Studies of *in vitro* propagation of *Rhynchostylis retusa* – promising foxtail orchid

A Dissertation

Submitted in partial fulfilment of the requirement for the award of degree of

Masters of Science

In

Biotechnology

UNDER THE SUPERVISION OF

Dr. ANIL KUMAR

(ASSOCIATE PROFESSOR)

DEPARTMENT OF BIOTECHNOLOGY

THAPAR INSTITUTE OF ENGINEERING & TECHNOLOGY

(Deemed to be University)

PATIALA, PUNJAB (147004)

SUBMITTED BY

KIRANDEEP KAUR

301701015

M.Sc. BIOTECHNOLOGY (2017-19)

July 2019

CERTIFICATE

This is to certify that the thesis entitled **"Studies of *in vitro* propagation of *Rhynchostylis retusa* – promising foxtail orchid"** submitted by Kirandeep Kaur (301701015) to the Thapar Institute of Engineering and Technology (Patiala) towards partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology. This work has been carried out under my supervision.

It is also certifying that this thesis or any part of this thesis has never been submitted, neither in part nor in full to this institute or any other institute for the award of any degree.

Dr. Anil Kumar

Supervisor

Department of Biotechnology

Thapar Institute of Engineering and Technology, Patiala

Declaration

I declare that the thesis entitles **"Studies of *in vitro* propagation of *Rhynchostylis retusa* – promising foxtail orchid"** is an authentic record of my own work, carried out during a period of six months. The work has been completed under the supervision and guidance of **Dr. Anil Kumar,** Associate Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology (Patiala).

I also declare that matter presented in this thesis has not been submitted by me in any other University/Institute for the award degree.

Date: 26/08/2019

Kirandeep Kaur

301701015

ACKNOWLEDGEMENT

I thank and praise GOD ALMIGHTY for his immense grace and blessings, which guided me in taking up this research project and gave me the confidence and sustained ability to complete this work successfully.

I have immense pleasure to express my deep sense of gratitude and indebtedness to my supervisor **Dr. Anil Kumar, Associate Professor, Department of Biotechnology, Thapar Institute of engendering and Technology (Patiala)** for his valuable advice, constant support, encouragement, unfailing patience and suggestions for completion of my research project. I am very thankful to **Dr. Anil Kumar** for giving me the opportunity and privilege to work in his **Plant Tissue Culture lab, TIFAC CORE, Thapar Institute of Engineering and Technology (Patiala).**

I thank Dr. Moushumi Ghosh, Professor and Head, Department of Biotechnology for giving me the opportunity of doing this project. I thank all the members of Department of Biotechnology for and TIFAC CORE for their cooperation and support.

I am always thankful for the help rendered by Amanpreet Kaur, Saloni Sharma, Davinder Singh and Shivani Guleria throughout my project period without which it would have been very difficult for me to achieve my goals.

I am highly thankful to the tissue culture staff with special mention Mr. Khem Raj, Mr. Rajesh Sharma, Mr. Bhupinder Singh, Mr. Baljit and Mr. Gurmeet Singh who helped me to understand minor details of the work.

Kirandeep Kaur

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ABBREVIATIONS

S.no	Abbreviations	Full form
1.	Mg	milli gram
2.	Ml	milli litre
3.	L	Litre
4.	mM	Millimolar
5.	µM	micromolar
6.	°C	degree Celsius
7.	v/v	Volume by volume
8.	w/v	weight by volume
9.	µl	Microliter
10.	Sec	Second
11.	Min	Minutes
12.	Fig	Figure
13.	PGRs	Plant Growth Regulators
14.	MS medium	Murashige and Skoog medium
15.	EDTA	Ethylenediaminetetraacetic acid
16.	NAA	1-Naphthaleneacetic acid
17.	2,4-D	2,4-Dichlorophenoxyacetic acid
18.	IAA	Indole-3-acetic acid
19.	BA	6-Benzylaminopurine
20.	CTAB	Cetyltrimethylammonium bromide
21.	RAPD	Random Amplification of Polymorphic DNA
22.	ISSR	Inter Simple Sequence Repeats

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Abstract

A simple and efficient shoot organogenesis protocol was developed from leaf explants of *Rhynchostylis retusa*. Initially, the protocorm-like bodies (PLB) culture was established using seeds of the plant. The surface disinfected seeds of *R. retusa* were cultured on basal MS medium to attain maximum number of PLBs per seed. However, the established PLB cultures were found to be devoid of any leaves which limits the regeneration studies, thus attempts were made to attain desired leaf growth from the cultures. For this purpose, PLBs were cultured on different concentration and combinations of PGRs (BA, NAA and GA_3). Among various combinations tested, MS medium fortified with 2.5 µM NAA, 1µM BA and 1µM GA_3 was found to be optimum for leaf growth from PLB clumps. These leaf explants were used to induce shoot organogenesis on MS medium variously supplemented with BA, NAA, IAA and 2,4-D. Maximum number of shoot buds were induced on MS medium supplemented with 2.5 µM BA after 28 days of the culture. It was important to note that any of the auxin was not able to induce shoot organogenesis in the explants except NAA (2.5 – 5.0 µM). The pathway of regeneration was also confirmed through histological studies which revealed that shoot buds differentiate from the leaf samples through intermediate callus phase. Further, clonal uniformity was analysed through Random amplified polymorphic DNA and Inter simple sequence repeat markers and the regenerated shoots found true to type.

Further, the PLBs were encapsulated in alginate matrix and effect of storage temperature and period on synthetic seeds viability was evaluated. The synthetic seed germination potential was found to decline with increasing storage period. Storage at 4 °C showed survival for a period of 60 days while synthetic seeds stored at 25 °C resulted in rapid deterioration before 60 days.

This indicated that storage at lower temperature is more effective for synthetic seed germination.

INTRODUCTION

“If nature ever showed her playfulness in the formation of flowers, this is visible in the most striking way among the orchids” (Davis *et al.*, 1983).

The orchids represent a group of botanically significant and commercially important flowers. Orchids are one of the most pampered plants and occupy top position among all the flowering plants. Orchids represents one of the highly evolved families of the monocotyledons with a 600-800 genera and 25,000 - 30,000 species, among which *Rhynchostylis* holds an important position due to its ornamental value and medicinal benefits. *Rhynchostylis* comprises of 6 species which are generally distributed in India, Sri Lanka, Malaysia, Indo-China region and the Philippines.

