PHYSIOLOGICAL APPROACHES FOR ENHANCING THE *EX VITRO* ESTABLISHMENT OF TISSUE CULTURED ORCHID (*Phalaenopsis* sp.)

By

SAYOOJ .S
(2017-11-088)

THESIS

Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture
Kerala Agricultural University

DEPARTMENT OF PLANT PHYSIOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM- 695 522
KERALA, INDIA
2019

DECLARATION

I, hereby declare that this thesis entitled **"PHYSIOLOGICAL APPROACHES FOR ENHANCING THE *EX VITRO* ESTABLISHMENT OF TISSUE CULTURED ORCHID (*Phalaenopsis* sp.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship or other similar title, of any other University or Society.

Place : Vellayani
Date : 04/09/2019

SAYOOJ .S
(2017-11-088)

CERTIFICATE

Certified that this thesis entitled **"PHYSIOLOGICAL APPROACHES FOR ENHANCING THE *EX VITRO* ESTABLISHMENT OF TISSUE CULTURED ORCHID (*Phalaenopsis* sp.)"** is a record of research work done independently by Mr. SAYOOJ.S under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Place : Vellayani
Date : 04/09/2019

Dr. Viji. M.M
(Major Advisor, Advisory Committee)
Professor and Head
Department of Plant Physiology
College of Agriculture, Vellayani

CERTIFICATE

We, the undersigned members of the advisory committee of Mr. SAYOOJ.S a candidate for the degree of **Master of Science in Agriculture** with major in Plant Physiology, agree that the thesis entitled **"Physiological approaches for enhancing the *ex vitro* establishment of tissue cultured orchid (*Phalaenopsis* sp.)"** may be submitted by Mr. SAYOOJ . S in partial fulfilment of the requirement for the degree.

Dr. Viji. M. M
Professor and Head
Department of Plant Physiology
College of Agriculture, Vellayani

Dr. Roy Stephen
Professor
Department of Plant Physiolgy
College of Agriculture, Vellayani

Dr. Swapna Alex
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani

Dr. Deepa S Nair
Assistant Professor and Head
Department of Plantation Crops and Spices
College of Agriculture, Vellayani

ACKNOWLEDGEMENT

First of all I wish to express sincere gratitude and indebtedness to ***Dr. viji M.M*** *, Professor and Head, Department of Plant Physiology, College of Agriculture, Vellayani and Chairperson of my advisory committee for her valuable guidance, suggestions, constant support and co-operation throughout the investigation and thesis preparation. This work would not have been possible without her valuable help and support.*

I am indebted to ***Dr. Roy Stephen*** *Professor, Department of Plant Physiology, College of Agriculture, Vellayani and member of my advisory committee for his ardent interest, expert advice and critical scrutiny of the manuscript. This task would not have been possible without his unexplainable help.*

With an overwhelming sense of pride and genuine obligation, I take this opportunity to express deep due sense of gratitude to ***Dr. Swapna Alex,*** *Professor and Head, Department of Plant Biotechnology, College of Agriculture, Vellayani and member of my advisory committee for her valuable sustained encouragement, necessary advices and contribution towards this work.*

With great pleasure I express my heartiest and esteem sense of gratitude to ***Dr. Deepa S Nair,*** *Assistant Professor and Head, Department of Plantation Crops and Spices, College of Agriculture, Vellayani, for her encouragement, wholehearted help and support throughout the period of research work.*

My heartful thanks to my beloved teachers, ***Dr.Manju.R.V*** *and* ***Dr. Beena .R*** *for their encouragement, valuable help and advice rendered during the course of study.*

I am thankful to my classmates ***Lakshmi, Aachi, Ammu pathy, Ragu, and Manikanta*** *for their friendship and kind help in times of need.*

A very special thanks to ***Vipin chettan, Anila chechi, Neethu chechi and Vishnu chettan*** *for their timely help and assistance.*

My special thanks goes to my seniors ***Yogesh bayya, Gayathri chechi, Rahul chettan, Amrutha chechi, Reshma chechi, Nithya chechi, and Manasa akka, Sreekanth Bayya and Saranya chechi*** *for their kind help, without which I may never have completed my research work.*

I would like to express my gratitude to my seniors ***K Raj, Nibin chettan, Sachin chettan, Ajilettan, Merin chechi, and Mithra chechi,*** *for their support and encouragement.*

I am thankful to my friends ***Anju, Sandy, Dhanu, Aju, Kuban, Thadiyan, Abu, Gopettan, Cibi, Sreekanth, Jaseel, Manu, Anand, Govind, Amal, Allen, Melvin, Suresh, John, Ahal, Thasni, Anargha, Liz, cubi, susu, Aisu, Swathi, Susan,*** *for their timely help and encouragement.*

Finally, I am thanking my juniors ***Sreerag, Sreevardhan, Amrutha, Bhavana and Sudha,*** *for their love and support during my PG programme.*

Mere words cannot express my profound indebtness to my beloved ***Achan,*** *my dear* ***Amma,*** *and my brother* ***Sarang,*** *for their unbounding love, unparalleled affection, constant prayers and encouragement throughout my career.*

Once again I express my cordial gratefulness collectively to everyone who helped me during my research work.

SAYOOJ . S

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ABA	:	Abscisic acid
AMF	:	Arbuscular mycorrhizal fungi
CD (0.05)	:	Critical difference at 5 % level
GA	:	Gibberellic acid
DAP	:	Days after planting
et al.	:	Co-workers/ Co-authors
Fig.	:	Figure
g	:	Gram
g^{-1}	:	Per gram
h	:	Hour
HCZ	:	Hexaconazole
HCl	:	Hydrochloric acid
kg	:	Kilogram
m^2	:	Square metre
mg	:	Milligram
mL	:	Millilitre
NS	:	Not significant
No.	:	Number
PBS	:	Phosphate buffered saline
PBZ	:	Paclobutrazol
SEm	:	Standard error of mean
SLA	:	Specific leaf area
TDM	:	Triadimefon
UCZ	:	Uniconazole
viz.,	:	Namely

LIST OF SYMBOLS

%	:	Per cent
@	:	at the rate of
µ	:	Micro

Introduction

1. INTRODUCTION

Orchids are the most valuable group of flowering plants in the nature and distributed throughout the world from the tropics to the high alpine mountains. They exhibit an incredible variety of diversity in terms of the size, shape and colour of their flowers. They are one of the main cut flowers of Kerala belonging to the Orchidaceae family, which includes about 800 genera and over 35,000 species. Orchids are famous for their mesmerising and beautiful flowers with long lasting shelf life. Also orchid flowers fetch high price in the international market.

Kerala has been blessed with a tropical and subtropical climate due to its elevation from the sea level to the western ghats. With the availability of sunlight, well distributed rainfall and high relative humidity, Kerala state has the potential to produce orchids in a large-scale on a commercial basis. Among orchids, phalaenopsis or moth orchids are the most common ones due to the ease of production and availability of flowering plants throughout the year. Phalaenopsis orchids are monopodial type and sometimes lithophytes with long and thick roots and short and leafy stems hidden by overlapping of foliar bases. The succulent leaves are usually oblong to elliptic in shape and arranged in two rows. Flowers are long lasting, fragrant and are seen in small to large racemes or panicles.

Since the conventional methods of orchid propagation are time consuming and laborious, nowadays people are going for tissue culture to produce more number of plants in a short period of time. In many countries, phalaenopsis plantlets are being produced using tissue culture techniques for commercial production.

The ability to transfer plants out of culture with high survival rates is the main success factor behind micropropagation on a commercial scale. But during *ex vitro* establishment the tissue cultured phalaenopsis plantlets usually show high rate of mortality due to sudden shock of environmental changes. So there is a need to standardise the *ex vitro* establishment of tissue cultured phalaenopsis orchids.

While transferring the *in vitro* grown plantlets to field conditions they are unable to compete with soil microbes and to survive in the environmental conditions. The *in vitro* culture conditions produce plantlets which are having altered physiology, morphology and anatomy. During hardening stage, efforts are focused on the control of both physical and chemical environment and biohardening of plantlets in order to increase growth and reduce mortality. Field grown plants are different from *in vitro* cultivated plants. High mortality is observed upon transfer of micro shoots of orchids to *ex vitro* conditions as the cultured plantles have non functional stomata, weak root system and poorly developed cuticle (Mathur *et al.,* 2008).

Within the *in vitro* system, the plantlets of orchids are heterotrophic and they are characterised by some physiological, morphological and anatomical peculiarities. During *ex vitro* establishment the plantlets have to switch over to autotrophic nutrition, involving normal photosynthetic activity. Normally the tissue cultured plantlets of orchids show high rate of mortality in *ex vitro* establishment. This is mainly because the *in vitro* derived plantlets will not be able to withstand the sudden shock of environmental changes and improper functioning of stomata (Brainerd and Fuchigami 1981; Wardle *et al.,* 1983).

Studies regarding the physiological changes that occur during *ex vitro* establishment of orchids and how they influence the plant growth and survival in the new environment are very much limited. Hence the present study was carried out with the following objectives,

- To study the physiological changes that occur during *ex vitro* establishment of orchid (*Phalaenopsis* sp.)
- To find out measures to overcome the field mortality
- To improve propagation efficiency

Review of Literature

2. REVIEW

The successful *ex vitro* establishment of phalaenopsis orchid plantlets raised *in vitro,* determines the quality of the end product (cut flowers or potted plants) in commercial production for economic gain. Due to sudden changes in the environment, *in vitro* derived plantlets may not survive well in *ex vitro* conditions if proper precautions (hardening) necessary for the plantlets to acclimatize are not given. The hardening of *in vitro* derived plantlets is by slowly subjecting them to higher light levels and relatively lesser humidity levels so as to facilitate more survival percentage. The most common approach to improve the survival of *in vitro* derived plantlets upon transfer to *ex vitro* environment is subjecting them to gradually adapt to the external environment. During hardening period, plants develop a fully functional root system and more control over their stomatal and cuticular transpiration and then gradually shift from a heterotrophic to an autotrophic growth.

The environmental changes that occur during transfer of *in vitro* derived plantlets to *ex vitro* conditions will create a stress for the plantlets. In order to adapt to the new environment, plantlets have to repair the structural and functional abnormalities occurred during their *in vitro* production (Ziv and Chen, 2008).The success of acclimatization mainly depends on the chemical and physical factors of both the *in vitro* as well as the *ex vitro* conditions.

This review encompasses the research works on physiological, morphological, biochemical, biometric and anatomical characteristics during *ex vitro* establishment of *in vitro* derived phalaenopsis plantlets.

4.1 ACCLIMATIZATION

Hardening contributes to a major portion of the production cost and it is a time consuming and labour intensive process. In commercial production, the quality of the end product is determined by the successful *ex vitro* acclimatization of *in vitro* derived plants (Conner and Thomas, 1982). Poole and

Conover (1983) reported that as a result of the change in environment, transplanted plantlets may wilt or desiccate and ultimately die. By accommodating substantial precautions, this problem can be avoided and this step is considered as the major limiting factor in commercial micropropagation.

Traditional process of acclimatization is the gradual adjustment of *in vitro* derived plants towards *ex vitro* conditions such as ambient relative humidity and light levels. *In vitro* derived plantlets must undergo a period of transitional development to escape from the influence of the *in vitro* environment and that period is specifically called as acclimatization (Donnelly and Vidaver 1984). Sutter (1984) opined that high mortality of plantlets is a result of abnormal morphology and physiology of the plants due to the induction of growth conditions inside the culture vials.

During the final stage of micropropagation, *in vitro* derived plantlets show high mortality due to transplantation shock (Dhawan and Bhojwani, 1986). Debergh (1988) stated that acclimatization is an expensive, challenging and labour intensive process. Temperature, humidity, irradiance, CO_2 concentration and air flow rate are the factors of acclimatization which need to be controlled (Hayashi *et al.,* 1988). Fila *et al.* (1998) suggested that the development of cuticle, epicuticular wax and effective stomatal regulation of water loss causes gradual reduction in cuticular transpiration rates during acclimatization to *ex vitro* conditions.

As a prerequisite to develop efficient transplantation protocols, these abnormalities should be analysed. To correct the abnormalities, a period of acclimatization is essential because, these plantlets might easily be impaired by sudden changes in environmental conditions (Pospisilova *et al.,* 1999). Hazarika (2003) reported that there is a necessary requirement of acclimatisation of micropropagated plantlets for better physiological and anatomical characters. Mathur *et al.* (2008) opined that environmental conditions should be controlled during acclimatization stage to compete with soil microflora and to increase the growth and reduce the mortality rate of plantlets. For successful establishment and

survival, the *in vitro* derived plantlets should be acclimatized, because they are being exposed to abiotic and biotic stress conditions in *ex vitro* environment (Deb and Imchen 2010).

4.2 EFFECT OF LIGHT INTENSITY ON *EX VITRO* ACCLIMATIZATION

Light intensity plays a significant role in *ex vitro* establishment of orchids. *In vitro* propagation is an efficient method to produce large amount of uniform plantlets. However, micropropagated plantlets are associated with several physiological and anatomical abnormalities during *in vitro* growth such as low photosynthesis, improper functioning of stomata, malfunctioning of water transport systems mainly due to the high humidity levels inside the culture vessel (Kozai, 1991). Once transferred to *ex vitro* condition, micropropagated plantlets are susceptible to photoinhibition because of lack of well developed physiological systems. Therefore, acclimatization of micropropagated plantlets to *ex vitro* condition is a crucial step to cope up with the new environment for better growth and development. These environmental changes associated with *in vitro* derived plantlets can be improved in *ex vitro* condition by controlling the light intensity and with improved air exchange (Amancio *et al.,* 1999; Hahn and Paek, 2001).

Gordon *et al.* (1994) reported that in *Posidonia sinuosa* plants leaf size decreased under low light conditions. Under high irradiance photosynthetic activity is depressed by photoinhibition (Osmond, 1994).Van-Huylenbroeck *et al.* (1995) examined the effect of light intensity in *Calathea louisae* and *Spathiphyllum floribundum* plantlets and pointed out that the exposure to high irradiance suddenly after transplantation caused chlorophyll photo bleaching and photoinhibition. Pospisilova *et al.* (1999) reported that when *in vitro* derived *Nicotiana tabacum* plantlets were acclimatized in two phases that is first in the greenhouse with low irradiance of 30-90 μ mol m^{-2} s^{-1} and then in the open air with high irradiance of 200-1,400 μ mol m^{-2} s^{-1} showed no photoinhibition. Samasya (2000) reported that the treatments of 50 per cent light intensity and 70 to 90 per cent humidity provided during *ex vitro* establishment of

orchid plantlets (*Dendrobium* sp.) recorded significantly higher survival rate and higher photosynthetic rate.

During *ex vitro* transfer, controlling the light intensity is a method to reduce the effect of environmental changes associated with *in vitro* plantlets (Hahn and Paek, 2001). Moraes *et al.* (2002) reported that when tissue cultured *Dendrobium nobile* plantlets were grown under a light intensity of 50%, they showed a survival rate ranging from 77.8 to 95.2%. Nayak *et al.* (2002) reported that in *Dendrobium sp.* survival rate of the plantlets enhanced during the acclimatization in a growth chamber for 8–10weeks before transfer to a field at 2590 lux and 80% relative humidity. Sunitibala and Kishor, (2009) reported that more than 90% survival rate was obtained when *Dendrobium transparens* plantlets were acclimatized by providing 50% shading.

The photosynthetic apparatus absorbs excessive light energy under high irradiance and results in the inactivation of the chloroplast reaction centers which contains chlorophyll (Bertamini *et al.,* 2006). Martin and Madassery (2006) when acclimatized dendrobium plantlets for one month in controlled conditions of (25 ± 2) °C, 85% ± 5% relative humidity and a 12 h photoperiod at 1850 lux, obtained over 80% survival rate. Dai *et al.* (2009) reported that in *Tetrastigma hemsleyanum*, light conditions had significant effect on leaf morphology. Larger leaves were produced by plants grown under 67% and 75% shade than other treatments. But smallest leaves, highest chlorophyll a, chlorophyll b and total chlorophyll content as well as the lowest chlorophyll a:b ratio were observed in 90% shade treatment.

In vitro derived plantlets are generally grown under a low light intensity of 1,200–3,000 lux and a temperature of 25 ± 2°C. So their direct transfer to high light intensity of 4,000–12,000 lux and temperature of 26–36°C might cause wilting and death of plantlets. Hence the process of hardening or acclimatization is necessary to habituate the plant with the natural conditions (Lavanya *et al.,* 2009). More than 90% survival rate was obtained when *Dendrobium transparens* plantlets were acclimatized by providing 50% shading

(Sunitibala and kishor, 2009). Winarto *et al.* (2013) pointed out that a high survival rate of 90–100% was obtained when *Cycas rumphii* plantlets were grown under reduced light intensity using 50% shading net (100–150 mol/m^2/s).

4.3 EFFECT OF HUMIDITY ON *EX VITRO* ACCLIMATISATION

Plantlets grown *in vitro* might be easily impaired by sudden changes in environmental conditions after *ex vitro* transfer. They usually need several weeks under shade and gradually decrease the air humidity to acclimate to the new conditions and to correct all abnormalities in their anatomy and physiology induced by special conditions of *in vitro* culture. Humidity has a significant role in *ex vitro* acclimatisation of tissue cultured orchid plantlets.

Ziv (1986) suggested that in carnation plantlets there was an increase in epicuticular wax development when the relative humidity was maintained at 50-70%, and the survival rates increased from 75 to 90%. Ritchie *et al.* (1991) opined that increased epicuticular wax, improved stomatal functioning and reduced leaf dehydration were shown by leaves of chrysanthemum and sugar beet, when they were acclimatized under a relative humidity below 100%.

Bolar *et al.* (1998) reported that *in vitro* derived plantlets usually demand some period of acclimatization with gradual lowering in air humidity during *ex vitro* establishment. Also it was reported that *in vitro* derived plantlets, when removed from culture vessels, they show high stomatal and cuticular transpiration of leaves due to the retarded development of the cuticle, epicuticular waxes as well as functional stomatal apparatus. Later the plantlets will develop cuticle and epicuticular waxes when they are transferred slowly from high to low humidity and the stomatal and cuticular transpiration rates will gradually decrease (Pospisilova *et al.,* 1999).

Sharma *et al.* (2007) opined that 60% survival rate was obtained when *Dendrobium microbulbon* plantlets were acclimatized by providing 80-90% relative humidity (RH). Tissue cultured *Dendrobium nanum* plantlets when acclimatized under 95% RH, showed 85% survival rate (Maridass *et al.,* 2010).

