MASS PROPAGATION AND *EX-SITU* CONSERVATION OF *CYMBIDIUM ALOIFOLIUM* (L.) SW., A THREATENED MEDICINAL ORCHID OF NEPAL THROUGH ARTIFICIAL SEED TECHNOLOGY

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF BOTANY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN BOTANY

BY

SHREETI PRADHAN

AUGUST 2017

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DECLARATION

Thesis entitled **"Mass Propagation and *Ex-situ* Conservation of *Cymbidium aloifolium* (L.) Sw., a Threatened Medicinal Orchid of Nepal through Artificial seed Technology"** which is being submitted to the Central Department of Botany, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Prof. Dr. Bijaya Pant, Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

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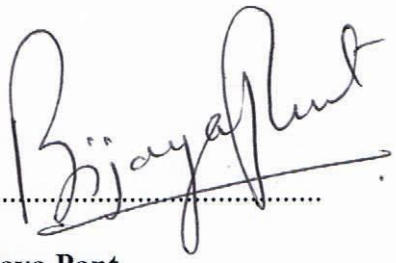
Shreeti Pradhan

RECOMMENDATION

This is to recommend that **Shreeti Pradhan** has carried out research entitled **"Mass Propagation and *Ex-situ* Conservation of *Cymbidium aloifolium* (L.) Sw., a Threatened Medicinal Orchid of Nepal through Artificial seed Technology"** for the award of Doctor of Philosophy (Ph.D.) in **Botany** under my supervision. To my knowledge, this work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.



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August 2017

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INSTITUTE OF SCIENCE AND TECHNOLOGY
CENTRAL DEPARTMENT OF BOTANY
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LETTER OF APPROVAL

Date: 25 July, 2017

On the recommendation of Prof. Dr. **Bijaya Pant**, this Ph.D. thesis submitted by **Shreeti Pradhan**, entitled **"Mass Propagation and *Ex-situ* Conservation of *Cymbidium aloifolium* (L.) Sw., a Threatened Medicinal Orchid of Nepal through Artificial seed Technology"** is forwarded by Central Department Research Committee (CDRC) to the Dean, IOST, T.U.

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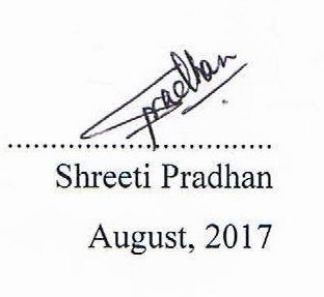

Shreeti Pradhan

August, 2017

ABSTRACT

Artificial seed technology is a rapidly growing area of research in plant cell and tissue culture. Application of this technology opens an alternative means for mass scale production, *ex situ* conservation and efficient delivery of cloned plantlets. *Cymbidium aloifolium* (L.) Sw. is one of the highly traded medicinal orchids of Nepal. This orchid species is also renowned for its beauty in nature and hence used in horticultural purpose too. This species is listed as an endangered category under Appendix II of CITES. So, efficient propagation technique is needed to conserve this orchid species.

In this respect, the present study was carried out to develop the efficient propagation technique using artificial seeds of *C. aloifolium*, production of virus free plantlets from *in vitro* explants and to obtain the genetic homogeneity between *in vitro* regenerated plantlets and *in vivo* grown mother plant. The *in vitro* germination and development of artificial seeds were compared with *in vitro* culture of seed derived protocorms. Two different media, liquid and solid Murashige and Skoog (MS) and Knudson C (KC) were used as the basal medium for *in vitro* germination and seedling development of above explants. Artificial seeds were prepared through protocorms and protocorms was derived from immature seeds. Hence, first of all, seeds were cultured *in vitro* on three different strength (i.e. full (1.0), half (½), quarter (¼) strength) of both solidified MS and KC medium and both media supplemented with various concentration of BAP (6-Benzyl aminopurine) (0.5-2mg/l) and NAA (α-Naphthalene acetic acid) (0.5-1mg/l).

Full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA was found to be most effective for *in vitro* seed germination (98.33%), large number of protocorm formation and seedling development of *C. aloifolium*. Twenty one days old protocorms (0.3-0.5 mm of diameter) were used as explants for *in vitro* culture of protocorms and artificial seeds. As MS medium gave the earliest response on *in vitro* seed germination and seedling development, further culture of single protocorm was carried out on liquid and solid MS medium alone and MS medium supplemented with BAP (0.5-2.0 mg/l) and NAA (0.5-1.0 mg/l). Hormone free full strength of liquid MS medium was more effective for earlier *in vitro* development and differentiation into seedling (20.5±0.34 weeks of culture) from a single protocorm rather than various hormone treated solid MS medium. As the increase concentration of BAP and NAA were found to be less effective for the development of seedling from a single protocorm, the further experiment was carried out on both MS and KC media supplemented with low concentration of hormones.

The artificial seeds were produced through encapsulation of protocorms in calcium alginate beads. Protocorms were encapsulated by using 2%, 3% and 4% sodium alginate and 0.2 M calcium chloride solution. Among different artificial seeds (2%, 3% and 4%), 3% alginate coated artificial seeds cultured on hormone free liquid MS medium was found to be more effective for the maximum proliferation of shoots

(10.5±1.96 shoots per culture), roots (5.33±0.92 roots per culture) and earlier development of complete seedling (18.5±0.84 weeks of culture) rather than other tested conditions of artificial seeds and protocorms (non encapsulated). For shoot multiplication, the shoot tip explants derived from *in vitro* culture of artificial seeds were sub-cultured on hormone free full strength of liquid and solid MS media. The maximum number of shoots was observed after 12 weeks of culture. All the artificial seeds and non encapsulated protocorms were stored at 4ºC and RT (21±2ºC) for 120 days for their viability test. The germination percentage was declined with prolonged storage time. It was found that 3% alginate coated artificial seeds showed higher conversion frequency (93.33%) after 30 days of storage at 4ºC on MS medium. Non-encapsulated protocorms stored for 30 to 120 days at 4ºC and RT became achlorophyllous, shrinked and completely lost their viability after certain period. Hence from the result, it was concluded that all the artificial seeds have higher regeneration capacity than non-encapsulated protocorms. For rooting, micro shoots (0.5-1.0cm) obtained from *in vitro* multiplied shoots was rooted on different concentrations of auxins i.e. IAA (Indole-3-acetic acid), IBA (Indole-3-butyric acid) and NAA (0.5mg/l to 2.0mg/l). The rooting was observed after 4 weeks of culture of micro shoots. MS medium supplemented with 1.0 mg/l IBA was found to be the most effective condition for the development of maximum number of healthy, thick and long roots (5.33±0.8 roots per culture).

In vitro regenerated plants derived from seeds, protocorms, shoot tips and artificial seeds (2%, 3% and 4% alginate coated) were tested for Cymbidium mosaic virus (CymMV) by using DAS-ELISA and was found to be completely free of virus (100% free) whereas the mother plant obtained from wild was highly infected with CymMV (83.33%) virus. Random Amplified Polymorphic Deoxyribonucleic acid (RAPD) and Inter Simple Sequence Repeats (ISSR) analysis was carried out to check for possible genetic alterations in plants regenerated form seeds, protocorms, shoot tip and artificial seeds (2%, 3% and 4% alginate coated). Both markers revealed 94% of genetic stability among the regenerants whereas mother plant showed 90% genetically similar with that of regenerated plants and only 10% showed genetic variation between them. The average PIC value of RAPD and ISSR markers was found to be 0.29 and 0.35 respectively depicting the low polymorphism and the average R_p value was found to be 3.2 indicating the efficiency of primers. The *in vitro* regenerated plantlets (4-5cm height) obtained from artificial seeds were successfully acclimatized in different potting mixture. Among them the potting mixture containing cocopeat along with litter or clay and sphagnum moss in 3:2:1 ratio was more suitable for acclimatization of plantlet with 86.67% survival rate whereas the potting mixture containing cocopeat, litter and with or without sphagnum moss was found to be more appropriate for directly regeneration of plantlet from 3% and 4% alginate coated artificial seeds of *C. aloifolium* with 73.33% and 46.67% survival rate respectively. Hence, the present investigation is important to develop an efficient protocol for mass propagation and *ex situ* conservation of a threatened medicinal orchid, *Cymbidium aloifolium* through artificial seeds.

LIST OF ACRONYMS AND ABBREVIATION

°C	Degree centigrade
µM	Micromolar
2,4-D	2,4- Dichlorophenoxyacetic acid
asl	Above sea level
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
BA	Benzyl Adenine
BAP	6-Benzylaminopurine
BM	Basal media
CITES	Convention on of International Trade in Endangered Species of Wild Fauna and Flora
cm	Centimeter
CTAB	Hexadecyltrimethyl Ammonium Bromide
CymMV	Cymbidium mosaic virus
DAMD	Directed Amplification of Minisatellite DNA regions
DAS-ELISA	Double Antibody Sandwich Enzyme Linked Immuno Sorbent Assay
D. Don	David Don
df	Degrees of freedom
DPR	Department of Plant Resources
EDTA	Ethylene Diamino Tetra Acetate
ELISA	Enzyme Linked Immuno Sorbent Assay
ERAPD	Extended Random Primer Amplified Polymorphic DNA
et al.	and others
etc	etcetera
eg	as an example
FCM	Flow Cytometry
Fig.	Figure
g	gram
HCl	Hydrochloric acid
HPLC	High Pressure Liquid Chromatography
IAA	Indole-3-acetic acid

IBA	Indole-3-butyric acid
ISSR	Inter Simple Sequence Repeats
KN	Kinetin
KC	Knudson C
l	Liter
Lindl.	John Lindley
M	Mitra *et.al.* medium
mg	milligram
ml	milliliter
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NHPL	National Herbarium and Plant Laboratory
NTSYSpc	Numerical Taxonomy and Multivariate Analysis System
PCR	Polymerase Chain Reaction
PIC	Polymorphic Information Content
PLBs	Protocorm-like bodies
ppm	Parts per million
psi	Pound per square inch
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
Rp	Resolving power
RT	Room temperature
Sig.	Significance level
SIMQUAL	Similarity for Qualitative Data Program
SPAR	Single Primer Amplification Reaction
SPSS	Statistical Package for Social Sciences
SSR	Simple Sequence Repeats
T.U.	Tribhuvan University
TC	Teixeira Cymbidium
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
viz.	namely
VW	Vacin and Went

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CHAPTER 1

1. INTRODUCTION

1.1 Introduction

Nepal with its unique geographical position and climate offers excellent growing conditions for orchid's biodiversity. They are popularly known as "sungava", "chandigava", and "sunakhari" in Nepal. Orchids as one of the most fascinating creations of nature belong to the family Orchidaceae which is one of the largest families of the flowering plants in the world (Dressler, 1993; Rajbhandari, 2015; Chase *et al*., 2015). It has been estimated about 25,000 to 30,000 species of orchids worldwide (Raskoti, 2009; Pant *et al*., 2016) and cover one tenth of the world's total flowering plant and distributed from tropic to high alpine. They exhibit incredible range of diversity in size, shape and colour of their flower. Approximately 25% of their species are terrestrial, 70% are epiphytic and about 5% are found on various other supports (lithophytes, saprophytes) (Pant & Raskoti, 2013).

Orchids have their name from the Greek word 'Orchis' that is "testicle", from the appearance of subterranean tuberoids of the Genus Orchis. The word "Orchis" was first used by Theophrastus, Father of Botany (372/371–278/286 BC), in his book "*De Historia Plantarum*" (The natural study of plants) (Jalal *et al*., 2008). Orchids resemble with some grasses in the form of their leaves, i.e., Monocotyledons. Orchids are cosmopolitan in their distribution, thus occur in every habitat. Though, Orchids are considered to be examples of the most advanced floral evolution due to their interactions with pollinators and their symbiosis with mycorrhizae fungi.

On the basis of habit, Orchids can be classified as Sympodials ("Joint-footed") and monopodials ("one-footed") while on the basis of habitat they are epiphytes, terrestrials and lithophytes. In sympodial Orchids, the new shoots arise from any axiliary bud present in any part of older shoots and spread out from a long rhizome, eg. *Bulbophyllum, Cypripedium, Dendrobium* etc. while in monopodial Orchids, the shoots undergo indefinite apical growth but lack rhizome pseudobulb, eg. *Aerides, Rhynchostylis, Vanda* etc. (Chugh *et al.*, 2009).

Orchids are found in almost every type of habitats ranging in condition from tropical to alpine zone. However, maximum number of orchid species occurs in sub-tropical

region where the majorities are epiphytes in forest. Most of the temperate and alpine genera are terrestrial (Raskoti, 2009). Numerous tropical species are grown around the world by florists and horticulturists, professionals and amateurs for their showy flowers of unusual structures and variety of colours. Most of the epiphytic orchids are common in eastern and central Nepal and terrestrial orchids (Ground orchids) are found more in western Nepal as climatic conditions are more suitable in those regions. The most important factor which determines distribution of orchids on Nepal is the monsoon (Rajbhandari & Bhattarai, 2001).

The human relation with orchid started from the fact that it is a fascinating plant with its unique and beautiful flower and most of the orchids have ornamental value. They exhibit beautiful comical mimics in nature because of their various flower shapes like birds, insects, dogs, butterfly etc. Attractive and long-lasting flowers of orchids gather the interests on nature lovers, scientists and horticulturists. They are highly valued as potted plants, foliage plants and cut flowers. They have a good price in national and international markets. Therefore, they are the one of the most expensive ornamental plants and dominate the international cut flower trade these days. Besides ornamental values, many orchids are also used in herbal medicines, food and cultural purposes. They comprise high amount of alkaloids, glycerides and other useful phytochemicals (Gutierrez, 2010; Nongdam & Chongtham, 2011). Orchids are not only important for aesthetically, medicinally, culturally but are also regarded as ecological indicators (Joshi *et al*., 2009).

The most beautiful orchids of Nepal belonging to genera are *Aerides, Arundina, Bulbophyllum, Calanthe, Coelogyne, Cymbidium, Dendrobium, Epigenecum, Eria, Esmeralda, Phaius, Phalaenopsis, Pleione, Rhynochostylis, Thunia, Vanda* and *Vandopsis,* (Rajbhandari & Bhattarai, 2001). Species cultivated for their ornamental purpose include: *Aerides multiflora, Ascocentrum ampullaceum, Bulbophyllum leopardinum, Calanthe masuca, C. plantagina, Coelogyne cristata, C. fuscescens, Cymbidium elegans, C. irridioides, Dendrobium densiflorum, D. nobile* etc.

Pant and Raskoti (2013) have reported 89 species of orchids having medicinal value in Nepal. However, Rajbhandari (2014) reported more than 100 species of Nepalese orchids are medicinal. They belong to the genera, *Acampe, Aerides, Anoectochilus, Arundina, Brachycorythis, Calanthe, Cephalanthera, Coelogyne, Conchidium,*

Crepidium, Cymbidium, Cypripedium, Dactylorhiza, *Dendrobium, Dienia, Epipactis, Eulophia*, *Flickingeria*, *Galearis, Goodyera, Gymnadenia, Habenaria, Herminium, Liparis, Luisia, Malaxis, Neottianthe, Nervilia, Oberonia, Otochilus*, *Papilionanthe, Peristylus*, *Phaius*, *Pholidota*, *Pinalia*, *Platanthera*, *Pleione*, *Ponerorchis*, *Rhynchostylis*, *Satyrium*, *Spiranthes, Thunia*, *Vanda* and *Zeuxine.*

There are 18 endemic orchid species reported from Nepal. They are *Bhutanthera fimbriata, Bulbophyllum nepalense, Eria annapurnensis*, *Eria baniaii, Eria nepalensis, Eria pokharensis, Herminium hongdeyanii*, *Liparis langtangensis, Liparis olivacea, Malaxis dolpensis*, *Malaxis tamurensis, Neottia chandrae, Neottia nepalensis, Oberonia nepalensis,Oreorchi porphyranthes, Panisea panchaseensis, Pleione coronaria* and *Sunipia nepalensis* (Subedi, 2002; Rajbhandari, 2014).

Some edible orchids are *Dactylorhiza hatagirea, Platanthera clavigera* and *Satyrium nepalensis,* (Dahal & Shakya, 1989). The orchids of Nepal having high export potential are mainly *Anoectochilus roxburghii, Dactylorhiza hetagirea* and species of *Cymbidium*, *Dendrobium*, *Calanthes*, *Coelogyne*, *Satyrium* (Pant, 2014).

Due to their high global demand for ornamental and medicinal purposes, they have been highly exploited from their natural habitat. There is a serious threat to the conservation of orchids in Nepal. Orchids are regarded as one of the most vulnerable commodities of extinction due to trade and commerce. Wild orchids are the main sources for rare varieties and new cross breeds. So, all orchid species are protected for the purpose of International commerce under Convention on International Trade in Endangered species of Wild Fauna and Flora (CITES) as potentially threatened or endangered in their natural habitat, with most species listed under Appendix II. A number of species and genera are afforded protection under Appendix I (Pant *et al*., 2016).

Nepal is the home to about 450 species of orchids belonging to 107 genera, out of which 18 species are endemic (Rajbhandari, 2014, 2015). Nepal is the treasure for many high valued medicinal orchids. The inventory of the entire region is yet to be investigated. People in the region are using orchids as medicinal, ornamental and even for edible purposes. Loss of habitat, deforestation, destructive collection technique, over exploitation of orchids with medicinal and ornamental values has depleted the

orchid wealth of Nepal (Subedi *et al*., 2013). Many orchids are now at the verge of extinction so it's high time to develop effective strategies to conserve the precious gems of nature (Pant, 2014).

In nature orchids are propagated by the germination of seeds and by vegetative propagation. Unlike other angiosperms, orchid seeds are very unique and have different physiological requirements for their germination. They are extremely small, dust like, and produced in large numbers (1,300-4,000,000 per capsule). Because of small size of the seed, the order to which orchids are placed has been named as *Microspermae*. The embryo has a rounded or spherical form without cotyledons, radicle and endosperm. In nature, the majority of orchid flowers are not pollinated, their ovules are not fertilized and capsule is rarely formed. Since, seeds lack endosperm; they must enter symbiotic relationship with mycorrhizal fungi to germinate. It is believed that the fungal association could provide physiological stimulus required for initiation of orchid growth (Sharma & Tandon, 1990; Ovando *et al*., 2005). All orchid species depend upon mycorrhizal associations with various fungi, mostly genus *Rhizoctonia* (Class Basidiomycetes), for at least part of their life cycle. So, under natural condition, the seeds of orchid have less than 5% germination rate, because of particular fungal requirement (Rao, 1997; Sharma & Tandon, 2010). Orchids that grow in nature can be propagated by both conventional and non-conventional techniques. Conventional breeding methods (budding and cutting) are time consuming due to slow reproduction cycle, need of mycorhiza in seed germination and slow rate of seed maturation. Any harsh physical environment might destroy the whole population. Therefore, an effective *ex-situ* conservation strategies need to be designed in order to save the valuable orchid species from the verge of extinction. Orchid seeds can be germinated and used to produce large amount of plantlets with the aid of tissue culture technique. Asymbiotic germination of seed culture will help in the production of quality planting materials in larger scale.

1.1.1 Plant tissue culture

An important aspect of orchid conservation is the conservation of population and species in their natural habitat (*in situ* conservation) by habitat protection. However there is no substitution for conservation of threatened species in their natural habitat by natural propagation method. Though large population of orchid is still confined in

their natural habitat, several orchid species are cultivated for their economic uses. The rich diversity and population of orchids in the world is decreasing due to human activities and high population pressure. Hence the rapid depletion of orchid population can be overcome by the *ex situ* conservation only. Many orchid species that approach to near extinction immediately require propagation and conservation (Pradhan & Pant, 2009).

In addition, the traditional method of propagation is very slow process to propagate a large quantity of clone orchids. Therefore, *ex-situ* conservation of highly exploited species can be done by tissue culture technique. Use of exogenous growth regulators in artificial nutrient media at suitable concentrations stimulates zygotic embryo to initiate protocorms that develop into plantlets (Pradhan *et al*., 2013). Micropropagation is propagation of explants *in vitro* to produce large number of clones of desired plants are produced in an artificial nutrient medium under aseptic condition in short time irrespective of physiological and climatic barriers. Explants can be a very small piece of plants such as embryos, seeds, protocorm, stem, shoot tip, meristems, root tips, single cell, protoplasts and pollen grains. Through this technique, from a very small part of plant, numerous plantlets can be produced in short duration with labour intensive process (Roy *et al*., 2011). It is useful in mass propagation of specific clones, production of pathogen free plants, clonal propagation of parental stock for hybrid seed production, year-round nursery production and germplasm preservation. Orchids are the very first flowering plants of commercial value to be propagated *in vitro* both through seeds and tissue culture (Pant, 2006).

In this direction, Bernard (1909) for the very first time inoculated orchid seeds and fungi in the culture tubes and gave the concept of *in vitro* symbiotic germination. Later, the asymbiotic germination of the orchid seeds was introduced by Knudson (1922) in culture tubes, eliminating the requirement of fungus for the germination. After that, there was a vigorous research to improve the Knudson medium for better germination rate. Morel (1960) tried to develop virus free *Cymbidium* by meristem culture. He produced more than 4,000,000 plants of *Cymbidium* in one year from single shoot apex. This attempt was the first important breakthrough in the clonal mass propagation of orchids (Rao, 1997). Since then tissue culture technique has become the most commonly applied biotechnological tools worldwide for the commercial production and mass propagation of many orchid species. This is the most efficient alternative for the germplasm conservation and mass propagation of

depleting orchid population.

In Nepal, local people as well as private nurseries have begun domestication of orchids although on a very low scale. A few commercial breeders are cultivating and using cut flowers of imported orchid hybrids in Nepal. Micropropagation of some of the Nepalese orchids is being done by National Herbarium and Plant Laboratory (NHPL), Godawari under Department of Plant Resources of Government of Nepal (DPR), Thapathali and institutions like Central Department of Botany, Tribhuvan University (T.U.) and Kathmandu University. Some private organization like Nepal Biotech Nursery, Standard Nursery, Parijat Nursery etc. are involved in the cultivation of endogenous orchid species in Nepal. There are ample opportunities of national economic gains if endogenous species are carefully selected and propagated *in vitro* for the commercial trade (Pant, 2014).

Hence, plant tissue culture techniques could be an alternative biotechnological tool for efficient conservation and commercialization of a number of rare, threatened and endangered orchid species. Several workers have successfully performed asymbiotic seed germination *in vitro* leading to the seedling development of many orchids (Chang *et al*., 2005; Pant & Gurung, 2005; Pradhan & Pant, 2009; Hossain *et al*., 2009). However more efforts need to be undertaken to understand the peculiarities of seed germination, protocorm formation and seedling development *in vitro* in response to varied combination and concentration of hormones and other additional substrates present in the nutrient medium for mass scale propagation of the orchid species. Based on the development of *in vitro* micropropagation techniques and hybrid production, orchid industries have been set up in many Asian countries including Thailand, Singapore, Japan and India.

1.1.2 *Cymbidium aloifolium*

Sixteen species of *Cymbidium* have been reported from Nepal (Rajbhandari, 2015). *Cymbidium aloifolium* (L.) Sw. is one of the threatened medicinal orchids of Nepal (Pant, 2014). It is commonly called as Aloe-leafed *Cymbidium* and locally called as Kamaru and Banharchhul. It has various synonyms, i.e., *Cymbidium pendulum* (Roxb.) Sw., *Cymbidium simulans* Rolfe., *Epidendrum aloifolium* L. They are either epiphytes on tree trunks or lithophytes on streamside cliffs on open mossy rocks, hot

to warm growing, like full sun, occur in dry lowland tropical to subtropical forest at elevations of 300-1600 m in eastern and central Nepal (Rajbhandari & Dahal, 2004; Rajbhandari, 2015). The plant body of *C. aloifolium* is characterized by the presence of ovoid pseudobulb enveloped by leaf bases; leaves leathery, linear-ligulate, apex bifid; inflorescence basal, pendant; flowers light yellow with reddish purple chestnut stripes; sepals subequal, oblong- elliptic, blunt; petals elliptic, blunt; labellum subovate, 3-lobed, lateral lobes erect, blunt, mid lobe recurved; ovate-oblong, disc with 2-lamellae, both ends dilated and capsule oblong-elliptic (Raskoti, 2009). It blooms from April to June and flower persists for approximately 20 days. The flower is yellowish with central radial stripes. Pod of *C. aloifolium* is large and greenish remains hanging arising from a very small pseudobulb enveloped by leaf bases. It has been used by local people in gardens for ornamental purposes (Paudyal & Subedi, 2001). It occupies a significant position in day to day life of tribal people of many countries because of its ornamental and medicinal values.

1.1.3 Medicinal use

Cymbidium aloifolium is a high valued medicinal herb in the indigenous system of medicine. It contains biologically active phytochemicals such as dihydrophenanthrene, phenanthraquinone (Cymbinodin A), tannins, alkaloids, phenols, flavonoids, carbohydrates and coumarins which indicates that this orchid has a potential source of useful drugs (Barua *et al*., 1990, Hossain *et al*., 2009). The plant is reported to have emetic and purgative properties.

The leaves, roots, rhizomes and even whole plant of *C. aloifolium* are used for treatment of different diseases. Tuberous roots are the good source of Saleb and used as nutrient and demulcent. Half spoon of mixture of dried root powder, dried ginger and black pepper with a cup of cow milk help to cure paralysis and chronic illness. Pastes of leaves are extensively used for styptic properties in the treatment of boils and fever and also for the treatment of otilis and inflammatory conditions (Das *et al*., 2008).

Some tribal people also use the small seeds of *C. aloifolium* for healing wound (Medhi & Chakrabarti, 2009). Whole plant of this species can also be used as tonic and for treating ear-ache, chronic illness, and weakness of eyes, vertigo, burns and sores (Hossain, 2011; Nongdam & Chongtham, 2011). The ethanolic leaf extract of *C.*

aloifolium possess CNS (Central nervous system) depressant activity. The different parts of this plant like leaf, root, seed and capsule cover showed antibacterial activities (Shubha & Srinivas, 2016). In Nepal, local people use the paste of rhizome and roots for joining fractured and dislocated bones (Pant, 2013).

Besides medicinal value, this orchid has a good command in floricultural market too because of its beautiful flower. Now, the population of this orchid species is decreasing day by day. The orchid population once lost from their natural habitat is very difficult to re-establish in nature. There are several factors which cause declination of this species at an alarming rate: indiscriminate harvesting of orchids directly from wild habitat for ornamental and medicinal purposes, specific host requirement of orchids and habitat destruction. Over grazing, road construction, deforestation, absence of pollinators due to indiscriminate use of pesticides, unauthorized trade, ruthless collection by orchid lovers and climate change are another serious cause for the loss of orchids (Chugh *et al.,* 2009; Mohanraj *et al.,* 2009).

1.1.4 Artificial seed technology

Artificial seed technology involves the production of embryogenic or non-embryogenic regenerative explants enclosed in a protective capsule (coating) where the ultimate product behaves like a seed for many functions including germination and plant regeneration and establishment. Artificial seeds have also been referred to as synthetic seed or synseed. The concept of Synseed or Artificial seed was first propounded by Murashige (1977) which was later described by several other investigators (Lambardi *et al*., 2006; Reddy *et al.*, 2012; Asmah *et al*., 2013).

Artificial seed or synthetic seed is defined as an artificially encapsulated somatic embryo, protocorm, shoot, node or any other meristematic tissue, which can develop into plant under *in vitro* and *in vivo* conditions (Kikowska & Thiem, 2011). Previously, the artificial seeds were largely developed by encapsulation of somatic embryo. However, the rate of production of uniform high quality embryo is lower in most species and as a result the preparation of high quality, artificial seeds has been successful in only few crops like carrot and alfalfa (Datta *et al.*, 1999). Bapat *et al.* (1987) proposed the making of synseeds through the encapsulation of *in vitro* derived non-embryogenic propagules such as axillary meristems as in *Morus indica*. This

concept was successfully tested in a number of species subsequently (Singh, 1991; Corrie & Tandon, 1993; Piccioni & Standardi, 1995; Chen *et al*., 1996; Yoshida, 1996).

A wide number of encapsulating agents have been tested in time for their capacity to produce beads such as agar, sodium alginate, agarose, carrageenan, gelrite, guar gum, polyacrylamide, polyox, sodium pectate, polymeric materials such as potassium starch acrylamide, copolymer of potassium acrylate and acrylamide. Cellulose based materials such as carboxymethyl cellulose, ethylcellulose, nitrocellulose have also been used for encapsulation of explants (Redenbaugh *et al*., 1986, 1987; Datta *et al*., 1999, Saiprasad, 2001; Khor & Loh, 2005).

The next innovation in artificial matrix was the concept of coating with polyethylene oxide homopolymer (polyox) or acrylic copolymer (Kitto & Janick, 1985; Kim & Janick, 1989). The plant tissues were also coated with a film composed of polyvinylchloride or polyvinylacetate (Singh *et al*., 2006). Out of these, sodium alginate is most popular gelling agent (Sarmah *et al*., 2010).

Sodium alginate is a naturally occurring anionic and hydrophilic polysaccharide product derived primarily from the cell wall of brown seaweed that grows in cold water regions. The natural function of alginate is to give flexibility to the seaweed. Due to its outstanding properties in terms of flexibility, quick gellation, biocompatibility, biodegradability, non toxic, non-antigenicity and chelating ability, sodium alginate has been widely used to make gel in presence of calcium, to stabilize emulsions or foams and to form films in food industry, acts as a supporting matrix or delivery system for tissue repair and regeneration and in a variety of biomedical applications including tissue engineering, drug delivery and in some formulations preventing gastric reflux (Sun & Tan, 2013).

The use of sodium alginate solution was first proposed by Redenbaugh *et al*. (1988) which could be turned into a hardened calcium alginate gel by ion-exchange reaction. Somatic embryos of alfalfa and celery were the first to be coated with alginate and reached over 85% germination in alfalfa. Since then, the alginate became more popular for synthetic seed preparation, there are various advantages of this compound such as the excellent water solubility and moderate viscosity at room temperature, easy availability at low cost, the long term storability, the easy use of calcium salts for

quick gellation and bead hardening at room temperature, no toxicity, the possibility to mix with a nutritive medium and obtain an artificial endosperm (Kikowska & Thiem, 2011).

Plant tissues (explants) encapsulated within the alginate matrix is protected from physical or environmental injury and allows germination and conversion into seedling. The encapsulated explants are kept in the 0.2 Molar solution of $CaCl_2$ for about half an hour for hardening the gel. The alginate matrix also reduces dehydration and grasps the explants inside the gel during storage (Ara *et al*., 2000; Mohanty & Das, 2013). Artificial seeds can be stored for short to long time in appropriate condition (Lambardi *et al*., 2006). However there are some limitations of using alginate solution. The alginate coated seeds are very moist, having sticky surface due to which seeds adhere to each other and difficult to separate. Redenbaugh and co-workers (1987) were the first to understand the necessity of a hydrophobic layer at the surface of the synthetic seeds, avoiding bead adhesion and preventing water loss.

Artificial seed technology provides a viable approach for *in vitro* germplasm conservations as it combines the advantages of clonal multiplication with those of seed propagation and storage (Mohanty & Das, 2013). However, successful plant recovery from encapsulated vegetative micropropagules mostly depends on the plant species, nutrient composition and period of storage. Protocorm and protocorm-like bodies are found to be the most efficient explants for artificial seed development due to their superior regenerative character (Saiprasad & Polisetty, 2003). Asymbiotic germination of artificial seed through protocorms provides a useful way to re-establish plants in the wild for germplasm preservation as well as for commercial propagation.

Different researchers have highlighted many significances of the artificial seed technology. Encapsulated *in vitro* derived plant tissues, due to small bead size, could be easy for handling and transportation. Artificial seeds are also very useful for the exchange of sterile material between laboratories (Redenbaugh, 1993; Gray *et al*., 1995; Piccioni & Standardi, 1995). Nursery and floriculture industries would take great advantage of this technique if proliferation, rooting and conversion are well controlled (Mathur *et al*., 1989; Bapat, 1993; Gray *et al*., 1995; Piccioni, 1997). Another potential application could be in germplasm conservation, with the development of storage techniques (Accart *et al.*, 1994; Bouafia *et al*., 1996; Na and

Kondo, 1996). The artificial seed technology has also a potential for mild or short term storage without losing viability and long term storage in liquid nitrogen (cryopreservation). It also maintains the clonal nature of the regenerated plants. Moreover; this technique offers space and equipment saving option for storage of artificial seeds at low temperature. It also reduces the labour cost as there is no need for transfer of explants to the fresh medium (Kikowska & Thiem, 2011). Hence, it is cheaper than tissue culture for farmers and horticulturists (Lambardi *et al*., 2006). Therefore, an attempt has been made to produce the artificial seed by encapsulating the protocorms of *C. aloifolium*.

The development of an artificial means of propagation is needed to reduce collection pressures on wild population. Nowadays, encapsulation technique for producing artificial seeds has become an important asset in micropropagation. Tissue culture techniques have been widely used for the *in vitro* mass multiplication of several commercially important orchids. Nayak *et al*. (1997), Hossain *et al.* (2009), Nongdam and Chongtham (2011) and Pradhan *et al.* (2013) had already reported the *in vitro* multiplication of *C. aloifolum* by using different explants. However, little is known about the artificial seed system for *C. aloifolium*. Artificial seeds technology, with its advantage of storage has enormous potential as a mass propagation system for *C. aloifolium* producing field-ready propagules. Therefore, the present investigation is aimed to develop a reliable protocol for production, short to mid-term storage and efficient regeneration of plantlets from artificial seeds containing protocorms of *C. aloifolium*. This protocol could be useful to facilitate the promotion of *ex situ* conservation and propagation of *C. aloifolium* through artificial seed in order to supply planting materials and to reintroduce back into the nature.

1.1.5 Viral diseases and production decline of orchids

Plant viruses and virus-like diseases damage almost all plants causing huge economic loss, through reduction in quality and quantity of products. Orchids are the host to a large number of viruses. They are highly susceptible to a variety of biotic and abiotic agents that can hinder their development and diminish the quality of their leaves and flowers. Some 25 different orchid infecting viruses have been reported in various regions of the world (Zettler *et al*., 1990). Out of them, Odontoglossum ring spot virus (ORSV) and Cymbidium mosaic virus (CymMV) are considered to be the most

important one due to their prevalence, worldwide occurrence and the severe symptoms they can induce in several orchid genera, often reducing their vigor, lowering flower quality and leading to economic losses (Zettler *et al.*, 1990; Wong *et al.*, 1994; Khentry *et al.*, 2006; Liu *et al.*, 2013).

CymMV virus was first described by Jensen in 1951 who found black necrotic spotting on *Cymbidium spp.* and named as Cymbidium Black Streak virus. CymMV virus is responsible for flower colour breaking and necrosis. It causes a mosaic of irregularly shaped chlorotic or necrotic lesions to appear on infected plants with sunken patches on leaves (Hu *et al.*, 1993). Besides *Cymbidium*, CymMV infects a number of other orchids and non-orchid plants. Certain infected plants may be symptomless but are still a source of infection for other neighbouring plants. A rod-shaped Odontoglossum ringspot virus (ORSV) was observed in *Odontoglossum grande* (Jensen & Gold, 1951). These viruses were later detected in *Cymbidium* where it was called Cymbidium diamond mottle and in Cattleya, where it causes flower variegation and ring spots on leaves (Jensen, 1970). Both viruses are highly infectious, stable, and are usually present in orchid juice in high concentrations. These viruses mostly spread through contaminated tools, pots and manual contact. Destruction of infected plant is the only way to prevent the spread of disease. These viruses are reported in a wide variety of orchid genera like *Cymbidium, Dendrobium, Oncidium, Phalaenopsis, Vanilla* etc. (Zhang *et al.*, 2005; Khentry *et al.*, 2006; Zeng *et al.*, 2009; Liu *et al.*, 2013; Chien *et al.*, 2015).

In Nepal, studies on orchids are mainly focused on their documentation, ornamental and medicinal uses (Shrestha, 2000; Vaidya *et al.*, 2002) but the quantitative study with regards to developing virus free plantlets is still lacking. It is possible to develop virus free orchid plantlets through *in vitro* meristem culture (Faccioli, 2001). It is, however essential to ensure that the supplied *in vitro* regenerated orchids are virus free. The detection of virus in plant is a critical requirement for the successful commercial production of orchids. Many certification authorities prefer visual detection of pathogen on the orchid plants which is however not always conclusive. In this context, Double Antibody Sandwich Enzyme Linked Immuno Sorbent Assay (DAS-ELISA) is used widely for virus detection (Clark & Adams, 1977). The advantage of this assay is that the virus particles are concentrated from the extracts by

the coating antibody.

1.1.5.1 ELISA test

The Enzyme Linked Immunosorbent Assay (ELISA), frequently used for virus detection is based upon the specific recognition of antigen by antibodies. ELISA can be applied to viruses of different morphological types both in purified preparations and crude extract (Mathew & Muniyappa, 1992). Primarily two types of ELISA technique exist: Direct and Indirect.

Direct ELISA or Double Antibody Sandwich ELISA (DAS-ELISA) technique uses antiviral antibodies to trap the virus from sample and also to detect virus. DAS ELISA is the most popular technique for the detection of plant viruses. The technique uses antigen, antibody, conjugated antibodies and substrate for the test reaction. The enzyme linked to the antibody reacts with specific substrate and forms enzyme substrate complex which gives a colour reaction. The colour is detected either by the naked eye for qualitative test or is quantified by a spectrophotometer. However, Indirect ELISA technique uses antiviral antibodies obtained from different animals as trapping (e.g., from mouse) and detecting antibodies (e.g., from rabbit).

Cymbidium Mosaic Virus (CymMV) is one of the most prevalent viruses that infects the cultivated orchids and are responsible for huge loss in its production (Khentry *et al*., 2006). Hence, efficient conservation strategies need to be designed not only to save the valuable species from the risk of extinction but also harnesses its wide range of economic potential. *In vitro* culture techniques such as shoot tip culture, meristem culture, micro-grafting, cryo-therapy etc. have been extensively used for elimination of pathogens from infected stocks to produce disease-free plants (Idowu *et al.*, 2009). Meristem culture technique provides a new dimension to more efficient conservation and commercialization of a number of rare and useful virus free orchid species (Vij & Aggarwal, 2003). The present investigation thus focussed on the detection of orchid virus on *in vitro* regenerated plantlets of orchids by using shoot tip and compare with mother plant before undergoing mass propagation.

1.1.6 Molecular techniques for genetic fidelity test

The significant advantage of *in vitro* propagation technique includes the production of

genetically identical materials. The increasing utilization of artificial seeds for clonal propagation necessitates the assessment of genetic stability of conserved seeds and recovered plants (Saiprasad, 2001; Srivastava *et al*., 2009). Traditionally, the identification of medicinal orchids has been based on the evaluation of phenotypic characteristics such as morphology, smell, taste, colour, texture, and size. These characteristics however have certain limitations (Beyene *et al*., 2005), including: insufficient variation among the samples; subjectivity in the analysis; plasticity of the character, which could be due to the influence of the environment and management practice and ability to be scored only at certain stages of the plant development (e.g., during flowering and/or fruiting) which may not coincide with the commercially important stage or organ (e.g., roots in ginseng production). More recently, traditional means of plant identification have been complemented by advanced analytical methods of phytochemistry such as High Pressure Liquid Chromatography (HPLC) and mass spectrometry (MS), as well as by molecular tools. Among these novel techniques, molecular markers based on DNA sequence variation have become increasingly important for the identification and authentication of medicinal plants and for the estimation of genetic diversity (Mondini *et al*., 2009).

In vitro propagation methods, such as somatic embryogenesis are very interesting approaches when compared with traditional propagation which presents serious drawbacks. Somatic embryogenesis is frequently regarded as the best system for propagation of superior genotypes (Kim, 2000) mostly because both root and shoot meristems are present simultaneously in somatic embryos. Most of the successful studies regarding somatic embryogenesis used juvenile materials (Gallego *et al*., 1997, Pinto *et al*., 2002).

Plantlets derived from *in vitro* culture might exhibit somaclonal variation (Larkin & Scowcroft, 1981) which is often heritable (Breiman *et al.*, 1987). Some reports claim that morphological, cytological and molecular variation may be generated *in vitro* (Larkin *et al.* 1989) due to the genotypes (Breiman *et al.*, 1987) and due to the protocols used during culture and plant regeneration *in vitro*. The genetic variation may be caused by changes in chromosomes structure and number or by changes in DNA structure. The species are more valuable for plant breeders as their variation has greatest potential for the plant improvement with the selection of desirable character

at cellular level (Nwauzoma & Jaja, 2013).

Somaclonal variant tissue produces phenotypic as well as genotypic variation. Somaclonal variation depend on the genotype of the material, duration of culture, types of regeneration, stress of the plant growth regulators, heat, osmotic stress and nutrient media concentration (Ngezahayo *et al,*. 2007; Sun *et al.,* 2013) and shows the morphological variation with the parent plant (control) in length of leaf, sheath, shoot tips, internode, stolon, width of leaf, thickness of node and number of inflorescence. In *Triticum durum*, deformed leaf, albino seedling, chimerical plantlet with leaves produced through the somaclonal variation (Bouiamrine, 2012). Similarly, *in vitro* grown tulip showed variation in the flower and leaf (Podwyszynska, 2005). *Populous deltoids* grown *in vitro* shows partial resistance to leaf rust diseases which are more beneficial parameters for the cultivation (Prakash & Thielges, 1989; Li *et al.,* 2010). The production of glabrous rice is also due to the effect of somaclonal variation (Yamamoto *et al.,* 1994). Somaclonal variation was also detected in *Allium sativum* (Saker & Sawahel, 1998), *Citrus limon* (Orbovic *et al.,* 2008), *Phalaenopsis bellina* (Khoddamzadeh *et al*., 2010) and *Oryza sativa* (Salehian *et al.,* 2013) by using different molecular markers.

Cytological studies can also help to detect the somaclonal variation by chromosomal studies, growth rates of plants and cell division (Raha & Roy, 2003; Ekanem & Osuji, 2006). Some genetic changes like polyploidy, aneuploidy, chromosomal breakdown, deletion, translocation, gene amplification and mutation are some causes of somaclonal variation observed in barley and oat plants (Jain, 2001; Satyanaryan, 2011).

For measuring genetic uniformity of regenerated plants, DNA marker based technology such as flow cytometry, restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphism (AFLPs), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs) and other molecular analysis have been used successfully. Flow cytometry (FCM) has increasingly been chosen for analysis of major ploidy changes in genetic stability assays. It thereby replaces other methods such as chromosome counting by flow cytometry provides unsurpassed rapidity, ease, convenience and accuracy. Both RFLPs and AFLPs are highly reproducible techniques but more costly and time consuming than SSRs, while

RAPDs show a lack of reproducibility either within or between laboratories (Jones *et al.*, 1997). In addition, microsatellites have high levels of polymorphism (Glaubitz & Moran, 2000) being extremely useful for fine scale genetic analysis. Molecular markers based on DNA sequence polymorphism, are independent of environmental conditions. They show higher levels of polymorphism and have proven to be effective tools for distinguishing closely related genotypes (Beyene *et al,*. 2005). There are several reports concerning the use of these molecular markers in micro propagated plants for genetic analysis such as *Gossypium hirsutum* (Jin *et al.*, 2008), *Bambusa balcooa* (Negi & Saxena, 2010), *Musa spp*. (Lu *et al.*, 2011) and *Oryza sativa* (Shan *et al.*, 2012).

RAPD technique is simple, easy, cost effective, does not require sequence information and a small amount of DNA sample is sufficient to yield quick result (Micheli *et al.*, 1994; Pathak & Jaroli, 2012). The marker is highly used for assessing genetic diversity of plant varieties but their producibility is affected by several factors (Kate-ngam & Lakote, 2008). RAPD technique is based on PCR reaction, require single short arbitrary oligonucleotides primer which anneals with multiple locations on genomic DNA and amplified randomly (Wiliams *et al*., 1990; Kumar & Gurusubramanian, 2011). It is most sensitive method for the identification of medicinal plant, and quality of herbal drugs (Khan *et al*., 2010). RAPD is one of the effective tools for the molecular studies which easily detect the variation within population, relationship, contrasting level of genetic diversity among population and also polymorphism in many plant species (Tivang *et al*., 1996; Xue *et al*., 2010). ISSR primers are designed from simple sequence repeats (SSR). SSR are highly scattered throughout the eukaryotic genomes. They use a single 16-20 bp long primer composed of a repeated sequence anchored at the 3' or 5' end of 2-4 arbitrary nucleotides (Zietkiewicz *et al.*, 1994; Javan *et al.*, 2012). ISSR markers have been used to generate gene-specific or trait-specific markers and for investigating variability at intraspecific level, genetic diversity studies and cultivar identification (Khattab *et al.*, 2014). They also requires small amount of DNA and doesn't require information on DNA sequence. ISSR markers have higher reproducibility than RAPD.

Hence in the present investigation, both RAPD and ISSR molecular markers are being

used to discriminate and detect somaclonal variations between *in vivo* and *in vitro* regenerated plantlets of *Cymbidium aloifolium.*

1.2 Rational

Orchids are the hidden heritage of nature. They are under considerable threat from continued habitat destruction for deforestation, road construction and agriculture practices, selective logging and timbering, illegal collection and trade and absence of pollinators due to indiscriminate use of pesticides or other modifications of the biome (Chugh *et al.,* 2009). Another reason for its decline is the slow germination rate in nature as it requires specific mycorrhizal fungi for successful seed germination (Mohanraj *et al.,* 2009). Weak implementation of conservation strategies is further adding the threatened number of this species. Though the collection of the orchid has been banned under the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES), implementation of regulations is poor and harvesting from wild sources still continues for commercial trade. Annually many orchid plants get exported to international market. If the proper sustainable utilization of the medicinal and ornamental orchids of Nepal is done, it may be helpful in uplifting the economics of the country.

As orchids are being used for various purposes, they have to be conserved for sustainable use. There are various methods of conservation of orchids. Among them, micro propagation is one of the important methods. In context of Nepal, cultivation of medicinal orchids is the effective way of income generation in those places where the utilization of agricultural land is limited**.** Thus to achieve the ultimate goal by sustainable use of medicinal orchids certain alternative ways for harvesting, consumption and protection are quite desirable. In this regard, plant tissue culture is one of the most suitable alternative tools to minimize the pressure on natural plant population. To maintain the same clones as mother stock and production of superior clone, *in vitro* technology is highly applicable. This technique is extensively employed in the production, conservation and improvement of plant research (Bairu & Kane, 2011).

Artificial seed technology is one of the most important applications of plant tissue culture which provides itself an important tool in orchid conservation. This technology is a good substitute to traditional seeds and micropropagation systems as

the establishment of germplasm repositories of micropropagated plants is costly and requires large space (Ara *et al.*, 2000). The exchange of stock cultures between laboratories is also problematic due to temperature fluctuations and chances of contamination. In this respect, artificial seed provide an alternative dependable way for mass scale production, short to mid term conservation, germplasm exchange of desirable genotypes and also for meeting the international quarantine requirements.

So, the present investigation is carried out for *ex-situ* conservation of orchid species *Cymbidium aloifolium* through artificial seed technology. There are ample opportunities of income generation if endogenous species are carefully selected and propagated *in vitro* for the commercial purpose (Pant, 2014). Thus it is possible to regenerate the threatened and commercially important orchid *C. aloifolium* through artificial seeds. The demand can be fulfilled only by the large scale propagation. Somaclonal variation may occur in plant species propagated through *in vitro*. Hence, the genetic stability is also important for the conservation of the threatened and rare orchid. The molecular technique such as RAPD and ISSR has been proved to be the valuable tools to check the genetic alterations between *in vivo* and *in vitro* regenerants of *C. aloifolium.*

1.3 Hypothesis

All the plant cells are totipotent in nature which can give identical mass of regenerants when provided with suitable environmental condition. Orchid seeds are small, dust like, non endospermic and rarely germinate in nature (only 2-3% germination rate) as they require appropriate mycorrhizal fungi for their seed germination. Development of asymbiotic germination methods through *in vitro* propagation techniques can overcome this problem. Hence, artificial seed technology is meant to be an alternative propagation technique for *ex situ* conservation of threatened plants. The genetic stability of artificial seed regenerated plants is also crucial for establishing clonal multiplication. Moreover, this technology is useful for mass production of commercially important species.

Therefore the hypothesis of present investigation is,

- Plantlets of *Cymbidium aloifolium* regenerated under *in vitro* conditions through artificial seeds have the ability for mass production and are genetically similar with their mother plant.

1.4 Objectives

Cymbidium aloifolium (L.) Sw. being highly medicinal orchids, its propagation and domestication is an urgent need. Thus, the primary objective of present research work is propagation of large quantity of good quality planting materials of *C. aloifolium* from elite mother plants within a short period of time and space through artificial seed technology thus supporting their *ex situ* conservation.

1.4.1 Specific objectives

1. To establish tissue culture system for mass propagation of *C. aloifolium* using different explants and artificial seeds.
2. To regenerate the virus free plantlets from different explants (artificial seeds, protocorms, shoot tips).
3. To analyze the genetic homogeneity among the *in vitro* regenerated and *in vivo* (mother plant) plantlets.

CHAPTER 2

2. LITERATURE REVIEW

A lot of works have been done regarding the *in vitro* culture of orchids. The important literatures are reviewed here:

2.1 Plant tissue culture

In vitro plant tissue culture technique is one of the most prominent biotechnological tools conducted in the well sophisticated lab under the aseptic condition. *In vitro* technology is highly applicable to maintain the same clones as mother stock and production of superior clone. The tissue culture techniques have high economic value and are widely used for the improvement of field crops, forest, horticulture and plantation crops for increased agricultural and forestry production (Arditti, 1977).

During the last five decades, tissue culture techniques have been extensively exploited not only for the rapid and large scale propagation of orchids but also for their *ex situ* conservation (Chugh *et al*., 2009). The procedure of symbiotic (i.e., dixenic) seed germination of orchids was first developed by Bernard (1909). He discovered the requirement of fungus infection for germination of orchid seeds. The fungus was found to be *Rhizoctonia* sp. which occurs in the roots of orchid plants. Later on, Burgeff (1909) carried on that work and demonstrated the association of fungal mycelium with orchid root structure and their role in orchid seed germination (Arditti, 1980). The concept of *in vitro* asymbiotic seed germination in Laelia-Cattleya by Knudson (1922) benchmarked the onset of orchid tissue culture. Since then protocols for rapid propagation of a number of orchid species through *in vitro* culture of various explants such as mature and immature seeds, protocorms, shoot tips, flower stalk nodes, buds, root tips and rhizome segments have been reported.

Murashige (1974) reported some 22 genera of orchids being propagated through tissue culture using shoot tip, root tip, young inflorescence, embryo and young leaf tip as explants. Morel (1960) produced a large clone of virus free plantlet from *Cymbidium* orchid through the meristem or shoot tip culture. He suggested the use of shoot meristem culture for the healthy plantlet production. This technique was used for several genera and found almost immediate practical application at the beginning

of 1970. Later, Wimber (1963) improved the method developed by Morel (1960) into a more efficient system suitable for commercial propagation of *Cymbidium* which allows the production of hundreds of seedlings from few shoot segments. Since then, the protocol has been extended to a number of other *Cymbidium* species as well as hybrids (Nayak *et al.*, 2006). The development of Murashige and Skoog (1962) or Linsmaier and Skoog (1965) medium were another breakthrough in plant research especially in procedures where the plant regeneration is the major objective. About 22 genera of orchids were propagated on this medium through tissue culture using shoot tip, root tip, young inflorescence and leaf as explants (Murashige & Skoog, 1962). Thus, this medium is widely used for propagation of other different orchids as they contain high salt compositions (Gamborg & Philips, 1995). Raghavan and Torrey (1964) also grew Cattleya seeds *in vitro* medium containing NH_4NO_3 as the sole source of nitrogen. The medium was best for germination of seeds and development to small plantlets. Arditti (1977) reviewed some 40 genera of orchid being propagated through shoot tip culture method. A significant number of identical clones can be raised from a single leaf through direct or callus mediated organogenesis.

In vitro seed germination and seedling development of *Cymbidium* are reported to be influenced by various factors (Prasad & Mitra, 1975; Bose & Mukherjee, 1976). The induction of high frequency shoot proliferation from shoot segments of *C. aloifolium* on MS medium supplemented with 2.2 μM thiadiazuron was reported by Nayak *et al.* (1997). They observed an average of 18 shoots regenerated from a single shoot within 8 weeks of culture. Similarly, Muralidhar and Mehta (1986) studied on the three basal media KC (Knudson, 1946), VW (Vacin & Went, 1949) and RT (Raghavn & Torrrey, 1964) with or without various concentration and combination of vitamins, hormones, aminoacids and micronutrient for the seed germination of *Cymbidium longifolium*. They found that the basal medium KC assessed the seed germination upto 30%, VW 65% and RT assessed upto 35% germination. Ueda and Torikata (1968) prepared protocorms by meristems culture of *Cymbidium insigne* and *C. pumilum* and finally developed plants on BM (Knudson C with Nitsch microelements) medium within 2 months. NAA, Bacto-trypton, α-arginine and L-aspartic acid also promoted their growth and development while yeast extract was inhibitory.

The combined effect of BAP and NAA on the formation of PLBs and the

development of shoot and root meristem culture of *Cymbidium* species was studied by Matsui *et al.* (1970). They reported that NAA alone had no effect upon the formation of PLB's, BAP (0.1 ppm) induced the greatest effect and 10 ppm of NAA and BAP had marked effect on shoot formation. Fonnesbech (1972) studied the effect of auxins (IAA, NAA and 2,4-D), cytokinin (Kinetin and BA) and gibberellins (GA) alone or in combination on the protocorm of *Cymbidium* species. He reported that IAA alone had no effect and NAA inhibited chlorophyll synthesis at high concentration. BA and Kinetin when used singly induced shoot formation in solid medium and callusing in liquid medium while GA induced shoots and leaf growth. NAA and Kinetin together resulted in maximum fresh weight. Besides the nutrients and vitamins available in the growth medium, the plant growth regulators like 6-Benzyl Amino Purine (BAP), α-Naphthalene Acetic Acid (NAA), Indole-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), N6-Benzyladenine (BA) and thiadiazuron along with organic additives including casein hydrolysate, yeast extract and coconut milk were documented to support and stimulate induction of germination to complete seedling development (Nayak *et al.,* 1998).