Rhynchostylis retusa, a member of genus *Rhynchostylis* is popularly known as ‘Kopoh phool’ in Assam, India. The flower has great cultural significance. The fragrant, beautiful rose-purple coloured flower of *R. retusa* is traditionally worn by women dancers during the Bihu festival in Assam. The plant is generally known as fox tail orchid due to its long tapering and densely packed inflorescences. The inflorescences of *R. retusa* have agile, pendulous racemes and produce sweet aroma. Owing to its beauty and attractiveness, it is praised as “Queen of Orchids” by Myanmar flower lovers and cultivators.

R. retusa is a promising orchid as it has both therapeutic and financial esteem. Many attractive *Rhynchostylis* orchids have become important in flowering decoration-based industries. In addition to its ornamental values the plant also shows noteworthy antiseptic activity against *Bacillus subtilis* and *Escherichia coli* (Hossain, 2011). Few therapeutic properties of the plant are described below: -

- Akhter et al. (2017) reported that *R. retusa* has been utilized by the tribal communities of different parts of south east Bangladesh for treatment of paralysis, rheumatism, piles, fever, fracture, hypersensitivity and inflammation.
- Leaf extract and aerial roots are used for treatment of auricle pain and ear cleaning (Basumtary *et al*., 2004).
- Roots are used to cure malarial fever and roots extracts are applied to cuts and wounds (Tiwari *et al*., 2012).

The orchid assets of the world are diminishing because of the destruction of natural habitat. Prevalent orchids of the province are confronting terrible possibility of destruction due to over-gathering by orchid seekers, deforestation, forest fires, shifting cultivation, continuous extension of crop cultivation, aimless wild accumulation and illicit exchange by the local people. Thus, preservation and sustainable utilization of orchids have more significant importance to save the diminishing orchid prosperity.

Due to these factors the plant, *R. retusa* is enlisted as endangered species in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Conventionally, *R. retusa* is cultivated through true seeds and vegetative propagation (using stem cuttings). However, under natural conditions, the germination rate of seeds is very low (0.2 – 0.3%) due to the absence of the endosperm. Further, the involvement of endophytic mycorrhizal fungi is required for stimulation of symbiotic seed germination (Arditti, 1967). This symbiotic association is mandatory throughout the lifetime of seedling. Moreover, the use of stem cuttings for traditional multiplication of the plant limits the growth of natural population of *R. retusa.*

Therefore, there is a need to find alternative approaches of plant multiplication and preservation. Plant tissue culture techniques such as in vitro propagation and regeneration

allows multiplication of the plant along with maintaining its clonal fidelity and minimising the chances of infection in this extremely wonderful orchid.

Micropropagation refers to an area of plant tissue culture for vegetative propagation of crops and decorative vegetations. This method involves multiplication of plant through variety of explants, such as auxiliary buds, apical shoot and meristem culture under specialized physiochemical conditions (Chandra *et al*., 2010). Micropropagation method has emerged as an efficient technique for the agriculture and horticulture (Standardi and Picconi, 1998).

Direct and indirect shoot organogenesis and somatic embryogenesis are most commonly used micropropagation techniques (Antony *et al*., 2014). Somatic embryogenesis in recent years has shown enormous potential in providing efficient clonal propagation system. Plant regeneration through somatic embryogenesis is one of the main pre-requisites for potential use of clonal propagation in woody plant (Park *et al*.,1998).

Although, small explant requirement and seasonal independence are some of the advantages associated with micropropagation strategy. But the associated disadvantages such as, unpredictable mutations leading to somaclonal variations had attracted research studies to find out alternative methods for germplasm conservation (Khoddamadeh *et al*., 2011).

One such method is artificial seed technology which has potential to overcome micropropagation based problems (Larkin and Scowcroft, 1981). Aside from lesser chances of somaclonal variation, various advantages of synthetic seed technology include easy handling and transportation, easy germplasm exchange and long-term storage etc. (Rai, 2008).

Synthetic seed is referred to as artificially encapsulated embryo or vegetative propagules like buds, shoot tips, protocorm like bodies or other tissues. It has potential to grow into whole plant

under in vitro and in vivo environments and retains this potential even after storage at low temperatures (Ara *et al*., 2000).

The principle of encapsulation involves the ion-exchange phenomena between the Na+ in sodium alginate and Ca^{2+} in calcium chloride solution. Round, firm capsules are formed when sodium alginate beads containing explant, are dropped into calcium chloride solution. Hence, the concentration of gelling matrix, concentration of calcium chloride and preservation time plays a significant role in determining texture and quality of synthetic seeds. (Sai prasad and Polisetty, 2003).

In synthetic seed technology major factor that influence encapsulation is concentration of gelling matrix (Mohanraj *et al*., 2009). For entrapment purpose, matrix or coating should be inert and should not have any adverse effect on the embryo or propagule under in vitro and ex vitro conditions. Matrix should also allow germination of the propagule (Sorvari *et al*., 1997). There are variety of gelling matrix which are utilized for encapsulation due to their capacity of formation of beads, for example agar, alginate, carboxy methyl cellulose etc. Among these, sodium alginate is used widely due to its various advantages like formation of a protective layer around somatic embryo (Malabadi *et al*., 2005), Further alginate have moderate viscosity and is available at low cost, which makes it a suitable gelling agent.

In view of the strategy for production, synthetic seeds are essentially characterized into two types: -

1)Encapsulated desiccated - In this sort of encapsulation strategy, propagules are hardened to withstand desiccation before encapsulation. Desiccation tolerance can be accomplished by gradually decreasing relative humidity, quickly unsealing petri dish for overnight to dry and by nutrient deprivation. This kind of synthetic seed was first reported in coated somatic embryo of carrot (Janick and Kitto, 1986).

2)Encapsulated hydrated - In this sort of encapsulation method, explants are coated by many coating agents, for example, sodium alginate, sodium pectate and carboxy methyl cellulose, which are tested as hydrogels. This type of hydrogel encapsulation of somatic embryos was first developed in alfalfa in 1988 (Redenbaugh *et al*., 1991).

