

***IN VITRO PROPAGATION OF ORCHID***  
***(Dendrobium formosum Roxb ex Lindl)***

A

THESIS SUBMITTED TO  
THE ORISSA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY  
BHUBANESWAR

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE  
DEGREE OF

**MASTER OF SCIENCE IN AGRICULTURE**  
**(FLORICULTURE & LANDSCAPING)**

BY

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This is to certify that the thesis entitled “*IN VITRO* PROPAGATION OF ORCHID (*Dendrobium formosum* Roxb ex Lindl)” submitted to the Orissa University of Agriculture and Technology, Bhubaneswar in partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE IN AGRICULTURE (FLORICULTURE & LANDSCAPING)** is a faithful record of *bonafide* research work carried out by **SAGARIKA MUNA** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma or published in any other form.

The assistance and help received during the course of investigation have been duly acknowledged.

Place: Bhubaneswar

Date: 31.7.2019


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This is to certify that the thesis entitled “*IN VITRO* PROPAGATION OF ORCHID (*Dendrobium formosum* Roxb ex Lindl)” submitted by SAGARIKA MUNA to the Orissa University of Agriculture and Technology, Bhubaneswar in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE IN AGRICULTURE** in the discipline of **FLORICULTURE & LAND SCAPING** has been approved by the Student’s Advisory Committee after an oral examination on the same in collaboration with the External Examiner.

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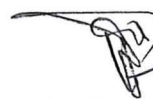
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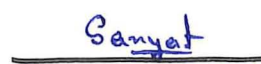
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## **ACKNOWLEDGEMENT**

*The precious gift of learning is a debt that is difficult to pay. Only gratitude can be felt. Mere words can never suffice to express the sense of gratitude to those whose assistance was indispensable for the completion of the present study. The piece of work assigned to me has come into reality with success and the success is nothing but the reflection of mutual efforts.*

*I would like to avail this opportunity of expressing my deep sense of gratitude and reverence to Dr. Sashikala Beura, Associate professor, Department of Floriculture and Landscaping and Director, Biotech-cum Tissue Culture Centre, College of Agriculture, OUAT, Bhubaneswar for suggesting the problem and providing all valuable guidance for undertaking the research work, this untiring attention, encouragement, expert advice and close monitoring during the entire period of investigation which were the pillars of success in this endeavour. I am very much thankful to him for his sustained interest and constant guidance to complete the study.*

*I am very much thankful to Dr. C.R. Mohanty, Professor and Head, Department of Floriculture and Landscaping College of Agriculture, OUAT, Bhubaneswar for his guidance, cooperation, encouragement and valuable suggestions during the course of investigation and preparation of this manuscript.*

*I am very much thankful to Dr. P.N. Jagdev, Professor, Department of Plant Breeding and Genetics, College of Agriculture, OUAT, Bhubaneswar for providing necessary facilities to carry out my research work,*

*I felt greatly indebted and convey my profound sense of gratitude to my esteemed teachers Professor, Dr. T.K. Das, Professor, Dr. J.N. Das, Department of Floriculture and Landscaping for their help and encouragement during my course of study.*

*I pay my due acknowledgement to the Dean, College of Agriculture, OUAT, Bhubaneswar for providing necessary facilities to carry out my research work,*

*Also I am thankful to Dolli and Pravati madam and Barika sir all the staff members of the Department and Tissue Culture Centre for their help during completion of my thesis work,*

*I pledge my heartiest appreciation to my seniors Anchal didi & Rasmita didi and my dearest friends Bina, Harapriya, Monalisha, Laxmi, Dibya, Jineeta, Swarnasarika, Puja and Roseline for their unhesitant help for giving the work final shape.*

*I would like to acknowledge my deep sense of regards to my sisters and brother for their constant encouragement and valuable friendly guidance.*

*Although it is like a drop in the ocean, yet, I do express my infinite gratitude to my much beloved parents and my late grandparents for their supreme sacrifice, lovable inspiration and eternal beautification in evaluation of this tiny personality and elders whose love, blessings and constant inspiration never gave me a chance to fall in this endeavour and paved the way for all successes in my career.*

*I bow down my head before my beloved parents with all veneration and devotion for their blissful blessing, selfless sacrifices and eternal benediction which has been instrumental in every success in my career. I still pray their blessing to be bestowed upon me on the every sphere of my life.*

*Last but not the least, with heartfelt devotion; I solicit the benedictions of almighty 'God' who is most benevolent to guide me to face the challenges in life.*

*I thank all those who directly or indirectly helped me during my research.*

Bhubaneswar

Date: 31.07.2014

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Degree for which the Thesis is Submitted : Master of Science in Agriculture (Floriculture and landscaping)

Year of submission : 2014

## **ABSTRACT**

An experiment was carried out in the Biotechnology- cum- Tissue Culture Centre Dept. of floriculture & Landscaping CA,OUAT Bhubaneswar, during the year 2013-2014 to study the “ *In vitro* propagation of orchid (*Dendrobium formosum* Rox ex Lindl) with objectives for Standardization of media supplements for shoot proliferation , Standardization of media supplements for root initiation and development of orchid(*Dendrobium formosum*) and standardization of media mixture for hardening of *in vitro* plantlets of *Dendrobium* hybrid Sonia-17.

The result revealed that MS medium fortified with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.1 mg/l GA<sub>3</sub> was found to be best for production of more no of PLBs/ explants (7.13 &9.27) and enhancement of length of PLBs (2.00 &2.03cm) and length of the leaf (1.16 &1.46) after an interval of 30 days of culture &sub culture respectively.

MS medium supplemented with 3.0 mg/l BAP, 1.5 mg/l IAA, and 0.1 mg/l NAA was found to be most potent combination for production of more no of shoot/explants (4.2 &8.0) more no of leaves /shoot mass (9.48 &11.48) and longer leaves (2.2 &2.36) after 60 and 90days of subcultures respectively.

As regards to root initiation & formation MS medium fortified with 1.0 mg/l IBA and 1.0 mg/l NAA was found to be best for obtaining higher number of root (3.5) and longer roots after 60 days of culture.

In another set of investigation for hardening of the *in vitro* grown *Dendrobium* cv. Sonia 17 plantlets it has been observed that 3 pieces coconut husk tied with the ex-agar plantlets and planted in the protray containing cocopeat significantly enhanced the formation of new leaves/plantlet (5.3), no of roots/plantlets(7.5), length of the root (2.5cm) and percentage of survival (96.67%) of the plantlets.

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## ABBREVIATIONS

% -	:	Percentage
@ -	:	At the rate of
/	:	Per
°C	:	Degree Celsius
L -	:	Linus
&	:	and
am	:	Ante meridiem
BAP	:	Benzyl Amino Purine
Ca	:	Calcium
C.D	:	Critical difference
cm -	:	Centimeter
cv.	:	Cultivars
DAI	:	Days After Inoculation
DAP-	:	Days After Planting
DAT	:	Days After Transplanting
DAE	:	Days After Establishment
Dm	:	Decimeter
d.f. -	:	Degree of freedom
e.g. -	:	Example
et al.	:	And others
Fig. -	:	Figure
g -	:	Gram
GA <sub>3</sub>	:	Gibberllic acid
HCL	:	Hydrochloric acid
IAA	:	Indole 3-Acetic Acid
IBA	:	Inodole 3-Buteric Aacid
i.e. -	:	That is
KC	:	Knudson C medium
KN	:	Kinetin
lt. -	:	Liter
m -	:	Meter
mg -	:	Miligram
ml-	:	Mili litre
MM	:	Modified Malmgren
MS	:	Murashige and Skoog
N	:	Normal
NAA	:	Napthalene Acetic Acid
NaOH	:	Sodium hydroxide
PBRs	:	Plant Bio Regulators
PLBs	:	Protocorm Like Bodies
S.E. -	:	Standard Error
Wt -	:	Weight
Z	:	Zeatin
2,4-D	:	2,4-Dichlorophenoxy acetic acid

## CHAPTER-I

---

# *Introduction*

# INTRODUCTION

---

Orchids, the most beautiful flowers in god's creation, comprise a unique group of plants. Taxonomically, they represent the most highly evolved family among monocotyledons with 600-800 genera and 25,000-35,000 species. Orchids exhibit an incredible range of diversity in size, shape and colour of their flowers. They are most pampered of the plants and occupy top position among all the flowering plants valued for cut flower production and as potted plants. They are known for their longer lasting and bewitchingly beautiful flowers which fetch a very high price in the international market.

Orchids are distributed all over the world except in the Antarctica. They are abundantly found in tropical countries in South East Asia, South and Central America and South Africa, also found distributed in China, Japan, Philippines, Europe and Australia. Epiphytic orchids are mainly distributed in the northern Andes of South America, Madagascar, Sumatra and Borneo while, Indo-China harbor both epiphytic and terrestrial species and South Western Australia believed to be the centre for terrestrial orchids. India is very rich in orchid flora. Orchids are found distributed in different parts of the country having different altitude, rainfall and temperature. They are located at an altitude up to 5000 meters from the sea level and in areas with rainfall ranging from 60 to 1100 cm per year. Epiphytic orchids are distributed up to 1800 meters and their frequency decreases with the increase in altitude. Most of the terrestrial orchids are limited to the temperate zone. In India, five major orchid zones such as plains, tropical, sub-tropical, temperate and sub-temperate, alpine and sub-alpine zone are classified based on their occurrence in different vegetations. Majority of orchids in India are distributed in the Eastern Himalayas, the Western and South Indian hills.

Orchids are unique group of plants that exhibit an incredible range of diversity in size, shape, structure; colour and fragrance of the flower. Many orchids are useful for small scale industries. Glue prepared from the species of *Cryptopodium*, *Geodorum nutans* and *Bletilla* are utilized for the manufacture of musical instruments. Yellow pseudostems of *Dendrobium utile* are used as ornaments in New Guinea by tribal people. *Dendrobium* stems are used in making baskets in the Philippines, Indonesia and New Guinea. In several parts of the world, orchids are used as food. Roasted Bulbs of *Gastrodiasesamoides* were used as food during emergency situation. Dried leaves of *Dendrobium salaccense* are used as flavoring agent during cooking of rice. In islands of Cyprus, milk custard drink is prepared from dried ground tubers of *Orchisanatolica*. Pseudobulbs of *Cymbidium madidum* and *Dendrobium speciosum* are also reported to be used as food. Root tubers of *Disa*, *Habenaria* and *Satyrium* are used to make food dish called Chikanda or Kinaka in Zambia. Powdered roots of *Vanda tessellate* are used as antidotes of poisoning, rheumatic pain and abdominal complaints. Different parts of orchid are used in treatment of various diseases. Juice obtained from the leaves of *Cymbidium aloifolium* are used for the treatment of otitis and other inflammatory conditions. In Malaysia, the decoction of leaves prepared from *Nervilia aragoana* is used as a protective medicine after childbirth. Roots of *Vanda tessellate* which forms constituents of medicated oils and used for rheumatic swellings and nervous disorders (Narasimhan, 1996). In the Khasi hills, juice prepared from the leaves of *Cymbidium giganteum* is used to prevent blood flow in wound, while powdered flower of *Vanda spathulata* is effective against hysteria. *Dendrobium fimbriatum* is used against liver upsets and nervous disability.

Orchids are valued for cut flowers due to their wonderful nature of flowers and extended keeping qualities. Orchids are mostly grown in parks, gardens and urban areas. Some orchids, flowers are long lasting for one to three months when remain attached to the plant while, cut flowers remains fresh for one to four weeks. It was

reported that flowers of *Dendrobium crumenatum* last for a day, while, the flowers of *Grammatophyllum multiflorum* last for about 270 days. Attri *et al.* (2008) reported that un-pollinated flowers of *Aerides multiflora* and *Rhynchostylis retusa* can remain fresh for 17 and 24 days, respectively.

*Dendrobium* is a huge genus of orchids. It was established by Olof Swartz in 1799 and today contains about 1,200 species. The genus occurs in diverse habitats throughout much of south, east and southeast Asia, including the Philippines, Borneo, Australia, New Guinea, Vietnam, Solomon Islands and New Zealand. The name is from the Greek *dendron* ("tree") and *bios* ("life"); it means "one who lives on trees", or, essentially, "epiphyte".

*Dendrobium* species are either epiphytic, or occasionally lithophytic. They have adapted to a wide variety of habitats starting, from the high altitudes in the Himalayan mountains to lowland tropical forests and even to the dry climate of the Australian desert.

This genus of sympodial orchids develop pseudo bulbs, and the length varies from less than a centimeter (e.g. *Dendrobium leucocyanum*) to several metres long (e.g. *Dendrobium discolor*), resembling canes. A orchids few grow into long reed like stems. Leaf bases form sheaths that completely envelope the stem. In the section *Formosae* (e.g. *Dendrobium infundibulum*), the sheaths and undersides of leaves are covered with fine short black hairs. Other species (e.g. *Dendrobium senile*), are covered with fine white hairs.

These orchids grow quickly throughout summer, but take a rest during winter. Dormant buds erupt into shoots from the base of the pseudo bulb mainly in spring, and a few species in autumn. This is then followed by rapid growth of new roots. Reproduction is usually through seed, but a few species reproduce asexually through keikis produced along the stem, usually after flowering and sometimes as a result of injury to the growing tip.

Generally, orchids are propagated both asexually and sexually. But the traditional asexual propagation is extremely slow which can give rise to 2-4 plants per year (Nasiruddin *et al.*, 2003). Micropropagation of orchids is the most frequently used convenient technique for their exploitation as a major trade in developed countries (Sagawa and Kunisaki, 1982). It is the only method currently available which initially starts in early 1960. Because commercially mass propagation is possible by producing millions of plantlets using tissue culture techniques (Lim *et al.*, 1985). Orchids can be rapidly propagated through tissue culture techniques by using shoot tips (Saiprasad *et al.*, 2002), leaf, and stem nodes (Pathania *et al.*, 1998). Due to difficulties in protocorm regeneration and plantlet formation, all procedures mentioned above have become inadequate for meeting commercial need for vegetative propagation. Some of these methods gave a lot of protocorms but they develop slowly or poorly to vital plants. Therefore, the present study was undertaken to investigate the concentrations and combinations of plant growth regulators is better for plantlet regeneration and the subsequent growth and development of *Dendrobium formosum* and *Dendrobium* hybrid Soni-17.

Large-scale multiplication of orchids, especially rare hybrids and endangered species using tissue culture techniques has helped orchids occupy a position as one of the top ten cut flowers. Orchid propagation by seed caused to the production of heterozygous plants. Thus, *in vitro* proliferation is a suitable alternative procedure for propagation of orchids. Different protocols have been established for micropropagation of orchids species through *in vitro* culture of various parts consisting shoot tip, root tip, stem, leaf, node, bud, inflorescence and rhizome, as well somatic embryo, callus and thin cell layer ( Park *et al.*, 2000, Ket *et al.*, 2004, Sheela *et al.*, 2004, Kalimuthu *et al.* 2006.).In comparison to plantlet development from seeds or adventitious shoots, the micropropagation through PLBs is more efficient because PLBs can be rapidly proliferated on solid or in liquid culture medium, and a large number of PLBs can be provided in a short period . Many studies have revealed

that the optimization of medium composition was an important approach to improve the micropropagation process of orchids by culturing PLBs that is species-specific. In order to stimulate efficient micropropagation PLB, much effort has been done to modify the culture media, mainly by inclusion of plant growth regulators (Nayak *et al.* 2002,) such as BA, thidiazuron (TDZ), N6-benzylaminopurine(BAP), NAA, 3-indoleacetic acid (IAA) and gibberellic acid (GA3) (Prakash *et al.*, 1996,Roy and Banerjee 2003, Saiprasad *et al.*, 2004, Roy *et al.*, 2011). *In vitro* protocols have been standardized for many orchids species and varieties however the techniques involved in terms of *in vitro* nutrient media, PBRs and media mixture for hardening are specific for specific orchids. Hence there is an argent need for further studies to standardize the techniques to protect high value endangered species for commercial mass multiplication.

Therefore keeping the above fact in view, the presented study on *In vitro* propagation of orchids (*Dendrobium formosum* Roxb *ex Lindl*) has been proposed with the following objectives--:

- ❖ Standardization of media supplements for shoot proliferation of orchid.
- ❖ Standardization of media supplements for root initiation and development.
- ❖ Standardization of media mixture for hardening of the *in vitro* plantlets.



CHAPTER-II

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*Review of Literature*

# REVIEW OF LITERATURE

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In this chapter the literature on in vitro propagation of Orchids which include media preparation and media supplements shoot proliferation, root formation and hardening. Conducted in different research ambiance of different scientist have reviewed.

## *Dendrobium*

*Dendrobium* Orchids are the most popular orchid genus, and the traditional asexual propagation is extremely slow which can give rise to 4 plants per year. *In vitro* culture has proved particularly useful with groups of plants, which are difficult to propagate using conventional techniques (Fay, 1994). When mass propagation of new hybrid or a variety is needed within a short time, advantage of tissue culture techniques is used for the purpose. Hence, mass propagation of orchid in commercial scale now a days is possible by tissue culture techniques (Lim *et al.*, 1985). Comparatively a rapid multiplication of orchid was reported by Intuwong and Sagawa (1974) using shoot tip of a plant, but excision of shoot tip or auxiliary bud can kill or damage the mother plants seriously. To avoid this problem, the multiple shoot proliferation techniques using different hormones tried (Sagawa, 1974). In recent times the application of tissue culture techniques has opened a new frontier for the clonal propagation of orchids (Morel, 1964).