Bopaiah and Jorapur (1986) sowed seeds of *Cymbidium aloifolium* Sw. on modified Knudson C medium (BM) supplemented with 100ml/l of coconut milk (CM) and 3 mg/l each of peptone (P) and Casein hydrolysate (CH). The PLBs obtained from 8 week old cultures, were subcultured on fresh medium containing all above nutrients and 200 g/l of banana pulp. The additional presence of either of the growth regulators like auxins (NAA, 2,4-D), cytokinin (Kn) and aminoacid (glycine), vitamins (thiamine HCl, niacin, pyridoxine) in different concentration in the nutrient medium comprising BM + CM + P + CH + banana pulp along with 1 mg/l each of thiamine HCl, niacin, glycine and kinetin was the most suitable for the normal and healthy seedling growth.

Shrestha and Rajbhandary (1988) regenerated plant through meristem of *Cymbidium giganteum* Wall ex. Lindl. The shoots were initiated on MS media, supplemented with BAP (5 mg/l), NAA (1 mg/l) and 10% coconut milk. The proliferation continued and plantlets survived in green house. Shrestha and Rajbhandary (1993) regenerated plant through meristem culture of *Cymbidium grandiflorum.* It was carried out on MS medium supplemented with BAP (2.2 mg/l), NAA (1.8 mg/l) and 10% coconut milk.

The protocorms developed gave shoots on subculture in the same medium but roots were developed when cultured in the basal medium containing only coconut milk.

Yasugi *et al.* (1994) observed the root segment culture in *Cymbidium.* They proved Kenny 'wine colour' as a useful method to induce PLB and plantlets from basal segments of root. Medium containing NAA (1 mg/l) and BA (1 mg/l) was most effective to induce PLB formation in *Cymbidium.* Devi *et al.* (1997) observed clonal propagation of *Cymbidium aloifolium* through shoot tip culture. The shoot tips were cultured in 5 different media viz. MS (1962), WI (Wimber, 1963), KC (Knudson, 1946), VW (Vacin & Went, 1949) and NI (Nitsch & Nitsch, 1969). Nitsch and Nitsch medium were found to be the best for formation and proliferation of PLBs. The duration for leaf and root differentiation from PLBs varied from 7-13 weeks in different media. Banerjee and Mandal (1999) germinated immature seeds of *Cymbidium* species obtained from green capsules on defined orchid culture medium supplemented with folic acid, NAA and organic adjuvants (Casein hydrolysate, peptone, coconut water and tryptophan) at varying rates. 2-5 mg/l of folic acid in VW medium induced maximum 85% germination while NAA (0.1 mg/l) induced 78% of seed germination. Vacin and Went (VW) medium supplemented with NAA (2 mg/l) were most appropriate for inducing roots.

Similarly, Nagaraju and Upadhyaya (2001) studied the *in vitro* morphogenetic response of *Cymbidium lunavian* Atlas. PLBs were cultured on three different basal media viz. KC, MS and Nitsch media. Among the media, Nitsch medium was found to be the best for growth of plantlets and supplementation of 0.3% activated charcoal brought about a marked effect on growth of shoots and roots. Karanjit (2002) cultured the seeds of *Cymbidium iridioides* D.Don. on MS medium and Gamborg B_5 (G-B_5) medium. The germination rate was noted vigorous on MS medium. However, the growth and development of seedlings of *C. iridioides* was favoured on MS medium supplemented with BAP (1 ppm) and NAA (1 ppm) as reported by Pant and Swar (2011). Das *et al.* (2007) reported that maximum number of protocorm (92.8%) was observed on B_5 medium. MS medium supplemented with BAP (1 mg/l) and NAA (1 mg/l) was the most favourable for multiple shoot induction from protocorm of *C. devonianum*. Hossain *et al.* (2009) found that Mitra *et al.* (M, 1976) medium supplemented with activated charcoal (2g/l) showed 100% seed germination and was

effective for induction of large protocorms of *C. aloifolium*. Half strength Phytamax (PM) and M media fortified with IAA (0.5 mg/l) induced strong and stout root system in *in vitro* plantlets. Nongdam and Chongtham (2011) reported that MS medium supplemented with BAP (1 mg/l) and activated charcoal was most effective condition for rapid propagation of *C. aloifolium*. The asymbiotic seed germination of *C. aloifolium* from immature embryos was reported by Deb and Pongener (2012). They observed the optimum regeneration on MS medium where as many as 12 shoot buds were developed per explants after 4 weeks of culture.

The inherent regenerative ability of roots in some naturally growing orchid species has been realized for the purpose of *in vitro* propagation. M medium enriched with NAA (0.5 mg/l) proved to be beneficial for root initiation. Shoot buds and PLBs were successfully regenerated from aerial root culture of two species *viz*. *Cymbidium aloifolum* and *C. iridioides*. MS medium supplemented with sucrose (3%) and BA (3 μM) was effective for development of multiple shoots in *C. aloifolium* (Deb & Pongener, 2012) while MS medium supplemented with sucrose (3%), BA (6 μM) and NAA (3 μM) was found to be the best for proliferation of shoots in *C. iridioides* (Pongener & Deb, 2010).

Plant regeneration from rhizomes has been reported in a number of *Cymbidium* species. A highly efficient methods for the induction of whole plantlets of *C. kanran* from both *in vitro* seed derived rhizomes and rhizomes induced directly from the axillary buds of pseudobulbs has been reported (Shimasaki & Uemoto, 1990). In *C. forrestii*, auxins stimulated rhizome growth by increasing branching and fresh weight but suppressed normal shoot formation (Paek & Yeung, 1991). However in BAP containing medium the rhizome growth and branching was reduced and some rhizome tips gradually turned up and developed into shoots. The highest frequency of shoot regeneration (91.5%) and the maximum number of shoot buds formation (3.5 shoots/rhizome) from rhizome segment of *C. aloifolium* were recorded on MS medium supplemented with BA (1 mg/l). Moreover, MS medium supplemented with NAA (1 mg/l) induced rooting in regenerated shoots (Nayak *et al.* 1998). Thin cross sections (TCSs) or TCLs of actively growing parts such as shoots, leaves, inflorescence stalks and developing PLBs have been successfully used by some workers for plantlet regeneration in a few orchids (Lakshmanan *et al.*, 1995; Teng *et*

al., 1997; Nayak *et al*., 2002; Da Silva *et al.*, 2005). According to Rout *et al.* (2006), the efficiency of thin cell layer culture is comparatively very high than the conventional technique of tissue culture. Nayak *et al.* (2002) fully exploited this culture system for propagation of *C. aloifolium*. An average number of 28.2 protocorm like bodies (PLBs) per section were developed within 8 weeks of culture on MS medium supplemented with either Zeatin riboside or BA.

Plant regeneration from flower buds was also reported by Shimasaski and Uemoto (1991) in *C. goeringii*. Apical flower buds cultured on MS medium supplemented with BA or NAA induced rhizomes. Medium supplemented with BA (0.1 mg/l) and NAA (10 mg/l) was found to be optimal concentration for initiating rhizomes and subsequent plant regeneration. Vij *et al.* (1994) reported that medium supplemented with BA found to be promotive for shoot multiplication from PLBs of nodal explants of *C. pendulum*.

Somatic embryogenesis also offers great potential for *Cymbidium* multiplication as well as genetic transformation since a single isolated cell can be induced to produce an embryo and subsequently a plant. Various researchers reported successful induction of somatic embryos from PLBs sections and regeneration of a number of normal plants of different species as well as hybrids (Begum *et al.*, 1994; Chang & Chang, 1998; Huan & Tanaka, 2004).

Besides *Cymbidium*, *in vitro* propagation of different orchids through tissue culture techniques have been reported by many researchers. Mitra (1971) reported the seeds germination and development of orchid *Arundina bambusifolia* Lindl. in Raghavan and Torrey (RT) medium supplemented with urea, peptone, casein hydrolysate without vitamin and casein hydrolysate with vitamin, yeast extract, ribonucleic acid and coconut water. Similarly, Reddy *et al*. (1992) studied *in vitro* seed germination and seedling development in four species of south Indian orchids and reported significant effect between the media and orchids. MS and RT media yielded best result on seedling development. They found that ground orchid *Spathoglottis plicata* gave best response in MS medium while epiphytic *Epidendrum radicans, Dendrobium crepidatum* and *Cymbidium aloifolium* gave better result on RT medium.

Likely, hormonal effect on *in vitro* germination of *Acampe longifolia* was studied on MS, KnC, VW, and B_5 (Gamborg) media supplemented with different concentrations

of NAA, IBA, and KN. Best germination was found on MS medium containing NAA (0.1 mg/l) and Kn (1 mg/l) while low germination was found on B_5 medium (Kalita & Sharma, 2001). Kosir *et al.* (2004) reported that MS medium supplemented with BAP (2 mg/l) and NAA (0.5 mg/l) was the most appropriate medium for the production of large number of vegetative shoots without callus of *Phalaenopsis* orchid. Sheela *et al.* (2006) also used cytokinin, BA (1 mg/l) and auxin, NAA (1 mg/l) on half strength MS for the production of PLBs of mutant *Dendrobium* species. Kongbangkerd and Wongsa (2007) cultured 1.5 cm-2 cm young shoot of *Dendrobium* hybrid on semisolid Vacin and Went medium supplemented with various types of cytokinin BA, TDZ (Thidiazuron), zip, and zeatin at different concentration for production of number of shoots. Highest shoot regeneration was observed on 5 mg/l zeatin and root in 2.5 mg/l zeatin.

Sunitibala and Kishor (2009) found the best germination of immature embryo of *Dendrobium transparens* on half strength of MS medium supplemented with NAA (1 mg/l) and BAP (2 mg/l) while full strength of MS medium supplemented with NAA (1 mg/l) and BAP (2 mg/l) was found to be best for shoot multiplication. Rahman *et al.* (2009) achieved maximum number of shoots on MS medium supplemented with NAA (0.5 mg/l) and BAP (1 mg/l) and induction of maximum number of roots on MS medium supplemented with NAA (1.5 mg/l) and BAP (1 mg/l) on *Vanda tessellata.* Pant and Pradhan (2010) reported the best medium for the seed germination of *Cymbidium elegans* was MS basal medium supplemented with BAP (1 mg/l) and also found the maximum number of shoot development on MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l). Similarly, maximum number of roots was reported on MS medium supplemented with IBA (0.5 mg/l). Niknejad *et al.* (2011) used leaf section of *Phalaenopsis gigantea* and cultured on Dogashima medium (NDM) supplemented with cytokinins (BAP, TDZ (Thidiazuron) and Kinetin) along with NAA. He found that TDZ with combination of auxin was best for induction of callus and PLBs.

Similarly, Pant and Shrestha (2011) also obtained maximum number of shoot on MS medium supplemented with BAP (1 mg/l) by using shoot tips of *Phaius tankervilleae.* Maximum induction of root was found on MS medium supplemented with 0.5 mg/l NAA. Pant and Thapa (2012) used shoot tips explants of *Dendrobium primulinum* for

the regeneration of plantlets. They found that MS medium supplemented with BAP (1.5 mg/l) and NAA (0.5 mg/l) was the most effective for the maximum multiplication of shoots and MS medium supplemented with IAA (0.5 mg/l) for inducing roots. Pradhan *et al.* (2013) cultured shoot tips of *Dendrobium densiflorum* on MS medium supplemented with different combination of BAP and NAA for plantlet regeneration. MS medium supplemented with BAP (2 mg/l) and NAA (0.5 mg/l) was the best condition for multiplication of shoots and maximum number of roots was induced on MS medium supplemented with IBA (1.5 mg/l).

For the mass production of *Dendrobium officinale*, Chen *et al*. (2014) used half strength of MS medium supplemented with BA (2 mg/l), NAA (0.1 mg/l) and potato extract (100 g/l) and increased the number of roots on half strength of MS medium supplemented with BA (0.2 mg/l) and NAA (1 mg/l). Julkiflee *et al*. (2014) studied effect of phytohormones for the production of protocorm like bodies of *Dendrobium sonia*-28, half strength MS medium produced highest PLBs compared to full and double strength MS medium in semisolid culture while the combination of BAP (4.411 µM or 8.88 µM) and NAA (8.88 µM) increased PLBs growth rate to 14%.

2.2 Artificial seed culture

Artificial seed technology is one of the most important applications of plant tissue culture which offers useful advantages on a commercial scale for mass propagation of elite species with high economic value and rare or endangered species (Ara *et al.,* 2000). The idea of artificial seeds was first proposed by Murashige (1978) and defined the artificial seed or synthetic seed as "an encapsulated single somatic embryo i.e., a clonal product that could be handled and used as a real seed for transport, storage and sowing and that therefore would eventually grow either *in vivo* or *ex vitro,* into a plantlet". This definition limited synseed manufacture to the use of somatic embryo. Later Bapat and co-workers (1987) broadened the technology to the encapsulation of various *in vitro* derived propagules, and they used axillary buds of *Morus indica* as a first example of this new application. This new concept paved the way for the encapsulation of explants other than somatic embryos and to the formulation of a new definition of synseed or artificial seed (Aitken-Christie *et al*., 1995) as "artificially encapsulated somatic embryos, shoots or other tissues which can be used for sowing under *in vitro* or *ex vitro* conditions (Lambardi *et al.,* 2006).

Standardi and Piccioni (1998) produced an extensive and well documented review concerning the use of non-embryogenic explants for the production of synthetic seeds. The artificial seed technology has been applied to a number of plant species especially vegetative crop plants and however very limited to the orchids.

Sodium alginate was found to be best gelling agent and protocorms were the best propagules for synthetic seed production (Nayak *et al*., 2006). Rai *et al.* (2008) reported that encapsulated vegetative propagules have the capacity to retain their viability after storage for a sufficient period. Corrie and Tandon (1993) reported synthetic or artificial seed production in *Cymbidium* sp which provides easy handling of propagules during transportation and potential for long term storage without losing viability. They encapsulated PLBs of *C. giganteum* with sodium alginate solution containing nutrients of MS medium, growth regulators, antimicrobial agents and hardened the beads in calcium chloride solution. Plantlets were successfully recovered from encapsulated PLBs on nutrient medium as well as on sterile sand and soil. The conversion frequency was high in both *in vitro* (100%) and *in vivo* (88%) conditions.

Similarly, Khor *et al.* (1998) developed two coat systems for encapsulation of *Spathoglottis plicata* seeds and protocorms. The encapsulated seeds and protocorms showed 64% and 40% regeneration, respectively. Sharma *et al.* (1992) successfully encapsulated protocorm like bodies of *Dendrobium wardianum* in calcium alginate solutions to get artificial seeds and recorded 100% of conversion frequency on MS medium after 45 days of culture. The regenerants were phenotypically similar to the mother plant. Martin (2003) produced artificial seeds of *Ipsea malabarica* through *in vitro* formed bulb and showed 100% germination on half strength MS medium without hormones or that supplemented with 6.97 mM kinetin.

The additional supplements such as nutrients, plant growth regulators, fungicides, pesticides and antibiotics to the gel matrix should have significant relationship with the efficiency of germination and increase the viability of encapsulated plant material (Kikowska & Thiem, 2011). Datta *et al.* (1999) produced artificial seeds through encapsulation of protocorm like bodies of *Geodorum densiflorum,* an endangered orchid. They found 88% germination of artificial seeds when cultured on Knudson C medium supplemented with coconut milk (15%), peptone (2g/l), BAP (2 mg/l) and NAA (1 mg/l). Artificial seeds incorporated with food preservative and fungicide in its encapsulating gel showed 28% viability when directly transferred to non sterile soil condition. Teixeira da Silva (2012) successfully produced artificial seeds from

protocorm like bodies of hybrid *Cymbidium* and cultured on new medium, Teixeira Cymbidium (TC) medium. The use of TC medium alone or incorporated with coconut milk into artificial seeds showed effective results on germination and short term preservation (1-6 month) of *Cymbidium* sp. germplasm. In addition, Sarmah *et al.* (2010) suggested that the germination percentage of encapsulated PLBs was influenced by the concentrations of complexing gel and duration of exposure of $CaCl_2.2H_2O$ solution. They produced the artificial seeds from protocorm like bodies (PLBs) obtained from leaf explants of *Vanda coerulea*. Among different concentrations tested, 3% sodium alginate and exposure to 100 mM $CaCl_2.2H_2O$ solution for 30 minutes produced clear, round and uniform beads and showed 94.9% germination percentage. Furthermore, encapsulated PLBs stored at 4ºC also retained their viability upto 100 days.

According to Saiprasad and Polisetty (2003), 3% sodium alginate upon complexation with 75mM $CaCl_2.2H_2O$ was effective for encapsulation of PLBs of some orchids. An encapsulation matrix prepared with MS medium supplemented with BA (0.44 mM) and NAA (0.54 mM) gave 100% conversion of encapsulated PLBs to plants after 60 days storage of *Dendrobium*, 45 days storage of *Oncidium* and 15 days storage of *Cattleya* at 4ºC. Zhang *et al.* (2011) revealed that PLBs was one of the most suitable propagules for encapsulation rather than axillary buds and adventitious shoots. They also found that maltose, active carbon and sodium alginate played the important role in germination of artificial seeds of *Dendrobium candidum*. Nagananda *et al.* (2011) found that single strength Burgeff's N3F basal medium fortified with sucrose (2%), agar (1%), adenine sulphate (2 mg/l) and IAA (1 mg/l) showed 95% germination of the encapsulated PLBs of *Flickingeria nodosa*. In addition, Mohanty and Das (2013) reported that 3% sodium alginate upon complexation with 100 mM $CaCl_2.2H_2O$ was the superior gel matrix for encapsulation of PLBs of *Dendrobium densiflorum* and showed 100% conversion of plantlets on MS medium supplemented with BAP (2 mg/l). They successfully stored the artificial seeds till 60 days at 8ºC with their conversion frequency of 95.5%.

2.3 Virus free plants in orchids

The production of virus free plants was revolutionized by Morel and Martin (1952) after recovering the virus free Dahlia plants from infected individuals by excising and culturing their shoot tips *in vitro*. While applying the technique of shoot-tip culture for

raising virus-free individuals of an orchid, Morel (1960) also realized the potential of this technique for the rapid propagation of these plants. Later, meristem culture, thermotherapy, chemotherapy, cryotherapy or combinations of these methods were also used for elimination of virus from plants (Milošević *et al*., 2012).

Most of the orchids have been reported to be infected with more than 25 viruses (Zettler *et al*., 1990). Cymbidium mosaic virus (CymMV) and Odontoglossum ringspot virus (ORSV) have been reported to be the two most prevalent and important viruses infecting orchids. Their high incidence in cultivated orchids has been attributed to the stability and ease of transmission of these two viruses through cultural practices (Sherpa *et al.,* 2006). Protocorms or protocorm like bodies (PLBs) are considered the best plant materials in the orchid industry due to their higher multiplication rate, ease of handling and transport (Chen, 2009). A large number of PLBs can be accommodated within a limited culture container space. Chien *et al*., (2015) demonstrated that some PLB lines selected at the first subculture as virus-free were found to be infected with virus at second subculture, however, re-occurrence of virus was never found in PLB lines at third subculture and onwards. Hence, at least 3 subcultures are necessary to authenticate that the cultures are free of viruses. They found that all the tissue culture-raised *Phalaenopsis* plants in the greenhouse derived from PLBs initiated through shoot tip culture, tested negative for the two viruses (CymMV and ORSV) by using Indirect enzyme-linked immunosorbent assay (ELISA) and one-step multiplex reverse transcription polymerase chain reaction (RT-PCR) techniques.

Cybularz-Urban and Hanus-Fajerska (2006) reported that addition of plant growth regulators (4.95 mg l-1 BA, 3.2 mg l-1 kinetin and 1.0 mg l-1 NAA) on modified MS medium and then further propagation on similar medium containing 0.2 mg l-1 zeatin was found to be effective to eliminate CymMV from cultures of the *Cattleya* hybrid. Pearson and Cole (1986) inoculated Odontoglossum ring spot virus (ORSV) and Cymbidium mosaic virus (CymMV) in two cultivars of *Cymbidium* species and found their infection rate of 70% and 20% respectively. Both viruses were found to reduce plant growth however the effects of CymMV being more severe than those of ORSV. However, cultivated orchid plantlets of Thailand derived from the micropropagation of axilliary bud and the shoot tip explants were infected with CymMV (27.6%) but

not infected with ORSV. This result revealed that CymMV is more prevalent than ORSV (Khentry *et al*., 2006).

The polyclonal antisera prepared from expressed CymMV coat proteins were useful for the detection of CymMV in an array of assays. The detection system developed is highly effective for detection of Indian strain of the virus in comparison to kits available in the international market (Sherpa *et al.*, 2012). Similarly, Lee and Chang (2008) obtained antisera against important orchid viruses like CymMV and ORSV using bacterially expressed recombinant capsid proteins (CP), instead of purified virus particles, as immunogens. These homemade antisera of CymMV and ORSV are suitable for the certification programme of orchids due to their low cost and high specificity.

Clark and Adams (1977) described the microplate method for the detection and assay of plan virus using enzyme labelled antibodies. The method enabled the highly sensitive detection of a number of morphologically different viruses in purified preparations and in unclarified extracts of herbaceous hosts and of infected crop plants. Khan *et al*. (2003) developed a reliable and sensitive method for virus identification of *in vitro* regenerated plantlets of six potato varieties such as, Cardinal, Diamant, Dhera, Multa, Cilena and Sieglinde. They detected PVA, PVY, PVV, PVM, PVS and PVX viruses by the DAS-ELISA test from *in vitro* raised infected and non-infected plants and reported that this method can be widely used for virus detection in potato and other related plants.

Hu *et al.* (1994) detected CymMV in fifty orchid samples and found that the results of ELISA and bioassay for detection were similar. However, ELISA is more rapid method for detecting CymMV than the mechanical inoculation bioassay. Porter *et al.* (1996) found that CymMV was not transmitted from CymMV-infected pod and pollen parents of 7,050 University of Hawaii (UH) *Dendrobium* hybrids seedlings and concluded that CymMV was not seed transmitted. Therefore, the use of seed-propagated cultivars and species shall be one of the most promising approaches to establish virus-free orchid plants and high quality germplasm.

2.4 Genetic fidelity of orchids

A number of DNA based reliable molecular markers have proven to be the most effective means to study the genetic fidelity in various plant species. Use of available molecular markers such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR) are frequently used to differentiate between the species and the cultivars and to determine the relationship between the species within the genus and other related groups. RAPD and ISSR are two simple and quick techniques; where the former detects nucleotide sequence and the latter permits detection of polymorphism in inter microsatellite loci using a primer designed from dinucleotide or trinucleotide simple sequence repeats (Parab & Krishnan, 2008).

In the past few years, efforts have been made to establish the phylogenetic relationship and genetic diversity of many orchid species however the assessment of the genetic fidelity of orchid is very limited. Obara-Okeyo and Kako (1998) successfully examined thirty six cultivars of *Cymbidium* sp. using RAPD markers and determining the levels of genetic variability. Choi *et al.* (2006) examined the genetic diversity and phylogenetic relationships among and within species of oriental *Cymbidiums* based on RAPD analysis. Zhu *et al.* (2008) suggested that RAPD markers were useful for cultivar identification and relationship analysis in *Cymbidium sinense*. Jian *et al.* (2013) demonstrated that the 3'-end extended random primer amplified polymorphic DNA (ERAPD) technique is a powerful tool for cultivar identification and establishment of genetic relationships of cultivars in *Cymbidium kanran*. Bhattacharyya and Kumaria (2015) studied the genetic structure of *Dendrobium nobile* from northeast India using RAPD. Their results revealed that variation amongst the populations was significantly higher than within the populations. They suggested that RAPD method was a valuable tool for estimation of genetic diversity and genetic relatedness of the *D. nobile* germplasm. Da Silva *et al.* (2006) reported that RAPD and mtDNA analysis of all resultant PLBs, callus or plants of hybrid *Cymbidium* showed them to be genetically identical however little variation obtained from abiotic factor treatment.

Similarly, Wang *et al.* (2004) used RAPD and AFLP to investigate the genetic

variation of 14 species of *Cymbidium*. The results obtained from both markers were similar and the genetic similarity was observed between *C. ensifolium* and *C. sinense, C. kanran* and *C. faberi* var. *omeiense, C. mastersii* and *C. eburneum* respectively. Cerasela and Lazar (2009) reported the slight polymorphism at molecular level between *in vitro* regenerants of *Cymbidium* species using RAPD markers. Khoddamzadeh *et al.* (2010) detected somaclonal variation in the plantlets (17% dissimilarity with the mother plant) obtained after six months subculture of PLBs of *Phalaenopsis bellina.* Sharma *et al.* (2011) reported that all the three SPAR (Single Primer Amplification Reaction) methods viz. RAPD, ISSR and DAMD, either independently and/or in combination, revealed wide range of genetic variation between and within five species of *Cymbidium* viz. *C. aloifolium*, *C. mastersii*, *C. elegans, C. eburneum* and *C. tigrinum*. They observed 96.6% polymorphism at inter-specific level and 51.2% to 77.1% polymorphism at intra specific level in five species of *Cymbidium*.

The genetic closeness of various species of *Vanda* using RAPD was observed by Lim *et al.* (1999). They suggested that terete leaved *Vanda teres* and *Vanda hookeriana* were classified in the separate genus *Papilionanthe* and that *Vanda sanderiana* should remain in the same genus. Roy *et al.* (2012) detected overall 5.81% genetic variation in the regenerated plantlets assessed by 18 RAPD primers. Pathak and Jaroli (2012) assessed the genetic variability and identification of eight species of *Dendrobium* using RAPD and ISSR markers. Mohanty and Das (2013) confirmed the genetic fidelity after observing the uniform RAPD banding profile among the plantlets derived from encapsulated PLBs of *Dendrobium densiflorum* following 60 days of storage. Pongsrila *et al.* (2014) reported that ISSR technique was effective for identification and determining genetic diversity of the genus *Dendrobium* at species level.

2.5 Summary and Research Gaps

Plant tissue culture technique has enormous potential for large scale propagation and conservation of rare, threatened and endangered orchids. Orchids require intensive care and habitat management because of their small size population of fungal partner for their seed germination and restricted distribution. Globally, they are very popular for potted plants, cut flower and medicinal purpose. Their increasing demands can

only be fulfilled by *in vitro* propagation technique through which a large number of identical clones can be raised from seeds or other different explants. Hence, this technique could be an important tool to minimize the pressure on natural population of orchids and their sustainable utilization.

Artificial seed technology is one of the major applications of plant tissue culture through which artificial seeds can be produced from both embryogenic as well as non-embryogenic tissues. This technology is very effective for orchids which cannot be easily reproduced by natural seeds. The advantage of this technology is to produce somatic embryos, closely resemble to the seed embryos and thus used as a unit for clonal propagation, short to midterm storage and easy for transportation. The scarcity and undesirable qualities of micropropagules, choice of coating material for making artificial seeds are some constraints of this technology for regeneration of plants from artificial seeds. Despite the limitations, the artificial seed technology provides an alternative method for mass propagation and germplasm conservation of economically important orchids which has been proven in some important orchid species.

Production of virus free plants from protocorm derived artificial seeds is another challenging works. It depends upon the viable and quality micropropagules used for artificial seed production. However, DAS-ELISA test or RT-PCR techniques are highly preferable to detect the virus from plants.

The occurrence of somaclonal variation in tissue cultured plants is also another limiting factor. DNA based molecular markers such as RAPD, ISSR, flow cytometry etc. have been successfully used to measure genetic stability of recovered plants from artificial seeds.

Hence from overall literature review, it was found that there is limited research on artificial seed development from orchids. However, the production and development of artificial seeds of *Cymbidium aloifolium*, production of virus free plants and testing of genetic fidelity for clonal propagation of this species is still lacking. The present study is an attempt to fullfil the research gaps on this species.

CHAPTER 3

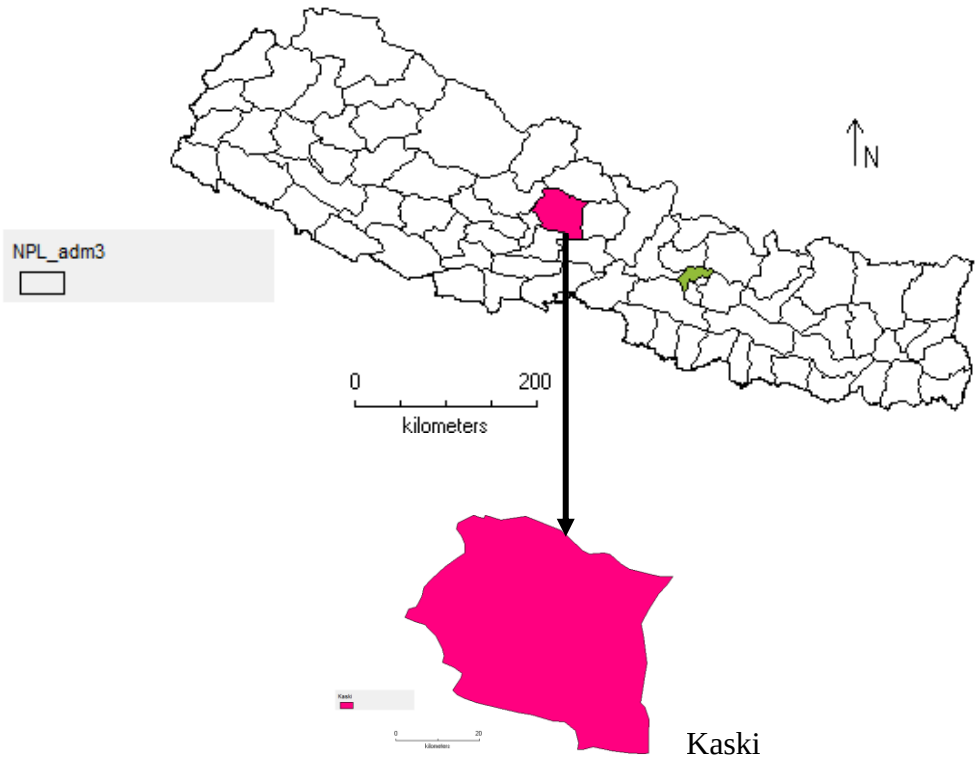
3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

The materials used for the present investigation were the, immature undehisced capsules of *Cymbidium aloifolium* (Fig. SG03), harvested from nature at an elevation of 500 meters from tropical region of Central Nepal (Pokhara). The explants used for present investigation are as follows:

- Immature green capsule was the primary explants for *in vitro* culture of seeds.
- Young medium sized three weeks old protocorms derived from *in vitro* culture of seeds were used as explants for plant regeneration; and production of artificial seed and its proliferation. The approximate size of protocorm was 0.3 to 0.5 mm in diameter.
- Young, healthy leaves derived from wild plants and tissue cultured plants were used as explants for DNA isolation and screening the CymMV virus.



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3.2 Methods

The methods applied for the *in vitro* seed germination, micropropagation; and production and proliferation of artificial seeds, test of Cymbidium mosaic virus, test of genetic homogeneity and acclimatization are described under following headings:

3.2.1 *In vitro* seed germination

The method for *in vitro* seed germination of C. *aloifolium* is described as under:

3.2.1.1 Preparation of stock solution

The MS (Murashige & Skoog, 1962) medium and Knudson C (Knudson, 1946) medium were used as the basal medium for the present investigation along and in combination with different concentration of hormones (BAP and NAA). The composition of the MS and KC media are as follows:

3.2.1.1.1 Preparation of Murashige and Skoog's medium (MS)

The Murashige and Skoog (1962) medium consist of macronutrients (stock A), micronutrients (stock B), iron source (stock C), vitamins (stock D), sucrose and agar (as gelling agent). The composition of the MS medium is as follows:

Table 1: Preparation of stock solution for MS medium

Stock A (macro-nutrient)-10X (g/lit)			
Components	**Composition of MS (Final conc.) mg/l**	**(10X) gm/l Stock Concentration**	**Volume to be taken for 1 litre medium**
Macronutrients			
Potassium nitrate (KNO_3)	1900	19.0	100 ml
Ammonium nitrate (NH_4NO_3)	1650	16.5	
Calcium chloride ($CaCl_2.2H_2O$)	440	4.4	
Magnesium sulphate ($MgSO_4 7H_2O$)	370	3.7	
Potassium dihydrogen phosphate (KH_2PO_4)	170	1.7	

Stock B (micro-nutrient)-100X (mg/100 ml)

Components	Composition of MS (Final conc.) mg/l	(100X) mg/100 ml stock Concentration	Volume to be taken for 1 litre medium
Micronutrients			
Boric acid (H_3BO_3)	6.2	620	
Manganese-sulphate ($MnSo_4$.4H2O)	22.3	2230	
Zinc sulphate ($ZnSO_4.7H_2O$)	8.6	860	1 ml
Sodium-molybdate ($Na_2MoO_4.2H_2O$)	0.25	25	
Cobalt Chloride ($CoCl_2.6H_2O$)	0.025	2.5	
Copper sulphate ($CuSO_4.5H_2O$)	0.025	2.5	
Potassium Iodine* (KI)	0.83	83	

Stock C (Iron source)-10X (mg/100 ml)

Components	Composition of MS (Final conc.) mg/l	(10X) mg/100 ml stock Concentration	Volume to be taken for 1 litre medium
Sodium ethylene diamine tetra acetate (Na_2EDTA)	37.3	373	10 ml
Ferrous sulphate ($FeSO_4$)	27.8	278	

Stock D (Vitamins)-100X (mg/100 ml)

Components	Composition of MS (Final conc.) mg/lit	(100X) mg/100 ml stock Concentration	Volume to be taken for 1 litre medium
Glycine	2.0	200	
Nicotinic acid	0.5	50	1 ml
Pyridoxin HCL	0.5	50	
Thiamin HCL	0.1	10	
Myo inositol**	100	10,000	

* 100 ml stock solution of KI was made separately and used as 1ml/l in MS medium.

** Myo-inositol was freshly made at the time of media preparation (100 mg/l).

E. Carbon source

Chemical	g/l
Sucrose	30 g

F. Gelling agent

Chemical	g/l
Agar	8 g

During the preparation of each stock solution, the above chemicals were weighed accurately and dissolved completely in distilled water. Myo inositol (which is considered as vitamin) was added directly while preparing medium (100 mg/l). To

dissolve the chemicals more readily, the solutions were stirred with magnetic stirrer. The final volume was mesh upto 1 litre for stock A and 100 ml for stock B, C and D. Due to light sensitivity, all stock solutions were kept in clean brown bottles and stored in the refrigerator at 4ºC.

3.2.1.1.2 Preparation of Knudson medium (KC)

The Knudson C (1946) was used as the basal medium for the present investigation alone and in combination with different concentrations of hormones. The KC medium consists of few nutrients and with no vitamins as compared to MS medium. It consists of macronutrients (stock A), micronutrients (stock B), iron source (stock C), sucrose and agar (as gelling agent). The composition of Knudson C (KC) medium is as follows:

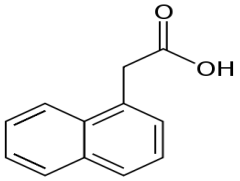
Table 2: Preparation of stock solution for KC medium

Chemical components **Stock solution of macronutrients (Stock A)**	**Composition of MS (final conc.) mg/l**	**(10X) g/l stock concentration**	**Volume to be taken for** **1 liter medium**
Calcium nitrate ($Ca(NO_3)_2.4H_2O$	1000	10.0	100 ml
Magnesium sulphate ($MgSO_4.7H_2O$)	250	2.5	
Potassium dihydrogen phosphate (KH_2PO_4)	250	2.5	
Ammonium sulphate ($(NH_4)_2SO_4$)	500	5.0	
Stock solution of micronutrients (Stock B)		**(100X) mg/100 ml stock concentration**	
Manganese sulphate ($MnSO_4.4H_2O$)	22.3	2230	1 ml
Stock solution of iron source (Stock C)		**(10X) mg/100 ml stock concentration**	
Ferrous sulphate ($FeSO_4.7H_2O$)	27.8	278	10 ml

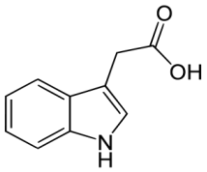
The amount of carbon source and agar on Knudson media was similar to that of MS media. The procedure for preparation of stock solutions and their amount for 1 litre media were also similar to MS medium.The pH of the both media (MS and KC) was maintained at 5.8 with the help of 0.1 N hydrochloric acid (HCl) and 0.1 N sodium hydroxide (NaOH).

3.2.1.1.3 Hormones used for the experiment

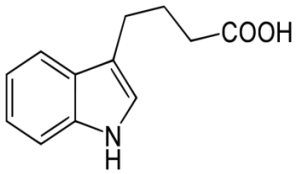
Plant hormones or phytohormones are organic substances which are naturally produced in plants and also found in synthetic form. These are of various types and differ in their activity but most of them stimulate the growth and development of plants. The growth hormones used in the present investigation for seed germination and seedling development are auxins, [NAA (α-Naphthalene Acetic Acid), IAA (Indole-3-Acetic Acid) and IBA (Indole-3-Butyric Acid)] and cytokinins [BAP (6-Benzyl Amino Purine)].



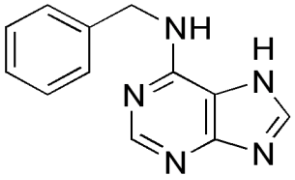
α-Naphthalene Acetic Acid



Indole-3-Acetic Acid



Indole-3-Butyric Acid



6-Benzylaminopurine

3.2.1.1.3a Preparation of hormones

In the present investigation, two plant growth hormones viz. cytokinin and auxins were used. For the preparation of cytokinin, i.e., BAP, 10 mg of it was dissolved in 2.5 ml of 0.5N NaOH and for the preparation of auxins, i.e., NAA, IAA and IBA, 10 mg of each was dissolved in 2.5 ml of 1N NaOH. After that the final volume was made 100 ml by addition of sterile water in each hormone separately. 1 ml of this hormone stock was used for 100 ml of media to make 1 mg/l of hormone concentration in the media.

3.2.1.1.4 Preparation of 1 litre media

The following protocol was applied for the preparation of 1 litre liquid (suspension) and solid media (static) of both MS and KC media:

- 1 litre sterilized conical flask was taken.

- 100 ml of Stock A, 1 ml Stock B, 10 ml of Stock C and 1 ml of Stock D (only for MS) were added one by one in about 400 ml sterile distilled water in sterilized conical flask.
- 30 g of sucrose was weighed and dissolved in stock solution.
- Sterile distilled water was added up to 900 ml and the solution was stirred by magnetic stirrer in order to mix the stock and sucrose solution more homogenously.
- The p^H of the media was maintained at 5.8 by using 0.1 N NaOH or HCl.
- Then, finally the volume was made 1000 ml by adding sterile distilled water.
- For the preparation of hormone medium, hormone stocks were added according to the media requirement in separate beakers to make 100 ml media in it.
- For the solid media, it was solidified by adding 0.8 g (0.8%) agar in each beaker containing 100 ml. It was heated with the help of heater up to boiling to melt the agar. When solution become clear, about 16 ml was poured in each of the 6 to 8 sterilized culture tubes. Then, each culture tube was enclosed by aluminum foil cap.
- The culture tubes containing media were sterilized in an autoclave at the temperature of 121ºC and pressure of 15 lb /sq inch for 20 minutes. After cooling down, the tubes were taken out and kept in slanting position (only solid) inside the culture room (25±2ºC).

Note:

- Suspension or liquid media was prepared by discarding solidifying substance, i.e., agar.
- ½ concentrations of media were prepared by taking, equal volume of media solution and sterile distilled water, i.e.,1:1 ratio.
- ¼th concentrations of media were prepared by taking media solution and sterile distilled water in the ratio of 1:3.
- But ½ and ¼th concentration of media required 0.8% agar for its solidification.

3.2.1.1.5 Sterilization procedures

Sterilization is the process of making contamination free environment for culture and growth of plant tissues. During *in vitro* culture, maintenance of aseptic environment is the most difficult task as the cultures are easily contaminated by microbes like fungi,

bacteria etc. The contaminants produce toxic metabolites which inhibit the growth of cultured plant tissues. Therefore, each part which is used for culture is necessary to handle aseptically and carefully. There are various techniques for sterilization of glasswares, equipment and plant tissues which are explained as below:

a. Dry heat sterilization: Sterilization is done through dry air in hot air oven at 160-180ºC for 2-4 hrs. Mostly glasswares like petriplates, vials, culture tubes, beakers, measuring cylinder, conical flasks, pipettes etc. and metallic instruments like forceps, scalpel etc. are sterilized through dry heat.

b. Wet sterilization: In this method, all the glasswares, metallic instruments and culture media are sterilized through steam by autoclave at 121ºC at 15psi for 30 minutes. This is the most reliable method of sterilization.

c. Flame sterilization: In this method, sterilization takes place by flaming. Metallic instruments eg. Forceps, scalpels, needles, spatula etc. are first dipped in 70% ethanol followed by flaming and cooling before using in culture.

d. Chemical sterilization: Sterilization takes place by means of chemical like detergent, spirit, ethanol (70%-95%), sodium hypochlorite (1%-2%). Working table, floor and walls of culture room and bench of laminar air flow cabinet are mostly cleaned with chemicals.

e. UV light: Exposure of UV light in large surface area is also one of the means of sterilization. The cabinet of laminar air flow is sterilized by exposing UV light for 30 minutes before working.

f. Surface sterilization: This is one of the important steps of culture before inoculation. Plant tissues which is to be cultured, is surface sterilized to remove the surface borne micro organisms.

3.2.1.1.5a Sterilization of glassware's and metal instruments

While preparing the media and even working with laminar air flow chamber, the necessary glassware's and metal instruments were subjected to wet and dry heat sterilization before their use. Glassware's such as beaker, petridish, culture tubes, pipettes, conical flasks, glass rod, measuring cylinder and metal instruments like forceps, scalpels, needle and scissors were dipped in detergent solution for 24 hours and washed with tap water. They were then steam sterilization with autoclave at

121°C for 20 minutes at 15 lb. pressure and dried with hot air oven at 150°C for 2 hours. Metal instruments were wrapped in aluminium foil before keeping inside the hot air oven for sterilization. Finally, flaming sterilization was done before using in a laminar air flow.

3.2.1.1.5b Surface sterilization of plant material

The plant materials used for the present investigation were green, immature undehisced pods or capsules of *C. aloifolium* (Fig. SG03). A single capsule contains a large number of orchid seeds. The freshly collected capsules were first thoroughly washed under running tap water for at least 30 minutes to remove the soil and other external particles attached on their surface. Then, the capsules were dipped in detergent water and Tween 20 (0.1%) for 15-20 minutes, shaken well and again washed in running tap water until all the detergents washed off clearly. The capsules were then rinsed with distilled water. After that, they were surface sterilized sequentially with 70% ethyl alcohol for 2 minutes and then dipped into 1% sodium hypochlorite solution for 15 minutes. Finally, the pods were rinsed thoroughly with sterile water for 5 times and ready for cut after drying in filter paper. The chemical surface sterilization process was carried out in laminar airflow cabinet.

3.2.1.1.6 Inoculation of seeds

The inoculation of seeds and explants was carried out in the laminar airflow cabinet. Before inoculation, the laminar airflow cabinet was cleaned with spirit or cotton soaked with 70% ethyl alcohol. The culture tubes containing media, sterile instruments and glassware's were exposed under ultraviolet (UV) radiation for 45 minutes to remove the possible contaminants presenting and around the transfer area. After turning off the UV light, the blower was kept running during the process of inoculation.

The surface sterilized capsules of *C. aloifolium* were put on sterile petridish containing sterile filter paper for soaking the surface moisture of orchid capsules. Then, each capsule was cut longitudinally into two halves by sterile surgical blade (Fig. SG04). The very tiny seeds of orchids were scooped out with the help of sterile spatula and inoculated on different strength of MS and KC media (full, half and quarter strength) supplemented with or without combination of BAP and NAA. After

that, all the cultures were maintained at 25±2ºC temperature and 16/8 hours (light/dark) photoperiods using white fluorescent tubes (Philips, India). The initiation and rate of seed germination was observed and recorded every week. The entire experiment was performed in aseptic condition under laminar air flow cabinet to prevent contaminations.

3.2.1.1.7 Histomorphological study

For histomorphological study, mass of protocorms obtained after 8 weeks of seed culture was fixed periodically in 1% acetocarmine for 24 hrs. Small piece of stained mass was mounted on glass slide with few drops of acetocarmine solution and squeezed gently. The slides were then examined under compound microscope. Photographs of the distinct stages were taken to confirm the different developmental stages of protocorm.

3.2.1.1.8 Inoculation of explants

The explants used in present investigation were protocorms, shoot tips and artificial seeds of *C. aloifolium*. The appropriate developmental stage of protocorms, obtained from *in vitro* culture of seeds were aseptically taken out from the culture vessels and cultured individually on different strength of MS and KC media (solid and liquid) supplemented with or without BAP and NAA (Tables 1 & 2). Protocorms were also used as primary explants to produce artificial seeds. They were cultured on similar medium to that of protocorms. After certain days, seedlings were developed. Small pieces of shoot tips (microshoots) (about 5 mm) were cut with the help of sterile surgical blade and inoculated on best optimized medium for shoot multiplication. Again, microshoots obtained from shoot tip culture were inoculated on MS medium supplemented with different concentration (0.5-2.0 mg/l) of auxins: IAA, IBA and NAA for induction of roots. All the cultures were maintained at 25±2ºC and 16/8 hrs photoperiod. They were observed regularly.

3.2.2 *In vitro* culture of artificial seeds

3.2.2.1 Production of artificial seeds

The methods for the production of artificial seeds from *in vitro* grown protocorms of *C. aloifolium* and their germination are explained under the following headings:

3.2.2.1a Artificial seed coat

Artificial seed coat is responsible for protection of explants during storage, handling and from mechanical injury. They should be capable of including nutrients and other growth and biological factors, non toxic and biodegradable. The artificial seed coat of *C. aloifolium* was prepared by intermixing of sodium alginate and calcium chloride solutions.

3.2.2.1b Preparation of sodium alginate (Na- alginate)

In the present investigation, 2%, 3% and 4% sodium alginate was used for the production of 2%, 3% and 4% encapsulated artificial seeds from *in vitro* grown protocorms of *C. aloifolium*. Thus, sodium alginate was weighed 2, 3 and 4 gram in electric balance and then dissolved in 100 ml sterile distilled water in well sterilized beaker separately with the help of glass rod. The mixture was slowly stirred with the help of glass rod to dissolve the solute completely and escape air bubbles. Finally it was allowed to stand for about 1-2 hours in laminar air flow under UV radiation treatment to prevent from contamination. Thus, the well sterile sodium alginate gel of 2%, 3% and 4% were prepared.

3.2.2.1c Preparation of calcium chloride ($CaCl_2$. $2H_2O$) solution

Calcium chloride solutions are most suitable to make gel with sodium alginate and formed firm, clear and isodiametric beads due to ion exchange between Na^+ in sodium alginate with Ca^{2+} in the $CaCl_2.2H_2O$ solution. Concentration of both solutions influences not only texture, size and shape of artificial seeds but mainly rate of plantlet conversion.

For the preparation of artificial seeds from protocorms, encapsulated gel hardening is very necessary to make rounded beads and for long term storage. The hardening of gel was done by using 0.2 M calcium chloride solutions. For this, 2.25 gram calcium chloride was weighed and dissolved in 100 ml sterile distilled water and finally made 0.2 M $CaCl_2$ solution. All the activities were done within laminar air flow cabinet.

3.2.2.1d Procedure for artificial seed production (encapsulation technique) and inoculation

The medium sized, green, twenty one days grown *in vitro* protocorms were separated with sterile forceps, dipped slowly in the sodium alginate gel (2%, 3% and 4%) and mixed well by sterile glass rod. The micropipette with sterilized tip was prepared ready for dropping gel with individual protocorms. The tips have been cut to make small rounded whole with the help of well sterilized blade or scissor. Then, micropipette was dipped into sodium alginate gel having separated protocorms; individual protocorms were filled in micropipette tip and poured drop wise from a height of about 20 cm in $CaCl_2$ solution. Finally, the rounded beads of artificial seeds were prepared (Fig. A11-A15).

The artificial seeds were then left in $CaCl_2$ solution for at least half an hour for hardening the seed coat or shell. Thus produced 2%, 3% and 4% alginate encapsulated artificial seeds were used as initial explants for the further investigation. They were inoculated on different strength of MS and KC media (solid and liquid) supplemented with or without BAP and NAA (Table 1 & 2). The freshly prepared artificial seeds (2%, 3% and 4%) were also stored at room temperature (RT) (21 ±2°C) as well as at 4°C for 120 days in order to check their viability.

3.2.2.2 Viability tests of stored artificial seeds

The explants or artificial seeds which have the capacity to germinate either in natural or artificial environment are known as viable explants or seeds. Normally, most of the explants have the totipotency to regenerate into complete plantlets. However, the stored plant seeds may be either viable or non viable. In present investigation, the artificial or synthetic seeds were stored for 120 days and their viability was tested under two different storage conditions, i.e., room temperature (RT) (21±2°C) and 4°C. The freshly prepared artificial seeds were stored in sterile petriplates and covered air tightly with the help of paraffin. They were labelled with date of artificial seed production, percentage of sodium alginate etc. They were sampled monthly and inoculated on full strength of liquid MS and KC media for germination and plantlet developmental studies.

During the viability test, stored artificial seeds were transferred in sterile petriplate

and washed at least two times with sterile distilled water. After washing, the seeds were soaked in sterile filter paper and finally inoculated in tested media with the help of sterile forceps. Finally the culture tubes containing artificial seed were transferred in culture room where temperature was maintained at 25±2ºC and photoperiod of 16/8 hrs. The cultures were observed regularly till the complete germination and development of shoots, leaves and roots from artificial seeds. All these works were done in laminar air flow cabinet.

The percentage of seed germination or viability of all explants (Hossain *et al*., 2009) was calculated as below:

$$\% \text{ of seed germination/viability} = \frac{\text{No. of seeds showing swelling of the embryo}}{\text{No. of total seeds used for study}} \times 100$$

3.2.3 Shoot multiplication from artificial seed derived shoot tip (microshoot)

For shoot multiplication, individual microshoots of about 5 mm were cut and transferred to the most appropriate media for mass propagation and shoot growth was recorded every week.

3.2.4 Rooting of artificial seed derived shoots

The microshoots (0.5-1.0 cm) obtained from the artificial seeds were transferred to the rooting media supplemented with different concentrations (0.5-2.0 mg/l) of rooting hormones such as IAA, IBA and NAA and recorded every week.

3.2.5 Detection of Cymbidium Mosaic Virus (CymMV) by DAS-ELISA

Double antibody sandwich enzyme linked immunosorbent (DAS-ELISA) method is widely used for detection of virus (CymMV) in *in vitro* and *in vivo* explants using antibodies of CymMV (Agdia, USA). DAS-ELISA was performed by following the protocol described by Clark and Adams (1977). According to this protocol, microtitre plate wells (96 wells; Agdia, USA) were coated with 200 µl of antibody of CymMV (dilute 1:200, Agdia, USA) diluted in coating buffer at 1:1000 ratio. The plate was incubated at 37°C for 4 hours. Following incubation, about 200 mg of fresh young

leaves containing midrib and vein were taken for plant extraction. Plant extracts were prepared by grinding leaf tissue in sterile mortar and pestle and homogenized in 5 ml of maceration buffer containing phosphate buffered saline (PBS) with 0.5ml/l Tween 20 and 2% polyvinylpyrrolidone (PVP). The homogenized sap were then centrifuged at 2000 rpm for 3 minutes and the supernatant was loaded into the ELISA plate at the rate of 200 µl per well. The plate was then incubated at 37°C for 4 hours. After incubation, the conjugate antibody (antibody linked with enzyme alkaline phosphatase; dilute 1:200; Agdia, USA) was mixed with the buffer at 1:1000 ratios and 200 µl of the mixture was added to each well.

The plate was again incubated at 37°C for 3 hours. Plates were thoroughly washed with 0.01M phosphate buffered saline (pH 7.4) at each stage after incubation for at least three times to remove the traces of soluble reactants that could cause non-specific reactions. The final step of DAS- ELISA is to add substrate which is p- nitro phenyl phosphate (pNPP; Agdia, USA). The substrate solution is freshly prepared in substrate buffer at the rate of 1 mg/ml and dispensed into the plate at the rate of 200 µl per well. These plates were incubated for 30-60 minutes for colour reaction. Strong yellow colour was observed in the positive control and CymMV positive samples. Finally, the optical density (OD) of the reaction mixture was recorded at 405 nm wavelength with the help of ELISA plate reader for quantitative result. The colour reaction was then stopped by adding 5 µl of 5% NaOH solution in each well. This experiment was performed in triplicates.

