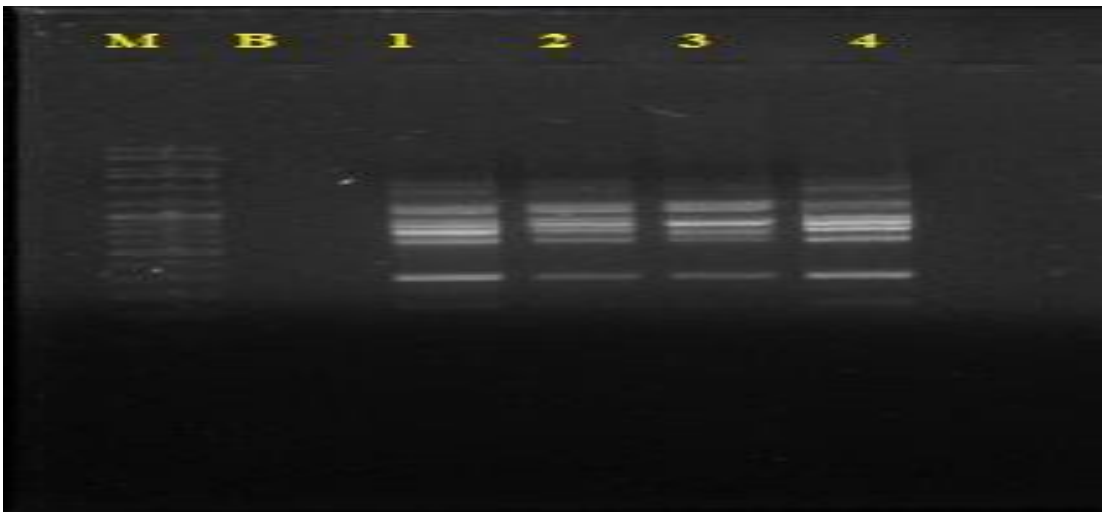
M- Marker (1Kb plus Ladder), B- Blank, 1- Mother plant, 2- 4 - Regenerants

Plate no.19: RAPD amplification pattern in M4 mother plant and regenerants from inflorescence node of *Phalaenopsis* with primer OPU15 and OPU16

a. Amplification with P12



b. **Amplification with P16**



M- Marker (1Kb plus Ladder), B- Blank, 1- Mother plant, 2- 4 - Regenerants

Plate no.20: RAPD amplification pattern in M4 mother plant and their regenerants from inflorescence node of *Phalaenopsis* with primer P12 and P16

4.1.8.6.1 Amplification pattern of M1 mother plant and M2 mother plant with regenerated clones using RAPD primers

Three clones were compared with the source mother plant using the six selected primers. Of the six primers tested, primers like OPU13, OPU15, OPU16 and P12 exhibited polymorphic bands in M1 mother plant and in M2 mother plant, primers like OPU10, OPU 15 and P12 show polymorphism. The average polymorphism was noticed in M1 mother plant at 19.7% and in M2 mother plant at 9.04%.

4.1.8.6.2 DNA Amplification pattern of M3 mother plant and M4 mother plant with regenerated clones using RAPD primers

Details of DNA amplification pattern in clones regenerated through inflorescence node are presented in Table 23 and Table 24. Three clones were compared with mother plant using RAPD assay. In M3 mother plant, out of the six selected primers tested, OPU 13, OPU16 and P16 exhibited polymorphism and in plant four, OPU10, OPU15 and P12 exhibited polymorphism. The average polymorphism observed was 8.15% in plant three and 15.8% in M4 mother plant.

4.1.8.7 Comparison of the mother plants with clones using NTSYS software

The similarity coefficients were calculated and the dendrogram based on UPGMA cluster analysis was constructed using NTSYS-pc software. The combined primer data show a significant variation with respect to mother and 3 clones and among clones as well.

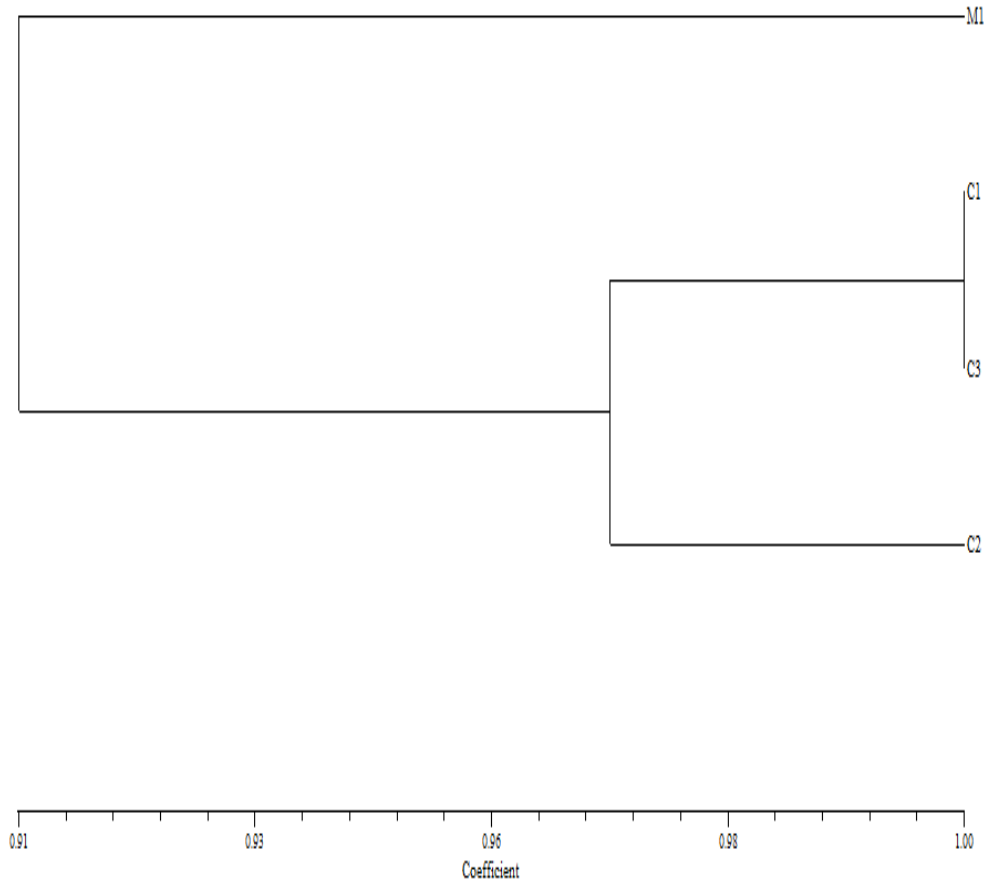
4.1.8.7.1 M1 mother plant and regenerated clones

The mother and regenerated clones were compared for genetic similarity using NTSYS software. The similarity coefficients between mother and clones and among clones were calculated.