Many ornamental and commercially significant plant species are desiccation sensitive and cannot be stored for an extensive stretch of time by traditional technique that leads to loss of viability. One of the widely used technique for in vitro germplasm conservation is slow-growth storage. In this technique, germplasm of plant is used to store at temperature of around 4 °C. At low temperature, metabolic activities and cellular divisions of plant cell gets slow. Plant material can be stored for an extended stretch of time without any genetic modification (Engelmann, 2008). Artificial seed have potential for germination after storage. In orchids, synthetic seeds are capable of germination without any loss in viability even after storage for 60 days at 4 °C (Mohanraj, 2009).

Multiplication and storage of plants like orchids through in-vitro propagation, organogenesis and artificial seeds may lead to somaclonal variations. Thus, establishing clonal fidelity is a major concern. Several molecular markers are available to check the genetic fidelity of regenerated plants for their molecular characterisation. Among various available molecular markers, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) has been widely used for detection of somaclonal variations, genetic characterization, germplasm evaluation, elucidation of phylogenetic relationship, construction of genetic linkage maps and to study clonal integrity (Mishra, 2011; Srivastava, 2011; Mandal., 2000). RAPD and ISSR are widely used because of its cost effectiveness and ease.

Depending on the gaps in the studies, the present work was divided into four major objectives listed below: -

- Development of micropropagation protocol using seed explants
- Shoot organogenesis from the various explants taken from microshoots and test of clonal fidelity of regenerated propagules.
- Histological studies of shoot organogenesis and shoot multiplication in *R. retusa*
- Preparation of artificial seeds and optimization of storage conditions for *R. retusa*

Review of Literature

Orchid plants are known for their wide range of beautiful flowers and have high commercial esteem, but they are under threat worldwide because of overexploitation by indiscriminate collection and deforestation (Swarts and Dixon 2009). Some reports suggested that orchid plants are mainly influenced by nutrient quality of soil which is declining due to human activities and mining (Ganeshiah *et al*. 1998; Sosa and Platas 1998). This prompt low seed germination rate of orchids under natural conditions. Plant tissue culture through micropropagation and regeneration is reported as an efficient method to overcome low germination rate in orchids (Gangaprasad *et al.* 1999). Various reports suggested that micropropagation of orchids can be achieved using different explants such as leaf segments, shoot apices, protocorm-like- bodies etc. (Arditi 2009; Wimber 1963; Kerbauy 1991).

Micropropagation is reported to be affected by various factors such as composition of medium, plant growth regulators (PGRs), light source and gelling agents. The effect of casein hydrolysate and PGRs (6- benzylaminopurine and indole-3-acetic) on in vitro propagation using green pod culture on Murashige and Skoog (1962; MS) medium was reported (Kumar *et al*.,2002). Casein hydrolysate, either alone or in combination BAP and/or IAA was found to improve percent seed germination on MS basal medium and the time taken for protocorm like bodies (PLBs) formation was also reduced. Effect of sucrose (0, 30 & 60 mM), and different light conditions (greenhouse, photosynthetically active radiations as the source of light and fluorescent light) was also studied in relation to growth and the chlorophyll content of the cultures. The growth of cultures was higher on medium containing sucrose as a source of carbohydrate.

In successive studies the effect of other PGRs (Thidiazuron and Naphthalene acetic acid) along with activated charcoal (AC) was studied (Naing, Park, Hwang, Chung 2010). Explants cultured on half strength MS medium containing TDZ in combination with NAA and AC show direct shoot organogenesis through culture of thin root section, leaf section and seedling. Seedling promoted only in shoot and root formation while no response was observed from other explants. The best response was recorded on the medium containing 1.0 mg/L NAA and 1.1 mg/L TDZ.

In another study, the effect of gelling agents (agar or phytagel) and light sources [cool fluorescent light (CFL) and photosynthetically active radiation (PAR)] was evaluated on *Rosa damascene* and *R. retusa* (Kumar and Palni, 2015). Phytagel (0.22%, w/v) and PAR was found to show better shoot multiplication as compared to cultures grown on medium gelled with agar (0.8%, w/v) and incubated under CFL. Light sources were also found to have an impact on differentiation of PLBs. The PAR light was found to be efficient in inducing PLB differentiation from root explants, whereas CFL was effective for differentiation of PLBs from leaf segment.

Regeneration is also reported to be affected by medium composition, PGRs, activated charcoal, peptone and coconut water. Nodal segments were used for high frequency regeneration (Sinha and Jahan, 2012). MS medium having 3% sucrose, 1.5 mg/L BA, 0.5mg/L NAA, 2g/L peptone, 10% (v/v) was found to be best nutrient medium, on which microshoots were induced from 89 percent cultures. Half strength MS medium supplemented with 2% sucrose, 2g/L peptone, 10% (v/v) coconut water and 150mg/l L-glutamine was found to be the best medium for further proliferation of microshoots, growth into shoots and formation of secondary microshoots.

The effect of PGRs (BA and kinetin) on regeneration of PLBs was also studied (Islam and Bhattacharjee, 2015). Somatic embryos cultured on MS medium having 1.0 mg/L BA and 1.5

mg/L kinetin show highest embryo induction from leaf. Germination of embryos was maximum on half strength MS medium having 1 mg/L BA. Multiplied shoots show maximum length and number on MS medium having 1.5 mg/L BAP and 1.0 mg/L NAA.

Although, large plant requirement is a major advantage associated with micropropagation but problem of somaclonal variations in regenerated plants attract the researchers to find out alternative methods of germplasm (Khoddamadeh *et al*., 2011).

Artificial seed technology is one of the methods that may avoid somaclonal variations and prove to be efficient in long term storage (Rai *et al*., 2009). Synthetic seed was firstly developed by encapsulating somatic embryo of carrot followed by desiccation (Janick and Kitto 1986). In this approach, various reagents were tested for encapsulation. Among them, polyoxymethylene was selected based on various parameters such as non-toxicity and solubility. The major drawback of this method was that the encapsulation process was only possible for desiccation sensitive plants. Later on, a procedure for encapsulation of desiccation resistant plants by testing a number of hydrogel coating matrix such as carrageenan, sodium pectate and alginate were developed (Redenbaugh *et al*., 1991). According to this report, alginate was found to be more appropriate for encapsulation of somatic embryo of alfa, due to its nontoxic and coating properties. Since, then different plant species have been tested for production of synthetic seeds including ornamental plants, fruits and cereals etc. (Ganapathi, 1992; Mandal, 2000; Rai, 2009).