The validity of asymbiotic seed germination was firmly establishment and the original medium developed by Knudson was improved to germinate the other species of orchids. Inorganic salts, carbohydrates, organic substances like vitamins, amino acids, growth hormones, water and proper pH along with optimum light and temperature were necessary for success in germination. The procedure of asymbiotic seed culture with respect to media, media supplements and other related factors influencing the germination of seeds and seedling growth are reviewed below.

## 2.1 CULTURE MEDIA

Several standardized media have been used for the germination of orchid seeds. The commonly used nutrient media for orchid seed culture are those proposed by Knudson (1946), Vacin and Went (1949), Murashige and Skoog (1962) and Nitsch (1967). Media such as Knudson C, Vacin and Went are broad spectrum used for seed germination in many orchid species.

Vacin and Went (1949) reported higher percentage and faster seed germination in liquid culture of orchid seed as compared to that cultured in semi solid VW medium.

Irawati *et al.* (1977) reported that the best growth and survival rates were obtained in *Dendrobium* when cultured in Knudson C medium.

Effect of media on seed germination has also been reported by Singh and Prakash (1985) who found that Knudson C medium or Vacin and Went medium supplemented with coconut water (150 ml) were most ideal for germination of tropical orchid seeds.

Devi *et al.* (1990) pointed out the preferred medium for *Dendrobium* seed germination which varied with the species. *Dendrobium farmari* and *Dendrobium primulinum* gave 50-60 percent higher seed germination on Vacin and Went medium.

Reddy (1992) reported that MS medium was rich in vitamin and micronutrients which gave better result than other media especially for tropical orchids.

Zhang *et al.* (1992) stated that since MS medium contained high ionic concentration of nutrients, half strength MS media could adequately support rapid protocorm production in orchids, as MS medium contained high ionic concentration of nutrients.

The morphological potential of shoot tip explants procured from axenic seedlings was investigated by Devi (1997) in *Dendrobium moschatum* and *Cymbidium aloifolium* for production of virus free orchids. Out of the five different media tested, Nitsch medium was found to be the best for formation and proliferation of protocorm like bodies. However, in a similar study Abhijit *et al.*(1998) reported that Knudson C medium was effective for inducing seed germination in *Dendrobium aphyllum*.

Pathania *et al.* (1998) reported that stem explants procured from shoots emerging from *Dendrobium cv.Sonia* pseudobulbs grown in Vacin and Went and Knudson C media favoured formation of protocorm like bodies(PLBs)and subsequent development of plantlets. KC medium supplemented with BAP (1.5mg/l), NAA (0.4 mg/l) and paclobutrazol (i.0 mg/l) was best for further multiplication of PLBs.

Sobhana and Rajeevan (2002) found that MS medium at  $\frac{1}{4}$  strength, Vacin and Went and Knudson C supplemented with Kinetin, BAP, NAA and IBA had profound influence on seedling growth as well as root length and number in *Dendrobium New pink* × *Emma White* hybrid seedlings.

Raju *et al.* (2003) observed that MS medium was the best for initial culture establishment of *Dendrobium osterholt*, 60 days after inoculation with a higher rate of multiple shoot production and higher average shoot length. The maximum rooting was recorded as a result of application of IBA 1mg/l+NAA 1mg/l with  $\frac{1}{2}$  strength MS medium.

Aktar *et al.* (2005) cultured PLBs of *Dendrobium* orchid. in Knudson C (KC), Vacin and Went (VW), half strength Murashige and Skoog ( $\frac{1}{2}$ MS) and New Phalaenopsis (NP) media supplemented with Sabri banana pulp (Sb), Charcoal (C) and Coconut water (Cw) organic extracts and stated that. KC, VW,  $\frac{1}{2}$ MS and NP

media significantly influenced the plantlet regeneration of *Dendrobium* orchid. The longest shoots were found in KC medium and maximum length of leaves was found in both KC and ½MS media.

Aktar, *et al.* (2008) reported that Interaction of different media and organic additives showed significant effects on growth and development of protocorm like bodies (PLBs) and subsequent plantlets regeneration of *Dendrobium* orchid in laboratory conditions. Except the length of shoots and leaves, the highest values of all parameters were obtained from the interaction of ½MS medium with Sabri banana pulp.

## **2.2 GROWTH REGULATORS AND THEIR EFFECTS ON:**

### **2.2.1 Seed germination**

Several growth regulators used in seed germination such as auxin, IAA, GA3, cytokinin, 2-4 D, IBA are most common

Sobhana and Rajeevan (2003) reported that very high percentage of germination was noticed in the cross of Emma white (EW)× New pink (NP), NP× HB, NP×EW and Sonia-28 NP of *Dendrobium* in *in vitro* condition..

Pradha *et al.*, 2009 two orchid species, viz., *Cymbidium elegant* Lind. And *Dendrobium densiflorum* Lind. ex Wal. on MS medium, supplemented with different concentrations of PGRs and stated that hormone free MS basal medium was found to be most effective for seed germination of *D. densiflorum*; whereas, basal medium supplemented with BAP (1mg/l) was effective for *C. elegans*

### **2.2.2 CALLUS INDUCTION**

Madera *et al.* (2003) conducted a study on somatic embryogenesis and plant regeneration with the use of plant growth regulators by using young leaves and nodes of *in vitro* cultured plantlets of *Grammatophyllum scriptum* and reported that

Modified Vacin and Went (MVW) medium supplemented with 2, 4-D (0 to 10 nM) induced callus formation.

Roy *et al.* (2003) cultured Shoot-tip explants of *Dendrobium fimbriatum* Lindl. Var. *Oculatum* Hk. F. on modified nutrient solution of Knudson's C supplemented with 10% (v/v coconut water, 0.5 mg l<sup>-1</sup> niacin, 0.5 mg l<sup>-1</sup> pyridoxine HCL, 0.5 mg l<sup>-1</sup> thiamine HCL and different concentration of N<sup>6</sup>- benzylaminopurine (BAP)

Huan *et al.* (2004) induced embryogenic calli from longitudinally bisected segments of protocorm like bodies (PLBs) of cymbidium twilight moon 'Day Light', on modified Vacin and Went medium supplemented with 0.1 mg/l NAA and 0.01 mg/l TDZ and calli proliferated very well when being subcultured every 4 weeks on the same medium.

Embryonic calli were produced from segments of young internodes of flower stalks of *Vanda Sanderiana* cultured *in vitro* on 1.2 times of Vacin and Went's basal medium supplemented with 1mg 6-benzylaminopurine (benzyladenine)/l, 0.1 mg kinetin/l and 20g sucrose/l & further cut internodes, 3 mm thick each, from 3-4 cm long flower stalks were the most suitable for the production of embryonic calli. (Linc, 2005).

Maridass *et al.* (2010) reported that *Dendrobium nanum* explants of rhizome buds cultured on MS basal medium with 2.0 μM/l-1NAA and 1.2 μM /l-1 kinetin induced callus & the maximum effect of shoot induction (15.78 ± 0.37mm) was observed on basal medium supplemented with 0.5 μM /l-1 BAP .

### 2.2.3 Protocorm formation

Park *et al.* (2000) immature seeds from unripe capsules of *calanthe sieboidii*, were sown on one of three sterilized media: MS; modified MSH (i.e., the inorganic salts of MS plus the organic elements of Schenk and Hildebrandt); and Hyponex and

stated that germination and protocorm development occurred on the MS and MSH media within eight weeks, however percent germination was low.

Efficiency of protocorm like bodies (PLBs) production as a function of various growth regulators was assessed in three orchid genera viz. *Dendrobium* 'soni', *Oncidium* 'grower ramsay' and *Cattleya leopoldii* by Saiprasad *et al.* (2002). They have stated that maximum number of PLBs were produced in BAP 1.0 mg/l and the relative ranking among plant growth regulators (highest to lowest) for obtaining maximum number of PLBs was BAP>Kinetin>NAA>2,4-D>GA<sub>3</sub>.

Talukdar *et al.* (2002) studied the embryo culture of two intergeneric hybrids of orchid. Such as *Spathoglottis plicata* x *Dendrobium densiflorum* and *Dendrobium muschatum* x *Arundina bambusifolia* and reported that embryo germinated with a very high frequency to form protocorm in Nitsch medium.

Yung *et al.* (2003) induced the totipotent callus of *Cypripedium formosanum* from seed derived protocorm segments on a quarter-strength MS medium containing 4.52 µM 2, 4-D and 4.54 µM thidiazuron. They have further reported that the callus proliferated well and was maintained by sub-culturing on the same medium and on an average, 13 protocorm-like bodies could be obtained from a piece of 4 mm callus after being transferred to the medium with 4.44 µM benzyladenine after 8 weeks of culture.

Nagaraju (2003) reported that MS medium was the best for better growth and rapid proliferation of protocorm which was regulated *in vitro* from meristem explants of *Cymbidium*. He further stated supplement of BA in the media increased total number of PLBs and BA with 0.5mg/l was optimum for the multiplication of PLBs.

Nasiruddin *et al.* (2003) conducted a study to investigate the effects of different concentrations of BAP (0, 2, 4 and 6 mg/l) and NAA (0, 0.5 and 1 mg/l) on formation of PLBs and plantlet regeneration from *Dendrobium formosum* leaf callus

and stated that the number of PLBs was the highest (10.52/explant) at 60 DAE and time required for PLB formation was the longest (32.72 days) with 0.50 mg/l 2, 4-D.

Kitsaki *et al.* (2004) investigated the effect of culture medium on the *in vitro* germination and development of protocorms and plantlets from seeds of 13 different *Ophrys* species (*O. apifera*, *O. attica*, *O. cornuta*, *O. delphinensis*, *O. ferrum-equinum*, *O. lutea*, *O. mammosa*, *O. speculum*, *O. spruneri*, *O. umbilicata*, *O. argolica*, *O. irricolor* and *O. tenthredinifera*). Mature seeds and immature seeds were cultured in a coconut milk-enriched or a pineapple enriched medium (CEM or PEM, respectively). They reported that highest percentage of protocorm formation (52%) was observed in mature seeds of *O. spruneri* in the CEM and protocorm formation was significantly lower in immature seeds than in mature seeds in both culture media.

Puchoa (2004) studied the potential of different media, supplements and conditions for reliable *Dendrobium* Orchid plantlet regeneration from *in vitro* grown leaf explants. In all three media under investigation, regeneration was via the production of protocorm-like bodies (PLBs). Maximum number of PLBs was obtained in Murashige and Skoog (MS) liquid medium agitated at 80 rpm and supplemented with 0.1 mg/l of BA, 1.0 mg/l of NAA and 15% (v/v) coconut water.

Seeds of *Cymbidium dayanum* Reichb. were sown *in vitro* and germinated to produce protocorms one month later. Protocorms elongated, and the unicellular cell absorbing hairs were initiated at the base. After two months, the protocorms grew, with the structure similar to that of rhizomes with nodes and lateral buds. (Chang and Chen *et al.*, 2005).

In *Rhynchosstylis retusa*, the Knudson C medium supplemented with peptone (1g/l), NAA (1.5 mg/l) gave best subsequent growth and development of protocorms and in *Spathoglottis plicata* on KC medium supplemented with Kin 0.1mg/l and NAA 0.1 mg/l resulted in healthier seedling growth (Madhusmita *et al.*, 2005).

Effects of six asymbiotic media (Modified Lucke, Murashige&Skoog, Lindemann, Vacin and Went, Malmgren Modified, Knudson C), four exogenous cytokinins (BA, Zea, Kin, 2-iP), and three photoperiods (0/24, 16/8, 24/0 h L/D) were examined on seed germination and early protocorm development of *Habenaria macroceratitis*. Protocorm development was enhanced on MM medium after both seven and 16 weeks & final percent of protocorm development was increased in the absence of light (0/24 h L/D) (Scott *et al.*, 2006)

Shylaraj *et al.* (2007) conducted a study to standardize a protocol for rapid mericlone of tropical Orchid *Dendrobium* cv. Sonia from the shoot tip explants. The shoot tip explants were cultured in agitated (80-100 rpm) liquid Vacin and Went medium supplemented with 3% sucrose, 2-2.5 mg/l BA and 0.5-1.0 mg/l NAA induced a large number of protocorms within a period of two weeks.

Ping *et al.* (2008) developed an efficient micro propagation protocol for *Dendrobium densiflorum* Lindl. ex Wall., through protocorm-like bodies (PLBs) from nodal stem segments using 6-benzylamino-purine (BAP) and the *Lanthanoid neodymium*. The highest percentage of explants producing PLBs (72%), with an average of 15 PLBs per explants, was induced by culturing stem segments on Murashige and Skoog (MS) medium supplemented with 5.0 mg/l BAP.

#### **2:2:4 Shoot Proliferation**

Paek and Edward (1991) cultured the rhizomes of *Cymbidium forrestii* on Murashige and Skoog medium supplemented with various growth substances. Leaf formation and development was retarded in the presence of auxin but benzyladenine (BA) could induce shoot formation *in vitro*. A 5% (w/v) sucrose concentration was most effective in shoot induction when combined with 5 mg/l BA.

Effect of growth regulators on *in vitro* seeding growth of *Spathoglottis plicata* was reported by Sangama and Singh (1994). They found that a lower level of NAA, GA3 and BA was required for higher germination of seeds and subsequent seedling

growth was better with the combination of a higher level of IBA, GA3 and BA each at 5mg/l in the KC medium.

High frequency direct shoot proliferation was induced in the shoot segments of three epiphytic Orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. A and *Dendrobium moschatum* (Buchham) Sw. on Murashige and Skoog's medium (MS) containing N6- benzyl adenine (BA) or thiadiazuron (TDZ) with the later being more effective at 2.2-4.5 $\mu$ M as observed by *Nayak et al.*(1997). In both *Dendrobium* species the number of shoot bud formation was greatly influenced by explants orientation.

An *in vitro* method was developed by Talukdar (2001) to regenerate multiple shoots of *Dendrobium aphyllum* Roxb. Upto a maximum of seven shoots were regenerated in Knudson medium supplemented with kinetin (10 mg/l), coconut water (15% v/v) and banana extract (6% v/v) within 13 weeks of culture.

Nagaraju and Das (2002) tested the shoots of *in vitro* grown cultures of *Ascocentrum ampullaceum*, *Dendrobium nobile* and *Cattleya maxima* for their response to Nitsch media with Triacontinal (0, 1, 2 and 3 mg/l). *C.maxima* showed better response with maximum number of shoots (9.2). *A.ampullaceum* showed better shoot growth (15.6 mm). Among the four concentrations, 2mg/l was optimum for number of shoots and leaves/shoots.

Pyati *et al.* (2002) cultured the nodal explants of *Dendrobium macrostachyum* on MS basal medium and MS medium supplemented with N6- Benzyladenine (BA- 2.22, 4.44 and 8.88  $\mu$ M), Kinetin (KN 2.32, 4.65 and 9.29  $\mu$ M) and coconut water (CW- 5, 10 and 15) individually or in combination with 2.69  $\mu$ M alpha-naphthalene acetic acid (NAA). Axillary shoots were induced directly from nodal explants in medium containing BA, KN or CW.

A study conducted at KAU by Sobhana and Rajeevan (2002) for refinement of culture media for growing *Dendrobium* New Pink × Emma white seedlings revealed that MS medium at 1/4<sup>th</sup> strength, Vacin and Went and Knudson-C media supplemented with Kinetin, BAP, NAA and IBA had pronounced influence on growth characters like height, leaf length and leaf number of seedlings.

Raju *et al.* (2003) observed that MS medium was the best for initial culture establishment of *Dendrobium osterholt*, 60 days after inoculation with a higher rate of multiple shoot production and higher average shoot length.

Talukder *et al.* (2003) investigated the effect of BAP (0, 1, 2.5 and 5 mg/l) and NAA (0, 0.1, 0.5 and 1 mg/l) on Orchid shoot regeneration with MS media. The best shoot proliferation, root formation, leaf number, increment of shoot length was obtained from 2.5 mg/l+ 0.5 mg/l NAA.

Ket *et al.* (2004) induced the multiple shoot proliferation of an elite cultivar of Jewel Orchid in shoot tip explants on Hyponex media supplemented with 1 mg/cubic dm benzyladenine or 1-2 mg/cubic thiadiazuron (TDZ). Addition of activated charcoal (1 g/cubic dm) to the TDZ containing medium protocorm multiple shoot formation (11.1 shoots per explant).

Leaf explants from flower stalk node derived shoots of *Dendrobium* hybrids Sonia 17 and 28 cultured on half-strength Murashige and Skoog (MS) medium supplemented with 44.4 µM N6-benzyladenine (BA) developed more than seven shoots per explants. Half-strength MS medium containing 6.97 µM kinetin (KN) facilitated conversion of more than 90% PLBs to shoots (Martin *et al.*, 2006).