Samples were considered positive when OD values were more than 1.0. In present investigation, the lypholized powder of *Cymbidium* species (obtained directly from Agdia, USA) which showed presence of CymMV was used as control [(+) ve]. The percentage of infection of CymMV of all explants was calculated as below:

$$\% \text{ of infection (CymMV)} = \frac{\text{No. of infected plants}}{\text{No. of total plants used for study}} \times 100$$

3.2.6 Marker analysis for genetic variation and homogeneity

3.2.6.1 Extraction and quantification of genomic DNA

Young and healthy leaves harvested from wild (non-tissue cultured mother plant collected from natural habitat) and eleven explants, each set derived from *in vitro* culture of seeds, protocorms, artificial seeds (encapsulated with 2%, 3% and 4% sodium alginate solution) and shoot tips grown on MS and KC media with or without

cytokinin (0.5 mg/l BAP) and auxin (0.5 mg/l NAA) were used as explants for DNA extraction of *C. aloifolium* (Appendix 1). Young leaves collected from wild mother plant were washed under running tap water and rinsed with 70% ethyl alcohol for surface sterilization. Leaves derived from *in vitro* culture were not subjected for surface sterilization. Total genomic DNA of *C. aloifolium* leaf (100 mg) was extracted using Hexadecyltrimethyl Ammonium Bromide (CTAB) method as well as Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. The quality and quantity of extracted DNA were determined by gel electrophoresis on 1% agarose gel and UV spectrophotometre. The DNA content of the isolate was adjusted to 10-20 ng/µl by TE buffer or DNA rehydration solution and stored at -20°C for further study.

3.2.6.1.1 Extraction of DNA from CTAB method

Genomic DNA of leaf samples of *Cymbdium* aloifolium were extracted by using CTAB (Hexadecyltrimethyl Ammonium Bromide) method (Doyle, 1991) with some modifications. Approximately 0.2 gm of leaf samples was taken and grinded to fine powder by using motor and pestle in liquid nitrogen. 500 µl of CTAB buffer (2% CTAB, 0.5M EDTA, 5M NaCl, 1M Tris-HCl, pH 8.0, 0.2% β-mercaptoethanol) was added to make the fine paste and transferred into clean sterilized micro-centrifuge tube (vol. 2.0 ml). Samples were incubated at 60^{o}C for 15 minutes in a recirculating water bath. After incubation, samples were centrifuged at 12,000 rpm for 5 minutes to spin down cell debris. Then supernatant were transferred to clean sterilized micro-centrifuge tube and equal volume of Chloroform: Isoamyl alcohol in the ratio of 24:1 was added and mixed gently by inversion for several times (5-8 minutes.) It was again centrifuged for 1 minute in 13,000 rpm and upper aqueous phase was transferred to sterilize micro-centrifuge tube. Approximately 50 µl of Ammonium Acetate (7.5M)) was added to each sample followed by addition of 500 µl of chilled ice cold absolute ethanol. The tube was slowly inverted several times to precipitate the DNA.

After that, DNA was spinned at 13,000 rpm for 1 minute to form the pellet. Supernatant was discarded and pellet was washed with ice cold 70% ethanol (500 µl volumes). Again, it was spinned at 13,000 rpm for 1 minute to get rid of salt. Then, ethanol was pipetted out and pellet was left to dry for 15 minutes. Finally, it was re-suspended in T.E. buffer (300 µl) and stored at 4^{o}C. The quality and quantity of

extracted DNA was estimated by electrophoresis on agarose gel (1%) and by UV spectrophotometry respectively. The concentration of extracted DNA was adjusted to 10-20 ng and finally DNA samples were stored at – 20ºC.

3.2.6.1.2 Extraction of DNA from genomic DNA purification kit (Promega)

Approximately 100 mg of young leaf was extracted from *in vivo* and *in vitro* plant materials. Leaves were washed with sterile water and finally rinsed with cold ethanol (2 ml). They were crushed in sterile sand by using sterile mortar and pestle. Cell lysis solution (600 µl) was added to make fine leaf paste. Then, the paste was poured in sterile 2 ml micro-centrifuge tubes and incubated for 10 minutes at room temperature. Then, the paste containing micro-centrifuge tube was spinned at 15,000 X g. for 1 minute. After centrifuging, the supernatant was discarded and remaining pellet was vortexed for 20 seconds. Then, nucleic lysis solution (600 µl) was added in pellet containing micro-centrifuge tube and kept in water bath (65ºC) for 15 minutes. The micro-centrifuge tubes were removed from hot water bath, RNAse solution (3ul) was added in the tubes, mixed the paste sample by inverting the tubes 2-5 times and again incubated in water bath (37ºC) for 15 minutes. Then, the sample was allowed to cool to room temperature for 5 minutes before proceeding further steps.

After cooling, protein precipitation solution (200 µl) was added in sample, vortexed vigorously at high speed for 20 seconds and spinned in centrifuge at 15,000 X g. for 3 minutes. Supernatant solution containing DNA was poured into new sterile micro-centrifuge tubes containing room temperature isopropanol (600 µl). Tubes were then inverted for several times until thread like strands of DNA form a visible mass and spinned at 15,000 X g. for 1 minute. The supernatant solution was discarded, added room temperature 70% ethyl alcohol (600 µl) and gently inverted several times to wash the DNA. After that, the tube was again spinned at 15,000 X g. for 1 minute. The supernatant was again discarded and dried the tube at room temperature for 15 minutes. Then, DNA rehydration solution (100 µl) was added in tube from top so that buffer slides down where the DNA had to precipitate. Again the tubes were kept in 65ºC hot water bath for 1 hrs. for complete solubilisation. Finally, the DNA samples containing micro-centrifuge tubes were stored at -20ºC for PCR amplification.

3.2.6.2 DNA amplification

A set of twenty six decamer RAPD and five ISSR primers were used to amplify DNA fragments of the selected explants (Appendix 2, 3 & 4). PCR reaction for RAPD and ISSR assays were conducted in 25 µl reaction volume by varying concentrations of $MgCl_2$ (1mM), DNA buffer (1X), dNTPs (0.4 mM), primers (10 µM), DNA template (3 µl) and Taq DNA polymerase (0.25 U/µl). Cycling condition followed by Cerasela and Lazar (2009) was also optimized for identifying best cycling conditions (45 cycles). DNA amplifications were performed in thermal cycler (BioRad) with the following PCR program: 5 min. of initial denaturation step at 94°C, 45 cycles comprising denaturation at 94°C for 1 min., annealing at 37°C for 1 min, and extension at 72°C for 2 min; followed by a final extension step of 10 min at 72°C.

3.2.6.3 Gel electrophoresis

The PCR amplified fragments were separated on 1% agarose gel using 1X TAE (Tris Acetic acid and EDTA) buffer and stained by ethidium bromide (1 µg/ml). The gel was running at 90 V constant for 1.5 to 2 hrs. and then visualised and photographed under UV light in a gel documentation system (UVITEC, Cambridge). The sizes of the amplification fragments were determined by comparisons with the 100bp DNA ladder marker (BioLabs). The PCR reactions were repeated thrice and only the clear bands were considered.

3.2.7 Acclimatization of plantlets

Acclimatization is a natural phenomenon in which an individual organism adjusts to a gradual change environment. The fully grown *in vitro* plantlets (4-5 cm long) derived from alginate coated artificial seeds with well developed roots of *Cymbidium aloifolium* was acclimatized in various substrates. Non encapsulated protocorm (single protocorm) as well as some freshly prepared and one week storage artificial seeds (2%, 3% and 4%) were also directly acclimatized in the same substrates. The following steps were carried out for acclimatization:

- At first, the culture tubes containing rooted plantlets were opened and kept at room temperature for 1 week.

- ❖ The plantlets grown in cultured conditions were picked out and washed with running water to remove the entire agar attached with it.
- ❖ The plantlets were dipped in fungicide, Bavistine (0.1%) for 5 minutes to minimize the chances of infection.
- ❖ Then, the plantlets were washed with distilled water for few minutes and kept in blotting paper for drying.
- ❖ The plantlets were then transferred to the cleaned earthen pots containing appropriate potting mixture.
- ❖ After one week of transplantation, the 5% Hyponex solution [Nitrogen, Phosphorus and potassium (NPK)] was sprayed once a week regularly for fastening their growth.
- ❖ The transferred plantlets were covered with transparent polythene sheets to control the humidity. The small holes were made into polythene sheet for aeration.
- ❖ The plants were kept in the greenhouse for several weeks and finally they were transferred to the natural environment to check their growth.

Note:

- ➢ Non encapsulated protocorms and alginate coated artificial seeds were first dipped in fungicide solution (0.1% Bavistine) for 2 minutes, washed with sterile water for few minutes and directly sown on substrates.

The potting mixture played an important role for successful acclimatization. Therefore following potting mixture (substrates) was used in present investigation for acclimatization process:

1. Coco peat along with sphagnum moss (to facilitate the holding of water) in ratio of 3:1 (A_1)
2. Coco peat along with litter in ratio of 3:1 (A_2)
3. Coco peat, litter and sphagnum moss in the ratio of 3:2:1 (A_3)
4. Coco peat, clay and sphagnum moss in the ratio of 3:2:1 (A_4)
5. Coco peat along with sand in ratio of 2:1(A_5)
6. Only sand (A_6)

3.2.8 Statistical analysis

Statistical analysis was done by using one way Analysis of Variance (ANOVA). The data obtained were first analysed using application software-Microsoft Excel. Then, the statistical analysis was carried out into three steps. They are:

1. Descriptive Analysis,
2. Normality Test and
3. Comparision of mean values

1. Descriptive Analysis: Mean and standard errors of growth parameters for germination (initiation of germination, shoot, leaf, root and seedling), proliferation (number of shoot, leaf and root and length of shoot, leaf and root) and rooting (number and length of shoot and root) were descriptively analyzed. Therefore, the values of different tested growth parameters were expressed as mean ± Standard error (SE).

2. Normality Test: The data were tested using Kolmogorov-Smirnov and Shapiro-Wilk test for normality. All the data showed the normal nature therefore paramateric test particularly one way ANOVA was used to compare the mean values.

3. Comparision of mean values: The one way ANOVA was carried out to compare the mean values of growth parameters of germination, proliferation and rooting. This analysis showed the comparision among the parameters only but not in two different parameters. Hence, those parameters which were significantly different among them at 5% level of significance ($p \leq 0.05$), were further tests for Tukey's Honestly Significant Difference (HSD) using Statistical Package of Social Science (SPSS) software version IBM SPSS 20.

3.2.8a RAPD and ISSR analysis

The RAPD and ISSR profiles were analysed on the basis of presence (1) or absence (0) of individual amplified DNA bands. Only clear and reproducible bands at a particular position were scored. By comparing the banding patterns of all the plant accessions, specific bands were identified and a marker system for genetic purity of *Cymbidium aloifolium* was established. The ability of the primers to distinguish between the individuals was determined by using genetic parameters viz. Polymorphic information content (PIC) and Resolving power (R_p). The PIC and R_p value was calculated using the formula,

PIC = $1- \sum pi^2$, where *pi* is the frequency of the ith allele (Smith *et al*., 1997)

R_p = $\sum Ib$, where *Ib* (band informativeness) = $1- 2|0.5-p|$, where *p* is the proportion of individuals containing the band (Prevost & Wilkinson, 1999).

NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) Version 2.1 software was used to perform the distance matrix and cluster analysis of the complete data set of the two markers employed (Rohlf, 1992). Genetic similarity amongst the different individuals based on Dice coefficient was calculated using SIMQUAL (Similarity for qualitative data program) in NTSYS module and was arranged into a similarity matrix. A dendrogram was constructed by following the UPGMA (Unweighted pair group method with arithmetic mean) option of the SAHN (Sequential, agglomerative hierarchical and nested clustering) module of NTSYSpc.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Results

The results of *in vitro* seed germination, culture of protocorm, artificial seeds (2%, 3% and 4%), shoot tips, rooting of shoots and acclimatization of *C. aloifolium* have been described under following headings:

4.1.1 *In vitro* culture of seeds of *Cymbidium aloifolium* in different media

Orchid seeds are very minute, produced in large number; contain undifferentiated embryos and lack endosperm. In present investigation, seeds reside in green capsules of *C. aloifolium* were inoculated on different strength (1, ½, ¼) of solid MS and KC media and full strength (1.0) of both media supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) (Table 3). On MS medium, more than 80% seeds were germinated whereas on KC medium, about 60% seeds were germinated in all conditions. In both media, full strength of medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) was found to be the effective condition for earlier germination of seed. The early response on seed germination was observed on full strength of MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) which took 7 weeks of primary culture in comparison to full strength of KC medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) which took 8 weeks of primary culture for *in vitro* seed germination.

The immature seeds undergo *in vitro* germination by developing green colour spherules, protocorms and finally initiating shoots and roots. The protocorms formed in different media were chlorophyllous and globular. The pattern of seed germination and seedling growth in the tested mediums of orchid, *C. aloifolium* is summarized in Table 3.

Table 3: Comparative effect of different strength and growth regulators supplemented MS and KC media on seed germination and seedling growth of *Cymbidium aloifolium* (L.) Sw.

Medium	Different strength of medium	Plant growth regulators (mg/l)		Observation taken in weeks					% of seed germination (mean)
		BAP	NAA	Initiation of germination	Development of protocorms	Differentiation of 1st shoot primordia	Differentiation of 1st root primordia	Seedling	
MS	1	–	–	10	12	16	23	30	93.33
	½	–	–	12	15	–	–	–	87.5
	¼	_	_	15	19	–	–	–	82.5
	1	0.5	0.5	7	10	13	21	27	98.33
KC	1	–	–	9	13	18	25	31	87.5
	½	–	–	10	15	21	–	–	70.83
	¼	–	–	15	19	–	–	–	65.0
	1	0.5	0.5	8	12	16	24	30	90.83

Culture conditions: - MS & KC solid media, 25 ± 2˚C, 32 weeks, 16/8hrs. photoperiod, 6 replicates were used in each condition.

The inoculation of seeds of *Cymbidium aloifolium* on different strength of MS and KC media supplemented with or without different hormonal concentration of BAP and NAA showed following changes:

Initiation of germination

All the tested conditions of MS and KC media responded more than 80% and 60% of seed germination respectively. The first visible sign of *in vitro* seed germination was observed as the swollen yellowish green spherules within 7 weeks of primary culture on full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. On this condition, about 98.33% of seeds were germinated. Whereas, the period of germination was late on full strength of KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA as compare to MS medium. On this condition, about 90.83% seeds were germinated after 8 weeks of culture. The quarter strength of MS medium took longer time for seed germination (15 weeks of culture) rather than half strength and full strength of MS medium which took 12 weeks and 10 weeks of culture (Fig. SG05) respectively for seed germination. Here, the germination period was decreased

with the increment of strength of medium. Similar trend was also observed on KC medium where quarter strength, half strength and full strength of KC medium took 15 weeks, 10 weeks and 9 weeks of culture respectively for germination of seeds (Table 3).

Development of protocorms

Green protocorms were developed on all the tested conditions of MS and KC media. The earlier development of large number of globular chlorophyllous protocorms was found on full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 10 weeks of culture (Fig. SG06) as compare to other tested conditions of MS and KC media. However, among the different tested conditions of KC medium, the earlier development of protocorm was observed on full strength of KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 12 weeks of culture for initiation of protocorm. Full strength, half strength and quarter strength of MS medium took 12 weeks (Fig. SG07), 15 weeks and 19 weeks of culture respectively for the development of protocrom which showed that the time taken for initiation of protocorm from seed was increased with decreasing the strength of medium. Similar results was also obtained on KC medium where protocorms were initiated on 13 weeks, 15 weeks and 19 weeks of culture on full strength, half strength and quarter strength of KC medium (Fig. SG08) respectively (Table 3).

Differentiation of first shoot primordia

The shoot primordia, developed from differentiation of protocorm were observed only on full strength of MS medium and MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. The earlier differentiation of shoot was found on full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 13 weeks of culture (Fig. SG09) rather than other tested conditions of MS and KC media whereas full strength of MS medium took 16 weeks of culture for initiation of first shoot primordia. However, the lower strength (half and quarter strength) of MS medium was not effective for the development of shoot. Among different tested conditions of KC medium, first shoot primordia was developed earlier on full strength

of KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA after 16 weeks of culture. This condition was followed by full strength and half strength of KC medium where first shoot primordia was initiated after 18 weeks and 21 weeks of culture respectively. The quarter strength of KC medium was not effective for shoot development (Table 3).

Differentiation of first root primordia

The development of root primordia was faster on MS medium rather than KC medium. However, the lower strength (half and quarter strength) of both MS and KC media were not effective for root development. First root primordia was emerged out earlier on 21 weeks of culture on full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. This condition was followed by full strength of MS medium only where initiation of root was observed on 23 weeks of culture. Similar trend was also found on KC medium where full strength of KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA and full strength of KC medium only took 24 weeks and 25 weeks of culture respectively (Table 3).

Development of seedling

On both MS and KC media, the complete seedling was found on only full strength of medium and hormone supplemented medium. The lower strength (half and quarter strength) of both medium were not effective for development of seedling. Complete seedling was developed earlier on 27 weeks of culture on full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA followed by hormone free full strength of MS medium which took 30 weeks of culture for seedling development (Fig. SG10). Similar results were also obtained on KC medium where only hormone free full strength of KC medium (Fig. SG11) and full strength of KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA (Fig. SG12) gave complete seedling which took 31 weeks and 30 weeks of culture respectively for their seedling development (Table 3).

Figure SG: Flowers, Pods and Seed Germination of *Cymbidium aloifolium* (L.) Sw.

Fig. SG 01 Naturally growing of *C. aloifolium*

Fig. SG 02 Blooming flower of *C. aloifolium*

Fig. SG 03 Immature capsule of *C. aloifolium*

Fig. SG 04 Longitudinal section of capsule of *C. aloifolium* showing yellowish tiny seeds

Fig. SG 05 Seed cultured on hormone free MS basal medium showing small green globular mass of small protocorms after 10 weeks of culture

Fig. SG 06 Seed cultured on full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA showing yellowish green globular hairy mass of protocorm

Fig. SG 07 Seed cultured on hormone free full strength of MS medium showing large number of green globular hairy mass of protocorm

Fig. SG 08 Seed cultured on quarter strength of KC medium showing the yellowish green globular hairy mass of protocorm with the formation of phenolic compound which affects the growth of seedlings

Fig. SG 09 Seed cultured on full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA showing small shoots developed from green globular protocorms

Fig. SG 10 Seed cultured on hormone free MS medium showing many green plantlets with small roots

Fig. SG 11 Seed cultured on KC medium showing the yellowish green plantlets with small roots

Fig. SG 12 Seed cultured on full strength of KC medium supplemented with 0.5mg/l BAP and 0.5 mg/l NAA showing the green plantlets with small roots

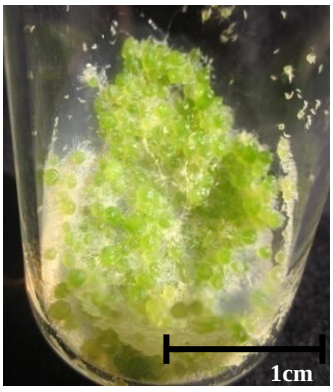
SG 01

SG 02

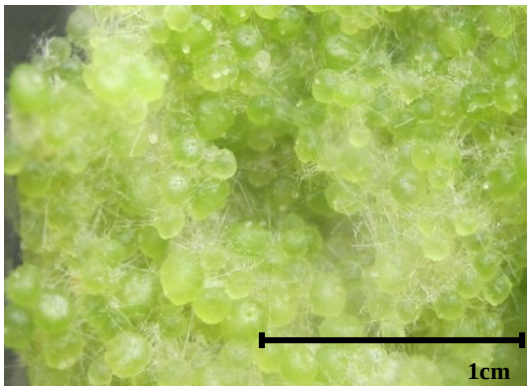
SG 03

SG 04

SG 05



SG 06

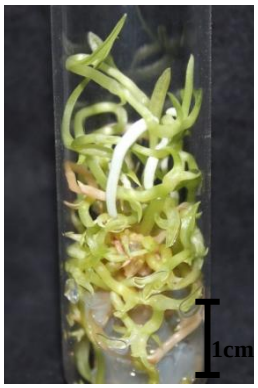


SG 07

SG 08

SG 09

SG 10

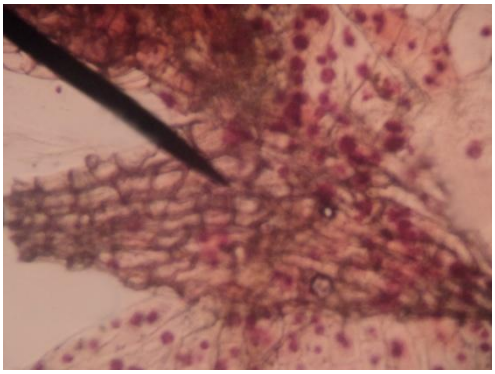


SG 11

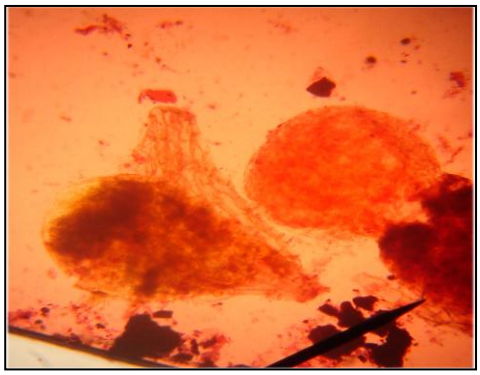
SG 12

4.1.2 Histomorphological study

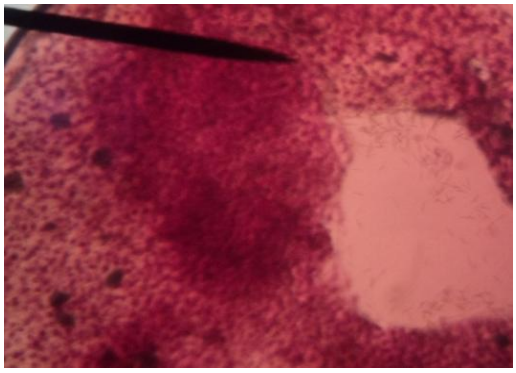
In vitro culture of seeds of *Cymbidium aloifolium* undergoes differentiation to produce globular embryo called protocorms which later undergoes various multiplication stages and finally developed seedlings. The histomorphological study of protocorms showed the serial developmental stages upto the seedling. In present investigation, globular hairy protocorms were formed after 3 weeks (twenty one days) of its initiation which was considered as the appropriate developmental stage of protocorm. After few weeks, single pair of leaf primordia emerged out from the promeristematic cells of proximal part of embryo which finally developed into embryonic photosynthetic leaves. Simultaneously, the marginal cells at the distal part of embryo gave rise to tubular and unicellular rhizoids (Fig. H).



a. Seed coat



b. Globular embryo emerging out of seed coat



c. Globular embryo with leaf primordia

Figure H: Sequential stages of proliferation of embryo to plantlets (a, b & c)

4.1.3 Micropropagation of *Cymbidium aloifolium*

4.1.3.1 *In vitro* culture of a single protocorm of *C. aloifolium*

Protocorms were the swelling of embryos that developed into green spherules. Protocorms were derived from *in vitro* culture of seeds. Three weeks old medium

sized protocorms (about 3±1 mm diameter) were used as explants for *in vitro* culture of *C. aloifolium*. They were inoculated on different strength of solid and liquid MS and KC media (full, half and quarter) supplemented with or without different concentrations of plant hormones viz. BAP and NAA and showed various responses. The culture of single protocorm was either differentiated into small protocorms, embryogenic callus and/or directly regenerated into seedling.

Differentiation of a single protocorm on different strength of solid and liquid MS and KC media supplemented with or without different concentrations of hormones was summarized on Table 4 and 5.

4.1.3.1.1 *In vitro* development and differentiation of single protocorm on solid MS medium

Individual protocorm were sub-cultured on agar fortified MS medium (solid) and MS medium supplemented with or without different concentrations and combinations of BAP (0.5 mg/l – 2.0 mg/l) and NAA (0.5 mg/l – 1.0 mg/l) (Fig. P01, Table 4). Each protocorm underwent differentiation however complete seedling was obtained only in few tested condition. The earlier response of protocorm differentiation was observed on MS + 0.5 mg/l BAP + 0.5 mg/l NAA where protocorm start to develop on 5 weeks of culture however earlier seedling development was observed on hormone free full strength of MS medium. On this medium, highest percentage (83.33%) of seedling was observed on shortest period of time (21.33±0.33 weeks of culture) (Fig. P05). One-way ANOVA showed that value of all the growth parameters varied significantly among different tested conditions of MS medium ($p \leq 0.05$; Table 4).

The following changes were found during the development of protocorms on different strength of solid MS medium and MS medium supplemented with or without BAP and NAA.

Development of a single protocorm

All the tested conditions of MS medium responded protocorms development. The earlier differentiation of protocorm was found on full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA where a single protocorm start to develop on 5weeks of culture followed by hormone free full strength of MS medium which took 5.17±0.31 weeks of culture for protocorm development (Fig. P02). The time taken for initiation of protcorm development was increased from 5.83±0.31

weeks of culture to 7.50±0.22 weeks of culture with increasing the concentration of BAP from 0.5 mg/l to 2.0 mg/l. Similarly, the combined effect of higher concentration of BAP (0.5 mg/l -2.0 mg/l) and NAA (0.5 mg/l - 1.0 mg/l) also elongate the time period from 5 weeks to 7.50±0.22 weeks of culture for protocorm development.

Table 4: Effect of agar fortified MS media on *in vitro* germination and seedling development from a single protocorm of *Cymbidium aloifolium*

Media	Plant hormones	Observation taken in weeks (Mean ± SE)					% of Seedling development
		Initiation of germination/ development	Initiation of shoot primordia	Initiation of leaf primordia	Initation of root primordia	Seedling	
MS		5.17±.31	8.33±.42	11.67±.42	16.17±.48	21.33±.33	83.33
½ MS		7.00±.26	11.83±.31	14.50±.43	18.50±.34	22.67±.42	66.67
¼ MS		7.50±.22	13.00±.52	17.00±.73	.00±0.00	.00±0.00	-
MS	0.5 BAP	5.83±.31	9.33±.33	13.00±.52	18.00±.58	23.00±.52	58.33
MS	1.0 BAP	6.83±.17	11.83±.17	15.50±.22	.00±0.00	.00±0.00	-
MS	1.5 BAP	7.17±.17	12.83±.31	16.33±.33	.00±0.00	.00±0.00	-
MS	2.0 BAP	7.50±.22	13.17±.17	17.00±0.00	.00±0.00	.00±0.00	-
MS	0.5 NAA	6.50±.22	11.00±.26	15.00±.26	18.50±.43	22.50±0.22	50
MS	0.5 BAP + 0.5NAA	5.00±0.00	8.50±.22	12.00±.26	16.17±.48	21.50±.50	80
MS	1.0 BAP + 0.5NAA	5.33±0.49	9.17±.48	13.00±.58	17.00±.52	22.00±.45	66.67
MS	1.5 BAP + 0.5NAA	6.00±.26	10.17±.17	13.00±.58	17.17±.54	22.17±.54	58.33
MS	2.0 BAP + 0.5NAA	6.00±.26	10.33±.33	13.50±.34	17.00±.45	22.83±.40	50
MS	1.0 NAA	7.50±.22	11.67±.21	16.00±.36	19.17±.40	23.00±.45	25
MS	0.5 BAP + 1.0NAA	5.50±.22	10.50±.22	14.33±.33	19.00±.36	23.00±.36	33.33
MS	1.0 BAP +1.0NAA	6.00±.26	9.17±.31	13.33±.33	.00±0.00	0.00±0.00	-
MS	1.5 BAP + 1.0NAA	6.50±.22	10.50±.22	14.67±.21	.00±0.00	0.00±0.00	-
MS	2.0 BAP +1.0NAA	6.83±.31	11.00±.36	15.33±.33	.00±0.00	0.00±0.00	-
F-value		10.329	23.815	16.766	642.288	1192.191	
p-value		.000	.000	.000	.000	.000	

Culture conditions: various concentrations of solid MS medium, 25±2˚C, 24 weeks, 16/8 hrs photoperiod; 6 replicates were used in each condition, ($p \leq 0.05$, F= 16, 85)

Initiation of shoot primordia

The earlier response on initiation of shoot primordia from protocorm was observed on hormone free full strength of MS medium which took 8.33±0.42 weeks of culture.

This condition was followed by full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 8.50±0.22 weeks of culture for shoot initiation. However, full strength of MS medium supplemented with 2 mg/l BAP took longer time, i.e., 13.17±0.17 weeks of culture for development of shoot than other treatments. The increase concentration of BAP alone and combined with NAA also suppressed the shoot initiation.

Initiation of leaf primordia

The development of leaf primordia was found earlier on hormone free full strength of MS medium on 11.67±0.42 weeks of culture than other tested conditions of MS medium. This condition was followed by full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA where leaf primordia was initiated on 12±0.26 weeks of culture. However, quarter strength of MS medium and hormone free MS medium supplemented with 2 mg/l BAP took 17 weeks of culture for leaf initiation.

Initiation of root primordia

The earlier development of root primordia was observed on 16.17±0.48 weeks of culture on hormone free full strength of MS medium and full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA (Fig. P04). Whereas full strength of MS medium supplemented with 1 mg/l NAA took longer time, i.e., 19.17±0.4 weeks of culture for root initiation than other tested conditions. However higher concentration of BAP alone and combined effect of higher concentration of BAP and NAA were not effective for the development of roots.

Development of seedling

The complete seedling was found earlier on hormone free full strength of MS medium (Fig. P05) on 21.33±0.33 weeks of culture with highest percentage of seedling development (83.33%) followed by full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA (Fig. P06) which took 21.5±0.5 weeks of culture with 80% of seedling development. Whereas full strength of MS medium supplemented with 1 mg/l NAA took 23±0.45 weeks of culture which was the longest time period for seedling development among other tested conditions. On this condition, only 25% of seedlings were developed. However, higher concentration of BAP alone and combined effect of higher concentration of BAP and NAA were not

effective for the development of seedlings.

4.1.3.1.2 *In vitro* development and differentiation of a single protocorm on liquid MS and KC media

Hormone free full strength of liquid MS medium was found to be the appropriate condition for earlier protocrom differentiation and elongation of mini-seedling. In this culture condition, the complete seedling was observed on shortest period of time (20.50±0.34 weeks of culture; $p< 0.001$) with highest percentage (83.33%) of seedling development compared to other tested conditions of MS medium (Table 5). One-way ANOVA showed that value of all the growth parameters except initiation of germination and shoot primordia varied significantly among different treatment conditions of MS medium ($p<0.01$). However, on KC medium, the result was significant only in the case of shoot and root initiation and seedling development ($p<0.01$; Table 5).

Table 5: Effect of MS and KC liquid media on *in vitro* germination and seedling development from a single protocorm of *Cymbidium aloifolium*

Media	Plant hormones	Observation taken in weeks (Mean ± SE)					% of seedling development
		Initiation of development	Initiation of shoot primordia	Initiation of leaf primordia	Initation of root primordia	Seedling	
MS		4.17 ± 0.17	6 ± 0.36	10 ± 0.68	13.83 ±0.65	20.5 ± 0.34	83.33
½ MS		4.83 ± 0.65	6.67 ± 0.71	10.5 ± 0.84	14.33 ±1.12	20.8 ± 0.3	66.67
¼ MS		4.67 ± 0.33	6.67 ± 0.42	11 ± 1.67	15 ± 0.63	21.33 ± 0.21	66.67
MS	0.5 BAP + 0.5 NAA	5.33 ± 0.42	8 ± 0.68	15 ± 0.57	23.5 ± 0.22	23.83 ±0.16	16.67
F-value		1.244	2.184	4.846	39.546	32.255	
p-value		.320	.122	.011	.000	.000	
KC		5.17 ± 0.30	6.67 ± 0.33	10.5 ± 1.56	14.5 ± 1.08	21.17 ± 0.79	66.67
½ KC		6 ± 0.36	8.17 ± 0.65	12.8 3 ± 1.85	15.33 ±0.88	21.67 ± 0.55	50
¼ KC		6.33 ± 0.5	7.5 ± 0.43	14.33 ± 2.29	0	0	0
KC	0.5 BAP + 0.5 NAA	6.5 ± 0.76	9.17 ± 0.47	17.5 ± 1.84	0	0	0
F-value		1.333	4.708	2.371	151.516	651.548	
p-value		.292	.012	.101	.000	.000	

Culture conditions: various strength of MS and KC liquid media, 25 ± 2˚C, 24 weeks, 16/8 hrs photoperiod; 6 replicates were used in each condition, ($p\leq0.05$, F= 3, 20)

The following changes were found during development of protocorms on different strength of liquid MS and KC media and both medium supplemented with or without 0.5 mg/l BAP and 0.5 mg/l NAA.

Development of a single protocorm

Among the various tested conditions of liquid MS and KC media, hormone free full strength of MS medium gave earliest response for initial development of protocorm on 4.17±0.17 weeks of culture. On this condition, single protocorm become green and elongate to produce miniseedling. The half strength and quarter strength of liquid MS medium took 4.83±0.65 weeks of culture and 4.67±0.33 weeks of culture respectively for initial differentiation of protocorm which time period was very close to full strength of liquid MS medium. However, protocorm was started to develop on 5.33±0.42 weeks of culture on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was quite longer than other strength of liquid MS medium. Hence, it was found that the time period for development of protocorm was increased with decreasing the strength of MS medium and medium supplemented with hormones. Similar trend was also obtained on liquid KC medium. A single protocorm was started to develop on 5.17±0.31 weeks of culture on hormone free full strength of liquid KC medium. This condition showed the earliest response on protocorm development than half strength, quarter strength and full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 6±0.365 weeks of culture, 6.33±0.5 weeks of culture and 6.5±0.76 weeks of culture respectively. A single protocorm also gave rise to multiple protocorms on half strength of liquid MS medium and full strength of KC medium whereas few amount of yellowish white callus was observed on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. The value of all the tested conditions of both media was not significant at 5% level of significance.

Initiation of shoot primordia

The shoot primordia were developed either directly from protocoroms or protocorm like bodies. Hormone free full strength of MS medium gave earliest response on

development of first shoot primordia on 6±0.36 weeks of culture (Fig. P07) than other tested conditions of liquid MS and KC media. The half strength and quarter strength of liquid MS medium took 6.67 ±0.71 weeks of culture and 6.67±0.42 weeks of culture respectively which was almost same time period for shoot initiation (Fig. P08). Whereas full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA took 8±0.68 weeks of culture for initiation of shoot primordia which were quite longer time period than different strength of MS medium (Fig. P09).

Among the different tested conditions of liquid KC medium, earliest response on initiation of shoot primordia was found on hormone free full strength of liquid KC medium which took 6.67±0.33 weeks of culture (Fig. P10). This condition was followed by quarter strength of liquid KC medium which took 7.5±0.43 weeks of culture for development of shoot (Fig. P11). Similarly, the half strength and full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA took 8.17±0.65 weeks of culture and 9.17±0.48 weeks of culture respectively for development of shoot. Later the shoots developed from protocorms on full strength of liquid KC medium supplemented with hormones become yellowish and lose their regeneration. Here, the values of all the tested conditions of liquid KC medium were significant at $p < 0.01$ however the values of liquid MS medium were not significant.

Initiation of leaf primordia

The development of leaf primordia was found earlier on 10±0.68 weeks of culture on hormone free full strength of liquid MS medium rather than other tested conditions of liquid MS and KC media. The half strength, quarter strength and full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA took 10.5±0.84 weeks of culture, 11±1.67 weeks of culture and 15±0.57 weeks of culture respectively for initiation of leaf primordia. Here, the time taken for development of leaf was increased with decreasing the strength of MS medium and medium supplemented with hormones.

Similar results was also obtained on liquid KC medium where full strength, half strength, quarter strength and full strength of liquid KC medium supplemented with

0.5 mg/l BAP and 0.5 mg/l NAA took 10.5±1.56 weeks of culture, 12.83±1.85 weeks of culture, 14.33±2.29 weeks of culture and 17.5±1.83 weeks of culture respectively for initiation of leaf primordia. Later, the leaves become non-chlorophyllous on above mentioned KC medium. The values of all the tested conditions of liquid MS medium were significant at p <0.01 however the values of liquid KC medium were not significant.

Initiation of root primordia

Almost all the tested conditions of liquid MS medium favoured the development of roots. However, among the different tested conditions of liquid KC medium, only hormone free full strength and half strength of liquid KC medium gave roots from protocorm. The earlier development of root primordia was observed on 13.83±0.65 weeks of culture on hormone free full strength of liquid MS medium as compare to other treatments. The half strength, quarter strength and full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA took 14.33±1.12 weeks of culture, 15±0.63 weeks of culture and 23.5±0.22 weeks of culture respectively for initiation of root primordia. From the result, it was found that lower strength of medium and medium supplemented with hormones required longer time for development of roots. Similar result was also obtained on liquid KC medium where hormone free full strength and half strength of liquid KC medium took 14.5±1.09 weeks of culture and 15.33±0.88 weeks of culture respectively for initiation of root. The quarter strength and full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA were not effective for the development of roots. The value of all the tested conditions of both medium was significant at p <0.05.

Development of seedling

All the tested conditions of liquid MS medium favoured the development of seedling. Hormone free full strength of liquid MS medium gave earliest response on complete development of seedling from a single protocorm on 20.5±0.34 weeks of culture (Fig. P12). On this condition, about 83.33% of seedlings were developed which was the highest seedling percentage compare to other treatments. However, the lowest

percentage of seedling development (16.67%) was found on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. The time taken for complete seedling development on half strength, quarter strength and full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA was 20.8±0.3 weeks of culture, 21.33±0.21 weeks of culture and 23.83 ±0.16 weeks of culture respectively which showed longer period of seedling development in lower strength of medium and medium supplemented with hormones. Similar trend was also found on liquid KC medium where hormone free full strength and half strength of liquid KC medium took 21.17±0.79 weeks of culture and 21.67±0.56 weeks of culture with 66.67% and 50% of seedling development respectively. However, quarter strength and full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA were not effective for the development of complete seedling till 24 weeks of culture.

From the result of protocorm development on different hormonal conditions on solid and liquid MS medium, it was found that the increased concentration of BAP and NAA inhibit the growth and development of seedling of *Cymbdium aloifolium*. All the tested conditions took long time for development of protocorm, initiation of shoot, root and also developed small seedlings on solid MS medium after 24 weeks of culture as compare to liquid MS medium. Hence, these data draw attention to the use of BAP and NAA for the further experiment. So, onwards low concentrations of BAP and NAA as well as low strength of MS and KC media were used in the present investigation in order to get the long and healthy seedlings of *C. aloifolium* for mass propagation.

Figure P: Development of a single protocorm of *Cymbidium aloifolium* on liquid and solid MS and KC media

Fig. P 01 *In vitro* culture of a single protocorm on hormone free full strength of solid MS medium

Fig. P 02 Development of shootbuds on hormone free full strength of solid MS medium

Fig. P 03 Development of small shoot on hormone free half strength of solid MS medium

Fig. P 04 Development of shoot with roots on hormone free full strength of solid MS medium

Fig. P 05 Development of many plantlets on hormone free full strength of solid MS medium

Fig. P 06 Development of plantlets on full strength of solid MS medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA

Fig. P 07 Development of many shootbuds on hormone free full strength of liquid MS medium

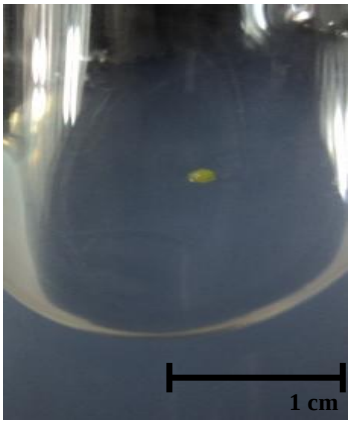
Fig. P 08 Development of multiple protocorms on hormone free half strength of liquid MS medium from a single protocorm.

Fig. P 09 Development of multiple shoots and small shoot buds on full strength of liquid MS medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA

Fig. P 10 Development of many shoot buds on hormone free half strength of liquid KC medium

Fig. P 11 Development of many shootbuds on hormone free quarter strength of liquid KC medium

Fig. P 12 Development of plantlets on hormone free full strength of liquid MS medium



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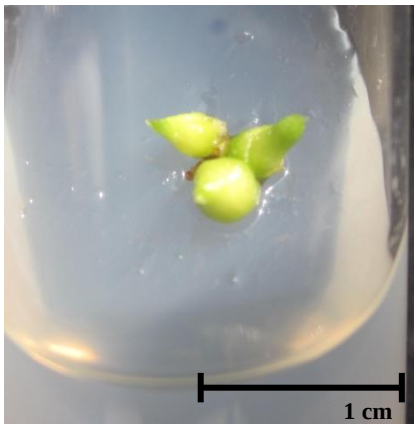

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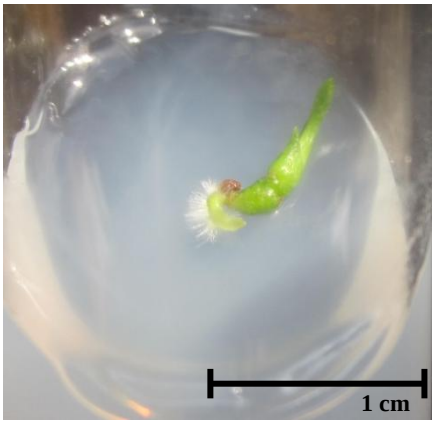

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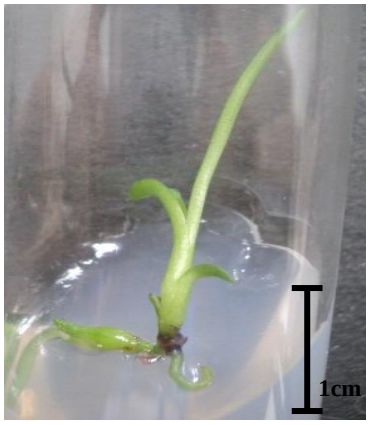

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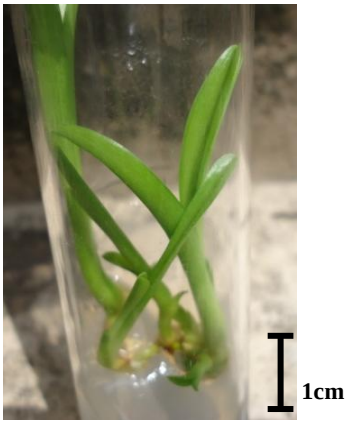

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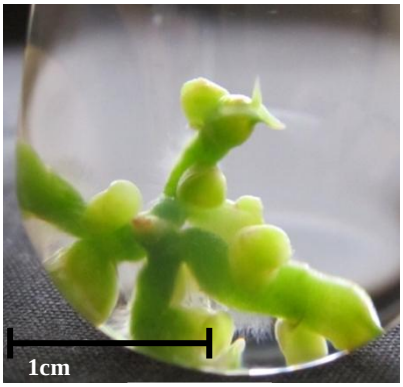

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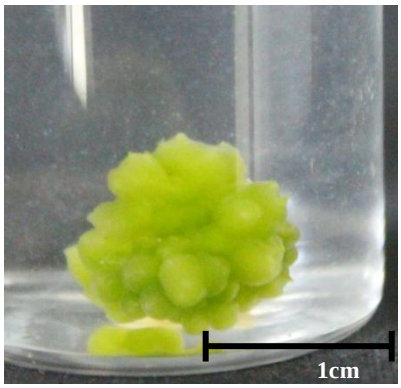

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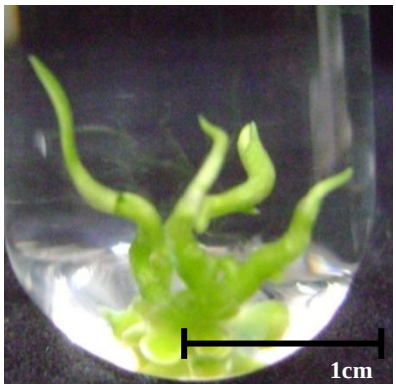

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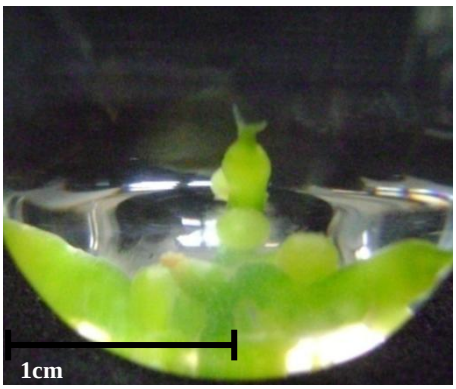

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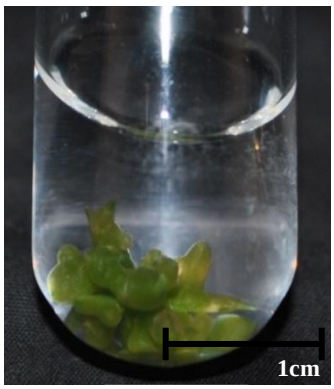

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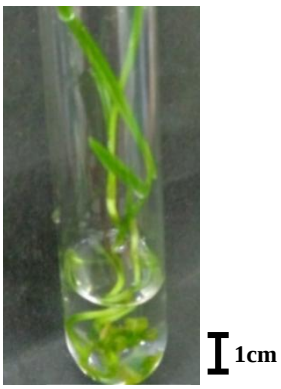


P 12

4.1.3.2 *In vitro* culture of artificial seeds of *Cymbidium aloifolium*

In vitro grown, young medium sized (approx. 3 ±1 mm diameters), three months old green protocorms were used as explants for production of different artificial seeds. Different percentage of sodium alginate (2%, 3% and 4%) and 0.2M calcium dihydrate solution ($CaCl_2.2H_2O$) were used for the production of round to somewhat elongated beads of artificial seeds. Thus produced artificial seeds were inoculated on four different conditions of liquid and solid MS and KC media supplemented with or without different concentrations of plant growth hormones.

4.1.3.2.1 *In vitro* production of artificial seeds in different concentration of sodium alginate

The 2%, 3% and 4% (w/v) alginate coated artificial seeds of *C. aloifolium* were produced in the laboratory by encapsulating young and green protocorm with 2%, 3% and 4% (w/v) sodium alginate and 0.2M $CaCl_2.2H_2O$ solution (Fig. 2AS1, 3AS1 & 4AS1).

4.1.3.2.2 *In vitro* germination of 2%, 3% and 4% alginate coated artificial seeds on liquid MS and KC media

For the *in vitro* germination, the freshly prepared 2%, 3% and 4% (w/v) alginate coated artificial seeds of *C. aloifolium* were inoculated on different strength of liquid MS and KC media supplemented with or without different concentrations of BAP and NAA. The data was observed and recorded till 24 weeks of culture. Full strength of liquid MS and KC media was found to be effective for earlier germination and seedling development from artificial seeds. One-way ANOVA for *in vitro* germination of 2% alginate coated artificial seeds on liquid medium showed that among the growth parameters studied only the initiation of leaf primordia and seedling development varied significantly among different conditions of liquid MS medium ($p \leq 0.05$; Appendix 5) whereas on liquid KC medium, initiation of germination, shoot and seedling development were significantly different than other tested conditions ($p < 0.05$; Appendix 6). Similarly, One-way ANOVA for *in vitro* germination of 3% alginate coated artificial seeds on liquid medium showed that among the growth parameters studied only the initiation of leaf primordia and seedling development varied significantly among different tested conditions of liquid MS medium ($p < 0.05$; Appendix 7). However, value of all the growth parameters

except initiation of germination varied significantly among different tested conditions of liquid KC medium ($p < 0.05$; Appendix 8). One-way ANOVA for *in vitro* germination of 4% alginate coated artificial seed on liquid medium showed that among the growth parameters studied; only the initiation of leaf primordia and seedling development were varied significantly among different tested conditions of liquid MS medium ($p < 0.05$; Appendix 9). However, value of all the growth parameters were varied significantly among different tested conditions of liquid KC medium ($p < 0.05$; Appendix 10).

The following responses were observed in different conditions during *in vitro* germination of 2%, 3% and 4% (w/v) alginate coated artificial seed of *C. aloifolium* (Figs. 1-5).

Initiation of germination

All the tested conditions of liquid MS and KC media favoured *in vitro* germination of artificial seeds of *Cymbidium aloifolium* (Fig. 1). 2% alginate coated artificial seeds were germinated quickly (4 weeks of culture) on hormone free full strength and half strength of liquid MS medium rather than 3% and 4% alginate coated artificial seeds. On hormone free quarter strength of liquid MS medium and full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA, *in vitro* germination of 2% alginate coated artificial seeds took 4.5±0.22 weeks and 4.83 ±0.48 weeks of culture respectively. It was found that germination period was longer on lower strength medium and medium supplemented with hormones. Similar trend of germination was also showed by 3% alginate coated artificial seeds which took 4.33±0.21 weeks, 4.67±0.33 weeks, 5±0.52 weeks and 5±0.45 weeks of culture for initiation of germination on hormone free full strength, half strength, quarter strength and full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA respectively. However, 4% alginate coated artificial seeds showed consistency result on their germination, i.e., they were germinated on 7 weeks of culture on all the tested conditions of liquid MS medium. Statistical analysis revealed that *in vitro* germination of 2%, 3% and 4% alginate coated artificial seeds were not varied significantly at $p \leq 0.05$ among all the tested conditions of liquid MS medium.

Among different tested conditions of liquid KC medium, hormone free full strength of KC medium was effective for earlier germination of 2%, 3% and 4% alginate coated artificial seeds. On this medium, 2% alginate coated artificial seeds showed fastest

germination (4 weeks of culture) than 3% (6.17±0.4 weeks of culture) and 4% (6.17±0.17 weeks of culture) alginate coated artificial seeds. The late germination of 2% and 4% alginate coated artificial seeds were observed on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 5.67±0.61 weeks of culture and 8.5±0.88 weeks of culture respectively. However, 3% alginate coated artificial seeds germinated lately on hormone free quarter strength of liquid KC medium which took 7.67±0.49 weeks of culture. Statistical analysis revealed that *in vitro* germination of 2% and 4% alginate coated artificial seeds showed significantly different at $p \leq 0.05$ among all the tested conditions of liquid KC medium however 3% alginate coated artificial seeds were not significant among similar tested conditions.

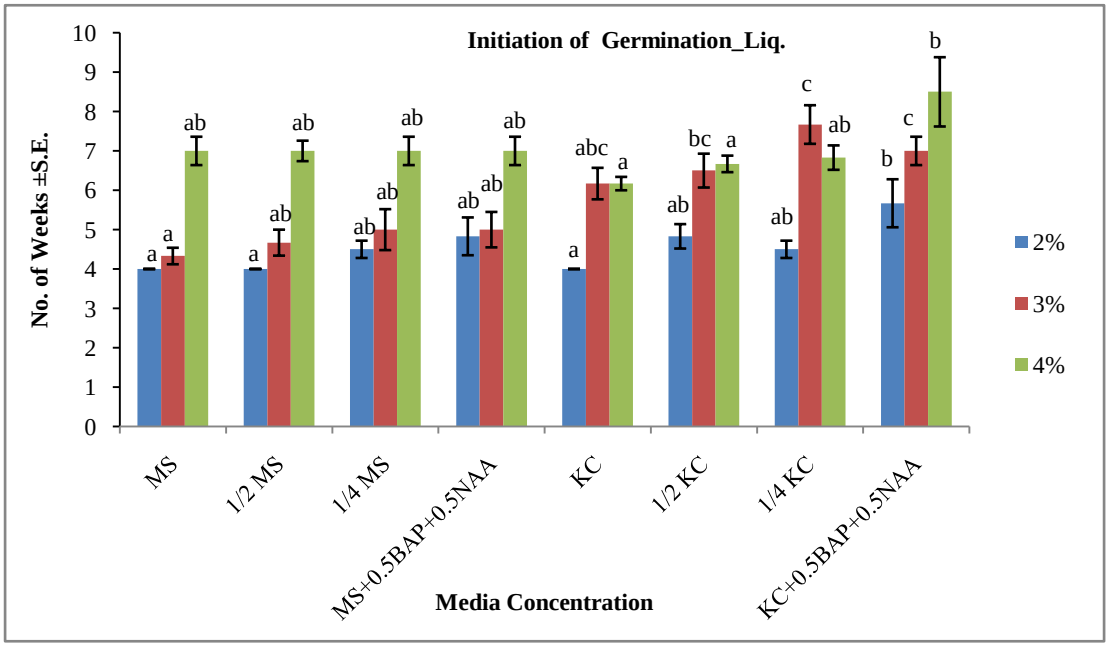


Figure 1: *In vitro* germination of artificial seeds of *C. aloifolium* after 24 weeks of culture on liquid MS and KC media. [The figure showed the statistical comparision on duration of initiation of germination of different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Initiation of shoot primordia

After germination, small green shoot primordia were developed from artificial seeds. The development of shoot was observed on all the tested condition of liquid MS and KC media (Fig. 2). 2% alginate coated artificial seeds gave the earliest response for initiation of first shoot primordia on 5±0.36 weeks of culture on hormone free full strength of liquid MS medium (Fig. 2AS2) than 3% and 4% alginate coated artificial seeds. 3% alginate coated artificial seeds also gave earlier response for development

of first shoot primordia on 5.67±0.42 weeks of culture on hormone free full strength of liquid MS medium (Fig. 3AS2) than other treatment conditions of liquid MS medium. However, 4% alginate coated artificial seeds gave the first shoot primordia quickly on 8±0.36 weeks of culture on hormone free full strength (Fig. 4AS2) and quarter strength of liquid MS medium than other treatment conditions. 2% and 4% alginate coated artificial seeds took longer time, i.e., 6.67±0.67 weeks and 8.33±0.42 weeks of culture respectively for initiation of shoot primordia on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Whereas 3% alginate coated artificial seeds developed shoot primordia lately on 7±0.68 weeks of culture on hormone free quarter strength of liquid MS medium. Statistically, the shoots initiated from all the artificial seeds were not significantly different at $p \leq 0.05$ among all the tested conditions of liquid MS medium.