Winarto and Silva, (2015) stated that the relative air humidity surrounding *in vitro* derived dendrobium plantlets upon transplantation is usually 70%–80%. But Das *et al.* (2008) reported it is occasionally 90% or higher in some of the orchid species *viz., Aerides odoratum, Cymbidium longifolium, Dendrobium aphyllum and Rhyncostylis retusa.*

4.4 EFFECT OF TRIAZOLE ON *EX VITRO* ACCLIMATISATION

The regulation of plant growth with synthetic plant growth regulators has become a common agricultural practice. Among the available synthetic plant growth regulators, triazoles are potent at low concentrations to inhibit shoot growth (Davis *et al.,* 1988). Paclobutrazol is a triazole derivative known to interfere with ent-kaurene oxidase activity in the ent-kaurene oxidation pathway leading to a decrease in endogenous GA levels and ABA catabolism (Rademacher, 1997).

Steffens *et al.*(1985) reported that treatments with paclobutrazol resulted in a shift in the partitioning of assimilates from the leaves to the roots and increased carbohydrates in all parts of apple seedlings. Also an increase in chlorophyll, soluble protein and mineral element concentration in leaf tissue was reported with paclobutrazol application. Triazole treatment was found to increase the root growth in cucumber and this was attributed to increased levels of endogenous cytokinins (Fletcher and Arnold, 1986). Smith *et al.* (1991) reported that with the use of paclobutrazol at a concentration of 0.5–4 mg L^{-1} in the rooting medium of apple an increase in epicuticular wax, reduction in shoot length, thickened roots and increased chlorophyll concentration per unit area of leaf were recorded. Samasya (2000) pointed out that, *in vitro* derived dendrobium orchid plantlets subjected to the treatment 5 mg L^{-1} of triazole had more number of roots than control plants.

Eliasson *et al.* (1994) noted that survival rate of *Prunus serotina,* four weeks after transfer to the soil was significantly improved by paclobutrazol. Sopher *et al.* (1999) suggested that application of paclobutrazol

increased the soluble protein content in maize and also increased the total foliar chlorophyll and carotenoid concentrations by 44 and 35%, respectively. Berova and Zlatev (2000) reported that in tomato plants, application of paclobutrazol as soil treatment (1 mg L^{-1}) and foliar treatment (25 mg L^{-1}) improved the photosynthetic activity. Sebastian *et al.* (2001) repored that paclobutrazol treatment increased the leaf tissue chlorophyll content in *Dianthus caryophyllus*.

Hazarika *et al.* (2002) reported that higher rate of water loss was incurred by *in vitro* grown citrus plants without any treatment (control) during acclimatization. But application of triazole during *ex vitro* establishment helped to overcome this limitation by reducing the water loss from the citrus plants. Thakur *et al.* (2006) reported that there was a significant increase in total chlorophyll content of lilium leaves when the plantlets were cultured in the presence of growth retardants at lower concentrations. Also it was reported that chlorophyll, carotenoid content and net photosynthetic rate increased and total leaf area decreased with triazole application. Triazole treated ornamental pepper plants typically appeared as darker green, which has been correlated with increased chlorophyll content (Grossi *et al.*, 2005).

Hazarika (2006) reported that in *ex vitro* conditions the plants treated with plant growth retardants like, uniconazole (UCZ), paclobutrazol (PBZ), triapenthenol (TPN) showed reduced size of plants, but retaining dark-green leaves and thick roots, helped in better survival and growth. Nazarudin *et al.* (2006) reported that one month after the treatment, the height of paclobutrazol treated (1.25, 2.50 and 3.75 g L^{-1}) *Syzygium campanulatum* plants were found to be inhibited and also had smaller leaves compared with the non-treated plants. Thakur *et al.* (2006) suggested that there are many reports on the successful acclimatization of *in vitro* cultivated plantlets using triazoles, namely paclobutrazole (PBZ), and subsequent transplantation to *ex vitro* environments with high survival percentage rates and rapid adaptation.

Sheena and Sheela (2010) studied the effect of triadimefon, (which is a triazole compound and a plant growth retardant) on the *ex vitro*

establishment of micropropagated gladiolus (*Gladiolus grandiflorus* L.). At one month after planting, these plants treated with 8 mg L^{-1} showed 55.83 per cent and untreated plants recorded 47.50 per cent survival and they also reported that triazole has a retarding effect on plant height. Untreated plants recorded maximum plant height of 15.81 cm while plants treated with triazole at 8 mg L^{-1} recorded minimum plant height (14.86 cm). The development of thicker epicuticular wax layer provides better protection against some plant pathogens and minor mechanical damage also (Kolattukudy, 1987).

4.5 EFFECT OF PGPR ON *EX VITRO* ACCLIMATISATION

Hayat *et al.* (2012) stated that plant growth promoting rhizobacteria (PGPR) are capable of promoting plant growth by colonizing their roots and they play an essential role in helping plants to establish and grow well. Plant growth-promoting rhizobacteria (PGPR) colonize roots of monocots and dicots, and enhance plant growth by direct and indirect mechanisms. Modification of root system architecture by PGPR implicates the production of phytohormones and other signals that lead mostly to enhance lateral root branching and development of root hairs. PGPR also modify root functioning, improve plant nutrition and influence the physiology of the whole plants (Vacheron *et al.,* 2013).

The PGPR produced higher percentages of root growth and better quality in terms of root length, diameter and root dry weight in kiwi fruits (Erturk *et al.,* 2010).

4.6 EFFECT OF AMF ON *EX VITRO* ACCLAMATISATION

Mycorrhizae can act as bio-regulators, bio-fertilizers and bioprotectors, making possible the production of healthy, high-quality plants with low chemical inputs (Lovato *et al.,* 1996).

Yano-Melo *et al.* (1999) observed that after inoculation with AMF (*Acaulospora scrobiculata* and *Glomus etunicatum*) there was an increase in the leaf area (57%) and height (32%) of micropropagated banana plants compared

to non-inoculated plants (control). Dry matter of shoots increased 45–64% in mycorrhizal associated plants. Also banana plantlets inoculated with *Acaulospora scrobiculata* and *Glomus etunicatum* recorded highest photosynthetic rates, being 45% more efficient than the non-inoculated plants.

Micropropagated guava plantlets (*Psidium guajava* L.) inoculated with mycorrhiza recorded increased gas exchange due to enhancement of stomatal opening (Estrada-Luna *et al.,* 2000). Rai (2001) reported that AMF improves bio-priming of micro propagated lucumo (*Pouteria lucuma*) plantlets and played a significant role in ensuring the health of plantlets. Borkowska (2002) reported that *in vitro* derived strawberry plants treated with mycorrhiza showed higher biomass accumulation and larger leaf area. Mycorrhized plants had a significantly larger root system with the plant development. At the beginning of colonization, shoot-to- root ratio and transpiration rate were significantly higher in AMF treated plants.

Krishna *et al.* (2005) reported that the mycorrhizal treatments showed about two times higher *ex vitro* survival than the control plantlets of grape (*Vitis vinifera* L.). Also they pointed out that the mycorrhizal inoculation of micropropagated grape plantlets significantly enhanced their photosynthetic rate compared to control (from 2.25 μmol m^{-2} s^{-1} to 6.39 μmol m^{-2} s^{-1}). Mycorrhizae form symbiotic associations between plant roots and certain soil fungi which play a key role in nutrient cycling in the ecosystem and also protect plants against environmental and cultural stress (Ortas, 2008).

Cha-um *et al.* (2009) stated that environmental conditions for *ex vitro* growth are quite different from those used for *in vitro* cultures. Plant growth retardants, i.e., uniconazole (UCZ), paclobutrazol (PBZ) triapenthenol (TPN), triadimefon (TDM) and hexaconazole (HCZ) have been reported as effective agents in reducing the size of plants, but retaining dark green leaves and thick roots, which define them as healthy plantlets, and aiding antiwilting, leading to better survival and growth in *ex vitro* condition.

Ruta *et al.* (2009) suggested that the improvement of *ex vitro* establishment is also favoured by the mycorrhization of the micropropagated plantlets of melon, oregano, artichoke and spanish broom. Arbuscular mycorrhizal fungi (AMF) are important soil microorganisms that can establish asymbiotic association with the roots of nearly 90% of plants (Smith *et al.*, 2010).

Due to endomycorrhizal inoculation, the micropropagated *Medicago truncatula* plants have a change in root morphology (Derelle *et al.*, 2012). Campanelli *et al.* (2014) suggested that in globe artichoke (*Cynara cardunculus* L. var. scolymus) *in vitro* derived plantlets treated with mycorrhiza showed higher *ex vitro* survival (91%) than the control plantlets (75%). Mycorrhizal symbiosis enhanced the leaf area, root density, fresh and dry shoot weight and fresh and dry root weight.

AMF symbiosis increases the absorption of nutrients like phosphorus and enhance the vigour of the plants. AMF also has the ability to reduce the bad effects of changes in pH, temperature and water stress (Siqueira, 1994). Healthy and vigourous plants with high transpiration and photosynthetic rate are the important advantages of AMF symbiosis. It also improved the uptake of water and nutrients and enhanced stress tolerance (Jaizme-Vega *et al.* 1997).

Jaizme-Vega *et al.* (1997) observed that improvement in root colonization by *Glomus mosseae* and *Glomus Fasciculatum* increased root fresh matter of micropropagated banana plantlets (*Musa acuminata* Colla AAA, subgroup Cavendish). Fortuna *et al.* (1992) reported that AMF can be used to promote plant growth rapidly. When plantlets were treated with AMF (*Glomus mosseae* and *Glomus coronatum*) the highest fresh and dry matter and height were observed.

4.7 ANATOMICAL PECULIARITIES DURING ACCLIMATIZATION

In dendrobium plantlets, epicuticular wax was found to be either reduced or absent in the *in vitro* leaves, leading to high rate of water loss (Samasya, 2000). In tissue cultured *Chrysanthemum morifolium* plantlets, the

density of wax deposition was found to increase during acclimatization (Wardle *et al.,* 1983). Fabri *et al.* (1986) reported that hardened leaves of tissue cultured strawberry plantlets enlarge due to increased cell size and increased epicuticular wax deposits on both adaxial and abaxial surfaces.

An increase in ABA level was observed in triazole treated plantlets, which stimulates the synthesis of lipid transfer proteins in barley, that play an important role in the formation of epicuticular waxes (Hollenbach *et al.,* 1997). The observed higher epicuticular wax deposition in treated leaves may be related to the increase in endogenous ABA levels in response to PBZ treatment (Rademacher, 1997). Tsegaw *et al.* (2005) reported that paclobutrazol treated potato plantlets showed thicker epicuticular waxes and it resulted in thicker cuticle.

Water potential of field transferred plantlets was stabilized due to development of cuticle, epicuticular waxes, and effective stomatal regulation of transpiration (Pospsilosova *et al.,* 1999). Ticha *et al.* (1999) observed that in *in vitro* derived tobacco plantlets, leaf thickness increased and leaf mesophyll differentiated into palisade and spongy parenchyma cells during hardening.

Materials and Methods

3. MATERIALS AND METHOD

The present study on "Physiological approaches for enhancing the *ex vitro* establishment of tissue cultured orchid (*Phalaenopsis* sp.) was conducted at the Department of Plant Physiology, College of Agriculture, Vellayani during the year 2017-2019. A brief account of materials and methodologies followed in this study are presented below.

3.1 EXPERIMENTAL DETAILS

3.1.1 Materials Utilized

The orchid cultivar chosen for the experiment was *Phalaenopsis sp. In vitro* derived plantlets of *Phalaenopsis sp.* derived from shoot meristem culture were used for this study and they were obtained from Biotechnology and Model Floriculture Centre (BMFC), Kazhakuttam.

3.1.2 Design of the Experiment:

The pot culture experiment was laid out in CRD with ten treatments and three replications.

3.1.3. *Ex vitro* Establishment

After opening the culture bottles the plantlets were taken out using sterilized forceps. The media adhering to the roots were removed by washing with running tap water. Then the plantlets were kept in a beaker of distilled water to completely remove the media. During this process, care was taken for not damaging the roots. Then the plantlets were subjected to fungicide treatment in 0.2 per cent bavistin by dipping in it for a period of 20 minutes. These plantlets were then planted directly into coconut husk balls. Before being used for planting, the coconut fibres were autoclaved. These fibres were then dipped in 0.2 per cent bavistin for 1hour and the water was squeezed off to remove excess fungicide. The coconut fibre balls were made by tying fibres at the centre. The plantlets were placed on the top of the coconut husk balls and the roots were spread on it.

Plate 1. Tissue culture derived Phalaenopsis orchid plantlets

3.1.4. Treatment Details

3.1.4.1. Triazole

Before placing the *in vitro* derived rooted plantlets of *Phalaenopsis sp.* on the husk balls, they were subjected to dip of 30 minutes in different concentrations of triazole as indicated below.

T_1: Plantlet dip with triazole @ 5ppm

T_2: Plantlet dip with triazole @ 10 ppm

T_3: Plantlet dip with triazole @ 5 ppm+ foliar application of triazole @ 5ppm after 15 days of *ex vitro* transfer

T_4: Plantlet dip with triazole @ 10 ppm+ foliar application of triazole @ 5ppm after 15 days of *ex vitro* transfer

Observations on different physiological, morphological, biochemical, biometric and anatomical characters were recorded.

3.1.4.2. Effect of Light Intensity and Humidity on *ex vitro* Establishment

Studies were conducted on the effect of light intensity and humidity on *ex vitro* establishment of *in vitro* derived plantlets of *Phalaenopsis sp.* The plantlets were grown in two different levels of light intensity and two different levels of humidity. The different levels of humidity treatments were given simultaneously along with light treatments.

They are as follows.

T_5: (L1H1) Plantlets providing with 40-50% light intensity and 60-70% humidity

T_6: (L1H2) Plantlets providing with 40-50% light intensity and 80-90% humidity

T_7: (L2H1) Plantlets providing with 70-80% light intensity and 60-70% humidity

T_8: (L2H2) Plantlets providing with 70-80% light intensity and 80-90% humidity

Plate 2. General View of the Experimental Site

Plate 3. Plantlets inside the growth chamber

Plate 4. Mist chamber

The two different levels of light intensity were provided by using different shade nets and control plants were maintained at normal light conditions inside the hardening chamber. For maintaining the two levels of humidity two chambers with polythene sheets were constructed within the shade nets. By adjusting misting frequency the required humidity was maintained. In the 80-90 per cent humidity chamber, the pots were placed in a tray with water so as to maintain high level of humidity. By using hygrometer, humidity was monitored throughout the day. Observations were recorded on different physiological, morphological, biochemical, biometric and anatomical characters.

3.1.4.3. Effect of PGPR

T_9: PGPR Mix I -5% (Plantlet dip)

Studies were conducted on the effect of PGPR on *ex vitro* establishment of *in vitro* derived plantlets of *Phalaenopsis sp.* Before placing the rooted plantlets on the husk ball, they were subjected to dip of 20 minutes in 5% concentration of PGPR Mix I and observations were recorded on different physiological, morphological, biochemical, biometric and anatomical characters.

3.1.4.4. Effect of AMF

T_{10}: Arbuscular Mycorrhizal Fungi (5g/plantlet)

Studies were conducted on the effect of AMF on *ex vitro* establishment of *in vitro* derived plantlets of *Phalaenopsis sp.* After placing the rooted plantlets on the husk ball, 5 g AMF was applied to the root zone of each plantlet and observations were recorded on different physiological, morphological, biochemical, biometrical and anatomical characters.

3.1.4.5. After Care of Plantlets

After planting out, the plants were irrigated daily. Bavistin 0.2% was sprayed twice weekly during the first two weeks of planting out and later at weekly intervals. In the mist chamber, the plantlets were given foliar spray of NPK mixture 19:19:19 (0.10%) on 15th day after planting and 30th day after

Plate 5. Plantlets inside the potting media

Plate 6. Coconut husk balls, the potting media used

planting. After 45th day the plants were potted in small pots containing charcoal, brick and coconut husk in the ratio of 1:1:1 and the plants were maintained in shade for upto 70 days.

3.2 OBSERVATIONS

Observations were recorded on physiological, morphological, biochemical biometric characters at 15 days intervals. Observations on anatomical characters were taken at 30 and 60 days of *ex vitro* transfer.

3.2.1. Physiological Parameters

3.2.1.1. Specific Leaf Area

It is the one sided area of a fresh leaf, divided by its oven dry mass, and it is calculated by using the equation

$$\text{Specific leaf area} = \frac{A}{M}$$

Where A is the area of a given leaf of a plant, and M is the dry mass of the leaf.

3.2.1.2. Photosynthetic Rate

It was measured directly by using LCA-4 (Leaf Chamber Analyser or portable CO_2 analyser) manufactured by Analytical Development Co. Ltd, UK. The values were expressed in μ mol $m^{-2}s^{-1}$

3.2.1.3. Transpiration Rate

Transpiration rate was measured directly by using the portable CO_2 analyser or leaf chamber analyser (LCA-4), manufactured by Analytical Development Co. UK. The values were expressed in μ mol $m^{-2}s^{-1}$.

3.2.1.4. Stomatal Index

Stomatal index is the ratio of number of stomata to total number of epidermal cells expressed in percentage. It is calculated by using following equation.

$$I = \frac{S}{E+S} \times 100$$

I = Stomatal index

S = No. of stomata per unit area

E = No. of epidermal cells in the same unit area

3.2.1.5. Stomatal Frequency

Stomatal frequency is the number of stomata per unit area. For calculating stomatal frequency a thick mixture of thermocol and xylene was prepared and to the upper surface of the leaf this solution was smeared and it was allowed to dry. Then after drying it was pealed gently and the peel was observed under microscope. The stomata were counted using a 40x objective and 10x eyepiece. The field of the microscope was measured using a stage micrometre and stomatal frequency per unit area was calculated using the formula given below,

$$\text{Stomatal frequency} = \frac{\text{Number of stomata}}{\text{Area of the microscopic field}}$$

3.2.2. Morphological Parameters

3.2.2.1. Height of the Plant

This was measured from the collar region to the shoot tip and the mean length was expressed in centimetre. The observations were made at 15 days interval.

3.2.2.2. Number of Leaves

The total number of fully opened leaves developed per plantlet was counted and the mean value was expressed at 15 days interval.

3.2.2.3. Number of Roots

Total number of roots per plantlet was counted and the mean value was recorded at 15 days interval.

3.2.2.4. Survival Percentage

In order to measure the per cent mortality the survival per cent of the hardened plants was noted at 15 days interval.