Martin *et al.* (2006) stated that leaf explants from flower stalk node derived shoots of *Dendrobium* hybrids Sonia 17 and 28 cultured on half strength MS medium supplemented with 44.4 µM N6- Benzyl adenine developed more than 7 shoots per

explants. They further stated that half strength of MS medium containing 6.97  $\mu$ M kinetin facilitated conversion of more than 90% PLBs to shoots.

Marcela Franco *et al.* (2007) reported that coconut fiber alone or mixed in equal parts with pine bark and coal was the most efficient substrate with percent survival ( $80 \pm SE=0.3742$ ), biomass and leaf length under green house condition in *Cattleya trianae*.

Kishor *et al.*, (2007) inoculated the pseudobulb segments of *Dendrobium* hybrid (*D. nobile* x *D. chrysotoxum* L.) on full strength and half strength MS medium with different concentrations and combinations NAA, BAP and Kinetin. An average of two shoot buds per explants were regenerated on  $\frac{1}{2}$  MS+2.0 mg/l KN and  $\frac{1}{2}$  MS+ 0.1 mg/l NAA+ 1.0 mg/l BAP.

Kurup *et al.* (2007) conducted a study on Plant regeneration from mesophyll protoplast of *Dendrobium* var. Sonia. The protoplast from the *Dendrobium* var. Sonia was successfully isolated and regeneration of shoots and roots were obtained in MS medium containing BA 5 mg/l+ NAA 1mg/l.

Parvin *et al.* (2009) conducted a study to investigate the effect of growth regulator NAA on *in vitro* shoot proliferation of *Dendrobium* Orchid. Among the different concentrations of NAA, the maximum increase in shoot weight (0.25 g) and shoot number (8.83) were observed from 0.1mg/l NAA. The highest shoot length (2.60 cm) and number of leaves (4.83) were obtained with 0.2 mg/l NAA at 60 DAT.

Parvin *et al.* (2009) reported that NAA at 0.1 mg/l enhanced the shoot weight (0.25g) and shoot number (8.83) in *Dendrobium* orchid. They further reported that highest shoot length (2.60cm) and number of leaves (4.83) were obtained with 0.2 mg/l NAA at 60 DAT.

Pradhan *et al* (2013) reported that the maximum number of healthy shoot was observed on MS+ BAP (2 mg/l) + NAA (0.5 mg/l) (4 shoots/ culture) in *Dendrobium*

*densiflorum* for inducing root, MS media supplemented with IBA (1.5 mg/l) was found to be most effective.

## **2:2:5 Root Formation**

Alam *et al.* (2002) obtained More number of roots per plantlet with highest root length in MS medium supplemented with NAA 1mg/l.

Pyati *et al.* (2002) reported that well-developed shoots rooted at an average 5 roots per shoot in half strength MS medium devoid of any growth regulators.

Sobhana and Rajeevan (2002) conducted a study on refinement of embryo culture media for growing *Dendrobium* New pink × Emma white hybrid seedlings and reported that MS medium at ¼ strength , Vacin and Went and Knudson C supplemented with Kinetin BAP, NAA and IBA had profound influence on seedling growth as well as root length and number.

Indhumati *et al.* (2003) reported that the root number and root length of micro shoots of *Dendrobium* hybrid Sonia-17 were the highest in KC medium supplemented with IBA 0.5 mg/l.

Nasiruddin *et al.* (2003) investigate the effect of different concentrations of BAP (0, 2, 4 and 6 mg/l) and NAA (0, 0.5 and 1 mg/l) on *Dendrobium formosum* leaf callus. Observations recorded on root formation, root length and number of roots per plantlet revealed that BAP at 2.5 mg/l showed the best results in combination with 1 and 2 mg/l NAA.

Raju *et al.* (2003) obtained maximum rooting of *in vitro* cultured *Dendrobium osterholt* in half strength MS media with IBA 1mg/l +NAA 1mg/l.

Le Van *et al.* (2004) conducted a trial on *Cymbidium* Twilight Moon 'Day Light' on modified Vacin and Went medium supplemented 1- naphthaleneacetic acid (NAA) or 2,4- dichlorophenoxyacetic acid (2,4-D) alone or in combination with N-

phenyl-N<sup>1,2,3</sup>-thiadiazol-5yl urea (TDZ) within one month. Shoots with well-developed roots were observed on the medium without plant growth regulators.

Ket *et al.* (2004) induced the shoot proliferation of an elite cultivar of Jewel Orchid on Hyponex media supplemented with 1 mg/cubic dm benzyl adenine or 1-2 mg/cubic dm thiadiazuron (TDZ). Rooting was induced in 100 percent of the regenerated shoots in the same media.

Martin *et al.* (2006) found that half strength MS medium with 2 g/l activated charcoal was the best for *in vitro* rooting of *Dendrobium* hybrids Sonia17 and 28.

Kalimuthu *et al.* (2007) achieved *in vitro* regeneration, multiplication and rooting of plantlets from immature seeds of *Oncidium* on MS medium supplemented with BAP (2mg/l) whereas Kurup *et al.* (2007) obtained regeneration of roots of *Dendrobium* var. Sonia in MS medium containing BA 5mg/l and NAA 1 mg/l.

Hossain (2008) induced strong and stout root system of *Epidendrum ibaguense* Kunth *in vitro* seedlings on half strength PM (Phytamax) and M (Mitra) media fortified with 0.5- 1.0mg/l IAA and well rooted seedlings were transferred to green house with 90 percent survival.

Ping *et al.* (2008) developed a protocol for *Dendrobium densiflorum* Lindl.ex Wall, through PLBs from the nodal stem segments using 6-BAP and lanthanoid neodymium. Shoots produced an average of 22 roots per plantlet when cultured on MS medium supplemented with 2.0 mg/l neodymium nitrate. The result suggested that thalanthanoids could be used effectively to initiate rooting in *D. densiflorum*.

Parvin *et al.* (2009) conducted a study to investigated the effect of growth regulator NAA on *in vitro* rooting of *Dendrobium* Orchid. The highest number of roots (5.15) with maximum root length (2.67 cm) were obtained with 0.2 mg/l NAA at 60 DAT.

Vyas *et al.* (2011) reported that morphologically uniform plantlets with elongated leaves and well developed roots of *Dendrobium lituiflorum* were formed on Knudson C (KC) medium supplemented with 20% (v/v) banana extract (BE).

### 2.2.6 Hardening

Pine-bark, cork-bark, tree fern pieces, sphagnum moss, gravel, charcoal pieces and coconut fibres are commonly used potting materials for Orchids (Suresh Kumar, 1992).

Sharma and Chauhan (1995), reported that the potting mixture comprising brick, charcoal, tree fern, bark pieces, leaf mould and dry sphagnum in 1:1:1:1:2 ratios with green sphagnum on top supported the maximum survival of *Dendrobium chrysanthum* seedlings.

According to Pathania *et al.* (1998) hardening medium containing bark pieces, brick pieces, moss, charcoal pieces (1:1:1:1) proved to be suitable for the establishment of *in vitro* rooted plantlets of Sonia-17.

The establishment of plantlets of *Dendrobium* Hybrid Sonia-17 in terms of plant height, leaf size, new growth and root length was good in the medium consisting of charcoal+brick+cocopeat (1:1:1) & root number was highest in pure charcoal medium. Spraying with half KC nutrient solution improved the growth and establishment of the *in vitro* derived plantlets (Indhumati *et al.*, 2003)

Arumugam and Jawaharlal (2004) reported that the vase life of *Dendrobium* cultivar Sonia-17 was found highest in coconut fibre medium (21.20 days). Early flowering (177.00 days) was recorded in coconut fibre medium while late flowering (198.66 days) was observed in brick pieces.

Mani and Nagaraju (2004) reported that cocopith alone and in combination with sand best suitable for the acclimatization of *in vitro* raised seedlings of *Cymbidium* hybrids.

Sugapriya *et al.* (2011) suggested among the different varieties of *Dendrobium* evaluated, Sonia-17 recorded maximum plant height, length of internode and number of pseudobulbs per plant under partially environment controlled Greenhouse.

Sahoo (2012) reported that the media combination sch brick pieces: charcoal: cocopeat: sphagnum moss: decay wood was found to be most suitable for hardening of *Dendrobium* cv. Sonia 17.

Vyas *et al.* (2011) stated that the tissue culture plantlets of *Dendrobium lituiflorum* are best hardened in on agar-agar gelled medium, *Luffa sponge* medium and cocopeat: perlite (9:1) as support matrices, each containing one-half strength KC major salts.



## CHAPTER-III

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# *Materials and Methods*

# MATERIALS AND METHODS

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The present investigation was under taken during the year 2013-14. The materials used and the techniques adopted during courses of investigation are described in this chapter.

## 3.1 EXPERIMENTAL SITE

The experiments on *In vitro* propagation of orchids (*Dendrobium formosum* Roxb ex Lindl) were carried out in the Tissue Culture Laboratory of the Biotechnology cum Tissue Culture Centre in OUAT, Bhubaneswar during 2013-14.

## 3.2 VARIETAL DESCRIPTION

The *Dendrobium formosum* was selected for this experiment. The important characteristics of the cultivar are presented as follows-

The *Dendrobium formosum* is an epiphytic and sympodial orchid. The flowers are white colour with combination of cream, yellow, orange colours. These plants need strong light and a humid atmosphere with regular feeding for ensured growth and flowering.

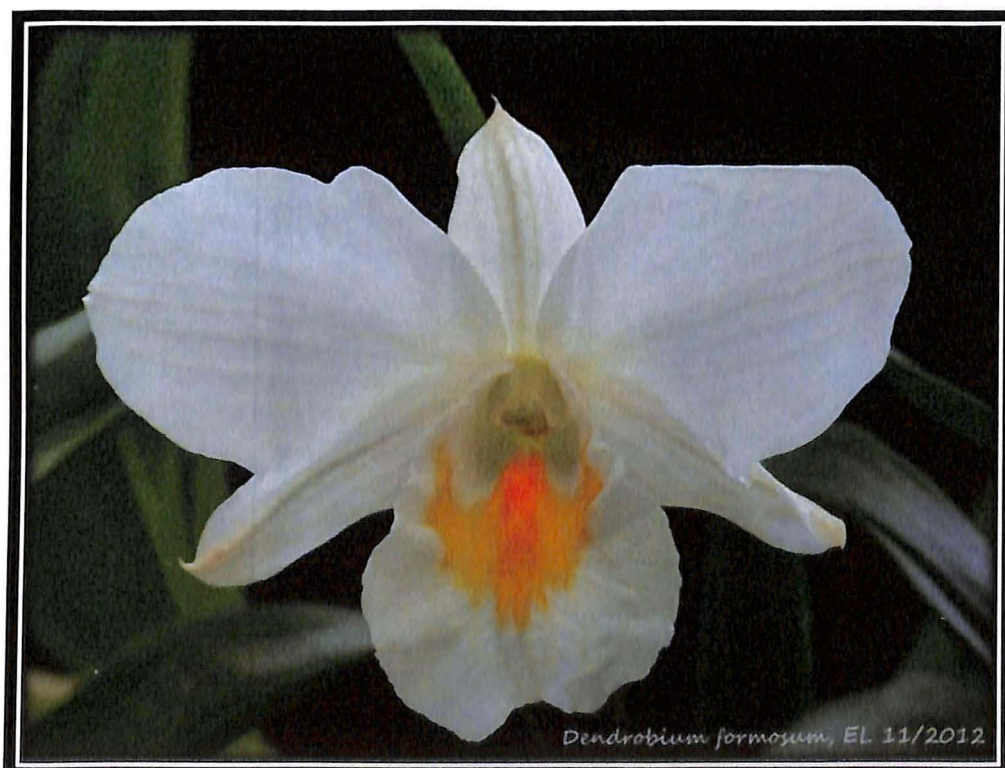


Plate 1: *Dendrobium formosum*

### 3.3 EXPLANTS

Greenish coloured calli mass derived from the germinated seeds of *Dendrobium formosum* previously present in Tissue Culture Laboratory, Bhubaneswar were taken as explants for further culture.

### 3.4 EXPERIMENTAL DETAILS

The following steps were undertaken for conducting the whole experiment-

- (a) Standardization of media supplements for shoot proliferation of orchids (*Dendrobium formosum* Roxb ex Lindl).
- (b) Standardization of media supplements for root initiation and development.
- (c) Standardization of media mixture for hardening of the *in vitro* plantlets

### 3.5 SOURCE OF CHEMICALS

The chemicals used for the study were analytical reagents of excel and grade of titan Biotech Ltd, Ranbaxy Laboratory Ltd, Merck (India), Qualigen , Fine Chemicals and Himedia Laboratories Ltd. (India). Auxins, Cytokinins, Myo-inositol and Fe-EDTA were supplied (USA) and Agar from Ranbaxy Laboratory Ltd.

### 3.6 GLASSWARE AND EQUIPMENTS

**Table .1 Details of glassware, equipments with name of their supplier company**

Glassware and equipments	Supplier Company
Culture tube, beakers, pipettes, measuring cylinders	Borosil, India
pH meter	Elico
Oven	Wiswo Instruments, India
Micropipettes	Borosil ,India
Digital balance	Sartorius Basic
Laminar air flow system	Klenzoids, India
Surgical Tools	Forceps, Scissor, Scalpel
Autoclave	Satyam

### 3.7 EXECUTION OF THE EXPERIMENT

#### 3.7.1 Formulation of media

The formulation of MS medium (Murashige and Skoog, 1962) was used as a basal medium for the entire study and the composition of the same is given as follows:

**Table.2 Inorganic and organic constituents of MS medium (Murashige and Skoog, 1962)**

Stock solution constituents	Concentration of stock solution (mg/l)	Volume of stock solution taken (mg/l)	Concentration in culture medium (mg/l)
<b>(Macronutrients (10 times concentrated))</b>			
NH <sub>4</sub> NO <sub>3</sub>	16500	100	1650
KNO <sub>3</sub>	19000		1900
CaCl <sub>2</sub> .2H <sub>2</sub> O	4400		440
MgSO <sub>4</sub> .7H <sub>2</sub> O	3700		370
KH <sub>2</sub> PO <sub>4</sub>	1700		170
<b>Micronutrients 100 times concentrated</b>			
KI	83	10	0.83
H <sub>3</sub> BO <sub>3</sub>	620		6.2
MnSO <sub>4</sub> .4H <sub>2</sub> O	2230		22.3
ZnSO <sub>4</sub> .7H <sub>2</sub> O	860		8.6
Na <sub>2</sub> MoO <sub>4</sub> 2 H <sub>2</sub> O	25		0.25
CuSO <sub>4</sub> 5H <sub>2</sub> O	2.5		0.025
CoCl <sub>2</sub> .6H <sub>2</sub> O	2.5		0.025
<b>Iron compound</b>			
FeSO <sub>4</sub> .7H <sub>2</sub> O	2780		27.8
Na <sub>2</sub> EDTA2H <sub>2</sub> O	3730		37.3
<b>Vitamins (100 times concentrated)</b>			
Myo-Inositol	10000	10	100
Glycine	200		2.0
Nicotinic acid	50		0.5
Pyridoxine-HCL	50		0.5
Thiamine-HCL	10		0.1
<b>Other substances</b>			
Sucrose			30000
Agar			7000
pH			5.7±0.1

### 3.7.2 Preparation of stock solution

The stock solution for macro and micro nutrients were prepared separately by dissolving the salt as mentioned against each stock in sterile double distilled water with due care. For the preparation of Fe-EDTA stock solution,  $\text{Na}_2\text{EDTA}$  and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  were dissolved separately in double distilled water, when both the solutes were dissolved then they were combined and final volume was made up with distilled water. Instead of  $\text{Na}_2\text{EDTA}$  and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , sodium ferric salt of EDTA was added fresh to the medium @40mg/l. Myoinositol and sucrose were also added to it. Fresh stock solution of vitamins like nicotine acid (50) thiamine HCl (10mg) pyridoxine HCL (50mg) and glycine (200mg) were separately dissolved in 100 ml of double distilled water and during media preparation 1 ml from each was added to the media. The stock solutions were stored in low temperature varying from 4°C to 20°C in refrigerator.

### 3.7.3 Preparation of stock solution for Plant Growth Regulators (PGRs):

#### (i) Auxin:-

##### a. IAA (Indole Acetic Acid):

100 mg of IAA were initially dissolved in a small quantity of 0.1 N NaOH and then the volume was made up to 100 ml by adding sterile double distilled water.

##### b. IBA (Indole-3-Butyric acid):

100 mg of IBA were first dissolved in small quantity of 0.1 N NaOH and then the volume was made up to 100 ml by adding sterile double distilled water.

##### c. NAA (Naphthalene Acetic acid):

100 mg of NAA were first dissolved in small quantity of 0.1 N NaOH and then the volume was made up to 100 ml by adding sterile double distilled water.

**(ii) Cytokinin:-**

BAP (Benzyl Amino Purine)

100 mg of BAP were dissolved initially in minimum quantity of 0.1 N HCL and volume was made up to 100 ml by adding sterile distilled water

**(iii) Gibberlic acid:-**

Gibberellic acid was prepared by dissolving 200 mg of GA3 in small amount of 0.1 N NaOH and final volume was made up to 200 ml with sterile distilled water.