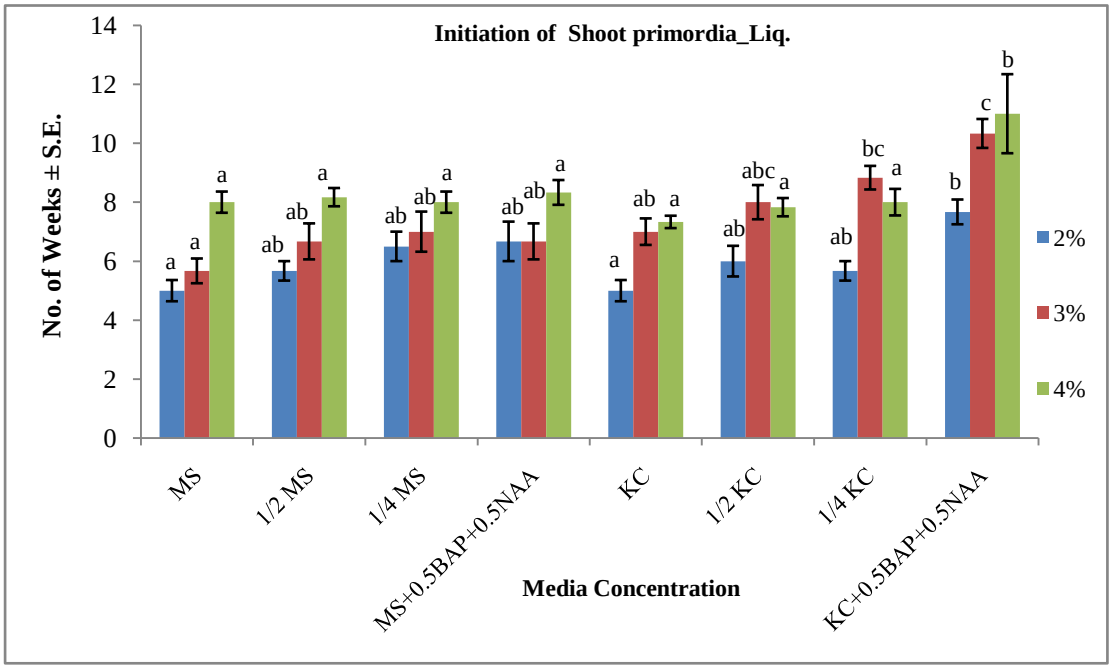


Figure 2: Initiation of shoot promordia from artificial seeds of *C. aloifolium* after 24 weeks of culture on liquid MS and KC media. [The figure showed the statistical comparision on duration of initiation of shoot primordia from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

On liquid KC medium, all the artificial seeds showed their quick response on development of first shoot primordia on hormone free full strength of KC medium. Among different artificial seeds, 2% alginate coated artificial seed gave earliest response for initiation of shoot on 5±0.36 weeks of culture than 3% and 4% alginate coated artificial seed which took 7±0.45 weeks and 7.33±0.21 weeks of culture respectively on hormone free full strength of KC medium. The longest time period for

initiation of shoot was observed on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. On this condition, 2%, 3% and 4% artificial seeds took 7.67±0.42 weeks, 10.33±0.49 weeks and 11±1.34 weeks of culture respectively for shoot initiation. Statistical analysis revealed that the shoot initiated from all the artificial seeds were significantly varied at $p \leq 0.05$ among all the tested conditions of liquid KC medium.

Initiation of leaf primordia

Green leaf primordia were developed on all the tested conditions of liquid MS and KC media (Fig. 3). Among different artificial seeds, 3% alginate coated artificial seeds gave the earliest response for development of first leaf primordia on 8±0.26 weeks of culture on hormone free full strength of liquid MS medium. On same condition, 2% alginate coated artificial seeds also showed earlier response for initiation of first leaf primordia on 8.33±1.12 weeks of culture than other tested conditions. However, earlier response for leaf initiation by 4% alginate coated artificial seeds was observed on 9.5±0.5 weeks of culture on hormone free quarter strength of liquid MS medium. All the artificial seeds (2%, 3% and 4%) showed their late response for initiation of leaf on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA and took 13±0.93 weeks, 10.83±0.79 weeks and 17.5±0.87 weeks of culture respectively for leaf initiation. Statistical analysis revealed that the leaf primordia developed from all the artificial seeds were significantly different at $p \leq 0.05$ among all the tested conditions of liquid MS medium.

On liquid KC medium, 2% artificial seeds gave earlier response for initiation of leaf on 7.5±0.99 weeks of culture on hormone free full strength of KC liquid medium than 3% and 4% alginate coated artificial seeds. However same condition was also effective for earlier development of leaf from 3% and 4% alginate coated artificial seeds which took 8.67±0.84 weeks and 16.67±1.85 weeks of culture respectively compare to other tested conditions of liquid KC medium. It was found that lower strength of medium and medium supplemented with hormones took longer time period for leaf initiation from 2% and 3% alginate coated artificial seeds. Whereas 4% alginate coated artificial seeds initiated leaf primordia lately on 19±1.61 weeks of culture on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and

0.5 mg/l NAA. Statistically, the leaf primordia developed from 2% alginate coated artificial seeds, were not significantly different while leaf primordia developed from 3% and 4% alginate coated artificial seeds were significantly different at $p \leq 0.05$ among all the tested conditions of liquid KC medium.

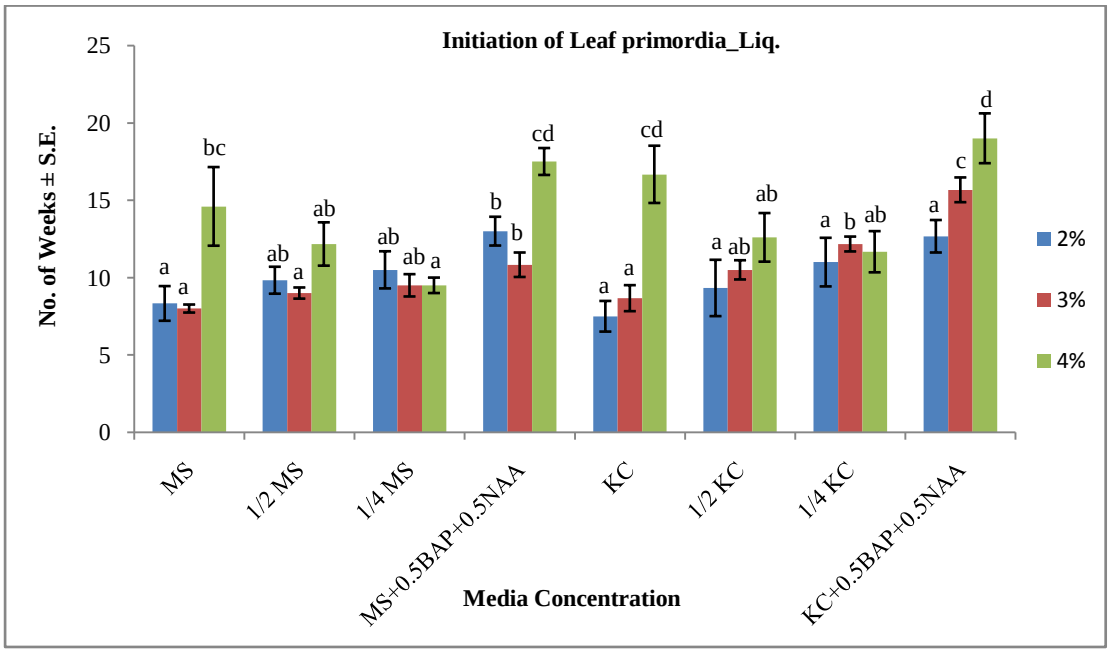


Figure 3: Initiation of leaf primordia from artificial seeds of *C. aloifolium* after 24 weeks of culture on liquid MS and KC media [The figure showed the statistical comparision on duration of initiation of leaf primordia from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Initiation of root primordia

The development of root was observed on all the tested conditions of liquid MS and KC media (Fig. 4). 2% alginate coated artificial seeds gave earliest response on 10±1.12 weeks of culture on full strength of liquid MS medium for initiation of first root primordia than 3% and 4% alginate coated artificial seeds. On same condition, 3% alginate coated artificial seeds took 13±0.93 weeks of culture for earlier initiation of root than other tested condition of liquid MS medium. Whereas 4% alginate coated artificial seeds showed quick response on 13±4 weeks of culture on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA for development of root. However, 2% alginate coated artificial seeds took longer time (16.5±0.67 weeks of culture) for initiation of root on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. 3% alginate coated artificial seeds gave root primordia lately on 15±0.68 weeks of culture on hormone free half strength of liquid MS medium. Similarly, 4% alginate coated artificial seeds

showed their late response on hormone free half strength of liquid MS medium which took 17.2±2.15 weeks of culture for root development. Statistical analysis revealed that root primordia developed from 2%, 3% and 4% alginate coated artificial seeds were not varied significantly at $p \leq 0.05$ among all the tested conditions of liquid MS medium.

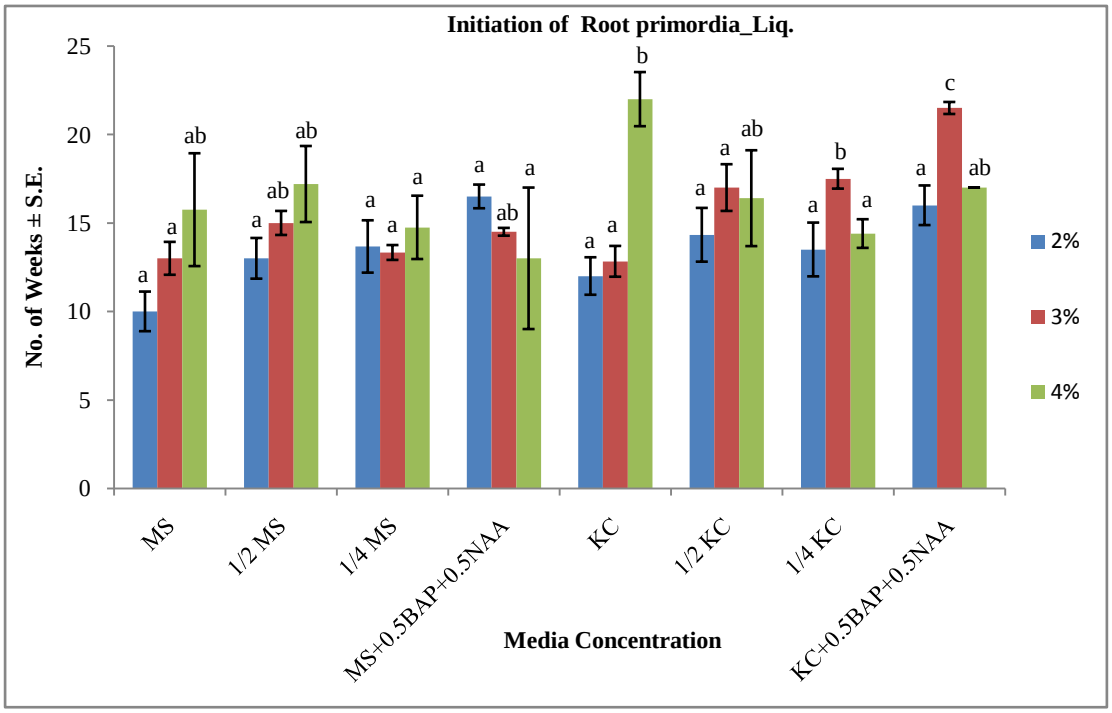


Figure 4: Initiation of root primordia from artificial seeds of *C. aloifolium* after 24 weeks of culture on liquid MS and KC media. [The figure showed the statistical comparision on duration of initiation of root primordia from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

On liquid KC medium, initiation of first root primordia was observed earlier from 2% alginate coated artificial seeds on hormone free full strength of KC medium on 12±1.06 weeks of culture. The time period for development of root was increased on lower strength of medium and medium supplemented hormones. Similar result was also obtained on 3% artificial seeds which took 12.83±.87 weeks of culture on hormone free liquid KC medium for earlier initiation of root while they showed late response for root development on 21.5±0.34 weeks of culture on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Similarly, 4% artificial seed took 14.4±0.81 weeks of culture on hormone free quarter strength of liquid KC medium for earlier initiation of root while they took 22±1.53 weeks of

culture on hormone free full strength of liquid KC medium which was late response as compare to other tested conditions for root initiation. Statistical analysis revealed that the root primordia developed from 2% alginate coated artificial seeds were not varied significantly however root primordia developed from 3% and 4% alginate coated artificial seeds were varied significantly at $p \leq 0.05$ among all the tested conditions of liquid KC medium.

Development of seedling

All the tested conditions of liquid MS and KC media gave complete seedling (Fig. 5). The quick response on development of seedling was observed on hormone free full strength of liquid MS medium showed by 3% alginate coated artificial seeds which took 18.5±0.84 weeks of culture (Fig. 3AS10) than other tested conditions of 2% and 4% artificial seeds. On hormone free half strength and quarter strength of liquid MS medium, 3% alginate coated artificial seeds took 20.5±0.7 weeks and 21.83±0.54 weeks of culture for development of seedling. However, they showed their late response on 22.5±0.22 weeks of culture on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Thus, it was found that lower strength of medium and medium supplemented with hormone increased the time period for seedling development. Similar trend was also found on 2% and 4% alginate coated artificial seeds which took 20.17±0.31 weeks of culture and 20±0.86 weeks of culture respectively for earlier seedling development on hormone free full strength of liquid MS medium whereas they developed seedling lately on 23±0.52 weeks of culture and 24 weeks of culture respectively on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA (Fig. 2AS9 & 4AS10). Statistically, the seedling development from all the artificial seeds was varied significantly at $p \leq 0.05$ among all the tested conditions of liquid MS medium.

Similar result was also obtained on liquid KC medium. 3% alginate coated artificial seeds gave earlier response on seedling development which took 19.33±0.95 weeks of culture on hormone free full strength of KC medium (Fig. 3AS7) rather than other tested conditions of 2% and 4% alginate coated artificial seeds. Among the different tested conditions of liquid KC medium, 2% alginate coated artificial seeds took 20±0.58 weeks of culture for earlier seedling development on hormone free full strength of liquid KC medium (Fig. 2AS10) whereas 4% alginate coated artificial

seed took 22±0.45 weeks of culture for earlier seedling development on hormone free quarter strength of liquid KC medium (Fig. 4AS6). However, all the artificial seeds took 24 weeks of culture for late seedling development on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Statistically, the seedlings developed from all the artificial seeds were significantly varied at $p \leq 0.05$ among all the tested conditions of liquid KC medium.

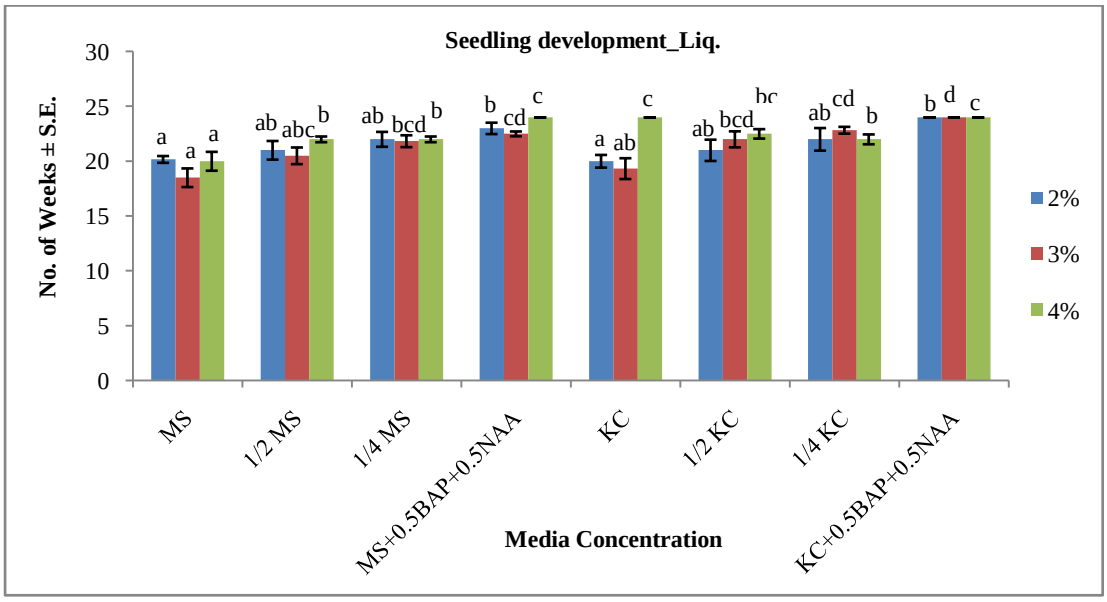


Figure 5: Development of seedling from artificial seeds of *C. aloifolium* after 24 weeks of culture on liquid MS and KC media. [The figure showed the statistical comparision on duration of seedling development from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

4.1.3.2.3 *In vitro* germination of 2%, 3% and 4% alginate coated artificial seeds on solid MS and KC media

For the *in vitro* germination, the freshly prepared 2%, 3% and 4% (w/v) alginate coated artificial seeds of *C. aloifolium* were inoculated on different strength of solid MS and KC media supplemented with or without different concentrations of BAP and NAA. The data was recorded and noted till 24 weeks. One-way ANOVA for *in vitro* germination of 2% alginate coated artificial seeds on solid medium showed that among the growth parameters studied only the initiation of germination varied significantly among different conditions of solid MS medium ($p < 0.05$; Appendix 11). However, value of all the growth parameters varied significantly among different tested conditions of solid KC medium at 5% level of significance (Appendix 12). One-way ANOVA for *in vitro* germination of 3% alginate coated artificial seeds on

solid medium showed that among the growth parameters studied, only the initiation of shoot, root and seedling development were varied significantly among different tested conditions of solid MS medium ($p < 0.05$; Appendix 13). However, value of all the growth parameters varied significantly among different tested conditions of solid KC medium ($p < 0.05$; Appendix 14). One-way ANOVA for *in vitro* germination of 4% alginate coated artificial seeds on solid medium showed that among the growth parameters studied, only the seedling development varied significantly among different tested conditions of solid MS and KC media ($p < 0.05$; Appendix 15 & 16).

The following responses were observed in different conditions during *in vitro* germination of 2%, 3% and 4% alginate coated artificial seeds of *C. aloifolium* (Fig. 6-10).

Initiation of germination

Among different artificial seeds, 2% alginate coated artificial seed showed earliest response on *in vitro* germination which took 4 weeks of culture on quarter strength of solid MS medium than other tested conditions (Fig. 6). However, 3% and 4% alginate coated artificial seeds germinated quickly on hormone free full strength of solid MS medium which took 4.83±0.31 weeks and 6.83±0.31 weeks of culture respectively than other tested conditions and also showed their delayed germination response on full strength of solid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 6.83±1.11 weeks and 7.83±0.87 weeks of culture respectively. Whereas 2% alginate coated artificial seeds germinated lately on hormone free full strength of solid MS medium which took 5.5±0.45 weeks of culture for initiation of germination. Statistical analysis revealed that the germination period of 3% and 4% alginate coated artificial seeds were not varied significantly however germination period of 2% alginate coated artificial seeds were significantly different at $p \leq 0.05$ among all the tested conditions of solid MS medium.

On solid KC medium, 2% alginate coated artificial seeds gave earlier germination response on 4.17±0.31 weeks of culture on hormone free quarter strength of KC medium than other tested conditions of 3% and 4% alginate coated artificial seeds. On the same condition, 3% alginate coated artificial seeds also germinated earlier on 5±0.82 weeks of culture than other tested conditions of solid KC medium. Whereas,

4% alginate coated artificial seeds showed their quick germination on hormone free full strength of KC medium which took 6.33±0.21 weeks of culture than other tested condition of solid KC medium. It was found that the duration for germination of 3% alginate coated artificial seeds were increased with increasing the salt concentration of medium. However, the germination period of 4% alginate coated artificial seed was increased in low salt concentration of medium and medium supplemented with hormones. All the artificial seeds (2%, 3% and 4%) showed their late germination on full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 6.17±0.31 weeks, 8.5±1.15 weeks and 8±1.41 weeks of culture respectively. Statistical analysis revealed that *in vitro* germination of 4% alginate coated artificial seeds were not varied significantly however 2% and 3% alginate coated artificial seeds were significantly varied at $p \leq 0.05$ among all the tested conditions of solid KC medium (Fig. 6).

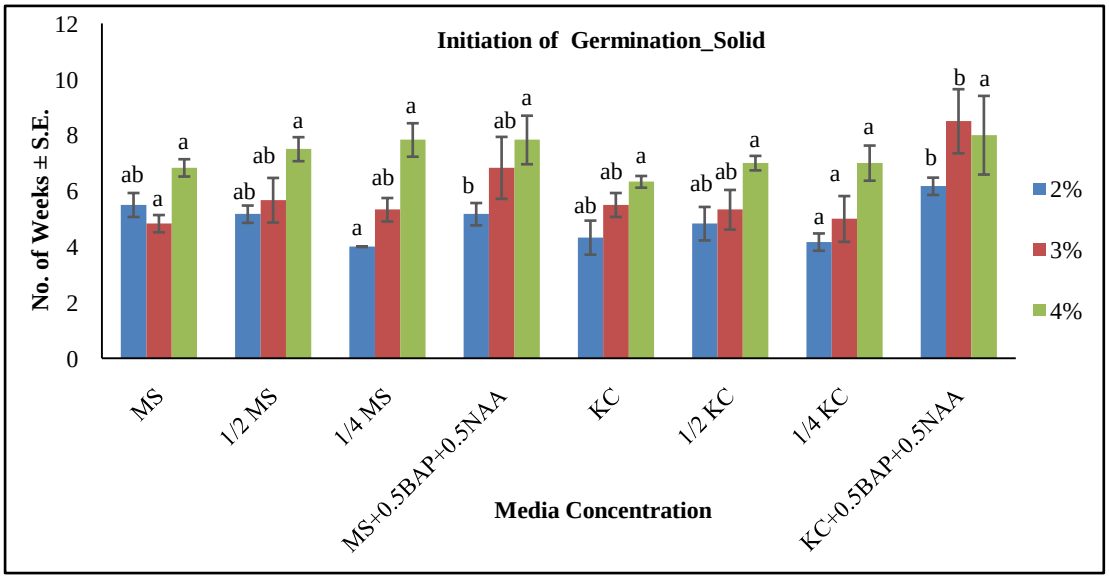


Figure 6: *In vitro* germination of artificial seeds of *C. aloifolium* after 24 weeks of culture on solid MS and KC media. [The figure showed the statistical comparision on duration of initiation of germination of different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Initiation of shoot primordia

All the tested conditions of solid MS and KC media developed shoots (Fig. 7). It was found that 2% alginate coated artificial seeds showed earlier response for shoot development on hormone free quarter strength of solid MS medium which took 6.17±0.54 weeks of culture for shoot initiation than other tested conditions (Fig.

2ASS3) whereas 3% and 4% alginate coated artificial seeds initiated first shoot primordia on hormone free full strength of solid MS medium which took 5.67±0.33 weeks and 7.83±0.31 weeks of culture respectively than other tested conditions (Fig. 3ASS1 & 4ASS1). The above results revealed that among different tested conditions of solid MS medium, 3% alginate coated artificial seeds showed earliest response for initiation of first shoot primordia than 2% and 4% alginate coated artificial seeds. The late response of 2% alginate coated artificial seeds for development of shoot primordia was observed on 8.33±0.8 weeks of culture on hormone free half strength of solid MS medium whereas 3% alginate coated artificial seeds took longer time, i.e., 8.83±1.11 weeks of culture for shoot development on full strength of solid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Similarly, hormone free quarter strength of solid MS medium gave late initiation of shoot from 4% alginate coated artificial seeds which took 9.67±0.67 weeks of culture for shoot development. Statistical analysis revealed that the shoot primordia developed from 2% and 4% alginate coated artificial seeds were not varied significantly while shoot primordia developed from 3% alginate coated artificial seeds were varied significantly at $p \leq 0.05$ among all the tested conditions of solid MS medium

On solid KC medium, 2% and 4% alginate coated artificial seeds gave earlier response for development of first shoot primordia on hormone free full strength of solid KC medium which took 5.5±0.56 weeks and 7.5±0.22 weeks of culture respectively. Whereas 3% alginate coated artificial seeds took 6±0.82 weeks of culture on hormone free quarter strength of solid KC medium for initiation of first shoot primordia than other tested conditions. This result revealed that among different tested conditions of solid KC medium, 2% alginate coated artificial seeds showed earlier response for initiation of first shoot primordia than 3% and 4% alginate coated artificial seeds. However, all the artificial seeds (2%, 3% and 4%) initiated shoot primordia lately on full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 7.67±0.33 weeks, 9.83±1.08 weeks and 10.67±1.72 weeks of culture respectively. Statistically, the shoot developed from 4% alginate coated artificial seeds were not varied significantly while shoot primordia developed from 2% and 3% alginate coated artificial seeds were significantly different at $p \leq 0.05$ among all the tested conditions of solid KC medium

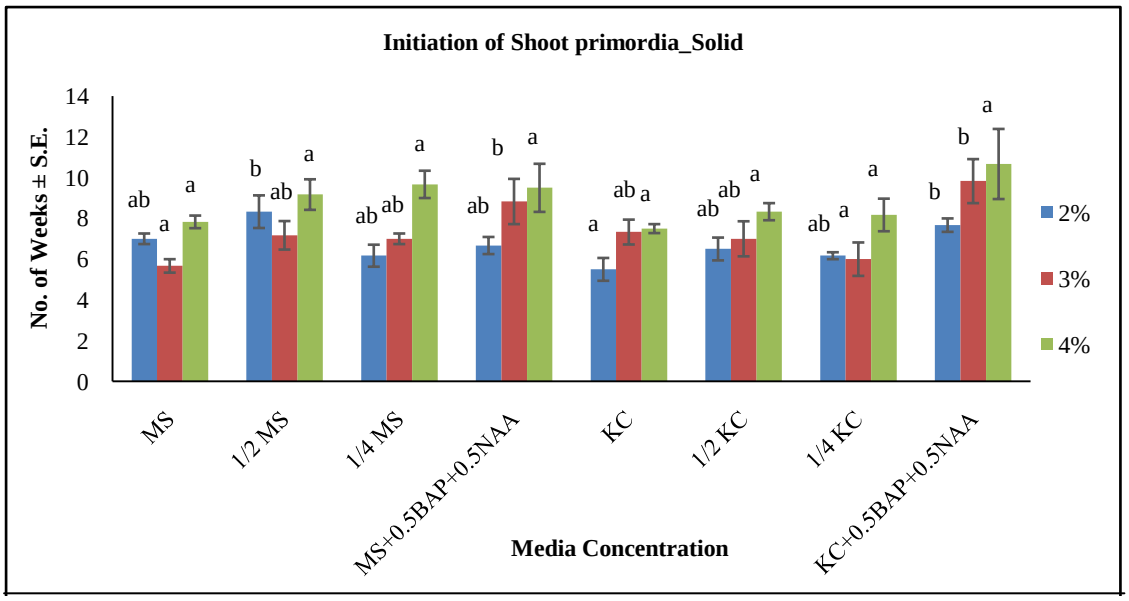


Figure 7: Initiation of shoot primordia from artificial seeds of *C. aloifolium* after 24 weeks of culture on solid MS and KC media. [The figure showed the statistical comparision on duration of initiation of shoot primordia from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Initiation of leaf primordia

The development of leaf was observed on all the tested conditions of solid MS and KC media (Fig. 8). 2% alginate coated artificial seeds gave earliest response for the development of first leaf primordia on hormone free quarter strength of solid MS medium which took 7.67±0.5 weeks of culture for initiation of leaf rather than 3% and 4% alginate coated artificial seeds. However, 3% and 4% alginate coated artificial seeds also took 11±0.68 weeks and 12±1.7 weeks of culture for earlier initiation of leaf on hormone free full strength of solid MS medium than other tested conditions of solid MS medium. The late response of 2% alginate coated artificial seeds for initiation of leaf was found on hormone free full strength of solid MS medium which took 10.67±1.12 weeks of culture for development of leaf. However, 3% and 4% alginate coated artificial seeds developed leaf primordia lately on 14.57±0.76 weeks and 17.17±1.92 weeks of culture respectively on hormone free quarter strength of solid MS medium. Statistical analysis revealed that the leaf primordia developed from 2%, 3% and 4% alginate coated artificial seeds were not varied significantly at $p \leq 0.05$ among all the tested conditions of solid MS medium.

On solid KC medium, the first leaf primordia developed from 2% and 4% alginate

coated artificial seeds were observed earlier on 7±0.68 weeks and 10±0.86 weeks of culture on hormone free full strength of solid KC medium whereas 3% alginate coated artificial seeds gave the earlier response for initiation of leaf on 11.17±1.05 weeks of culture on hormone free quarter strength of solid KC medium than other tested conditions of solid KC medium. This result revealed that 2% alginate coated artificial seeds gave the earliest response for initiation of first leaf primordia on solid KC medium rather than 3% and 4% alginate coated artificial seeds. However, all the artificial seeds (2%, 3% and 4%) showed their late response for initiation of leaf on full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 14±1.32 weeks, 16.5±0.85 weeks and 12.5±1.5 weeks of culture respectively for development of leaves. Statistical analysis revealed that the leaf primordia developed from 4% alginate coated artificial seeds were not significantly different while leaf primordia developed from 2% and 3% alginate coated artificial seeds were significantly varied at $p \leq 0.05$ among all the tested conditions of solid KC medium.

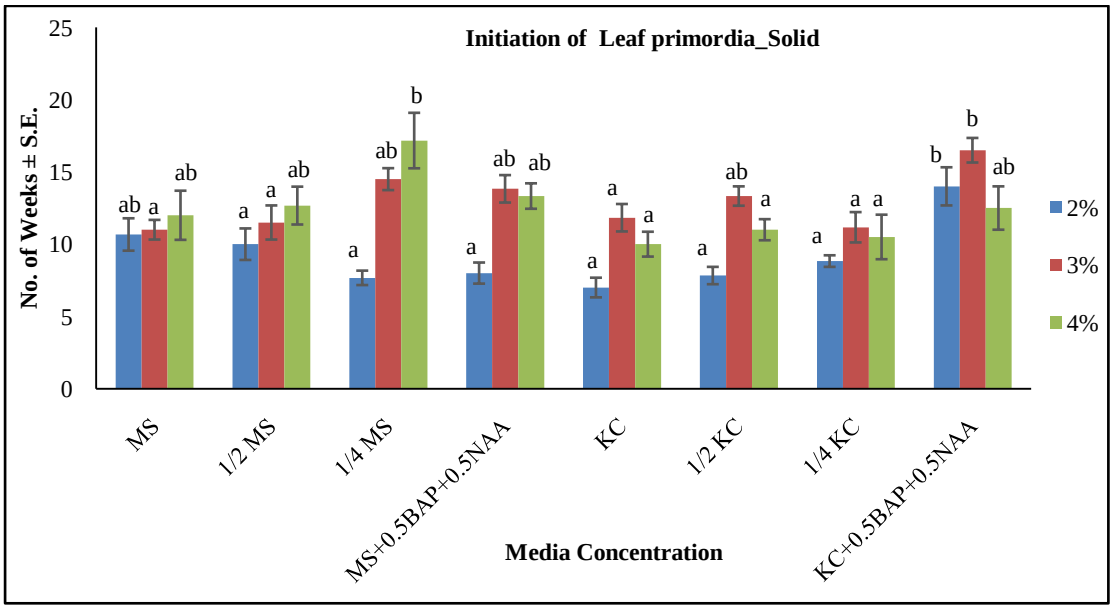


Figure 8: Initiation of leaf primordia from artificial seeds of *C. aloifolium* after 24 weeks of culture on solid MS and KC media. [The figure showed the statistical comparision on duration of initiation of leaf primordia from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Initiation of root primordia

Among the different artificial seeds, 2% alginate coated artificial seeds gave the earliest response for initiation of first root primordia on 11±0.52 weeks of culture on

hormone free quarter strength of solid MS medium rather than 3% and 4% alginate coated artificial seeds (Fig. 9). However, 3% alginate coated artificial seeds showed their earlier response for initiation of first root primordia on 12.67±0.56 weeks of culture on hormone free full strength of solid MS medium whereas 4% alginate coated artificial seeds gave quick response for development of root primordia on full strength of solid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which took 15±0.58 weeks of culture than other tested conditions of solid MS medium. 2% alginate coated artificial seeds developed root primordia lately on hormone free full strength of solid MS medium which took 13.67±1.05 weeks of culture for root initiation. Whereas 3% and 4% alginate coated artificial seeds initiated root primordia slowly on hormone free quarter strength of solid MS medium which took 18.5±0.22 weeks and 22.33±1.2 weeks of culture respectively than other tested conditions of solid MS medium. Statistically, the root primordia developed from 2% and 4% alginate coated artificial seeds were not varied significantly however root primordia developed from 3% alginate coated artificial seeds were significantly varied at $p \leq 0.05$ among all the tested conditions of solid MS medium.

On solid KC medium, all the artificial seeds (2%, 3% and 4%) gave their earlier response for root development on hormone free full strength of solid KC medium which took 12.5±1.61 weeks, 14.33±0.84 weeks and 11±1.09 weeks of culture respectively. Here, the time taken for initiation of root was longer on lower salt concentration of medium and medium supplemented with hormones. 2% alginate coated artificial seeds initiated root primordia lately on 15±0.45 weeks of culture on hormone free half strength of solid KC medium. However, roots were not developed from 2% alginate coated artificial seeds on full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Whereas on same condition, 3% and 4% alginate coated artificial seeds initiated root primordia lately on 21.33±0.56 weeks and 16±1 weeks of culture respectively. Statistical analysis revealed that the root initiated from 4% alginate coated artificial seeds were not varied significantly however root primordia developed from 2% and 3% alginate coated artificial seeds were significantly varied at $p \leq 0.05$ among all the tested conditions of solid KC medium (Fig. 9).

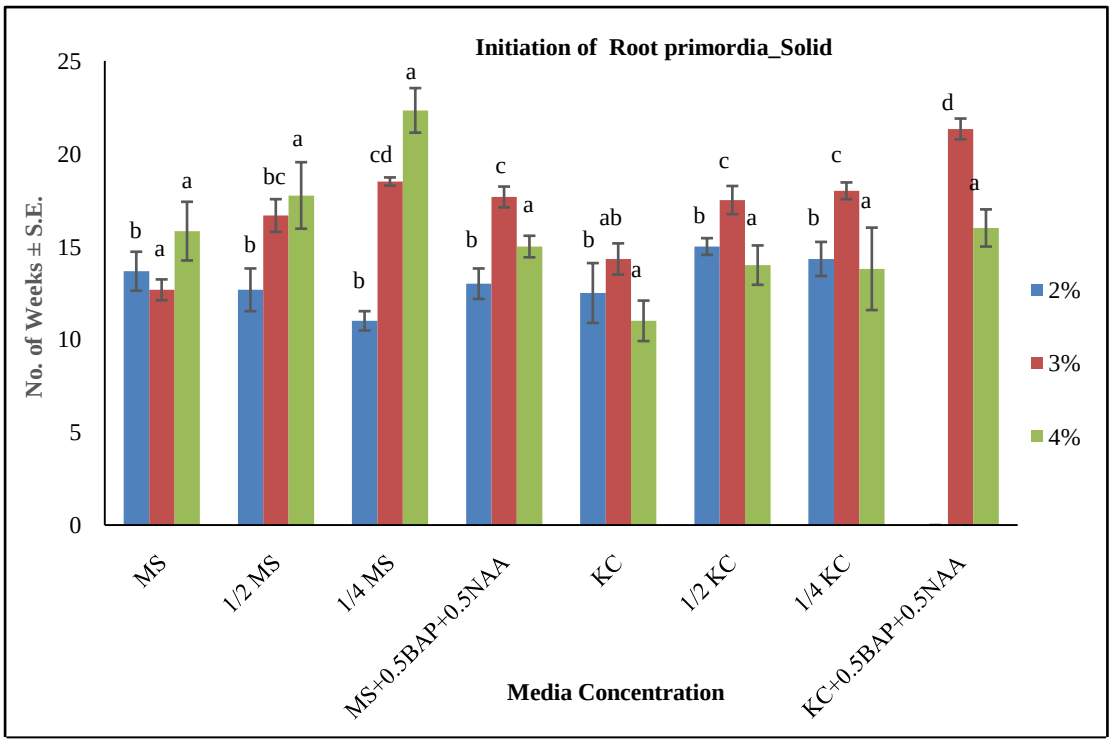


Figure 9: Initiation of root primordia from artificial seeds of *C. aloifolium* after 24 weeks of culture on solid MS and KC media. [The figure showed the statistical comparision on duration of initiation of root primordia from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Development of seedling

The earlier development of seedling from 2% alginate coated artificial seeds was observed on 20±0.58 weeks of culture on hormone free quarter strength of solid MS medium (Fig. 2ASS7). However, 3% and 4% alginate coated artificial seeds took 19±0.52 weeks and 21±0.26 weeks of culture for earlier development of seedling than other tested conditions of solid MS medium (Fig. 3ASS6 & 4ASS7). This result revealed that 3% alginate coated artificial seeds gave earliest response for complete seedling development than 2% and 4% alginate coated artificial seeds. 2% alginate coated artificial seeds developed seedling lately on 22±0.68 weeks of culture on full strength of solid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. However, 3% and 4% alginate coated artificial seeds showed late response for seedling development on 23±0.26 weeks and 24 weeks of culture respectively on hormone free quarter strength of solid MS medium. Statistical analysis revealed that

the seedling developed from 2% alginate coated artificial seeds was not varied significantly however seedling developed from 3% and 4% alginate coated artificial seeds were significantly different at $p \leq 0.05$ among all the tested conditions of solid MS medium (Fig. 10).

On solid KC medium, all the artificial seeds (2%, 3% and 4%) gave complete seedling earlier on 21±0.96 weeks, 20±0.86 weeks and 21±0.68 weeks of culture respectively on hormone free full strength of KC medium (Fig. 2ASS10, 3ASS9 & 4ASS9). Here, the time taken for seedling development was increased on lower strength of medium and medium supplemented with hormones. Hormone free quarter strength of solid KC medium gave late response on 23±0.96 weeks of culture for seedling development from 2% alginate coated artificial seeds. Full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA were not effective for the development of seedling from 2% alginate coated artificial seeds till 24 weeks of culture. However on same condition, 3% and 4% alginate coated artificial seeds showed their late response for seedling development on 24 weeks and 23±0.45 weeks of culture respectively. Statistically, seedling developed from all the artificial seeds was significantly different ($p \leq 0.05$) among all the tested conditions of solid KC medium (Fig. 10).

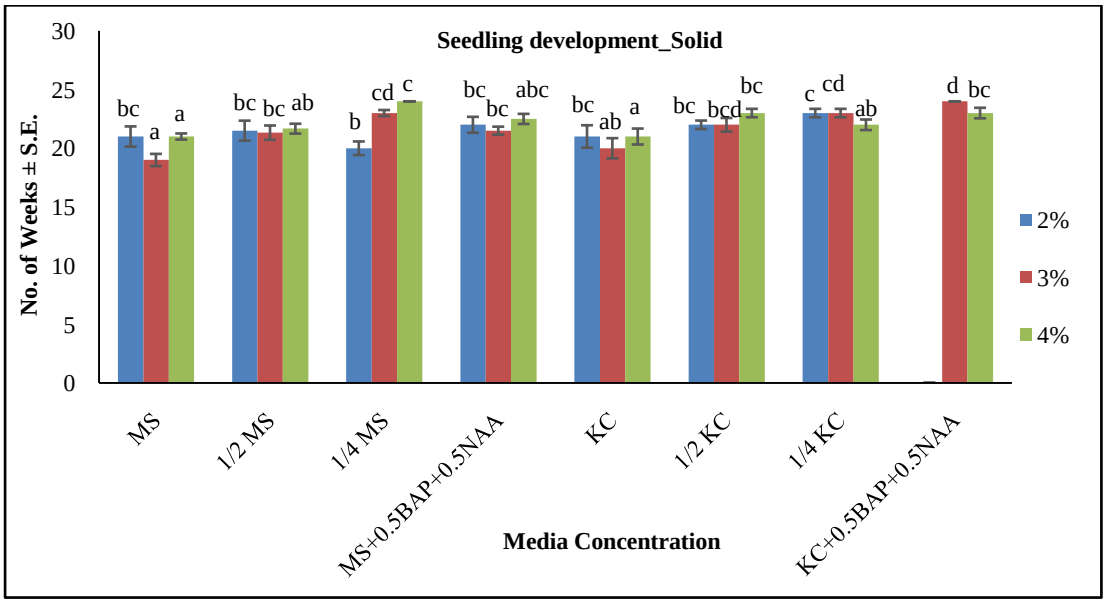


Figure 10: Development of seedling from artificial seeds of *C. aloifolium* after 24 weeks of culture on solid MS and KC media. [The figure showed the statistical comparision on duration of seedling

development from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

4.1.3.2.4 Development of artificial seeds on liquid MS and KC media

The freshly prepared artificial seeds were inoculated on two different liquid media (MS and KC) for the development and growth of shoots, leaves and roots. Each media comprises the four concentrations (viz. full, half, quarter strength and full strength of media supplemented with BAP and NAA) and each has 6 replicates. The data was recorded and noted till 24 weeks. Hormone free full strength of MS media was found to be effective for development of healthy seedlings. One-way ANOVA for development of 2% alginate coated artificial seeds on liquid medium showed that value of all the growth parameters except number and length of shoot varied significantly among different conditions of liquid MS medium ($p < 0.05$; Appendix 17). However on liquid KC medium, the result was significant only in the case of number and length of shoot and length of root ($p < 0.05$; Appendix 18). One-way ANOVA for development of 3% alginate coated artificial seeds on liquid medium showed that the value of all the growth parameters except length of root were significantly different among different tested conditions of liquid MS medium ($p < 0.05$; Appendix 19) whereas on liquid KC medium, the value of all the growth parameters were varied significantly among different tested conditions at 5% level of significance (Appendix 20). One-way ANOVA for development of 4% alginate coated artificial seeds on liquid medium showed that the value of all the growth parameters except number of shoot, root and length of root were significantly different among different tested conditions of liquid MS medium ($p < 0.05$; Appendix 21). However on liquid KC medium, the value of all the growth parameters except number of root, were varied significantly among different tested conditions at 5% level of significance ($p < 0.05$; Appendix 22).

The following responses were observed in different conditions during development of 2%, 3% and 4% (w/v) alginate coated artificial seeds of *C. aloifolium* (Figs. 11-16; Fig. 2AS, 3AS & 4AS)

Number of shoots

All the artificial seeds gave rise to large number of shoots on liquid MS medium

rather than liquid KC medium (Fig. 11). 3% and 4% alginate coated artificial seeds developed large number of shoots, i.e., 10.5±1.96 shoots and 9.83±2.52 shoots per culture respectively on hormone free full strength of liquid MS medium (Fig. 3AS12 & 4AS11) than other tested conditions of liquid MS medium. Both artificial seeds decreased their shoot number on lower strength of medium and medium supplemented with hormones. Whereas 2% alginate coated artificial seeds developed many shoots, i.e., 9±2.67 shoots per culture on hormone free quarter strength of liquid MS medium than other tested conditions. All the artificial seeds (2%, 3% and 4%) gave average number of lowest shoots, i.e., 2±0.45 shoots, 4±0.36 shoots and 3.17±0.31 shoots per culture respectively on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Statistical analysis revealed that the shoots developed from 2% alginate coated artificial seeds were not varied significantly however shoots developed from 3% and 4% alginate coated artificial seeds were significantly different at $p \leq 0.05$ among all the tested conditions of liquid MS medium.

On liquid KC medium, 2% and 3% alginate coated artificial seeds gave maximum number of shoots, i.e., 4.5±0.76 shoots and 9.5±0.56 shoots per culture respectively on hormone free quarter strength of liquid KC medium (Fig. 2AS4 & 3AS8) than other tested conditions. Whereas, 4% alginate coated artificial seeds gave maximum number of shoots, i.e., 9.17±1.8 shoots per culture on hormone free half strength of liquid KC medium (Fig. 4AS7) than other tested conditions of liquid KC medium. However, the multiplication of shoots was lower on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA where 2%, 3% and 4% artificial seeds gave only 1.2±0.31 shoots, 2.2±0.48 shoots and 2.5±0.34 shoots per culture respectively. The multiplication of shoot from 2% alginate coated artificial seeds were very low (ranging from 1.2 to 4.5 shoots per culture) on all the tested conditions of liquid KC medium as compare to 3% and 4% alginate coated artificial seeds. Statistically, the number of shoot developed from all the artificial seeds were significantly different ($p \leq 0.05$) among all the tested conditions of liquid KC medium.

Hence, this result showed that 3% alginate coated artificial seeds produced highest number of shoots on both liquid MS and KC media rather than 2% and 4% alginate

coated artificial seeds.

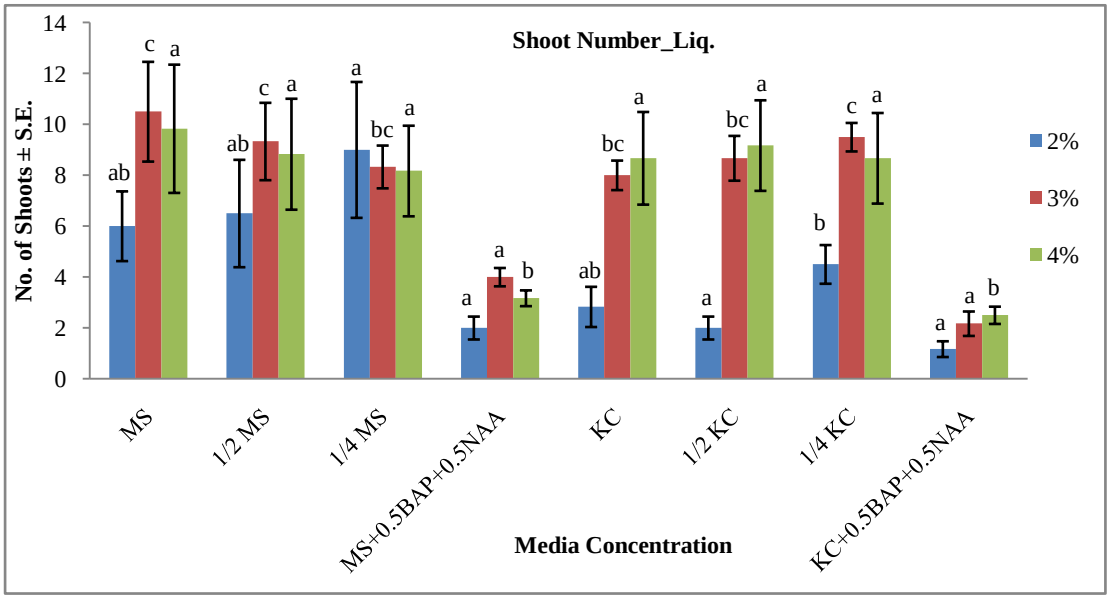


Figure 11: Average number of shoots derived from artificial seeds of *C. aloifolium* on different concentrations of liquid MS and KC media. [The figure showed the statistical comparision on number of shoots developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Length of shoot

On liquid MS medium, the maximum shoot length obtained from 2% and 3% alginate coated artificial seeds were observed on hormone free full strength of liquid MS medium which was found to be 6.4±1.31 cm and 8.5±1.22 cm shoot length per culture respectively whereas 4% alginate coated artificial seeds showed maximum shoot length, i.e., 7.57±0.66 cm per culture on hormone free quarter strength of liquid MS medium than other tested conditions of liquid MS medium (Fig. 12). It was found that the shoot length of 2% and 3% alginate coated artificial seeds were decreased on lower strength of medium while shoot length of 4% alginate coated artificial seeds decreased on higher strength of medium. However, all the artificial seeds (2%, 3% and 4%) showed their least development of shoot length on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 2.37±0.59 cm, 2.3±0.28 cm and 1.65±0.36 cm shoot length per culture respectively. Statistical analysis revealed that the shoot length of 2% alginate coated artificial seeds were not varied significantly however shoot length of 3% and 4% alginate coated artificial seeds were significantly different at $p \leq 0.05$ among all the tested conditions of liquid MS medium.

On liquid KC medium, 2% alginate coated artificial seeds showed their maximum shoot length on hormone free half strength of liquid KC medium which was found to be 6.5±1.21 cm shoot length per culture than other tested conditions of liquid KC medium (Fig. 12). However, 3% and 4% alginate coated artificial seeds showed their maximum shoot length on hormone free quarter strength of liquid KC medium which was found to be 7.08±0.35 cm and 5.62 ±0.82 cm shoot length per culture respectively than other tested conditions of liquid KC medium. The least development of shoot from 2% and 3% alginate coated artificial seeds were observed on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which gave 1.95 ±0.29 cm and 1.32 ±0.34 cm shoot length per culture respectively while 4% alginate coated artificial seeds gave least shoot length, i.e., 1.82 ±0.71 cm per culture on hormone free full strength of liquid KC medium. Statistical analysis revealed that the shoot length of all the artificial seeds were significantly different ($p \leq 0.05$) among all the tested conditions of liquid KC medium.

Hence among the different artificial seeds, 3% alginate coated artificial seeds gave the maximum development of shoot on hormone free full strength of liquid MS medium rather than other tested conditions of liquid MS and KC media.

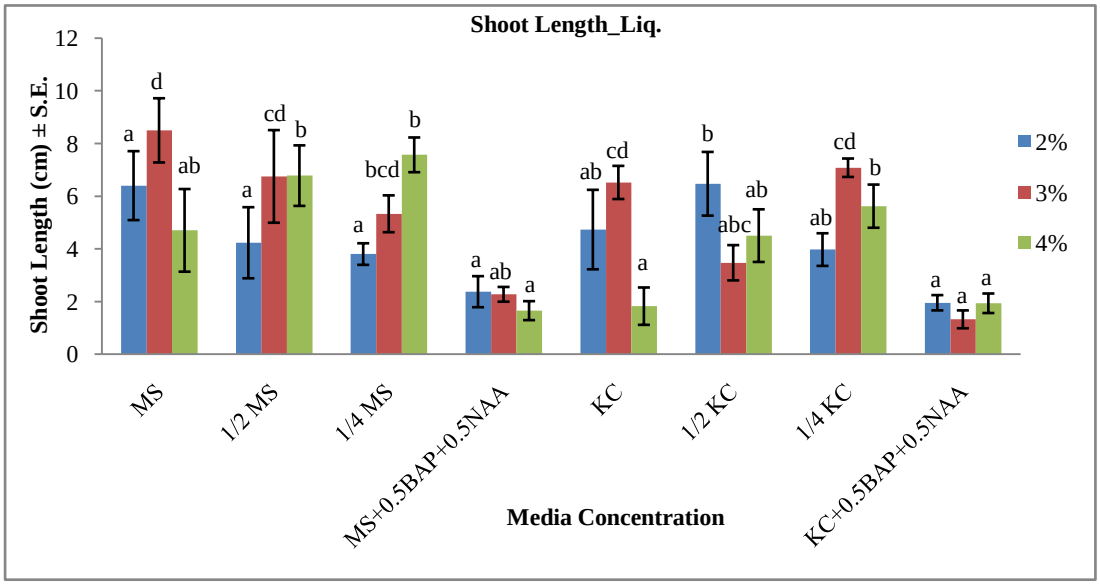


Figure 12: Average length of shoot derived from artificial seeds of *C. aloifolium* on different concentrations of liquid MS and KC media. [The figure showed the statistical comparision on length of shoots developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Number of leaf

On liquid MS medium, the maximum proliferation of leaf from 2% and 4% alginate coated artificial seeds was found to be 13.33±4.21 leaves and 10.67±1.67 leaves per culture on hormone free quarter strength of liquid MS medium while 3% alginate coated artificial seeds gave maximum number of leaf on hormone free full strength of liquid MS medium which was found to be 14.5±1.78 leaves per culture than other tested conditions of liquid MS medium (Fig. 13). However, all the artificial seeds (2%, 3% and 4%) developed least number of leaves on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 2±0.45 leaves, 4.67±0.61 leaves and 1.5±0.62 leaves per culture respectively. Statistically, the leaves developed from all the artificial seeds were significantly different ($p \leq 0.05$) among all the tested conditions of liquid MS medium.

On liquid KC medium, 2% and 3% alginate coated artificial seeds gave maximum proliferation of leaf on hormone free quarter strength of liquid KC medium which was found to be 7±2.13 leaves and 12.5±1.54 leaves per culture respectively while 4% alginate coated artificial seed gave maximum number of leaf, i.e., 10.5±2.74 leaves per culture on hormone free half strength of liquid KC medium than other tested conditions of liquid KC medium (Fig. 13). However, all the artificial seeds (2%, 3% and 4%) gave least number of leaves on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 1.33±0.33 leaves, 1.2±0.4 leaves and 1.67±0.71 leaves per culture respectively. Statistical analysis revealed that the number of leaf developed from 2% alginate coated artificial seeds were not varied significantly however leaves developed from 3% and 4% alginate coated artificial seeds were significantly different at $p \leq 0.05$ among all the tested conditions of liquid KC medium.

Hence among the different artificial seeds, 3% alginate coated artificial seeds gave the maximum proliferation of leaf on hormone free full strength of liquid MS medium than other tested conditions of liquid MS and KC media.