3.2.3. Biochemical Characters

3.2.3.1. Chlorophyll Content (Chlorophyll a, Chlorophyll b, total Chlorophyll and carotenoids) of leaves

Photosynthetic pigments namely chlorophyll a, chlorophyll b, total chlorophyll and carotenoids were estimated by the following method described by Arnon (1949).

A representative sample of 100 mg of leaf tissue was weighed and ground with 10 ml of 80 per cent acetone using a pestle and mortar. The homogenate was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and was made up to 10 ml with 80 per cent acetone. The optical density (O.D.) value of the extract was measured at 663, 645 and 480 nm using 80 per cent acetone as the blank in spectrophotometer (Systsonics UV-VIS spectrophotometer 118). The amount of the pigments were calculated using the following formulae and expressed as mg of pigments g^{-1} of fresh leaf.

Chlorophyll 'a' = [(12.7 x A663) – (2.69 x A645)] x V/1000 x W

Chlorophyll 'b'= [(22.9 x A645) – (4.68 x A663)] x V/1000 x W

Total chlorophyll = [(20.2 x A645) + (8.02 x A663)] x V/1000 x W

Carotenoid = [(7.6 x A480) – (1.49 x A510)] x V/1000 x W

Where,

A = Absorption at given wavelength

V = Total volume of sample in extraction medium

W = Weight of sample

3.2.3.2. Estimation of Protein (mg g^{-1})

Total soluble proteins were estimated using Bradford method (Bradford,1976). The assay is based on the ability of proteins to bind coomassie brilliant blue G 250 and a complex is formed whose extinction coefficient is much greater than that of free dye. The total soluble protein from 500 mg of plant samples were extracted using 10 ml of phosphate buffered saline (PBS) solution. The extracts were collected and 0.1 ml of extract was taken and made up to 3 ml by adding PBS. A known volume (5 ml) of dye binding solution was added to each sample. The solution was mixed well and allowed to develop blue colour. The red dye turned blue when it bound with protein and its absorbance was read at 595 nm. Bovine serum albumin was used as the protein standard and the protein concentration was calculated and expressed as mg g^{-1} fresh weight of plant tissue.

3.2.3.3. Estimation of Total Carbohydrate (mg g^{-1})

Total carbohydrate was estimated by Anthrone method (Hedge and Hofreiter, 1962). Leaf samples of 100 mg each were weighed out and hydrolysed with 5 ml of 2.5 N hydrochloric acid (HCI at 100°C in a water bath).The hydrolysate was neutralised with solid sodium carbonate until the effervescence ceased. The volume was made up to 100ml and centrifuged at 5000 rpm for 15 minutes. From the supernatant 0.5 ml aliquot was taken and made up to 1 ml by adding distilled water. To this 4 ml of anthrone reagent was added and heated for 8 minutes at 100°C in a water bath. This was cooled rapidly and absorbance was measured at 630nm in a spectrophotometer (Systonics UV-VIS spectrophotometer 118). The amount of carbohydrate present was calculated from standard graph

using glucose and expressed in terms of mg of glucose equivalent per gram of leaf tissue on fresh weight basis.

3.2.4. Biometric Characters

3.2.4.1. Leaf Area

Leaf area was measured by using graph paper method. Area of leaf lamina is expressed in cm^2.

3.2.4.2. Root Length

Length of root from the collar region to the root tip was measured and the mean was expressed in cm.

3.2.4.3. Root: Shoot Ratio

This was calculated by using the formula,

$$\text{Root: Shoot ratio} = \frac{\text{Root length}}{\text{Shoot length}}$$

3.2.4.4. Total Fresh Weight and Dry Weight (g)

Fresh weight of individual plants were recorded and then those plants were dried at 70°C for 48 hr and the dry weight was recorded. These parameters were taken at 15 days interval for each treatment.

3.2.4. Anatomical Characters

3.2.4.1. Cuticle Thickness

Cuticle thickness was observed and measured using LEICA DM 2000 image analyser available at Department of Botany, Karyavattom campus under the University of Kerala.

3.2.4.2. Number of Mesophyll Layers and Type of Cells

Number of mesophyll layers in the leaf cross section were counted and type of cells were observed using compound microscope with 40x objective and 10x eye piece and observations were recorded.

Results

4. RESULTS

The investigation on "Physiological approaches for enhancing the *ex vitro* establishment of tissue cultured orchid (*Phalaenopsis* sp.)" was carried out at College of Agriculture, Vellayani during November 2018 to February 2019. The objective of the experiment was to study the physiological changes that occur during *ex vitro* establishment of orchid (*Phalaenopsis* sp.) and to find out measures to overcome the field mortality rate and improve propagation efficiency. The results obtained after the statistical analysis of the data are presented in this chapter.

4.1. Physiological Characters

The data on physiological characters *viz.*, specific leaf area, photosynthetic rate, transpiration rate, stomatal index and stomatal frequency were statistically analysed and presented in Table 1, 2, 3 and 4.

4.1.1. Specific Leaf Area

Data on specific leaf area of tissue cultured phalaenopsis orchid at 15, 30, 45 and 60 days of *ex vitro* transfer are furnished in Table 1.

Specific leaf area was significantly influenced by growth regulators, growth stimulants, light and humidity during the *ex vitro* establishment of tissue cultured phalaenospis plantlets. Results revealed that the treatment, T_6 (519.30, 530.10, 542.66, 550.83 cm^2 g^{-1} respectively) recorded significantly higher specific leaf area at all the four stages of observation. The lowest specific leaf area was recorded by T_4 (400.50, 449.10, 469.99, 482.46 cm^2 g^{-1} respectively) at 15, 30, 45 and 60 days of *ex vitro* transfer)

4.1.1.2 Photosynthetic Rate

Data related to photosynthetic rate is presented in Table 2.

Photosynthetic rate was favourably influenced by growth regulators, growth stimulants, light and humidity.

Table 1. Effect of growth regulators, growth stimulants, light and humidity on specific leaf area of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Specific leaf area, $cm^2 g^{-1}$			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	448.20	460.80	500.52	518.15
T_2	443.25	454.50	478.59	496.22
T_3	428.40	455.85	474.72	493.21
T_4	400.50	449.10	469.99	482.46
T_5	504.90	515.25	531.48	538.79
T_6	519.30	530.10	542.66	550.83
T_7	462.60	482.85	505.68	513.85
T_8	450.90	474.30	500.09	509.12
T_9	438.30	449.10	475.58	488.48
T_{10}	474.30	495.90	525.46	541.37
C	456.30	474.75	492.35	502.24
SE m (±)	3.67	5.37	5.86	3.67
CD (0.05)	1.244	1.818	1.985	1.242

Table 2. Effect of growth regulators, growth stimulants, light and humidity on photosynthetic rate of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Photosynthetic rate, μ mol CO_2 $m^{-2}s^{-1}$			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	0.287	1.187	1.853	1.933
T_2	0.590	1.410	2.093	2.330
T_3	0.400	1.300	1.957	2.037
T_4	0.583	1.360	1.973	2.067
T_5	0.700	1.500	2.233	2.447
T_6	0.707	1.607	2.443	2.667
T_7	0.207	1.107	1.807	1.887
T_8	0.357	1.257	1.880	1.960
T_9	0.168	1.070	1.770	1.850
T_{10}	0.677	1.577	2.277	2.627
C	0.143	1.043	1.743	1.843
SE m (±)	0.047	0.112	0.168	0.263
CD (0.05)	0.0160	0.0380	0.0570	0.0890

At 15 DAP, T_6 (0.707 μ mol CO_2 m^{-2} s^{-1}) recorded significantly higher photosynthetic rate but it was statistically on par with T_5 (0.700 μ mol CO_2 m^{-2} s^{-1}). Control plants (0.143 μ mol CO_2 m^{-2} s^{-1}) recorded significantly lower photosynthetic rate.

At 30 and 45 DAP also, photosynthetic rate was significantly influenced by growth regulators, growth stimulants, light and humidity. Among the treatments, T_6 (1.607, 2.443 μ mol CO_2 m^{-2} s^{-1} respectivley) recorded the highest photosynthetic rate. The control (1.043, 1.743 μ mol CO_2 m^{-2} s^{-1} respectively) recorded the lowest photosynthetic rate.

Similarly, at 60 DAP, T_6 (2.667 μ mol CO_2 m^{-2} s^{-1}) recorded the highest photosynthetic rate and was on par with T_{10} (2.627 μ mol CO_2 m^{-2} s^{-1}).

4.1.1.3 Transpiration Rate

The data related to transpiration rate is presented in Table 3.

Results revealed that, at 15 and 30 DAP, treatment T_3 (0.418, 0.351 m mole H_2O m^{-2} s^{-1} respectively), registered the lowest transpiration rate but it was statistically on par with T_1 (0.424, 0.384 m mole H_2O m^{-2} s^{-1} respectively).

At 45 and 60 DAP also T_3 (0.304, 0.364 m mol CO_2 m^{-2} s^{-1} respectively) recorded significantly lower transpiration rate and it was statistically superior to all other treatments.

4.1.1.4 Stomatal Index

The data on stomatal index of tissue cultured phalaenopsis orchid at 15, 30, 45 and 60 DAP are furnished in Table 4.

Results revealed that stomatal index was not significantly influenced by growth regulators, growth stimulants, light and humidity during the *ex vitro* establishment of tissue cultured phalaenospis plantlets.

4.1.1.5 Stomatal Frequency

The influence of growth regulators, growth stimulants, light and humidity on stomatal frequency at 15, 30, 45 and 60 days of *ex vitro* transfer are given in Table 5.

Table 3. Effect of growth regulators, growth stimulants, light and humidity on transpiration rate of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Transpiration rate(m.mole H_2O m^{-2} s^{-1})			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	0.424	0.384	0.432	0.476
T_2	0.510	0.484	0.530	0.551
T_3	0.418	0.351	0.304	0.364
T_4	0.559	0.528	0.469	0.499
T_5	0.576	0.535	0.495	0.445
T_6	0.594	0.553	0.513	0.463
T_7	0.650	0.594	0.554	0.504
T_8	0.714	0.641	0.601	0.551
T_9	0.750	0.705	0.665	0.615
T_{10}	0.830	0.788	0.766	0.748
C	0.816	0.766	0.716	0.666
SE m (±)	0.012	0.011	0.012	0.014
CD (0.05)	0.037	0.032	0.036	0.041

Table 4. Effect of growth regulators, growth stimulants, light and humidity on stomatal index of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Stomatal index			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	18.667	20.767	22.567	24.767
T_2	18.333	20.333	22.233	24.133
T_3	19.000	20.900	22.900	25.200
T_4	17.667	19.767	21.867	23.467
T_5	19.000	21.000	22.900	25.000
T_6	18.667	20.767	22.667	24.167
T_7	18.333	20.833	22.833	24.733
T_8	18.333	20.433	22.333	24.333
T_9	18.000	20.100	22.000	23.850
T_{10}	18.667	20.867	22.767	24.867
C	18.000	20.300	22.200	24.000
SE m (±)	0.433	0.436	0.438	0.440
CD (0.05)	NS	NS	NS	NS

Table 5. Effect of growth regulators, growth stimulants, light and humidity on stomatal frequency of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Stomatal frequency			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	108.61	119.61	130.11	140.51
T_2	86.14	95.14	104.14	113.44
T_3	104.87	120.59	134.62	145.82
T_4	93.63	101.63	109.83	118.83
T_5	93.63	102.13	112.13	122.23
T_6	89.89	99.89	109.39	118.39
T_7	86.14	94.84	103.84	113.04
T_8	93.63	102.63	111.73	121.33
T_9	89.89	97.89	106.99	116.29
T_{10}	78.65	87.75	96.85	106.05
C	78.65	86.75	95.85	104.85
SE m (±)	4.92	4.92	4.79	4.79
CD (0.05)	14.530	14.528	14.145	14.145

Stomatal frequency was markedly influenced by growth regulators, growth stimulants, light and humidity. Results revealed that at 15 DAP, T_1 (108.61) recorded the highest stomatal frequency but it was statistically on par with T_3 (104.87).

However, at 30, 45 and 60 DAP, T_3 (120.59, 134.62, 145.82 respectively) recorded the highest stomatal frequency among the treatments which was statistically comparable with T_1 (119.61, 130.11, 140.51).

The control (C) registered significantly lower stomatal frequency at 15 DAP (78.65), 30 DAP (86.75), 45 DAP (95.85) and 60 DAP (104.85).

4.2. Morphological Parameters

4.2.1. Plant Height

The data on plant height of tissue cultured phalaenopsis orchid at 15, 30, 45 and 60 days of *ex vitro* transfer are furnished in Table 6. Results revealed that plant height was significantly influenced by growth regulators, growth stimulants, light and humidity during their *ex vitro* establishment.

T_{10} (4.92, 5.06 cm respectively) recorded the highest plant height at 15 and 30 days after *ex vitro* transfer and it was statistically comparable with T_5 (4.70, 4.86 cm respectively), T_6 (4.84, 4.98 cm respectively), T_7 (4.35, 4.49 cm respectively) and T_8 (4.26, 4.52 cm respectively).

However, at 45 and 60 DAP, T_6 (5.94 cm, 6.30 cm respectively) recorded the highest plant height which was statistically comparable with T_5 (5.88 cm, 6.28 cm respectively) and T_{10} (5.61 cm, 5.77 cm respectively).

The treatment T_4 (3.32, 3.38, 3.67, 3.86 cm respectively) registered significantly lower plant height at 15, 30, 45 and 60 DAP.

4.2.2. Number of Leaves

The data pertaining to the effect of growth regulators, growth stimulants, light and humidity on number of leaves are presented in Table 7.

Growth regulators, growth stimulants, light and humidity significantly influenced the leaf number of tissue cultured phalaenopsis orchid.

Table 6. Effect of growth regulators, growth stimulants, light and humidity on height of the plant of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Plant height (cm)			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	3.79	3.99	4.18	4.46
T_2	3.39	3.57	3.73	4.00
T_3	3.62	3.63	3.84	4.21
T_4	3.32	3.38	3.67	3.86
T_5	4.70	4.86	5.88	6.28
T_6	4.84	4.98	5.94	6.30
T_7	4.35	4.49	4.86	5.08
T_8	4.26	4.52	5.00	5.31
T_9	3.59	3.77	4.21	4.42
T_{10}	4.92	5.06	5.61	5.77
C	3.91	4.02	4.30	4.61
SE m (±)	0.22	0.20	0.11	0.19
CD (0.05)	0.660	0.656	0.335	0.564

Table 7. Effect of growth regulators, growth stimulants, light and humidity on number of leaves of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Number of leaves			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	2.67	4.00	4.33	5.67
T_2	2.77	3.44	3.78	5.11
T_3	3.22	4.22	4.56	5.89
T_4	2.18	2.89	3.22	4.56
T_5	3.11	4.44	4.78	6.11
T_6	4.22	5.22	5.56	6.89
T_7	2.44	3.11	3.45	4.78
T_8	2.33	3.67	4.00	5.33
T_9	2.89	4.11	4.44	5.78
T_{10}	3.22	4.56	4.89	6.22
C	2.10	2.78	3.11	4.44
SE m (±)	0.39	0.49	0.48	0.49
CD (0.05)	1.175	1.462	1.465	1.463

Among the treatments, T_6 (4.22) recorded the highest number of leaves at 15 DAP and it was statistically at par with T_3 (3.22), T_5 (3.11) and T_{10} (3.22).

At 30, 45 and 60 DAP, the highest number of leaves were registered in the treatment T_6 (5.22, 5.56, 6.89 respectively) and was at par with T_1 (4.00, 4.33, 5.67), T_3 (4.22, 4.56, 5.89), T_5 (4.44, 4.78, 6.11) T_9 (4.11, 4.44, 5.78) and T_{10} (4.56, 4.89, 6.22).

The control plants (2.10, 2.78, 3.11, 4.44 respectively) registered the lowest number of leaves at all the four stages of observation.

4.2.3. Number of Roots

The influence of growth regulators, growth stimulants, light and humidity on number of roots of tissue cultured phalaenopsis orchid are presented in Table 8.

Results on number of roots revealed that, T_3 (6.45, 8.45 respectively) recorded the highest number of roots at 15 DAP and 30 DAP. It was comparable with T_5 (6.12, 7.78 respectively) and T_6 (5.67, 8.00 respectively).

Similarly, at 45 and 60 DAP, T_3 (10.33, 10.67 respectively) registered the highest number of roots and it was statistically on par with T_6.

The control (3.22, 5.34, 6.67, 6.99 respectively) registered the lowest number of roots at 15, 30, 45 and 60 days after *ex vitro* transfer.

4.2.3. Survival Percentage

Data pertaining to the effect of growth regulators, growth stimulants, light and humidity on survival percentage are presented in Table 9. Growth regulators, growth stimulants, light and humidity favourably influenced the survival percentage at all the four stages of observation.

Among the treatments, T_6 (80.66%) recorded the highest survival percentage at 15 days after *ex vitro* transfer. It was statistically at par with T_1 (79.66%), T_3 (80.00%), T_4 (76.33%) and T_5 (76.00%).

Similarly at 30, 45 and 60 DAP, T_6 (76%, 72.33%, 66.33% respectively) registered the highest survival percentage which was statistically at par with T_1

Table 8. Effect of growth regulators, growth stimulants, light and humidity on number of roots of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Number of roots			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	4.11	6.44	8.34	8.67
T_2	4.89	7.22	9.00	9.33
T_3	6.45	8.45	10.33	10.67
T_4	5.56	7.33	9.33	9.60
T_5	6.12	7.78	9.34	9.67
T_6	5.67	8.00	10.00	10.34
T_7	4.33	6.44	7.67	7.78
T_8	4.89	7.00	8.44	8.78
T_9	4.67	6.44	7.89	8.22
T_{10}	5.11	7.22	9.02	9.35
C	3.22	5.34	6.67	6.99
SE m (±)	0.24	0.31	0.23	0.22
CD (0.05)	0.699	0.925	0.680	0.679

Table 9. Effect of growth regulators, growth stimulants, light and humidity on survival percentage of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Survival percentage, %			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	79.66	74.00	68.66	63.66
T_2	73.33	69.00	64.00	59.66
T_3	80.00	73.66	69.33	66.00
T_4	76.33	70.33	64.66	60.67
T_5	76.00	71.33	67.66	62.67
T_6	80.66	76.00	72.33	66.33
T_7	59.00	54.00	50.00	45.33
T_8	62.66	58.66	53.33	48.00
T_9	47.66	42.33	37.33	33.33
T_{10}	57.33	53.33	48.66	45.33
C	44.33	40.33	36.00	32.66
SE m (±)	1.87	1.79	1.61	1.31
CD (0.05)	5.526	5.299	4.756	3.879

(74.00%, 68.66%, 63.66%), T_3 (73.66%, 69.33%, 66.00%), T_5 (71.33%, 67.66%, 62.67%).