The result obtained from the combined primers dendrogram based on similarity coefficient showed that the source mother plant had 0.91 (91%)

similarity coefficients with the clones. The NTSYS software results show that the similarity coefficient among clones ranged from 0.96 (96%) to 1.0(100). Clone (C1) and clone (C3) recorded 100% similarity (Figure 1).

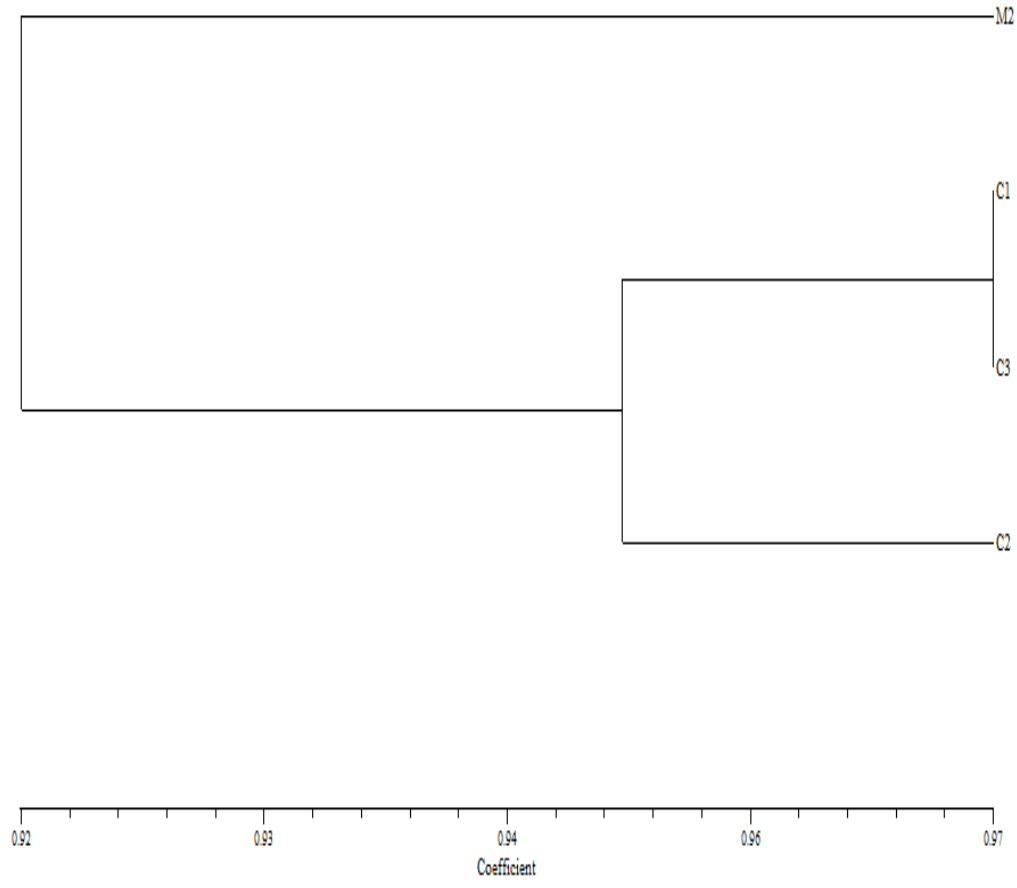
Figure 1: Dendrogram for M1 mother plant and regenerants



4.1.8.7.2 M2 mother plant and regenerated clones

The similarity coefficient obtained NTSYS pc software showed that the mother plant and clones exhibited 92% (0.92) similarity. The regenerated clones C1 and C3 exhibited the maximum similarity coefficient of 0.97. Based on the dendrogram obtained using NTSYS software, the percentage of variability between mother plant and clones was 8 %.(Figure 2).

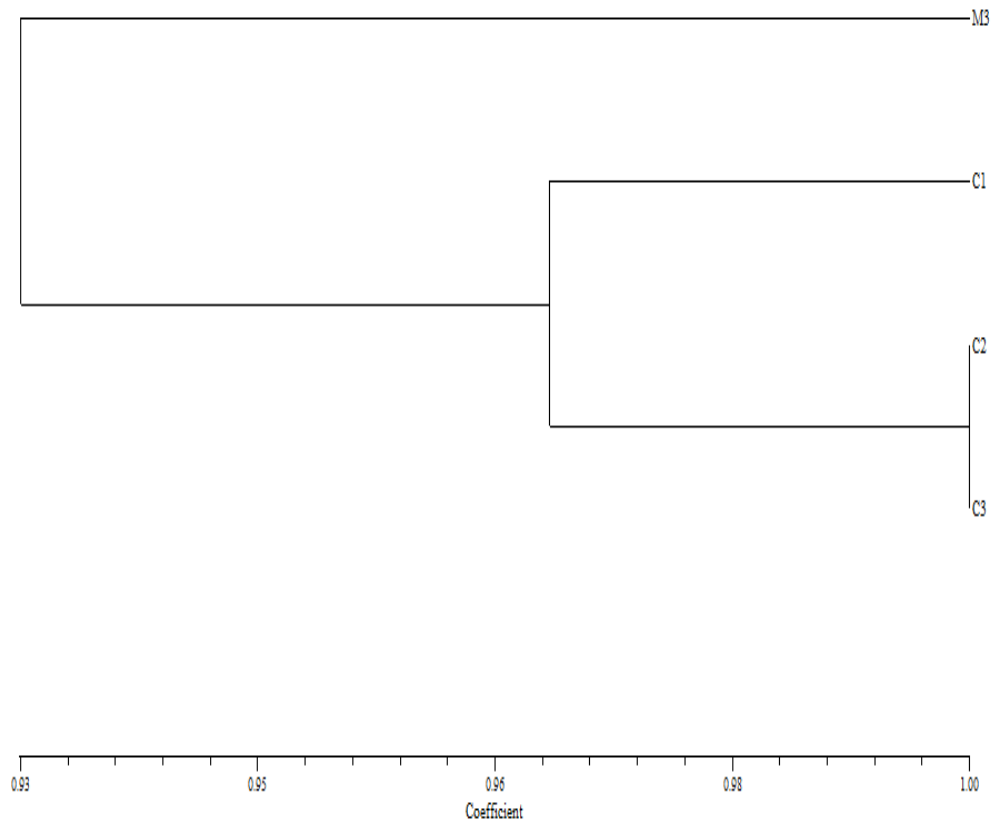
Figure 2: Dendrogram for M2 mother plant and regenerated clones



4.1.8.7.3 M3 mother plant and regenerated clones

The data had shown that the similarity coefficient between mother plant and clones exhibited was 0.93 (93%). The regenerated clones like C2 and C3 exhibited the maximum similarity coefficient of 1.0 (100%). Based on the dendrogram obtained using NTSYS pc, the variation between the clones and the mother plant was 7% (Figure 3).