In encapsulation process, type of explant has been reported to play an important role in synthetic seed formation. Earlier, somatic embryo was widely used as an explant. In recent years, many reports suggested the possibility of using other propagules as an explant such as nodal segments, auxiliary buds, protocorm- like bodies etc. (Nhut *et al*., 2005; Rihan *et al*., 2011).

In orchids, most widely used explant for synthetic seed production are protocorm-like-bodies which are equivalent to somatic embryos (Reddy *et al*., 2012). Corrie and Tandon (1993), reported that PLBs are converted into platelets when they were transferred either on sterile soil or nutrient media. It was found that encapsulated protocorms like bodies showed conversion frequency of 100% and 84% under in vitro and in vivo conditions respectively. These studies highlighted the possible for transplant of those propagules directly to soil giving an advantage of easy acclimatization.

In encapsulation process, coating matrix associated with plant material plays an important role to achieve higher conversion frequency. Earlier reports suggested that majority of spherical beads were obtained with 3% sodium alginate and 100 mM $CaCl_2$ solution (Nayak, 1997; Faisal *et al*., 2012). The regeneration potential of encapsulated explant is highly influenced by coating matrix.

Gaintait (2012) reported alginate encapsulation (protocorm like bodies) of orchid, by using different concentrations of sodium alginate (1-4%) in complexing with 75 mM calcium chloride. In this study, it was found that superior coating was observed by using 3 percent sodium alginate with 75 mM $CaCl_2$ resulting in higher conversion frequency. In another report, encapsulation of protocorm-like-bodies was carried out by using 3% sodium alginate with 75 mM calcium chloride that resulted in maximum conversion of synthetic seed and emergence of shoot when supplemented on MS medium (Mohanraj et al. 2009). Few reports suggested that lower concentration of sodium alginate (1-2%) were responsible for asymmetric and fragile beads. On the other hand, very, hard capsules were produced when high concentration of sodium alginate (4%) was used (Ray and Bhattacharya 2010). Effect of exposure time of calcium chloride on encapsulation was also studied. It was observed that exposure time of 30 minutes to calcium chloride solution absorb optimized amount of calcium ions that leads to round and firm beads formation and results in maximum emergence of roots and shoots,

whereas the exposure time above 30 minutes proved to be detrimental for shoot emergence from beads (Nagesh *et al*., 2009).

Synthetic seed technology plays an important role in the short-term storage for the conservation of germplasm. Saiprasad and Polisetty (2003) reported that the encapsulated protocorm-like-bodies on MS medium supplemented with 3.4 μM BAP resulted in maximum conversion frequency when stored at 4 °C and retained viability up to 75 days of storage. In another study, it was suggested that at the storage temperature of 4 °C, encapsulated protocorm-like-bodies showed higher conversion on MS medium containing IAA in comparison with 25 °C storage (Mohanraj *et al*., 2009). Several reports concluded the fact that storage at 4°C for synthetic seed are more preferable than storage of 25 °C (Chand and Singh 2004; Mandal *et al*., 2000).

Parab *et al*., (2008) studied genetic variation among population of *R. retusa*, using RAPD and ISSR markers. Among 35 tested markers, 12 RAPD and 7 ISSR were studied. Total number of 74 RAPD and 30 ISSR fragments were generated from 12 RAPD primers and 7 ISSR primers. Polymorphism was recorded both in RAPD (75.11%) than ISSR (61.4%). Thus, RAPD was recorded with high level of polymorphism compared to ISSR.

In view of this literature review, the present study was focused to develop a simple and efficient regeneration system in economically important plant *R. retusa*. The study also includes synthetic seed technology as a plant conservation strategy and the effect of storage temperature and duration on seed viability was evaluated.

Materials and Methods

Culture initiation and plant material preparations.

Cultures were established using seeds of *R. retusa* procured from Palampur, India. Seeds were used for culture initiation following a procedure described by Kumar *et al*., 2003. The surface disinfection procedure includes washing of pods thoroughly under running tap water, treating the explants with an aqueous solution of Bavistin (0.5%, w/v) containing 0.02% (w/v) Tween 20 for 20 minutes. Under laminar air flow cabinet, the pods were dipped in 70% ethanol and flamed for 10 sec and put into the sterile distilled water. Following surface disinfection, pods were dissected to take out seeds and these seeds were cultured on Murashige and Skoog (MS) medium supplemented. The cultured seeds were incubated at 25±2 °C under white cool fluorescent light under 42 µmol m^{-2} s^{-1} light intensity with 16hr light and 8hr dark photoperiod cycle.

Chemicals and glassware

Culture initiation and other plant tissue culture related experiments were carried out in 300 ml tissue grade culture bottles purchased from Kasablanka Corporation, (Mumbai India). Plant growth regulators (PGR) and tissue culture grade chemicals were purchased from Hi-media Laboratories Pvt. Ltd. Mumbai. Enzymes, dNTPs required for amplification were purchased from Thermo fermentas (Mumbai, Ltd).

Preparation of culture medium and stock solutions

MS medium containing essential micronutrients, macronutrients, 3% (w/v) sucrose and gelled with 0.75% (w/v) agar (basal MS medium) was prepared fresh for each experiment. Various plant growth regulators (PGRs) like BA, NAA, GA_3 were added to the medium in various concentrations and combinations. The stock solutions of all the macronutrients, micronutrients and vitamins were prepared individually, which were further used to prepare the medium. The 2.5 mM stock solutions of plant growth regulators (PGRs) were prepared by dissolving these in respective solvents (1N HCl, 1N KOH and water) and the final volume was made by distilled water. The stock solutions were stored at 4 °C. The pH of the medium was adjusted to 5.8 with 1N HCl or 1N KOH using pH meter before autoclaving.

Table 1 - Composition of Murashige and Skoog (1962) medium

Components	Amount (mg/L)
Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Micronutrients	
H_3BO_3	6.2
$MnSO_4.4H_2O$	16.9
$ZnSO_4.H_2O$	8.6
KI	0.83
$NaMO_4$ $.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2$ $.6H_2O$	0.025
Vitamins	
Nicotinic acid	0.5
Pyridoxine HCL	0.5
Thiamine HCL	0.1
Myo-inositol	100
Others	
Glycine	2
Sucrose	3%(w/v)
Agar	0.7%(w/v)

Preparation of explants

PLB cultures initiated from seeds of *R. retusa* were not having any leaves, so in order to obtain leaf explants for the regeneration studies, PLBs were cultured on MS medium supplemented with various combinations and concentrations of BA (0µM, 1µM, 2.5µM), NAA (0µM, 1µM, 2.5µM) and GA_3 (0µM, 1µM, 2.5µM). The cultures were incubated under tissue culture conditions and results were recorded after 4 weeks.