**3.7.4 Preparation of culture medium:**

For preparation of culture medium required quantities of macronutrients, micronutrients, Fe-EDTA, vitaminins and growth regulators were taken from the stock solution and mixed in distilled water. Required quantities of sucrose dissolved in water was also added fresh to the solution prepared. The pH of the solution was adjusted to 5.7 using 0.1 N NaOH or 0.1 N HCL. Then the final volume was made up to one litre with distilled water. After the volume was made up, the required of agar was added and boiled in beaker till it got dissolved and looked transparent. The hot medium was distributed to sterilized culture bottle.

**3.7.5 Sterilization of culture medium:**

The culture bottle with culture medium was autoclaved for 20-25 minutes at 121°C and 15 psi pressure. The autoclaved medium was kept in a laminar air flow bench for cooling.

**3.7.6 Sterilization of glassware and Instruments:**

All the glassware were dipped in detergent solution overnight and washed under running tap water. The glassware were again rinsed with distilled water and dried in an oven for two hours at 150°C. Petridishes, scalpel and forceps were cleaned

properly with alcohol, wrapped with a paper and were kept in a clean sterilized base followed by sterilization in autoclave at 15 psi and 121°C for 30 minutes.

### **3.7.7 Sterilization of laminar air flow cabinet:**

The laminar air flow cabinet was cleaned by wiping with absolute ethyl alcohol. Filtered air (80-100 cft/min) was blown for five minutes to remove the dirt particles from the working area. All the instruments that were to be used were kept inside the chamber and exposed to UV light for 20-25 minutes. While executing the inoculation work, filtered air was continuously passed through the laminar air flow cabinet.

### **3.7.8 Culture room**

The temperature of the culture room was maintained at 25°C and relative humidity of 80% by providing air conditioner in the room and 16 hour photo period (3000-3200lux) supplied by fluorescent tubes exclusively for maintaining the light requirements.

## **3.8 STAGE OF *IN VITRO* CULTURE**

### **3.8.1 Standardization of media supplements for shoot proliferation of orchids (*Dendrobium formosum*).**

The existing greenish colour calli mass derived from germinated seeds of *Dendrobium formosum* maintained at the tissue culture laboratory were cut into pieces of more or less uniform size with the help of a sterilized sharp blade and pieces were inoculated as explant at the rate of five pieces per bottle in MS medium containing different concentration of Auxin (IAA& NAA), Gibberelic acid (GA<sub>3</sub>) and Cytokinins (BAP) for eliciting shoot regeneration from calli mass for shoot proliferation. In the experiment were 17 treatments replicated thrice with three bottles/replication for *Dendrobium formosum*.

**Table. 3 Impact of Plant Bioregulators on shoot proliferation of orchid (*Dendrobium formosum* Roxb ex Lindl)**

Treatment	Basal medium-MS Concentration of Plant Bioregulators (mg/l)			
	BAP	IAA	NAA	GA <sub>3</sub>
T <sub>1</sub>	-	-	-	-
T <sub>2</sub>	2	0.5	-	-
T <sub>3</sub>	2	1.0	-	-
T <sub>4</sub>	3	1.0	-	-
T <sub>5</sub>	4	1.0	-	-
T <sub>6</sub>	2	1.5	-	-
T <sub>7</sub>	3	1.5	-	-
T <sub>8</sub>	4	1.5	-	-
T <sub>9</sub>	2	1.0	0.1	-
T <sub>10</sub>	3	1.0	0.1	-
T <sub>11</sub>	4	1.0	0.1	-
T <sub>12</sub>	2	1.5	0.1	-
T <sub>13</sub>	3	1.5	0.1	-
T <sub>14</sub>	4	1.5	0.1	-
T <sub>15</sub>	2	1.0	-	0.1
T <sub>16</sub>	3	1.0	-	0.1
T <sub>17</sub>	4	1.0	-	0.1

### Observation recorded

Observations were recorded on 30 days of culture and 30, 60, & 90 days of sub cultures, on no. and length of PLBs, no. of shoots/explant, no., length and colour of leaves.

**Number of leaves/PLB:** It was obtained by dividing the total no. of leaves with no. of PLBs used in the culture bottle.

**Length of leaves:** The average length of the leaves in the culture bottles was measured with the help of scale from outside the bottle and recorded in centimeter.

**Colour of leaf:** visually observed colour of leaf under various treatments in the whole phase was noted.

**Number of PLBs/explant:** The average length of the PLBs in the culture bottles was measured with the help of scale from outside the bottle and recorded in centimeter.

**Length of PLBs:** The average length of the PLBs in the culture bottles was measured with the help of scale from outside the bottle and recorded in centimeter.

### 3.8.2 Standardization of media supplements for root initiation and development

The shoots developed in culture bottles were transferred into the MS medium supplemented with different concentration of IBA and NAA alone or in combination, for initiation and better development of roots. There were 16 treatments which were replicated thrice in this experiment.

**Table. 4 Effect of plant bioregulators on root initiation and development in *Dendrobium formosum* Roxb ex Lindl.**

Treatment	Concentration of Plant Bioregulators (mg/l)	
	IBA	NAA
T <sub>1</sub>	-	-
T <sub>2</sub>	0.5	-
T <sub>3</sub>	1.0	-
T <sub>4</sub>	1.5	-
T <sub>5</sub>	-	0.5
T <sub>6</sub>	-	1.0
T <sub>7</sub>	-	1.5
T <sub>8</sub>	0.5	0.5
T <sub>9</sub>	1.0	0.5
T <sub>10</sub>	1.5	0.5
T <sub>11</sub>	0.5	1.0
T <sub>12</sub>	1.0	1.0
T <sub>13</sub>	1.5	1.0
T <sub>14</sub>	0.5	1.5
T <sub>15</sub>	1.0	1.5
T <sub>16</sub>	1.5	1.5

### 3.8.3 Standardization of media mixture for hardening of the *in vitro* raised plantlets of *Dendrobium* cv. Sonia-17

The well developed seedlings of 2.5-3 cm height were taken and thoroughly washed with tap water to remove adhering medium completely without causing any damage to the roots. The media mixture are prepared with charcoal and cocopeat in equal proportions and filled in the protrays. Than the *in vitro* plantlets are tied with coconut husk and planted in portrays. The components of the media were sterilized with 0.2% bavistin for 30 minutes before used. The seedling were planted carefully in the respective medium so that the roots of seedlings passed through the media and kept under the green house. The half MS liquid medium was fertigated to the seedlings at an interval of two days up to two weeks and then one fourth MS liquid medium was applied at an interval of two days the next two weeks. Then irrigation was given with the drippers present in green house. For conducting the experiment total eight treatments replicated thrice with 10 plants per replication were taken. The observations were recorded on plant height, no. of leaf, no. of root/plant, length of root.

**Plant height:** The average height of the plantlets present in culture bottle.

**Number of roots/plantlet:** It was computed by dividing the total no. of roots with the no. of plantlets develops in the bottle.

**Root length:** the root with maximum length were measured with a scale and recorded in cm.

**Table.5 Effect of different composition of potting media on hardening and survivability percentage of the seedling after 60 days of hardening**

Treatments	Media composition
T <sub>1</sub>	Cocopeat
T <sub>2</sub>	Cocopeat + 1 pieces coconut husk
T <sub>3</sub>	Cocopeat +2 pieces coconut husk
T <sub>4</sub>	Cocopeat + 3 pieces coconut husk
T <sub>5</sub>	Cocopeat + charcoal
T <sub>6</sub>	Cocopeat + charcoal+ 1 pieces coconut husk
T <sub>7</sub>	Cocopeat+ charcoal +2 pieces coconut husk
T <sub>8</sub>	Cocopeat + charcoal +3 pieces coconut husk

The observations were recorded on plant height, no. of leaf, no. of root/plant, length of root, survival percentage.

### **3.9 STATISTICAL ANALYSIS**

The data recorded from the experiments were analyzed following the method of Gomez and Gomez (1984) using one way ANOVA in Completely Randomized Design (CRD) with 3 replications each consisting of 9 cultures bottles for shoot proliferation, rooting and for hardening 30 pots per replication were used. The analysis of variance (ANOVA) table was prepared. The treatment effect was tested by 'F' test at 5% level of significance. The critical difference at 5% level was calculated for comparing the treatments means.

$$CD = SE (m) \times t, \text{ where } SE (m)$$

$$= \sqrt{\text{Standard Error Mean/Number of replication}}$$

T= two sided table value at error degree of freedom at 5% level of Significance.

### **3.10 SCORING DATA AND PHOTOGRAPHY**

The important event of development in the course of the investigation, such as PLBs and shoot formation, root formation and development as well as acclimatization of plantlets were photographed by using a good close up digital camera with good quality of film like Kodak colour VR-100. Colour negative film was used for the purpose to provide illustrative evidence. For each treatment at least three replication were used depending on the different experiment and for confirmation of the result. The cultures were examined at weekly intervals. In each case the average value of the data was recorded from PLBs production to hardening of the orchids.



## CHAPTER-IV

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# *Experimental Findings*

## EXPERIMENTAL FINDING

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The experimental results of the present investigation“ *In vitro* propagation of orchid (*Dendrobium formosum* Rox ex Lindl)” with objectives for standardization of media supplements for shoot proliferation , standardization of media supplements for root initiation and development of orchid( *Dendrobium formosum*) and, standardization of media mixture for hardening of *in vitro* plantlets are presented as follows:

### 4.1. STANDARDIZATION OF MEDIA SUPPLEMENTS FOR SHOOT PROLIFERATION OF ORCHID (*Dendrobium formosum*)

The data presented in Table 6, plate 2 and Fig. 1 showed the significant effect of various concentrations of BAP on protocorm formation after 30 days of culture.

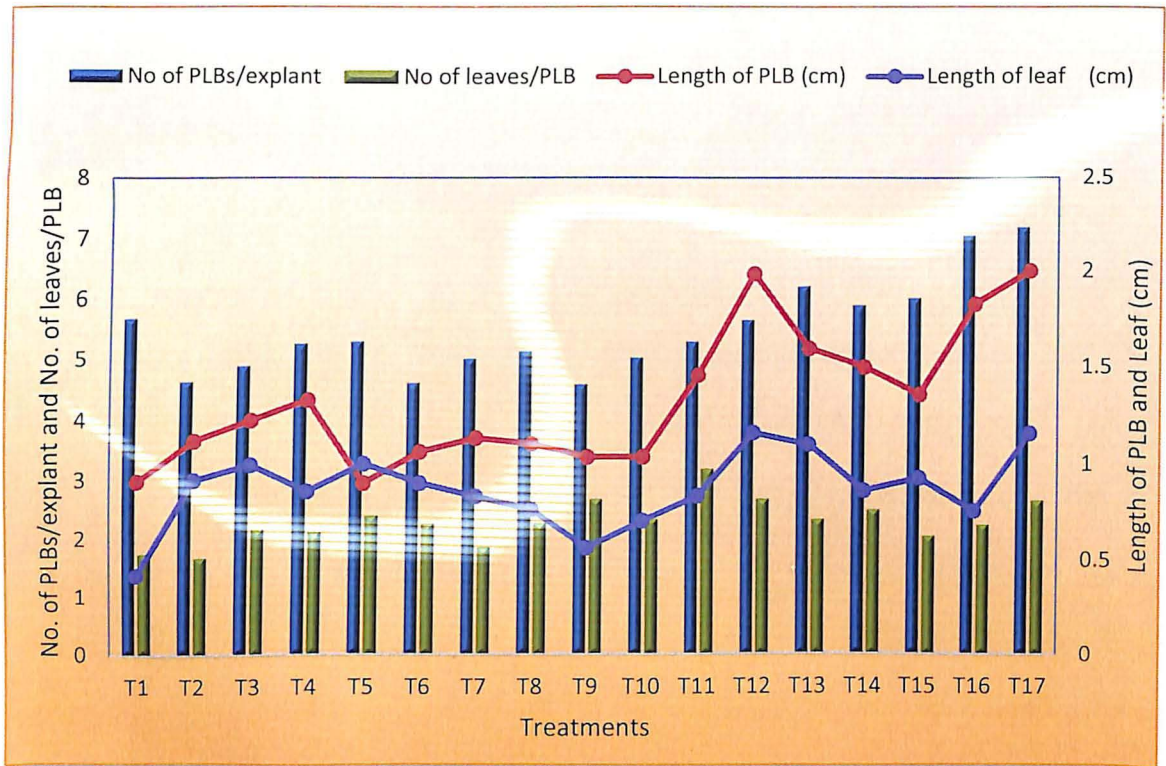
It was evident from the data presented in the Table 6 that MS medium supplemented with 4.0mg/l BAP, 1.0mg/l IAA, and 0.1 mg/l GA<sub>3</sub> significantly enhanced the no of PLBs per explant (7.13) and the data remained at par with the treatment, where MS medium was supplied with 3.0 mg/l BAP, 1.0 mg/l IAA, & 0.1mg/l GA<sub>3</sub>. The length of PLBs was significantly maximum (2.0cm) in the treatment T<sub>12</sub> (MS+ 2.0mg/l BAP +1.5 mg/l IAA +0.1 mg/l NAA) and T<sub>17</sub> (MS +4.0 mg/l BAP +1.0 mg/l IAA +0.1 mg/l GA<sub>3</sub>) and the data remained at par with the T<sub>16</sub> (MS + 3.0 mg/l BAP +1.0 mg/l IAA +0.1 mg/l GA<sub>3</sub>). Significantly maximum no of leaves per PLBs (3.10) was reported in the treatment T<sub>11</sub> (MS + 4.0 mg/l BAP 1.0 mg/l IAA +0.1 mg/l NAA ) and the data stood at par with the treatment T<sub>9</sub> (MS +2.0mg/l BAP +1.0 mg/l IAA 0.1 mg/l NAA), T<sub>12</sub> (MS + 2.0 mg/l BAP+ 1.5 mg/l IAA +0.1 mg/l NAA) and T<sub>17</sub> (MS +4.0 mg/l BAP + 1.0 mg/l IAA + 0.1 mg/l GA<sub>3</sub>) . Length of leaf was significantly higher (1.16cm) in the treatment T<sub>17</sub> (MS + 4.0 mg/l BAP +1.0 mg/l IAA + 0.1 GA<sub>3</sub>). MS medium fortified with 2-4 mg/l BAP in combination with 1.0 mg/l IAA alone or in combination with 0.1 mg/l GA<sub>3</sub> produced deep green leaves. MS medium supplied with 4.0 mg/l BAP, 1.5 mg/l IAA and 0.1 mg/l NAA also produced

deep green leaves. Except the treatments T<sub>4</sub>, T<sub>5</sub>, T<sub>14</sub>, T<sub>16</sub>, and T<sub>17</sub> all other treatments produced light green coloured leaves.

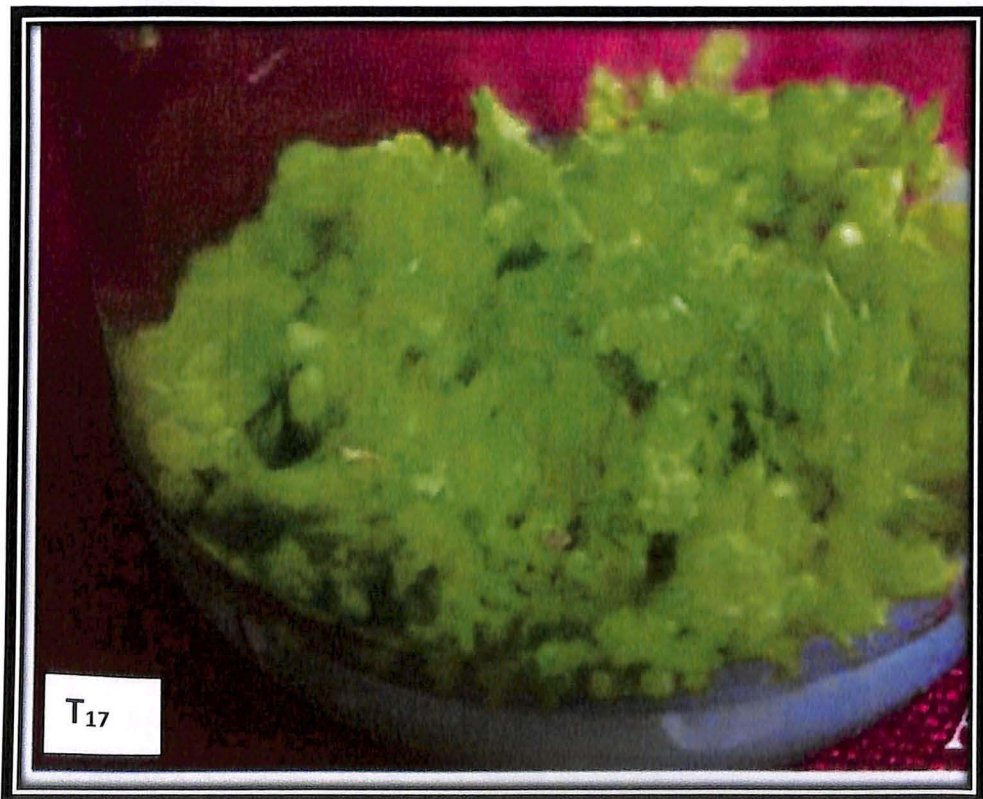
Considering the above mentioned characters after 30 days of culture, it has been concluded that the treatment T<sub>17</sub> where MS medium fortified with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.1 mg/l GA<sub>3</sub> significantly produced more no of PLBs per explant (7.13), and enhanced the length of PLBs (2.0cm) as well as length of the leaf (1.16cm) and the data remained at par with the treatment T<sub>11</sub> for no of leaves per PLBs.