The control (44.33%, 40.33%, 36.00%, 32.66%) registered the lowest survival percentage at 15, 30, 45 and 60 days of *ex vitro* transfer.

4.3. Biochemical Characters

4.3.1. Chlorophyll content

The data related to total chlorophyll, chlorophyll a and b content of leaves are presented in Table 10, 11 and 12.

4.3.1.1. Chlorophyll a Content

The effect of growth regulators, growth stimulants, light and humidity on chlorophyll a content at 15, 30, 45 and 60 DAP is shown in Table 10.

At 15 DAP, chlorophyll a content was not significantly influenced by growth regulators, growth stimulants, light and humidity. Whereas at 30, 45 and 60 days after *ex vitro* transfer it showed significant effect. The treatment, T_3 (0.332 mg g^{-1}) recorded significantly higher chlorophyll a content at 30 DAP.

Similarly, at 45 DAP, T_3 (0.418 mg g^{-1}) registered the highest chlorophyll a content and it was statistically comparable with T_1 (0.380 mg g^{-1}).

At 60 DAP, T_3 (0.446 mg g^{-1}) recorded the highest chlorophyll a content and it was statistically comparable with T_1 (0.384 mg g^{-1}), T_5 (0.345 mg g^{-1}) and T_6 (0.417 mg g^{-1}).

Control (0.174 mg g^{-1}, 0.176 mg g^{-1}, 0.182 mg g^{-1}, 0.183 mg g^{-1} respectively) registered the lowest chlorophyll a content during 15, 30, 45 and 60 days after *ex vitro* transfer.

4.3.1.2 Chlorophyll b Content

Chlorophyll b content was also significantly influenced by growth regulators, growth stimulants, light and humidity (Table 11).

At 15, 30, 45 and 60 DAP, the highest chlorophyll b content was registered in the treatment T_6 (0.172 mg g^{-1}, 0.191 mg g^{-1}, 0.186 mg g^{-1}, 0.192 mg g^{-1} respectively) and was statistically on par with T_3 (0.156 mg g^{-1}, 0.177 mg g^{-1}, 0.183 mg g^{-1}, 0.190 mg g^{-1} respectively).

Table 10. Effect of growth regulators, growth stimulants, light and humidity on chlorophyll a content of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Chlorophyll a (mg g^{-1})			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	0.221	0.267	0.380	0.384
T_2	0.218	0.229	0.238	0.292
T_3	0.332	0.399	0.418	0.446
T_4	0.108	0.127	0.130	0.185
T_5	0.264	0.316	0.34	0.345
T_6	0.121	0.273	0.293	0.417
T_7	0.206	0.263	0.265	0.269
T_8	0.201	0.214	0.224	0.266
T_9	0.241	0.254	0.263	0.270
T_{10}	0.183	0.229	0.249	0.256
C	0.174	0.176	0.182	0.183
SE m (±)	0.046	0.012	0.018	0.044
CD (0.05)	NS	0.034	0.052	0.130

Table 11. Effect of growth regulators, growth stimulants, light and humidity on chlorophyll b content of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Chlorophyll b (mg g^{-1})			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	0.121	0.128	0.136	0.149
T_2	0.091	0.095	0.100	0.100
T_3	0.156	0.177	0.183	0.190
T_4	0.058	0.060	0.064	0.074
T_5	0.138	0.145	0.146	0.147
T_6	0.172	0.191	0.186	0.192
T_7	0.099	0.121	0.127	0.131
T_8	0.126	0.141	0.159	0.164
T_9	0.099	0.113	0.124	0.148
T_{10}	0.089	0.102	0.118	0.126
C	0.077	0.082	0.098	0.103
SE m (±)	0.011	0.009	0.008	0.006
CD (0.05)	0.033	0.027	0.024	0.018

Control (0.077 mg g^{-1}, 0.082 mg g^{-1}, 0.098 mg g^{-1}, 0.103 mg g^{-1} respectively) recorded the lowest chlorophyll b content at all the four stages of observation.

4.3.1.3 Total Chlorophyll Content

Total chlorophyll content of leaves was positively influenced by growth regulators, growth stimulants, light and humidity.

At 15 and 30 DAP, T_3 (0.488, 0.576 mg g^{-1}respectively) recorded significantly higher total chlorophyll content in leaves and it was significantly superior to other treatments.

At 45 DAP, T_3 (0.601 mg g^{-1}) registered the highest total chlorophyll content and it was on par with T_1 (0.516 mg g^{-1}). Similarly, at 60 DAP also T_3 (0.638 mg g^{-1}) recorded the highest total chlorophyll content which was statistically comparable with T_6 (0.607 mg g^{-1}).

4.3.2. Carotenoid Content

The effect of growth regulators, growth stimulants, light and humidity on carotenoid content is shown in Table 13.

At 15 DAP, growth regulators, growth stimulants, light and humidity treatments significantly influenced the carotenoid content. The treatment, T_6 (0.131 mg g^{-1}) recorded significantly higher carotenoid content and was statistically comparable with T_2 (0.124 mg g^{-1}) and T_5 (0.128 mg g^{-1})

Whereas, at 30 days of *ex vitro* establishment, the treatment T_6 (0.137 mg g^{-1}) recorded the highest carotenoid content and it was statistically on a line with T_4 (0.134 mg g^{-1}) and T_5 (0.136 mg g^{-1})

Similarly, T_6 (0.189 mg g^{-1}) registered the highest carotenoid content at 45 DAP, which was statistically superior to all other treatments.

At 60 days of *ex vitro* transfer, the treatment T_6 (0.204 mg g^{-1}) recorded the highest carotenoid content which was statistically comparable with T_1 (0.190 mg g^{-1}), T_2 (0.197 mg g^{-1}), T_3 (0.185 mg g^{-1}), T_4 (0.181 mg g^{-1}) and T_5 (0.186 mg g^{-1}).

Table 12. Effect of growth regulators, growth stimulants, light and humidity on total chlorophyll content of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Total chlorophyll (mg g^{-1})			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	0.342	0.395	0.516	0.533
T_2	0.309	0.324	0.338	0.392
T_3	0.488	0.576	0.601	0.638
T_4	0.166	0.187	0.194	0.259
T_5	0.402	0.461	0.486	0.492
T_6	0.293	0.464	0.479	0.607
T_7	0.305	0.384	0.392	0.400
T_8	0.327	0.355	0.383	0.430
T_9	0.34	0.367	0.387	0.418
T_{10}	0.272	0.331	0.367	0.382
C	0.251	0.258	0.28	0.286
SE m (±)	0.027	0.007	0.030	0.020
CD (0.05)	0.080	0.019	0.088	0.059

Table 13. Effect of growth regulators, growth stimulants, light and humidity on carotenoid content of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Carotenoid content (mg g^{-1})			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	0.113	0.122	0.174	0.190
T_2	0.124	0.131	0.178	0.197
T_3	0.111	0.124	0.176	0.185
T_4	0.116	0.134	0.167	0.181
T_5	0.128	0.136	0.169	0.186
T_6	0.131	0.137	0.189	0.204
T_7	0.094	0.105	0.146	0.157
T_8	0.091	0.098	0.149	0.166
T_9	0.076	0.082	0.120	0.136
T_{10}	0.082	0.092	0.144	0.160
C	0.072	0.079	0.131	0.147
SE m (±)	0.003	0.002	0.001	0.009
CD (0.05)	0.008	0.005	0.003	0.026

4.3.3. Protein Content

The data related to the influence of growth regulators, growth stimulants, light and humidity on protein content of leaves is presented in Table 14.

Growth regulators, growth stimulants, light and humidity had significant effect on protein content. Results revealed that, at 15 DAP, T_1 (0.081 mg g^{-1}) recorded significantly higher protein content and it was statistically at par with T_2 (0.069 mg g^{-1}), T_3 (0.077 mg g^{-1}) T_5 (0.072 mg g^{-1}) and T_6 (0.068 mg g^{-1}).

However, at 30 days after *ex vitro* transfer, T_3 (0.087 mg g^{-1}) recorded the highest protein content, which was statistically on par with T_1 (0.086 mg g^{-1}), T_2 (0.077 mg g^{-1}), T_4 (0.067 mg g^{-1}), T_5 (0.072 mg g^{-1}) and T_6 (0.068 mg g^{-1}).

At 45 and 60 DAP, the highest protein content were registered in T_3 (0.096, 0.120 mg g^{-1}) and it was comparable with T_1 (0.094, 0.113 mg g^{-1}), T_5 (0.090, 0.110 mg g^{-1}) and T_6 (0.088, 0.108 mg g^{-1}).

The control (0.048, 0.050, 0.057, 0.062 mg g^{-1} respectively) registered the lowest protein content at all the four stages of observation.

4.3.4. Total Carbohydrate

The data pertaining to the effect of growth regulators, growth stimulants, light and humidity on total carbohydrate at 15, 30, 45 and 60 DAP are depicted in Table 15.

Total carbohydrate was markedly influenced by growth regulators, growth stimulants, light and humidity. Results revealed that at 15 and 30 DAP, T_3 (0.055 mg g^{-1}, 0.068 mg g^{-1}) recorded the highest total carbohydrate and it was statistically on par with T_6 (0.052 mg g^{-1}, 0.065 mg g^{-1}).

However, at 45 and 60 days after *ex vitro* transfer, T_3 (0.073, 0.075 mg g^{-1} respectively) recorded the highest total carbohydrate content among the treatments, which was statistically superior to other treatments.

Table 14. Effect of growth regulators, growth stimulants, light and humidity on protein content of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Protein content (mg g^{-1})			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	0.081	0.086	0.094	0.113
T_2	0.069	0.077	0.082	0.106
T_3	0.077	0.087	0.096	0.120
T_4	0.067	0.075	0.080	0.103
T_5	0.072	0.084	0.090	0.110
T_6	0.068	0.081	0.088	0.108
T_7	0.064	0.069	0.075	0.103
T_8	0.060	0.065	0.073	0.098
T_9	0.056	0.060	0.067	0.092
T_{10}	0.056	0.059	0.063	0.095
C	0.048	0.050	0.057	0.062
SE m (±)	0.004	0.005	0.003	0.004
CD (0.05)	0.013	0.016	0.010	0.012

Table 15 Effect of growth regulators, growth stimulants, light and humidity on total carbohydrate content of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Total carbohydrate content (mg g^{-1})			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	0.050	0.061	0.063	0.067
T_2	0.041	0.042	0.047	0.051
T_3	0.055	0.068	0.073	0.075
T_4	0.037	0.040	0.043	0.046
T_5	0.038	0.040	0.043	0.045
T_6	0.052	0.065	0.067	0.071
T_7	0.045	0.054	0.060	0.064
T_8	0.034	0.038	0.042	0.043
T_9	0.030	0.035	0.040	0.041
T_{10}	0.035	0.037	0.041	0.042
C	0.021	0.026	0.029	0.035
SE m (±)	0.001	0.001	0.001	0.001
CD (0.05)	0.004	0.004	0.002	0.002

The control (C) registered significantly lower total carbohydrate content at 15 DAP (0.021 mg g^{-1}), 30 DAP (0.026 mg g^{-1}),45 DAP (0.029 mg g^{-1}) and 60 DAP (0.035 mg g^{-1}), respectively.

4.4. Biometric Characters

4.4.1. Leaf Area

Effect of growth regulators, growth stimulants, light and humidity on leaf area are presented in Table 16.

Growth regulators, growth stimulants, light and humidity treatments significantly influenced the leaf area at 15, 30, 45 and 60 days of *ex vitro* transfer. T_6 (11.54, 11.78, 12.62 cm^2 respectively) recorded significantly higher leaf area and it was superior to all other treatments at 15, 30 and 45 DAP.

Whereas, at 60 DAP also, the treatment T_6 (12.81 cm^2) recorded higher leaf area and it was on par with T_5 (12.53 cm^2) and T_{10} (12.59 cm^2).

The lowest leaf area was recorded by the treatment T_4 (8.90, 9.98, 10.93, 11.22 cm^2 respectively) at all the four stages of observation.

4.4.2. Root Length

The influence of growth regulators, growth stimulants, light and humidity treatments on root length at 15, 30, 45 and 60 DAP are given in Table 17.

Results revealed that growth regulators, growth stimulants, light and humidity treatments significantly influenced root length at all the four stages of observation. At 15, 30 and 45 DAP, T_6 (5.11, 5.51, 5.79 cm respectively) was found to be the highest which was significantly superior to other treatments.

Similarly at 60 DAP, T_6 (6.09 cm) recorded the highest root length which was statistically comparable with T_9 (5.72 cm).

Lesser root length was registered in the control plants (2.97, 3.30, 3.75, 4.07 cm respectively) at all the four stages of observation.

Table 16. Effect of growth regulators, growth stimulants, light and humidity on leaf area of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Leaf area (cm^2)			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	9.960	10.240	11.640	12.050
T_2	9.850	10.100	11.130	11.540
T_3	9.520	10.130	11.040	11.470
T_4	8.900	9.980	10.930	11.220
T_5	11.220	11.450	12.360	12.530
T_6	11.540	11.780	12.620	12.810
T_7	10.280	10.730	11.760	11.950
T_8	10.020	10.540	11.630	11.840
T_9	9.740	9.980	11.060	11.360
T_{10}	10.540	11.020	12.220	12.590
C	10.140	10.550	11.450	11.680
SE m (±)	0.028	0.040	0.046	0.030
CD (0.05)	0.081	0.119	0.136	0.090

Table 17. Effect of growth regulators, growth stimulants, light and humidity on root length of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Root length (cm)			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	4.40	4.80	5.08	5.38
T_2	4.21	4.60	4.89	5.19
T_3	4.46	4.98	5.22	5.52
T_4	4.19	4.75	4.90	5.20
T_5	4.49	4.89	5.17	5.47
T_6	5.11	5.51	5.79	6.09
T_7	4.30	4.53	4.98	5.28
T_8	4.05	4.45	4.73	5.03
T_9	4.73	5.14	5.42	5.72
T_{10}	3.60	4.00	4.27	4.57
C	2.97	3.30	3.75	4.07
SE m (±)	0.13	0.18	0.09	0.17
CD (0.05)	0.392	0.527	0.273	0.526

192644

4.4.3. Root Shoot Ratio

The data related to the influence of growth regulators, growth stimulants, light and humidity treatments on root shoot ratio is presented in Table18. Growth regulators, growth stimulants, light and humidity treatments had significant effect on root shoot ratio at 15, 30, 45 and 60 DAP.

Results revealed that at 15 DAP, plantlets dipped in 5% PGPR Mix I (T_9) recorded significantly higher root shoot ratio (1.409) and it was statistically on par with T_1 (1.163) T_2 (1.243), T_3 (1.260) and T_4 (1.230).

Similarly, at 30 DAP also T_9 (1.438) recorded the highest root shoot ratio which was statistically at par with T_2 (1.290), T_3 (1.407) and T_4 (1.382). At 45 and 60 days after *ex vitro* transfer, the highest root shoot ratio was registered in T_3 (1.347, 1.362) and it was comparable with T_1 (1.215, 1.207), T_2 (1.312, 1.299), T_4 (1.363, 1.312) and T_9 (1.321, 1.322). The treatment T_{10} (0.734, 0.789, 0.778, 0.792) registered the lowest root shoot ratio at all the four stages of observation.

4.4.4. Total Fresh Weight

The data pertaining to the effect of growth regulators, growth stimulants, light and humidity treatments on total fresh weight at 15, 30, 45 and 60 days after *ex vitro* transfer are depicted in Table 19.

Total fresh weight was markedly influenced by growth regulators, growth stimulants, light and humidity treatments. Results revealed that, T_{10} (3.774, 4.176, 4.378, 4.626 g respectively) recorded the highest total fresh weight but it was statistically on par with T_6 (3.753, 4.153, 4.328, 4.581 g respectively) at all the four stages of observation.

The treatment T_4 (3.521, 3.741, 4.091, 4.385 g respectively) registered significantly lower total fresh weight at 15, 30, 45 and 60 days after *ex vitro* transfer.

Table 18. Effect of growth regulators, growth stimulants, light and humidity on root shoot ratio of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Root shoot ratio			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	1.163	1.202	1.215	1.207
T_2	1.243	1.290	1.312	1.299
T_3	1.260	1.407	1.347	1.362
T_4	1.230	1.382	1.363	1.312
T_5	0.955	1.005	0.879	0.870
T_6	1.057	1.109	0.975	0.967
T_7	0.988	1.009	1.025	1.040
T_8	0.953	0.987	0.946	0.947
T_9	1.409	1.438	1.321	1.322
T_{10}	0.734	0.789	0.778	0.792
C	0.759	0.821	0.871	0.882
SE m (±)	0.083	0.079	0.056	0.060
CD (0.05)	0.246	0.233	0.166	0.176

Table 19. Effect of growth regulators, growth stimulants, light and humidity on fresh weight of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Fresh weight (g)			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	3.703	3.924	4.294	4.481
T_2	3.535	3.776	4.126	4.406
T_3	3.659	3.788	4.138	4.418
T_4	3.521	3.741	4.091	4.385
T_5	3.718	3.960	4.310	4.524
T_6	3.753	4.153	4.328	4.581
T_7	3.701	3.916	4.266	4.546
T_8	3.680	3.865	4.215	4.495
T_9	3.635	3.753	4.175	4.425
T_{10}	3.774	4.176	4.378	4.626
C	3.622	3.831	4.123	4.519
SE m (±)	0.014	0.012	0.018	0.024
CD (0.05)	0.040	0.034	0.053	0.071

4.4.4. Total Dry Weight

The data regarding to the influence of growth regulators, growth stimulants, light and humidity treatments on total dry weight are presented in Table 20.

At 15 DAP, the highest total dry weight (0.754 g) was recorded in the treatment T_{10}. It was statistically at par with T_5 (0.751 g) and T_6 (0.734 g).

However, at 30 DAP also T_{10} (0.868 g) recorded the highest total dry weight which was statistically at par with T_1 (0.826 g) T_2 (0.774 g), T_5 (0.848 g), T_6 (0.859 g), T_8 (0.775 g) and T_9 (0.819 g). At 45 DAP, T_{10} (0.948 g) recorded the highest total dry weight which was statistically comparable with T_1 (0.902 g), T_5 (0.928 g) and T_6 (0.939 g). Similarly, at 60 DAP also T_{10} (0.972 g) recorded the highest total dry weight and it was at par with T_1 (0.960 g), T_2 (0.921 g), T_5 (0.958 g), T_6 (0.962 g) and T_9 (0.932 g).