Effect of PGRs on shoot organogenesis

Leaf explants from microshoots of *R. retusa* were excised and cultured on MS medium supplemented with various concentrations of BA (2.5µM to 12.5µM), NAA (2.5µM to 12.5µM), IAA (2.5µM to 12.5µM) and 2-4D (2.5µM to 12.5µM). Leaf explants were cut along the mid-rib region and care was taken that the adaxial side of explants is in contact with the medium.

Clonal Fidelity Studies

The Microshoots regenerated from the explants were maintained individually as regenerated lines. DNA was isolated from each line and mother culture to establish the clonal fidelity.

Isolation, quantitative and qualitative analysis of Genomic DNA

Genomic DNA was isolated from mother plant and regenerated lines, using the CTAB method (Doyle and Doyle 1990). Fresh tissue (2.0 gm) was grounded into fine powder using liquid nitrogen. Prewarmed (60 °C) CTAB extraction buffer (10.0ml) was added to each sample to make slurry following the incubation at 60 °C for 1 hr in the water bath. Chloroform and isoamyl alcohol (24:1 v/v) were added in equal volume to the slurry and mixed for about 3 minutes. Following the centrifugation at 5000 rpm for 10 min, the aqueous phase was removed

with the help of wide-bore pipette and transferred to clean eppendorf tubes. The chloroform extraction procedure was repeated twice. DNA was precipitated with 0.66 volume of cold isopropanol followed by incubation for 1 hr at -20 °C. The supernatant was discarded after centrifugation (10,000 X g for 15 min) and the pellet was dissolved in 1 ml TE buffer and transferred to microfuge tube. 2µl of preheated RNase solution (10 mg/ml stock) was added and incubated at 37 °C for 1hr. Equal volume of phenol chloroform (1:1 v/v) solution was added to samples followed by gentle shaking and centrifugation (10000 X g for 10 min). Aqueous layer was retained, and 0.3 volume of 3 M sodium acetate and 0.6 volume of chilled isopropanol was added. After incubation at -20 °C/hr, samples were centrifuged (1000 X g for 10 min) and the pellet was retained, dried and dissolve in TE buffer and stored at -20 °C.

Qualitative estimation of DNA was performed through agarose gel electrophoresis. DNA was checked on 0.8% agarose gel (w/v). Gel was prepared by adding 0.32 g of agarose (Life Technologies India Pvt. Ltd.) in 30 ml 0.5 X TAE buffer (Tris-Acetate-EDTA) buffer. The agarose gel solution was boiled until clear. The molten agarose was cooled to 45°C and 1µl of ethidium bromide (10 mg/ml) was added and poured into the casting tray inserted with combs. At room temperature the gel was solidified. After loading DNA sample, electrophoresis was carried out in electrophoresis apparatus (Amersham Bioscience, USA) at 50 volts for one 1 hr and bands were visualized on U.V. transilluminator (Viber Loumart, France).

The extracted DNA samples were qualified by spectrophotometric measurement using Nano Drop 1000 Spectrophotometer (Thermo Scientific, Wilmington DE, USA) at wavelength of 260 nm. The qualitative estimation of DNA was also done by taking the ratio of absorbance at 280 nm and 260 nm.

Testing clonal fidelity using molecular markers

Primers (15 each) of Random Amplification of Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) were checked for the presence of amplified bands. The 20 µl of polymerase chain reaction (PCR) mixture contain 50 ng of genomic DNA, 1.0 U Taq polymerase, 100 mM dNTPs mixture, 2.0 µl reaction buffer (10 X) and 10 nM primer, Milli Q water (Millipore India, Bangalore) was added to make up the final volume of 20 µl. Amplification was performed in thermal cycler model Gene Amp 9700. Under conditions of initial denaturation at 94°C for 5 min; 41 cycle of 94°C for 60 sec, 36° C (55°C in case of ISSR) for 90 sec; with final extension at 72°C for 5 min. Amplified products were resolved on Ethidium bromide stained agarose gel and visualized under UV light. The pattern showing bands were photographed as digital picture using Geldoc system (BioRad).

Statistical analysis

Each experiment was repeated three times with five replicates each. The data was analysed using Analysis of Variance (ANOVA) and the means were compared through Newman Kuel's test at $P<0.05$. Statistical analysis and graph plotting were carried out GraphPad Prism 5 software.

Histological studies

Following the procedure described by Johansen (1940), tissue sample showing various stages of morphogenesis were fixed in formalin, glacial acetic acid and 50% aqueous ethanol (FAA) in a ratio of (5:5:90 v/v) for 1 week. The fixed samples were dehydrated using *t*-butyl alcohol series (Table 2).

Table 2: Tertiary butyl alcohol (*t*-BA) series for dehydration of samples

S.No. (ml)	Rectified alcohol (ml)	Tertiary butyl alcohol (ml)	Water (ml)	Time (h)
A	30	20	50	3-4
B	50	20	50	3-4
C	50	35	15	3-4
D	45	55	-	3-4
E	25	75	-	3-4
F	-	100	-	3-4

Glass vial containing samples and TBA was kept in an oven at 60 °C and pellets of wax were added to it after short intervals of time. Sample was kept in oven until the smell of the TBA disappeared. Samples in proper orientation was embedded in filtered wax and block were made. Sections of 10 µm were cut using rotatory microtome. The sections were placed on slide and stretched over water at 60 °C. Sections were dried for about 5-6 days. According to the method described by Sharma and Millam (2004) (Table 3) staining of sections was carried out with Toluidine blue (0.2% w/v) dye. Sections were mounted with DPX (distyrene, a plasticizer and xylene) and observed under light microscope.

Table 3: The de-waxing, staining and mounting procedure for prepared microscopic slides (Sharma and Millam, 2004).