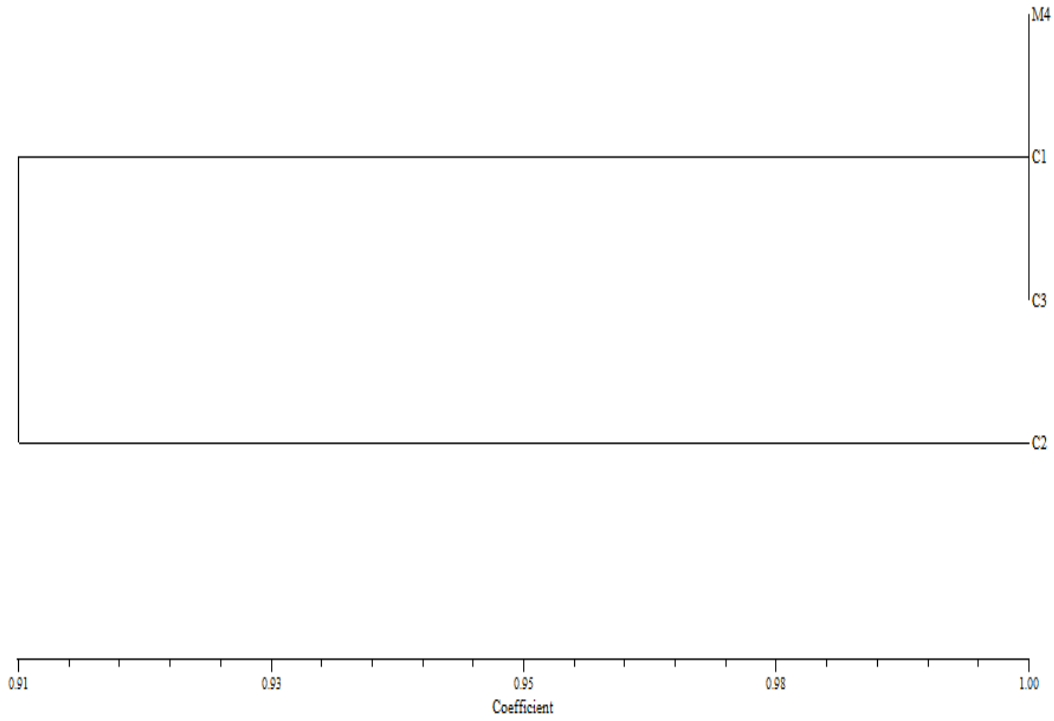
Figure 3: Dendrogram for M3 mother plant and regenerated clones



4.1.8.7.4 M4 mother plant and regenerated clones

The similarity coefficient obtained from NTSYS pc software showed that the mother plant and the first C1 and third C3 clones exhibited 100% (1.0) similarity. The clone C2 exhibited 91% (0.91) similarity coefficient with clones C1, C3 and source mother plants.

Figure 4: Dendrogram for M4 mother plant and regenerated clones



Discussion

5. Discussion

The present study "Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid Winter Spot with molecular marker" was carried out in order to regenerate tissue culture plants of *Phalaenopsis* through viable protocols and to examine suitability of the same for micropropagation with genetic stability analysis using RAPD markers. The results of the experiments are discussed in this chapter.

5.1 Studies on micropropagation

The micropropagation protocols reported by Thida *et al*., 2009 and Rittirat *et al*., 2012 were used as the basis for regeneration of plantlets from three explants - Inflorescence node, transverse thin cell layer of leaf and root tip. Flowering mother plants of *Phalaenopsis* hybrid Winter Spot maintained at PPNMU were used as explants source.

5.1.1 Standardization of surface sterilization of *Phalaenopsis* explants

In inflorescence node, the surface sterilization treatment T3 ($HgCl_2$ 0.1%7 min) recorded maximum percentage (55 %) of explants survival followed by treatment T4 ($HgCl_2$ 0.1% 8min) with 40 per cent. Least contamination was recorded in the treatment T4 ($HgCl_2$ 0.1% 8min) which was 20 per cent, closely followed by T3 ($HgCl_2$ 0.1% 7min) with 45 per cent.

Though contamination rate was lowest in T4, explants survival percentage and percentage of responding culture were maximum in T3. Increased exposure to mercuric chloride might have reduced the survival rate.

In leaf explants, the per cent of contamination was observed maximum (75%) in treatment T1 (5 minutes 0.1 per cent $HgCl_2$) closely followed by T2 (6 minutes 0.1 per cent $HgCl_2$) with 55per cent. The treatment T4 (8 minutes 0.1 per

cent $HgCl_2$) recorded maximum per cent of explant survival (65 %) with least contamination (35 per cent) followed by T3 with 50 per cent explant survival and 50% contamination. Based on the observations treatment T4 (8 minutes 0.1 per cent $HgCl_2$) was followed the further surface sterilization of leaf explants.

In *Phalaenopsis*, Chen *et al.* (1998) obtained better culture survival when dormant bud dissected from flower stalk was surface sterilized by immersion in 70 % ethanol for 1 min, then in a10% sodium hypochloride solution for 15 min. wei *et al.*, (2009) reported that seed sterilized with detergent and 10% $NaCl_2$ were best suitable for seed germination and protocorm formation.

In *Phalaenopsis*, Young *et al.* (2000) observed that flower stalk section containing bud were sterilized in 0.2% $HgCl_2$ for 10 min.

5.1.2Effect of basal media for culture establishment of *Phalaenopsis* explants

Full strength and half strength of MS basal media tried for culture establishment using different explants like inflorescence nodes, leaf segment and root segments. Shoot regeneration was observed in the explant of inflorescence node with the medium of full strength MS with $2mgl^{-1}$ of TDZ.

Park *et al*. (2002) tested few media such as MS (Murashige and Skoog 1962) and VW (Vacin and Went, 1949) for proliferation and differentiation of PLBs in various *Phalaenopsis* hybrids. Survival percentage of the explants and multiplication of PLBs was high on MS media followed by VW media. In the present study also better shoot regeneration was observed in MS medium.