The treatment T_4 (0.448, 0.561, 0.641, 0.660 g respectively) registered significantly lower total dry weight at 15, 30, 45 and 60 days after *ex vitro* transfer.

4.5. Anatomical Characters

4.5.1. Cuticle Thickness

The data pertaining to the effect of growth regulators, growth stimulants, light and humidity treatments on cuticle thickness at 30 DAP and 60 DAP are depicted in Table 21.

Cuticle thickness was markedly influenced bygrowth regulators, growth stimulants, light and humidity treatments both at 30 DAP and 60 DAP. Results revealed that at 30 and 60 DAP, the treatment T_3 (1.763 and 1.852 µm) recorded the highest cuticle thickness, but it was statistically on par with T_1 (1.703 and 1.830 µm).

Table 20. Effect of growth regulators, growth stimulants, light and humidity, growth stimulants on dry weight of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Dry weight (g)			
	15 DAP	30 DAP	45 DAP	60 DAP
T_1	0.682	0.826	0.902	0.960
T_2	0.631	0.774	0.854	0.921
T_3	0.586	0.729	0.809	0.876
T_4	0.448	0.561	0.641	0.660
T_5	0.715	0.848	0.928	0.958
T_6	0.734	0.859	0.939	0.962
T_7	0.563	0.707	0.787	0.820
T_8	0.662	0.775	0.855	0.889
T_9	0.675	0.819	0.899	0.932
T_{10}	0.754	0.868	0.948	0.972
C	0.563	0.707	0.787	0.853
SE m (±)	0.022	0.034	0.024	0.023
CD (0.05)	0.066	0.101	0.072	0.069

Table 21. Effect of growth regulators, growth stimulants, light and humidity on cuticle thickness of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Cuticle thickness (µm)	
	30 DAP	60 DAP
T_1 (Plantlet dip with Triazole @ 5ppm)	1.703	1.830
T_2 (Plantlet dip with Triazole @ 10 ppm)	1.103	1.267
T_3 (Triazole @ 5ppm+ FA Triazole @ 5ppm)	1.763	1.852
T_4 (Triazole @ 10 ppm+ FA Triazole @ 5ppm)	1.223	1.333
T_5 (40-50% light intensity and 60-70% humidity)	1.420	1.503
T_6 (40-50% light intensity and 80-90% humidity)	1.427	1.513
T_7 (70-80% light intensity and 60-70% humidity)	1.150	1.233
T_8 (70-80% light intensity and 80-90% humidity)	1.100	1.267
T_9 (PGPR Mix I -5%)	1.240	1.367
T_{10}(Arbuscular Mycorrhizal Fungi :5g/plantlet)	1.153	1.227
Control	1.067	1.200
SEm (±)	0.023	0.049
CD (0.05)	0.069	0.144

4.5.2. Number of Mesophyll Layers and Type of Cells

The data on number of mesophyll layers of tissue cultured phalaenopsis orchid at 30 DAP and 60 DAP are furnished in Table 22. Results revealed that mesophyll layers of tissue cultured phalaenopsis orchid was not significantly influenced by growth regulators, growth stimulants, light and humidity treatments at both the stages of observation.

With regard to the type of cells observed from the cross sectional view of leaves, at 30 DAP they possessed chlorenchymatous parenchyma cells and a large number of inter cellular spaces or vacuoles. At 60 DAP, density of chlorenchyma cells was more in the leaves. Cells were more differentiated than before.

4.6 Correlation Analysis

Correlation analysis of the present study clearly revealed that there are several parameters which are positively correlated to survival percentage and the parameters are stomatal frequency, number of roots, chlorophyll a content, carotenoid content, protein, carbohydrate content, cuticle thickness and number of mesophyll layers. Survival percentage is negatively correlated with transpiration rate.

Table 22. Effect of growth regulators, growth stimulants, light and humidity on number of mesophyll layers of tissue cultured orchid (*Phalaenopsis* sp.)

Treatments	Number of mesophyll layers	
	30 DAP	60 DAP
T_1 (Plantlet dip with Triazole @ 5ppm)	8.66	9.67
T_2 (Plantlet dip with Triazole @ 10 ppm)	9.00	9.25
T_3 (Triazole @ 5ppm+ FA Triazole @ 5ppm)	8.33	10.00
T_4 (Triazole @ 10 ppm+ FA Triazole @ 5ppm)	8.66	9.18
T_5 (40-50% light intensity and 60-70% humidity)	8.000	8.66
T_6 (40-50% light intensity and 80-90% humidity)	8.33	8.66
T_7 (70-80% light intensity and 60-70% humidity)	7.66	9.00
T_8 (70-80% light intensity and 80-90% humidity)	7.66	8.66
T_9 (PGPR Mix I -5%)	8.00	8.66
T_{10}(Arbuscular Mycorrhizal Fungi :5g/plantlet)	7.66	8.66
Control	7.66	8.00
SEm (±)	0.333	0.362
CD (0.05)	NS	NS

Table 23. Correlation analysis

Parameters	Survival percentage
Specific leaf area	0.242^{NS}
Photosynthetic rate	0.456^{NS}
Transpiration rate	-0.620^{*}
Stomatal index	0.310^{NS}
Stomatal frequency	0.642^{*}
Height of the plant	0.086^{NS}
Number of leaves	0.399^{NS}
Number of roots	0.821^{**}
Survival percentage	1
Chlorophyll a	0.686^{*}
Chlorophyll b	0.298^{NS}
Total chlorophyll	0.592^{NS}
Carotenoid content	0.950^{**}
Protein content	0.842^{**}
Total carbohydrate	0.673^{*}
Leaf area (cm^2)	0.242^{NS}
Root length (cm)	0.555^{NS}
Root: shoot ratio	0.266^{NS}
Total fresh weight	-0.171^{NS}
Total dry weight	0.508^{NS}
Cuticle thickness	0.660^{*}
Number of mesophyll layers	0.665^{*}

Discussion

5. DISCUSSION

The present investigation on the "Physiological approaches for enhancing the *ex vitro* establishment of tissue cultured orchid (*Phalaenopsissp.*)" was carried out at the Department of Plant Physiology, College of Agriculture, Vellayani, during 2017-2019. The outcome of the study is discussed in this chapter. A critical discussion with the background material available from the literature is presented and developed in the following pages.

Orchids, the super ordinate among ornamental are very distinctive plants. Taxonomically they represent the most highly evolved family (Orchidaceae) among monocotyledons, and the largest of all flowering plant families of about 800 genera and more than 35,000 species (Garay, 1960 and Chadha, 1992). Orchids account for 7 per cent of the total species of flowering plants of the world which exhibit an incredible range of diversity in size, shape and colour of plants. The conventional methods of propagation through symbiotic seed germination and division of black bulbs, off shoots and keikis are very slow (Blower, 1964). So the use of micropropagation techniques have revolutionised the commercial orchid industry which made it possible to multiply unlimited number of desirable clones. Most of the commercially grown monopodial orchids belong to genus *Phalaenopsis* and they perform excellently well in the humid tropical climatic conditions of Kerala.

The *ex vitro* stage of tissue cultured (*in vitro* derived) plants is characterized by gradual acclimatization to the *ex vitro* environment and transformation into complete autotrophs, accompanied by anatomical and physiological changes which complement the process. Micropropagation of orchid plantlets is seriously handicapped by the high rate of field mortality during planting out.

The present study was taken up with the objective of understanding physiological, morphological, biochemical, biometric and anatomical changes associated with ex vitro establishment of *in vitro* derived plantlets of phalaenopsis orchid. The result of this study will help in evolving measures to overcome the

field mortality and to improve propagation efficiency of not only orchids but also of various other crops.

In the present study, the *in vitro* derived plantlets of phalaenopsis orchids were collected from Biotechnology and Model Floriculture Centre (BMFC) Kazhakuttam and their physiological, morphological, biochemical, biometric and anatomical changes were evaluated during their *ex vitro* establishment. Significant variations were observed for most of the parameters studied and the results obtained are discussed in this chapter with appropriate support from previous studies.

5.1. EFFECT OF GROWTH REGULATORS, GROWTH STIMULANTS, LIGHT AND HUMIDITY ON PHYSIOLOGICAL CHARACTERS

In this study, various physiological parameters were studied during *ex vitro* establishment of phalaenopsis orchids and this section explains the basis of the results obtained.

Growth regulators, growth stimulants, light and humidity treatments significantly influenced physiological characters *viz.*, specific leaf area (SLA), photosynthetic rate, transpiration rate, stomatal index and stomatal frequency (at 15,30,45 and 60 days after planting).

In general, growth regulators, growth stimulants, light and humidity treatments recorded higher specific leaf area than control. Specific leaf area is the ratio of leaf area to leaf weight. Higher SLA indicates, more leaf area per unit biomass which result in higher photosynthetic rate. In this experiment, the treatment T_6 (plantlets provided with 40-50% light intensity and 80-90% humidity) recorded significantly higher specific leaf area at all the four stages of observation.

Matsoukis *et al.* (2007) reported a similar kind of result in *Lantana camara* L. (subsp. camara) wherein specific leaf area was found highest with 60% shading. This might be due to the reduction in leaf thickness of plants grown under shade nets. As the leaf thickness reduces, the leaf dry weight also reduces. Plants grown under shaded condition, expands the leaf area per unit of leaf biomass. Juraimi *et al.* (2004) also observed that in *Cynodon dactylon,* 65%

shading enhanced the specific leaf area. Generally, triazole treatments recorded lesser values for specific leaf area and this might be due to the reduction of leaf area due to triazoles (Davis *et al.,* 1988).

Growth regulators, growth stimulants, light and humidity treatments had significant effect on photosynthetic rate at 15, 30, 45 and 60 days of *ex vitro* transfer. In the present study, photosynthetic rate was significantly higher in plantlets provided with 40-50% light intensity and 80-90% humidity (T_6). This might be due to the impact of high humidity. High relative humidity reduced the extent of leaf damage and chlorophyll damage during the acclimatization of eucalyptus plants (James and Bell, 2000). Samasya (2000) reported a similar trend in photosynthetic rate during *ex vitro* establishment of dendrobium orchid. Orchid plantlets (*Dendrobium sp.)* provided with 50 per cent light intensity and 70 to 90 per cent humidity recorded significantly higher photosynthetic rate.

Transpiration is an important physiological process that affects the growth and establishment of tissue cultured orchids. Transpiration rate was the lowest in triazole treated plantlets. Fila *et al.* (1998) suggested that the development of cuticle, epicuticular wax and effective stomatal regulation of water loss causes gradual reduction in cuticular transpiration rates during acclimatization to *ex vitro* conditions. This might be the reason for the lowest transpiration rate in triazole treated plantlets, since cuticle thickness was the highest in these plantlets. Similar result was reported by Gopi *et al.* (2005) who states that at all stages of growth, the rate of transpiration was lowered in triazole treated elephant foot yam plants.

Stomatal frequency is defined as number of stomata per unit leaf area (Salisbury, 1927). Stomatal frequency was higher in plants with triazole treatment. Triazole compounds cause an increase in the number of stomata per unit area when compared to the control plants. This might be due to the effect of the triazoles on the hormone balance and the increased number of stomata in the triazole treated plants can be attributed to higher cell divisions induced through the increased cytokinin content (Fletcher and Arnold, 1986).

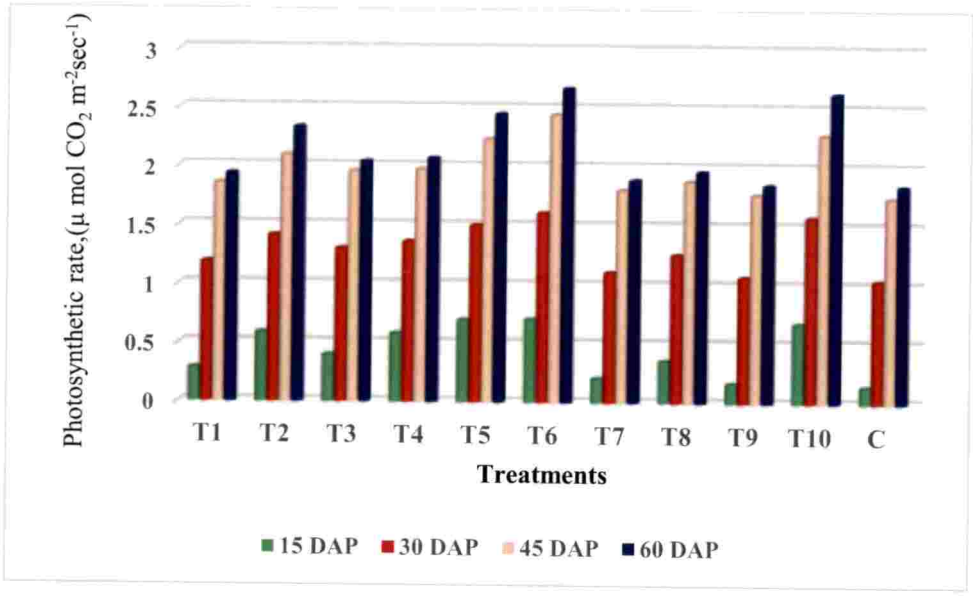

Fig.1 Effect of growth regulators, growth stimulants, light and humidity on photosynthetic rate of tissue cultured orchid (*Phalaenopsis* sp.)

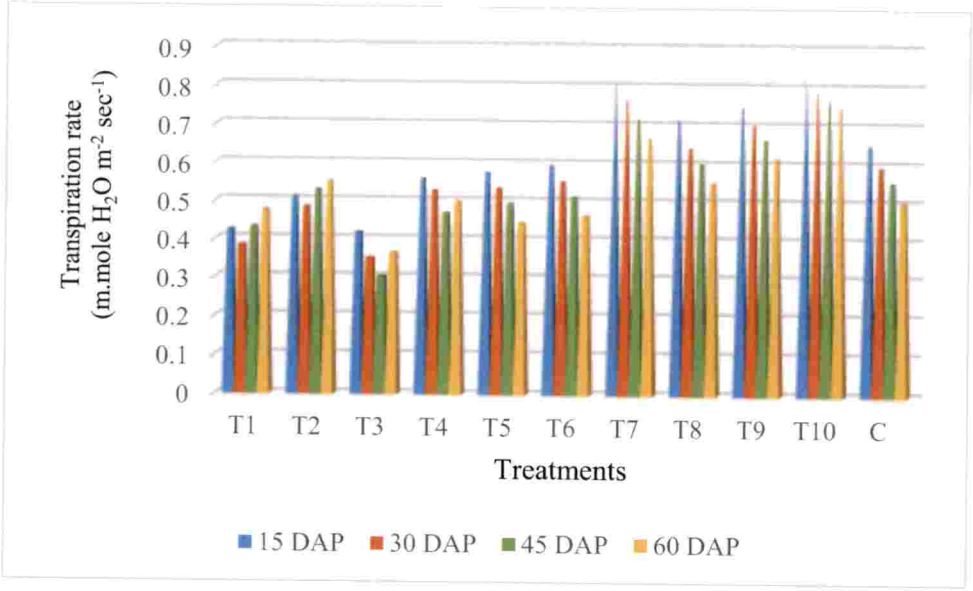

Fig.2 Effect of growth regulators, growth stimulants, light and humidity on transpiration rate of tissue cultured orchid (*Phalaenopsis* sp.)

The result of the present study is in conformity with the findings of Gao *et al.* (1988) who reported that triadimefon (TDM) treatment increased the stomatal number per unit area in wheat leaves.

5.2. EFFECT OF GROWTH REGULATORS, GROWTH STIMULANTS, LIGHT AND HUMIDITY ON MORPHOLOGICAL CHARACTERS

Growth regulators, growth stimulants, light and humidity treatments significantly influenced the morphological characters. Plantlets treated with AMF show the highest plant height at earlier stages. This might be due to the effect of AMF symbiosis formed with the root system of *in vitro* derived plantlets and which in turn result in vigorous plants and improved absorption of nutrients and water (Jaizme-Vega *et al.*, 1997).

This result reinforce those findings obtained by Yano-melo *et al.* (1999) who pointed out that height of the banana plantlets inoculated with AMF were approximately 32% higher, than non-inoculated plants.

In the present study, highest number of leaves were observed in plantlets provided with 40-50% light intensity and 80-90% humidity (T_6). This result is in conformity with the findings of Tibbitis and Bottenberg, (1976) who reported that when relative humidity (RH) was increased from 50% to 85%, leaf number also increased significantly in lettuce. Also in mandarin orange, number of leaves, shoot length and dry matter were reported to increase with increase in humidity level (Mageed *et al.,* 1988).

Samasya, (2000) pointed out that *in vitro* derived dendrobium orchid plantlets subjected to the treatment 5 mg L^{-1} of triazole had more number of roots than control plants. In the present study, T_3 (plantlets dipped with triazole @ 5 ppm + foliar application of triazole @ 5ppm after 15 days of *ex vitro* transfer) recorded the highest root number. This was owing to the fact that the shift in partitioning of assimilates from the leaves to the roots due to the action of triazoles (Steffens *et al.*, 1985).

In the present investigation, maximum survival percentage was noted in the plantlets subjected to 40-50% light intensity and 80-90% humidity (T_6). This might be attributed to the high leaf area, rooting, more number of leaves, high

Plate 7. Variation in plant height at 60DAP

Plate 8. Variaton in number of roots at 60 DAP

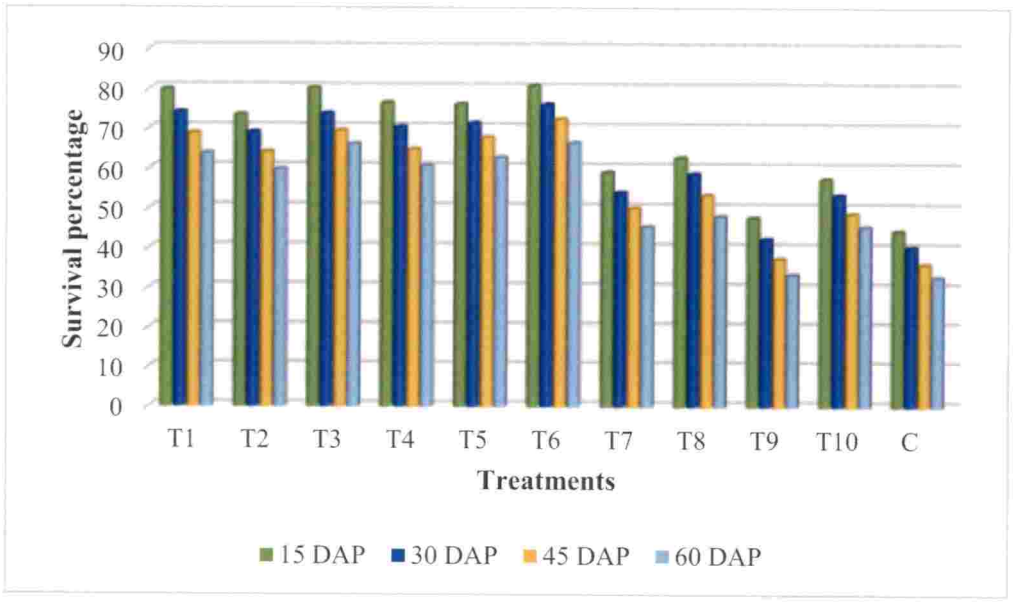

Fig.3 Effect of growth regulators, growth stimulants, light and humidity on survival percentage of tissue cultured orchid (*Phalaenopsis* sp.)