S. No.	Treatment	Duration (min)
Dewaxing		
1.	Toluene	10
2.	Toluene	10
3.	Ethanol (99.7%)	3
4.	Ethanol (99.7%)	3
5.	Ethanol (95%)	3
6.	Ethanol (95%)	3
7.	Ethanol (85%)	3
8.	Ethanol (85%)	3
9.	Ethanol (70%)	3
10.	Distilled water	3
11.	Distilled water	3
12.	Distilled water	3
Staining		
13.	Toluidine blue (0.2%)	2
14.	Washing under running tap water	10-15
15.	Distilled water	1
Mounting		
16.	Ethanol (85%)	Quick dip
17.	Ethanol (99.7%)	Quick dip
18.	Ethanol (85%)	Quick dip
19.	Toluene	2
20.	Toluene	2
21.	Air drying	>120
22.	DPX mounting and coverslips	Processing of individual slide

Synthetic seed synthesis

Protocorm-like bodies (PLBs) of smaller size (2-3 mm diameter) were individually separated from 21 days old PLB cultures of *R. retusa* under aseptic conditions. PLBs were suspended in 3% (w/v) autoclaved sodium alginate (alginic acid sodium salt from brown algae) gelling agent and aseptically dropped in autoclaved calcium chloride (100 mM) solution using 50 ml wide-bore pipette (5mm diameter). The beads were kept immersed in calcium chloride solution for 10 minutes to achieve polymerization. The percolated beads were washed with sterile distilled water 5-6 times to remove any excess calcium chloride.

Storage of encapsulated Protocorm like bodies (PLBs)

The beads were stored at cold temperature (4 °C) and room temperature (25 °C) for durations (15, 30, 45 and 60 days) to evaluate the effect of storage temperature and various storage period on the artificial seed viability. After the storage, beads were cultured on MS medium and results were recorded after 10 to 12 days.

Results

Culture initiation and multiplication

Cultures were established using seeds of *R. retusa* as explants. The seeds were cultured on MS medium and Protocorm like bodies (PLBs) were obtained (Figure 1A). PLBs grown from the seed explants were multiplied and maintained on the same medium with a regular subculture of 21 days. The cultures were incubated at 25±2 °C under photoperiod regime of 16/8-h light/dark. Actively multiplying PLBs were found to be devoid of any leaves.

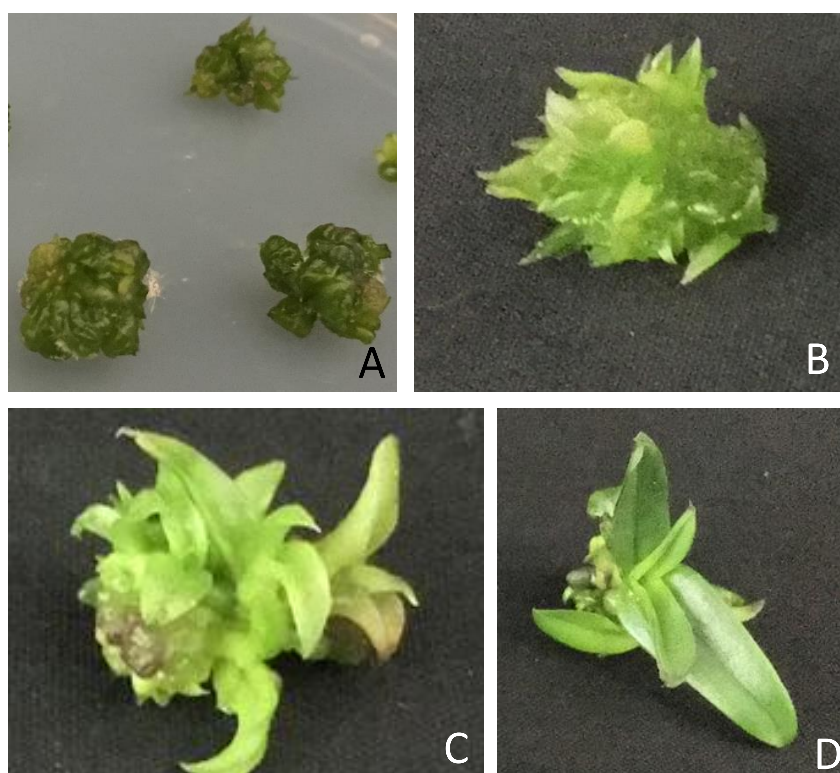

Figure 1: The effect of various plant growth regulators (BA, NAA, GA_3) on leaf formation and its growth from protocorm-like bodies of *Rhynchostylis retusa*. **A** PLBs cultured on basal MS medium **B** leaf initiation from PLBs on MS medium supplemented with BA (2.5 µM) **C-D** Leaf expansion on MS medium supplemented with BA (1.0 µM), NAA (2.5 µM) and GA_3 (1 µM).

Effect of Plant Growth Hormones on leaf growth

The protocorm like cultures established using seed explants were devoid of any leaves. Thus, to obtain leaf explants, PLBs of *R. retusa* were cultured on the MS medium containing different concentrations and combinations of NAA, BA and GA_3 to evaluate the effect of PGRs on leaf expansion. Although leaf growth was initiated on all the medium compositions but maximum number of leaves per PLB clump was observed on medium containing 2.5 µM NAA, 1 µM BA and 1 µM GA_3 (Table 4, Figure 1B-D). It was also noteworthy that the desired expansion of leaves was also obtained on the same medium combination. The expanded leaves (Figure 1D) from elongated shoots were used as explants for the induction of shoot organogenesis.

Table 4 The effect of Plant Growth Regulators on leaf expansion from Protocorm-like bodies of *R. retusa*

PGR concentration (µM)			**No. of leaves per PLB clump**	**Length of leaf**
NAA	**BA**	**GA_3**	**Mean ±SEM**	**Mean ±SEM**
0	0	0	5.67±0.38 [b]	0.06±0.01 [a]
1	0	0	5.67±1.00 [b]	0.22±0.11 [a]
2.5	0	0	5.01±1.54 [b]	0.11±0.01 [a]
0	1	1	9.78±1.93 [a]	0.20±0.03 [a]
1	1	1	9.89±2.02 [a]	0.25±0.10 [a]
2.5	1	1	10.34±0.51 [a]	0.30±0.06 [a]
0	2.5	2.5	7.12±1.09 [a]	0.21±0.06 [a]
1	2.5	2.5	7.56±1.61 [a]	0.18±0.06 [a]
2.5	2.5	2.5	8.11±1.63 [a]	0.30±0.05 [a]

Data were recorded after 30 days of culture and analysed using ANOVA. Means were compared using Newman Keul's test at $P<0.05$. The values followed by same lowercase letters are not significantly different at $P<0.05$

Effect of Plant growth regulators (PGRs) on shoot organogenesis

To evaluate the effect of various PGRs on shoot organogenesis in *R. retusa*, leaf explants were cultured on MS medium supplemented with different concentrations of BA, NAA, 2,4-D and IAA. Leaves were cut along the midrib and utmost care was taken to remove the petiole before culture.