Tanaka and Sakanishi, (1980) used solid and liquid media cultures for proliferation of PLBs and found that solid media cultures had yielded better results than liquid cultures and also found that the type of hormone for induction is

dependent on the species studies and rate of proliferation can differ based on the composition of the media.

Effect of culture media on germination, multiplication and differentiation of *Phalaenopsis* seeds were studied by Wei *et al.* (2009). The best suitable medium for seed germination was full strength of MS with NAA 0.5 mgl^{-1}.

Gnasekaran *et al*. (2010) demonstrated that shoot tip of *Phalaenopsis violacea* produced maximum growth of PLB, when cultured on Murashige and Skoog (MS) semi-solid medium.

5.1.3 Response of different explants to various growth regulators combination

Various combination of growth regulators like BA at (1-5mgl^{-1}), IAA.(0.2mgl^{-1}),NAA (0.2mgl^{-1}) , TDZ (1-2mgl^{-1}) and kinetin (1-5mgl^{-1}) were used for the study. MS medium supplemented with BA and TDZ was found to give good shoot regeneration from the explants of inflorescence node.

It was found that MS with TDZ at 2 mgl^{-1} TDZ recorded highest percentage (80%) of culture establishment, followed by 4.5 mgl^{-1} of BA (55%).

In MS medium supplemented with various concentration of auxin *viz*, IAA (0.2 mgl^{-1}) and NAA (0.1mgl^{-1}) along with various concentration of cytokinin BA (1-5mgl^{-1}) observed the bulging of dormant bud of the inflorescence node.

Cytokinin generally promotes cell division, adventitious bud and axillary bud formation and inhibits root formation.

TDZ was first used for micropropagation of orchids, specifically *Phalaenopsis* (Ernst, 1994). The result of the present study were in close agreement with those of Thida *et al.*, 2006 reported that thin section of leaf explants cultured on low concentration (4.54- 9.08 μm) of Thidiazuron were

observed to be more effective than high concentration of benzyl adenine (BA: 44.4μm) to induce shoot buds within 2 weeks

Kanchanapoom, (2014) found that VW medium supplemented with 9 mM nitrogen and 10 mg l^{-1} BA gave the best leaf length, 3.4 cm, and width, 1.3 cm, with an average of three leaves per plantlet

Kuo *et al.*, (2005) evaluated the effects of 2,4-D (0.45, 2.26, 4.52 mM), kinetin (2.32, 4.65, 13.95mM), BA (2.22, 4.44, 13.32mM), and TDZ (2.27, 4.54, 13.62mM) on cultured leaf segment of the orchid sp. *Phalaenopsis* 'Little Steve' for the induction of direct somatic embryogenesis. After 20–30 d of culture, clusters of somatic embryos formed from leaf surfaces on half-strength Murashige and Skoog medium supplemented with BA and TDZ.

In the present study, MS medium supplemented with BA and TDZ was found to give good shoot regeneration from the explants of inflorescence node.

5.1.4 Effect of different additives for shoot regeneration from inflorescence node

The inflorescence node with dormant bud was cultured on MS medium with TDZ along with different additives like, coconut water, banana pulp, activated charcoal and adenine sulphate

With coconut water at 10%, shoot regeneration was observed in15 days after inoculation. With 15% and 20% of coconut water, the sprouting was observed within 20 days of inoculation. Percentage of inflorescence node explants established and sprouted was 100 per cent in all the media containing coconut water as additive. Addition of banana pulp in the medium did not induce any sprouting of inflorescence node.

The liquid endosperm of the coconut promotes growth and differentiation. A number of cell division factors are present in CW including diphenyl urea , 9-B-ribofuranozyl Zeatin (Letham, 1974) and a compound which co-chromatographs with Zeatin riboside (Van Staden and Drewes, 1975). Coconut water is a complex additive which contains many nutritional and/or hormonal substances (Dix and Van Staden, 1982). It also contains a large number of free amino acids including phenylalanine which has the cell division activity in soybean assays (Van Staden and Drewes, 1975).

Bhaskar, (1996) observed that in *Phalaenopsis*, ½ MS liquid medium with BA 5mgl^{-1} + NAA 2mgl^{-1} +2,4-d 2 mgl^{-1} + CW 15% recorded the minimum number of days for nodal swelling and bud development. Similar trend was observed in the present study.

The highest frequency of protocorm-like body (PLB) formation in *Phalaenopsis* hybrid was obtained when basal stem explants were cultured on VW medium containing 30 gl^{-1} sucrose, 500 mgl^{-1} activated charcoal, 150 mll^{-1} coconut water, 1 mgl^{-1} NAA, 5 mgl^{-1} 2iP and 2.5 gl^{-1} gelrite (Jo *et al.*, 2007).

In *Phalaenopsis*, high frequency regeneration obtained by inoculating the leaf segment on gelrite gelled (1/2) MS medium supplemented with 2% sucrose, 2.0 mgl^{-1} BA, 0.5 mgl^{-1} NAA, 10% coconut water (CW), 2 gl^{-1} peptone and 1 gl^{-1} activated charcoal (Sinha *et al.*, 2010)

Lakshmanan *et al*. (1995) reported the formation of PLBs from thin sections of shoot tips of *Aranda* Deborah on treatment with CW (5–25%, v/v). In *Dendrobium*, it was observed that the best medium for maximum number of PLB formation was 0.1 mg l^{-1} of BA, 1.0 mgl^{-1} of NAA and 15% (v/v) coconut water in MS Liquid medium (Puchooa, 2004).

In present study, in MS media with addition of activated charcoal (1-5gl^{-1}), bulging of dormant bud of the inflorescence node was observed after 28 days of inoculation.

Rittirat *et al.* (2012) reported development of protocorm–like bodies (PLBs) of *Phalaenopsis*. in ½ MS medium supplemented with 0.1µm each of NAA and TDZ. They observed plant conversion of PLB's in MS medium with 0.2% activated charcoal.

Eymar *et al*. (2000) observed that the addition of AC increased and maintained pH levels during culture, increased the nitrogen uptake and improved growth and visual aspects of the explants and reduced the inhibitory effect of exogenous cytokinin on root growth.

In *Dendrobium nobile* the asymbiotic seed germination was observed on 1/2 MS medium supplemented with 0.05 gl^{-1} AC (Men *et al*., 2003).

In *Rhynchostylis retusa* the *in vitro* seedling growth was promoted by the addition of AC in the medium. Seedling growth was maximum on MS medium supplemented with BA, NAA and 1 gl^{-1}AC (Thomas and Michael, 2007).