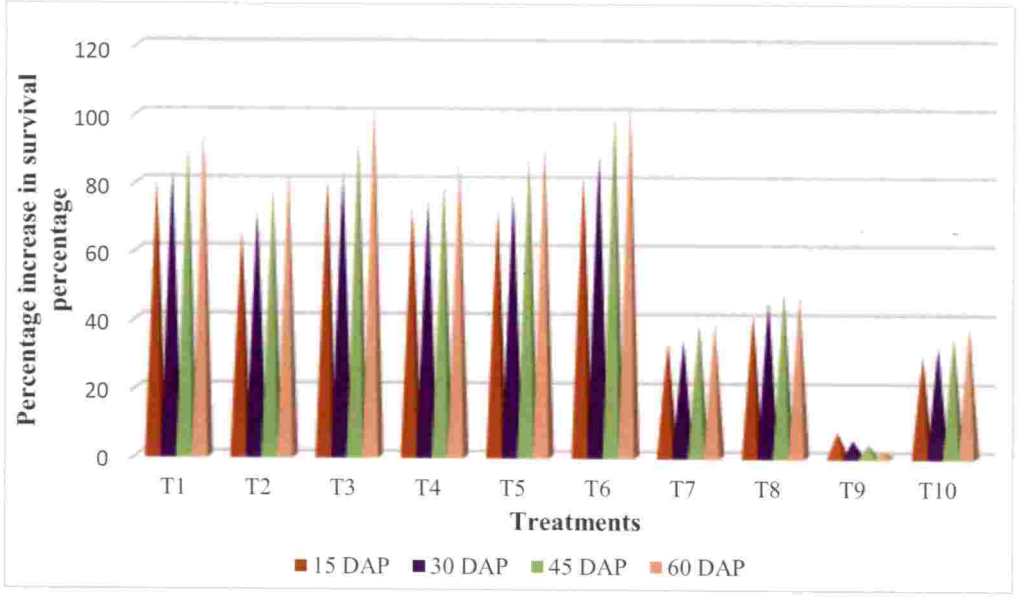

Fig.4 Effect of growth regulators, growth stimulants, light and humidity on percentage increase in survival percentage of tissue cultured orchid (*Phalaenopsis* sp.)

photosynthetic rate and more dry matter accumulation. Improvement in survival percentage was also observed by Moraes *et al.* (2002) who reported that when tissue cultured *Dendrobium nobile* plantlets were grown under a light intensity of 50%, they showed a survival rate ranging from 77.8 to 95.2%. Sunitibala and Kishor, (2009) reported that more than 90% survival rate was obtained when *Dendrobium transparens* plantlets were acclimatized by providing 50% shading.

Correlation analysis of the present study clearly revealed that there are several parameters which are positively correlated to survival percentage and the parameters are stomatal frequency, number of roots, chlorophyll a content, carotenoid content, protein, carbohydrate content, cuticle thickness and number of mesophyll layers. Survival percentage is negatively correlated with transpiration rate.

5.3. EFFECT OF GROWTH REGULATORS, GROWTH STIMULANTS, LIGHT AND HUMIDITY ON BIOCHEMICAL CHARACTERS

Triazole treated plants typically appeared as darker green, which has been correlated with increased chlorophyll content (Grossi *et al.,* 2005). In the present study a similar trend was noticed wherein growth regulators, growth stimulants, light and humidity treatments significantly influenced the chlorophyll content of leaves. The treatment T_3 (plantlets dipped with triazole @ 5 ppm + foliar application of triazole @ 5ppm after 15 days of *ex vitro* transfer) recorded the highest chlorophyll content. This was owing to the fact that retardant action of triazole retards chlorophyll destruction or accelerates its synthesis. A similar study was carried out in *Solenostemon* plants and the increase in abscisic acid and cytokinin contents due to triazole treatment is opined to be the reason for increased pigment contents in triazole treated plants (Fletcher *et al.,* 2000). However, it is not clear whether increased chlorophyll content is due to enhanced chlorophyll biosynthesis or is simply a "concentrating effect" due to reduced leaf expansion (Davis *et al.,* 1988).

These findings are in line with Borkowski *et al.* (1989) who found out that paclobutrazol (PBZ) treatment increased chlorophyll content in leaves of tomato plants and its effect was more apparent in earlier phases of plant growth. Gopi *et*

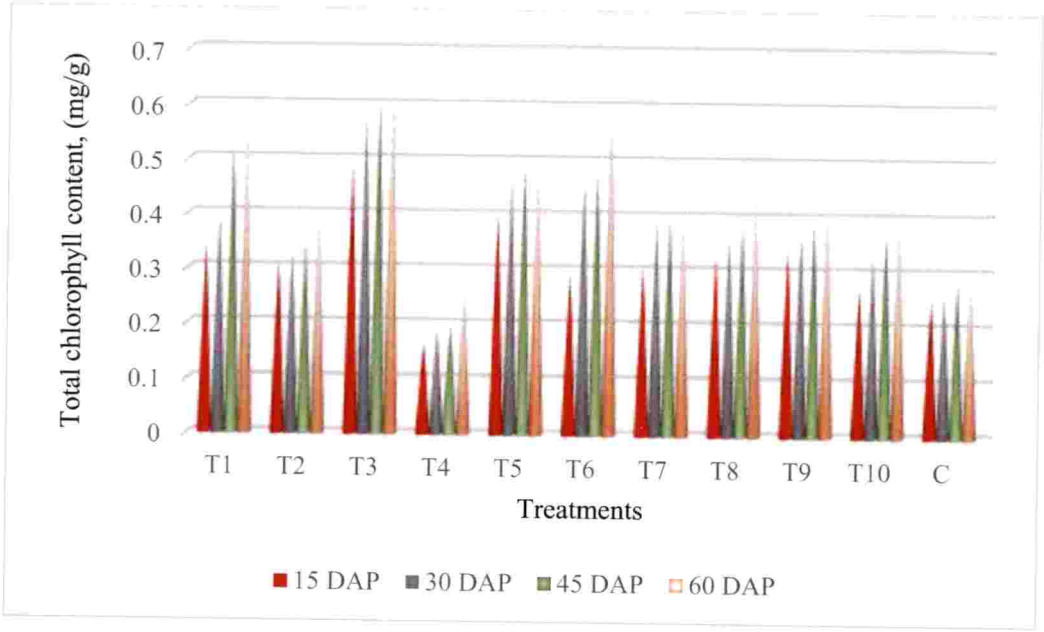

Fig.5 Effect of growth regulators, growth stimulants, light and humidity on total chlorophyll content of tissue cultured orchid (*Phalaenopsis* sp.)

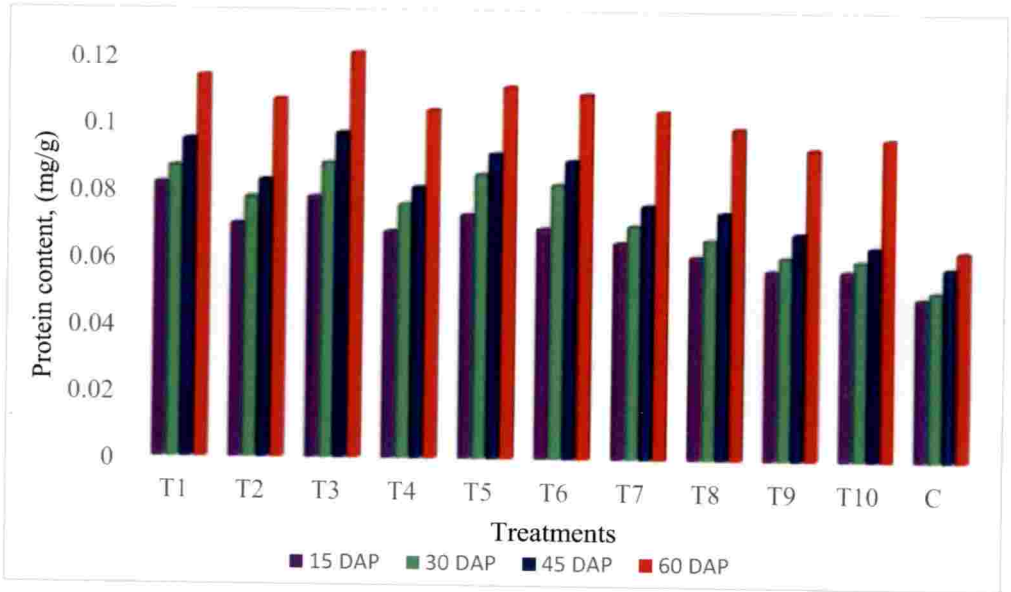

Fig.6 Effect of growth regulators, growth stimulants, light and humidity on protein content of tissue cultured orchid (*Phalaenopsis* sp.)

al. (2005) also observed a significant variation in chlorophyll content of *Amorphophallus campanulatus* (Elephant Foot Yam) after triazole application.

Sopher *et al.* (1999) reported that the application of paclobutrazol increased the soluble protein content in maize. In the present study, triazole treated *in vitro* derived phalaenopsis plantlets showed significantly higher protein content during the *ex vitro* establishment. Similar results were obtained by Sairam (1995) who reported an increase of protein content in the case of triazole treated wheat.

Triazole compounds are known to alter the carbohydrate status in various plants. Triadimefon (TDM) and hexaconazole (HEX) are reported to inhibit the gibberellin biosynthesis and increase the cytokinin level (Fletcher *et al.*, 2000) and this decreased gibberellin and increased cytokinin status induced by triazoles might be the reason for the increased starch content in the tubers of *Solenostemon rotundifolius*.

These findings are in line with Kapur *et al.* (1993) who reported that triadimefon treatment, uniconazole and ectaconazole increased the total non-structural carbohydrates in potato and *Poa pratensis*. Paclobutrazol was also reported to increase the starch and sorbitol concentration in apple (Wieland and Wample,1985).

5.4. EFFECT OF GROWTH REGULATORS, GROWTH STIMULANTS, LIGHT AND HUMIDITY ON BIOMETRIC CHARACTERS

Growth regulators, growth stimulants, light and humidity treatments significantly influenced the leaf area. James and Bell, (2000) reported that leaf size increased with decreasing irradiance from full sunlight to 50% sunlight, and then declined with further reduction in light availability. Similar trends have been found for individual leaf area and total plant leaf area in eucalyptus plants (Stoneman and Dell, 1993). In the present study *in vitro* derived phalaenopsis plantlets provided with 40-50% light intensity and 80-90% humidity (T_6) registered higher leaf area during the *ex vitro* establishment. Similar results were

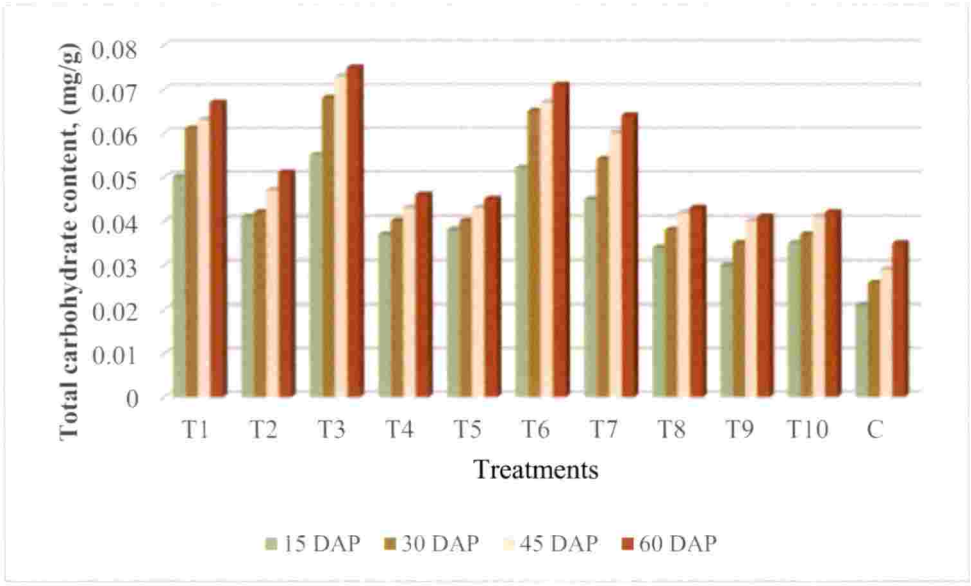

Fig.7 Effect of growth regulators, growth stimulants, light and humidity on total carbohydrate content of tissue cultured orchid (*Phalaenopsis* sp.)

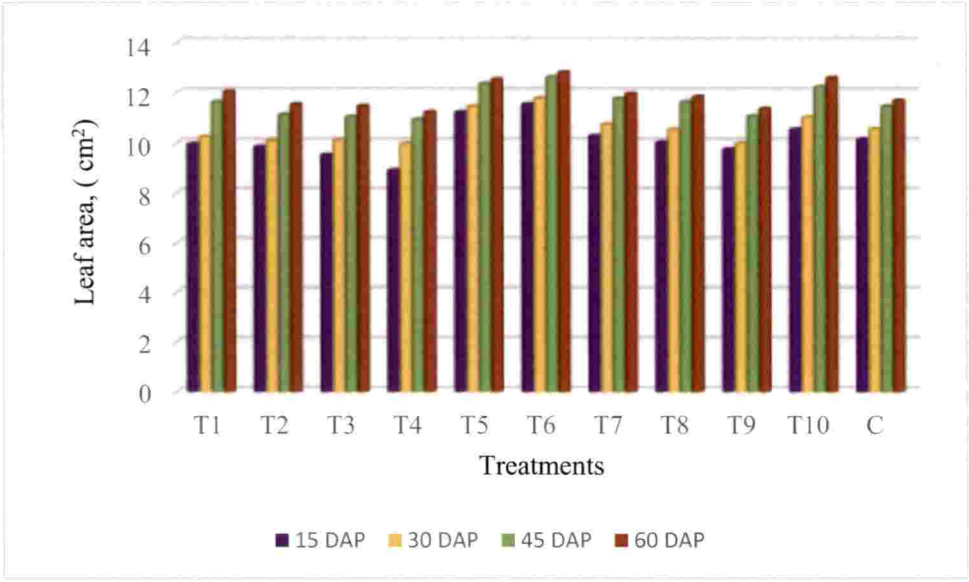

Fig.8 Effect of growth regulators, growth stimulants, light and humidity on leaf area of tissue cultured orchid (*Phalaenopsis* sp.)

obtained by Samasya (2000) who reported that dendrobium orchid plantlets subjected to 50 per cent light intensity and below 70 per cent humidity recorded maximum leaf area.

At 50 per cent light intensity there is a tendency to produce increased root growth of plants (Tester *et al.,* 1986). The result of the present study also corroborates this findings. Plantlets provided with 40-50% light intensity and 80-90% humidity (T_6) recorded the highest root growth. Similar result was obtained in the study carried out by Samasya (2000).

Plant growth-promoting rhizobacteria (PGPR) colonize roots of monocots and dicots, and enhance plant growth by direct and indirect mechanisms. Modification of root system architecture by PGPR implicates the production of phytohormones and other signals that lead mostly to enhanced lateral root branching and development of more root hairs. PGPR also modify root functioning, improve plant nutrition and influence the physiology of the whole plants (Vacheron *et al.,* 2013). The PGPR produced higher percentages of root growth and better quality in terms of root length, diameter, root dry weight *etc* in kiwi fruits (Erturk *et al.,* 2010). In the present study, PGPR treated *in vitro* derived phalaenopsis plantlets showed highest root shoot ratio during the *ex vitro* establishment. This might be attributed to better root growth.

AMF inoculation is reported to have tremendous beneficial effect on plant growth by enhancing nutrient and water uptake (Davies *et al.,* 1993). A similar result was noticed in the present study also. The highest fresh weight and dry weight were reported in AMF inoculated plantlets. This result is in conformity with the findings of Yano-Melo *et al.* (1999) in *in vitro* derived banana plantlets.