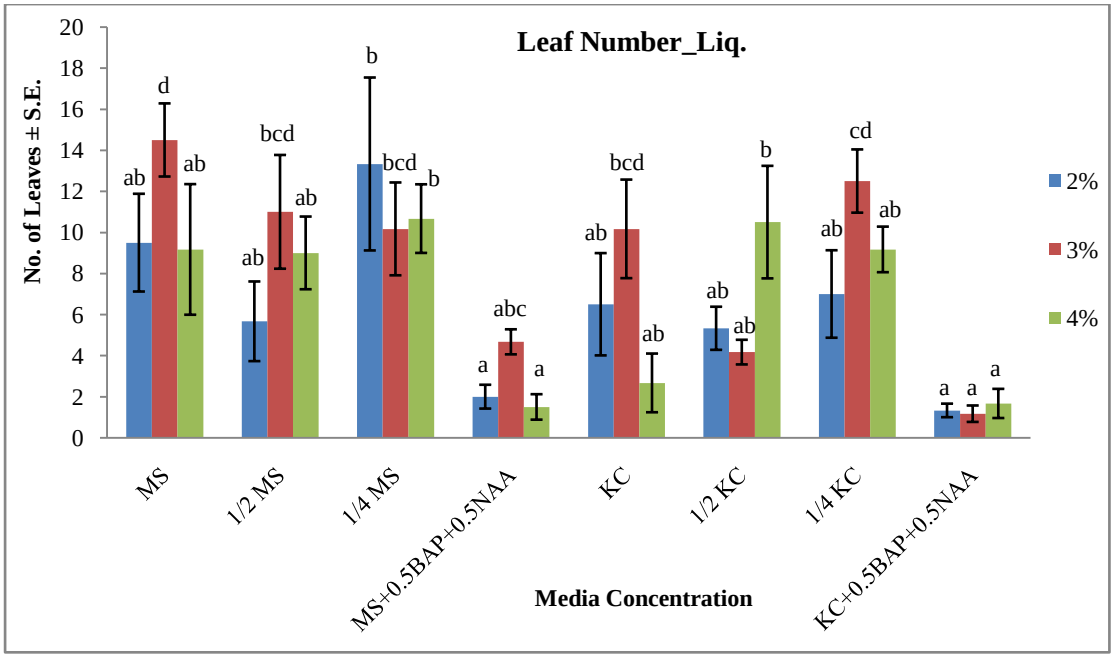


Figure 13: Average number of leaves derived from artificial seeds of *C. aloifolium* on different concentrations of liquid MS and KC media. [The figure showed the statistical comparision on number of leaves developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Length of leaf

On liquid MS medium, 2% and 3% alginate coated artificial seeds gave maximum development of leaf on hormone free full strength of liquid MS medium which was found to be 5.22±0.89 cm and 6.17±1.22 cm leaf length per culture respectively while 4% alginate coated artificial seeds gave maximum development of leaf, i.e., 3.63±0.6 cm leaf length per culture on hormone free quarter strength of liquid MS medium than other tested conditions of liquid MS medium (Fig. 14). Here, the leaf length of 2% and 3% alginate coated artificial seeds were decreased on lower strength of medium while vice versa on 4% alginate coated artificial seed, i.e., leaf length increased on lower strength of medium. However, all the artificial seeds (2%, 3% and 4%) gave least development of leaf on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 0.77±0.32 cm, 0.83±0.17 cm and 0.33±0.13 cm leaf length per culture respectively. Statistical analysis revealed that the leaf length of all artificial seeds were significantly different ($p \leq 0.05$) among all the tested conditions of liquid MS medium.

Similar results were also obtained on liquid KC medium. The maximum development

of leaf of 2% alginate coated artificial seeds were observed on hormone free half strength of liquid KC medium which was found to be 3.6±0.74 cm leaf length per culture whereas 3% and 4% alginate coated artificial seeds gave their maximum development of leaf on hormone free quarter strength of liquid KC medium which was found to be 4.25±0.46 cm and 3.23±0.63 cm leaf length per culture respectively than other tested conditions of liquid KC medium (Fig. 14). However, all the artificial seeds (2%, 3% and 4%) gave least development of leaf on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 0.65±0.19 cm, 0.62±0.28 cm and 0.5±0.16 cm leaf length per culture respectively. Statistical analysis revealed that the leaf length of 2% alginate coated artificial seeds were not varied significantly however leaf length of 3% and 4% alginate coated artificial seeds were significantly varied at $p \leq 0.05$ among all the tested conditions of liquid KC medium.

Hence among the different artificial seeds, 3% alginate coated artificial seeds showed the maximum growth of leaf on hormone free full strength of liquid MS medium than other tested conditions of liquid MS and KC media.

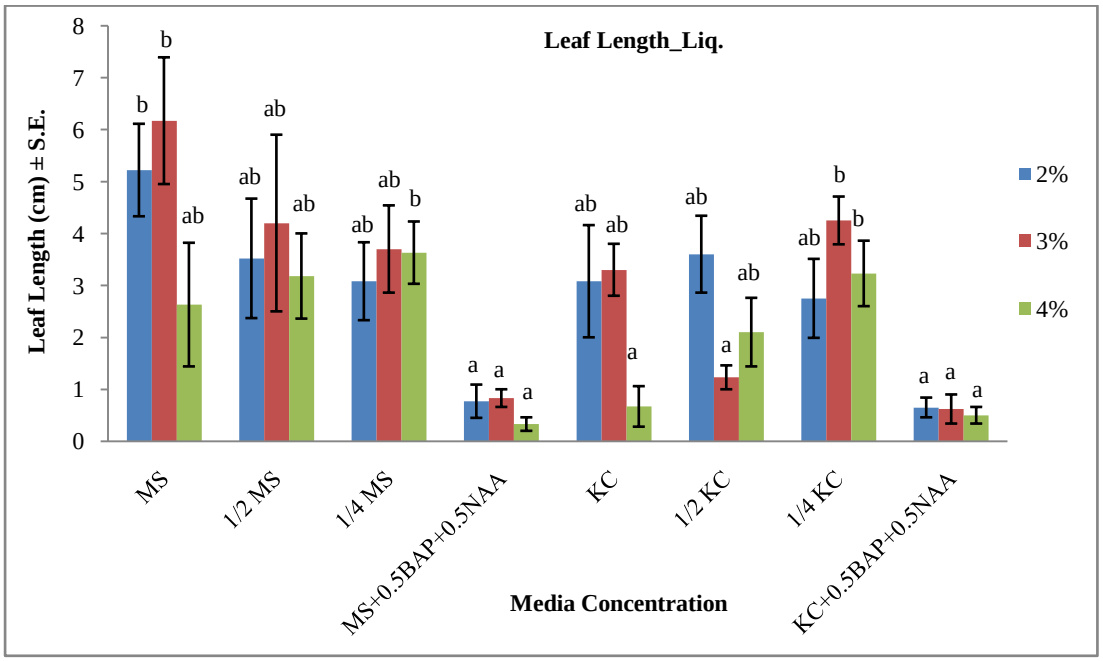


Figure 14: Average length of leaf derived from artificial seeds of *C. aloifolium* on different concentrations of liquid MS and KC media. [The figure showed the statistical comparision on length of leaf developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Number of root

On liquid MS medium, the maximum proliferation of root of 2%, 3% and 4% alginate coated artificial seeds were observed on hormone free full strength of liquid MS medium which was found to be 4.5±0.99 roots, 5.33±0.92 roots and 2.67±1.08 roots per culture respectively than other tested conditions of liquid MS medium (Fig. 15). Here, all the artificial seeds decreased their number of root on lower strength of medium. The least proliferation of root from 2% and 4% alginate coated artificial seeds were observed on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 1.12±0.6 roots and 0.33±0.21 roots per culture respectively while 3% alginate coated artificial seeds gave least number of root, i.e., 2.17±0.87 roots per culture on hormone free quarter strength of liquid MS medium. Statistical analysis revealed that the roots developed from 4% alginate coated artificial seed was not varied significantly however roots developed from 2% and 3% alginate coated artificial seeds were significantly varied at $p \leq 0.05$ among all the tested conditions of liquid MS medium.

On liquid KC medium, 2% alginate coated artificial seeds gave maximum proliferation of root on hormone free full strength of liquid KC medium which was found to be 2.83±1.38 roots per culture than other tested conditions of liquid KC medium (Fig. 15). Similarly, 3% alginate coated artificial seeds proliferate maximum root, i.e., 3.2±0.48 roots per culture on hormone free quarter strength of liquid KC medium while 4% alginate coated artificial seeds gave maximum root on hormone free half strength of liquid KC medium which was found to be 2.5±1.0 roots per culture than other tested conditions of liquid KC medium. However, all the artificial seeds (2%, 3% and 4%) showed their least proliferation of root on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 0.17±0.17 roots, 0.33±0.21 roots and 0.5±0.34 roots per culture respectively. Statistical analysis revealed that the roots developed from 2% and 4% alginate coated artificial seeds were not varied significantly however roots developed from 3% alginate coated artificial seeds were significantly varied at $p \leq 0.05$ among all the tested conditions of liquid KC medium.

Figure 2AS: *In vitro* germination and development of 2% alginate coated artificial seeds of *Cymbidium aloifolium* on liquid MS and KC media

Fig. 2AS1 Production of artificial seeds coated with 2% alginate

Fig. 2AS2 Development of shoot buds and small shoot on full strength MS medium

Fig. 2AS3 Development of multiple shoot buds and small shoots on hormone free full strength MS medium

Fig. 2AS4 Development of shoot buds and shoots on hormone free quarter strength KC medium

Fig. 2AS5 Development of roots on hormone free full strength MS medium

Fig. 2AS6 Development of roots on hormone free half strength MS medium

Fig. 2AS7 Development of shoot and roots on hormone free half strength MS medium

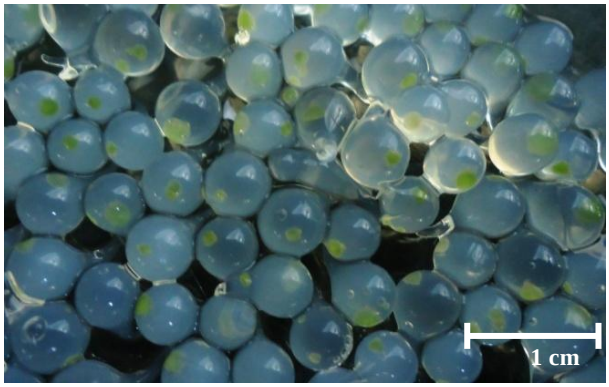
Fig. 2AS8 Development of shoot buds and shoots on hormone free KC medium

Fig. 2AS9 Development of plantlet on full strength MS medium supplemented with BAP 0.5mg/l + NAA 0.5mg/l

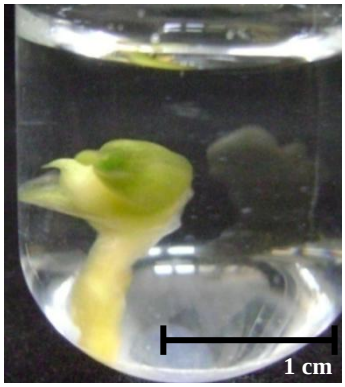
Fig. 2AS10 Development of plantlet on full strength KC medium

Fig. 2AS11 Development of plantlet on full strength KC medium supplemented with BAP 0.5mg/l and NAA 0.5mg/l

Fig. 2AS12 Development of plantlet on full strength MS medium



2AS1



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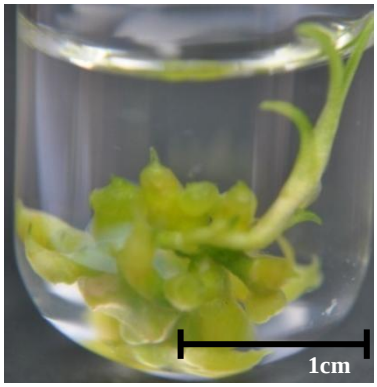

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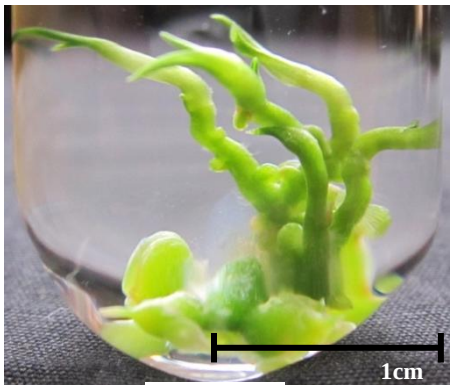
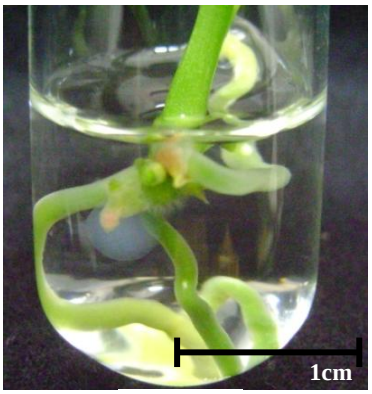


2AS4

2AS5

2AS6



2AS7

2AS8

2AS9

2AS10

2AS11

2AS12

Hence among the different artificial seeds, 3% alginate coated artificial seeds showed the maximum proliferation of root on hormone free full strength of liquid MS medium than other tested conditions of liquid MS and KC media.

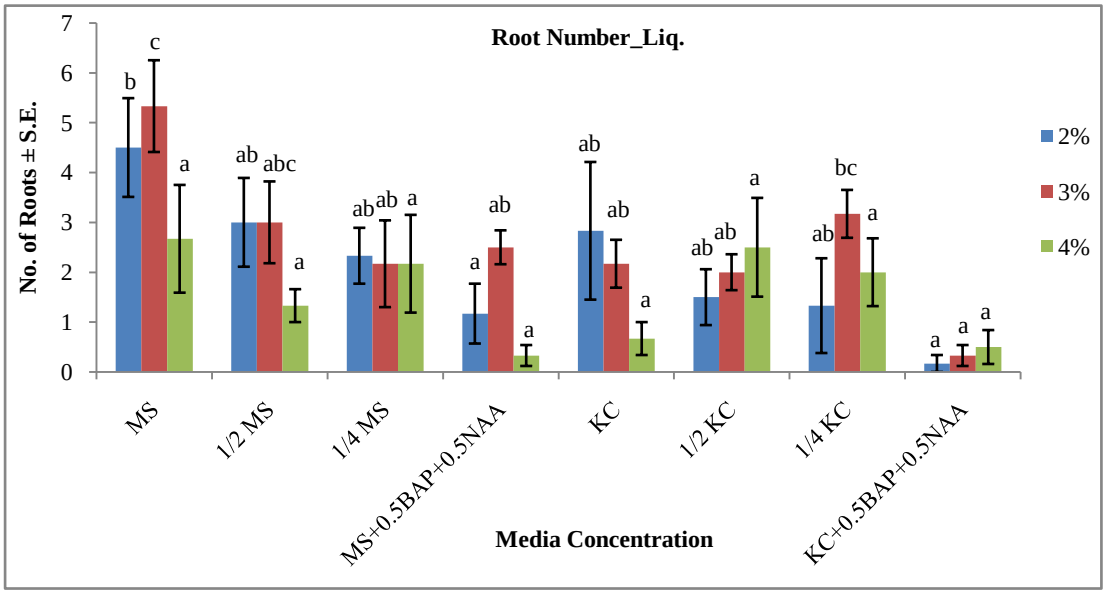


Figure 15: Average number of roots derived from artificial seeds of *C. aloifolium* on different concentrations of liquid MS and KC media. [The figure showed the statistical comparision on number of roots developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Length of root

On liquid MS medium, the maximum development of root of 2%, 3% and 4% alginate coated artificial seeds were observed on hormone free full strength of liquid MS medium which was found to be 4.9±1.36 cm, 4.72±0.88 cm and 3.03±1.61 cm root length per culture respectively than other tested conditions of liquid MS medium (Fig. 16). Here, the root length of all the artificial seeds was decreased on lower strength of medium. The least development of root from 2%, 3% and 4% alginate coated artificial seeds were obtained on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 0.83±0.41 cm, 2±0.29 cm and 0.27±0.17 cm root length per culture respectively. Statistical analysis revealed that the root length developed from 3% and 4% alginate coated artificial seeds were not varied significantly however root length developed from 2% alginate coated artificial seeds was significantly varied at $p \leq 0.05$ among all the tested conditions of liquid MS medium

Figure 3AS: *In vitro* germination and development of 3% alginate coated artificial seeds of *Cymbidium aloifolium* on liquid MS and KC media

Fig. 3AS 1 Production artificial seeds coated with 3% alginate

Fig. 3AS 2 Development of many shoot buds and small plantlets on hormone free full strength of MS medium

Fig. 3AS 3 Development of shoot buds from artificial seed on hormone free half strength of MS medium

Fig. 3AS 4 Development of single shoot on hormone free quarter strength of KC medium

Fig. 3AS 5 Development of shoot buds on hormone free half strength of KC medium

Fig. 3AS 6 Development of shoot buds and small root on hormone free quarter strength of MS medium

Fig. 3AS 7 Development of shoot buds and plantlets on hormone free full strength of KC medium

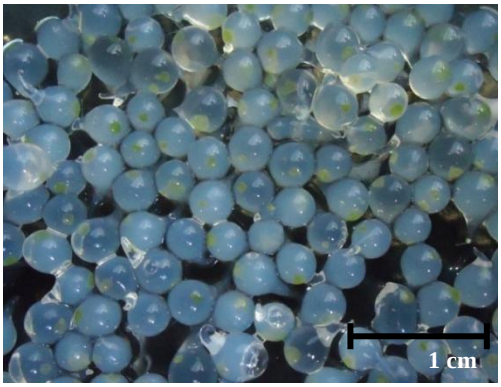
Fig. 3AS 8 Development of shoot buds and shoot on hormone free quarter strength of KC medium

Fig. 3AS 9 Development of shoot buds and small plantlet on full strength of KC medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA

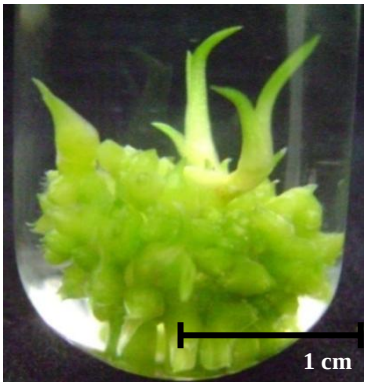
Fig. 3AS10 Development of plantlet on hormone free full strength of MS medium

Fig. 3AS11 Development of plantlet and roots on full strength of MS medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA

Fig. 3AS12 Development of plantlet on hormone free full strength of MS medium



3AS1



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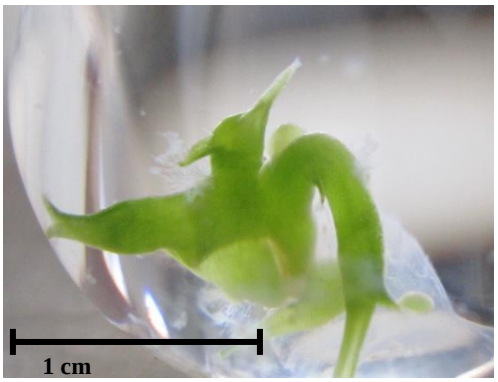

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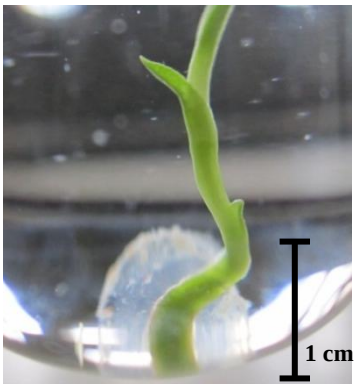

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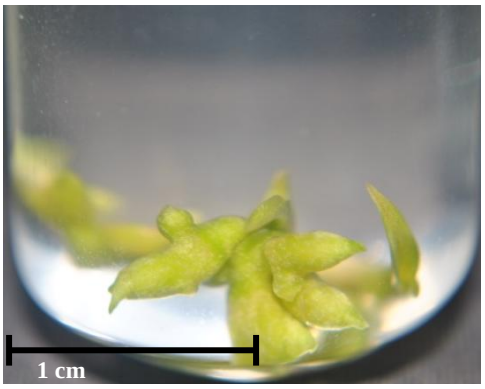

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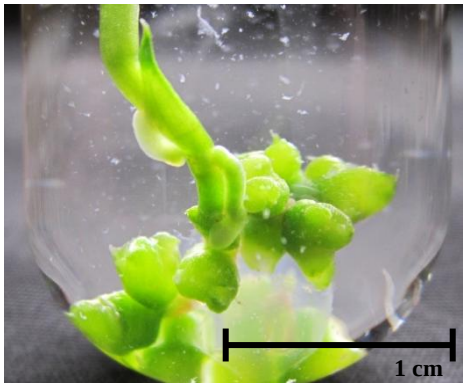

3AS6

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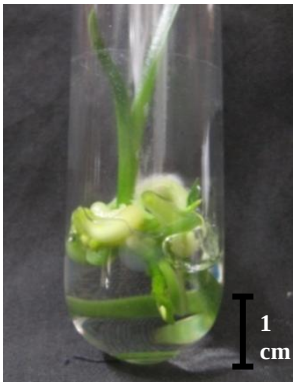


3AS11

3AS12

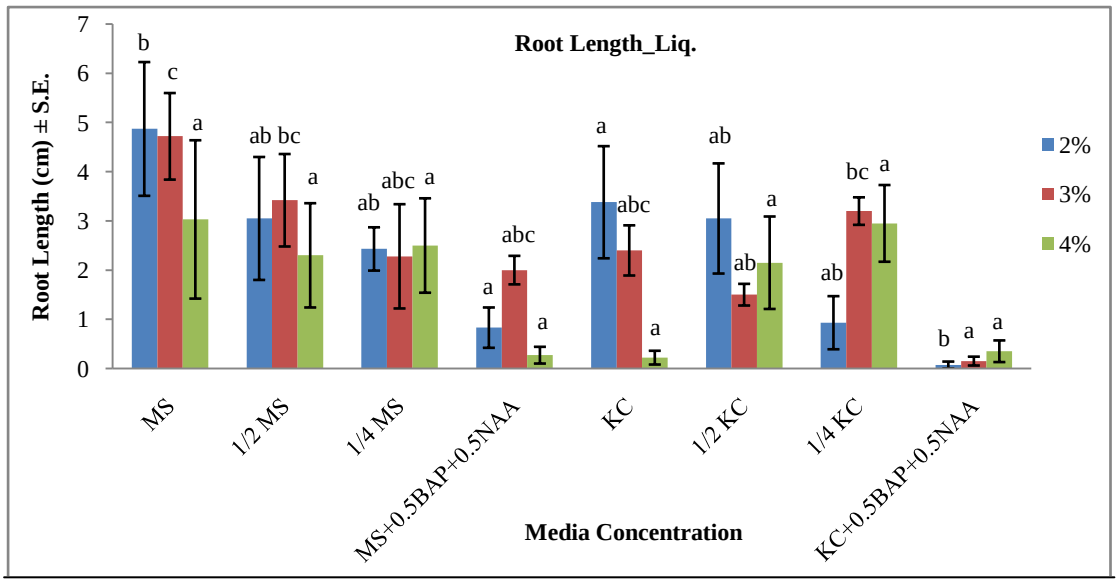


Figure 16: Average length of root derived from artificial seeds of *C. aloifolium* on different concentrations of liquid MS and KC media. [The figure showed the statistical comparision on length of root developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

On liquid KC medium, 2% alginate coated artificial seeds showed maximum root growth (3.38±1.14 cm root length per culture) on hormone free full strength of liquid KC medium while 3% and 4% alginate coated artificial seeds gave maximum development of root on hormone free quarter strength of liquid KC medium which was found to be 3.2±0.28 cm and 2.95±0.78 cm root length per culture respectively than other tested conditions of liquid KC medium (Fig. 16). However, the least development of root from 2% and 3% alginate coated artificial seeds were observed on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 0.07±0.07 cm and 0.15±0.09 cm root length per culture respectively whereas 4% alginate coated artificial seeds gave least root growth, i.e., 0.22±0.14 cm root length per culture on hormone free full strength of liquid KC medium. Statistical analysis revealed that the root length developed from 4% alginate coated artificial seeds were not varied significantly however root length developed from 2% and 3% alginate coated artificial seeds were significantly varied at $p \leq 0.05$ among all the tested conditions of liquid KC medium. Hence among the different artificial seeds, 2% alginate coated artificial seeds showed the maximum growth of root followed by 3% alginate coated artificial seeds on hormone free full strength of liquid MS medium than other tested conditions of liquid MS and KC media.

Figure 4AS: *In vitro* germination and development of 4% alginate coated artificial seeds of *Cymbidium aloifolium* on liquid MS and KC media

Fig. 4AS 1 Production of artificial seeds coated with 4% alginate

Fig. 4AS 2 Development of small shoot buds from artificial seed on hormone free full strength of MS medium

Fig. 4AS 3 Development of small shoot buds from artificial seed on hormone free half strength of KC medium

Fig. 4AS 4 Development of shoot buds on hormone free quarter strength of MS medium

Fig. 4AS 5 Development of multiple shoot buds from single artificial seed on hormone free full strength of MS medium

Fig. 4AS 6 Development of shoot buds, shoots and roots on hormone free quarter strength of KC medium

Fig. 4AS 7 Development of shoot buds and shoots on hormone free half strength of KC medium

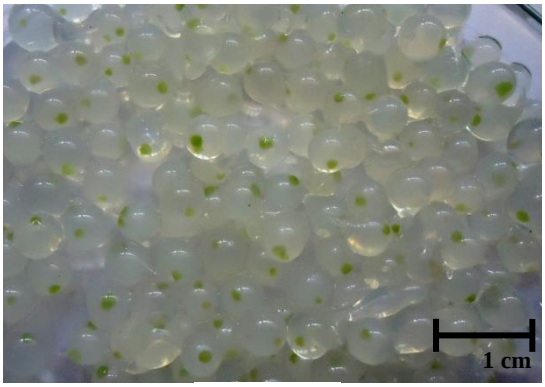
Fig. 4AS 8 Development of shoots and roots on hormone free full strength of MS medium

Fig. 4AS 9 Development of plants with roots (seedling) on hormone free full strength of MS medium

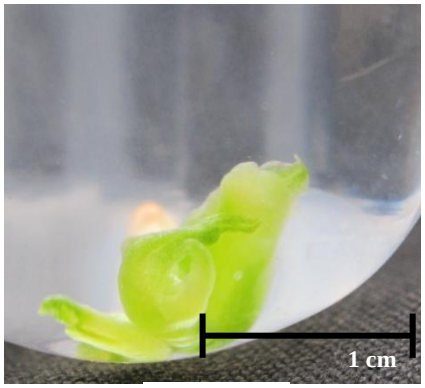
Fig. 4AS10 Development of plantlet and roots on quarter strength MS medium

Fig. 4AS 11 Development of multiple shoot buds and shoots on hormone free full strength of MS medium

Fig. 4AS12 Development of shoot buds, plantlets and roots on full strength of MS medium



4-AS1



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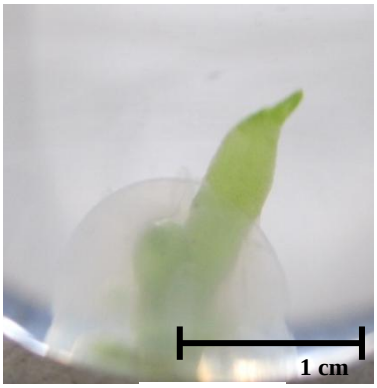

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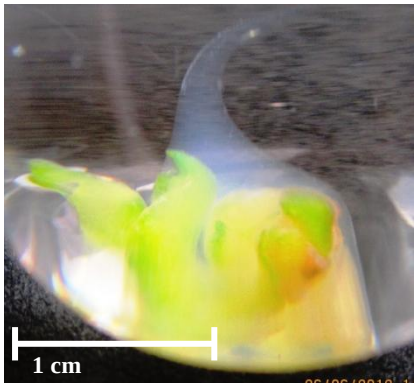

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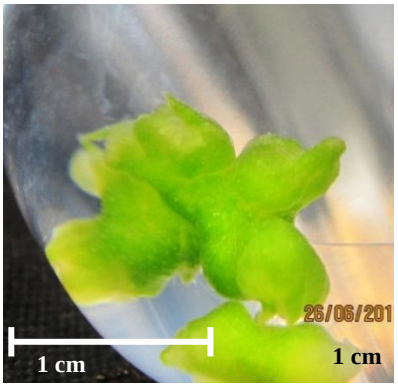

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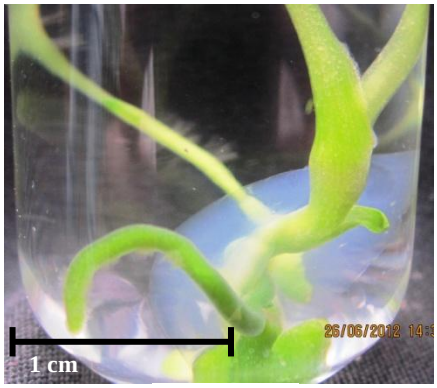

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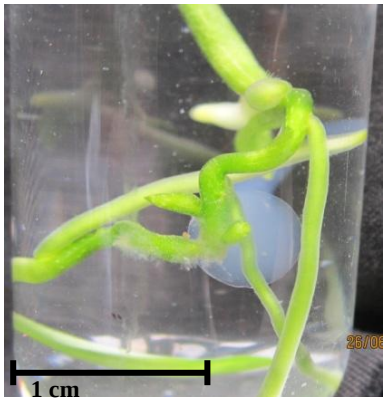

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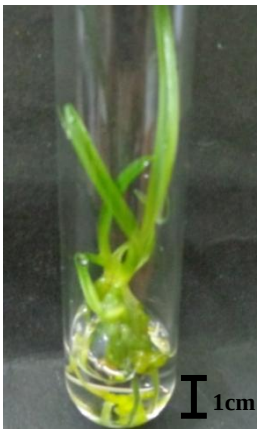
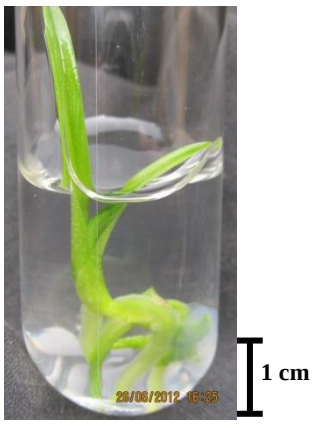


4-AS9

4-AS10

4-AS11



4-AS12

4.1.3.2.5 Development of artificial seeds of on solid MS and KC media

The freshly prepared artificial seeds were inoculated on two different solid media (MS and KC) and their hormonal concentrations for the development and growth of shoots, leaves and roots. Each media comprises the four concentrations and each has 6 replicates. The data was recorded and noted till 24 weeks. One-way ANOVA for development of 2% alginate coated artificial seed on solid medium showed that the value of all the growth parameters were not significantly different among different tested conditions of solid MS medium at 5% level of significance (Appendix 23) whereas on solid KC medium, the value of all the growth parameters except number of shoots varied significantly among different tested conditions at 5% level of significance (Appendix 24). One-way ANOVA for development of 3% alginate coated artificial seeds on solid medium showed that among the growth parameters studied only the length of root varied significantly among different conditions of solid MS medium ($p < 0.05$; Appendix 25). However, value of all the growth parameters except number of shoot, leaf and root varied significantly among different tested conditions of solid KC medium ($p < 0.05$; Appendix 26). One-way ANOVA for development of 4% alginate coated artificial seeds on solid medium showed that among the growth parameters studied only the length of shoot varied significantly among different tested conditions of solid MS medium ($p < 0.05$; Appendix 27). However, value of all the growth parameters except number of shoot and root varied significantly among different tested conditions of solid KC medium ($p < 0.05$; Appendix 28).

The following responses were observed in different conditions during development of 2%, 3% and 4% (w/v) alginate coated artificial seeds of *C. aloifolium* (Figs. 17-22; Fig. 2ASS, 3ASS & 4ASS).

Number of shoot

On solid MS medium, all the artificial seeds (2%, 3% and 4%) showed their maximum proliferation of shoot on hormone free full strength of solid MS medium (Fig. 17) which was found to be 8±3.01 shoots, 9.83±2.27 shoots and 9.5±5.22 shoots per culture respectively than other tested conditions of solid MS medium (Fig. 2ASS8, 3ASS8 & 4ASS8). However, the least proliferation of shoot from 2%, 3% and 4% alginate coated artificial seeds was observed on hormone free quarter strength

of solid MS medium which was found to be 2±0.68 shoots, 6±0.97 shoots and 2±0.45 shoots per culture respectively. Statistical analysis revealed that the shoots developed from all the artificial seeds were not varied significantly ($p \leq 0.05$) among all the tested conditions of solid MS medium.

Similar results were also obtained on solid KC medium (Fig. 17). The maximum proliferation of shoot from 2%, 3% and 4% alginate coated artificial seeds was observed on hormone free full strength of solid KC medium which was found to be 5±1.12 shoots, 9±3.23 shoots and 9.5±2.28 shoots per culture respectively than other tested conditions of solid KC medium (Fig. 2ASS10, 3ASS10 & 4ASS9). The least proliferation of shoot from 2% and 3% alginate coated artificial seeds were observed on full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 1.67±0.42 shoots and 3±0.58 shoots per culture respectively while 4% alginate coated artificial seeds showed their least proliferation of shoot, i.e., 5±1.29 shoots per culture on hormone free quarter strength of solid KC medium. Statistically, the shoots developed from all the artificial seeds were not varied significantly ($p \leq 0.05$) among all the tested conditions of solid KC medium

Hence among different artificial seeds, 3% alginate coated artificial seeds produced maximum number of shoot on hormone free full strength of solid MS medium rather than other tested conditions of solid MS and KC media.

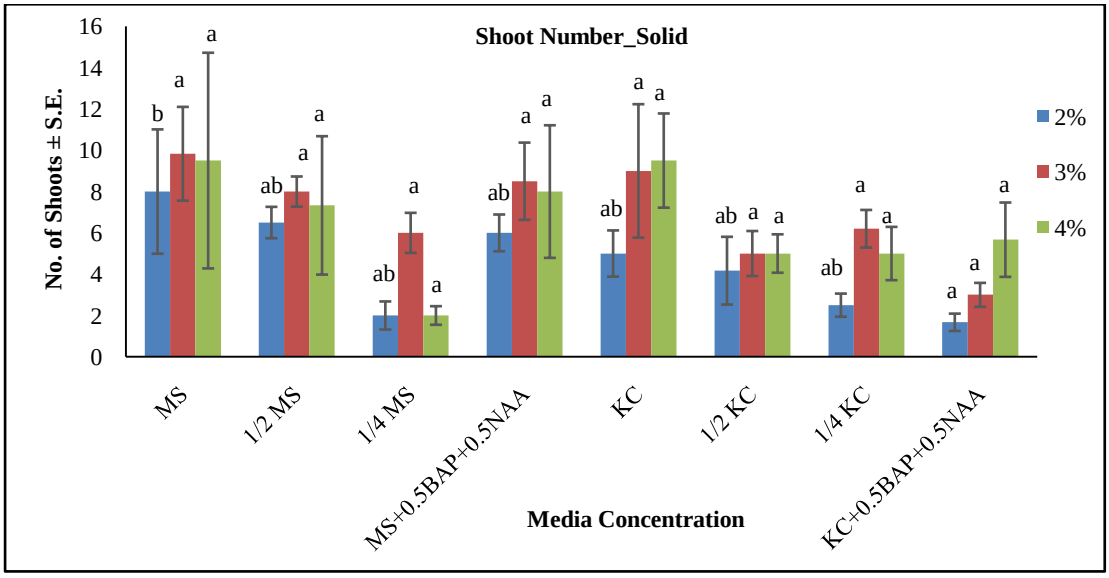


Figure 17: Average number of shoots derived from artificial seeds of *C. aloifolium* on different concentrations of solid MS and KC media. [The figure showed the statistical comparision on number of shoot developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)]

Length of shoot

Among the different tested conditions of solid MS medium, all the artificial seeds (2%, 3% and 4%) showed their maximum development of shoot on hormone free full strength of solid MS medium which was found to be 5.5±0.53 cm, 7±0.36 cm and 6.92±1.22 cm shoot length per culture respectively (Fig. 18). Here, the length of shoot was decreased on lower strength of medium. The least development of shoot from 2%, 3% and 4% alginate coated artificial seeds was observed on hormone free quarter strength of solid MS medium which was found to be 4.67±0.98 cm, 4.77±0.54 cm and 2.68±0.55 cm shoot length per culture respectively. Statistical analysis revealed that the shoot length of 2% and 3% alginate coated artificial seeds were not varied significantly while shoot length of 4% alginate coated artificial seeds were varied significantly ($p \leq 0.05$) among all the tested conditions of solid MS medium.

On solid KC medium, 2% alginate coated artificial seeds gave maximum shoot length, i.e., 6.05±0.33 cm shoot length per culture on hormone free half strength of solid KC medium than other tested conditions of solid KC medium (Fig. 18). While 3% alginate coated artificial seeds showed their maximum shoot length, i.e., 6.2±0.75 cm shoot length per culture on hormone free full strength of solid KC medium whereas 4% alginate coated artificial seeds gave maximum shoot length on hormone free quarter strength of solid KC medium which was found to be 6.13±1.03 cm shoot length per culture than other tested conditions of solid KC medium. However, the least development of shoot from 2%, 3% and 4% alginate coated artificial seeds were observed on full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 2.12±0.31 cm, 1.7±0.13 cm and 2.32±1.02 cm shoot length per culture respectively. Statistically, the shoot length of all the artificial seeds were varied significantly ($p \leq 0.05$) among all the tested conditions of solid KC medium.

Hence among different artificial seeds, 3% alginate coated artificial seeds developed maximum shoot length on hormone free full strength of solid MS medium rather than other tested conditions of solid MS and KC media.

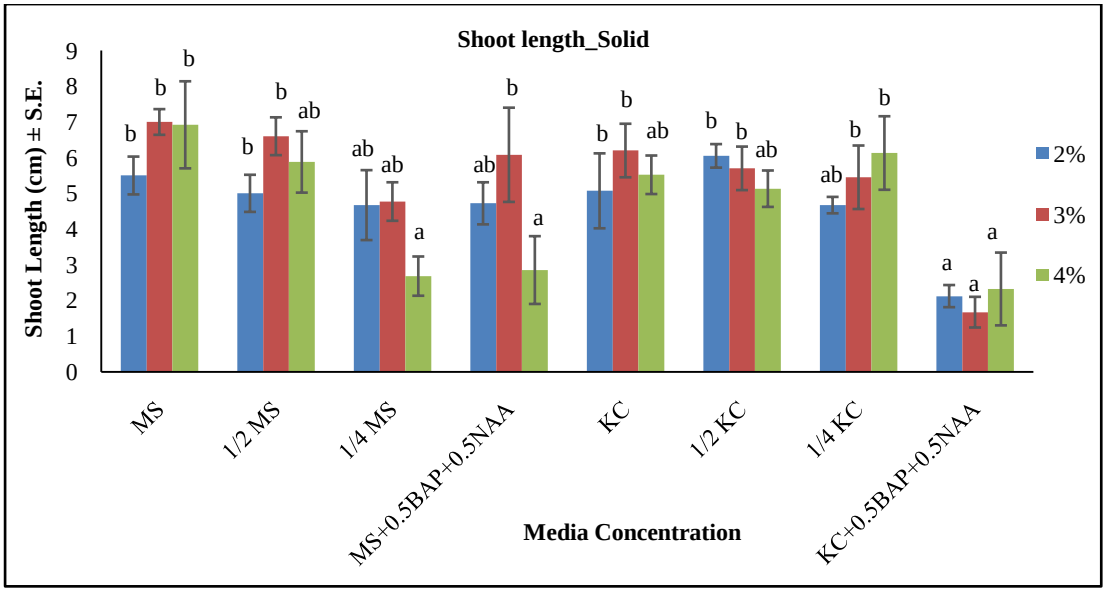


Figure 18: Average length of shoot derived from artificial seeds of *C. aloifolium* on different concentrations of solid MS and KC media. [The figure showed the statistical comparision on length of shoot developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Number of leaf

On solid MS medium, the number of leaf was decreased on lower strength of medium. All the artificial seeds (2%, 3% and 4%) showed their maximum proliferation of leaf on hormone free full strength of solid MS medium which was found to be 13.2±3.49 leaves, 13±1.98 leaves and 14.67±5.64 leaves per culture respectively than other tested conditions of solid MS medium (Fig. 19). The least proliferation of leaf from 2% and 4% alginate coated artificial seeds were observed on hormone free quarter strength of solid MS medium which was found to be 4.33±1.2 leaves and 4±1.03 leaves per culture respectively whereas 3% alginate coated artificial seeds developed least number of leaf, i.e., 11±3.52 leaves per culture on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Statistically, the leaves developed from all the artificial seeds were not varied significantly ($p \leq 0.05$) among all the tested conditions of solid MS medium.

Similar results were also obtained on solid KC medium (Fig. 19). All the artificial seeds (2%, 3% and 4%) showed their maximum proliferation of leaf on hormone free full strength of solid KC medium which was found to be 10±2.19 leaves, 14.33±6.47 leaves and 16.67±2.29 leaves per culture respectively than other tested conditions of solid KC medium. The least proliferation of leaf from 2%, 3% and 4% alginate coated

artificial seeds were observed on full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which gave 2.5±0.56 leaves, 4±0.58 leaves and 5±2.41 leaves per culture respectively. Statistical analysis revealed that the leaves developed from of 3% alginate coated artificial seeds were not varied significantly while leaves developed from of 2% and 4% alginate coated artificial seeds were varied significantly ($p \leq 0.05$) among all the tested conditions of solid KC medium.

Hence among the different artificial seeds, 4% alginate coated artificial seeds gave the maximum proliferation of leaf on hormone free full strength of solid MS and KC media than other tested conditions of solid MS and KC media.

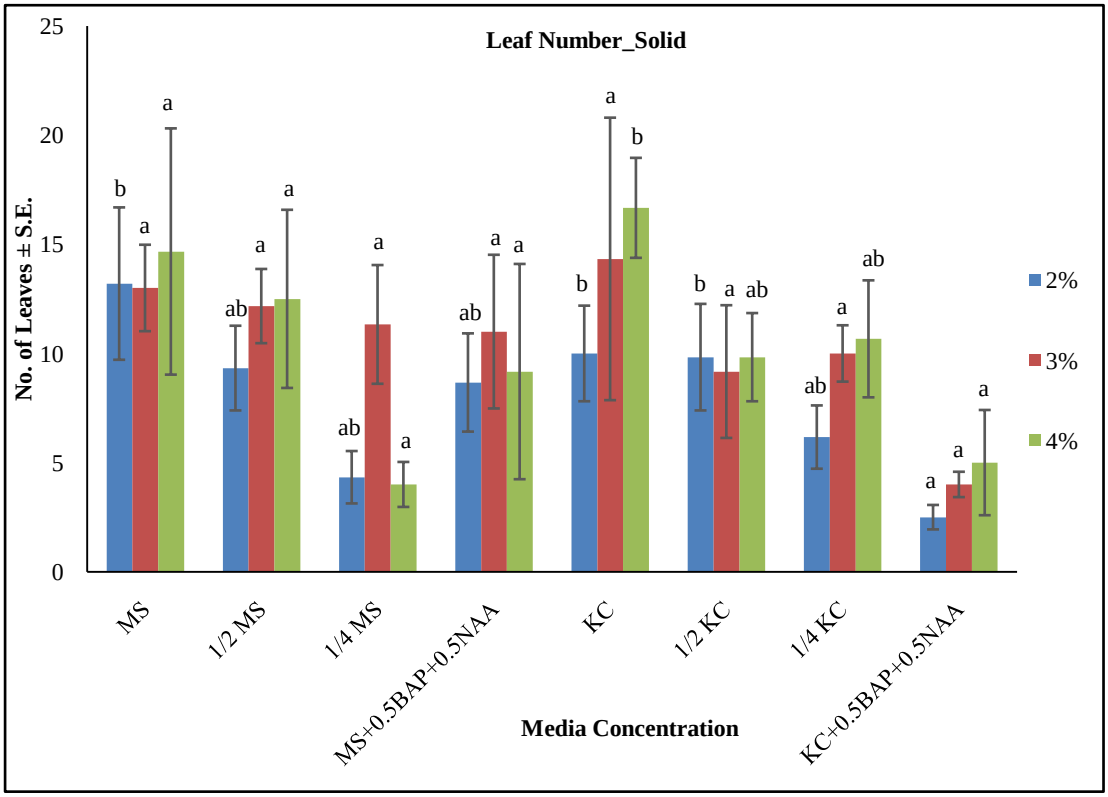


Figure 19: Average number of leaves derived from artificial seeds of *C. aloifolium* on different concentrations of solid MS and KC media. [The figure showed the statistical comparision on number of leaves developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Length of leaf

On solid MS medium, 2% alginate coated artificial seeds gave maximum leaf growth, i.e., 3.03±0.62 cm leaf length per culture on hormone free quarter strength of solid MS medium than other tested conditions of solid MS medium (Fig. 20). Similarly, 3% alginate coated artificial seeds gave maximum development of leaf, i.e., 3.77±1.01 cm leaf length per culture on full strength of liquid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA while 4% alginate coated artificial seeds showed maximum leaf growth, i.e., 2.85±0.55 cm leaf length per culture on hormone free full strength of solid MS medium. The least development of leaf from 2% alginate coated artificial seeds were observed on hormone free full strength of solid MS medium which was found to be 2.05±0.37 cm leaf length per culture. However, 3% and 4% alginate coated artificial seeds showed their least development of leaf on hormone free quarter strength of solid MS medium which was found to be 2.55±0.37 cm and 1.17±0.35 cm leaf length per culture respectively. Statistically, the leaf length of all the artificial seeds were not varied significantly ($p \leq 0.05$) among all the tested conditions of solid MS medium.

On solid KC medium, the maximum development of leaf from 2% and 4% alginate coated artificial seeds were observed on hormone free full strength of solid KC medium which was found to be 4.03±0.89 cm and 3.6±0.45 cm leaf length per culture respectively whereas 3% alginate coated artificial seeds showed their maximum development of leaf, i.e., 2.98±0.44 cm leaf length on hormone free quarter strength of liquid KC medium than other tested conditions of solid KC medium respectively (Fig. 20). However, all the artificial seeds (2%, 3% and 4%) gave least development of leaf on full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 0.93±0.24 cm, 0.55±0.16 cm and 0.98±0.54 cm leaf length per culture respectively. Statistical analysis revealed that the leaf length of all the artificial seeds were varied significantly ($p \leq 0.05$) among all the tested conditions of solid KC medium.

Hence among different artificial seeds, 3% alginate coated artificial seeds showed the maximum growth of leaf on full strength of solid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA while 2% alginate coated artificial seeds developed maximum length of leaf on hormone free full strength of solid KC medium rather than other tested conditions of solid MS and KC media.

Figure 2ASS: *In vitro* germination and development of 2% alginate coated artificial seeds of *Cymbidium aloifolium* on solid MS and KC media

Fig. 2ASS1 Development of plantlet from artificial seed on hormone free full strength of MS medium

Fig. 2ASS2 Development of shoot buds and small plantlets on hormone free half strength of MS medium

Fig. 2ASS3 Development of shoots and aerial root on hormone free quarter strength of MS medium

Fig. 2ASS4 Development of shoots and roots on MS medium supplemented with 0.5mg/l BAP + 0.5mg/l NAA

Fig. 2ASS5 Development of plantlets and roots on hormone free half strength of KC medium

Fig. 2ASS6 Development of small shoots and aerial roots on hormone free quarter strength of KC medium

Fig. 2ASS7 Development of small plantlets on hormone free quarter strength of MS medium

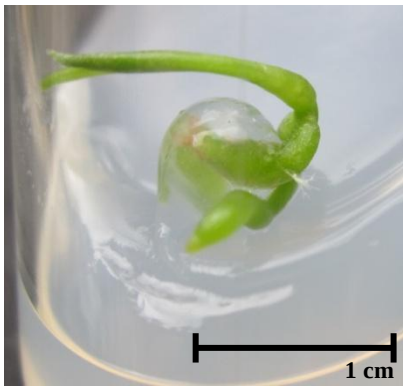
Fig. 2ASS8 Development of strong plantlet and aerial roots on hormone free full strength of MS medium

Fig. 2ASS9 Development of plantlets and roots on MS medium supplemented with 0.5mg/l BAP + 0.5mg/l NAA

Fig. 2ASS10 Development of plantlet on hormone free full strength of KC medium

Fig. 2ASS11 Development of plantlets on hormone free quarter strength of KC medium

Fig. 2ASS12 Development of plantlets on KC medium supplemented with 0.5mg/l BAP + 0.5mg/l NAA



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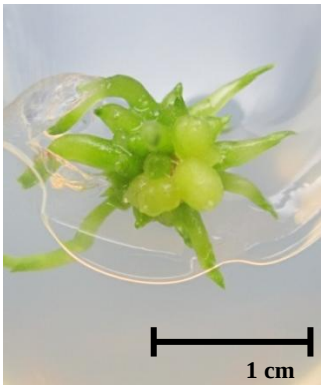

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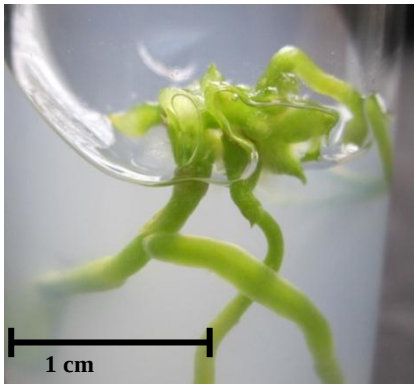
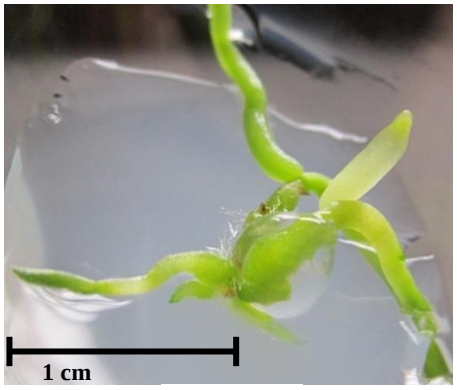
2ASS3

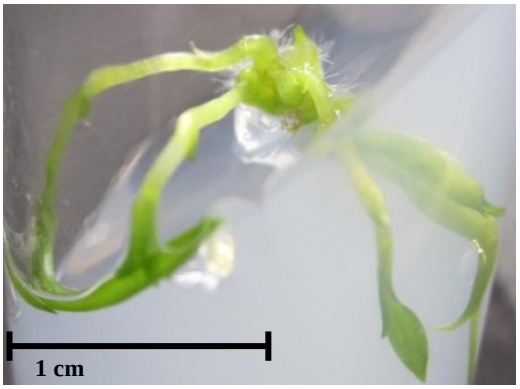


2ASS4

2ASS5



2ASS6



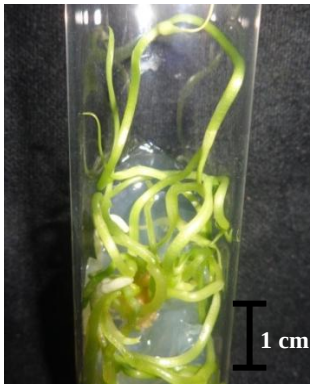
2ASS7

2ASS8

2ASS9

2ASS10

2ASS11



2ASS12

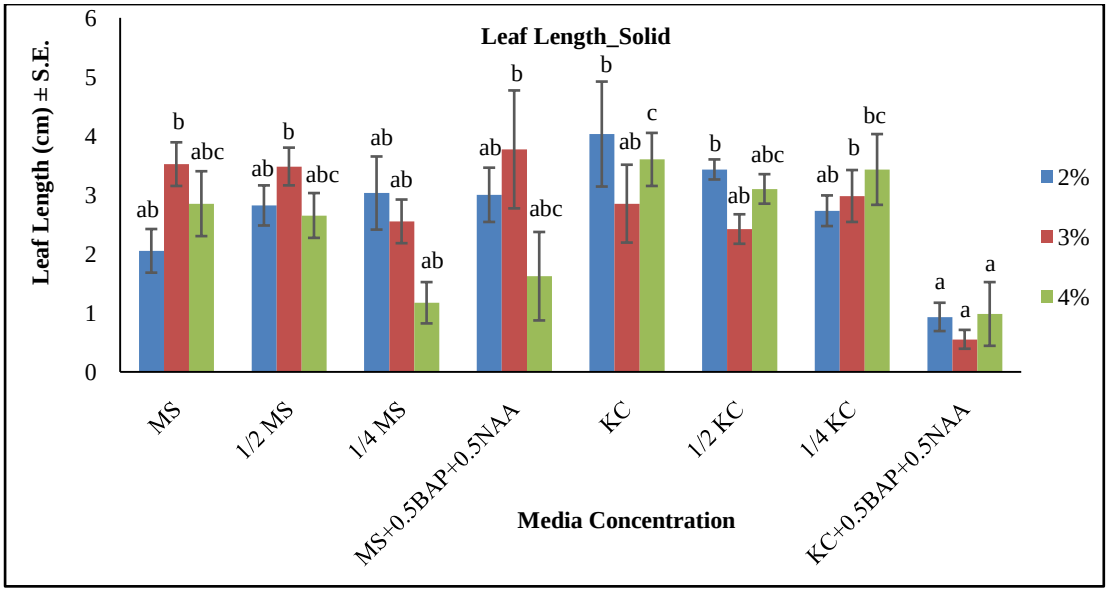


Figure 20: Average length of leaf derived from artificial seeds of *C. aloifolium* on different concentrations of solid MS and KC media. [The figure showed the statistical comparision on length of leaf developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Number of root

On solid MS medium, the maximum proliferation of root from 2% and 4% alginate coated artificial seeds were observed on full strength of solid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 3±1.12 roots and 4±2.11 roots per culture respectively while 3% alginate coated artificial seeds showed highest root number, i.e., 4.5±1.12 roots per culture on hormone free full strength of solid MS medium than other tested conditions of solid MS medium (Fig. 21). However, the least proliferation of root from 2%, 3% and 4% alginate coated artificial seeds were observed on hormone free quarter strength of solid MS medium which was found to be 1.5±0.34 roots, 2±0.26 roots and 0.67±0.33 roots per culture respectively. Statistical analysis revealed that the roots developed from all the artificial seeds were not varied significantly ($p \leq 0.05$) among all the tested conditions of solid MS medium.