In the present study with addition of coconut water at 10%, shoot regeneration was observed in15 days after inoculation. With addition of 15% and 20% of coconut water, the sprouting was observed within 20 days of inoculation. MS media with addition of activated charcoal (1-5gl^{-1}), bulging of dormant bud of the inflorescence node was observed after 28 days of inoculation.

5.1.5 Effect of different Growth regulators for multiple shoot induction from inflorescence node explants

MS medium supplemented with 4.5 mgl^{-1} BA resulted in the highest average number of multiple shoot (4.15) followed by MS supplemented with 2 mg l^{-1} BA and 4.5 mgl^{-1} BA+ 0.1mgl^{-1} IAA (4.0).

Among the different multiplication media tested in the present study, percentage of culture exhibiting multiple shoot induction recorded for MS + 4.5 mg l^{-1} BA, MS+ 4.5 mg l^{-1} BA+ 0.1mg l^{-1} IAA and MS + 2 mg l^{-1} BA were 86.6, 80 and 33.3 respectively within the period of 45 days.

Among the growth regulators, cytokinins, like BA and TDZ were found to be the most effective in regenerating plantlets in a number of orchids (Nayak *et al*., 1997 ; Seeni and Latha, 1992). Thida *et al.,* 2006 reported that MS medium supplemented with BA, recorded the 28.0% of multiple shoot induction. Similar results were recorded in present study and BA at 4.5mgl^{-1} was found better.

Huaiyiu, (1998) reported that an MS medium supplemented with 0.5-5mgl^{-1} BA or 0.5-5mgl^{-1} BA + lmgl^{-1} NAA yielded high shoot multiplication rate from adventitious buds (65%).

In *Phalaenopsis*, indole acetyl glycine (0.5 mg l^{-1}) added to MS medium promoted the formation of a large number of shoots from protocorms derived from inflorescence nodal buds (53%) (Mahmood and Chew, 2008)

The effects of 6-benzyladenine (BA) on growth of *Phalaenopsis* orchid were studied by Kanchanapoom, (2014). VW medium supplemented with 9 mM nitrogen and 10 mg l^{-1} BA gave the best leaf length, 3.4 cm, and width, 1.3 cm, with an average of three leaves per plantlet.

Tanaka and Sakanishi, (1978) observed that, in *Phalaenopsis* cultured buds which had remained dormant were stimulated to sprout by addition of BA ($5mgl^{-1}$) to the medium.

Pant and Thapa, (2012) reported that maximum numbers of rootless healthy shoots were observed on MS medium fortified with BAP 1.5 mgl^{-1} with an average value of 4.5 shoots per culture where shoot multiplication was initiated after 5 weeks of culture of shoot tip.

In the present study MS medium supplemented with 4.5 mgl^{-1} BA resulted in the highest number of multiple shoots (80%) and highest average number of multiple shoot (4.15).

5.1.6 Effect of different growth regulators for shoot elongation and rooting

In different treatment given, the rooting was found maximum in MS + BA $4.5mgl^{-1}$ +IAA $1mgl^{-1}$ (80 % rooting) followed by MS +Kinetin $0.1mgl^{-1}$ +$1mgl^{-1}$ NAA (65%). The number of days taken for *in vitro* rooting initiation ranged from 50-56 days. Among different media tried, IAA with BA was found to cause early root induction in 50 days after inoculation.

In *Phalaenopsis*, Hempfling and Preil, (2005) reported that *in vitro* proliferated shoots produced maximum number of roots on the MS medium supplemented with 1.0 mg l^{-1} IAA. The highest root number obtained was 3.7 roots per shoot.

MS medium supplemented with various concentrations of IAA and IBA were found to be effective for rooting of *D. primulinum* in comparison to NAA. The best rooting response was observed on MS medium with exogenous supply of IAA 0.5 mgl^{-1} (Pant and Thapa, 2012).

In the present study, IAA medium recorded highest rooting response (80%) with good shoot length 2.98cm.

5.1.7 Hardening of *in vitro* plantlets of *Phalaenopsis*

The potting medium of charcoal, brick pieces and sphagnum moss were used for hardening micropropagated plantlets of *Phalaenopsis*. The survival rate of 100% was observed with mean plant height of 2.98 cm. The mean number of leaves recorded was 3.

Williams, (1885) suggested rough fibrous peat and live sphagnum moss as a medium for epiphytic orchids. Growth and flowering of *Phalaenopsis* were greater when commercial medium was mixed with 20% sphagnum moss (Hwang *et al.*, 2004).

An efficient hardening technique for orchid seedling was reported on chips of charcoal, bricks and decayed wood (Deb and Imchen, 2010). Plants grown in a medium consisting of 1:1 chunky peat: perlite or 2:1:1 perlite: chunky peat: coconut coir had fewer aerial roots than plants grown in a bark-based or 1:1 perlite: charcoal media. Plants grown in the chunky peat: perlite mix or chunky peat: charcoal mix had the greatest increase in leaf span compared to the other media (Blanchard and Runkle, 2008).

In *Dendrobium* sp., Indhumathi *et al*. (2003) reported that the establishment of plantlets in terms of plant height, leaf size, new growth and root length was good in the medium consisting of charcoal + brick pieces + cocopeat (1:1:1).

Saiprasad and Polisetty. (2003) showed that the orchids *Dendrobium*, *Oncidium* and *Cattleya* had complete survival or establishment in potting media having wood charcoal pieces alone and wood charcoal + brick pieces, which allowed good circulation of air and excellent drainage.

5.2.1 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 Doyle and Doyle, (1990) with 4x 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 Oyarzum, 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. Sreenath *et al*. 1992 used β- Mercaptoethanol and PVP to remove high phenol content in coffee. 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 dissolved and stored as was reported by Sambrook *et al*. (1989).

A DNA sample has been reported as good quality if it had a band of high molecular weight with a low amount of RNA (Wettasinghe and Peffley, 1998). The use of RNase for removal of RNase from DNA was reported by several

workers [Wettasinghe and Peffley (1998) and Gallego and Martinez (1996)]. In the present study, RNase treatment was given and it yield good quality DNA.

5.2.2 Quality and Quantity of genomic DNA

Good quality DNA isolated from mother plants and regenerants were used for RAPD marker analysis. The quality and quantity of DNA was calculated using the absorbance ration as OD at 260/280 for the various samples using Nano drop spectrophotometer. Those samples with ratio between 1.8-2 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 136.80 ng/µl to 1032 ng/µl and the purity (A_{260}/ A_{280}) of DNA ranged from 1.9 to 2.1.