5.5. EFFECT OF GROWTH REGULATORS, GROWTH STIMULANTS, LIGHT AND HUMIDITY ON ANATOMICAL CHARACTERS

In dendrobium plantlets, epicuticular wax has been found to be either reduced or absent in the *in vitro* leaves, leading to high rate of water loss (Samasya, 2000). In tissue cultured *Chrysanthemum morifolium* plantlets, the density of wax deposition was found to increase during acclimatization (Wardle *et*

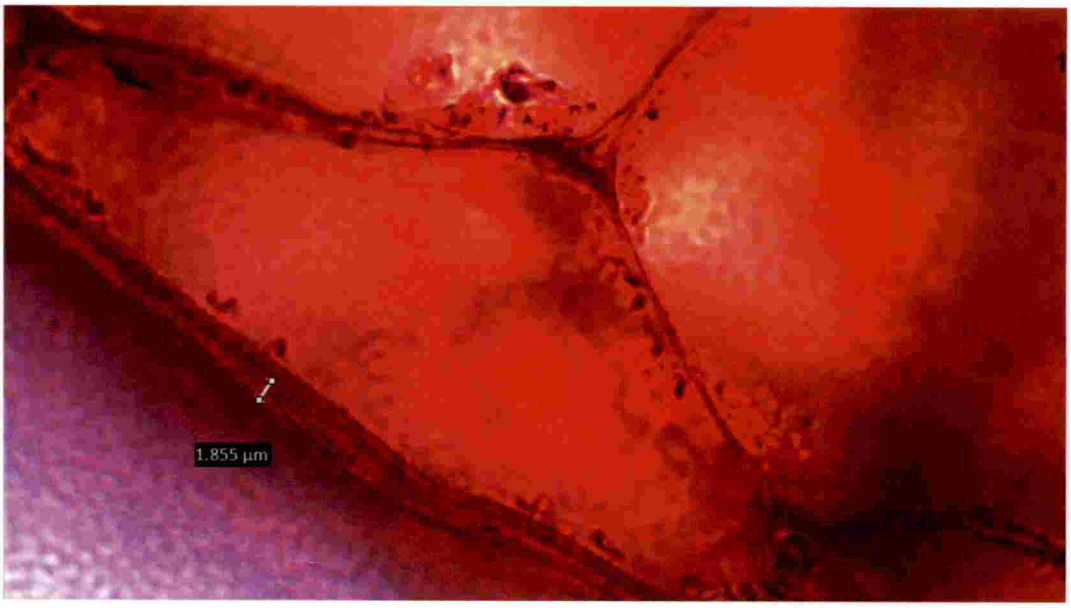

Plate 9. Leaf cuticle thickness at 60 DAP

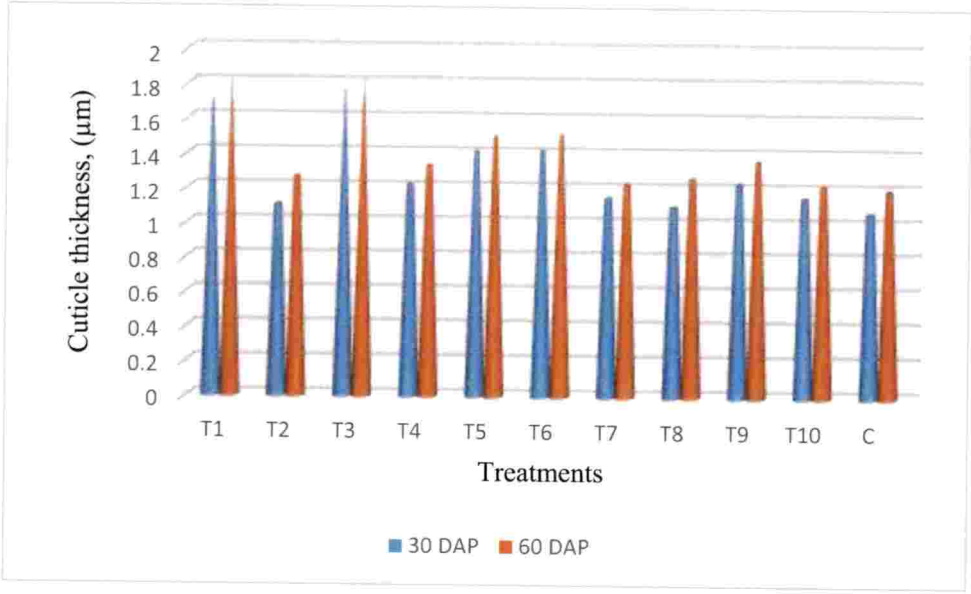

Fig.9 Effect of growth regulators, growth stimulants, light and humidity on cuticle thickness of tissue cultured orchid (*Phalaenopsis* sp.)

al., 1983). Fabri *et al.* (1986) reported that hardened leaves of tissue cultured strawberry plantlets enlarge due to increased cell size and increased epicuticular wax deposits on both adaxial and abaxial surfaces.

Tsegaw *et al.* (2005) reported that paclobutrazol treated potato plants showed thicker epicuticular waxes and it resulted in thicker cuticle. The observed higher epicuticular wax deposition in triazole treated leaves may be related to the increase in endogenous ABA levels in response to PBZ treatment (Rademacher, 1997). An increase in ABA stimulates the synthesis of lipid transfer proteins in barley, that play an important role in the formation of epicuticular waxes (Hollenbach *et al.,* 1997). The development of thicker epicuticular wax layer provides better protection against some plant pathogens and minor mechanical damage (Kolattukudy, 1987).The present study also revealed that triazole treated plants showed higher cuticle thickness. Similar result was reported by Samasya (2000) in *in vitro* derived dendrobium plantlets.

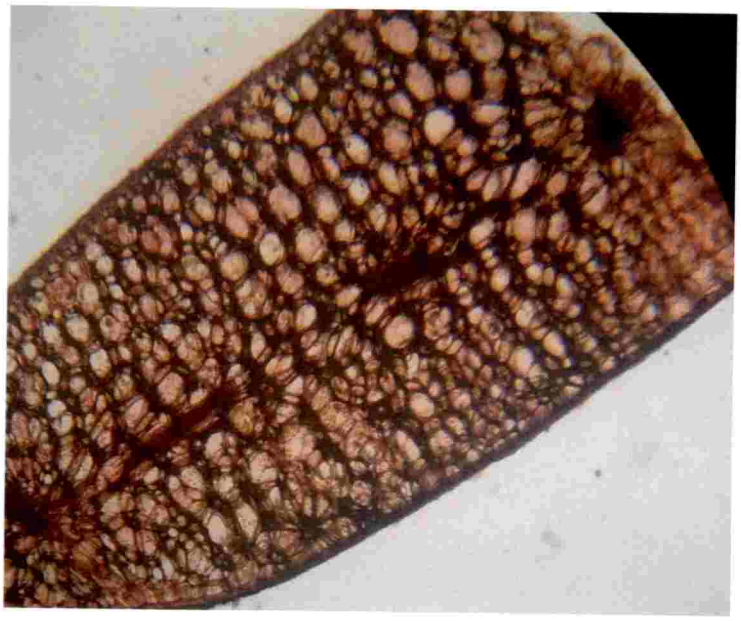

Plate 10. Number of mesophyll layers

Summary

6. SUMMARY

The research work entitled "Physiological approaches for enhancing the *ex vitro* establishment of tissue cultured orchid (*Phalaenopsis sp.*)" was conducted with an objective to study the physiological changes that occur during *ex vitro* establishment of orchid *Phalaenopsis sp.* and to find out measures to overcome the field mortality rate and improve propagation efficiency. The experiment was carried out during 2017-2019 at College of Agriculture, Vellayani, Thiruvananthapuram. Salient findings of the experiment are summarized below.

The orchid cultivar chosen for the experiment was *Phalaenopsis sp.* *In vitro* derived plantlets from shoot meristem culture were used for this study, and they were obtained from Biotechnology and Model Floriculture Centre (BMFC) Kazhakuttam. The experiment was laid out in completely randomized block design with three replications of ten treatments as well as a control. The ten treatments were T_1 and T_2 (plantlet dip with triazole @ 5 ppm and 10 ppm respectively), T_3 and T_4 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm after 15 days of planting and plantlet dip with triazole @ 10 ppm + foliar application of triazole @ 5ppm after 15 days of planting respectively) , T_5 and T_6 (plantlets providing with 40-50% light intensity & 60-70% humidity and 40-50% light intensity & 80-90% humidity respectively) T_7 and T_8 (plantlets providing with 70-80% light intensity & 60-70% humidity and plantlets providing with 70-80% light intensity and 80-90% humidity respectively) , T_9 (plantlet dip of PGPR mix I -5%) , T_{10} (root zone application of arbuscular mycorrhizal fungi - 5g/plantlet) and a control (C). The crop was maintained in the hardening chamber for 70 days and observations were taken at 15, 30, 45 and 60 days of *ex vitro* transfer.

Growth regulators, growth stimulants, light and humidity treatments had significant effect on physiological parameters *viz.*, specific leaf area, photosynthetic rate , transpiration rate , stomatal index , stomatal frequency at 15 DAP, 30 DAP, 45 DAP and at 60 DAP. During their *ex vitro* establishment, physiological parameters like specific leaf area and photosynthetic rate showed significantly higher response at all the four stages of observation, in the treatment provided with 40-50% light intensity and 80-90% humidity (T_6) whereas transpiration rate was the lowest in T_3 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm, after 15 days of planting).

Among the stomatal characters, stomatal index was not significantly affected by growth regulators, light intensity, humidity and growth stimulants. However, highest stomatal frequency was recorded in T3 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm, after 15 days of planting).

Growth regulators, growth stimulants, light and humidity treatments had significant effect on morphological characters *viz.,* plant height, number of leaves, number of roots and survival percentage. Plantlets treated with arbuscular mycorrhizal fungi (T_{10}) recorded the highest plant height during 15 and 30 DAP. However at 45 and 60 DAP, plantlets which were provided with 40-50% light intensity and 80-90% humidity (T_6) recorded the highest plant height. Number of leaves per plantlet and survival percentage were also found highest in T_6. But in the treatment, T_3 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm) maximum number of roots was observed at all the four stages of observation. Survival percentage is considered to be the most important factor during the *ex vitro* establishment of tissue cultured orchids. Among the different treatments, T6 (plantlets provided with 40-50% light intensity and 80-90% humidity) recorded the highest plantlet survival percentage at 15, 30, 45 and 60th day of observation (80, 76, 72, 66 percentage respectively) compared to control.

The results also showed that growth regulators, growth stimulants, light and humidity treatments had significant effect on biochemical characters *viz.*, chlorophyll, carotenoid, protein and carbohydrate content. Chlorophyll a and total chlorophyll content of the leaves were maximum in T_3 whereas in T_6, the highest chlorophyll b and carotenoid content were found at all the four stages of observation. Also T_3 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm) recorded the highest protein content and carbohydrate content.

Growth regulators, growth stimulants, light and humidity treatments had significant effect on biometric characters *viz.,* leaf area, root length, root shoot ratio, fresh weight and dry weight. Leaf area and root length were also found to be significantly higher in T6 (plantlets provided with 40-50% light intensity and 80-90% humidity). Root shoot ratio was the highest in T_9 at 15 (1.409) and 30 (1.438) DAP and T_3 recorded the highest root shoot ratio at 45 (1.347) and 60 (1.362) DAP. Fresh weight and dry weight of the plantlets were recorded maximum in T_{10} (plantlets treated with arbuscular mycorrhizal fungi). There was no significant difference in the number of mesophyll layers of orchid leaves during the *ex vitro* establishment. However, cuticle thickness was found highest in T_3 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm, after 15 days).

Considering the physiological, morphological, biochemical, biometric and anatomical characters, treatment T_6 (plantlets provided with 40-50% light intensity and 80-90% humidity) is adjudged as the best physiological approach to overcome field mortality and improve propagation efficiency of tissue cultured orchid *Phalaenopsis sp.* during *ex vitro* establishment.

Future line of work

- For better result, integration of all the treatments included in the current study can be taken up as a separate programme to decide the recommendation for the *ex vitro* establishment of tissue cultured orchid (*Phalaenopsis sp.)*

174644

KERALA AGRICULTURAL UNIVERSITY CENTRAL LIBRARY

7. REFERENCES

Amancio, S., Rebordao, J. P., and Chaves, M. M. 1999. Improvement of acclimatization of micropropagated grapevine: photosynthetic competence and carbon allocation. *Plant Cell Tissue Organ Cult.* 58(1): 31-37.

Arnon, D. I. 1949. Copper enzymes in isolated chloroplast: Polyphenol oxidase in *Beta vulgaris. Plant Physiol.* 24: 1-15.

Berova, M. and Zlatev, Z. 2000. Physiological response and yield of paclobutrazol treated tomato plants (*Lycopersicon esculentum* Mill.). *Plant Growth Reg.* 30(2): 117-123.

Bertamini, M., Muthuchelian, K., and Nedunchezhian, N. 2006. Shade effect alters leaf pigments and photosynthetic responses in Norway spruce (*Picea abies* L.) grown under field conditions. *Photosynthetica* 44(2): 227-234.

Blower, J. W. 1964. Meristem propagation and possible effects. *Orchid Rev.* 72: 407-408.

Bolar, J., Norelli, J. L., Aldwinckle, H. S., and Hanke, V. 1998. An efficient method for rooting and acclimation of micropropagated apple cultivars. *Hortic. Sci.* 33(7): 1251-1252.

Borkowska, B. 2002. Growth and photosynthetic activity of micropropagated strawberry plants inoculated with endomycorrhizal fungi (AMF) and growing under drought stress. *Acta Physiologiae Plant* 24(4): 365-370.

Borowski, J., Dobrzanski, A., and Bakowski, J. 1989. The effect of paclobutrazol: The growth and cropping of tomatoes. *Biuletyn Warzywniczy* 2: 121-124.

Braford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilising the principle of protein dye binding. *Ann. Biochem.* 72: 248-251.

Brainerd, K. E. and Fuchigami, I. H. 1981. Acclimatization of aseptically cultured apple plants to low relative humidity. *J. Am. Soc. Hortic. Sci.* 106(4): 515-518.

Campanelli, A., Ruta, C., Tagarelli, A., Morone-Fortunato, I., and de Mastro, G. 2014. Effectiveness of mycorrhizal fungi on globe artichoke (*Cynara cardunculus* L. var. *scolymus*) micropropagation. *J. Plant Interactions* 9(1):100-106.

Chadha, K. L. 1992. The Indian orchid scenario. *J. Orchid Soc. India.* 6(1): 1-4.

Cha-um, S., Puthea, O., and Kirdmanee, C. 2009. An effective in-vitro acclimatization using uniconazole treatments and *ex-vitro* adaptation of Phalaenopsis orchid. *Sci. Hortic.* 121: 468-473.

Conner, A. J. and Thomas, M. B. 1982. Re-establishing plantlets from tissue culture a review. *Comb. Proc. Int. Pl. Prop. Soc.* 31: 342-357.

Dai, Y., Shen, Z., Liu, Y., Wang, L., Hannaway, D., and Lu, H. 2009. Effects of shade treatments on the photosynthetic capacity, chlorophyll fluorescence, and chlorophyll content of *Tetrastigma hemsleyanum* Diels et Gilg. *Environ. Exp. Bot.* 65: 177-182.

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

Davies Jr. F. T., Potter, J. R., and Linderman, R. G. 1993. Drought resistance of mycorrhizal pepper plants independent of leaf P concentration response in gas exchange and water relations. *Physiol. Plant* 87: 45-53.

Davis, T. D., Steffens, G. L., and Sankhla, N. 1988. Triazole plant growth regulators. *Hortic. Rev.* 10: 63-105.

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

Debergh, P. 1988. Micropropagation of woody species state of the art on *in vitro* aspects. *Acta Hortic.* 2(27): 287-295.

Derelle, D., Declerck, S., Genet, P., Dajoz, I., and van Aarle, I. M. 2012. Association of highly and weakly mycorrhizal seedlings can promote the extra-and intraradical development of a common mycorrhizal network. *FEMS Microbiol. Ecol.* 79(1): 251-259.

Dhawan, V. and Bhojwani, S. S. 1986. Micropropagation in crop plants. *Glimpses Plant Res.* 7: 1-75.

Donnelly, D. J. and Vidaver, W. E. 1984. Leaf anatomy of red raspberry transferred from culture to soil. *J. Am. Soc. Hortic. Sci.* 109: 177-181.

Eliasson M. K., Beyl C. A., and Barker P. A. 1994. *In vitro* responses and acclimatization of *Prunus serotina* with paclobutrazol. *J. Plant Growth Reg.*13: 137-142.

Erturk, Y., Ercisli, S., Haznedar, A., and Cakmakci, R. 2010. Effects of plant growth promoting rhizobacteria (PGPR) on rooting and root growth of kiwifruit (*Actinidia deliciosa*) stem cuttings. *Biol. Res.* 43: 91-98.

Estrada-Luna, A. A., Davies Jr, F. T., and Egilla, J. N. 2000. Mycorrhizal fungi enhancement of growth and gas exchange of micropropagated guava plantlets (*Psidium guajava* L.) during *ex vitro* acclimatization and plant establishment. *Mycorrhiza* 10(1): 1-8.

Fabri, A., Sutter, E., and Dunston, S. K. 1986. Anatomical changes in persistent leaves of tissuecultured strawberry plants after removal from culture. *Sci. Hortic.* 28: 331-337.

Fila, G., Ghashghaie, J., and Hoarau, J. 1998. Photosynthesis, leaf conductance and water relations of *in vitro* cultured grapevine rootstock in relations to acclimatization. *Physiologia Plant.* 102: 411-418.

Fletcher, R. A. and Arnold, V. 1986. Stimulation of cytokinins and chlorophyll synthesis in cucumber cotyledons by triadimefon. *Physiol. Plant* 66: 197-201.

Fletcher, R. A., Gilley, A., Sankhla, N., and Davis, T. D. 2000. Triazoles as plant growth regulators and stress protectants. *Hortic. Rev.* 24: 55-138.

Fortuna, P., Citernesi, S., Morini, S., Giovannetti, M., and Loreti, F. 1992. Infectivity and effectiveness of different species of arbuscular mycorrhizal fungi in micropropagated plants of Mr S2/5 plum rootstock. *Agron.* 12: 825-830.

Gao, J., Hofstra, G., and Fletcher, R. A. 1988. Anatomical changes induced by triazoles in wheat seedlings. *Canadian J. Bot.* 66(6): 1178-1185.

Garay, L. 1960. On the origin of orchidaceae. In: *Proceedings of third world orchid conference*, pp. 172-195.

Gopi, R., Sridharan, R., Somasundaram, R., Lakshmanan, G. A., and Panneerselvam, R. 2005. Growth and photosynthetic characteristics as affected by triazoles in *Amorphophallus campanulatus* Blume. *General Appl. Plant Physiol.* 31(1): 3-4.

Gordon, D. M., Grey, K. A., Chase, S. C., and Simpson, C. J. 1994. Changes to the structure and productivity of a *Posidonia sinuosa* meadow during and after imposed shading. *Aquatic Bot.* 47(4): 265-275.

Grossi, J. A. S., de Moraes, P. J., de Araujo Tinoco, S., Barbosa, J. G., Finger, F. L., and Cecon, P. R. 2005. Effects of paclobutrazol on growth and fruiting characteristics of Pitanga ornamental pepper. *Acta Hortic.* 68(3): 333-337.

Hahn, E. J. and Paek, K. Y. 2001. High photosynthetic photon flux and high CO2 concentration under increased number of air exchanges promote growth and photosynthesis of four kinds of orchid plantlets *in vitro*. *In vitro Cell Dev. Biol.* 37: 678–682.

Hayashi, M., Nakayama, M., and Kozai, T. 1988. An application of the acclimatization unit for growth of carnation explants and rooting and acclimatization of the plants. *Acta Hortic.* 230: 189-194.

Hayat, R., Ahmed, I., and Sheirdil, R. A. 2012. An overview of plant growth promoting rhizobacteria (PGPR) for sustainable agriculture. In: *Crop production for Agricultural Improvement.* Springer, Dordrecht, pp. 557-579.

Hazarika, B. N. 2003. Acclimatization of tissue cultured plants. *Curr. Sci.* 85: 1704-1712.

Hazarika, B. N. 2006. Morpho-physiological disorders in *in vitro* culture of plants. *Sci. Hortic.* 108: 105-120.