On solid KC medium, the number of root was decreased on lower strength of medium (Fig. 21). The maximum proliferation of root from 2%, 3% and 4% alginate coated artificial seeds were observed on hormone free full strength of solid KC medium which was found to be 2.5±0.67 roots, 2.5±0.62 roots and 5±0.58 roots per culture than other tested conditions of solid KC medium. 2% alginate coated artificial seeds

proliferate least number of root, i.e., 1±0.36 roots per culture on hormone free quarter strength of solid KC medium while they were not able to produce any single roots on full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. However, 3% and 4% alginate coated artificial seeds showed their least proliferation of root on full strength of liquid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 0.5±0.22 roots and 1.83±1.64 roots per culture respectively. Statistical analysis revealed that the roots developed from 3% and 4% alginate coated artificial seeds were not varied significantly while roots developed from 2% alginate coated artificial seeds were varied significantly ($p \leq 0.05$) among all the tested conditions of solid KC medium.

Hence among the different artificial seeds, 3% alginate coated artificial seeds showed the maximum proliferation of root on hormone free full strength of solid MS medium while 4% alginate coated artificial seeds showed the maximum proliferation of root on hormone free full strength of solid KC medium than other tested conditions of solid MS and KC media.

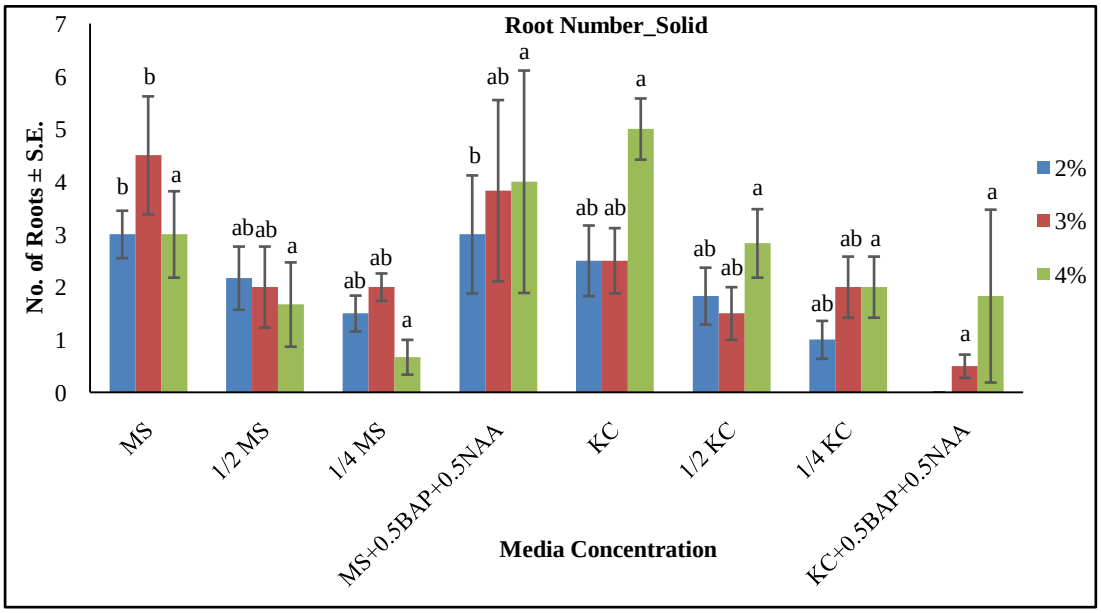


Figure 21: Average number of roots derived from artificial seeds of *C. aloifolium* on different concentrations of solid MS and KC media. [The figure showed the statistical comparision on number of roots developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Figure 3ASS: *In vitro* germination and development of 3% alginate coated artificial seeds of *Cymbidium aloifolium* on solid MS and KC media

Fig. 3ASS1 Development of shoot buds from artificial seed on hormone free full strength of MS medium

Fig. 3ASS2 Development of small plantlets on hormone free half strength of MS medium

Fig. 3ASS3 Development of small plantlets on hormone free quarter strength of MS medium

Fig. 3ASS4 Development of shoots and roots on full strength of MS medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA

Fig. 3ASS5 Development of shoot buds and small plantlets on full strength of KC medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA

Fig. 3ASS6 Development of healthy plantlets with green aerial roots on hormone free full strength of MS medium

Fig. 3ASS7 Development of plantlets on hormone free half strength of MS medium

Fig. 3ASS8 Development of plantlets with aerial roots on hormone free full strength of MS medium

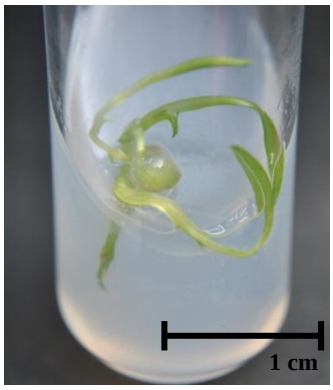
Fig. 3ASS9 Development of plantlets on hormone free full strength of KC medium

Fig. 3ASS10 Development of plantlets on hormone free full strength of KC medium

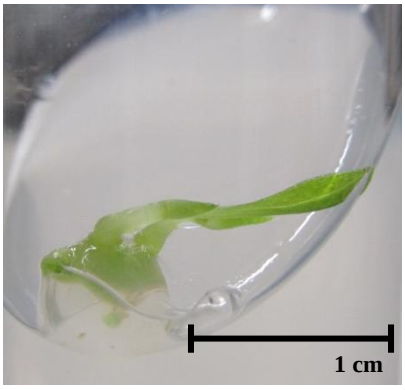
Fig. 3ASS11 Development of shoot buds and plantlets on hormone free half strength of KC medium

Fig. 3ASS12 Development of multiple shoots on full strength of KC medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA

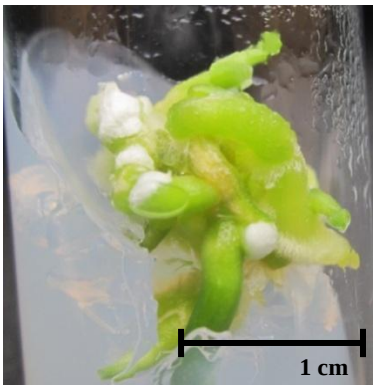
3ASS1



3ASS2



3ASS3



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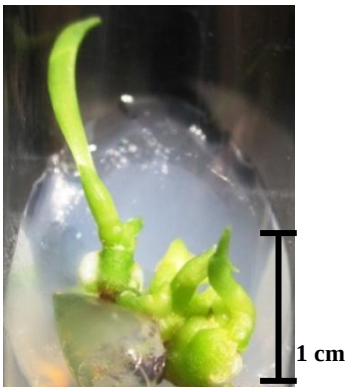
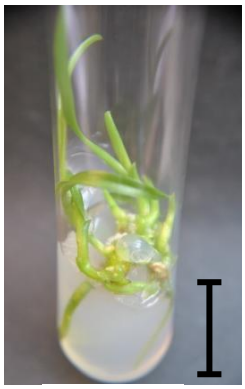


3ASS5

3ASS6



3ASS7

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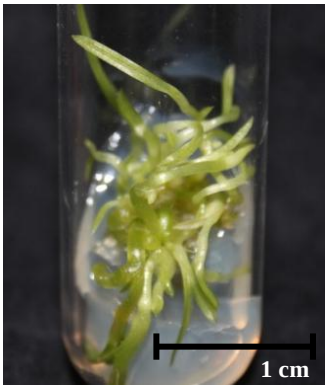

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3ASS11

3ASS12

Length of root

On solid MS medium, 2% alginate coated artificial seeds showed maximum development of root, i.e., 2.77±0.79 cm root length per culture on hormone free quarter strength of solid MS medium while 3% and 4% alginate coated artificial seeds showed their maximum root growth on hormone free full strength of solid MS medium which was found to be 5.15±0.68 cm and 2.62±0.68 cm root length per culture respectively than other tested conditions of solid MS medium (Fig. 22). However, the least development of root from 2% alginate coated artificial seeds were observed on full strength of solid MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 1.87±0.62 cm root length per culture whereas 3% and 4% alginate coated artificial seeds showed least development of root on hormone free quarter strength of solid MS medium which was found to be 1.5±0.18 cm and 0.18±0.11 cm root length per culture respectively. Statistical analysis revealed that the root length of 2% and 4% alginate coated artificial seeds were not varied significantly while root length of 3% alginate coated artificial seeds showed significantly different ($p \leq 0.05$) among all the tested conditions of solid MS medium.

On solid KC medium, the length of root was decreased on lower strength of medium and medium supplemented with hormones (Fig. 22). Here, all the artificial seeds (2%, 3% and 4%) showed their maximum root growth on hormone free full strength of solid KC medium which was found to be 3.2±1.01 cm, 3.63±1.39 cm and 5.17±0.64 cm root length per culture than other tested conditions of solid KC medium. The least development of root from 3% and 4% alginate coated artificial seeds were observed on full strength of solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA which was found to be 0.18±0.12 cm and 0.78±0.65 cm root length per culture respectively whereas 2% alginate coated artificial seeds gave least root growth, i.e., 1.67±0.71 cm root length per culture on hormone free quarter strength of solid KC medium and was not able to produce any root on solid KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Statistically, root length of all the artificial seeds were varied significantly ($p \leq 0.05$) among all the tested conditions of solid KC medium.

Figure 4ASS: *In vitro* germination and development of 4% alginate coated artificial seeds of *Cymbidium aloifolium* on solid MS and KC media

Fig. 4ASS1 Development of elongated shoot buds from artificial seed on hormone free full strength of MS medium

Fig. 4ASS2 Development plantlet with root on hormone free full strength of MS medium

Fig. 4ASS3 Development of small plantlets with aerial roots on full strength of MS medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA

Fig. 4ASS4 Development of small plantlets with roots on full strength of MS medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA

Fig. 4ASS5 Development of shoot buds and plantlets on hormone free full strength of MS medium

Fig. 4ASS6 Development of multiple shoots on hormone free half strength of MS medium

Fig. 4ASS7 Development of healthy plantlets on hormone free full strength of MS medium

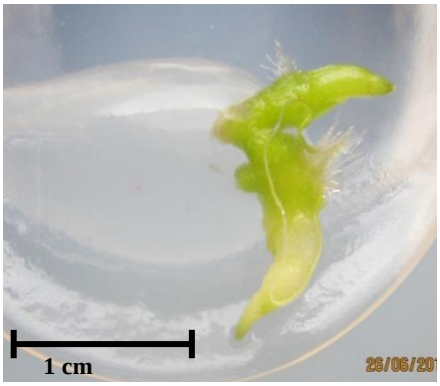
Fig. 4ASS8 Development of plantlets on hormone free full strength of MS medium

Fig. 4ASS9 Development of healthy green plantlets with roots on hormone free full strength of KC medium

Fig. 4ASS10 Development of plantlets with root on hormone free half strength of KC medium

Fig. 4ASS11 Development of plantlet with green root on hormone free half strength of KC medium

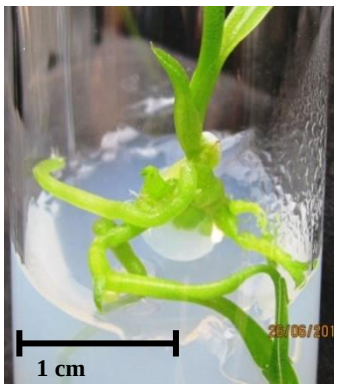
Fig. 4ASS12 Development of shoot buds and small plantlets on full strength of KC medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA



4-ASS1

4-ASS2

4-ASS3



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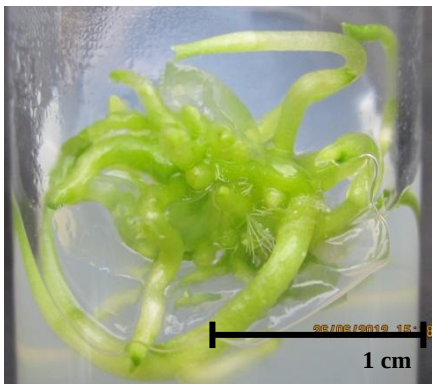
4-ASS5



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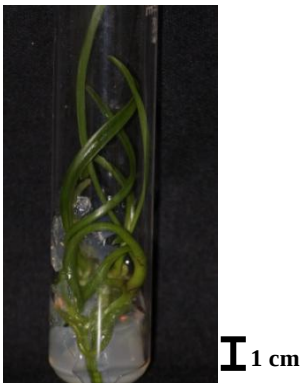
4-ASS8



4-ASS9

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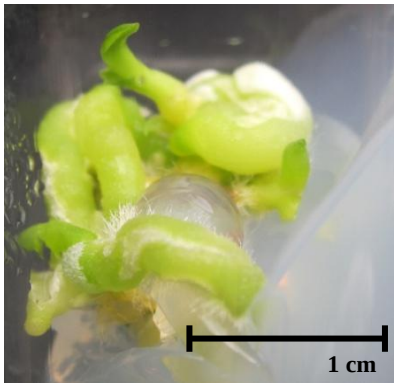
4-ASS11



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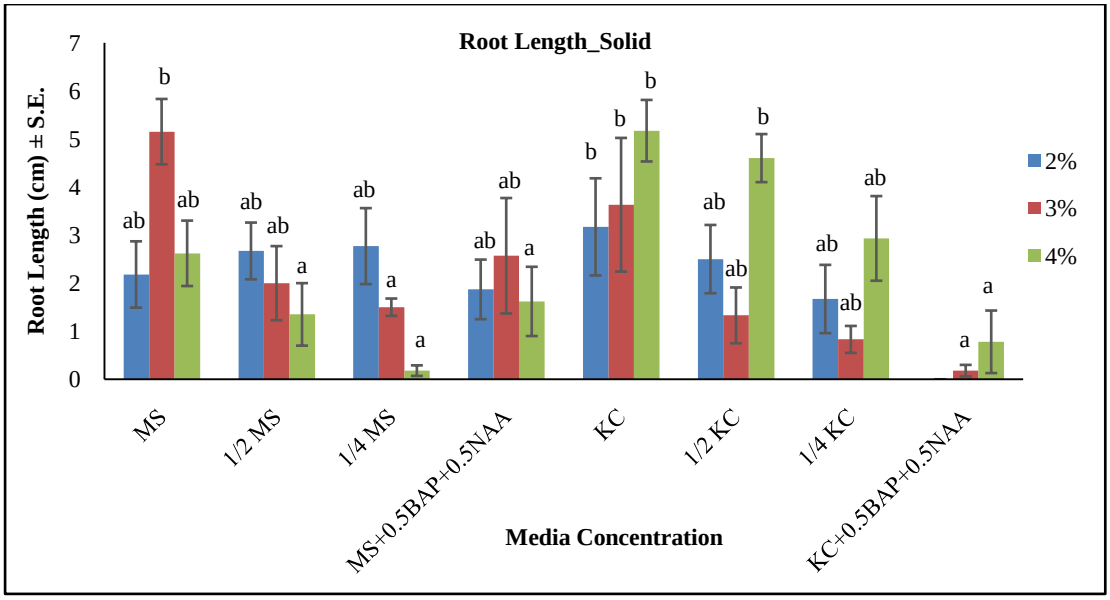


Figure 22: Average length of root derived from artificial seeds of *C. aloifolium* on different concentrations of solid MS and KC media. [The figure showed the statistical comparision on length of root developed from different types of artificial seeds on different media concentration, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)].

Hence among the different artificial seeds, 3% alginate coated artificial seeds showed the maximum growth of root on hormone free full strength of solid MS medium while 4% alginate coated artificial seeds showed the maximum growth of root on hormone free full strength of solid KC medium than other tested conditions of solid MS and KC media.

4.1.3.2.6 Effect of storage conditions (temperature and time period) for viability of artificial seeds

In present investigation, the coating gel of artificial seeds contained only calcium alginate (after ionization between sodium and calcium ions) and was devoid of any other nutrients or plant growth regulators. In both MS and KC liquid media, 2%, 3% and 4% alginate coated artificial seeds stored at 4ºC were green, healthy and remained intact inside the calcium alginate beads until 120 days; whereas those stored at room temperature (RT, 21 ±2°C) became pale and reduced their size. On MS medium, all the artificial seeds ruptured slowly after 9-10 weeks of inoculation at RT and most of them become contaminated; while at 4ºC the germination was started within 7-9 weeks of inoculation. In case of KC medium, stored artificial seeds germinated within

8-10 weeks of culture at 4ºC and 10-12 weeks of culture at RT. On MS medium, 2%, 3% and 4% alginate coated artificial seeds showed 70%, 93.33% and 83.33% viability respectively when stored for 30 days at 4ºC (Figs. V01, V10 to V14) while their viability percentage decreased to 6.67%, 36.67 and 26.67% on 120 days of storage (Fig. V09). At RT, 3% and 4% alginate coated artificial seeds showed 40% and 30% viability respectively after 30 days of storage (Fig. V02) and no regeneration were occurred after 60 days of storage.

However on KC medium, 2%, 3% and 4% alginate coated artificial seeds showed 26.67%, 46.67% and 33.33% of viability respectively when stored for 30 days at 4ºC (Fig. V03) and their viability percentage decreased to 0%, 16.67% and 13.33% on 120 days of storage respectively. The 2% alginate coated artificial seeds showed only 10% viability after 90 days (Fig. V06) and no viability or regeneration was observed after 120 days of storage at 4ºC. The 3% and 4% alginate coated artificial seeds showed only 20% and 10% viability when stored for 30 days at RT and no regeneration was observed after 60 days of storage.

The 2% alginate coated artificial seeds did not give any regeneration response from 30 to 120 days at RT on both MS and KC media. It may be due to the low concentration of sodium alginate which may reduce the growth of protocorm. Non-encapsulated protocorms also failed to give regeneration response when stored at 4ºC and RT for 30 to 120 days. It may be due to the desiccation (Table 6).

Table 6: Effect of storage temperature and duration of storage on regeneration of plantlets of *Cymbidium aloifolium* from different artificial seeds

Artificial seed	Media	Storage Temperature	Duration of Storage (days)	Total no. of tested artificial seeds	No. of Unruptured artificial seeds	No. of Ruptured artificial seeds	Conversion of artificial seeds into plantlets (%)
2%	**MS**	4ºC	30	30	10	21	70
			60	30	18	12	40
			90	30	21	9	30
			120	30	28	2	6.67
		RT	30	30	30	0	-
			60	30	30	0	-
			90	30	30	0	-
			120	30	30	0	-
	KC	4ºC	30	30	22	8	26.67
			60	30	24	6	20
			90	30	27	3	10
			120	30	30	0	-
		RT	30	30	30	0	-
			60	30	30	0	-
			90	30	30	0	-
			120	30	30	0	-
3%	**MS**	4ºC	30	30	2	28	93.33
			60	30	10	20	66.67
			90	30	14	16	53.33
			120	30	19	11	36.67
		RT	30	30	18	12	40
			60	30	30	0	-
			90	30	30	0	-
			120	30	30	0	-
	KC	4ºC	30	30	16	14	46.67
			60	30	20	10	33.33
			90	30	23	7	23.33
			120	30	25	5	16.67
		RT	30	30	24	6	20.00
			60	30	30	0	-
			90	30	30	0	-
			120	30	30	0	-
4%	**MS**	4ºC	30	30	5	25	83.33
			60	30	14	16	53.33
			90	30	17	13	43.33
			120	30	20	8	26.67

		RT	30	30	21	9	30.00
			60	30	30	0	-
			90	30	30	0	-
			120	30	30	0	
	KC	4ºC	30	30	20	10	33.33
			60	30	23	7	23.33
			90	30	25	5	16.66
			120	30	26	4	13.33
		RT	30	30	27	3	10.00
			60	30	30	0	-
			90	30	30	0	-
			120	30	30	0	-
Non encapsulated protocorm	**MS, KC**	4ºC	30	30	0	0	0
			60	30	0	0	0
			90	30	0	0	0
			120	30	0	0	0
	MS, KC	RT	30	30	0	0	0
			60	30	0	0	0
			90	30	0	0	0
			120	30	0	0	0

Figure V: Test of viability of artificial seeds stored at 4ºC and room temperature (21±2°C) cultured on liquid and solid MS and KC media

Fig. V 01 Development of multiple shoot buds and shoot from 30 days storage 4% alginate coated artificial seed at 4ºC on liquid MS medium

Fig. V 02 Development of multiple shoot buds from 30 days storage 3% alginate coated artificial seed at room temperature on liquid MS medium

Fig. V 03 Development of multiple shoot buds from 30 days storage 2% alginate coated artificial seed at 4ºC on liquid KC medium

Fig. V 04 Development of shoot and roots from 60 days storage 4% alginate coated artificial seed at 4ºC on liquid KC medium

Fig. V 05 Development of shoot buds and plantlets from 60 days storage 3% alginate coated artificial seed at 4ºC on liquid MS medium

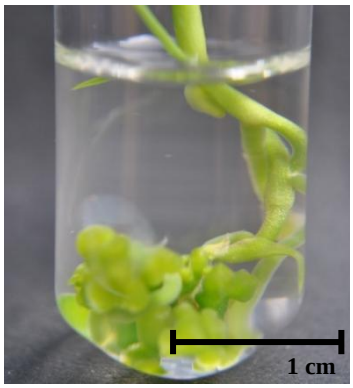
Fig. V 06 Development of shoots from 90 days storage 2% alginate coated artificial seed at 4ºC on liquid MS medium

Fig. V 07 Development of plantlets and roots from 90 days storage 3% alginate coated artificial seed at 4ºC on liquid MS medium

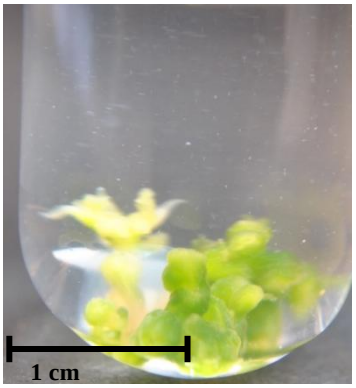
Fig. V 08 Development of multiple shoot buds and single shoot from 90 days storage 3% alginate coated artificial seed at 4ºC on liquid KC medium

Fig. V 09 Development of plantlets and roots from 120 days storage 3% alginate coated artificial seed at 4ºC on liquid MS medium

Fig. V10-V14 Development of multiple shoot buds and plantlets from 30 days storage 3% alginate coated artificial seed at 4ºC on solid MS medium



V 01



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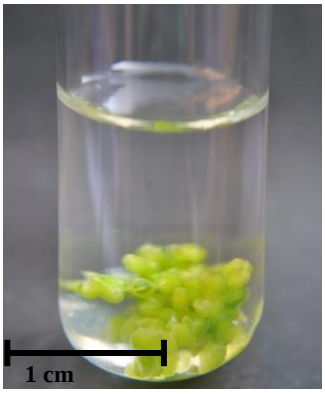

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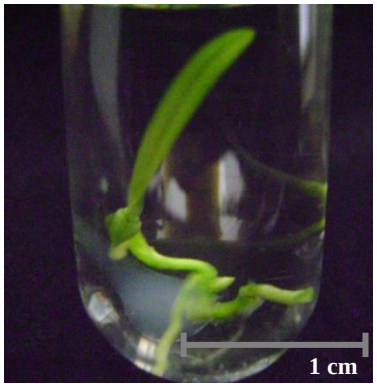

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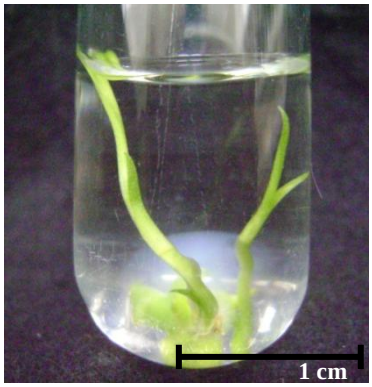

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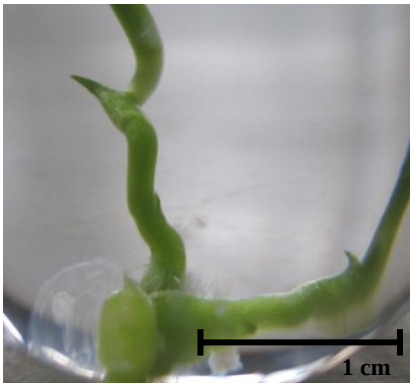
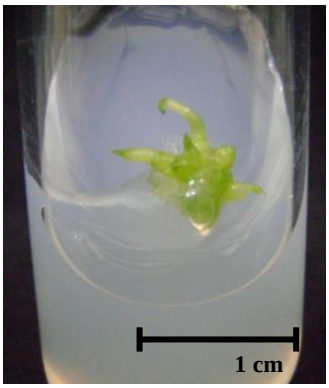


V 06

V 07

V 08

V 09



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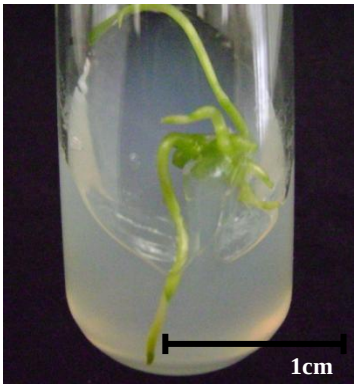
V 11



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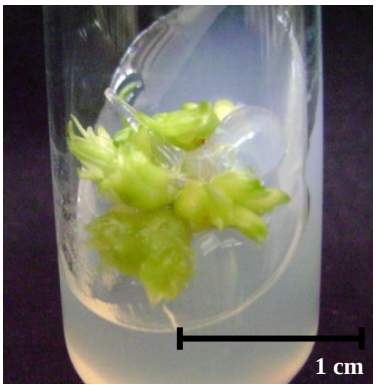

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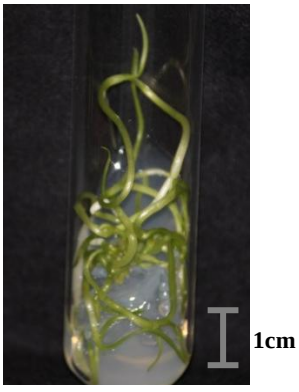


V 14

4.1.3.3 Shoot multiplication

For shoot multiplication, the shoot tips (about 5 mm) derived from *in vitro* culture of 2%, 3% and 4% alginate coated artificial seeds were sub cultured on hormone free full strength of liquid and solid MS medium as this medium was found to be effective for the development of large number of seedlings of *Cymbidium aloifolium*. The maximum number of shoots with strong root was observed on both medium after 12 weeks of culture. The micro shoots obtained from multiple shoots were used as explant for induction of large number of roots on rooting medium. The shoots after attaining the height of about 4 to 5 cm were also used for acclimatization and mass propagation (Fig. A01 to A04).

4.1.3.4 Rooting of artificial seed derived shoots

The micro shoots obtained from shoot tip culture were subcultured on MS medium alone (control) and MS medium supplemented with different auxins (IAA, IBA and NAA) in different concentrations (0.5-2.0 mg/l) for the root induction (Fig. R). In present study, it was found that the different concentrations of auxins showed varied responses on development of shoots and roots from microshoot of *C. aloifolium*. MS medium supplemented with 1.0 mg/l IBA was found to be effective for development of thick roots as compare to MS medium supplemented with different concentrations of NAA and IAA and MS medium alone. One-way ANOVA showed that the value of all the growth parameters except number of root were significantly different among different tested conditions of MS medium supplemented with various concentrations (0.5-2.0 mg/l) of auxins, i.e., IAA, IBA and NAA ($p < 0.05$; Appendix 29).

The following responses were obtained during rooting of *C. aloifolium* after 24 weeks of culture of shoot tip derived from artificial seeds (Figs. 23 & 24).

Number of shoot

All the tested condition of rooting favoured the multiplication of shoots from micro shoot derived from shoot multiplication. The maximum proliferation of shoot was observed on full strength of MS medium supplemented with 1 mg/l IBA which was found to be 6.33±1.23 shoots per culture than other tested conditions of rooting. These shoots were green and fleshy. This condition was followed by MS medium supplemented with 1 mg/l IAA where the proliferation of shoot was found to be 3.67±0.88 shoots per culture. However the least multiplication of shoots was observed on hormone free full strength of MS medium which was found to be 1.33±0.21 shoots

per culture. The statistical analysis revealed that all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different auxins (Fig. 23).

Length of shoot

Among different tested conditions of rooting, the maximum development of shoot was obtained on MS medium supplemented with 1.5 mg/l IAA which was found to be 6.17±0.82 cm shoot length per culture after 24 weeks of culture. This condition was followed by MS medium supplemented with 1 mg/l IAA and MS medium supplemented with 1 mg/l IBA where the shoot length was 5.92 cm than other tested conditions. However the least development of shoots was observed on full strength of MS medium supplemented with 1.5 mg/l NAA which was found to be 2.33±0.25 cm shoot length per culture. Statistically, all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different auxins (Fig. 23).

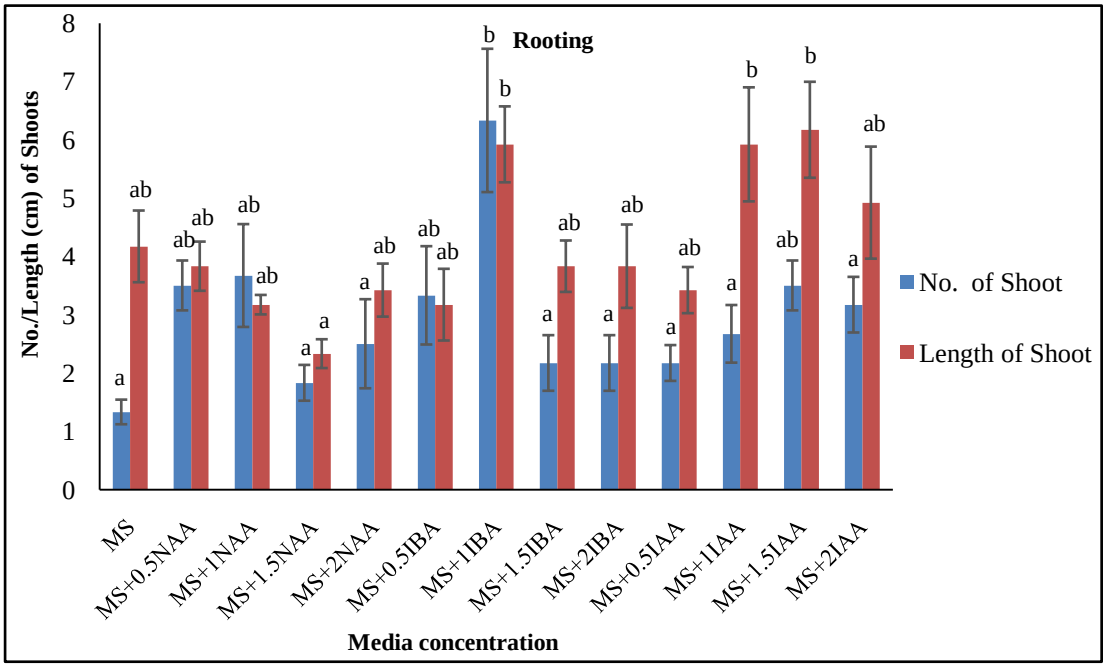


Figure 23: Average number and length of shoots of *C. aloifolium* on different concentrations of auxins [The figure showed the statistical comparision on number and length of shoot on different media concentration of rooting, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)]

Number of root

All the tested condition of rooting favoured the multiplication of roots from micro

shoot derived from shoot multiplication. The highest proliferation of root was observed on full strength of MS medium supplemented with 1 mg/l IAA which was found to be 5.83±0.95 roots per culture than other tested conditions of rooting. These roots were thin. This condition was followed by MS+1.5 mg/l IAA, MS+2 mg/l IAA, MS+2 mg/l NAA and MS+1 mg/l IBA where the proliferation of shoot was found to be about 5 roots per culture. Whereas the least multiplication of roots was observed on full strength of MS medium supplemented with 0.5 mg/l IBA which was found to be 3.17±1.04 roots per culture. However the roots were thick and fleshy on all the tested conditions of IBA. The statistical analysis revealed that all the values were not significant ($p \leq 0.05$) among different tested conditions of MS medium supplemented with different auxins (Fig. 24).

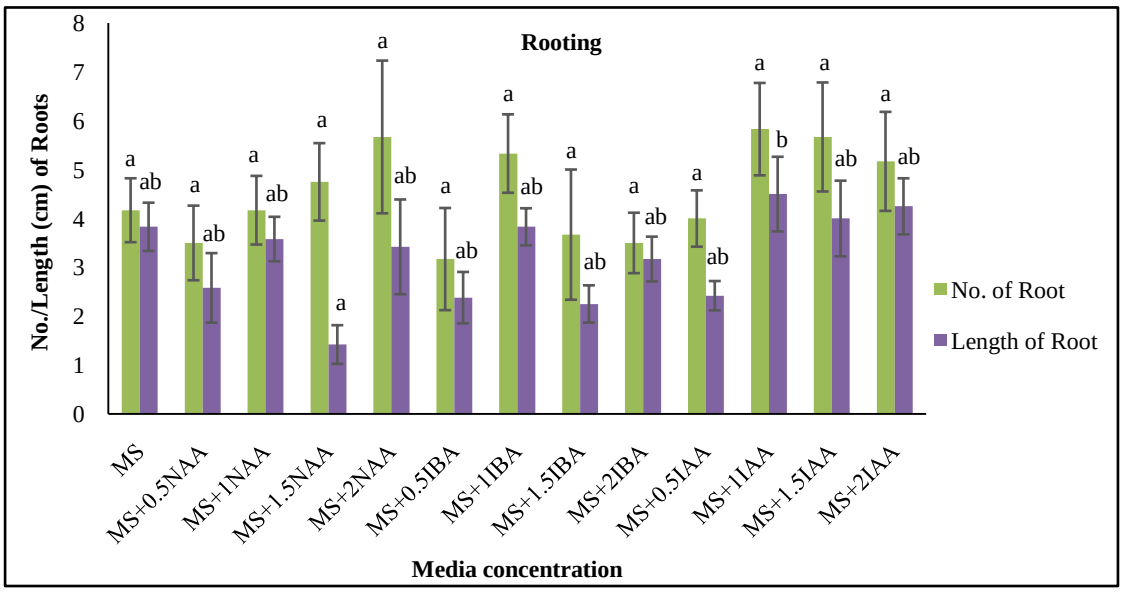


Figure 24: Average number and length of roots of *C. aloifolium* on different concentrations of auxins [The figure showed the statistical comparision on number and length of root on different media concentration of rooting, same alphabets above the bar indicates no significant different at 5% level of significance ($p \leq 0.05$)]

Length of root

Among different tested conditions of rooting, the maximum development of root was obtained on MS medium supplemented with 1.0 mg/l IAA which was found to be 4.5±0.76 cm root length per culture after 24 weeks of culture. The root length was decreased from 4.5 to 3.2 cm on higher concentration of IAA. This condition was followed by MS medium supplemented with 1 mg/l IBA where the root length was 3.83±0.38 cm per culture than other tested conditions.

Figure R: Development of roots on different hormone concentrations of auxins after 10 weeks of culture of shoot tip derived from artificial seeds

Fig. R 01 Root formation on MS medium.

Fig. R 02 Root formation on MS medium supplemented with IAA 1.0 mg/l.

Fig. R 03 Root formation on MS medium supplemented with IAA 1.5 mg/l.

Fig. R 04 Root formation on MS medium supplemented with IAA 2 mg/l.

Fig. R 05 Root formation on MS medium supplemented with IBA 0.5 mg/l.

Fig. R 06 Root formation on MS medium supplemented with IBA 1 mg/l.

Fig. R 07 Root formation on MS medium supplemented with IBA 1.5 mg/l.

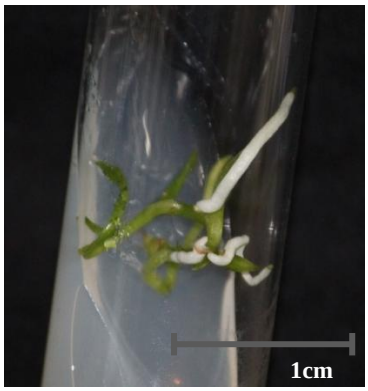
Fig. R 08 Root formation on MS medium supplemented with IBA 2 mg/l.

Fig. R 09 Root formation on MS medium supplemented with NAA 0.5 mg/l.

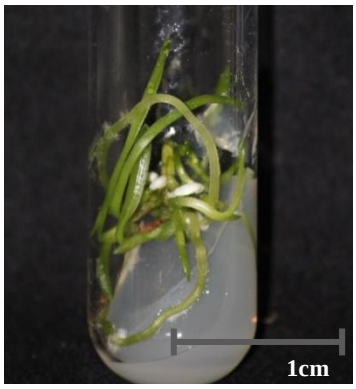
Fig. R 10 Root formation on MS medium supplemented with NAA 1 mg/l.

Fig. R 11 Root formation on MS medium supplemented with NAA 1.5 mg/l.

Fig. R 12 Root formation on MS medium supplemented with NAA 2 mg/l.



R 01



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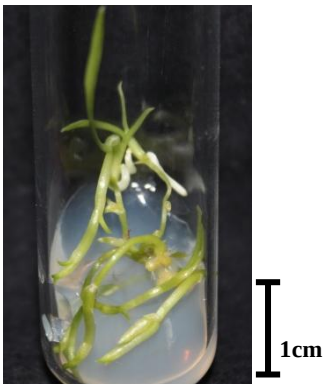

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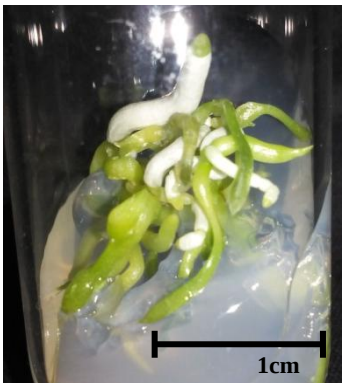

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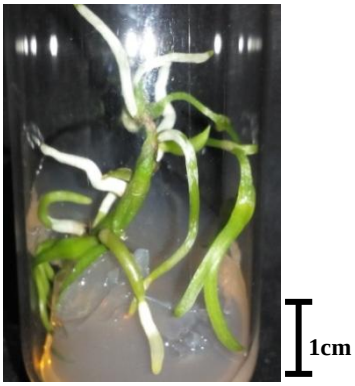
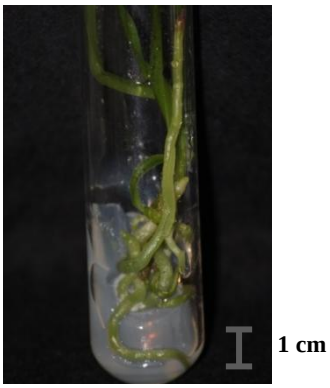


R 05

R 06

R 07

R 08



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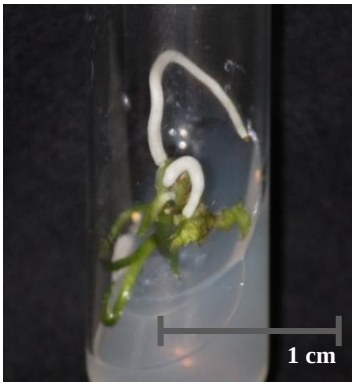


R 10

R 11

R 12

However the least development of root was observed on full strength of MS medium supplemented with 1.5 mg/l NAA which was found to be 1.42±0.39 cm root length per culture. Statistically, all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.01$) supplemented with different auxins (Fig. 24).

4.1.3.5 DAS-ELISA test of the plant samples

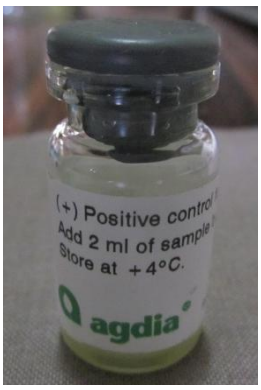
DAS ELISA test for Cymbidium mosaic virus was carried out in an aseptic condition. Young *in vitro* plantlets (Fig. VR01) of *Cymbidium aloifolium* obtained from different culture explants *viz.* seeds, protocorm, artificial seeds (2%, 3% and 4%) and shoot tip were tested for CymMV virus and compared with mother plant obtained from wild. The result showed that all the *in vitro* plantlets were 100% free from CymMV virus whereas mother plant showed 83.33% of virus infection (Table 7). There were no characteristic symptoms of CymMV in all the *in vitro* plantlets which might be due to the absence of causal agent of CymMV. The mean absorbance value at 405 nm optical density (OD) of the leaflet sample obtained from seed was found to be 0.18±0.05, similarly the mean absorbance value of leaflet sample from protocorm was found to be 0.16±0.07, leaflet sample from 2%, 3% and 4% alginate coated artificial seed was found to be 0.18±0.05, 0.17±0.05 and 0.17±0.05 respectively and the sample from shoot tip was found to be 0.19±0.06. Whereas the mean absorbance value of the mother plant and control used for the present test was found to be 1.09±0.23 and 1.702±0.59 respectively (Table 7). Here, the control was the CymMV virus itself (obtained directly from Agdia, USA). Yellow colour reaction was observed in case of positive which was seen on mother plant and control (Fig. VR08). The result of the present test is given below:

Figure VR: Test of Cymbidium mosaic virus (CymMV) on *in vivo* (wild) and *in vitro* plantlets of *Cymbidium aloifolium*

Fig. VR 01	*In vitro* plantlets of *C. aloifolium* for CymMV test
Fig. VR 02	Extraction of *in vivo* and *in vitro* leaf samples
Fig. VR 03-VR 05	Different chemicals used for CymMV test
Fig. VR 06	Loaded plant samples along with substrate in ELISA plate
Fig. VR 07	ELISA plate reader to detect optical density of loaded samples
Fig. VR 08	Colour reaction in plant samples after adding antigen/antibody for CymMV test

VR 01

VR 02



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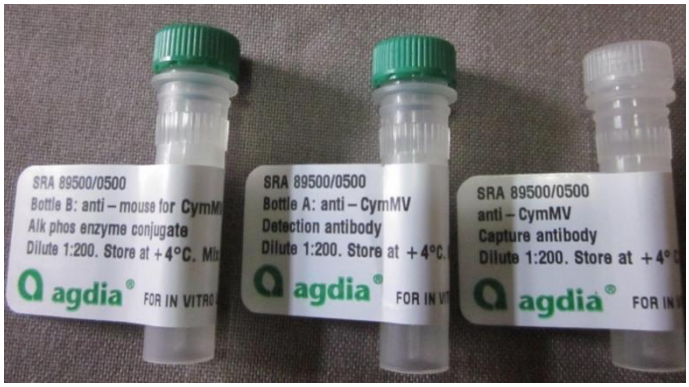

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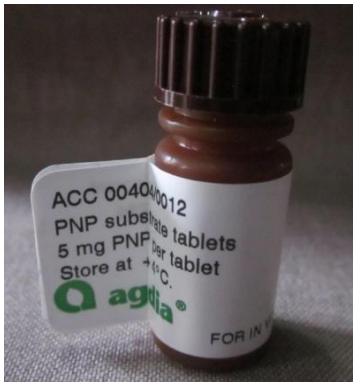

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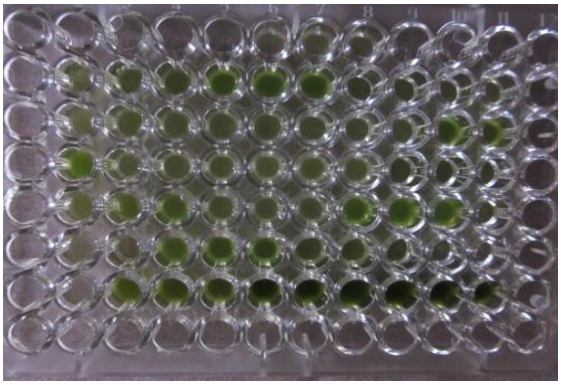

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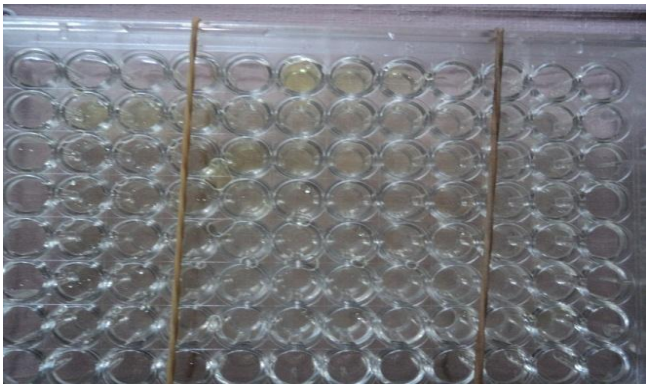


VR 08

Table 7: Detection of Cymbidium mosaic virus (CymMV) in wild (mother plant) and *in vitro* explants of *Cymbidium aloifolium*

Explants	Antigen	Absorbance (405 nm) mean ±SD	Percent (%) of infection (CymMV)	Inference
Control	CymMV	1.70±0.59	100	Severely Positive
Mother plant	"	1.09±0.23	83.33	Severely Positive
Seed	"	0.18±0.05	0	Negative
Protocorm	"	0.16±0.07	0	Negative
Shoot tips	"	0.19±0.06	0	Negative
Artificial seed (2%)	"	0.18±0.05	0	Negative
Artificial seed (3%)	"	0.17±0.05	0	Negative
Artificial seed (4%)	"	0.17±0.05	0	Negative

6 replicates were used for each plant sample, 3 replicates were used for control, Virus index: < 0.3 = negative; 0.3-0.99 = mild positive; >1.0 = severely positive

4.1.3.6 Extraction of genomic DNA

Total genomic DNA of *in vitro* and *in vivo* plant material of *Cymbidium aloifolium* was extracted by CTAB method and Genomic DNA Purification Kit (Promega). CTAB method gave rise to highly concentrated DNA (50 ng/ul) than Kit (30 ng/ul). DNA samples were diluted in TE buffer (to make 25 ng) and subjected to gel electrophoresis in 1% agarose gel (w/v). DNA was stained by gel immersion in ethidium bromide (1 μg/ml) solution for 45 min. After sometime, clear smear of DNA bands were observed. Extracted DNA thus obtained was then used for PCR amplification.

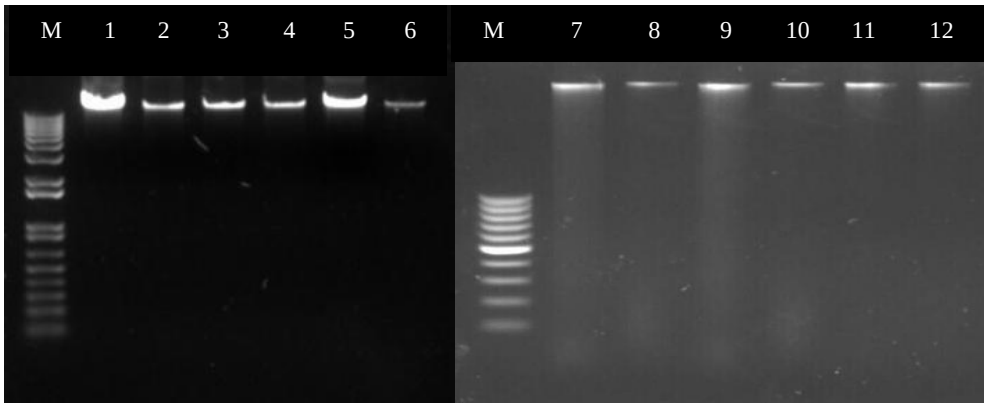
In the present investigation, PCR-based molecular technique of RAPD and ISSR markers were used to assess the genetic variations among the eleven randomly chosen tissue cultured plantlets and compared with the non-tissue cultured source plant of *C. aloifolium* collected from the wild (mother plant).

Gel electrophoresis

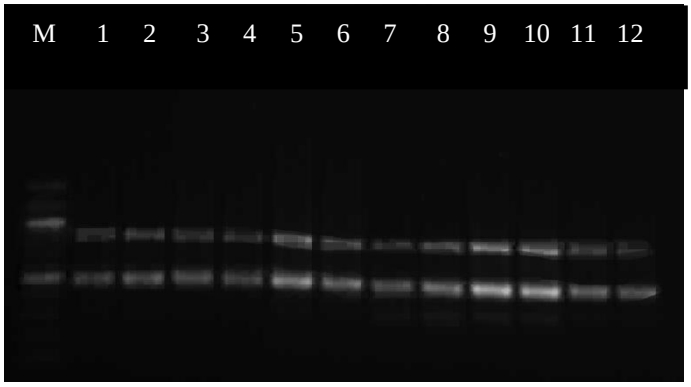
PCR Machine

In vivo leaf sample

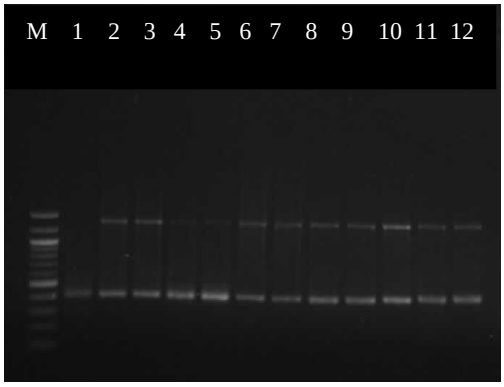
In vitro leaf sample



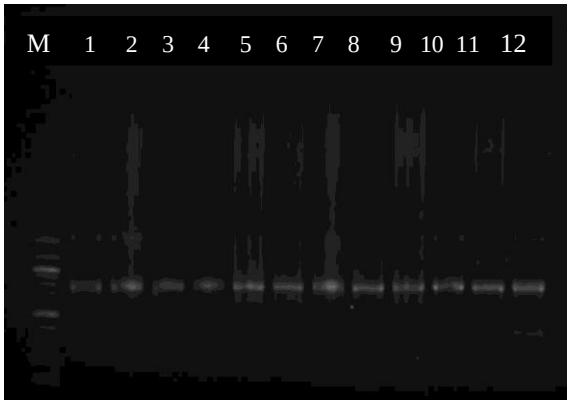
DNA bands



ISSR 807



ISSR 815



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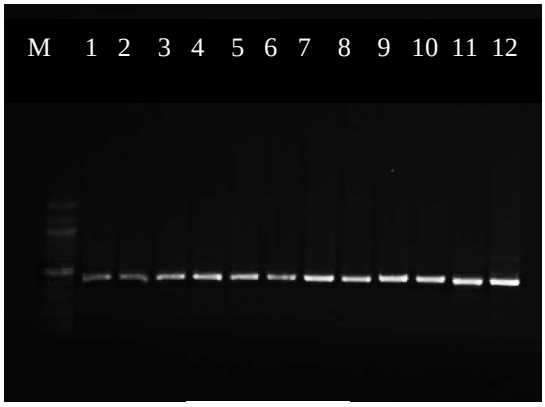


ISSR 825

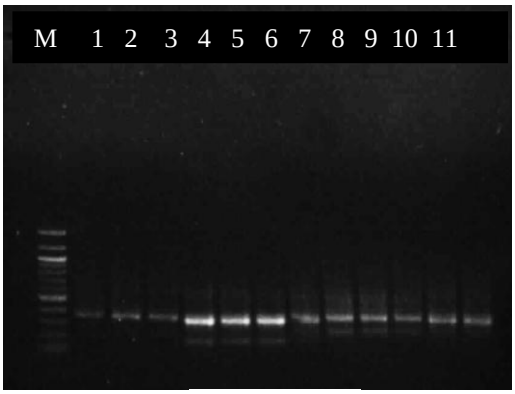
Fig. 25. DNA bands and PCR bands of *in vivo* (wild) and *in vitro* plant samples of *C. aloifolium* by using ISSR primers

4.1.3.6.1 RAPD analysis

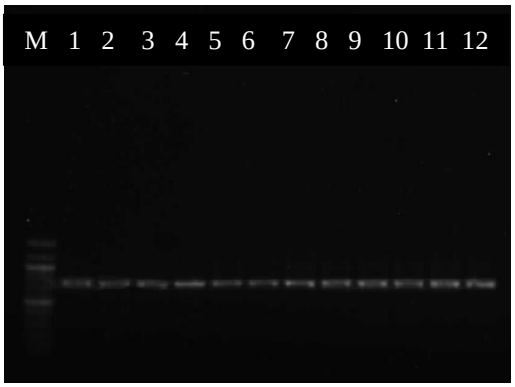
A total of ten arbitrary decamers of twenty six RAPD primers were used for initial screening, out of which twenty one primers gave reproducible bands. Both monomorphic and polymorphic bands were observed on all *in vitro* regenerated plants. A total of 483 clear, distinct and reproducible bands with the size ranging from 300 to 1400bp were scored from twenty one RAPD primers and rest of five primers were not give effective result (Appendix 2 & 3). The number of bands varied from 12 to 56 with an average of 18.58 bands per RAPD primer and their R_p values ranged from 2 to 7.5. The average PIC value of RAPD marker system was found to be 0.29 (Table 8). Out of 483 amplified bands, only 51 bands were polymorphic and the rest were monomorphic. The polymorphism level which was calculated as the number of polymorphic bands per primer ranged from 0 to 45.45%. Hence, the RAPD results showed average polymorphisms of 10.55% and similarity of 87.11% across the 12 plant samples (Table 8; Fig. 26).

4.1.3.6.2 ISSR analysis

Five ISSR primers were used to determine the genetic variability of tissue cultured and mother plant of *C. aloifolium*. All the ISSR primers were able to produce clear PCR bands. A total of 99 bands with the size ranging from 300 to 1400bp with an average of 19.8 bands per ISSR primer were scored. The R_p value of ISSR primers ranged from 2 to 4 (Appendix 4). The average PIC value of ISSR marker system was found to be 0.35 (Table 8). ISSR primers 807 and 816 amplified the highest number of fragments (24 bands) while ISSR primer 825 produced the lowest fragments (12 bands). All the banding patterns of *in vitro* derived regenerants and elite mother plants were found to be monomorphic in three ISSR primers *viz*. 807, 816 and 825 while the rest of two primers 815 and 827, displayed few polymorphic bands (15). Figure 25 depicts the amplification products using ISSR primers 815, 825 and 827. The number of polymorphic bands per ISSR primer ranged from 0 to 47.83% (Appendix 4). Thus, all the ISSR primers showed an average of 15.15% polymorphisms and 84.85% similarity within and between the regenerants and mother plants (Table 8).