**Table -6 Efficacy of plant Bioregulators on shoot initiation of *Dendrobium formosum* after 30 days of culture**

Treatment	Concentration of PBRs (mg/l)				No of PLBs/ explant	Length of PLB (cm)	No of leaves/ PLB	Length of leaf (cm)	Colour of leaf
	BAP	IAA	NAA	GA <sub>3</sub>					
T <sub>1</sub>	-	-	-	-	5.66	0.93	1.75	0.43	Light green
T <sub>2</sub>	2	0.5	-	-	4.60	1.13	1.66	0.93	Light green
T <sub>3</sub>	2	1.0	-	-	4.83	1.23	2.13	1.00	Light green
T <sub>4</sub>	3	1.0	-	-	5.20	1.33	2.06	0.86	Deep green
T <sub>5</sub>	4	1.0	-	-	5.23	0.90	2.33	1.00	Deep green
T <sub>6</sub>	2	1.5	-	-	4.53	1.06	2.20	0.90	Light green
T <sub>7</sub>	3	1.5	-	-	4.93	1.13	1.80	0.83	Light green
T <sub>8</sub>	4	1.5	-	-	5.06	1.10	2.20	0.76	Light green
T <sub>9</sub>	2	1.0	0.1	-	4.50	1.03	2.60	0.56	Light green
T <sub>10</sub>	3	1.0	0.1	-	4.96	1.03	2.26	0.70	Light green
T <sub>11</sub>	4	1.0	0.1	-	5.25	1.46	3.10	0.83	Light green
T <sub>12</sub>	2	1.5	0.1	-	5.61	2.00	2.60	1.16	light green
T <sub>13</sub>	3	1.5	0.1	-	6.16	1.60	2.26	1.10	Light green
T <sub>14</sub>	4	1.5	0.1	-	5.83	1.50	2.43	0.86	Deep green
T <sub>15</sub>	2	1.0	-	0.1	5.95	1.36	2.00	0.93	Light green
T <sub>16</sub>	3	1.0	-	0.1	7.00	1.83	2.20	0.76	Deep green
<b>T<sub>17</sub></b>	<b>4</b>	<b>1.0</b>	<b>-</b>	<b>0.1</b>	<b>7.13</b>	<b>2.00</b>	<b>2.60</b>	<b>1.16</b>	<b>Deep green</b>
SE(m)					0.26	0.09	0.21	0.08	
CD (%)					0.75	0.26	0.59	0.23	



**Fig. 1** Efficacy of plant Bioregulators on shoot initiation of *Dendrobium formosum* after 30 days of culture



**Plate 2:** PLBs formation after 30 days of culture of *Dendrobium formosum* (T<sub>17</sub> MS medium + 4.0 mg/l BAP, 1.0 mg/l IAA, 0.1 mg/l GA<sub>3</sub>)

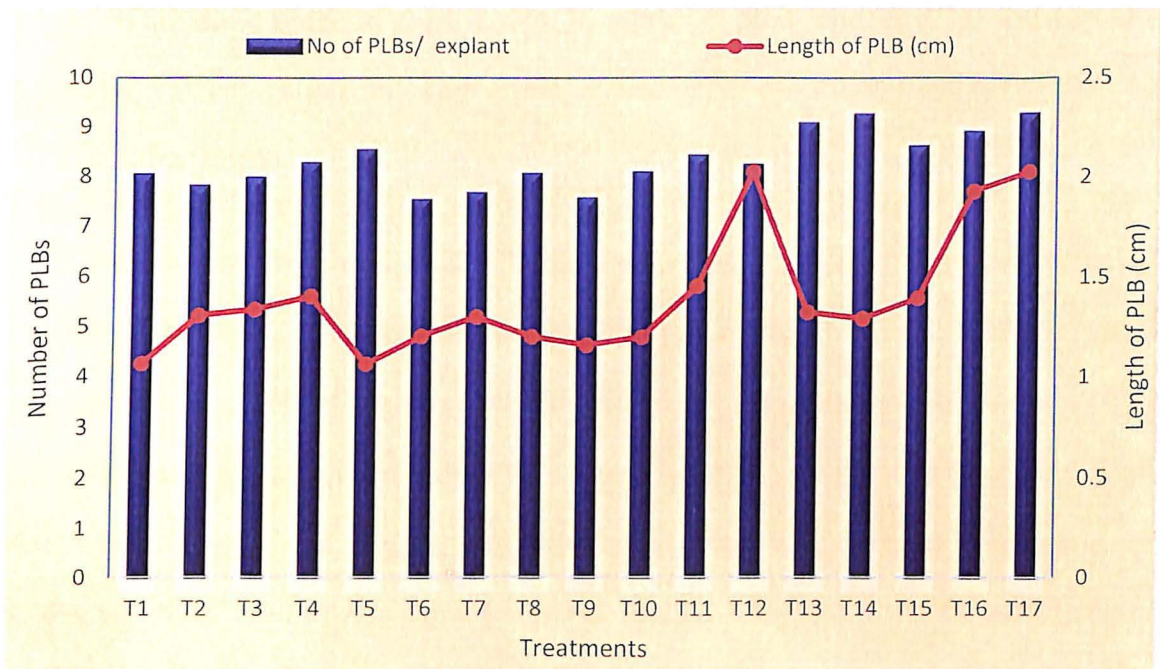
The data presented in Table 7, plate 3 and Fig. 2 showed the significant effect of various concentrations of BAP on protocorm multiplication after 30 days of subculture.

The data presented in Table 7 after 30 days of subculture revealed that the treatment T<sub>17</sub>(MS medium + 4.0 mg/l BAP + 1.0 mg/l IAA + 0.1 mg/l GA<sub>3</sub>) significantly increased the no of PLBs per explant (9.27) and the data stood at par with in the treatment T<sub>13</sub>, T<sub>14</sub> and T<sub>16</sub>. Significantly longer PLBs (2.03) were recorded in both the treatments T<sub>12</sub> and T<sub>17</sub> and the data stood at par with T<sub>16</sub>. The treatment T<sub>2</sub> (MS medium fortified with 2.0mg/l BAP+ 0.5 mg/l IAA) significantly increased the no of leaves per PLBs (5.66) and the data remained at par with treatments T<sub>4</sub>, T<sub>12</sub>, T<sub>13</sub>, T<sub>15</sub>, T<sub>16</sub>. Significantly longer leaves per PLBs (1.46) were recorded in both the treatment T<sub>8</sub> and T<sub>13</sub> and the data stood at par with the treatment T<sub>2</sub>, T<sub>9</sub> and T<sub>17</sub>. The treatment T<sub>4</sub>, T<sub>5</sub>, T<sub>6</sub>, T<sub>11</sub>, T<sub>12</sub>, T<sub>14</sub>, T<sub>16</sub>, and T<sub>17</sub> produced deep green leaf coloured leaves.

Considering the above mentioned characters it has been concluded that the treatment T<sub>17</sub>(4.0 mg/l BAP + 1.0 mg/l IAA + 0.1 mg/l GA<sub>3</sub>) significantly increased the no of PLBs per explant (9.27) and length of PLBs (2.03cm) and the treatment T<sub>2</sub> significantly increased the no of leaves per PLBs (5.66). Both treatments T<sub>8</sub> and T<sub>13</sub> increase the length of leaf (1.46cm).

**Table 7 Developmental stage of PLBs of *Dendrobium formosum* after 30 days of subculture**

Treatment	Concentration of PBRs (mg/l)				No of PLBs/ explant	Length of PLB (cm)	No of leaves/P LB	Length of leaf(cm)	Colour of leaf
	BAP	IAA	NAA	GA <sub>3</sub>					
T <sub>1</sub>	-	-	-	-	8.03	1.06	4.41	1.13	Light green
T <sub>2</sub>	2	0.5	-	-	7.80	1.30	5.66	1.36	Light green
T <sub>3</sub>	2	1.0	-	-	7.97	1.33	4.46	1.06	Light green
T <sub>4</sub>	3	1.0	-	-	8.27	1.40	5.13	1.03	Deep green
T <sub>5</sub>	4	1.0	-	-	8.53	1.06	3.10	1.00	Deep green
T <sub>6</sub>	2	1.5	-	-	7.53	1.20	1.96	1.00	Deep green
T <sub>7</sub>	3	1.5	-	-	7.67	1.30	4.23	1.00	Light green
T <sub>8</sub>	4	1.5	-	-	8.07	1.20	2.15	1.46	Light green
T <sub>9</sub>	2	1.0	0.1	-	7.58	1.16	4.48	1.20	Light green
T <sub>10</sub>	3	1.0	0.1	-	8.10	1.20	4.48	0.90	Light green
T <sub>11</sub>	4	1.0	0.1	-	8.43	1.46	3.31	1.00	Deep green
T <sub>12</sub>	2	1.5	0.1	-	8.25	2.03	5.25	1.16	Deep green
T <sub>13</sub>	3	1.5	0.1	-	9.08	1.33	5.48	1.46	Light green
T <sub>14</sub>	4	1.5	0.1	-	9.25	1.30	4.23	0.86	Deep green
T <sub>15</sub>	2	1.0	-	0.1	8.61	1.40	5.43	1.03	Light green
T <sub>16</sub>	3	1.0	-	0.1	8.90	1.93	5.33	1.00	Deep green
<b>T<sub>17</sub></b>	<b>4</b>	<b>1.0</b>	<b>-</b>	<b>0.1</b>	<b>9.27</b>	<b>2.03</b>	<b>4.16</b>	<b>1.46</b>	<b>Deep green</b>
SE(m)					0.18	0.08	0.19	0.09	
CD (%)					0.519	0.23	0.57	0.26	



**Fig. 2** Developmental stage of PLBs of *Dendrobium formosum* after 30 days of subculture



**Plate 3:** PLBs multiplication after 30 days of sub culture of *Dendrobium formosum* (T<sub>17</sub> MS medium + 4.0 mg/l BAP, 1.0 mg/l IAA, 0.1 mg/l GA<sub>3</sub>)

The data presented in Table 8, plate 4 & 5 and Fig. 3 showed the significant effect of various concentrations of BAP on shoot formation after 60 days of subculture.

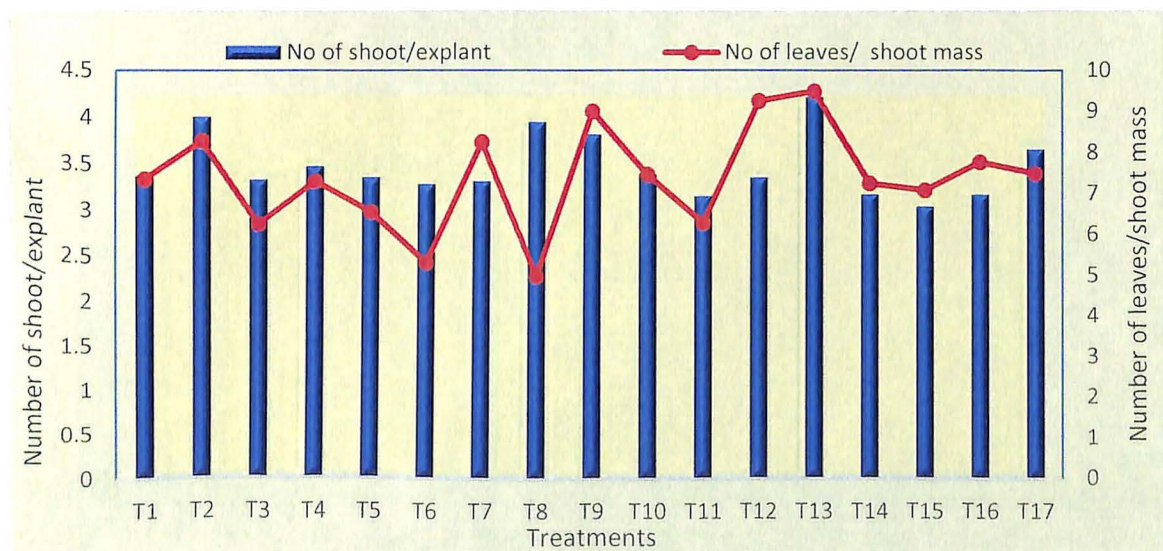
The data presented in the Table 8 after 60 days of subculture revealed that the treatment T<sub>13</sub> where MS medium supplied with 3.0 mg/l BAP, 1.5 mg/l IAA and 0.1 mg/l NAA significantly increased the no of shoots per explant (4.2) and data stood at par with treatment T<sub>2</sub> where MS medium fortified with 2.0 mg/l BAP and 1.0 mg/l IAA. Again the treatment T<sub>13</sub> significantly enhanced the no of leaves per shoot mass (9.48) and length of leaf (2.2cm) and the data stood at par with the treatment T<sub>9</sub> & T<sub>12</sub> for no of leaf per shoot mass and with the treatments T<sub>2</sub> and T<sub>8</sub> for length of the leaf. The treatment T<sub>3</sub> (MS medium + 2.0 mg/l BAP+ 1.0 mg/l IAA), T<sub>5</sub>(MS medium + 4.0 mg/l BAP + 1.0 mg/l IAA), T<sub>6</sub>(MS medium + 2.0 mg/l BAP + 1.5 mg/l IAA), T<sub>9</sub>(MS medium + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.1 mg/l NAA), T<sub>10</sub>(MS medium + 3.0 mg/l BAP + 1.0 mg/l IAA + 0.1 mg/l NAA), T<sub>12</sub>(MS medium + 2.0 mg/l BAP + 1.5 mg/l IAA + 0.1 mg/l NAA), and T<sub>13</sub> (MS medium + 3.0 mg/l BAP + 1.5 mg/l IAA + 0.1 mg/l NAA) produced deep green coloured leaves.

Considering all the above mentioned characters it has been concluded that after 60 days of sub culture MS medium supplied with 3.0 mg/l BAP, 1.5 mg/l IAA and 0.1 mg/l NAA significantly increased the no of shoots per explant (4.2), no of leaves per shoot mass (9.48) and the length of the leaf (2.2cm) and produced deep green coloured leaves.

**Table.8** Impact of plant Bioregulators on shoot proliferation of *Dendrobium formosum* after 60 days of sub culture

Basal medium-MS

Treatment	Concentration of PBRs ( mg/l)				No of shoot/ explants	Length of leaf(cm)	No of leaves/ shoot mass	Colour of leaf
	BAP	IAA	NAA	GA <sub>3</sub>				
T <sub>1</sub>	-	-	-	-	3.37	1.36	7.41	Light green
T <sub>2</sub>	2	0.5	-	-	4.00	2.00	8.31	Light green
T <sub>3</sub>	2	1.0	-	-	3.30	1.30	6.23	Deep green
T <sub>4</sub>	3	1.0	-	-	3.43	1.43	7.25	Light green
T <sub>5</sub>	4	1.0	-	-	3.30	1.30	6.48	Deep green
T <sub>6</sub>	2	1.5	-	-	3.23	1.23	5.25	Deep green
T <sub>7</sub>	3	1.5	-	-	3.27	1.26	8.23	Light green
T <sub>8</sub>	4	1.5	-	-	3.93	1.93	4.98	Light green
T <sub>9</sub>	2	1.0	0.1	-	3.80	1.80	9.00	Deep green
T <sub>10</sub>	3	1.0	0.1	-	3.37	1.10	7.48	Deep green
T <sub>11</sub>	4	1.0	0.1	-	3.13	1.36	6.31	Light green
T <sub>12</sub>	2	1.5	0.1	-	3.33	1.33	9.25	Deep green
<b>T<sub>13</sub></b>	<b>3</b>	<b>1.5</b>	<b>0.1</b>	-	<b>4.20</b>	<b>2.20</b>	<b>9.48</b>	<b>Deep green</b>
T <sub>14</sub>	4	1.5	0.1	-	3.13	1.13	7.23	Light green
T <sub>15</sub>	2	1.0	-	0.1	3.00	1.00	7.06	Light green
T <sub>16</sub>	3	1.0	-	0.1	3.13	1.13	7.75	Light green
T <sub>17</sub>	4	1.0	-	0.1	3.63	1.63	7.48	Light green
SE(m)					0.08	0.08	0.15	
CD (%)					0.22	0.23	0.43	