5.2.3 RAPD marker analysis

Genomic DNA isolated from mother plants and regenerants from various protocols were subjected to RAPD analysis. Twelve RAPD primers were screened with good quality DNA isolated from mother plants. Based on amplification six primers were selected for RAPD analysis.

RAPD, which is the simplest version of PCR with arbitrary primers used for detecting DNA variation and is popular due to its convenience, low cost and simplicity (Sun and Wong, 2001; Bairu *et al.*, 2011), was used to find molecular differences to support physiological differences in tissue culture plantlets.

RAPD analysis is a fingerprinting method using short, random, oligonucleotide primers to search for variation in the entire genomic DNA (Williams *et al*., 1990) and has been widely employed in evaluating genetic distances in many diverse plant genera, e.g., *Acacia* (Casiva *et al*., 2002); *Cicer*

(Sudupak *et al*., 2002); *Cupressus* (Rushforth *et al*., 2003); *Linum* (Fu *et al*., 2002) and *Rhizophora* (Lakshmi et al., 2002).

Genomic DNA isolated from mother plants and regenerants from various protocols were subjected to RAPD analysis.

5.2.4 Clonal fidelity analysis of M1 mother plant

M1 mother plant and their regenerants were subjected to RAPD assay. Three clones were compared with the source mother plant using the six selected primers. Of the six primers tested, primers like OPU13, OPU15, OPU16 and P12 exhibited polymorphic bands in M1 mother plant. The six primers produced 30 distinct and scorable bands in the size range of 100 bp to 8000 bp. The number of scorable bands for each primer varied from 5 to 6 with an average of 5 bands per primer.

5.2.4.2 Clonal fidelity analysis of M2 mother plant

M2 Mother plant and their regenerants were subjected to RAPD assay. Out of six primers tested, primers like OPU10, OPU 15 and P12 show polymorphism. The six RAPD primers produced 29 distinct and scorable bands in the size range of 100 bp to 8000 bp. The number of scorable bands for each primer varied from 2 to 7 with an average of 4.8 bands per primer. A total of 29 bands were generated from mother plant and regenerants, out of which 25 were monomorphic and 4 were polymorphic.

5.2.4.3 Clonal fidelity analysis of M3 mother plant

M3 Mother plant and their regenerants were subjected to RAPD assay. Out of six primers tested, OPU 13, OPU16 and P16 exhibited polymorphism while the remaining gave monomorphic amplicons. The six RAPD primers produced 34 distinct and scorable bands in the size range of 200 bp to 6000 bp. The number of

scorable bands for each primer varied from 5 to 8 with an average of 5.6 bands per primer. A total of 34 bands were generated from mother plant and regenerants, out of which 31 were monomorphic and 3 were polymorphic.

5.2.4.4 Clonal fidelity analysis of M4 mother plant

M4 Mother plant and their regenerants were subjected to RAPD assay. Out of the six selected primers tested, OPU 10 and OPU15 exhibited polymorphism. The six RAPD primers produced 29 distinct and scorable bands in the size range of 100 bp to 6000 bp. The number of scorable bands for each primer varied from 4 to 6 with an average of 4.85 bands per primer. A total of 29 bands were generated from mother plant and regenerants, out of which 27 were monomorphic and 2 were polymorphic.

Somaclonal variations in flower and inflorescence axis were investigated among the plants micropropagated through protocorm-like bodies induced by flower stalk bud culture of various cultivars of *Phalaenopsis* and *Doritaenopsis*. The frequencies of these somaclonal variations in each genotype ranged from 0 to 100%, but most of the cultivars showed variations less than 10% (Tokuhara and Mii, 1993). Although the direct formation of plant structures without any intermediate callus phase, minimises the possibility of uncertainty (Karp, 1994).

In some reports, plants derived from organised meristems are not always genetically true-to-the type in many crops (Devarumath *et al*., 2002). Plants regenerated from adventitious shoots from axillary buds or from other well developed meristematic tissue showed the lowest tendency for genetic variation (Joshi and Dhawan, 2007).

The occurrence of somaclonal variation during culturing is a frequent and consistent event. The frequency of variation depends on the genotype, culture

medium, growth hormones and the way of multiplication. Somaclonal variations not only can be distinguished by their morphological traits (Gamborg *et al.*, 1977) but also by their biochemical, physiological and genetic characteristics. Several reports in the literature indicate that variations can be detected by identifying chromosome numbers (Ogura, 1990), isozyme patterns (Pereira *et al.*, 1996) and random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) in many crops.

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. Martin *et al*. (2005) 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 and variation was observed.

It appears that, the presence of variations *in vitro* is common and what have to be determined are the frequencies in relation to the type of growth regulator and the subculture period. Some species are more amenable to variations than others and understanding this will enable the producer to limit the subculture cycles in order to maintain the clonal characteristics.

Some authors have indicated that the treatment 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.

Khoddamzadeh *et al.* (2010) reported that Twelve decamer random amplified polymorphic DNA (RAPD) primers were used to study somaclonal variation among the mother plant, the initially induced PLBs .Eight out of twelve primers produced 172 bands with 18 polymorphic bands in all the treatments. The amplified products varied between 125 to 8000 bp. The range of similarity coefficient was from 0.83 to 1.0 among the different sub-cultures and mother plant (MP). It was reported that no change occurred between the mother plant and the PLBs produced after 3 months of culture, but 17% dissimilarity reported after 6 months of subculture.

The genetic distance and relationships of 149 accessions representing 46 species in the genus *Phalaenopsis* and four species in *Paraphalaenopsis* were studied using random amplified polymorphic DNA (RAPD) markers. A total of 20 random primers were screened and out of these, six random primers provided 123 polymorphic bands and zero monomorphic bands (Goh *et al*., 2005).

In the present study, M1 mother plant recorded the highest average polymorphism of 19.7% and M3 mother plant recorded the least average polymorphism of 8.18%.

5.2.5 Comparison of the M1 mother plants with clones using NTSYS software

Using NTSYS software, the similarity coefficients for first, second and third plant between M1 mother plant, M2 mother plant and M3 mother plant and corresponding regenerants were 0.91, 0.92 and 0.93 respectively. In fourth plant, the similarity coefficient exhibited 100% similarity between mother plant, the first clone C1 and third clone C3.

These high variations could possibly be due to the naturally occurring variation or due to the accumulation of mutation by various factors such as *in vitro*

regeneration process and its duration, auxin to cytokinin ratio (hormonal balance), and *in vitro* stress induced by added biochemicals all of which are known to induce somaclonal variation (Devarumath *et al.,* 2002).