OPA 07



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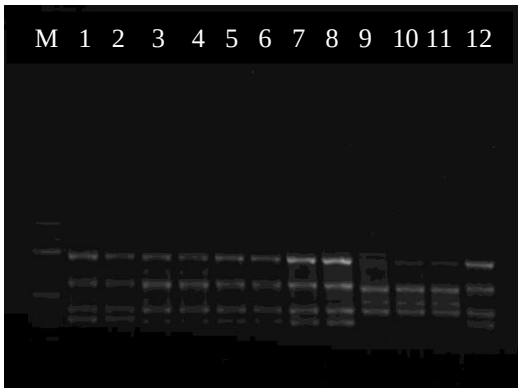
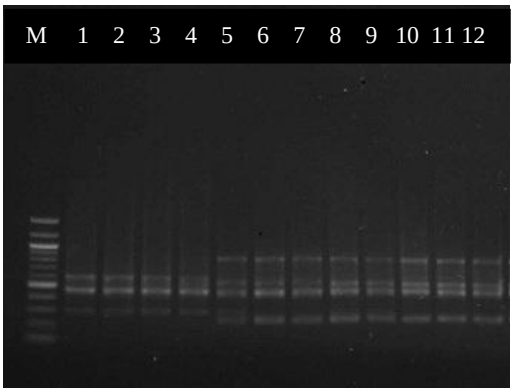
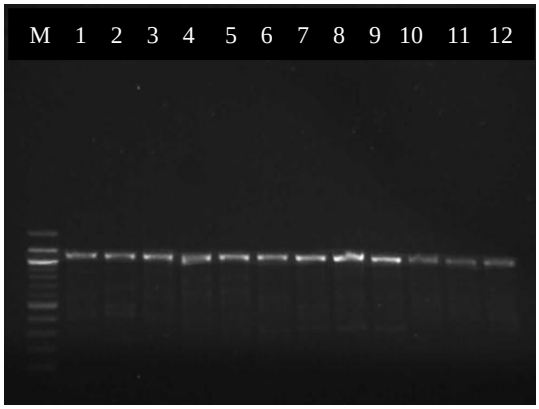


OPA 13



OPI 09



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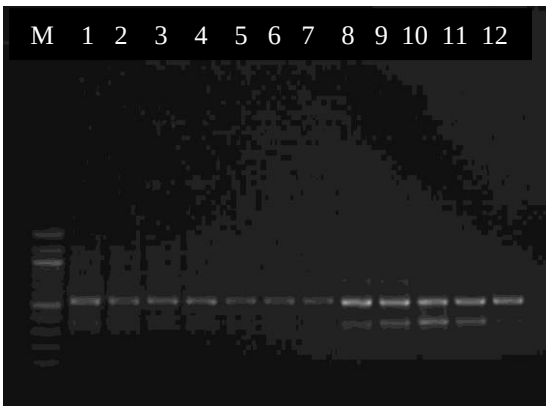

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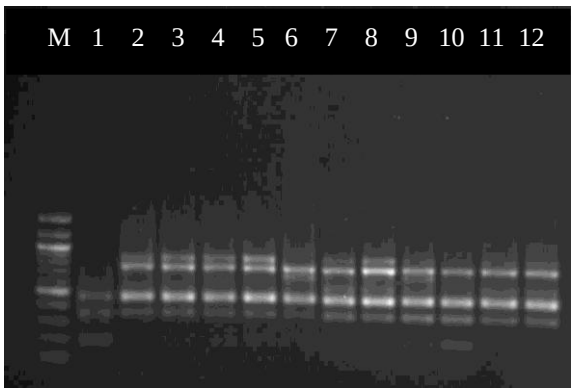

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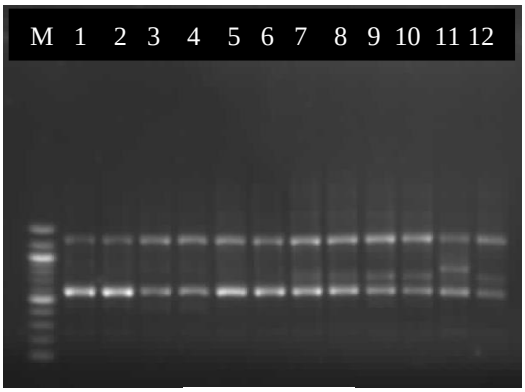


OPP 08

Fig. 26. PCR bands of *in vivo* (wild) and *in vitro* plant samples of *C. aloifolium* by using RAPD primers

Table 8: Comparative study of RAPD and ISSR markers individually as well as collectively

SN	Marker	No. of primer used	Total no. of amplified bands	Average bands/ Primer	Total no. of polymorphic bands	Percent (%) of polymorphism	Distance range (dice coefficient)	Polymorphic information content (PIC)	Resolving power (R_p)
1.	RAPD	26	483	18.58	51	10.55	0.87-1.0	0.29	3.09
2.	ISSR	5	99	19.8	15	15.15	0.87-1.0	0.35	3.3
3.	RAPD + ISSR	14	582	25.36	48	13.52	0.84-1.0	0.32	3.2

4.1.3.6.3 Dendrogram analysis

The similarity matrix and cluster analysis of RAPD and ISSR markers were performed individually using Dice similarity coefficient. They showed that the genetic relationships between the *in vitro* regenerants and the elite mother plant were varied from 0.87 to 1.00. The similarity index was shown in Appendix 30 and Appendix 31. The dendrogram constructed by using DICE coefficient through UPGMA analysis revealed 90% similarity amongst the *in vitro* regenerants and the mother plant using RAPD markers and divide the plant accession into 3 groups (Fig. 27) while ISSR markers (Fig. 28) showed 91% similarity between mother and tissue cultured plants and divided the plants into two groups. Both the markers showed that all the *in vitro* cultured plants were about 94% genetically similar with each other.

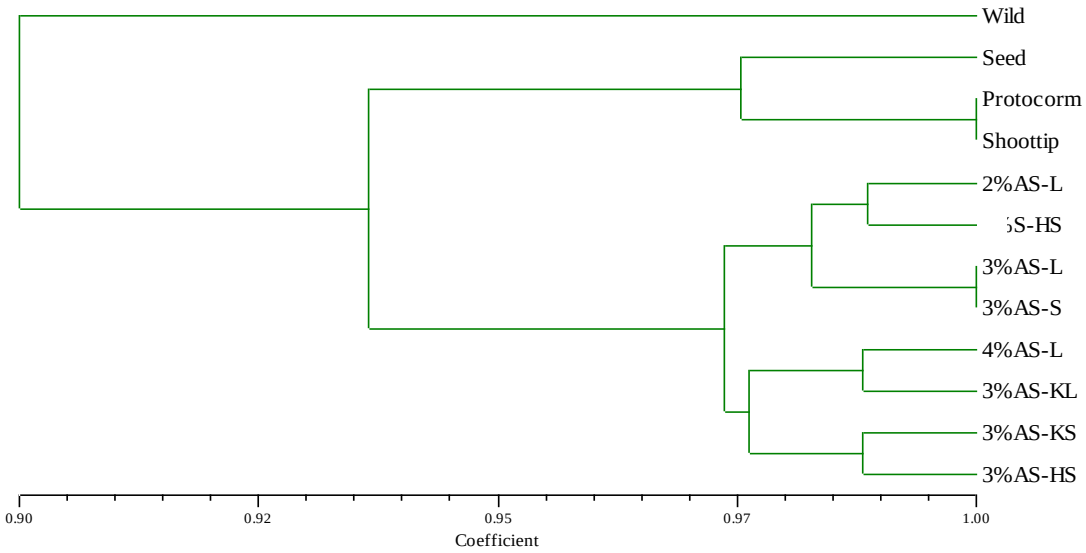


Figure 27: UPGMA dendrogram based on Dice similarity indices from RAPD primers, showing the genetic relationship between *in vivo* (wild) plants and *in vitro* regenerants of *Cymbidium aloifolium* (AS –L: artificial seed cultured on liquid MS medium, AS-S: artificial seed cultured on solid MS medium, AS-HS: artificial seed cultured on solid MS medium supplemented with hormones, BAP and NAA, AS-KS: artificial seed cultured on solid KC medium; AS-KL: artificial seed cultured on liquid KC medium; S-HS: seed cultured on solid MS medium supplemented with hormones, BAP and NAA)

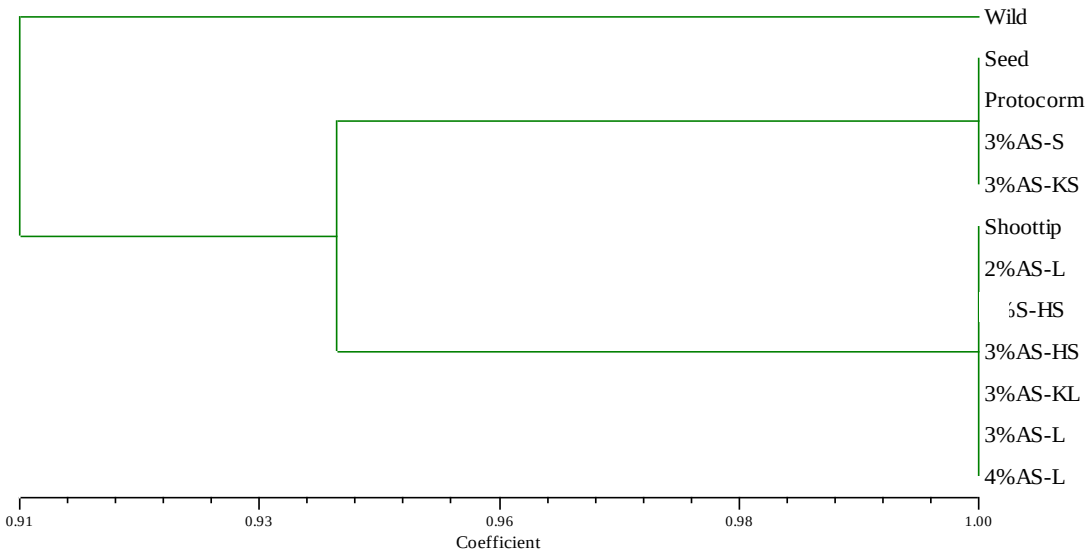


Figure 28: UPGMA dendrogram based on Dice similarity indices from ISSR primers, showing the genetic relationship between *in vivo* (wild) plants and *in vitro* regenerants of *Cymbidium aloifolium* (AS –L: artificial seed cultured on liquid MS medium, AS-S: artificial seed cultured on solid MS medium, AS-HS: artificial seed cultured on solid MS medium supplemented with hormones, BAP and NAA, AS-KS: artificial seed cultured on solid KC medium; AS-KL: artificial seed cultured on liquid KC; S-HS: seed cultured on solid MS medium supplemented with hormones, BAP and NAA)

4.1.2.7 Acclimatization

The rooted plantlets derived from artificial seeds measuring about 4 to 5 cm were transferred in pot containing different potting mixture for acclimatization (Fig. A06). All the potting mixture showed varied response on growth and development of plant. It was found that the potting mixture containing coco peat along with litter and sphagnum moss (Fig. A08) and cocopeat containing clay and sphagnum moss in the ratio of 3:2:1 showed 86.67% of survivability of plantlets (Fig. A09) whereas potting mixture containing cocpeat along with sphagnum moss in the ratio of 3:1 showed 80% of survivability (Fig. A07) and potting mixture containing cocopeat along with litter in the ratio of 3:1 showed 73.33% survivability of plantlets (Fig. A10). However potting mixture containing cocopeat along with sand and only sand did not give any response on growth of plant (Table 9).

Non-encapsulated protocorm as well as freshly prepared and one week storage at 4°C of 2%, 3% and 4% alginate coated artificial seeds were also acclimatized directly on different potting mixture for acclimatization (Fig. A17 to A20). The directly sown freshly prepared and one week stored at 4°C of 2% alginate coated artificial seeds and non-encapsulated protocorm did not give any response on regeneration of plantlet in all the tested acclimatization substrates.

It was found that the potting mixture containing cocopeat along with litter in the ratio of 3:1 was more appropriate for the acclimatization of 3% alginate coated freshly prepared artificial seeds which showed 73.33% of survivability (Figs. A22 & A23). The potting mixture containing cocopeat, litter and sphagnum moss (3:2:1) (Fig. A21) and cocopeat along with sphagnum moss (3:1) also showed 66.67% and 60% of survivability respectively whereas the potting mixture containing cocopeat, clay and sphagnum moss (3:2:1) showed only 20% of survivability. However, the potting mixture containing cocopeat along with sand and only sand did not response on their development. Similarly, 3% alginate coated artificial seeds stored at 4°C for one week showed 53.33% and 46.67% of survivability on potting mixture containing cocopeat, litter and sphagnum moss (3:2:1) and cocopeat along with litter (3:1) respectively while other potting mixture were not effective for their development on *in vivo* condition (Table 10).

Figure A1: Shoot multiplication and acclimatization of *Cymbidium aloifolium* on different potting mixture

Fig. A 01 Shoot multiplication on hormone free full strength of MS medium

Fig. A 02 Shoot multiplication on culture tubes and jars

Fig. A 03 Development of healthy shoots with roots on hormone free full strength of MS medium

Fig. A 04 Measurement of healthy plant with roots before acclimatization

Fig. A 05 Chemicals used for acclimatization

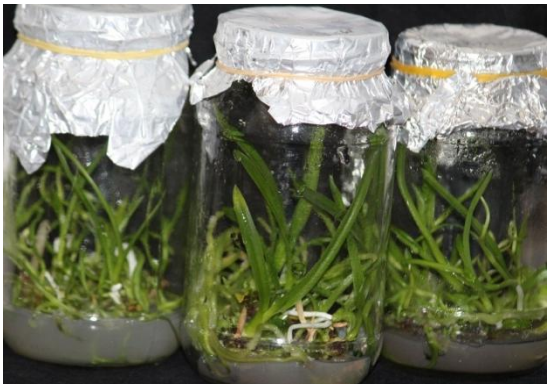
Fig. A 06 Plantlets acclimatized on different potting mixture

Fig. A 07 Plantlets acclimatized on potting mixture containing cocopeat and sphagnum moss (3:1)

Fig. A 08 Plantlets acclimatized on potting mixture containing cocopeat along with litter and sphagnum moss in the ratio of 3:2:1

Fig. A 09 Plantlets acclimatized on potting mixture containing cocopeat along with clay and sphagnum moss in the ratio of 3:2:1

Fig. A 10 Plantlets acclimatized on potting mixture containing cocopeat along with litter in the ratio of 3:1



A 01

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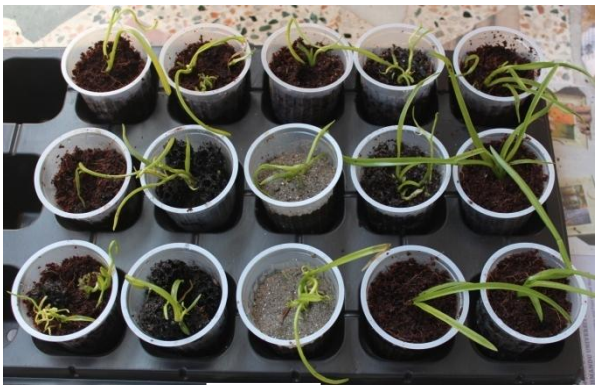

A 06

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Table 9: Effect of different potting mixture on acclimatization of plant derived from artificial seeds of *Cymbidium aloifolium*

SN	Potting mixture	Total no. of acclimatized plant	Total no. of survivable plant	Percentage (%) of survivability
1.	A_1 [Cocopeat + sphagnum moss, 3:1]	15	12	80
2.	A_2 [Cocopeat + litter, 3:1]	15	11	73.33
3.	A_3 [Cocopeat + litter + sphagnum moss +, 3:2:1]	15	13	86.67
4.	A_4 [Cocopeat + clay + sphagnum moss, 3:2:1]	15	13	86.67
5.	A_5 [Cocopeat + sand, 3:1]	15	0	0
6.	A_6 [Sand]	15	0	0

The directly sown freshly prepared 4% alginate coated artificial seeds showed 46.67% survivability on cocopeat along with litter and sphagnum moss and 26.67% survivability on cocopeat along with sphagnum moss. Whereas, one week stored 4% alginate coated artificial seeds at 4°C showed only 33.33% of survivability on potting mixture containing cocopeat along with litter and sphagnum moss which was quite lower than results obtained from 3% alginate coated artificial seeds. Other treatments were not effective for acclimatization of both freshly prepared as well as stored 4% alginate coated artificial seeds (Table 11).

Table 10: Effect of different potting mixture on acclimatization of directly sown freshly prepared and stored 3% alginate coated artificial seeds of *Cymbidium aloifolium*

SN	Potting mixture	Freshly prepared 3% alginate coated artificial seeds			7 days storage at 4°C of 3% alginate coated artificial seeds		
		Total no. of acclimatized plant	Total no. of survivable plant	Percentage (%) of survivability	Total no. of acclimatized plant	Total no. of survivable plant	Percentage (%) of survivability
1.	A_1 [Cocopeat + sphagnum moss, 3:1]	15	9	60	15	0	0
2.	A_2 [Cocopeat + litter, 3:1]	15	11	73.33	15	7	46.67
3.	A_3 [Cocopeat + litter + sphagnum moss +, 3:2:1]	15	10	66.67	15	8	53.33
4.	A_4 [Cocopeat + clay sphagnum moss, 3:2:1]	15	3	20	15	0	0
5.	A_5 [Cocopeat + sand, 3:1]	15	0	0	15	0	0
6.	A_6 [Sand]	15	0	0	15	0	0

Table 11: Effect of different potting mixture on acclimatization of directly sown freshly prepared and stored 4% alginate coated artificial seeds of *Cymbidium aloifolium*

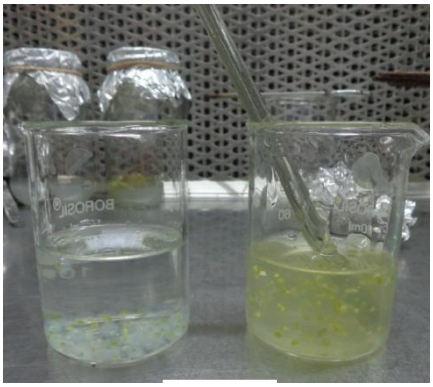
SN	Potting mixture	Freshly prepared 4% alginate coated artificial seeds			7 days storage at 4°C of 4% alginate coated artificial seeds		
		Total no. of acclimatized plant	**Total no. of survivable plant**	**Percentage (%) of survivability**	**Total no. of acclimatized plant**	**Total no. of survivable plant**	**Percentage (%) of survivability**
1.	A_1 [Cocopeat + sphagnum moss, 3:1]	15	4	26.67	15	0	0
2.	A_2 [Cocopeat + litter, 3:1]	15	7	46.67	15	0	0
3.	A_3 [Cocopeat + litter + sphagnum moss +, 3:2:1]	15	7	46.67	15	5	33.33
4.	A_4 [Cocopeat + clay sphagnum moss, 3:2:1]	15	0	0	15	0	0
5.	A_5 [Cocopeat + sand, 3:1]	15	0	0	15	0	0
6.	A_6 [Sand]	15	0	0	15	0	0

Figure A2: Acclimatization of artificial seeds of *Cymbidium aloifolium* on different potting mixture

Fig. A 11	Development of protocorms from *in vitro* culture of seeds and used for making artificial seeds
Fig. A 12-A14	Procedure for making alginate beads in laminar air flow cabinet
Fig. A 15	Storage of artificial seeds on pertriplates
Fig. A 16	Storage of artificial seeds on Ziploc
Fig. A 17	Acclimatization of artificial seeds on potting mixture containing cocopeat along with litter and sphagnum moss in the ratio of 3:2:1
Fig. A 18-A20	Different steps of development of artificial seed on potting mixture
Fig. A 21	Development of small plantlets from artificial seeds, acclimatized on potting mixture containing cocopeat, litter and sphagnum moss (3:2:1)
Fig. A 22-23	Development of small plantlets from artificial seeds, acclimatized on potting mixture containing cocopeat and litter (3:1)

A 11

A 12



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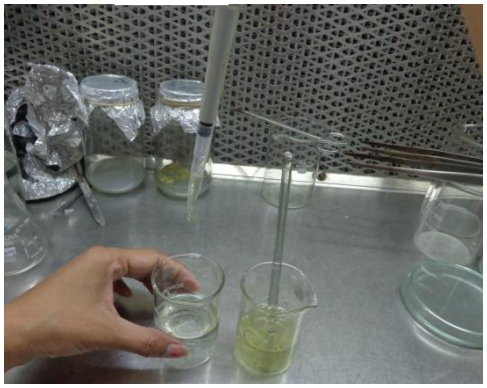

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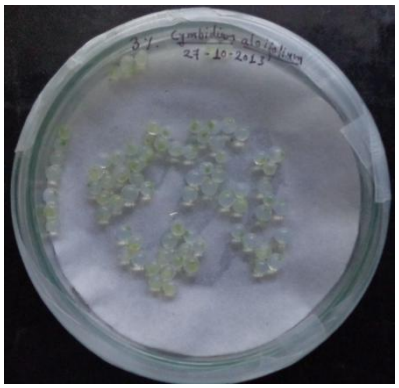

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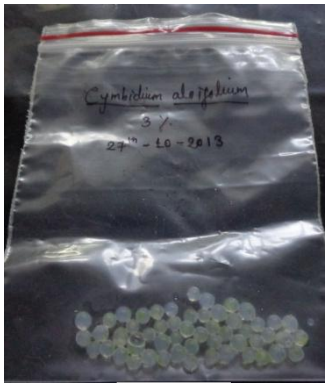

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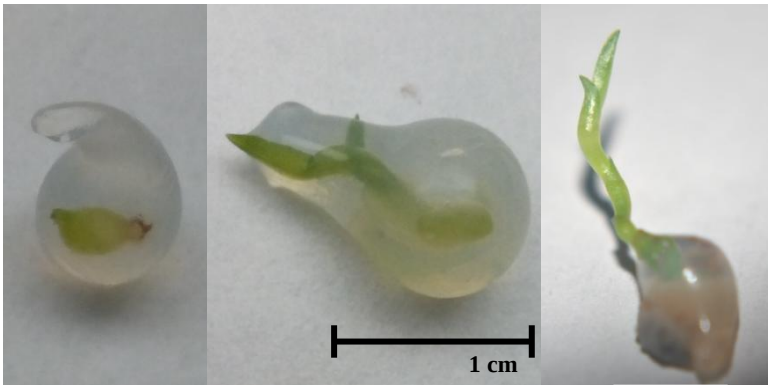

A 18 A 19 A 20

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4.2 Discussions

Tissue culture technique has become the most commonly applied biotechnological tools worldwide for the commercial production of many orchid species. Artificial seed technology is one of the most efficient alternative of tissue culture for the germplasm conservation and mass propagation of deplicating orchid population.

4.2.1 *In vitro* seed germination of *Cymbidium aloifolium*

In the present investigation, immature green pods were taken for *in vitro* seed germination of *Cymbidium aloifolium*. Immature seeds of *C. aloifolium* undergo various developmental stages during germination. Germination of orchid seeds is different from other seeds. Orchid seeds are produced in large numbers inside a pod. Due to non-endospermic nature of orchid seed, their germination in nature is a unique phenomenon and requires fungal infection. In certain orchids self-pollination is not possible and even if it is possible as in the case of *Vanda*, one has to wait for 4-6 month for pod development (Fitch, 1981). Germination is much more successful in *in vitro* conditions. Cells of immature seeds first turned into smooth walled globular structures called spherules with sticky hair after seven weeks of seed culture due to the swelling of embryos. This indicates the first visible sign of germination of cultured seeds. Light yellowish cultured seeds first changed into light green and finally to green in colour during development of protocorms. Protocorms later differentiated into shoot and root with or without undergoing callus formation in *C. aloifolium*.

In the present investigation, all tested conditions of both MS and KC media showed seed germination however MS and KC media supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) was the most effective condition for enhancing seed germination and seedling development. Comparative effects of different strength of MS and KC media supplemented with or without BAP and NAA on seed germination are shown on Table 3. Two media employed in present study were different from one another in their chemical composition. MS medium is highly enriched with macro and micro-elements with different vitamins whereas KC medium contained comparatively low concentration of macro and micro elements without vitamins (Hossain *et al*., 2009). Due to this, maximum percentage of seed germination was observed in different conditions of MS medium rather than Knudson medium which lack vitamins. There

are several reports explaining enhancement of germination and seedling growth and development by vitamins on different orchids. Addition of various vitamins into the medium was reported to be promotive for seed germination and seedling growth of *Cymbidium elegans* and *Coelogyne punctulata* (Sharma *et al*., 1991). Mariat (1949) reported that vitamin-B favoured germination and differentiation in *Cattleya* seedlings. He showed that thiamine, nicotinic acid and biotin were most effective in *Cattleya* hybrids production. Pyridoxine was shown to be essential for chlorophyll synthesis and combination of nicotinic acid and biotin favoured better germination of *Orchis laxiflora* seeds (Mead & Bulard, 1979).

In present investigation, ½ and ¼ strength of both MS and KC media were less effective for plantlet formation as they contain low amount of macro and micro elements. Full strength of MS and KC media gave satisfactory result but the time taken for germination and seedling development was quite longer than hormone supplemented MS and KC media. On *in vitro* culture, cytokinins like BAP, BA and Kinetin are generally known to induce both axillary and adventitious shoots formation and auxins like NAA, IAA and IBA for root induction (Jafari *et al*., 2011). Hormone like BAP was known to enhance the germination frequency of *Cypripedium candidum* (De Pauw *et al*., 1995), protocorm multiplication and shoot formation in *Cymbidium pendulum* (Pathak *et al.,* 2001). The synergistic effect of cytokinin and auxin in germination and plantlet development as found in present study has also been reported in *Phaius tancarvilleae* (Pant & Shrestha, 2011), *Cymbidium aloifolium* (Nongdam & Chongtham, 2011), *C. elegans* (Pant & Pradhan, 2010) and *C. iridiodes* (Pongener & Deb, 2010). Hossain *et al.* (2009) reported 100% seed germination of *C. aloifolium* on M-medium supplemented with activated charcoal (AC).

Protocorms produced in present investigation were globular, hairy and chlorophyllous (green) in all testing conditions of MS medium (Fig. SG 07) whereas on KC medium, they were light yellowish in colour (Fig. SG 08). The number of protocorms was also lesser on Knudson medium than MS medium but size of protocorms was superior on Knudson medium. Low amount of macro and micro elements of Knudson medium could have been effective for enlargement of protocorms due to nutritional stress but not for increasing their number. Production of large number of protocorms on MS medium indicates that culture seeds require sufficient amount of nutrients. Thus, the nutrient regime for orchid culture is species specific and no single culture medium is

universally applicable for all the orchid species. From above result, it was concluded that MS medium enriched with high concentration of nutritional compounds was suitable for earlier germination, large number of protocorm formation and seedling development rather than Knudson medium. Similar findings were reported on *Cleisostoma racemiferum* (Deb, 2007), *Malaxix khasiana* (Deb and Temjensangba, 2006), *Coelogyne suaveolens* (Deb, 2008) where MS medium was shown to be the most suitable medium over other nutrient media.

4.2.2 *In vitro* development and differentiation of single protocorm

In the present investigation, using single protocorm as an explant, showed significant regeneration capacity depending upon the nutrient media and plant growth regulators fortified at varied concentrations (singly or in combination) (Table. 4 & 5). It was found that a single protocorm first gave rise to multiple protocorms and shoot buds, which later underwent differentiation into leaf, root and finally developed complete seedling. The result of present study was also analogous to multiple shoot proliferation from protocorm of *Dendrobium candidum* (Yin & Hong, 2009), *Cleisostoma racemiferum* (Deb, 2007) and protocorm proliferated into plantlets of *Vanda helvola* (David *et al*. 2008). Protocorm apices elongate to form rhizomes that continue to grow to give branches with several nodes. After the elongation of the rhizome, the terminal bud grows upward and differentiates into shoots and roots, which was similar to the findings of Chang and Chang (1998).

Among various hormonal conditions of solid MS medium, MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA was found to be effective for earlier development of shoot, leaf, root and seedling (21.33±0.33 weeks of culture) from protocorm of *C. aloifolium*. However, most of the medium supplemented with hormone retarded the growth of seedling and become dormant for long time. Hence, low concentrations of hormone and low strength of media were used for further experiment.

In the present investigation the most appropriate medium was selected on the basis of time taken for initiation of protocorms and their growth and development. Here, the most effective culture condition for the *in vitro* development and differentiation of single protocorm of *C. aloifolium* was found to be hormone free full strength of liquid MS medium as this medium gave earliest response to proliferate protocorms on 4

weeks of culture. The same medium also proved to be effective for the formation of maximum number of shoots, leaves and roots. It may be due to their effect on physiological processes or presence of high salt concentration on the medium. On this medium, the complete seedling was obtained after 20.5±0.34 weeks of culture (Fig. P01). However on liquid KC medium, only full strength of KC and ½ KC medium were successfully proliferate the protocorm. The hormone free quarter strength and full strength of KC medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA developed only shoots; roots were not developed till 24 weeks of culture. Hence complete seedlings were not developed on these two conditions of KC medium. It may be due to the presence of low amount of nutrients present in KC medium and habituation effect of BAP and NAA. The present finding was supported by the study of Hossain *et al*. (2009). In their study, only 50% seed germination of *C. aloifolium* was obtained on KC medium.

In present investigation, the duration for first shoot, leaf and root differentiation from protocorm varied from 6-13 weeks of culture on MS medium. This finding was supported by the result of Devi *et al*. (1997). They found that the duration of leaf and root differentiation from PLBs varied from 7-13 weeks in different media such as MS, KC, VW, NI etc. Similarly, Chen *et al*. (2002) developed protocol for PLBs induction, multiplication, shooting and rooting. Shoot and root were induced from PLBs. Kalita and Sharma (2001) concluded that MS media was best for the development of PLBs than KC, VW and BS media in *Acampe longifolia* which also supported the present study.

The result of present investigation was also supported by the findings made by Matsui *et al.* (1970) who reported that the combination of BAP and NAA induced greatest effect on development and differentiation of PLB's (protocorm like bodies) in *Cymbidium* sp. It might be due to their physiological process or interaction between the two hormones. Hormones have stimulatory effect on rapid clonal multiplication *in vitro* but their higher amount sometimes causes phenotypical variations in propagules (Fujii et al. 1999). Similarly, Pant and Pradhan (2010) also reported that MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) was most effective for the multiplication of protocorms in *Cymbidium elegans*. In present investigation, higher concentration of BAP when employed in the medium individually as well as combined with NAA led to reduce the multiplication and elongation of shoots, leaves

and roots. Low concentration of hormones may be biologically effective because of the sensitivity of the whole plants to extremely low doses of hormones. Induction of maximum number of shoots at low concentration of BAP in the medium was reported in species of *Cymbidium* and *Cattleya* by Nagaraju *et al*. (2003). Shimura and Koda (2004) reported that the intensity of BAP was crucial for vegetative growth of *Cymbidium* sp. Das *et al.* (2007) found that protocorm cultured on MS medium supplemented with 1mgl BAP and 1mgl IAA was effective for proliferation of shoot (5.1 shoots per plantlet) and MS medium supplemented with 1mgl BAP and 0.5mgl IAA was proved to be best for multiplication of root (3.9 roots per plantlet) in *Cymbidium devonianum*.

The present investigation also induced the embryonic yellowish white callus from the single protocorm on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Callus induction was vigorous from protocorm due to the presence of growth hormones. This finding is consistent with Huan and Tanaka (2004) who also observed embryogenic callus induction and plant regeneration in *Cymbidium* from longitudinally bisected segments of PLB's within one month on VW medium supplemented with NAA (1.0 mg/l).

4.2.3 *In vitro* germination and development of artificial seeds

The propagation of medicinal plant species through seed has not been always successful due to heterozygosity of seed, minute seed size, presence of reduced endosperm and the requirement of seed with mycorrhizal fungi association for germination and also in some seedless varieties plants. Some of these species can be propagated by vegetative means. However, these techniques are time consuming, expensive and the propagules carry the diseases and pest from the mother plant to the seedlings. Hence, development of artificial seed producing technology is currently considered as an effective and efficient alternate method of propagation in several commercially important agronomical and horticultural plants (Islam & Bari, 2014). The artificial seed technology, based on vegetative propagules like protocorm, shoot tip, bud, nodal explants, etc., may be useful for large-scale propagation, short- to long-term conservation and germplasm exchange of desirable species (Kikowska & Thiem, 2011). Encapsulation of protocorms offers efficient techniques for clonal propagation of elite genotypes as protocorms have high cell dividing capacity and

tolerance against encapsulation and conversion related stresses (Sanada *et al.*, 1993). In the present investigation, the rounded and somewhat elongated beads of 2%, 3% and 4% alginate coated artificial seeds of *Cymbidium aloifolium* were successfully produced by encapsulating the in *vitro* grown three weeks old, green protocorm with sodium alginate (2%, 3% and 4%) and 0.2M calcium chloride solution ($CaCl_2.2H_2O$). For *in vitro* germination and seedling development, the freshly prepared artificial seeds were inoculated on different strength of liquid and solid MS and KC media supplemented with or without different concentrations of BAP and NAA and showed varied responses.

4.2.3.1 Comparative study of production, *in vitro* germination and seedling development of 2%, 3% and 4% alginate coated artificial seeds

In present investigation, *in vitro* propagation of *C. aloifolium* from 2%, 3% and 4% alginate coated protocorms (artificial seeds) were carried out to evaluate the capability of protocorm to break the gel coat and to continue the nomal growth resulting in the emergence of shoots and roots. The artificial seeds obtained from different concentrations of alginate solution showed variable germination response and also required different time intervals. Based on the result, it was obvious that the concentration of sodium alginate and complexing agent ($CaCl_{2.}2H_2O$) influenced the frequency of artificial seeds to plant conversion. In present investigation, the artificial seeds produced by using lower concentration of sodium alginate (2%) were not uniform, delicate resulting in reduction of germination and seedling development after certain level. At higher concentrations of sodium alginate (4%), the artificial seeds were somewhat elongated, beaked, hard and suppressed the emergence of shoot and root apices. As a result 4% alginate coated artificial seeds delayed the germination and plant regeneration process. At the concentration of 3% sodium alginate, the artificial seeds thus formed were rounded, firm, transparent and suitable for efficient germination and growth of plant. Hence in present investigation, 3% sodium alginate was found to be the optimal concentration for encapsulation and production of artificial seeds. Similar results were reported by Mohanraj *et al.* (2009) for the encapsulation of PLBs in *Coelogyne breviscapa* and Saiprasad and Polisetty (2003) for the encapsulation of PLBs in *Dendrobium*, *Oncidium* and *Cattleya* species.

A comparative study on *in vitro* germination and seedling development of 2%, 3% and 4% alginate coated artificial seeds revealed that hormone free full strength of liquid MS medium was more appropriate for *in vitro* germination and successful conversion into plantlet of 2% and 3% alginate coated artificial seeds while hormone free full strength of solid KC medium was found to be more effective for *in vitro* germination and seedling development of 4% alginate coated artificial seeds. However maximum multiplication of shoot and root was observed on liquid MS medium than other tested conditions of solid MS and KC media. The reasons for these results was the fact that the aeration system of liquid medium and the close contact of explants with medium may facilitate the uptake of oxygen, nutrients, phytohormones and consequently lead to increase the rate of germination and development. Though 2% alginate coated artificial seeds gave the earlier response on *in vitro* germination, the development of complete seedling and the maximum proliferation of shoot and root was found earlier on 3% alginate coated artificial seeds. It may be due to the impaired gelling ability of the low concentrations of alginate which suppress the growth of encase propagule.

The present investigation was supported by the finding of many researchers. Sarmah *et al*. (2010) found 94.9% conversion frequency from 3% sodium alginate encapsulated PLBs of *Vanda coerulea.* Awal *et al.* (2007) reported that a concentration of 3% sodium alginate and 100 mM $CaCl_2.2H_2O$ was found to be effective for encapsulation of micro shoots of *Begonia* X Hiemalis Fotch and obtained 90.48% germination. Soneji *et al.* (2002) reported that 3% sodium alginate was most effective for shoot encapsulation in *Ananas cosmosus*.

The appropriate concentration of alginate seems to vary with the species and the nature of the propagule. 1.5% sodium alginate was used to encapsulate the somatic embryo of *Daucus carota* (Sakamoto *et al*. 1995), 2% for alfalfa somatic embryos (Redenbaugh *et al*., 1987), 2.5% for *Spathoglottis plicata* protocorms (Singh, 1991), and *Phaius tankervilliae* PLBs (Malemnganba *et al*., 1996), 3% each for *Dendrobium* cultivars (Saiprasad & Polisetty, 2003; Siew *et al*., 2014) and *Aranda* hybrid PLBs (Gantait *et al.*, 2012), 4% for *Spathoglottis plicata* PLBs (Nayak *et al*., 1998), *Geodorum densiflorum* PLBs (Datta *et al*., 1999) and *Renanthera imschootiana* PLBs (Gupta, 2016) and 5-6% for *Atropa belladona, Dioscorea floribunda, Hyoscyamus muticus, Mentha arvensis, Picrorhiza kurroa,* and *Valeriana wallichii* shoot-buds

(Ahuja *et al*., 1989; Gupta, 2016). The required concentration may also vary with the alginate quality, which differs from brand to brand (Nayak *et al*., 1998) and batch to batch (Ahuja *et al*., 1989).

The present investigation also reported that the seedlings were green, healthy on all the conditions of solid and liquid MS medium and also showed their earliest development whereas on solid and liquid KC medium, seedlings were green on initial 4-16 weeks but later they were gradually turned into yellowish green which decreased their growth. It may be due to the lack of vitamins and low amount of nutrients on KC medium which retarded the development of seedling. In the present investigation, 100% of all artificial seeds undergone differentiation *in vitro* to develop complete seedlings of *C. aloifolium* on hormone free full strength of MS and KC media. Similar results were also reported by various researchers. Gantait *et al*. (2012) obtained 96.4% conversion of 3% alginate coated PLBs of *Aranda* × *Vanda coerulea*. Nagananda *et al*. (2011) achieved 95% conversion of encapsulated PLBs of *Flickingeria nodosa* into plantlets after 3 months of storage at 4°C. Similarly, Corrie and Tandon (1993) reported 100% conversion of 4% alginate encapsulated protocorm-like bodies (PLBs) into plantlet under *in vitro* condition in *Cymbidium giganteum*. Mohanraj *et al*. (2009) obtained 95% conversion of 3% encapsulated PLBs of *Coelogyne breviscapa* into plantlets.

The requirement of exogenous auxins and/or cytokinins for regeneration of protocorm like bodies (PLBs) or shoot buds and plantlet development has been reported for many orchid species (Pradhan *et al.*, 2013; 2014). However, the combinations, concentrations, and the ratio between them are usually critically important. The ratio of auxin to cytokinin for shoot buds or protocorm formation varies from species to species. In present investigation, the synergistic effect of BAP and NAA along with MS and KC media supplement delayed the development of seedling from artificial seed of *C. aloifolium*. The hormonal effect on the frequency of cell division and direction of cell wall formation of this plant might be one of the reasons for their late development.

The present findings were dissimilar to the reports obtained by other researchers. Mohanty and Das (2013) found that MS medium supplemented with 2 mg/l BAP was efficient for 100% conversion of encapsulated PLB's into plantlets on *Dendrobium densiflorum*. Zhang *et al.* (2011) reported that Maltose 4%, 6-BA/NAA 12:1, active

carbon 0.3%, sodium alginate 4% and time of ion exchange for 5 minutes were optimum condition for artificial seed production with PLB's in *Dendrobium candidum*. Similarly, Qin *et al.* (2008) found that modified MS with 4% maltose and BA (1 mg/l) and NAA (1 mg/l) favoured germination and seedling growth of *Dendrobium huoshanense* by using 4% sodium alginate. Further, Saiprasad and Polisetty (2003) reported 100% conversion of encapsulated PLBs when cultured on MS medium supplemented with BA and NAA on *Dendrobium* and NAA alone on *Oncidium* and *Cattleya*. Datta *et al*. (1999) reported 88% of conversion of artificial seeds into plantlets in *Geodorum densiflorum* on modified Knudson medium supplemented with BAP (1 mg/l) and NAA (1 mg/l). The differences in these studies may be due to the differences in species and growth conditions of encapsulated protocorm.

4.2.3.2 A comparative study on germination and development of a single protocorm and artificial seeds

In the present investigation, it was found that all the artificial seeds (2%, 3% & 4%) took longer time for germination than a single protocorm (non-encapsulated protocorm). It may be due to the delay on rupturing of the alginate matrix to emerge the tissue outside and came in contact with nutrient medium. However, artificial seeds (2% and 3% alginate coated) gave earlier response on their conversion into complete seedling than a single protocorm. Plantlets derived from artificial seeds were multiplied vigorously than a single protocorm. Hence, in terms of plant development, artificial seeds or encapsulated protocorms were more efficient than non encapsulated ones. The reasons for these results may be attributed to the rigidity of the alginate capsule which may not only provided better protection but also facilitated their growth by providing nourishment to the encased protocorm (Redenbaugh *et al*., 1987). Similar results were reported by Mohanty and Das (2013) in artificial seeds of *Dendrobium densiflorum*. Thus, based on the results, it was found that encapsulated protocorm or artificial seed is highly relevant for germplasm conservation *in vitro* than non encapsulated ones.

4.2.3.3 Effect of storage conditions (temperature and duration) for viability of artificial seeds

In present investigation, 2%, 3% and 4% alginate coated artificial seeds and non

encapsulated protocorms were stored for 120 days in two different temperatures, i.e., room temperature (RT, 21±2ºC) and 4ºC and cultured on two different liquid media, i.e., hormone free full strength of MS and KC medium. Their viability was checked at regular interval of 30 days.

In both hormone free full strength of liquid MS and KC medium, it was found that the viability percentage of artificial seeds (2%, 3% and 4%) of protocorms decreased gradually with increase in storage time. The decline in germination and plant development as a result of prolonged storage of tissue could be due to the inhibited respiration of tissues (Danso and Ford-Lloyd, 2003; Mohanty and Das, 2013). The maximum percentage (93.33%) of viability was observed in 30 days storage artificial seeds at 4ºC, inoculated on MS medium. This result revealed that the germination and conversion capacity of the stored artificial seeds at 4ºC was quite higher than that of artificial seed stored at RT (21±2ºC) (Table 6). It might be due to the chilling effect of low temperature, which not only store the calcium alginate beads for prolonged period by reducing their growth but also enhance their regeneration capacity when transferred to re-growth medium containing essential nutrients. This result was supported by the findings obtained by various researchers: Pradhan *et al.* (2014) successfully stored the artificial seed of *Cymbidium aloifolium* upto 28 days on MS medium with 97.5% conversion rate. Nagananda *et al.* (2011) encapsulated the PLBs of *Flickingeria nodosa* and achieved 95% conversion after 3 months storage at 4°C. Zhang *et al.* (2011) produced artificial seeds of *Dendrobium candidum* with protocorm like bodies as propagator and achieved the germination rate of 87.70±0.46 after 5 days and 14.87±0.31 after 20 days of storage in refrigerator at 4ºC which was lower compared to the present investigation. Mohanraj *et al.* (2009) reported 91% viability of encapsulated PLBs of *Coelogyne breviscapa* when stored for 30 days at 4ºC. Similarly, Datta *et al.* (1999) found 86% viability of artificial seeds of *Geodorum densiflorum* when stored at 4°C and 44% viability when stored at room temperature for 120 days.

Nutrient medium also plays a crucial role on germination and development of stored artificial seed. In present investigation, MS medium was found to be the most favourable condition for higher conversion frequency of plantlet from fresh as well as stored artificial seed of *C. aloifolium.* The use of MS medium not only purpose for regeneration of plantlet from stored artificial seed but also provides a way to reduce

the costs by eliminating the need for plant hormones.

Non-encapsulated protocorms stored for 30 to 120 days at 4ºC and RT became achlorophyllous, shrinked and lost their viability completely. Hence from the result, it was concluded that all the artificial seeds have higher regeneration capacity than non-encapsulated protocorms (Table 6). Similar results have been found in *Coelogyne breviscapa* (Mohanraj *et al*., 2009). The coating of alginate on artificial seeds not only protects them from desiccation but also enhance their growth on nutrient media. Thus, artificial seeds can be stored for short- to mid-term for germplasm preservation and can be easily delivered from laboratory to nursery. Out of different percentage of alginate coated stored artificial seeds, the 3% alginate coated artificial seed showed higher conversion frequency (93.33%) after 30 days of storage at 4ºC on MS medium. It may be due to the concentration of sodium alginate which forms more or less firm and uniform beads at 3% and gave sufficient aeration to encase protocorm resulting their earlier development however soft and very hard ones when sodium alginate was used at lower concentrations and very hard in texture at higher concentrations, showed a poor conversion frequency due to delay on breaking the gel.

4.2.4 Rooting of artificial seed derived shoots

The shoot tip explants cultured on MS basal medium and MS medium supplemented with different concentrations (0.5–2 mg/l) of root initiating hormones (IAA, IBA and NAA) showed varied responses on rooting. In present investigation, MS basal medium favoured root induction after 4 weeks of culture. Similar result was obtained by Shrestha and Rajbhandary (1988) in which MS basal medium induced good rooting in *Cymbidium giganteum* within 2 months. Shrestha and Rajbhandary (1994) again gave similar results in *C. longifolium.*

In present investigation, all the rooting hormones favoured the root induction of *C. aloifolium*. The highest number of shoot was obtained on MS medium supplemented with 1 mg/l IBA (6.33±1.23 shoots per culture) (Fig. R06) while the maximum proliferation of shoot was found on MS medium supplemented with 1.5 mg/l IAA (6.2±0.82 cm). The highest number of roots (5.83±0.94 roots per culture) and root length (4.5±0.76 cm) was observed on MS medium supplemented with 1.0 mg/l IAA (Fig. R02). However, the shoot and root were found to be thin and number of shoot

was also very few (2.67±0.5 shoots per culture) on this condition. The healthy and strong rooted shoot was found on MS medium supplemented with 1.0 mg/l IBA where the number of root was found to be 5.33±0.8 roots per culture and length of shoot and root was found to be 5.92±0.65 cm and 3.83±0.38 cm after 24 weeks of culture respectively. Hence in the present investigation, on the basis of development of strong shoot and root system, MS medium supplemented with 1.0 mg/l IBA was considered as the most effective condition for rooting. It may be due to the presence of enhanced levels of auxin (IBA) and related compounds in the medium which has strong absorption power of inhibitory compounds. Statistically, the number of shoot and length of shoot and root were found to be significant at 5% level of significance (Appendix 29).

There are several reports that support the present work. Pant and Swar (2011) obtained the highest number of roots on MS medium supplemented with IBA (1 mg/l) in *Cymbidium iridioides*. Pradhan and Pant (2009) reported that MS medium supplemented with IBA (1.5 mg/l) favoured the rooting of *Dendrobium densiflorum*. Koirala (2007) found that MS medium supplemented with IBA (1 mg/l) was best for rooting in *Coelogyne fuscescens*. Asghar *et al.* (2011) obtained maximum number of roots on modified MS medium fortified with various concentration of IBA than in NAA in *Dendrobium nobile.* Nayak *et al.* (1997) reported that MS medium supplemented with 10.8 μM was most effective for inducing strong root from *Cymbidium aloifolium, Dendrobium aphyllum* and *Dendrobium moschatum*. Nongdam and Chongtham (2011) found that MS basal medium alone and MS medium supplemented with 0.5 mg/l IBA or NAA was effective for maximum development of roots in *C. aloifolium.*

The present result was similar to the findings of Pant and Gurung (2005), who reported the highest number of roots in *Aerides odorata* on MS medium supplemented with IAA (0.5 mg/l). Similarly, Hossain *et al.* (2010) reported that half strength of PM fortified with 0.5 mg/l IAA was found to be most effective for inducing strong and stout root system from *Cymbidium giganteum.* Pant and Thapa (2012) found the best rooting on MS medium supplemented with 0.5 mg/l IAA in *Dendrobium primulinum*.

4.2.5 DAS ELISA test of the *in vitro* and *in vivo* (wild) plant samples of *Cymbidium aloifolium*

It has been reported that orchids are affected by at least 25 different orchid viruses around the world (Liu *et al*., 2013). Of these, CymMV virus is one of the most prevalent and economically important followed by Odontoglossum ringspot virus (ORSV) (Khentry *et al*., 2006; Wong *et al*., 1994; Zettler *et al*., 1990). CymMV is stable and widespread infection can occur, once the virus is introduced into a nursery. So, it is necessary to test the plant materials for the presence of viruses before being used for mass propagation by tissue culture technique. Serological (ELISA), biological indexing and molecular methods (RT PCR) are generally used for detection and identification of plant viruses (Taskin *et al*., 2013). DAS ELISA technique has been used by different researchers because of its simplicity, accuracy and cost effectiveness (Chien *et al*., 2015; Milosevic *et al*., 2012; Khan *et al*., 2003; Hu *et al*., 1993).

In the present investigation, CymMV virus was not found in all the *in vitro* regenerated plantlets (100% CymMV free) used. However, the source mother plant was highly infected with CymMV (83.33%) (Fig. VR09). It has been reported that CymMV was not seed transmitted (Khentry *et al.*, 2006). Hence the use of seed propagated plantlets as explants should be one of the most prominent approaches to establish the virus free orchid plants and high quality germplasm. This showed that *in vitro* culture of seed, protocorm, artificial seed and shoot tip could eliminate CymMV virus which could be confirmed by the use of CymMV antibodies in DAS-ELISA. The lack of virus infection of plantlets regenerated *in vitro* in present investigation also supports this hypothesis.

When the plants were infected with virus, chemical therapy won't be effective to control it. Meristem culture technique is the most common method for obtaining virus-free plants. Different researchers have also reported that meristem culture is effective for obtaining virus-free plants of different crops (Cybularz-Urban & Hanus-Fajerska, 2006; Fidan *et al*., 2009; Kumar *et al*., 2009; Milosevic *et al*., 2012; 2011). However, extracting the shoot meristem and regenerating them into plants is difficult, time consuming and requires crop-specific expertise mostly in orchids. Therefore protocorm, shoot tip and artificial seed culture can be used as an alternative to produce virus-free plants of orchids. The result of present work are similar to Chien *et*

al. (2015) who successfully obtained virus free plant from shoot tip derived PLBs of *Phalaenopsis*. Liu *et al*. (2013) reported that virus free orchids showed faster and healthier growth and produce larger inflorescences. In present investigation, the virus free *in vitro* plantlets were multiplied (Fig. A01) and transferred to the earthen pots in greenhouse for acclimatization (Fig. A07).

4.2.6 Molecular analysis (genetic fidelity)

Culture systems of plants *in vitro* usually result of somaclonal variations (Larkin & Scowcroft, 1981; Taji *et al*., 2002). *In vitro* factors such as nutritional composition, stress, hormonal balance, duration of culture are factors contributing to *in vitro* variability (Khan *et al*., 2011). Thus, it is necessary to evaluate whether the *in vitro* regenerants are genetically identical to the mother plant or not. RAPD and ISSR markers were successfully used to determine the genetic uniformity on various species of micropropagated plants such as *Tylophora indica* (Sharma *et al*., 2014), *Spilanthes calva* (Razaq *et al*., 2013), *Picea* sp. (Ramya & Kabwe, 2012), banana (Khan *et al*., 2011) and *Swertia chirayita* (Joshi & Dhawan, 2007).

In the present investigation, RAPD and ISSR markers were tested to assess the genetic uniformity of *in vitro* regenerants of *Cymbidium aloifolium* derived from *in vitro* culture of seed, protocorm, artificial seed or, shoot tip and their source plant collected from the wild (Fig. 25 & 26). Out of twenty six RAPD primers tested, the twenty one RAPD markers generated 51 polymorphic bands while rest of five RAPD primers were not generated bands however all five ISSR markers gave 15 polymorphic bands (Table 8). Dendrograms based on UPGMA analysis revealed two and three main clusters for ISSR and RAPD, respectively (Fig. 27 & 28). According to Dice coefficient, RAPD and ISSR analysis revealed that the genetic distance levels range from 0.87 to 1.00 with a mean value 0.93 between non-tissue cultured source plant and tissue cultured derived plants (Appendix 30 & 31) indicating the genetic similarity at moderate to high level.

Greater polymorphisms are indicative of greater genetic variations. However the present investigation revealed the presence of higher genetic similarity than polymorphism. RAPD markers showed 10.55% of polymorphism while ISSR markers showed 15.15% variation among and within the *in vitro* cultured plants. It is hard to

explain the exact reason(s) for this variation but may be associated with the intrinsic factors associated with biological materials and some of the culture conditions as shown in different species (Smith *et al*., 1997; Lakshmanan *et al*., 2006). This finding was supported by Khoddamzadeh *et al*. (2010) who reported 17% polymorphism in *Phalaenopsis bellina* using RAPD primers among the *in vitro* regenerants. Similarly, small differences of genetic fingerprints of *Cymbidium sp*. using RAPD primers were reported by Cerasela and Lazâr (2009), while Bhattacharyya and Kumaria (2015) found 5.95% of variability within the propagated plants of *Dendrobium nobile* using RAPD and SCoT markers. Similar findings were recorded for *Cymbidium giganteum* (Roy *et al*., 2012), *Dendrobium* species (Zha *et al*., 2009; Pongsrila *et al*., 2014) and *Vanda* (Lim *et al.*,1999).