**Fig. 3** Impact of plant Bioregulators on shoot proliferation of *Dendrobium formosum* after 60 days of sub culture

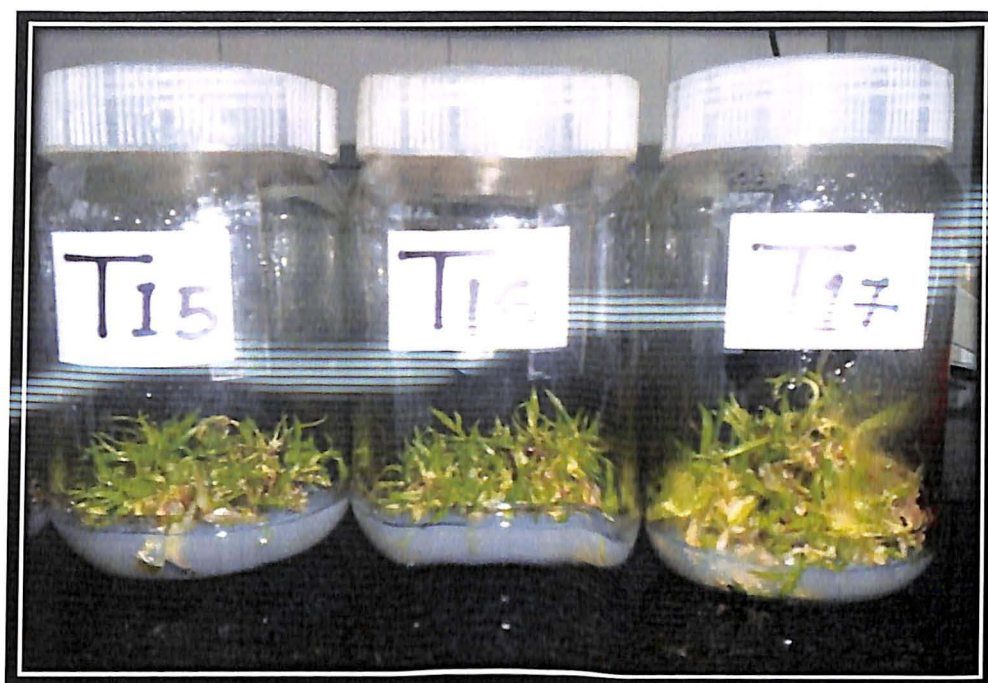


Plate 4



Plate 5

Plate 4 & 5 : shoot proliferation After 60 days of sub culture of *Dendrobium formosum* (T<sub>15</sub> MS medium + 2.0 mg/l BAP, 1.0 mg/l IAA, 0.1 mg/l GA<sub>3</sub>, T<sub>16</sub> MS medium + 3.0 mg/l BAP, 1.0 mg/l IAA, 0.1 mg/l GA<sub>3</sub>, T<sub>17</sub> MS medium + 4.0 mg/l BAP, 1.0 mg/l IAA, 0.1 mg/l GA<sub>3</sub>, )

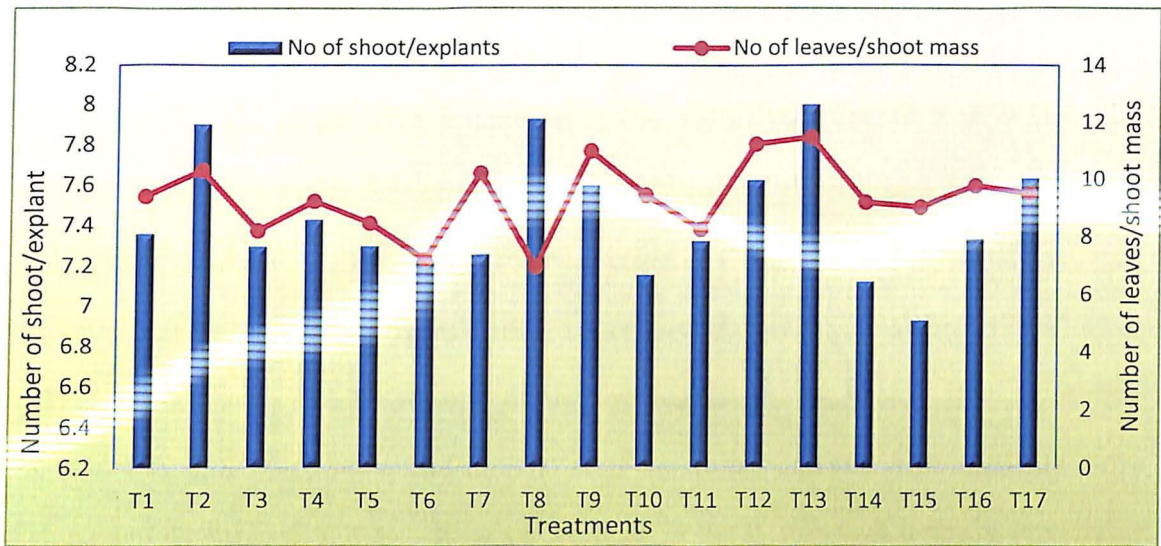
The data presented in Table 9 and Fig.4 showed the significant effect of various concentrations of PBRs on shoot formation after 90 days of subculture.

The perusal of the data presented in Table 9 revealed that MS medium fortified with 3.0 mg/l BAP, 1.5 mg/l IAA and 0.1 mg/l NAA significantly increased the no of shoots per explants (8.0) and data was found to be at par with treatment T<sub>2</sub> (MS medium + 2.0 mg/l BAP + 0.5 mg/l IAA), T<sub>8</sub>( MS medium + 4.0 mg/l BAP + 1.5 mg/l IAA), T<sub>12</sub>(MS medium + 2.0 mg/l BAP + 1.5 mg/l IAA + 0.1 mg/l NAA) and T<sub>17</sub>(MS medium + 4.0 mg/l BAP + 1.0 mg/l IAA + 0.1mg/l GA<sub>3</sub>). Again the same treatment T<sub>13</sub> significantly produced maximum no of leaves per shoot mass (11.48) and the data remained at par with the treatments T<sub>9</sub> and T<sub>12</sub>. The treatment T<sub>13</sub> (MS medium + 3.0 mg/l BAP + 1.5 mg/l IAA + 0.1 mg/l NAA) significantly increased the length of leaf (2.36cm) and the data stood at par with T<sub>2</sub>, T<sub>8</sub>, and T<sub>9</sub>. The treatments T<sub>5</sub>, T<sub>6</sub>, T<sub>7</sub>, T<sub>9</sub>, T<sub>12</sub>, T<sub>13</sub>, and T<sub>17</sub> produced deep green coloured leaves.

It has been concluded from the above mentioned characters that after 90 days of subculture the treatment T<sub>13</sub>, where MS medium was fortified with 3.0 mg/l BAP, 1.5 mg/l IAA and 0.1 mg/l NAA significantly increased the no of shoots per explant (8.00), no of leaves per shoot mass (11.48), and length of the leaf (2.36 cm) and produced deep green coloured leaves.

**Table 9 Standardization of media supplements for shoot proliferation of orchids. After 90 days of subculture**

Treatment	Concentration of PBRs( mg/l)				No of shoot/explants	Length of leaf(cm)	No of leaves/shoot mass	Colour of leaf
	BAP	IAA	NAA	GA <sub>3</sub>				
T <sub>1</sub>	-	-	-	-	7.36	1.67	9.41	Light green
T <sub>2</sub>	2	0.5	-	-	7.90	2.13	10.31	Light green
T <sub>3</sub>	2	1.0	-	-	7.30	1.65	8.23	Light green
T <sub>4</sub>	3	1.0	-	-	7.43	1.73	9.25	Light green
T <sub>5</sub>	4	1.0	-	-	7.30	1.63	8.48	Deep green
T <sub>6</sub>	2	1.5	-	-	7.23	1.63	7.25	Deep green
T <sub>7</sub>	3	1.5	-	-	7.26	1.63	10.23	Deep green
T <sub>8</sub>	4	1.5	-	-	7.93	2.13	6.98	Light green
T <sub>9</sub>	2	1.0	0.1	-	7.60	2.10	11.00	Deep green
T <sub>10</sub>	3	1.0	0.1	-	7.16	1.50	9.48	Light green
T <sub>11</sub>	4	1.0	0.1	-	7.33	1.66	8.31	Light green
T <sub>12</sub>	2	1.5	0.1	-	7.63	1.63	11.25	Deep green
<b>T<sub>13</sub></b>	<b>3</b>	<b>1.5</b>	<b>0.1</b>	-	<b>8.00</b>	<b>2.36</b>	<b>11.48</b>	<b>Deep green</b>
T <sub>14</sub>	4	1.5	0.1	-	7.13	1.33	9.23	Light green
T <sub>15</sub>	2	1.0	-	0.1	6.93	1.20	9.06	Light green
T <sub>16</sub>	3	1.0	-	0.1	7.33	1.40	9.75	Light green
T <sub>17</sub>	4	1.0	-	0.1	7.63	1.83	9.48	Deep green
SE(m)					0.13	0.08	0.14	
CD (%)					0.39	0.24	0.43	



**Fig. 4** Standardization of media supplements for shoot proliferation of orchids. After 90 days of subculture



**Plate 6:** The cultures for shoot proliferation maintained in the culture room

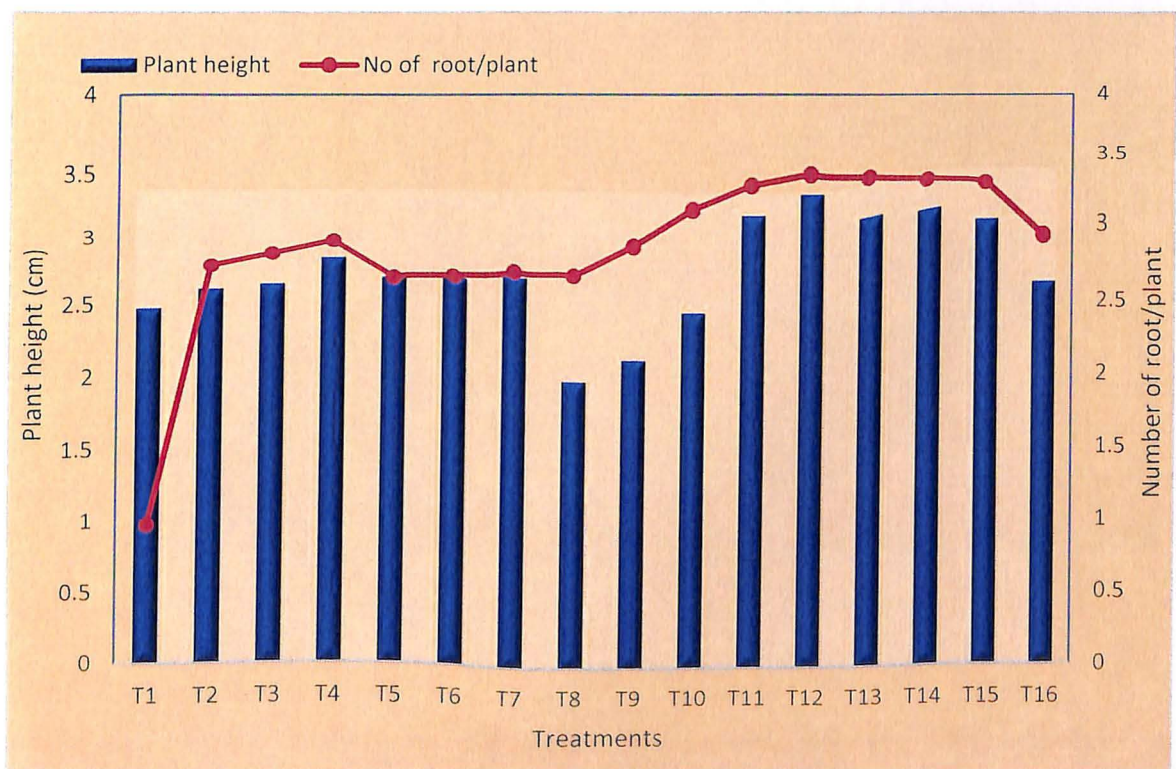
## 4.2 STANDARDIZATION OF MEDIA SUPPLEMENTS FOR ROOT INITIATION AND DEVELOPMENT

The perusal of the data presented in the Table 10, plate 7, 8 & 9 and fig. 5 revealed that MS medium supplemented with 1.0 mg/l IBA and 1.0 mg/l NAA significantly enhanced the vegetative growth of the plant as well as root production. The treatment significantly increased the plant height (3.37cm) after 60 days of culture. The treatment further significantly increased the no of leaf /plant (6.13) and the data stood at par with the treatment T<sub>8</sub> (MS medium +0.5 mg/l IBA + 0.5 mg/l NAA), T<sub>9</sub> (MS +1.0 mg/l IBA + 0.5mg/l NAA), T<sub>10</sub>(MS +1.5 mg/l IBA +0.5 mg/l NAA), T<sub>11</sub>(MS + 0.5 mg/l IBA + 1.0 mg/l NAA), T<sub>13</sub>(MS + 1.5 mg/l IBA +1.0 mg/l NAA), T<sub>14</sub> to T<sub>16</sub> where MS medium fortified with 0.5 mg/l or 1.0 mg/l or 1.5 mg/l IBA with 1.5 mg/l NAA. The no of roots/plant was significantly maximum (3.5) in the treatment T<sub>12</sub> i.e. MS medium fortified with 1.0 mg/l IBA along with 1.0 mg/l NAA and the data remained at par with the treatment T<sub>11</sub> (MS medium + 0.5 mg/l IBA + 1.0 mg/l NAA) and T<sub>13</sub> (MS + 1.5 mg/l IBA + 1.0 mg/l NAA). The same treatment significantly enhanced the length of the root (0.47cm) after 60 days of culture.

It has been concluded that MS medium enriched with 1.0 mg/l IBA along with 1.0 mg/l NAA was most suitable combination for initiation and development of root in *Dendrobium formosum* after 60 days of culture, where the combination produced more no (3.50) of longer roots /plants (0.47cm).

**Table 10 Standardization of media supplements for root initiation and development**

Treatment	Concentration of PBRs (Mg/l)		Plant height	No of leaf	No of root/plant	Length of root
	IBA	NAA				
	T <sub>1</sub>	-				
T <sub>2</sub>	0.5	-	2.60	5.03	2.77	0.31
T <sub>3</sub>	1.0	-	2.63	5.00	2.87	0.32
T <sub>4</sub>	1.5	-	2.83	5.07	2.97	0.36
T <sub>5</sub>	-	0.5	2.67	4.97	2.67	0.32
T <sub>6</sub>	-	1.0	2.73	4.83	2.73	0.32
T <sub>7</sub>	-	1.5	2.83	4.93	2.87	0.35
T <sub>8</sub>	0.5	0.5	2.07	5.27	2.83	0.32
T <sub>9</sub>	1.0	0.5	2.23	5.53	3.03	0.33
T <sub>10</sub>	1.5	0.5	2.57	5.80	3.27	0.37
T <sub>11</sub>	0.5	1.0	3.23	5.60	3.43	0.38
<b>T<sub>12</sub></b>	<b>1.0</b>	<b>1.0</b>	<b>3.37</b>	<b>6.13</b>	<b>3.50</b>	<b>0.47</b>
T <sub>13</sub>	1.5	1.0	3.17	5.53	3.43	0.38
T <sub>14</sub>	0.5	1.5	3.13	5.40	3.33	0.37
T <sub>15</sub>	1.0	1.5	3.03	5.67	3.27	0.33
T <sub>16</sub>	1.5	1.5	2.63	5.60	2.93	0.31
SE(m)			0.03	0.26	0.04	0.01
CD%			0.10	0.75	0.11	0.03



**Fig. 5 Standardization of media supplements for root initiation and development**

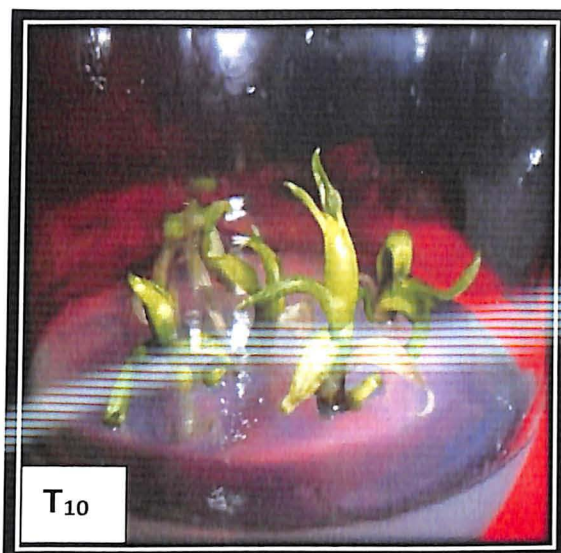


Plate 7

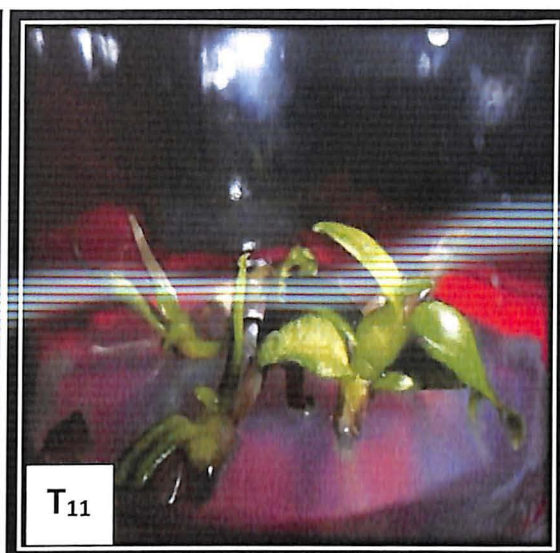


Plate 8



Plate 9

Plate 7, 8, & 9. Root initiation and development observed after 60 days in *Dendrobium formosum* (T<sub>12</sub> MS medium + 1.0 mg/l IBA, 1.0 mg/l NAA, T<sub>11</sub> MS medium + 0.5 mg/l IBA, 1.0 mg/l NAA, T<sub>10</sub> MS medium + 1.5 mg/l IBA, 0.5 mg/l NAA, )