In the present study, the multiple shoots produced from inflorescence node are adventitious form and the time interval between the extraction of DNA from mother plants and regenerants were three to four weeks interval. This might have been some factors leading to the variations.

The genetic distance and relationships of 149 accessions representing 46 species in the genus *Phalaenopsis* and four species in *Paraphalaenopsis* were studied using random amplified polymorphic DNA (RAPD) markers. Pairwise genetic distances between accessions were estimated according to Nei and Li, (1979). Cluster analysis of data using the UPGMA algorithm placed the species in seven groups based on morphological characters (Goh *et al*., 2005).

Khoddamzadeh *et al.,* (2010) reported that RAPD primers were used to study somaclonal variation among the mother plant, the initially induced PLBs and proliferated. Eight out of twelve primers produced 172 bands with 18 polymorphic bands in all the treatments. The range of similarity coefficient was from 0.83 to 1.0 among the different sub-cultures and mother plant (MP). It was reported that no change occurred between the mother plant and the PLBs produced after 3 months of culture, but 17% dissimilarity reported after 6 months of subculture.

Random amplified polymorphic DNA (RAPD) analysis was conducted to determine their genetic distances and relationships. Among 20 different primers used for RAPD analysis, 10 primers showed polymorphism and 26 to 54 DNA fragments were amplified. The highest value of Similarity index was 0.28 between *Ph. Violacea malaysia* and *Ph. violacea witte*. The dendrogram resulting from UPGMA (Unweighted Pair Group Method using Arithmetic average) hierarchical

cluster analysis separated the 20 *Phalaenopsis* species into three groups (Atienzar *et al*., 2002)

Chang and Veilleux, (2009) studied the genetic variability of 16 *Phalaenopsis* species using amplified fragment length polymorphism (AFLP) markers. Ten AFLP primer combinations amplified 1353 DNA fragments ranging in size from 100 to 350 bp and 1285 (95%) of them were polymorphic. The genetic similarity among *Phalaenopsis* species and hybrids ranged from 0.298 to 0.774 based on Dice coefficient.

Huang *et al*. (2009) were used a set of 13 expressed sequence tag (EST)-derived simple sequence repeat (SSR) to analyze 103 cultivars of six species of Chinese orchid (*Cymbidium* spp.). The 13 SSR primer pairs generated a total of 168 polymorphic bands, with an average of 12.92 bands per primer and a range of 6–24 bands which clearly revealed the difference between cultivars inter- or intra-species of Chinese orchid. Cluster analysis based on UPGMA method showed a dendrogram with three basic clusters.

Moe *et al*. (2010) studied polymorphic analysis using 14 newly developed SSRs, a total of 201 alleles across 96 *Cymbidium* accessions were detected with an average of 14.4 per locus. The average gene diversity and polymorphism information content values were 0.394 and 0.639, respectively. The mean genetic similarity coefficient was 0.4297, indicating a wide genetic variation among the *Cymbidium* accessions.

In the future, Performance of regenerants can be compared with mother plants with respect to floral characters. Present protocol can be used with suitable modification for large scale production of other *Phalaenopsis* varieties

Summary

6. SUMMARY

The study entitled "Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid Winter Spot with molecular marker" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2013- February 2016, with the objective to regenerate tissue culture plants of *Phalaenopsis* through viable protocols and examine suitability of the same for micropropagation with genetic stability analysis using RAPD markers.

The findings of the study are as follows:

- Among the explants namely viz, inflorescence node, leaf segment and root segments used for tissue culture study hybrid Winter spot of *Phalaenopsis*, inflorescence node was the best with respect to culture response.
- The best surface sterilization treatment for leaf identified was treatment 20 minutes 0.1% bavistin + 2 drops prill , 1 minute dip in 70 per cent ethanol and 8 minutes 0.1 per cent HgC_{l2} which give maximum per cent of culture survival and minimum contamination rate.
- The best surface sterilization treatment for flower stalk identified was 20 minutes 0.1% bavistin + 2 drops prill , 1 minute dip in 70 per cent ethanol and $HgCl_2$ 0.1% 7 min.
- From different basal media (full MS and ½ MS) tried, response was observed only in the medium of Full MS with $2mgl^{-1}$ of TDZ, for the explant of inflorescence node. In other media, inflorescence node exhibited bulging of bud and later turned to brown. Leaf explants remained green up to 2 weeks, thereafter started drying. Root segment remained as such without any change

- MS medium supplemented with BA and TDZ was found to give good shoot regeneration from the explants of inflorescence node. In the medium MS + 2 mgl^{1}

TDZ recorded highest percentage (80%) of culture establishment, followed by MS + 4.5 mgl^{-1} of BA with 55 per cent of sprouting.

- Among the explants tried, inflorescence node sprouted and established. Root segment remained as such without any change, whereas leaves explants remained green up to 2 weeks, thereafter started drying in all the growth regulators combination.
- For establishment of shoot, MS medium supplemented with coconut water at 10%, shoot regeneration was observed in15 days after inoculation. Addition of activated charcoal (1-5gl^{-1}), bulging of dormant bud of the inflorescence node was observed after 28 days of inoculation. With the addition of adenine sulphate and banana pulp in the medium, there was no remarkable growth promotion for explants.
- For induction of multiple shoot, MS medium supplemented with 4.5 mgl^{-1} BA resulted in the highest average number of multiple shoot (4.1). The percentage of multiple shoot induction was observed maximum 86.6 % in MS + 4.5 mgl^{-1} BA followed by MS+ 4.5 mgl^{-1} BA+ 0.1mgl^{-1} IAA with 80 per cent. The number of days taken for multiple shoot induction ranged from 40 to 45 days.
- For elongation and rooting, MS medium supplemented with BA 4.5mgl^{-1} +IAA 1mgl^{-1}, 80 % rooting was observed. Root initials were observed 50 days after inoculation. Plantlets recorded good root characters with 3 roots per plant with mean root length of 2.98cm. In the media MS +Kinetin 0.1mgl^{-1} + 1mgl^{-1} NAA recorded good root formation.
- The potting media, charcoal, brick pieces and sphagnum moss ratio of 1:1:1 was found ideal for hardening of *Phalaenopsis* winter spot with 100% survival.
- Good quality DNA was isolated from tender leaf using the protocol suggested by Doyle & Doyle, (1990) was used for extraction of genomic DNA from young tender leaves.
- The yield of DNA in the present study ranged from 136.80 ng/µl to 1032 ng/µl and the purity (A_{260}/ A_{280}) of DNA ranged from 1.9 to 2.0.