Callus initiation was observed from the explants cultured on both auxin and cytokinin rich medium. However, shoot regeneration was observed only on few selected medium combinations (Figure 2). Maximum shoot organogenesis frequency (80%) was observed on the MS medium fortified with 2.5 µM BA (Table 5). It was important to note that the same medium also resulted in regeneration of maximum number of shoots per leaf explant.

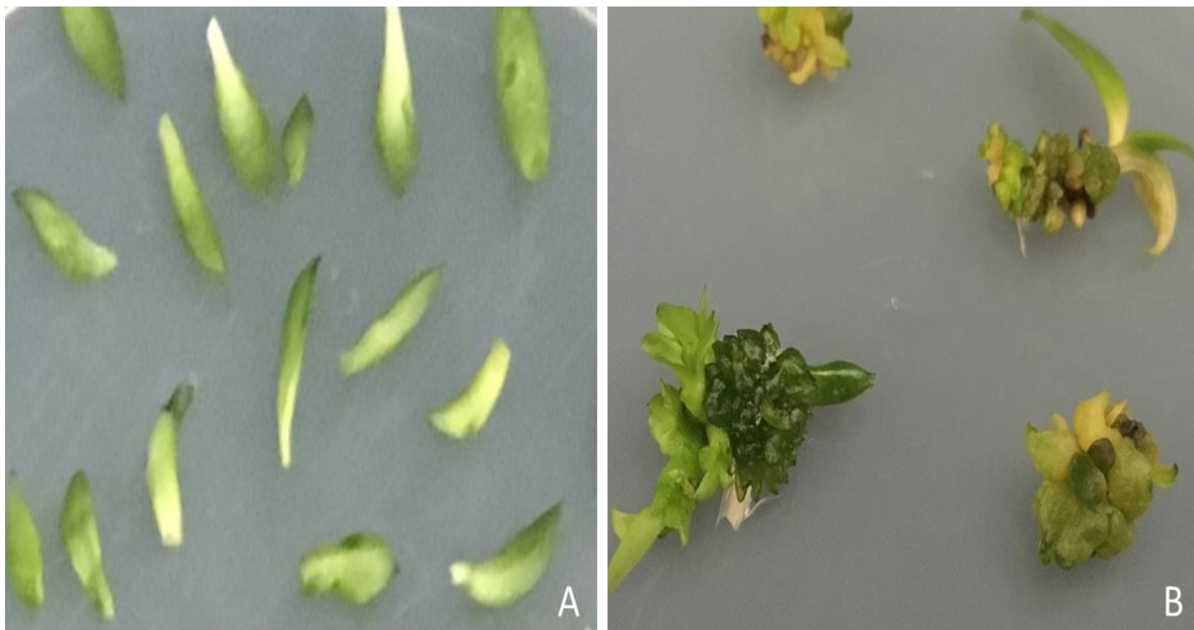

Figure 2: The effect of BA on shoot organogenesis from **A** leaf explants of *Rhynchostylis retusa* **B** shoot regeneration from leaf explants on MS medium supplemented with 2.5 µM BA

Table 5: The effect of BA on shoot organogenesis from leaf explants of *R. retusa*

BA (µM)	**Percent explants showing shoot organogenesis Mean ±SEM**	**No. of shoots per explant Mean ±SEM**
0	0.00±0.00[d]	0.00±0.00[b]
1	20.00±11.54[c]	1.33±.88[a]
2.5	80.00±11.54[a]	2.25±0.38[a]
5.0	60.00±11.54[b]	1.89±.11[a]
7.5	53.00±13.33[b]	2.10±0.23[a]
10.0	46.67±6.67[b]	1.78±.36[a]
12.5	26.67±17.64[c]	0.78±.40[b]

Data were recorded after 30 days of culture and analysed using ANOVA. Means were compared using Newman Keul's test at $P<0.05$. The values followed by same lowercase letters are not significantly different at $P<0.05$.

It was important to note that the addition of auxins alone to the medium could not result in shoot regeneration from leaf explants of *R. retusa.* Among all the auxins (NAA, 2,4-D and IAA) and their concentrations (0µM, 2.5µM, 5µM, 7.5µM, 10µM and 12.5µM) tested, shoot organogenesis was observed only in the presence of 2.5 µM and 5 µM of NAA (Table 6). Intense callus initiation was observed from leaf explants cultured on MS medium supplemented with NAA (2.5 – 5 µM) and shoot buds differentiate from this callus within 3-4 weeks. It was

also noteworthy that the explants cultured on other medium combinations showed symptoms of necrosis and turned brown eventually.

Table 6 : The effect of NAA on regeneration from leaf explant of *R. retusa*

NAA (μM)	**Percent explants showing shoot organogenesis Mean ±SEM**	**No. of shoots per explant** **tion per explant (%)** **Mean ±SEM** **Mean ±SEM**
0.0	0.00±0.00[a]	0.00±0.00[a]
1.0	0.00±0.00[a]	0.00±0.00[a]
2.5	6.67±6.67[a]	1.00±1.00[a]
5.0	6.67±6.67[a]	0.33±0.33[a]
7.5	0.00±0.00[a]	0.00±0.00[a]
10.0	0.00±0.00[a]	0.00±0.00[a]
12.5	0.00±0.00[a]	0.00±0.00[a]

Data were recorded after 30 days of culture and analysed using ANOVA. Means were compared using Newman Keul's test at $P<0.05$. The values followed by same lowercase letters are not significantly different at $P<0.05$.

Clonal fidelity of regenerated shoots

The RAPD and ISSR profiles of regenerated shoots derived from leaves and those of the mother plants were found to be similar, thus indicating the clonal nature of these plants. Of the 30 primers used (15 each of RAPD and ISSR), 11 RAPD and 2 ISSR primers resulted in the

amplification of DNA fragments (Figure 3) . These primers produced a maximum of 7 and a minimum of 1 band. Of the total 35 markers obtained, 23 markers were scored with using RAPD (Table 7) and 12 markers were scored with ISSR primers (Table 8). The size of the amplified markers ranged from 450 to 2,000 bp. No polymorphic band was observed, and all the markers were found to be similar to the mother plant (Figure 4). This indicated that there is no change in genetic makeup during regeneration from leaf explant and proved clonal fidelity.