### 4.3 STANDARDIZATION OF MEDIA MIXTURE FOR HARDENING OF THE *IN VITRO* PLANTLETS

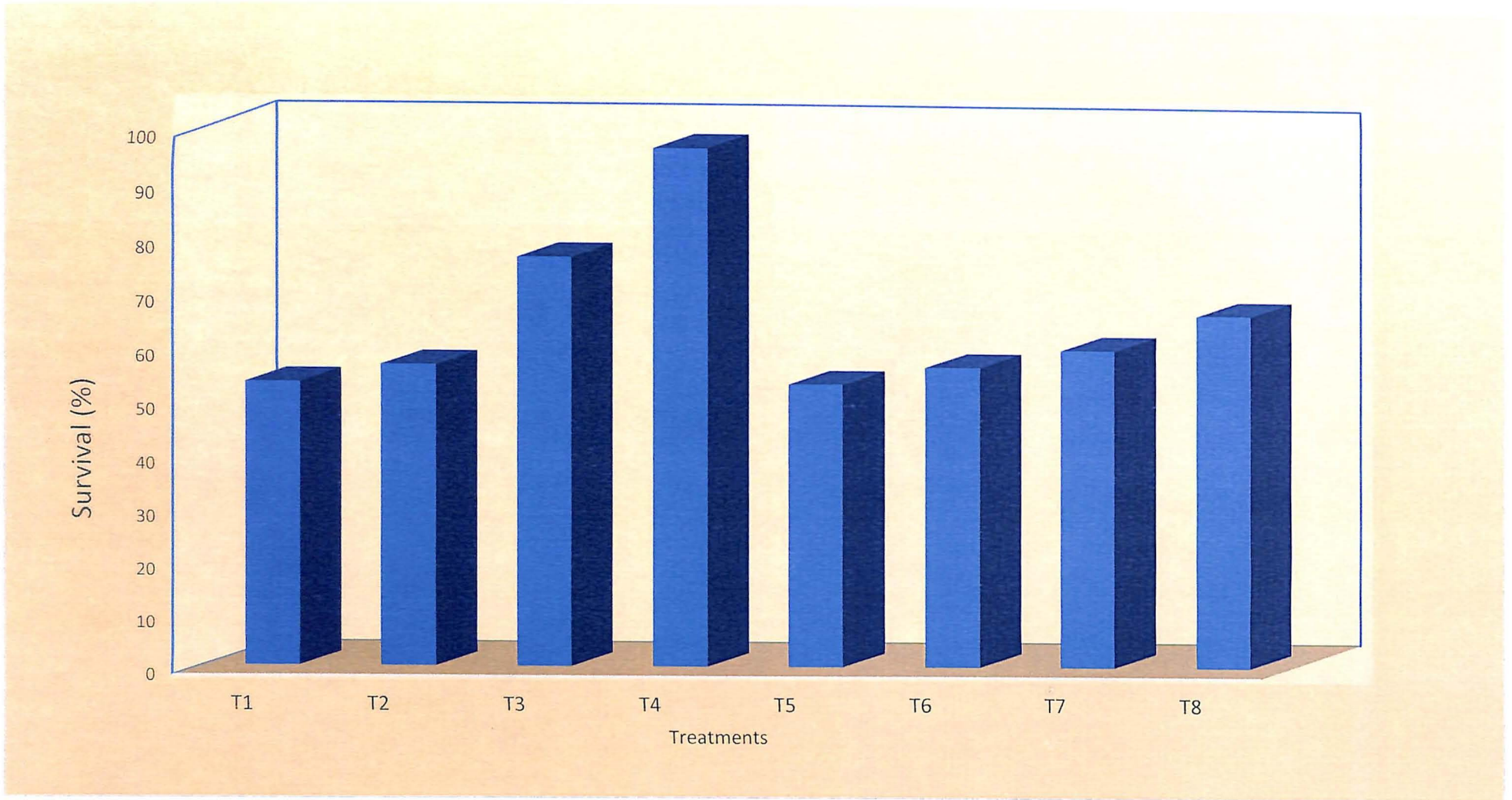
The data presented in the Table 11, plate 10 & 11 and fig. 6 revealed that the *Dendrobium* plantlets tied with 3 pieces coconut husk and planted in protray containing cocopeat significantly enhanced the plant height(27.33cm) after 60 days of hardening. Same combination significantly increased the no of leaf/plant (5.30) and data stood at par with the T<sub>3</sub> (cocopeat + 2 pieces cocpnut husk) and T<sub>8</sub> (cocopeat + charcoal + 3 pieces coconut husk ). Significantly maximum no of roots/ plant (7.50) was recorded in the same treatment. Further the treatment T<sub>4</sub> (cocopeat + 3 pieces coconut husk ) significantly produced longer root (2.5cm) and the data stood at par with the treatment T<sub>8</sub> survival percentage was significantly higher (96.67%) in the treatment T<sub>4</sub> i. e. cocopeat + 3pieces coconut husk.

Considering the above mentioned character it has been concluded that the *Dendrobium* plantlets tied with 3 pieces coconut husk and planted in protray containing cocopeat was found to be most suitable for hardening of the plantlets, where after 60 days of hardening it has increased the plant height (27.33), and produced more no of (7.5) and longer roots (2.5cm) with maximum survival percentage (96.67%).

**Table 11 Standardization of media mixture for hardening of the *in vitro* plantlets of *Dendrobium* hybrid Sonia-17**

Treatment	Plant height	No of leaf	No of root/plant	Length of root	Survival %
T <sub>1</sub> - cocopeat	6.67	4.17	6.33	1.83	53.33 (43.22)
T <sub>2</sub> - cocopeat +1pc coconut husk	19.67	4.60	7.03	2.07	56.66 (44.32)
T <sub>3</sub> - cocopeat +2pc coconut husk	21.67	5.10	7.23	2.37	76.66 (51.48)
T <sub>4</sub> - cocopeat +3pc coconut husk	27.33	5.30	7.50	2.50	96.67 (79.37)
T <sub>5</sub> - cocopeat + charcoal(1: 1)	8.33	4.17	6.43	1.83	53.33 (43.22)
T <sub>6</sub> - cocopeat + charcoal + 1pc coconut husk	18.00	4.60	6.93	2.17	56.66 (44.32)
T <sub>7</sub> - cocopeat + charcoal + 2pc coconut husk	19.67	4.87	7.06	2.27	60. 00 (45.42)
T <sub>8</sub> - cocopeat + charcoal + 3pc coconut husk	21.67	4.93	7.16	2.43	66.66 (47.73)
SE(m)	0.42	0.12	0.03	0.04	1.90
CD%	1.28	0.37	0.11	0.11	5.76

(Fig. in parenthesis indicate angular/arsenic value)



**Fig. 6** Standardization of media mixture for hardening of the *in vitro* plantlets of *Dendrobium* hybrid Sonia-17

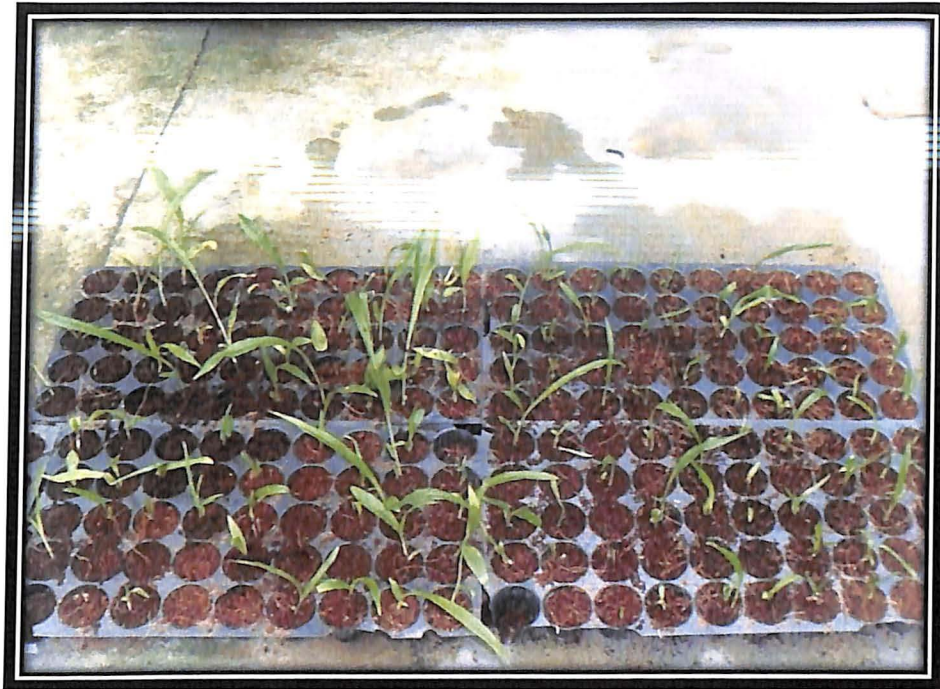


Plate 10



Plate 11

Plate 10 & 11: hardening of the *in vitro* *Dendrobium* Sonia-17 plantlets tied with coconut husk and planted in portrays containing cocopeat: charcoal in 1:1 ratio



## CHAPTER-V

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# *Discussion*

# DISCUSSION

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Generally, orchids are propagated both by asexually and sexually. But the traditional asexual propagation is extremely slow which can give rise to 2-4 plants per year (Nasiruddin *et al.*, 2003). Orchid capsule contains 2-3 millions seeds which look just like dust. The capsule usually takes 4-17 months to mature. Due to lack of any metabolic machinery and functional endosperm though the seeds are functional they are not able to germinate properly. However these seeds need a symbiotic relationship with a fungus called as orchid mycorrhiza and about 0.2-0.3 % of seeds in a capsule germinate in nature. Asexually orchids propagated by division of rhizomes, air layering, separation of offshoots & keikis. Considering the aforementioned propagation methods, it has been seen that both the processes of propagation are very slow, tedious and time consuming. These problems necessitate an alternate method of propagation which helps in bypassing the slow rate of multiplication, bulking of true to type disease free stocks and production of huge quantity of planting materials in shorter span of time. Though *in vitro* techniques have been standardized for different Orchid species by various workers (Indhumati *et al.*(2003), Parvin *et al.* (2009) & Vyas *et al.*(2011), ). The work on standardization of media supplements and plant bioregulators for shoot proliferation, rooting and media mixture of a hardening of a specific Orchid species or varieties is meagre. Therefore, in present investigation attempts are made to standardize the media combinations for shoot proliferation , root initiation and development of *Dendrobium formosum* and to standardize the media combinations for hardening of Orchid *Dendrobium* cv. Sonia-17 and the results obtained so far have been discussed in this chapter.

## 5.1 STANDARDIZATION OF MEDIA SUPPLEMENTS FOR SHOOT PROLIFERATION OF ORCHID (*Dendrobium formosum*)

In the present investigation greenish colour calli mass derived from the seed culture of the *Dendrobium formosum* where inoculated in 17 types of media combination. After 30 days of culture MS medium fortified with 4.0 mg/l BAP, 1.0

mg/l IAA and 0.1 mg/l GA<sub>3</sub> produced more no longer PLBs with deep green colour prominent rudimentary leaf. Same trend was also observed after 30 days of sub culture where MS medium supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.1 mg/l GA<sub>3</sub> enhanced the no of PLBs & length of the PLBs and produced more no of & longer deep green prominent leaves. However after 60 and 90 days of sub cultures, MS medium fortified with 3.0 mg/l BAP, 1.5 mg/l IAA and 0.1 mg/l NAA increased the no of multiple shoots/explant and produced more no of longer deep green coloured leaves. The effect was spectacular at 90 days of sub culture where the treatment produced 8 no of multiple shoots/ explant.

The result is in confirmity with the findings obtained by Rasmussen (1995) who reported a positive effect of adding cytokinins to the media. It might be due to the enhancement of cell division normally observed by action of cytokinin. Similar effect of BAP on stimulation of shoot growth in *Oncidium* was reported by Kalimuthu *et al.* (2007), who observed that culture of immature seeds of *Oncidium spp* in MS medium supplemented with BAP at 2.0 mg/l stimulated shoot multiplication and recorded maximum number of shoots/bottle.

The result corroborated with the findings of Talukder *et al.* (2003) who observed improvement in shoot proliferation, leaf number and shoot length in *Dendrobium* Orchid in MS medium supplemented with BAP @ 1.0 mg/l and 2.5 mg/l as compared to control i.e. 0 mg/l BAP and the most effective treatment was 2.5 mg/l BAP. Salam (2010) reported similar result with application of BAP 2.0 mg/l to MS medium.

The results corroborated with the findings of Patra (2009) who reported that kinetin 2.0 or 2.5 with ads 75 or 100 mg/l produced longer shoots in Gerbera and Sahoo (2012) in orchids. In Tobacco, the presence of adenine or kinetin in the medium leads to promotion of bud differentiation and development (Chawala 2002). Pattnaik (2007) reported that the longer shoots in gerbera cv. common Red. Pyati *et al* 2002 stated that axillary shoots were induced directly in medium containing BA, kinetin and coconut water.

## **5.2 STANDARDIZATION OF MEDIA SUPPLEMENT FOR ROOT INITIATION AND DEVELOPMENT**

*In vitro* root formation study in *Dendrobium formosum* has shown a very interesting result. It has been clearly indicated that MS medium supplement with 1.0 mg/l IBA and 1.0 mg/l NAA was found to be most potent combination for vegetative growth as well as root initiation and development after 60 days of culture, where the combination produced more no of longer roots/plant.

The results corroborated with the findings of Raju *et al.* (2003) who obtained maximum number of root in *Dendrobium osteroholt* in addition of IBA 1.0 mg/l and NAA 1.0 mg/l. The result was in agreement with Salam (2010) who reported that MS medium supplemented with IBA 1.0 mg/l on rooting of *Dendrobium* cv. banayat pink. Ancora *et al.* (1981) reported the effectiveness of IBA and NAA in root induction of *in vitro* produced plants in Globe artichoke. Sit and Tiwari (1997-98) stated that half MS medium supplemented with IBA successfully produced roots in Turmeric. MS medium supplemented with NAA developed healthy roots in Turmeric (Meenakhi *et al.*, 2001).

Treatments with auxins stimulate and show an increase in peroxidase activity, as observed by Palai (2001). It was suggested that the auxin entered through the cut surfaces of the proliferated shoots and rapidly absorbed in the cell walls by pH trapping (Rubery and Sheldrake, 1973) and by influx carriers (Delbarre *et al.*, 1996). Epstein and Ludwig-Muller (1993) reported that during the process of root induction, there were two major pathway of conversion: oxidation and conjugation. Exogenous auxins applied to micro-cuttings get oxidized, resembled the enzymes involved in the wounding reaction as reported by De Klerk *et al.* (1999). They also indicated that IAA or IBA oxidation caused by non-specific peroxidase was related to the rooting response.

## **5.3 STANDARDIZATION OF MEDIA SUPPLEMENT FOR HARDENING OF *IN VITRO* PLANTLETS**

The success of tissue culture depends on the sustainability of the *in vitro* produced plants under the natural conditions. Under *in vitro* conditions, the plantlets

are heterotrophs. So they have to be gradually converted into autotrophs. The pre-hardening and hardening are the processes for making the *in vitro* raised plantlets into autotrophs and adapt them to the outside environment (Beura, 1998). The existing rooted plantlets of *Dendrobium Sonia-17* varying from height 2.5-3cm were taken for hardening in 8 different combinations of media i.e. 1. Cocopeat, 2. Cocopeat + 1 pieces coconut husk, 3. Cocopeat + 2 pieces coconut husk, 4. Cocopeat + 3 pieces coconut husk, 5. Cocopeat + charcoal (1:1), 6. cocopeat + charcoal + 1 pieces coconut husk, 7. Cocopeat + charcoal + 2 pieces coconut husk, 8. Cocopeat + charcoal + 3 pieces coconut husk in portrays. The plants were hardened up to 60 days. The half MS liquid medium was fertigated to the seedlings at an interval of two days up to two weeks and then one fourth MS liquid medium was applied at an interval of two days for the next two weeks. Different observations such as plant height, leaf number, leaf length and percentage of survival have been recorded after 60 days of hardening. the data indicated very specific results that the plantlets tied with 3 pieces coconut husk and planted in portray containing cocopeat media combinations significantly increase plant height, leaf number, leaf length and percentage of survival after 60 days of hardening followed by the plantlets tied with 2 pieces coconut husk and planted in portray containing cocopeat and tied with 2 or 3 pieces coconut husk and planted in portray containing cocopeat and charcoal in 1:1 ratio for plant height, leaf number, leaf length concerned.