- Out of twelve RAPD primer reported Khoddamzadeh *et al*. (2011), six primers were selected based on DNA amplification pattern. Three clones were compare with their source mother plant were subjected to RAPD assay.
- In M1, mother plant and regenerants from inflorescence node exhibited 19.7 per cent polymorphism when DNA was resolved with primer OPU13, OPU15, OPU16 and P12.
- In M2, mother plant and regenerants exhibited 11.4 per cent polymorphism with primers OPU10, OPU 15 and P12.
- In M3 mother plant, out of the six selected primers tested, OPU 13, OPU16 and P16 exhibited polymorphism and in M4 mother plant, primers like OPU10, OPU15 and P12 exhibited polymorphism. The average polymorphism observed was 8.15% in M3 and 15.8% in M4.
- The combined RAPD primers data in comparing the mother plants and regenerants using NTSYS software, the similarity coefficients were 0.91 for M1 mother plant, 0.92 for M2 mother plant and 0.93 for M3 mother plant.
- In M4 mother plant, the similarity coefficient obtained from NTSYS pc software showed that the mother plant and the first C1 and third C3 clones exhibited 100% (1.0) similarity. The clone C2 exhibited 0.91 (91%) similarity coefficient with clones C1, C3 and mother plants.

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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 USA	:	Nanodrop®Technologies lnc.

ANNEXURE II

Chemical composition of the Murashige and Skoog medium

Stock	Chemical	mg/litre	Stock concentration	Stock
I	$(NH_4)NO_3$	1,650	50 X	82.5g l^{-1}
	KNO_3	1,900		95.0g l^{-1}
	KH_2PO_4	170		8.5g l^{-1}
	$MgSO_4.7H_2O$	370		18.5g l^{-1}
II	$CaCl_2.2H_2O$	440	50 X	22.0g l^{-1}
	(Prepare the stock separately or it may precipitate)			
III	Na_2EDTA	37.3	100 X	3.7g l^{-1}
	$FeSO_4.7H_2O$	27.8		2.8g l^{-1}
(Remember to prepare this as described under stock solution preparation)				
IV	$MnSO_4.7H_2O$	22.3	100 X	2.23g l^{-1}
	$ZnSO_4.7H_2O$	8.6		860mgl^{-1}
	H_3BO_3	6.2		620mgl^{-1}
	Kl	0.83		83.0mgl^{-1}
	$Na_2MoO_4.2H_2O$	0.250		25.0mgl^{-1}

	$CuSO_4.5H_2O$			
	$CoCl_2.2H_2O$			
V	Vitamins			
	Glycine	2.0	100X	$200mgl^{-1}$
	Nicotinic acid	0.5		$50mgl^{-1}$
	Pyridoxine acid – HCL	0.5		$50mgl^{-1}$
	Thiamine – HCL	0.1		$10mgl^{-1}$

$100\ mg\ l^{-1}$ myo – inositol

$30\ g\ l^{-1}$ sucrose

$2\ g\ l^{-1}$ CleriGel

pH 5 – 7 – 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 0ºC 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.

Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid winter spot with molecular marker

By

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ABSTRACT OF THESIS

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Abstract

Phalaenopsis “Moth Orchids” are among the most beautiful flowers in the world. This genus has economic value for pot plant and cut flower production and is distributed throughout Southeast Asia. Most popular method of propagation for orchid is through *in vitro* propagation, as it produces large number of clones in relatively short duration. Despite its potential to produce numerous plants from a single leaf segment, it is liable to unpredictable mutations or somaclonal variation during the process of multiplication. Variation can arise due to many reasons such as type of media, plant growth regulators and its concentration, type of explants and number of subculture cycles. The percentage of the variation can range from 0-100% depending on varieties with an average of 10% among *Phalaenopsis* (Tokuhara and Mii, 1993).

So the present investigation on “Development of an *in vitro* regeneration system and validation of genetic stability in *Phalaenopsis* hybrid Winter Spot with molecular marker” was taken up at the Center for Plant Biotechology and Molecular Biology, College of Horticulture from 2013-2016.

Flowering mother plants of *Phalaenopsis* hybrid Winter Spot were used as explant source**.** Among the explants namely inflorescence node, transverse thin cell layer of leaf and root segments used for tissue culture study in this orchid, inflorescence node was the best with respect to culture response.

The best surface sterilization treatment for leaf explants identified was treatment 0.1% bavistin + prill 2 drops (30 min) and 0.1 per cent $HgCl_2$ (8 min) which give maximum per cent of culture survival and minimum contamination rate. The best surface sterilization treatment for inflorescence node identified was treatment with 0.1% bavistin + 2 drops prill (30 min) , one minute dip in 70 per cent ethanol and 0.1% $HgCl_2$ (7 min).

From different basal media (full MS and ½ MS) tried, response was observed only in the medium of Full MS for inflorescence node. Among the different growth regulators tried, MS medium supplemented with BA and TDZ was found to give good shoot regeneration from inflorescence node explants. MS +2mgl^{-1} TDZ recorded highest percentage (80%) of culture establishment, followed by MS + 4.5 mgl^{-1} of BA (55%) per cent of sprouting. Among the explants tried, only inflorescence node responded with sprouting. Root segment remained as such without any change, whereas leaf explants remained green up to 2 weeks, thereafter started drying in all the growth regulators combination.

For induction of multiple shoot, MS medium supplemented with 4.5 mgl^{-1}BA resulted in the highest average number of multiple shoot (4.15). Elongation and rooting was observed in MS medium supplemented with BA 4.5mgl-1 +IAA 1mgl^{-1} with 80 % rooting. Root initials were observed 50 days after inoculation. The potting media, charcoal, brick pieces and sphagnum moss in the ratio of 1:1:1 was found ideal for hardening of *Phalaenopsis* hybrid winter spot with 100% survival.

Genetic stability studies using RAPD marker were carried out with the mother plants along with three regenerants each. Six primers were selected based on DNA amplification pattern. In RAPD assay, M1 mother plant recorded the highest average polymorphism of 19.7% and M3 mother plant recorded the least average polymorphism of 8.18%.

Using NTSYS software, the similarity coefficients for first, second and third plant between M1 mother plant, M2 mother plant and M3 mother plant and corresponding regenerants were 0.91, 0.92 and 0.93 respectively. In fourth plant, the similarity coefficient exhibited 100% similarity between mother plant, the first clone C1 and third clone C3.

The established micropropagation protocol can be used with suitable modification for large scale production of other *Phalaenopsis* varieties.