Morphological analysis of *in vitro* derived plants of *Cymbidium aloifolium* didn't show any noticeable variations among each other and with that of mother plant. However, the evaluation of somaclonal variability of *C. aloifolium* using RAPD and ISSR markers showed slight polymorphism. Polymorphism was established with the presence of either new bands in genotypes or loss of original bands in mother plants (Chandrika & Rai, 2010; Ramesh *et al.*, 2011). Genetic changes due to somaclonal variation in micropropagated plant is beneficial for plant breeding and crop improvement (Taji *et al.*, 2002; Mallaya & Ravishankar, 2013). Genetic variation influenced by genetic and epigenetic mechanism can be reflected in the banding profile developed by different molecular marker systems (Phillips *et al.*, 1994). This system helps to detect the reliability of the plant tissue culture protocol for large scale exploitation and species restoration programme. In RAPD analysis, all the *in vitro* regenerated plants obtained from seed, protocorms, shoot tip and artificial seeds showed 94% genetic similarity with each other and 90% genetic similarity with that of mother plant (Fig. 27) whereas ISSR analysis revealed 91% similarity amongst mother and all tissue cultured plants and 94% genetic similarity between all *in vitro* propagated plants (Fig. 28). This difference between RAPD and ISSR data may be due to the fact that PCR profiles are amplified from non-repetitive and repetitive regions of the genome in the two marker systems (Thormann *et al*., 1994).

ISSR markers are considered as effective and reliable genetic markers for assessing the degree of genetic variation of many crop plants such as Yacon (Viehmannova *et al.*, 2014), *Pilosocereus robinii* (Khattab *et al*., 2014), *Stevia rebaudiana* (Lata *et al*.,

2013), *Lilium orientalis* (Liu & Yang, 2012), *Salvia* sp. (Song *et al*., 2010) and Banana (Rout *et al*., 2009). As compared to other molecular markers, ISSR could reveal high polymorphism and found to be potentially useful for studying genetic variation, diversity, introgression analysis and identification of germplasm. High genetic similarity and variation was also reported by RAPD markers in different crop and cultivated populations (Horejsi *et al*., 1999; Bahraminejad *et al*., 2012; Mallaya & Ravishankar, 2013; Khaled *et al.*, 2015; Singh & Sengar, 2015). However, sometimes RAPD markers fail to reveal changes in the repetitive regions of the genome of some species (Palombi & Damiano, 2002). Therefore, the two marker systems employed in the present investigation is used to crosscheck and validate the degree of genetic variability of test genotypes (Bhattacharyya & Kumaria, 2015). The PIC and R_p values further determine the efficacy of the markers used in the genetic fingerprinting assay. R_p provides the quantitative data of a primer to correlate strongly with its ability to distinguish between genotypes (Prevost & Wilkinson, 1999). PIC provides an estimate of the discriminatory power of a gene or locus in a population (Smith *et al*., 1997). The average PIC value of RAPD and ISSR was found to be 0.32 demonstrating the low polymorphism and R_p value i.e., 3.2 indicating the efficiency of primers used. The PIC and R_p values of the primers used in present investigation strengthen the significance of present findings. Hence, the data reported here based on the two marker systems was found to be almost similar and revealed the existence of low genetic variation among and within the mother and *in vitro* regenerated plants of *C. aloifolium*.

4.2.7 Acclimatization

The *in vitro* raised seedlings developed from artificial seeds were successfully established in different potting medium. The substrates or potting mixture used in present investigations were cocopeat + sphagnum moss (3:1), cocopeat + litter (3:1), cocopeat + litter + sphagnum moss (3:2:1), cocopeat + clay + sphagnum moss (3:2:1), cocopeat + sand (3:1) and only sand (Table 9). The survival rate of plantlets of *Cymbidium aloifolium* was reached about 86.67% in the greenhouse on the potting mixture containing cocopeat along with litter or clay and sphagnum moss in the ratio of 3:2:1. Hence, this potting medium was found to be more favourable for acclimatization of plantlet of *C. aloifolium* (Table 9). Similar results were reported by

Nagananda *et al.* (2011) for the plantlets regenerated from artificial seeds in *Flickingeria nodosa*. They obtained 85% survivability of these plantlets in potting mixture containing brick, charcoal and moss. Sharma *et al*. (1992) achieved 65% survivability of the plantlets regenerated through artificial seeds of *Dendrobium wardianum* in the potting mixture containing charcoal pieces, brick bats, coconut fibres and a layer of mosses which was lower in comparision to the present study.

However, the potting mixture containing cocopeat, litter and with or without sphagnum moss was found to be more appropriate for directly regeneration of plantlet from one week stored (4º C) and freshly prepared 3% and 4% alginate coated artificial seeds of *C. aloifolium* (Tables 10 & 11). It was found that 4% alginate coated artificial seeds showed lower regeneration potential (46.67%) than 3% coated artificial seeds (73.33%) due to the formation of hard seed coat surface which delay and disturb the development process of plantlet (Tables 10 & 11). The 2% alginate coated artificial seeds gave unsatisfactory results on conversion of plantlets. It may be due to the low concentration of sodium alginate solution which prolongs the polymerization duration and causes fragility to the seed coat resulting the desiccation of plantlet. Hence the ability of 3% alginate coated artificial seeds to establish plantlets under *in vivo* condition showed promise for direct field planting. The non encapsulated protocorm was also unsuccessful to grow directly under *in vivo* conditions. It may be due to the lack of proper growth conditions and supply of nutrients. The present findings was consistent with the results obtained from Corrie and Tandon (1993) who reported the failure of regeneration of plantlet from non encapsulated protocorm but they reported that the conversion frequency of 4% encapsulated protocorm under *in vivo* condition was found to be 64% in soil mixture.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The regeneration of orchid plants through encapsulation of tissue culture derived propagules in a nutrient gel has initiated a new line of research on artificial seeds. Hence, the present investigation was focussed on mass propagation and *ex situ* conservation of medicinally and commercially important species, *Cymbidium aloifolium* (L.) Sw. through artificial seed technology. The present finding offers an opportunity to local growers, commercial nurseries for large scale propagation, short to midterm storage and easy transfer from lab and nursery/land.

From the present investigation, following conclusions have been made:

The plantlets of *C. aloifolium* regenerated from artificial seeds were propagated mass scale in suitable culture condition. Effective tissue culture protocols were developed during mass propagation of this orchid species. It was found that full strength of MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA was found to be more effective with respect to other concentrations of MS and KC media for *in vitro* seed germination, large number of protocorm formation and seedling development of *C. aloifolium.* For in *vitro* development and differentiation of a single protocorm, hormone free full strength of MS medium was the most effective culture condition. Among different concentration of sodium alginate solution (2%, 3% & 4%), 3% sodium alginate was found to be the optimal concentration for encapsulation and production of artificial seeds. Among 2%, 3% and 4% alginate coated artificial seeds, the 3% alginate coated artificial seeds were most favourable for the maximum proliferation of shoot, root and development of complete seedling of *C. aloifolium* on MS basal medium. Hence, overall MS medium was found to be the most effective nutrient medium than KC medium for *in vitro* development of *C. aloifolium.*

All the artificial seeds have higher regeneration capacity than non-encapsulated protocorms (single protocorm). They were remained viable when stored at 4ºC for 120 days. The 3% alginate coated artificial seeds showed higher conversion frequency (93.33%) after 30 days of storage at 4ºC on MS medium. MS medium supplemented

with 1.0 mg/l IBA favoured the development of maximum number of healthy shoots with thick and long roots from artificial seed derived shoot tip of *C. aloifolium.* This rooting medium was followed by MS medium supplemented with 1.0 mg/l IAA.

For acclimatization of plantlet of *C. aloifolium*, the most favourable condition was found to be the potting mixture containing cocopeat along with litter or clay and sphagnum moss in the ratio of 3:2:1. On this potting mixture, about 86.67% of plantlets were survived. However, the potting mixture containing cocopeat, litter and with or without sphagnum moss was found to be more appropriate for directly regeneration of plantlet from one week stored (4ºC) and freshly prepared 3% alginate coated artificial seeds of *C. aloifolium* which showed 73.33% of their survivability.

The virus free plantlets were regenerated from different explants, i.e., artificial seeds, protocorms, shoot tips. All the *in vitro* regenerated plantlets were 100% free from CymMV virus whereas the source mother plant was highly infected with CymMV (83.33%) virus. The plantlets regenerated directly from seeds also showed 100% CymMV virus free.

High genetic homogeneity (90%) was found among and within the mother and *in vitro* regenerated plantlets of *C. aloifolium* and analyzed by using RAPD and ISSR markers.

5.2 Recommendations

Following recommendations are made from the present research work:

- The mass propagation of economically important orchids can be started by using artificial seed technology in order to reduce the time, space and cost.
- Additional research work related to addition of nutrients in coating matrix of artificial seed can be tested to increase the efficiency of tissue viability.
- Further study on phytochemistry and biological activity of artificial seed derived plantlets of *Cymbidium aloifolium* might be the new area of research to authenticate their medicinal properties.
- Technology developed from this research findings can be transformed for its commercialization and *ex situ* conservation which will be helpful to upgrade the economic status of rural people by cultivation of tissue cultured raised *C. aloifolium* and reintroduction from lab to land (nature) can be done for species restoration programme.

CHAPTER 6

6. SUMMARY

Cymbidium aloifolium (L.) Sw., is one of the threatened orchids of Nepal, mainly used for horticulture as well as medicinal purposes. It is epiphytic in nature and it remains attached to the bark of old trees. Whole plants as well as different parts viz. roots, small seeds and leaves of this orchid are used in medicine. Apart from its medicinal importance, this orchid commands a good price in floricultural market because of its highly attractive beautiful flowers. Currently, population of this orchid species is decreasing from nature due to habitat destruction, over exploitation and ruthless collection for trade, consumption and to fulfil market demand. Hence a fast method of growing and conserving it in the green house is an urgent need.

To meet this objective, the present research aimed to produce artificial seeds for propagation of this orchid species in mass scale and compare their regeneration potential with that of seed derived protocrom culture *in vitro*; to develop the technique and production of Cymbidium mosaic virus (CymMV) free plantlets (by using DAS-ELISA) through protocorms and protocorm derived explants and to produce genetically similar tissue cultured plants of this species.

6.1 Media

The basal media used for the present investigation were MS and KC media. The different explants (seeds, protocorms, artificial seeds) of *C. aloifolium* were inoculated on different strength (1, ½, ¼) of hormone free liquid and solid MS and KC media and full strength (1.0) of both media supplemented with BAP (0.5mg/l) and NAA (0.5mg/l) for their *in vitro* seed germination and seedling development. Individual protocorm was also cultured on MS medium supplemented with different concentrations of BAP (0.5-2.0 mg/l) and NAA (0.5-1.0 mg/l). For rooting, MS medium supplemented with various concentrations (0.5-2.0 mg/l) of auxins, i.e., IAA, IBA and NAA were used for the development of long and thick roots.

6.2 Production of Artificial seeds

The artificial encapsulation of explants which may be embryo, protocorms, protocorm like bodies, shoot tip, node, pseudobulb etc. or any other meristematic tissues in a protecting gel and can develop under *in vitro* and *in vivo* conditions is commonly called as synthetic seed or artificial seed or synseed. Sodium alginate was used as a gelling agent and protocorms were used as explants for production of artificial seeds. During artificial seed production, a single protocorm was dipped in various concentrations (2%, 3% and 4%) of sodium alginate and hardened in 0.2 M calcium chloride solution. After half an hour, 2%, 3% and 4% alginate coated beads were formed, looks like a true seed.

6.2.1 *In vitro* germination and development of 2%, 3% and 4% alginate coated artificial seeds

All the tested conditions showed varied response on *in vitro* germination and seedling development of 2%, 3% and 4% alginate coated artificial seeds. Different treatment on liquid conditions was found to be more suitable for earlier germination and development of all the artificial seeds except in germination of 4% alginate coated artificial seeds. Among the two media employed, MS medium was found to be most effective for germination, development of protocorms and regeneration of plantlet rather than KC medium. It may be due to the presence of high salt concentrations on MS medium which was required for the development of artificial seeds of *Cymbidium aloifolium*. In present investigation, hormone free full strength of liquid MS medium was found to be most effective condition for *in vitro* germination and seedling development of 2% and 3% alginate coated artificial seeds of *C. aloifolium* whereas hormone free full strength of solid KC medium was found to be most favourable for *in vitro* germination and seedling development of 4% alginate coated artificial seeds.

Among 2%, 3% and 4% alginate coated artificial seeds, though 2% alginate coated artificial seeds germinated on shortest period of time (4 weeks of culture) compared to other culture conditions, the 3% alginate coated artificial seeds were more favourable for the maximum proliferation of shoot (10.5±1.96 shoots per culture), root (5.33±0.92 roots per culture) and development of complete seedling (18.5±0.84 weeks

of culture) of *C. aloifolium*. Among different concentration of sodium alginate solution (2%, 3% & 4%), 3% sodium alginate was found to be the optimal concentration for encapsulation and production of uniform artificial seeds and their development.

6.2.2 *In vitro* germination and development of true seeds into plantlets and compared with artificial seed derived plantlets

Germination of orchid seeds is different from other seeds. The production of orchid seedling from seeds involves the sequential phases of germination, protocorm formation and seedling development. *In vitro* culture of true seeds of *Cymbidium aloifolium* took much longer time for their germination and proliferation as compare to artificial seeds. MS solid medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) was found to be effective for *in vitro* germination (7 weeks of primary culture) and seedling development (27 weeks of culture) of *in vitro* culture of seeds. Here, hormonal medium was important for seed culture to promote orchid seeds germination and seedling growth. However, hormone free full strength of MS solid medium was found to be effective for quick *in vitro* germination (4 weeks of culture) and seedling development (19 weeks of culture) of artificial seeds (3%).

6.2.3 *In vitro* development of a single protocorm and compared with development of artificial seeds

A single protocorm gave their earlier proliferation response and converted into seedlings on hormone free full strength of MS medium rather than other tested conditions of MS and KC media. On this medium, the complete seedling was obtained after 20.5±0.34 weeks of culture. By comparing the regeneration potential of artificial seeds and protocorms, it was found that all the artificial seeds took longer time for germination than a single protocorm (non-encapsulated protocorm). It may be due to the delay on rupturing of the alginate matrix to emerge the tissue outside and came in contact with nutrient medium. However, artificial seeds (2% and 3% alginate coated) gave earlier response on their conversion into complete seedling (18.5±0.84 weeks of culture) than a single protocorm. Artificial seeds gave rise to many shoot buds (>10) and shoots on liquid MS medium whereas a single protocorm hardly gave rise to 4 shoot buds and shoots on same medium. From the present result, it was

concluded that artificial seeds undergoes maximum proliferation to give multiple shoots than a single protocorm.

6.2.4 Effect of storage conditions (temperature and duration) for viability of artificial seeds

Storage of artificial seed is a crucial factor for exchange and preservation purposes. Therefore, appropriate storage conditions and definite storage periods required to maintain viability during exchange of germplasm are prerequisites for artificial seed technology and its commercial applications. In present investigation, 3% and 4% alginate coated artificial seeds were remained viable for 120 days at 4ºC and for 30 days only at room temperature (RT, 21±2ºC) on both liquid MS and KC media. In case of 2% alginate coated artificial seeds, they were viable for 120 days on MS medium and 90 days on KC medium at 4ºC while at room temperature, they were non viable on both liquid MS and KC media. In both temperatures, the germination percentage of artificial seeds gradually declined with increased in storage time. However, non encapsulated seeds (single protocorm) were not responded on both temperatures when stored for 120 days on both liquid medium. The highest viability percentage (93.33%) was found on 3% alginate coated artificial seeds when stored for 30 days at 4ºC on liquid MS medium.

6.3 DAS-ELISA test of the plant samples

DAS ELISA technique was found to be more effective for detection of CymMV virus on *in vivo* and *in vitro* plantlets of *C. aloifolium*. With the aid of this technique, all the *in vitro* regenerated plantlets derived from different explants such as seeds, protocorms, shoot tips and 2%, 3% and 4% alginate coated artificial seeds were found to be 100% free from CymMV virus whereas the source mother plant was highly infected with CymMV (83.33%) virus.

6.4 Molecular analysis (Genetic fidelity)

DNA based molecular markers, i.e., RAPD and ISSR were found to be effective to determine the genetic uniformity on tissue cultured plants of *C. aloifolium.* Dendrogram based on UPGMA analysis revealed 90% and 91% genetic homogeneity by RAPD and ISSR markers respectively between mother and *in vitro* regenerated plants derived from different explants such as seeds, protocorms, shoot tips and 2%,

3% and 4% alginate coated artificial seeds. However, both markers showed around 94% genetic homogeneity within *in vitro* regenerated plantlets. The PIC and R_p values of both primers used in present investigation further strengthen the significance of present findings.

As the *in vitro* regenerated plantlets of *C. aloifolium* were found to be CymMV virus free and also showed higher genetic uniformity with mother plants, they were selected for clonal multiplication and *ex-situ* conservation which was the major goal of the present study.

6.5 Shoot Multiplication

The shoot tips derived from *in vitro* culture of 2%, 3% and 4% alginate coated artificial seeds were sub cultured on hormone free full strength of solid and liquid MS medium for shoot multiplication *in vitro*. The maximum number of shoots with strong root was observed on this medium after 12 weeks of culture. The micro shoots obtained from multiple shoots were used as explant for induction of large number of roots on rooting medium. The shoots with strong root system after attaining the height of 4 to 5cm as well were used for acclimatization.

6.6 Rooting of artificial seed derived shoots

The micro shoots obtained from artificial seed derived shoot tip culture were sub cultured on MS medium supplemented with different concentrations (0.5 mg/l – 2 mg/l) of auxins, i.e., IAA, IBA and NAA for the root induction. MS medium supplemented with 1.0 mg/l IBA was found to be the more effective condition for development of thick and long shoots and roots in comparison to MS medium alone and MS medium supplemented with other different concentrations of NAA and IAA.

6.7 Acclimatization

Acclimatization is one of the crucial steps of micropropagaiton. The *in vitro* raised seedlings were successfully established in the potting medium. The potting mixture played an important role to make success the acclimatization process of any plants. Among different potting mixture tested, the potting mixture containing cocopeat along with litter or clay and sphagnum moss in the ratio of 3:2:1 was found to be more suitable for acclimatization of plantlet of *C. aloifolium* with 86.67% survival

rate whereas the potting mixture containing cocopeat, litter and with or without sphagnum moss was found to be more appropriate for directly regeneration of plantlet from one week stored (4ºC) and freshly prepared 3% alginate coated artificial seeds of *C. aloifolium* with 73.33% survival rate.

6.8 List of Published Articles Based on the Present Research

Followings are the list of published articles and full articles are available in annex.

1. Pradhan, S., Regmi, T., Ranjit, M. & Pant, B. (2016). Production of virus-free orchid *Cymbidium aloifolium* (L.) Sw. by various tissue culture techniques. *Heliyon*, 2(10), e00176 (Elsevier). [https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5079657/].

2. Pradhan, S., Tiruwa, B. L., Subedee, B. R. & Pant, B. (2016). Efficient plant regeneration of *Cymbidium aloifolium* (L.) Sw., a threatened orchid of Nepal through artificial seed technology. *American Journal of Plant Sciences*, **7**, 1964-1974. [http://file.scirp.org/pdf/AJPS_2016101214595694.pdf].

3. Pradhan, S., Regmi, T. & Pant, B. (2015). Comparative study of encapsulated and non-encapsulated protocorms for the propagation of *Cymbidium aloifolium* (L.) Sw. on two different media. *Botanica Orientalis-Journal of Plant Science*, 9, 40-48.
4. Pradhan, S., Tiruwa, B. L., Subedee, B. R. & Pant, B. (2014). Micropropagation of *Cymbidium aloifolium* (L.) Sw., a medicinal orchid by artificial seeds technology. *Journal of Natural History Museum*, 28, 42-48. [http://www.nepjol.info/index.php/JNHM/article/view/14166].
5. Pradhan, S., Tiruwa, B., Subedee, B. R. & Pant, B. (2014). *In vitro* germination and propagation of a threatened medicinal orchid, *Cymbidium aloifolium* (L.) Sw. through artificial seed. *Asian Pacific Journal of Tropical Biomedicine*. 4 (12), 971-976. (Elsevier). [http://www.sciencedirect.com/science/article/pii/S2221169115301131].
6. Pradhan, S., Regmi, T., Parmar, G. & Pant, B. (2013). Effect of different media on *in vitro* seed germination and seedling development of *Cymbidium aloifolium* (L.) Sw. *Nepal Journal of Science & Technology*, 14(1), 51-56. [http://www.nepjol.info/index.php/NJST/article/view/8878].

7. Pradhan, S. (2013). *Cymbidium aloifolium* (L.) Sw.: A medicinally important orchid of Nepal. *Hamro Sampada*, 12(10), 105-106.

8. Pradhan, S., Pathak, J. & Pant, B. (2013). *In vitro* propagation of *Cymbidium elegans* Lindl. from shoot tip culture. *Journal of Nepal Biotechnology Association*, 3, 45-48.

9. Regmi, T., Pradhan, S. & Pant, B. (2017). *In vitro* mass propagation of an epiphytic orchid, *Cymbidium aloifolium* (L.) Sw. through protocorm culture. (Accepted: Biotechnology Journal International).

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APPENDIX

Appendix 1: Plant samples used for DNA extraction

Plant	No. of samples	Sample source	Locality/Concentration
Cymbidium aloifolium	1.	*In vivo* (wild) plant	Natural Habitat
	2.	Plant from Seed culture	*In vitro* plant regenerated on MS solid medium
	3.	Plant from Protocorm culture	*In vitro* plant regenerated on MS solid medium
	4.	Plant from Shoot tip culture	*In vitro* plant regenerated on MS solid medium
	5.	Plant from Artificial seed (2%)	*In vitro* plant regenerated on MS liquid medium
	6.	Plant from Artificial seed (4%)	*In vitro* plant regenerated on MS liquid medium
	7.	Plant from Artificial seed (3%)	*In vitro* plant regenerated on MS liquid medium
	8.	Plant from Artificial seed (3%)	*In vitro* plant regenerated on MS solid medium
	9.	Plant from Artificial seed (3%)	*In vitro* plant regenerated on solid KC medium
	10.	Plant from Artificial seed (3%)	*In vitro* plant regenerated on liquid KC medium
	11.	Plant from Artificial seed (3%)	*In vitro* plant regenerated on solid MS + 0.5 mg/l BAP + 0.5 mg/l NAA
	12.	Plant from seed culture	*In vitro* plant regenerated on solid MS + 0.5 mg/l BAP + 0.5 mg/l NAA

Appendix 2: The sequences of oligonucleotide primers used for RAPD analysis and PCR amplicons obtained from *in vivo* and *in vitro* grown *Cymbidium aloifolium*

SN	Primer	Primer sequence (5'-3')	Total no. of PCR amplicons	No. of polymorphic band	Polymorphism (%)	Polymorphic information content (PIC)	Resolving power (R_p)	Range of PCR amplicons (bp)
1	OPA 01	CAGGCCCTTC	22	10	45.45	0.49	3.67	0.4-0.5
2	OPA 06	GGTCCCTGAC	-	-	-	-	-	-
3	OPA 07	GAAACGGGTG	12	0	0	0	2	0.4
4	OPA 11	CAATCGCCGT	20	8	40	0.44	3.33	0.6-1.4
5	OPA 12	TCGGCGATAG	12	0	0	0	2	0.7
6	OPA 13	CAGCACCCAC	45	9	20	0.75	7.5	0.3-1.0
7	OPI 07	CAGCGACAAG	36	0	0	0.67	6	0.3-0.6
8	OPI 09	TGGAGAGCAG	44	7	15.9	0.78	7.33	0.3-0.8
9	OPI 11	ACATGCCGTG	12	0	0	0	2	1.1
10	OPI 12	AGAGGGCACA	36	0	0	0.67	6	0.3-0.8
11	OPI 13	CTGGGGCTGA	12	0	0	0	2	0.5
12	OPI 14	TGACGGCGGT	56	9	16.07	0.75	7.33	0.4-0.9
	Total		307	43	14.0	4.55	49.16	

Appendix 3: The sequences of oligonucleotide primers used for RAPD analysis and PCR amplicons obtained from *in vivo* and *in vitro* grown *Cymbidium aloifolium*

SN	Primer	Primer sequence (5'-3')	Total no. of PCR amplicons	No. of polymorphic band	Polymorphism (%)	Polymorphic information content (PIC)	Resolving power (R_p)	Range of PCR amplicons (bp)
13.	OPI 15	TCATCCGAGG	-	-	-	-	-	No result
14	OPI 16	TCTCCGCCCT	-	-	-	-	-	No result
15	OPI 17	GGTGGTGATG	-	-	-	-	-	No result
16	OPI 18	TGCCCAGCCT	24	0	0	0.5	4	0.7-1.0
17	OPI 19	AATGCGGGAG	-	-	-	-	-	No result
18	OPI 20	AAAGTGCGGG	12	0	0	0	2	0.6
19	OPP 01	GTAGCACTCC	24	0	0	0.5	4	0.4-0.5
20	OPP 02	TCGGCACGCA	12	0	0	0	2	0.4
21	OPP 03	CTGATACGCC	12	0	0	0	2	0.4
22	OPP 04	GTGTCTCAGG	24	0	0	0.5	4	0.4-0.7
23	OPP 07	GTCCATGCCA	24	0	0	0.5	4	0.5-0.6
24	OPP 08	ACATCGCCCA	12	0	0	0.5	4	0.6
25	OPP 09	GTGGTCCGCA	20	8	40	0.54	3.33	0.5-1.0
26	OPP 10	TCCCGCCTAC	12	0	0	0	2	0.3
	Total		176	8	2.63	3.04	31.33	
	All total		483	51	10.55	0.29	3.09	

Appendix 4: The sequences of oligonucleotide primers used for ISSR analysis and PCR amplicons obtained from *in vivo* and *in vitro* grown *Cymbidium aloifolium*

SN	Primer	Primer sequence (5'-3')	Total no. of PCR amplicons	No. of polymorphic band	Polymorphism (%)	Polymorphic information content (PIC)	Resolving power (R_p)	Range of PCR amplicons (bp)
1	807	AGAGAGAGAGAGAGAGT	24	0	0	0.5	4	0.5-0.9
2	815	CTCTCTCTCTCTCTCTG	23	11	47.83	0.5	3.83	0.4-1.4
3	816	CACACACACACACACAA	24	0	0	0.5	4	0.4-0.8
4	825	ACACACACACACACACT	12	0	0	0	2	0.5
5	827	ACACACACACACACACC	16	4	25	0.25	2.67	0.5-0.7
	Total		99	15	15.15	0.35	3.3	

Appendix 5: One way ANOVA for *in vitro* germination of 2% alginate coated artificial seeds (MS-liquid) of *Cymbidium aloifolium*

Growth parameters (2% MS-liquid)		Sum of squares	Df	Mean square	F	Sig.
Germination	Between groups	3.000	3	1.000	2.400	0.098
	Within groups	8.333	20	.417		
	Total	11.333	23			
Shoot	Between groups	10.792	3	3.597	2.554	0.084
	Within groups	28.167	20	1.408		
	Total	38.958	23			
Leaf	Between groups	92.125	3	30.708	5.041	0.009
	Within groups	121.833	20	6.092		
	Total	213.958	23			
Root	Between groups	67.125	3	22.375	2.928	0.059
	Within groups	152.833	20	7.642		
	Total	219.958	23			
Seedling	Between groups	30.000	3	10.000	3.704	0.029
	Within groups	54.000	20	2.700		
	Total	84.000	23			

Appendix 6: One Way ANOVA for *in vitro* germination of 2% alginate coated artificial seeds (KC-liquid) of *Cymbidium aloifolium*

Growth parameters (2% KC-liquid)		Sum of squares	df	Mean square	F	Sig.
	Between groups	8.833	3	2.944	3.759	.027
Germinaion	Within groups	15.667	20	.783		
	Total	24.500	23			
	Between groups	23.167	3	7.722	7.473	.002
Shoot	Within groups	20.667	20	1.033		
	Total	43.833	23			
	Between groups	88.458	3	29.486	2.497	.089
Leaf	Within groups	236.167	20	11.808		
	Total	324.625	23			
	Between groups	50.125	3	16.708	1.585	.224
Root	Within groups	210.833	20	10.542		
	Total	260.958	23			
	Between groups	52.500	3	17.500	5.000	.010
Seedling	Within groups	70.000	20	3.500		
	Total	122.500	23			

Appendix 7: One Way ANOVA for *in vitro* germination of 3% alginate coated artificial seeds (MS-liquid) of *Cymbidium aloifolium*

Growth parameters (3% MS-liquid)		Sum of squares	df	Mean square	F	Sig.
	Between groups	1.833	3	.611	.655	.589
Germination	Within groups	18.667	20	.933		
	Total	20.500	23			
	Between groups	6.000	3	2.000	.952	.434
Shoot	Within groups	42.000	20	2.100		
	Total	48.000	23			
	Between groups	25.000	3	8.333	4.132	.020
Leaf	Within groups	40.333	20	2.017		
	Total	65.333	23			
	Between groups	16.125	3	5.375	2.295	.109
Root	Within groups	46.833	20	2.342		
	Total	62.958	23			
	Between groups	56.000	3	18.667	7.568	.001
Seedling	Within groups	49.333	20	2.467		
	Total	105.333	23			

Appendix 8: One Way ANOVA for *in vitro* germination of 3% alginate coated artificial seeds (KC-liquid) of *Cymbidium aloifolium*

Growth parameters (3% KC-liquid)		Sum of squares	df	Mean square	F	Sig.
	Between groups	7.667	3	2.556	2.359	.102
Germination	Within groups	21.667	20	1.083		
	Total	29.333	23			
	Between groups	35.792	3	11.931	8.471	.001
Shoot	Within groups	28.167	20	1.408		
	Total	63.958	23			
	Between groups	159.500	3	53.167	18.023	.000
Leaf	Within groups	59.000	20	2.950		
	Total	218.500	23			
	Between groups	226.125	3	75.375	17.163	.000
Root	Within groups	87.833	20	4.392		
	Total	313.958	23			
	Between groups	70.792	3	23.597	10.223	.000
Seedling	Within groups	46.167	20	2.308		
	Total	116.958	23			

Appendix 9: One Way ANOVA for *in vitro* germination of 4% alginate coated artificial seeds (MS-liquid) of *Cymbidium aloifolium*

Growth parameters (4% MS-liquid)		Sum of Squares	df	Mean Square	F	Sig.
	Between Groups	.000	3	.000	.000	1.000
Germination	Within Groups	18.000	20	.900		
	Total	18.000	23			
	Between Groups	.458	3	.153	.152	.927
Shoot	Within Groups	20.167	20	1.008		
	Total	20.625	23			
	Between Groups	210.792	3	70.264	14.171	.000
Leaf	Within Groups	99.167	20	4.958		
	Total	309.958	23			
	Between Groups	55.125	3	18.375	1.109	.369
Root	Within Groups	331.500	20	16.575		
	Total	386.625	23			
	Between Groups	48.000	3	16.000	12.308	.000
Seedling	Within Groups	26.000	20	1.300		
	Total	74.000	23			

Appendix 10: One Way ANOVA for *in vitro* germination of 4% alginate coated artificial seeds (KC-liquid) of *Cymbidium aloifolium*

Growth parameters (4% KC-liquid)		Sum of Squares	df	Mean Square	F	Sig.
	Between Groups	18.458	3	6.153	6.652	.003
Germiantion	Within Groups	18.500	20	.925		
	Total	36.958	23			
	Between Groups	49.792	3	16.597	11.004	.000
Shoot	Within Groups	30.167	20	1.508		
	Total	79.958	23			
	Between Groups	212.000	3	70.667	22.083	.000
Leaf	Within Groups	64.000	20	3.200		
	Total	276.000	23			
	Between Groups	183.000	3	61.000	6.524	.003
Root	Within Groups	187.000	20	9.350		
	Total	370.000	23			
	Between Groups	19.125	3	6.375	11.087	.000
Seedling	Within Groups	11.500	20	.575		
	Total	30.625	23			

Appendix 11: One Way ANOVA for *in vitro* germination of 2% alginate coated artificial seeds (MS-solid) of *Cymbidium aloifolium*

Growth parameters (2% MS-solid)		Sum of squares	Df	Mean square	F	Sig.
	Between groups	7.792	3	2.597	3.945	.023
Germination	Within groups	13.167	20	.658		
	Total	20.958	23			
	Between groups	15.458	3	5.153	2.903	.060
Shoot	Within groups	35.500	20	1.775		
	Total	50.958	23			
	Between groups	39.167	3	13.056	2.701	.073
Leaf	Within groups	96.667	20	4.833		
	Total	135.833	23			
	Between groups	23.167	3	7.722	1.534	.236
Root	Within groups	100.667	20	5.033		
	Total	123.833	23			
	Between groups	13.125	3	4.375	1.296	.303
Seedling	Within groups	67.500	20	3.375		
	Total	80.625	23			

Appendix 12: One Way ANOVA for *in vitro* germination of 2% alginate coated artificial seeds (KC-solid) of *Cymbidium aloifolium*

Growth parameters (2% KC-solid)		Sum of squares	df	Mean square	F	Sig.
	Between groups	14.792	3	4.931	3.543	.033
Germination	Within groups	27.833	20	1.392		
	Total	42.625	23			
	Between groups	14.792	3	4.931	4.257	.018
Shoot	Within groups	23.167	20	1.158		
	Total	37.958	23			
	Between groups	178.167	3	59.389	14.544	.000
Leaf	Within groups	81.667	20	4.083		
	Total	259.833	23			
	Between groups	895.125	3	298.375	54.832	.000
Root	Within groups	108.833	20	5.442		
	Total	1003.958	23			
	Between groups	2190.000	3	730.000	405.556	.000
Seedling	Within groups	36.000	20	1.800		
	Total	2226.000	23			

Appendix 13: One Way ANOVA for *in vitro* germination of 3% alginate coated artificial seeds (MS-solid) of *Cymbidium aloifolium*

Growth parameters (3% MS-solid)		Sum of squares	df	Mean square	F	Sig.
	Between groups	13.000	3	4.333	1.347	.287
Germination	Within groups	64.333	20	3.217		
	Total	77.333	23			
	Between groups	30.333	3	10.111	3.548	.033
Shoot	Within groups	57.000	20	2.850		
	Total	87.333	23			
	Between groups	53.125	3	17.708	3.548	.33
Leaf	Within groups	99.833	20	4.992		
	Total	152.958	23			
	Between groups	120.125	3	40.042	18.410	.000
Root	Within groups	43.500	20	2.175		
	Total	163.625	23			
	Between groups	49.125	3	16.375	13.188	.000
Seedling	Within groups	24.833	20	1.242		
	Total	73.958	23			

Appendix 14: One Way ANOVA for *in vitro* germination of 3% alginate coated artificial seeds (KC-solid) of *Cymbidium aloifolium*

Growth parameters (3% KC-solid)		Sum of squares	df	Mean square	F	Sig.
	Between groups	47.500	3	15.833	3.942	.023
Germination	Within groups	80.333	20	4.017		
	Total	127.833	23			
	Between groups	47.792	3	15.931	3.614	.031
Shoot	Within groups	88.167	20	4.408		
	Total	135.958	23			
	Between groups	101.458	3	33.819	7.158	.002
Leaf	Within groups	94.500	20	4.725		
	Total	195.958	23			
	Between groups	147.792	3	49.264	18.190	.000
Root	Within groups	54.167	20	2.708		
	Total	201.958	23			
	Between groups	52.500	3	17.500	9.722	.000
Seedling	Within groups	36.000	20	1.800		
	Total	88.500	23			

Appendix 15: One Way ANOVA for *in vitro* germination of 4% alginate coated artificial seeds (MS-solid) of *Cymbidium aloifolium*

Growth parameters (4% MS-Solid)		Sum of Squares	df	Mean Square	F	Sig.
	Between Groups	4.000	3	1.333	.606	.619
Germination	Within Groups	44.000	20	2.200		
	Total	48.000	23			
	Between Groups	12.458	3	4.153	1.058	.389
Shoot	Within Groups	78.500	20	3.925		
	Total	90.958	23			
	Between Groups	96.458	3	32.153	2.537	.086
Leaf	Within Groups	253.500	20	12.675		
	Total	349.958	23			
	Between Groups	47.500	3	15.833	.563	.646
Root	Within Groups	562.333	20	28.117		
	Total	609.833	23			
	Between Groups	30.125	3	10.042	15.649	.000
Seedling	Within Groups	12.833	20	.642		
	Total	42.958	23			

Appendix 16: One Way ANOVA for *in vitro* germination of 4% alginate coated artificial seeds (KC-solid) of *Cymbidium aloifolium*

Growth parameters (4% MS-Solid)		Sum of Squares	df	Mean Square	F	Sig.
	Between Groups	8.500	3	2.833	.733	.545
Germination	Within Groups	77.333	20	3.867		
	Total	85.833	23			
	Between Groups	34.333	3	11.444	2.026	.143
Shoot	Within Groups	113.000	20	5.650		
	Total	147.333	23			
	Between Groups	21.000	3	7.000	.773	.522
Leaf	Within Groups	181.000	20	9.050		
	Total	202.000	23			
	Between Groups	76.125	3	25.375	2.277	.111
Root	Within Groups	222.833	20	11.142		
	Total	298.958	23			
	Between Groups	16.500	3	5.500	3.667	.030
Seedling	Within Groups	30.000	20	1.500		
	Total	46.500	23			

Appendix 17: One Way ANOVA for *in vitro* development of 2% alginate coated artificial seeds (MS-Liquid) of *Cymbidium aloifolium*

Growth parameters (2% MS-Liquid)		Sum of Squares	df	Mean Square	F	Sig.
	Between Groups	151.125	3	50.375	2.460	.092
No of shoot	Within Groups	409.500	20	20.475		
	Total	560.625	23			
	Between Groups	50.173	3	16.724	2.734	.071
Length of shoot	Within Groups	122.327	20	6.116		
	Total	172.500	23			
	Between Groups	429.458	3	143.153	3.474	.035
No of leaf	Within Groups	824.167	20	41.208		
	Total	1253.625	23			
	Between Groups	60.541	3	20.180	4.784	.011
Length of leaf	Within Groups	84.358	20	4.218		
	Total	144.900	23			
	Between Groups	34.833	3	11.611	3.152	.048
No of root	Within Groups	73.667	20	3.683		
	Total	108.500	23			
	Between Groups	50.015	3	16.672	2.934	.058
Length of root	Within Groups	113.655	20	5.683		
	Total	163.670	23			

Appendix 18: One Way ANOVA for *in vitro* development of 2% alginate coated artificial seeds (KC-liquid) of *Cymbidium aloifolium*

Growth parameters (2% KC-liquid)		Sum of squares	df	Mean square	F	Sig.
No of shoot	Between groups	36.458	3	12.153	5.381	.007
	Within groups	45.167	20	2.258		
	Total	81.625	23			
Length of shoot	Between groups	63.085	3	21.028	3.325	.041
	Within groups	126.495	20	6.325		
	Total	189.580	23			
No of leaf	Between groups	118.792	3	39.597	2.211	.118
	Within groups	358.167	20	17.908		
	Total	476.958	23			
Length of leaf	Between groups	30.201	3	10.067	2.861	.063
	Within groups	70.378	20	3.519		
	Total	100.580	23			
No of root	Between groups	21.458	3	7.153	1.514	.242
	Within groups	94.500	20	4.725		
	Total	115.958	23			
Length of root	Between groups	46.868	3	15.623	3.679	.029
	Within groups	84.930	20	4.247		
	Total	131.798	23			

Appendix 19: One Way ANOVA for *in vitro* development of 3% alginate coated artificial seeds (MS-liquid) of *Cymbidium aloifolium*

Growth parameters (3% MS-liquid)		Sum of squares	df	Mean square	F	Sig.
No of shoot	Between groups	144.792	3	48.264	4.593	.013
	Within groups	210.167	20	10.508		
	Total	354.958	23			
Shoot length	Between groups	125.185	3	41.728	5.383	.007
	Within groups	155.042	20	7.752		
	Total	280.226	23			
No of leaf	Between groups	298.167	3	99.389	4.059	.021
	Within groups	489.667	20	24.483		
	Total	787.833	23			
Length of leaf	Between groups	87.298	3	29.099	3.778	.027
	Within groups	154.047	20	7.702		
	Total	241.345	23			
No of root	Between groups	36.833	3	12.278	3.426	.037
	Within groups	71.667	20	3.583		
	Total	108.500	23			
Length of root	Between groups	27.545	3	9.182	2.127	.129
	Within groups	86.325	20	4.316		
	Total	113.870	23			

Appendix 20: One Way ANOVA for *in vitro* development of 3% alginate coated artificial seeds (KC-liquid) of *Cymbidium aloifolium*

Growth parameters (3% KC-liquid)		Sum of squares	df	Mean square	F	Sig.
	Between groups	200.167	3	66.722	26.868	.000
No of shoot	Within groups	49.667	20	2.483		
	Total	249.833	23			
	Between groups	131.431	3	43.810	26.830	.000
Length of shoot	Within groups	32.658	20	1.633		
	Total	164.090	23			
	Between groups	494.000	3	164.667	12.667	.000
No of leaf	Within groups	260.000	20	13.000		
	Total	754.000	23			
	Between groups	52.208	3	17.403	19.412	.000
Length of leaf	Within groups	17.930	20	.897		
	Total	70.138	23			
	Between groups	24.833	3	8.278	8.713	.001
No of root	Within groups	19.000	20	.950		
	Total	43.833	23			
	Between groups	30.791	3	10.264	17.142	.000
Length of root	Within groups	11.975	20	.599		
	Total	42.766	23			

Appendix 21: One Way ANOVA for *in vitro* development of 4% alginate coated artificial seeds (MS-liquid) of *Cymbidium aloifolium*

Growth parameters (4% MS-liquid)		Sum of Squares	df	Mean Square	F	Sig.
	Between Groups	158.667	3	52.889	2.452	.093
No of Shoot	Within Groups	431.333	20	21.567		
	Total	590.000	23			
	Between Groups	125.748	3	41.916	6.437	.003
Length of Shoot	Within Groups	130.237	20	6.512		
	Total	255.985	23			
	Between Groups	306.167	3	102.056	4.151	.019
No of Leaf	Within Groups	491.667	20	24.583		
	Total	797.833	23			
	Between Groups	38.711	3	12.904	3.511	.034
Length of Leaf	Within Groups	73.508	20	3.675		
	Total	112.220	23			
	Between Groups	18.792	3	6.264	1.820	.176
No of Root	Within Groups	68.833	20	3.442		
	Total	87.625	23			
	Between Groups	26.458	3	8.819	1.254	.317
Length of Root	Within Groups	140.687	20	7.034		
	Total	167.145	23			

Appendix 22: One Way ANOVA for *in vitro* development of 4% alginate coated artificial seeds (KC-liquid) of *Cymbidium aloifolium*

Growth parameters (4% KC-liquid)		Sum of Squares	df	Mean Square	F	Sig.
No of Shoot	Between Groups	181.500	3	60.500	4.130	.020
	Within Groups	293.000	20	14.650		
	Total	474.500	23			
Length of Shoot	Between Groups	64.583	3	21.528	6.198	.004
	Within Groups	69.470	20	3.474		
	Total	134.053	23			
No of Leaf	Between Groups	361.000	3	120.333	7.099	.002
	Within Groups	339.000	20	16.950		
	Total	700.000	23			
Length of Leaf	Between Groups	29.978	3	9.993	6.487	.003
	Within Groups	30.807	20	1.540		
	Total	60.785	23			
No of Root	Between Groups	17.500	3	5.833	2.318	.106
	Within Groups	50.333	20	2.517		
	Total	67.833	23			
Length of Root	Between Groups	32.800	3	10.933	4.637	.013
	Within Groups	47.153	20	2.358		
	Total	79.953	23			

Appendix 23: One Way ANOVA for *in vitro* development of 2% alginate coated artificial seeds (MS-Solid) of *Cymbidium aloifolium*

Growth parameters (2% MS-solid)		Sum of squares	df	Mean square	F	Sig.
No of shoot	Between groups	118.125	3	39.375	2.405	.098
	Within groups	327.500	20	16.375		
	Total	445.625	23			
Length of shoot	Between groups	2.628	3	.876	.314	.815
	Within groups	55.762	20	2.788		
	Total	58.390	23			
No of leaf	Between groups	235.792	3	78.597	2.329	.105
	Within groups	674.833	20	33.742		
	Total	910.625	23			
Length of leaf	Between groups	3.808	3	1.269	1.005	.411
	Within groups	25.257	20	1.263		
	Total	29.065	23			
No of root	Between groups	9.500	3	3.167	1.086	.378
	Within groups	58.333	20	2.917		
	Total	67.833	23			
Length of root	Between groups	3.201	3	1.067	.390	.761
	Within groups	54.728	20	2.736		
	Total	57.930	23			

Appendix 24: One Way ANOVA for *in vitro* development of 2% alginate coated artificial seeds (KC-Solid) of *Cymbidium aloifolium*

Growth parameters (2% KC-solid)		Sum of squares	df	Mean square	F	Sig.
No of shoot	Between groups	41.667	3	13.889	2.078	.135
	Within groups	133.667	20	6.683		
	Total	175.333	23			
Length of shoot	Between groups	50.575	3	16.858	8.222	.001
	Within groups	41.010	20	2.051		
	Total	91.585	23			
No of leaf	Between groups	227.458	3	75.819	3.837	.025
	Within groups	395.167	20	19.758		
	Total	622.625	23			
Length of leaf	Between groups	32.460	3	10.820	7.495	.001
	Within groups	28.873	20	1.444		
	Total	61.333	23			
No of root	Between groups	21.000	3	7.000	5.316	.007
	Within groups	26.333	20	1.317		
	Total	47.333	23			
Length of root	Between groups	33.667	3	11.222	3.673	.030
	Within groups	61.107	20	3.055		
	Total	94.773	23			

Appendix 25: One Way ANOVA for *in vitro* development of 3% alginate coated artificial seeds (MS-Solid) of *Cymbidium aloifolium*

Growth parameters (3% MS-solid)		Sum of squares	df	Mean square	F	Sig.
No of shoot	Between groups	45.500	3	15.167	.997	.415
	Within groups	304.333	20	15.217		
	Total	349.833	23			
Length of shoot	Between groups	17.025	3	5.675	1.543	.234
	Within groups	73.562	20	3.678		
	Total	90.586	23			
No of leaf	Between groups	14.458	3	4.819	.121	.947
	Within groups	798.167	20	39.908		
	Total	812.625	23			
Length of leaf	Between groups	5.145	3	1.715	.824	.496
	Within groups	41.625	20	2.081		
	Total	46.770	23			
No of root	Between groups	29.500	3	9.833	1.344	.288
	Within groups	146.333	20	7.317		
	Total	175.833	23			
Length of root	Between groups	47.441	3	15.814	4.160	.019
	Within groups	76.028	20	3.801		
	Total	123.470	23			

Appendix 26: One Way ANOVA for *in vitro* development of 3% alginate coated artificial seeds (KC-Solid) of *Cymbidium aloifolium*

Growth parameters (3% KC-solid)		Sum of squares	df	Mean square	F	Sig.
No of shoot	Between groups	113.125	3	37.708	1.960	.153
	Within groups	384.833	20	19.242		
	Total	497.958	23			
Length of shoot	Between groups	77.823	3	25.941	9.058	.001
	Within groups	57.277	20	2.864		
	Total	135.100	23			
No of leaf	Between groups	323.458	3	107.819	1.353	.286
	Within groups	1594.167	20	79.708		
	Total	1917.625	23			
Length of leaf	Between groups	22.833	3	7.611	7.144	.002
	Within groups	21.307	20	1.065		
	Total	44.140	23			
No of root	Between groups	13.125	3	4.375	2.869	.062
	Within groups	30.500	20	1.525		
	Total	43.625	23			
Length of root	Between groups	40.541	3	13.514	3.802	.026
	Within groups	71.088	20	3.554		
	Total	111.630	23			

Appendix 27: One Way ANOVA for *in vitro* development of 4% alginate coated artificial seeds (MS-solid) of *Cymbidium aloifolium*

Growth parameters (4% MS-solid)		Sum of Squares	df	Mean Square	F	Sig.
No of Shoot	Between Groups	192.125	3	64.042	.871	.473
	Within Groups	1470.833	20	73.542		
	Total	1662.958	23			
Length of Shoot	Between Groups	82.493	3	27.498	5.317	.007
	Within Groups	103.440	20	5.172		
	Total	185.933	23			
No of Leaf	Between Groups	388.167	3	129.389	1.167	.347
	Within Groups	2217.667	20	110.883		
	Total	2605.833	23			
Length of Leaf	Between Groups	11.798	3	3.933	2.298	.109
	Within Groups	34.232	20	1.712		
	Total	46.030	23			
No of Root	Between Groups	38.667	3	12.889	1.459	.256
	Within Groups	176.667	20	8.833		
	Total	215.333	23			
Length of Root	Between Groups	18.018	3	6.006	2.799	.066
	Within Groups	42.920	20	2.146		
	Total	60.938	23			

Appendix 28: One Way ANOVA for *in vitro* development of 4% alginate coated artificial seeds (KC-solid) of *Cymbidium aloifolium*

Growth parameters (4% KC-solid)		Sum of Squares	df	Mean Square	F	Sig.
No of Shoot	Between Groups	84.125	3	28.042	1.716	.196
	Within Groups	326.833	20	16.342		
	Total	410.958	23			
Length of Shoot	Between Groups	51.402	3	17.134	4.303	.017
	Within Groups	79.643	20	3.982		
	Total	131.045	23			
No of Leaf	Between Groups	412.458	3	137.486	4.107	.020
	Within Groups	669.500	20	33.475		
	Total	1081.958	23			
Length of Leaf	Between Groups	26.578	3	8.859	6.378	.003
	Within Groups	27.782	20	1.389		
	Total	54.360	23			
No of Root	Between Groups	38.167	3	12.722	2.239	.115
	Within Groups	113.667	20	5.683		
	Total	151.833	23			
Length of Root	Between Groups	69.735	3	23.245	8.332	.001
	Within Groups	55.795	20	2.790		
	Total	125.530	23			

Appendix 29: One Way ANOVA for *in vitro* rooting of artificial seed derived shoots of *Cymbidium aloifolium*

Growth parameters (rooting)		Sum of squares	df	Mean square	F	Sig.
No of shoot	Between groups	112.462	12	9.372	3.973	.000
	Within groups	153.333	65	2.359		
	Total	265.795	77			
Length of shoot	Between groups	105.038	12	8.753	3.738	.000
	Within groups	152.208	65	2.342		
	Total	257.247	77			
No of root	Between groups	63.872	12	5.323	.963	.493
	Within groups	359.375	65	5.529		
	Total	423.247	77			
Length of root	Between groups	61.039	12	5.087	2.484	.010
	Within groups	133.100	65	2.048		
	Total	194.139	77			

Appendix 30: Dice similarity matrix of *in vitro* regenerated plants of *Cymbidium aloifolium* compared with elite mother plant (RAPD)

	M	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**
M	1											
C1	0.947	1										
C2	0.921	0.974	1									
C3	0.921	0.974	1	1								
C4	0.9	0.951	0.951	0.951	1							
C5	0.897	0.925	0.925	0.925	0.976	1						
C6	0.871	0.925	0.95	0.95	0.976	0.975	1					
C7	0.871	0.925	0.95	0.95	0.976	0.975	1	1				
C8	0.871	0.925	0.925	0.925	0.976	0.975	0.975	0.975	1			
C9	0.883	0.911	0.911	0.911	0.963	0.987	0.962	0.962	0.987	1		
C10	0.883	0.936	0.936	0.936	0.963	0.962	0.962	0.962	0.987	0.975	1	
C11	0.886	0.938	0.938	0.938	0.988	0.987	0.987	0.987	0.987	0.975	0.975	1

M- Mother plant (wild), C1- C11- *in vitro* regenerated plants derived from [C1: seed culture on MS medium, C2: protocorm culture on MS medium, C3: shoot tip culture on MS medium, C4: 2% alginate coated artificial seed cultured on liquid MS medium, C5: 4% alginate coated artificial seed cultured on liquid MS medium, C6: 3% alginate coated artificial seed cultured on liquid MS medium, C7: 3% alginate coated artificial seed cultured on solid MS medium, C8: 3% alginate coated artificial seed cultured on solid KC medium, C9: 3% alginate coated artificial seed cultured on liquid KC medium, C10: 3% alginate coated artificial seed cultured on solid MS medium supplemented with BAP and NAA, C11: seed cultured on solid MS medium supplemented with BAP and NAA]

Appendix 31: Dice similarity matrix of *in vitro* regenerated plants of *Cymbidium aloifolium* compared with elite mother plant (ISSR)

	M	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**
M	1											
C1	0.875	1										
C2	0.875	1	1									
C3	0.933	0.941	0.941	1								
C4	0.933	0.941	0.941	1	1							
C5	0.933	0.941	0.941	1	1	1						
C6	0.933	0.941	0.941	1	1	1	1					
C7	0.875	1	1	0.941	0.941	0.941	0.941	1				
C8	0.875	1	1	0.941	0.941	0.941	0.941	1	1			
C9	0.933	0.941	0.941	1	1	1	1	0.941	0.941	1		
C10	0.933	0.941	0.941	1	1	1	1	0.941	0.941	1	1	
C11	0.933	0.941	0.941	1	1	1	1	0.941	0.941	1	1	1

M- Mother plant (wild), C1- C11- *in vitro* regenerated plants derived from [C1: seed culture on MS medium, C2: protocorm culture on MS medium, C3: shoot tip culture on MS medium, C4: 2% alginate coated artificial seed cultured on liquid MS medium, C5: 4% alginate coated artificial seed cultured on liquid MS medium, C6: 3% alginate coated artificial seed cultured on liquid MS medium, C7: 3% alginate coated artificial seed cultured on solid MS medium, C8: 3% alginate coated artificial seed cultured on solid KC medium, C9: 3% alginate coated artificial seed cultured on liquid KC medium, C10: 3% alginate coated artificial seed cultured on solid MS medium supplemented with BAP and NAA, C11: seed cultured on solid MS medium supplemented with BAP and NAA]