The results of the present investigation are in partially agreement with that of Sharma (1995) who reported that the media combination comprising of brick: charcoal: tree fern: bark pieces: leaf mould: dry sphagnum moss 1:1:1:1:2 in the ratio was considered suitable for maximum survival of *Dendrobium* & chrysanthemum seedlings. Indhumati *et al.*, (2003) reported that charcoal+brick+cocopeat in the ratio 1:1:1 was found suitable for establishment of plantlets of *Dendrobium* Hybrid Sonia-17. According to Pathania *et al.* (1998) hardening medium containing bark pieces: brick pieces: moss: charcoal pieces (1:1:1:1) proved to be suitable for the establishment of *in vitro* rooted plantlets of Sonia-17.



CHAPTER-VI

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*Summary & Conclusion*

## **SUMMARY AND CONCLUSION**

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The present investigation entitled “*In vitro* propagation of orchid (*Dendrobium formosum* Rox ex Lindl)” was carried out in the Biotechnology- cum- Tissue Culture Centre, Dept of floriculture & Landscaping CA, OUAT Bhubaneswar, during the year 2013-2014. The following steps were undertaken for conducting the whole experiment:

1. Standardization of media supplements for shoot proliferation.
2. Standardization of media supplements for root initiation and development of orchid (*Dendrobium formosum*).
3. Standardization of media mixture for hardening of *in vitro* plantlets.

The result of the experiments were summarized as follow:

1. **Standardization of media supplements for shoot proliferation of orchid *Dendrobium formosum***
  - i) MS medium supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.1 GA<sub>3</sub> significantly produced more no of PLBs per explant (7.13), and enhanced the length of PLBs (2.0cm) as well as length of the leaf (1.16cm), after 30days of culture.
  - ii) After 30 days of subculture MS medium supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.1 GA<sub>3</sub> significantly increased the no of PLBs per explant (9.27), length of PLBs (2.03cm), no of leaves per PLBs (5.66) and increase the length of leaf (1.46cm).
  - iii) After 60 days of sub culture MS medium supplied with 3.0 mg/l BAP, 1.5 mg/l IAA and 0.1 mg/l NAA significantly increased the no of shoot per explant (4.2), no of leaves per shoot mass (9.48) and the length of the leaf (2.2cm) and produced deep green coloured leaves.

iv) After 90 days of subculture MS medium fortified with 3.0 mg/l BAP, 1.5 mg/l IAA and 0.1 mg/l NAA significantly increased the no of shoot per explant (8.00), no of leaves per shoot mass (11.48), and length of the leaf (2.36 cm) and produced deep green coloured leaves.

**2. Standardization of media supplements for root initiation and development of orchid (*Dendrobium formosum*)**

i) MS medium supplemented with 1.0 mg/l each of IBA and NAA had positive effect on improving vegetative as well root growth in orchid.

ii) MS medium enriched with 1.0 mg/l IBA along with 1.0 mg/l NAA was most suitable combination for initiation and development of root in *Dendrobium formosum* after 60 days of culture, where the combinations produced more no of roots /plant (3.50) longer roots /plants (0.47cm).

**3. Standardization of media mixture for hardening of *in vitro* plantlets of *Dendrobium Sonia-17*, tissue cultured.**

i) *Dendrobium* plantlets tied with 3 pieces coconut husk and planted in protray containing cocopeat was found to be most suitable for hardening of the plantlets, where after 60 days of hardening it has increased the plant height (27.33), and produced more no of (7.5) and longer roots (2.5cm) with maximum survival percentage (96.67%).

## CONCLUSION

The findings of the present investigations are expected to give a new orientation to the thoughts of the future workers for the large scale multiplication of the precious orchids through *in vitro* culture. The work on *in vitro* culture *Dendrobium formosum* an orchid native to state of Odisha appears to be quite innovative as there has been no work, reported so far on this prized species. The unique protocol developed for *in vitro* propagation of *Dendrobium formosum* will guide the researchers, scientists and entrepreneurs in a great way.

The work on *in vitro* culture of *Dendrobium spp* cv. Sonia-17 has given very promising results in respect of hardening of *in vitro* plantlets. The tissue cultured plantlets of *Dendrobium* Sonia-17 tied with 3 pieces coconut husk and planting in portrays containing cocopeat was found to be the most suitable for hardening. The outcome of the observations made during the protocol development of *Dendrobium formosum* and hardening studies of the *Dendrobium spp* cv. Sonia-17 give the author enough confidence to recommend the suitable medium used for plantlets production and as well.



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# *Bibliography*

## BIBLIOGRAPHY

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- Alam, M.K., Rashid, M.H., Hossain, M.S., Salam, M.A and Rouf, M.A. (2002). *In vitro* seed propagation of *Dendrobium (D.transparens)* Orchid as influenced by different media Biotechnology.Asian Network for Scientific Information, 2002.1 (2-4): 111-115.
- Akhtar,S. Nasiruddin, K.M. and Hossain, K. (2008). Effects of different media and organic additives on *in vitro* regeneration of *Dendrobium* Orchid. *Journal of Agriculture and Rural Development*.6 (1 & 2): 69-74.
- Arditti,J.(1979). Aspect of the physiological of Orchids. *Adv. Bot. Rev.*33: 1-97.
- Arumugam, T. and Jawaharlal, M.(2004).Effect of shade levels and growing media on growth and yield of *Dendrobium*Orchid cultivar Sonia-17.*Journal of Ornamental Horticulture*,7(1): 107-110.
- Abhijit,T and Handique, A.K.(1998). Phytohormone induced variation in *in vitro* seed culture of *Dendrobium aphyllum (Roxb.)*. *Journal of Phytological Res.* 11 (1): 19-22.
- Devi,J.,Natum, Deuli, M. and Deka, P.C (1990). Effect of different media on germination and growth of some north Indian species of *Dendrobium*.*Journal of Orchid Society of India*.4 : 45-49
- Hossain, M.M. (2008). Asymbiotic seed germination and *in vitro* seedling development of *Epidendrum ibagueme* Kunth (Orchidaceae).*African Journal of Biotechnology*.Vol. 7 (20): 3614-3619, 20 October, 2008.
- Huan, L.V.T., Takamura,T. and Tanaka, M.( 2004). Callus formation and plant regeneration from callus through somatic embryo structures in *Cymbidium* Orchid. *Plant Science*.166 (6): 1443-1449.

- Indhumati, K., Kannan M., Jawaharlal, M., and Amarnath V.(2003). Standardization of prehardening and hardening techniques for *in vitro* derived plantlets of *Dendrobium* Orchid Hybrid Sonia-17. *Journal of Ornamental Horticulture*, 6 (3) : 212-216
- Irawati, S.S,(1977). Effect of culture( Knudson) media in best growth of *Dendrobium*. *Orchid Review*.85:138-142.
- Kishor, R., Meitei, W.L. and Sharma, G.J. (2007). Clonal propagation of a *Dendrobium* hybrid Orchid (*D.nobile* L. X *D.chrysotoxum*L) using pseudobulb segment. *Recent Trends in Horticultural Biotechnology*.315-317.
- Kurup, S.S., John, S., Rejimol, K.R. and Mathew, J.C. (2007). Plant Regeneration from Mesophyll Protoplast of *Dendrobium* var. Sonia. *Recent Trends in Horticultural Biotechnology (Eds.)*.RaghunathKeshvachandran, et al.425- 427.
- Kalimuthu, K., Senthikumar, R., and Vijayakumar,S. (2007). *In vitro* micropropagation of Orchid, *Oncidium* sp.(dancing doll). *African Jour.Of Biotechnology*.6(10) : 1171 -1174.
- Ket, N.V., Hahn,E.J., Park, S.Y., Chakrabarty, D., Paek, K.Y.E.(2004). Micropropagation of an endangered Orchid *Anoectochilm formosanns*, Biological Plantarum (Czech Republic).48(3) 339-344: Sep 2004.
- Kitsaki, C.K., Zygouraki, S., Ziobora, M. and Kintzios, S. (2004). *In vitro* germination, protocorm formation and plantlet development of mature versus immature seeds from several *Ophryss* species (Orchidaceae). *Plant Cell Reports*.23 (5):284-290.
- Knudson, L. (1946). A new nutrient solution for the germination of Orchid seed. *American Orchid Society Bulletin*.15: 214-217.

- Le Van, T. H., Takamura, T. and Tanaka, M. (2004). Callus formation and plant regeneration from callus through somatic embryo structures in *Cymbidium* Orchid. *Plant Science*. **166** (6): 1443-1449.
- Lin, C.C. (2005). Histology and morphology studies on induced somatic embryos: protocorm-like bodies formation and plants regeneration from callus in *Vanda sanderiana* (Orchidaceae) flower stalks internodal segments *in vitro* culture. Proceedings-of-the-18th-World-Orchid-Conference, Dijon, France, -11-20-March, -2005. 2005; 311-317.
- Morel, G.M. (1974). Clonal multiplication of Orchids. In: The Orchids: Scientific Studies. Withner, C.L. (Ed.), Wiley New York. 513-523.
- Murashige, T. and F. Skoog. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. **15**: 473-497.
- Martin, K.P. and Madassery, J. (2006). Rapid *in vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. *Scientia Horticulturae*. **108**(1):95-99
- Mani, S.K. and Nagaraju V. (2004). Influence of potting media on acclimatization and growth of *in vitro* plantlets of *Cymbidium* hybrids. *Journal of Ornamental Horticulture*, **7** (1) :23-30
- Nitsch, C, Nitsch, J.P. (1967). The induction of flowering *in vitro* on stem segments of *Plumbago indica* L., The production of reproductive buds, *Planta*, 1967. **72**:371.
- Nayak, N.R., Rath, S.P. and Patnaik, S. (1997). *In vitro* propagation of three epiphytic Orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch-Ham) Sw. through thidiazuron-induced high frequency shoot proliferation. *Scientia Horticulturae*. **71**(3-4):243-250.

- Nasiruddin, K.M., Begum, R. and Yasmin, S. (2003). Protocorm like bodies and plantlet regeneration from *Dendrobium formosum* leaf callus. *Asian Journal of Plant Sciences*. 2(13):955-957.
- Nagaraju, V., Das, S.P. (2003), *In vitro* morphogenetic response of Orchids to tricontanol. *Journal of Ornamental Horticulture*. New Series-2003.6:16-19.
- Nagaraju, V., and Mani, S.K. (2007). Coconut water mediated multiplication of *Cymbidium Showgirl Cooks bridge* *in vitro*. *Jour. Of Ornamental Horticulture*. 10(2): 101-104.
- Paek, K.Y. and Edward C, Yeung, (1991). The effects of 1-naphthaleneacetic acid and N<sup>6</sup>-benzyladenine on the growth of *Cymbidium forrestii* rhizomes *in vitro*. *Plant Cell, Tissue and Organ Culture*. 24 (2): 65-71.
- Patra, S. 2009, Rapid clonal multiplication of commercial varieties of gerbera (*G. jamsonii*, Bolus) through *in vitro* techniques. Msc. (Ag.) thesis submitted to department of horticulture CA, OUAT, Bhubaneswar. pp134
- Ping, L. J., Wang, Y., Zha, X.Q. and Huang, L. (2008). Micropropagation of *Dendrobium densiflorum* Lindl. ex Wall, through protocorm-like bodies: Effects of plant growth regulators and lanthanoids. *Plant Cell, Tissue and Organ Culture*. 93(3): 333-340.
- Pradha, S., Bijaya, P., (2009). *In vitro* seed germination in *Cymbidium elegans* Lindl. and *Dendrobium densiflorum* Lindl. ex Wall. (Orchidaceae). *Botanical Orientalis- Journal of Plant Science*. (6): 100-102.
- Pathania, N.S; Shegal, O.P., Debojit, P. Dutta, B.S and Paul, D. (1998). Studies on micropropagation in *Dendrobium* cv Sonia. *J. Orchid. Soc.* 12 (1-2): 35-38.
- Puchooa, D. (2004). Comparison of Different Culture Media for the *In vitro* Culture of *Dendrobium*. *International Journal Of Agriculture & Biology*. 884-888.

- Pyati,A.N., Murthy,H.N., Hahn,E.J., Paek,K.Y .(2002) . *In vitro* propagation of *Dendrobiummacrostachyum*Lindl.*Indian Journal of Experimental Biology*.**40** (5).
- Ping, L. J., Wang,Y., Zha, X.Q. and Huang, L. (2008) . Micropropagation of *Dendrobiumdensiflorum*Lindl.ex Wall, through protocorm-like bodies: Effects of plant growth regulators and lanthanoids. *Plant Cell, Tissue and Organ Culture* .**93**(3): 333-340.
- Park, S.Y., Murthy, H.N, and Paek, K.Y. (2000). *In-vitro* seed germination of *Calanthesieboldii*, an endangered Orchid species.*Journal of Plant Biology*.**43** (3):158-161.
- Parvin,M.S., Haque,M.E., Moniruzzaman,F.A. and Khaldun,A.B.M.(2009). Effect of different levels of NAA on *in vitro* growth and development of shoots of *Dendrobium* Orchid. *Bangladesh J. Agril. Res.* **34**(3): 411-416.
- Reddy,P,V.(1992). Study of the effect of-culture media on seed germination of *Dendrobium* Orchid. *Journal of OrchidSociety, India*.**6**: 75-78.
- Raju, B., George, A., Jaganath, J., Jayaprasad K.V. and Honabyraiah,(2003). Micropropagation of *Dendrobium*. National Symposium on Recent Advances in Indian Floriculture.12-14.
- Roy, J. and Banerjee, N. (2003). Induction of callus and plant regeneration from shoot-tip explants of *Dendrobium fimbriatum* Lindl. var. *oculatum*Hk. f. . *Scientia Horticulturae*.**97**(3-4):133-140.
- Saiprasad,G.V.S.,Raghuveer,P.,Khetrapal,S.,Chandra, R.(2002). Effect of various growth regulators on the production of protocorm like bodies in three Orchid genera. *Indian-Journal-of-Plant-Physiology* (India). (Jan-Mar 2002). **7**(1): 35-39.

- Sangama and Singh, F. (1994). Effect of growth regulators on *in vitro* seed germination of *Spathoglottis plicata*. In :J. Prakash and K.R. Bhunday(Eds). Floriculture Technology, trades and trends oxford & IBH publishing co pvt. Ltd. 389:391.
- Sagawa, Y. and Kunisakhi, J.T.(1982). Clonal propagation of Orchids by tissue culture. In A. Fujiwara Plant Tissue Culture Maruzen, Tokyo.683-688.
- Sahoo, S. 2012, High frequency multiplication of orchid through *in vitro* culture Msc. (Ag.) thesis submitted to department of Floriculture and Landscaping, CA, OUAT, Bhubaneswar.pp77
- Singh, F and Prakash D.(1985). Suspension Culture technique for the culture of Orchid embryos. *GartenbauWissenSchaft*. **50**: 236- 238.
- Scott L. S. and Kane, M.E. (2006). Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial Orchid. *Plant Cell, Tissue and Organ culture*. **86**(2):147-158.
- Shylaraj, K.S., Deepthy, R. and Korah, P.L.(2007). High Frequency Mericlone from Shake Cultures of Tropical Orchid *Dendrobium* cv. Sonia. *Recent Trends in Horticultural Biotechnology (Eds.)* Raghuvanath Keshvachandran, et al., 327-332.
- Sobhana, A, and Rajeevan, P.K.(2002). Refinement of embryo culture medium in *Dendrobium* proceedings of the Abstract National Symposium on Indian Floriculture in the New Millennium. 65.
- Sharma, J. and Chauhan, Y.S.,(1995). Establishment of *in vitro* raised seedlings of *Dendrobium* and *Paphiopedilum*. *Journal of the Orchid Society of India*, **9** (1-2) : 37-41.

- Sugapriya,S.,Mathad,J.C., Patil, A. A., Hegde,R.V., Lingaraju,S., and Biradar,M.S(2011).Evaluation of *Dendrobium* Orchids for growth and yield grown under greenhouse. *Karnataka J. Agric. Sci.*, **25** (1) : (104-107) 2012
- Talukdar, A.(2001). Multiple shoot induction in *Dendrobium aphyllum* Roxb.*J. Orchid Soc. India.***15**: 35-38.
- Vacin, E. and F. Went, (1949). Some pH changes in nutrient solution. *Bot. Gaz.*,**110**: 605-13.
- Vyas,S., Kapoor-Pandey,P.,Guha, S. and Usha Rao, I.,(2011).Synchronous Plantlet Formation by Using Banana Extract and *In vitro* Hardening in Orchid, *Dendrobium lituiflorum* Lindl. *Journal of Ornamental and Horticultural Plants*, **1**(3): 175-184, December, 2011
- Yung,L.,Lee,N.A.,Lee,Y.,Lee,N.A., (2003). Plant regeneration from protocorm-derived callus of *Cypripedium formosanum*. Plant regeneration from protocorm-derived callus of *Cypripedium formosanum*.*In-Vitro-Cellular-and-Developmental-Biology-Plant*.2003, **39**: 5, 475-479; 11 ref.
- Zhang,Z.G.Liu,Y.,WangL.,,Cai,Z.G.,Han.,X.Z.,Zhao)L.H.,andLuo, Z.J.(1992). Study on factors protocorm proliferation of *Dendrobium candidum*. *Chinese Traditional and Herbal Drugs*.**23** (8):431-433.