Hazarika, B. N., Parthasarathy, V. A., and Nagaraju, V. 2002. Action of paclobutrazol in acclimatizing micropropagated citrus plantlets. *Indian J. Agric. Res.* 36: 57-60.

Hedge, J. E. and Hofreiter, B. T. 1962. In: Whistler, R. L and Be Miller, J. N. (eds), *Carbohydrate Chemistry.* Academic press, New York, 17p.

Hollenbach, B., Schreiber, L., Hartung, W., and Dietz, K. J. 1997. Cadmium leads to stimulated expression of the lipid transfer protein genes in barley: implications for the involvement of lipid transfer proteins in wax assembly. *Planta* 203(1): 9-19.

Huylenbroeck, J. V., Huygens, H., Debergh, P. C., Boettinger, J. L., and Southard, R. J. 1995. Photo inhibition during acclimatization of micropropagated *Spathiphyllum*" Petite" plantlets Plant. *In vitro Cell Dev. Biol.* 31(3):160-164.

Jaizme-Vega, M. C. and Pinochet, J. 1997. Growth response of banana to three mycorrhizal fungi in *Pratylenchus goodeyi* infested soil. *Nematropica* 27(1): 69-76.

James, S. A. and Bell, D. T. 2000. Leaf orientation, light interception, and conductance of *Eucalyptus globulus* ssp. *globulus* leaves. *Tree Physiol.* 20(12): 815-823.

Juraimi, Donald S. H., Drennan, and Anuar, N. 2004.The effects of shading on the growth, development and partitioning of biomass in Bermuda grass (*Cynodon dactylon* (L.) Pers.). *J. Biol. Sci.* 4(6): 756-762.

Kapur, K. K., Narula, S. L., and Upadhyaya, M. D. 1993. Modification of physiological and biochemical responses in potato with CCC and triazole. In: *Proceedings of the National Symposium on Plant Physiology*, NBRL, Lucknow, India, pp. 361-369.

Kolattukudy, P. E. 1987. Lipid derived defensive polymers and waxes and their role in plant microbe interaction. In: Stumpf, P. K. (Ed.), *The Metabolism, Structure and Function of Plant Lipids.* Plenum Press, New York, pp. 291-314.

Kozai, T. 1991. Photoautotrophic micropropagation. *In Vitro Cell. Dev. Biol.* 27(1): 47-51.

Krishna, H., Singh, S. K., Sharma, R. R., Khawale, R. N., Grover, M., and Patel, V. B. 2005. Biochemical changes in micropropagated grape (*Vitis vinifera* L.) plantlets due to arbuscular-mycorrhizal fungi (AMF) inoculation during *ex vitro* acclimatization. *Sci. Hortic.* 106: 554-567.

Lavanya, M., Venkateshwarlu, B., and Devi, B. P. 2009. Acclimatization of neem microshoots adaptable to semi-sterile conditions. *Indian J. Biotechnol.* 8: 218-222.

Lovato, P. E., Gianinazzi-Pearson, V., Trouvelot, A., Trouvelet, A., and Gianinazzi, S. 1996. The state of art of mycorrhizas and micropropagation. *Adv. Hortic. Sci.* 1: 46-52.

Bertamini, M., Muthuchelian, K., and Nedunchezhian, N. 2006 Shade effect alters leaf pigments and photosynthetic responses in Norway spruce (*Picea abies* L.) grown under field conditions. *Photosynthetica* 44(2): 227-234.

Mageed, K. J. A., Sharma, B., and Balakrishnan, K. A. 1988. Association of growth characters in mandarin orange with consumptive water use. *Indian J. Agric. Sci.* 55(6): 406-408.

Maridass, M., Mahesh, R., Raju, G., Benniamin, A., and Muthuchelian, K. 2010. *In vitro* propagation of *Dendrobium nanum* through rhizome bud culture. *Int. J. Biol. Technol.* 1(2): 50-54.

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. *Sci. Hortic.* 108: 95-99.

Mathur, A., Mathur, A. K., and Verma, P. 2008. Biological hardening and genetic fidelity testing of micro-cloned progeny of *Chlorophytum borivilianum*. *Afr. J. Biotechnol.* 7: 1046-1053.

Matsoukis, A., Gasparatos, D., and Chronopoulou-Sereli, A. 2007. Specific leaf area and leaf nitrogen concentration of Lantana in response to light regime and triazole treatment. *Commun. Soil Sci. Plant Anal.* 38(18): 2323-2331.

Moraes, L. M., Cavalcante, L. C. D. and de Faria, R. T. 2002. Substratos para aclimatizaçao de plântulas de *Dendrobium nobile* Lindl. (Orchidaceae) propagadas *in vitro*. *Acta Scientiarum. Agron.* 24: 1397-1400.

Nayak, N. R., Sahoo, S., Patnaik, S., and Rath, S. P. 2002. Establishment of thin cross section (TCS) culture method for rapid micropropagation of *Cymbidium aloifolium* (L.) and *Dendrobium nobile* Lindl. (Orchidaceae). *Sci. Hortic.* 94: 107-116.

Nazarudin, M. A., Fauzi, R. M., and Tsan, F. Y. 2006. Effects of paclobutrazol on the growth and anatomy of stems and leaves of *Syzygium campanulatum*. *J. Tropical Forest Sci.* 19(2): 86-91.

Ortas, I. 2008. Field trials on mycorrhizal inoculation in the eastern Mediterranean horticultural region. In: Feldmann, F., Kapulnik, Y., and Baar, J. (eds), *Mycorrhiza*, pp. 56-77.

Osmond, C. B. 1994. What is photoinhibition: Some insights from comparisons of sun and shade plants. In: Baker, N. R. and Bowyer, J. R. (eds), *Photoinhibition of Photosynthesis: From Molecular Mechanisms to the Field.* BIOS Scientific Publishers, Oxford, pp. 95-110.

Poole, R. T. and Conover, C. A. 1983. Establishment and growth of *in vitro* cultured Dieffenbachia. *Hortic. Sci.* 18: 185-187.

Pospisilova, J., Ticha, I., Kadlecek, S., Haisel, D., and Pizakova, S. 1999. Acclimatization of micropropagated plants in *ex vitro* conditions. *Biologia Plant* 42: 481-497.

Rademacher, W. 1997. Bioregulation in crop plants with inhibitors of gibberellin biosynthesis. *Proc. Plant Growth Reg. Soc. America.* 24(1): 27-31.

Rai, M. K. 2001. Current advances in mycorrhization in micropropagation. *In Vitro Cell. Dev. Biol. Plant.* 37: 158-167.

Ritchie, G. A., Short, K. C., and Davey, M. R. 1991. *In vitro* acclimatization of chrysanthemum and sugar beet by treatment with paclobutrazol and exposure to reduced humidity. *J. Exp. Bot.* 42: 1557–1563.

Ruta, C., Perrini, R., Tagarelli, A., and Morone-Fortunato, I. 2009. Use of arbuscular mycorrhiza for acclimatization of micropropagated plantlets of melon, oregano, artichoke and spanish broom. *Acta Hortic.* 812: 473-478.

Sairam, R. K., Shukla, D. S., and Deshmukh, P. S. 1995. Effect of triazole-triadimefon on tolerance to moisture stress in wheat (*Triticum aestivum*). *Indian J. Agric. Sci.* 65: 483-489.

Salisbury, E. J. 1927. On the causes and ecological significance of stomatal frequency with special reference to the woodland flora. *Phyl. Trans. R. Soc. Ser.* 21(6): 1-65.

Samasya, K. S. 2000. Physiological aspects of *ex vitro* establishment of tissue cultured orchid (*Dendrobium* sp. Var. Sonia 17) plantlets. M.Sc.(Ag) thesis, Kerala Agricultural University, Thrissur, 140p.

Sebastian, B., Gonzalez, A., Cano, E. A., Franco J. A., and Fernandez, J. A. 2001. Growth, development and colour response of potted *Dianthus caryophyllus* cv. Mondarin to paclobutrazol treatment. *Sci. Hortic.* 94: 371-377.

Sharma, U., Rama Rao, V., Mohan, J. S. S., and Reddy, A. S. 2007. *In vitro* propagation of *Dendrobium microbulbon* A. Rich - A rare ethnomedicinal herb. *Indian J. Biotechnol.* 6: 381-384.

Sheena, A. and Sheela, V. L. 2010. Effects of the growth retardant Triadimefon on the *ex vitro* establishment of Gladiolus (*Gladiolus grandiflorus* L.) cv. Vinks Glory. *Plant Tissue Cult. Biotechnol.* 20(2): 171-178.

Short, K. C., Warburton, J., and Roberts, A. V. 1987. *In vitro* hardening of cultured cauliflower and chrysanthemum to humidity. *Acta Hortic.* 212: 329–340.

Siqueira, J. O. 1994. Micorrizas. In: Araujo, R. S. and Hungria, M. (eds), Microrganismos de importância agricola. Embrapa-CNPAF, Embrapa-CNPSo, Embrapa-SPI, Brasilia, pp. 151–194.

Smith, E. E., Facelli, E., Pope, S., and Smith, F. A. 2010. Plant performance in stressful environments- Interpreting new and established knowledge of the roles of arbuscular mycorrhizas. *Plant Soil* 32(6): 3-20.

Smith, E. F., Roberts, A. V., Mottley, J., and Denness, S. 1991. The preparation *in vitro* of chrysanthemum for transplantation to soil- The effects of eleven growth retardants on wilting. *Plant Cell Tiss. Org. Cult.* 27: 309-313.

Sopher, C. R., Krol, M., Huner, N. P. A., Moore, A. E., and Fletcher, R. A. 1999. Chloroplastic changes associated with paclobutrazol induced stress protection in maize seedlings. *Can. J. Bot.* 77: 279-90.

Steffens, G. L., Wang, S.Y., Faust, M., and Byun, J. K. 1985. Growth, carbohydrate and mineral element status of shoot and spur leaves and fruit of 'Spartan' apple trees treated with paclobutrazol. *J. Am. Soc. Hortic. Sci.* 110(6): 850-855.

Stoneman, G. L. and Dell, B. 1993. Growth of *Eucalyptus marginata* (Jarrah) seedlings in a greenhouse in response to shade and soil temperature. *Tree Physiol.* 13(3): 239-252.

Sunitibala, H. and Kishor, R. 2009. Micropropagation of *Dendrobium transparens* L. from axenic pseudobulb segments. *Indian J. Biotechnol.* 8: 448-452.

Sutter, E. 1984. Chemical composition of epicuticular wax in cabbage plants grown *in vitro*. *Can. J. Bot.* 62: 74-77.

Tester, M., Smith, S. E., Smith F. A., and Walker, N. A. 1986. The effects of photon irradiance on the growth of shoots and roots on the rate of initiation of mycorrhizal infection and on the growth of infection units in *Trifolium subterraneum*. *New Phy.* 103(2): 375-390.

Thakur, R., Sood, A., Nagar, P. K., Pandey, S., Soboti, R. C., and Ahuja P. S. 2006. Regulation of growth of lilium plantlets in liquid medium by application of paclobutrazol or ancymidol, for its amenability in a bioreactor system: Growth parameters. *Plant Cell Rep.* 25: 382–391.

Tibbitts, T. W. and Bottenberg, G. 1976. Growth of lettuce under controlled humidity levels. *J. Am. Soc. Hortic. Sci.* 101: 70-73.

Tsegaw, T., Hammes, S., and Robbertse, J. 2005. Paclobutrazol-induced leaf, stem, and root anatomical modifications in potato. *Hortic. Sci.* 40(5): 1343-1346.

Vacheron, J., Desbrosses, G., Marie-Lara, B., Touraine, B., Moenne-Loccoz, Y., and Muller, D. 2013. Plant growth-promoting rhizobacteria and root system. *Frontiers Plant Sci.* 3(1): 1-19.

van-Huylenbroeck, J. M., Huygens, H., and Debergh, P. C. 1995. Photoinhibition during acclimatization of micropropagated *Spathiphyllum* "Petite" plantlets. *In Vitro Cell. Dev. Biol.* 31: 160-164.

Wardle, K., Dalsou, V., Simpkins, I., and Short, K. C. 1983. Redistribution of rubidium in plants of *Chrysanthemum morifolium* Ram cv. Snowdon derived from tissue cultures and transferred to soil. *Ann. Bot.* 1: 261-264.

Wieland, W. F. and Wample, R. L. 1985. Effect of paclobutrazol on growth, photosynthesis and carbohydrate content of Delicious apples. *Sci. Hortic.* 26: 139-147.

Winarto, B. and Silva, T. J. A. 2015. Use of coconut water and fertilizer for *in vitro* proliferation and 758 plantlet production of Dendrobium 'Gradita 31'. *In Vitro Cell Dev. Biol.* 51: 303–314.

Winarto, B., Rachmawati, F., Santi, A., and da Silva, J. A. T. 2013. Mass propagation of Dendrobium 'Zahra FR 62', a new hybrid used for cut flowers, using bioreactor culture. *Sci. Hortic.* 161: 170-180.

Wright, I. J. and Westoby, M. 2001. Understanding seedling growth relationships through specific leaf area and leaf nitrogen concentration: Generalisations across growth forms and growth irradiance. *Oecologia*. 127:21-29.

Yano-Melo, A. M., Maia, L. C., Saggin Jr, O. J., Lima-Filho, J. M., and Melo, N. F. 1999. Effect of arbuscular mycorrhizal fungi on the acclimatization of micropropagated banana plantlets. *Mycorrhiza* 9(2): 119-123.

Ziv, M. 1986. *In vitro* hardening and acclimatization of tissue culture plants. In: Withers, L. A. and Alderson, P. G. (eds), *Plant Tissue Culture and its Agricultural Applications*, pp. 187–196.

Ziv, M. and Chen, J. 2008. The anatomy and morphology of tissue cultured plants. In: George, E. F., Hall, M. A., and de Klerk, G. J. (eds), *Plant Propagation by Tissue Culture* (3rd Ed.). Springer, Dordrecht, pp. 465-467.

PHYSIOLOGICAL APPROACHES FOR ENHANCING THE *EX VITRO* ESTABLISHMENT OF TISSUE CULTURED ORCHID (*Phalaenopsis* sp.)

By

SAYOOJ .S
(2017-11-088)

ABSTRACT

Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture
Kerala Agricultural University

DEPARTMENT OF PLANT PHYSIOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM- 695 522
KERALA, INDIA
2019

ABSTRACT

The study entitled "Physiological approaches for enhancing the *ex vitro* establishment of tissue cultured orchid (*Phalaenopsis sp.*)" was undertaken during 2017-2019, at College of Agriculture, Vellayani, Thiruvananthapuram, with the objective to study the physiological changes that occur during *ex vitro* establishment of orchid *Phalaenopsis sp.* and to find out measures to overcome the field mortality rate and improve propagation efficiency.

The experiment was laid out in completely randomized block design with three replications of ten treatments as well as a control. *In vitro* derived plantlets of *Phalaenopsis sp.* obtained from Biotechnology and Model Floriculture Centre, Kazhakuttam were used for the study. Ten treatments were T_1 and T_2 (plantlet dip with triazole @ 5ppm and 10 ppm respectively), T_3 and T_4 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm after 15 days of planting and plantlet dip with triazole @ 10 ppm + foliar application of triazole @ 5ppm after 15 days of planting respectively) , T_5 and T_6 (plantlets providing with 40-50% light intensity & 60-70% humidity and 40-50% light intensity & 80-90% humidity respectively) T_7 and T_8 (plantlets providing with 70-80% light intensity & 60-70% humidity and plantlets providing with 70-80% light intensity and 80-90% humidity respectively) , T_9 (plantlet dip of PGPR mix I -5%) , T_{10} (root zone application of arbuscular mycorrhizal fungi -5g/plantlet) and a control (C). The crop was maintained in the hardening chamber for 70 days and observations were taken at 15, 30,45 and 60 days of *ex vitro* transfer.

Results revealed that growth regulators, growth stimulants, light and humidity had significant effect on physiological, morphological, biochemical, biometric and anatomical characters during the *ex vitro* establishment of tissue cultured orchid *Phalaenopsis sp.*

During their *ex vitro* establishment, physiological parameters like specific leaf area and photosynthetic rate showed significantly higher response at all the four stages of observation, in the treatment provided with 40-50% light intensity and 80-90% humidity (T_6) whereas transpiration rate was the lowest in T_3 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm after 15 days of

planting and plantlet dip with triazole @ 10 ppm). Among the stomatal characters, stomatal index was not significantly affected by growth regulators, light intensity, humidity and growth stimulants. However, highest stomatal frequency was recorded in T_3 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm, after 15 days of planting).

Plantlets treated with arbuscular mycorrhizal fungi (T_{10}) recorded the highest plant height during 15 and 30 DAP. However at 45 and 60 DAP, plantlets which were provided with 40-50% light intensity and 80-90% humidity (T_6) recorded the highest plant height. Number of leaves per plantlet and survival percentage were also found highest in T_6. But in the treatment, T_3 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm) maximum number of roots was observed at all the four stages of observation.

The results also showed that chlorophyll a and total chlorophyll content of the leaves were maximum in T_3 whereas in T_6, the highest chlorophyll b and carotenoid content were found at all the four stages of observation. Also T_3 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm) recorded the highest protein content and carbohydrate content.

Leaf area and root length were also found to be significantly higher in T_6 (plantlets provided with 40-50% light intensity and 80-90% humidity). Root shoot ratio was the highest in T_9 at 15 and 30 DAP and T_3 recorded the highest root shoot ratio at 45 and 60 DAP. Fresh weight and dry weight of the plantlets were recorded maximum in T_{10} (plantlets treated with arbuscular mycorrhizal fungi).

There was no significant difference in the number of mesophyll layers of orchid leaves during the *ex vitro* establishment. However, cuticle thickness was found highest in T_3 (plantlet dip with triazole @ 5 ppm + foliar application of triazole @ 5ppm, after 15 days).

Survival percentage is considered to be the most important factor during the *ex vitro* establishment of tissue cultured orchids. Among the different treatments, T_6 (plantlets provided with 40-50% light intensity and 80-90% humidity) recorded the highest survival percentage at all the four stages of observation (80, 76, 72, 66 percentage respectively) compared to control.

Considering the physiological, morphological, biochemical, biometric and anatomical characters, treatment T_6 (plantlets provided with 40-50% light intensity and 80-90% humidity) is adjudged as the best physiological approach to overcome field mortality and improve propagation efficiency of tissue cultured orchid *Phalaenopsis* sp. during *ex vitro* establishment.

14644

CENTRAL LIBRARY