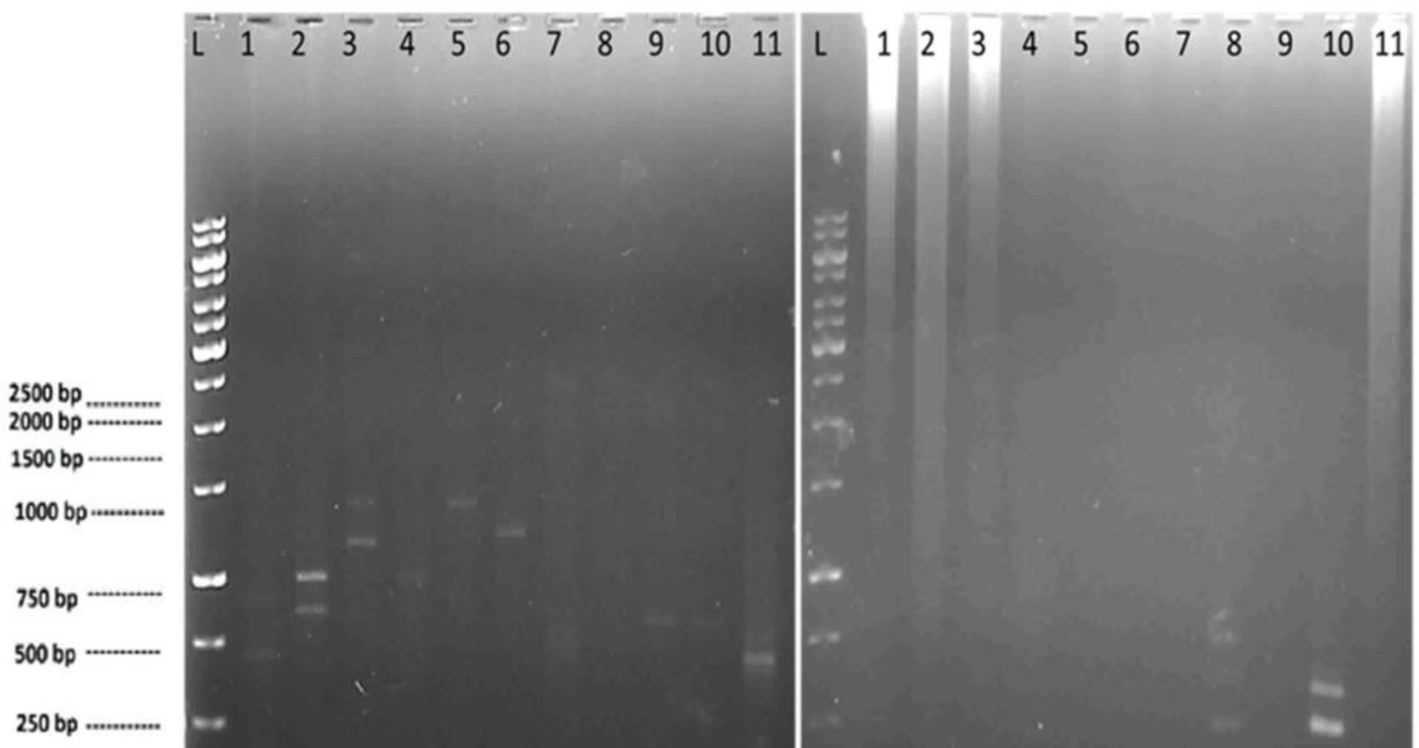

Figure 3 Agarose gel electrophoresis of RAPD and ISSR for scanning of primers resulting in maximum number of amplified bands

Table 7 : List of RAPD primers used for assessment of clonal fidelity of regenerated leaves of *R. retusa* , their sequence number and range of amplified bands.

S. no.	Primer	Primer sequence	No. of bands	No. of polymorphism	Size range
1	RAPD 1	AGCGCCATTG	2	No	450bp
2	RAPD 4	CTGGGGGACT	2	No	750-500bp
3	RAPD 5	ACCGCGAAGG	2	No	1000-750bp
4	RAPD 6	GGACCCAACC	1	No	750bp
5	RAPD 8	TCTGGTGAGG	1	No	1000bp
6	RAPD 10	GTGATCGCAG	1	No	900bp
7	RAPD 11	TTGCACGGG	-	No	-
8	RAPD 12	GTGTGCCCCA	-	No	-
9	RAPD 13	CTCTGGAGAC	-	No	-
10	RAPD 14	TCTGTGCTGG	1	No	500bp
11	RAPD 15	GGGGTGACGA	2	No	500bp
12	RAPD 17	GACCGCTTGT	1	No	750bp
13	RAPD 20	ACCCGGTCAC	7	No	1500-500bp
14	RAPD 21	CAGGCCCTTC	2	No	1000-750bp
15	RAPD 22	TGCCGAGCTG	1	No	2000bp

Table 8 : List of ISSR primers used for assessment of clonal fidelity of regenerated leaves of *R. retusa* , their sequence number and range of amplified bands.

S. no.	Primer	Primer sequence	No. of bands	No. of polymorphism	Size range
1	ISSR 5	$(AC)_8$	-	No	-
2	ISSR 6	$(CA)_8TG$	-	No	-
3	ISSR 7	$(GC)_8A$	-	No	-
4	ISSR 8	$(GA)_8TA$	-	No	-
5	ISSR 9	$(GC)_8T$	-	No	-
6	ISSR 10	$(GC)_8A$	-	No	-
7	ISSR 11	$(GC)_8AT$	-	No	-
8	ISSR 12	$(AT)_8GC$	6	No	1000-250bp
9	ISSR 13	$(CT)_8A$	-	No	-
10	ISSR 14	$(GA)_8TG$	6	No	500-250bp
11	ISSR 15	$(GT)_8A$	-	No	-
12	ISSR 16	$(GT)_8C$	-	No	-
13	ISSR 17	$(AT)_8C$	-	No	-
14	ISSR 18	$(GA)_8CC$	-	No	-
15	ISSR 19	$(AT)_8GC$	-	No	-

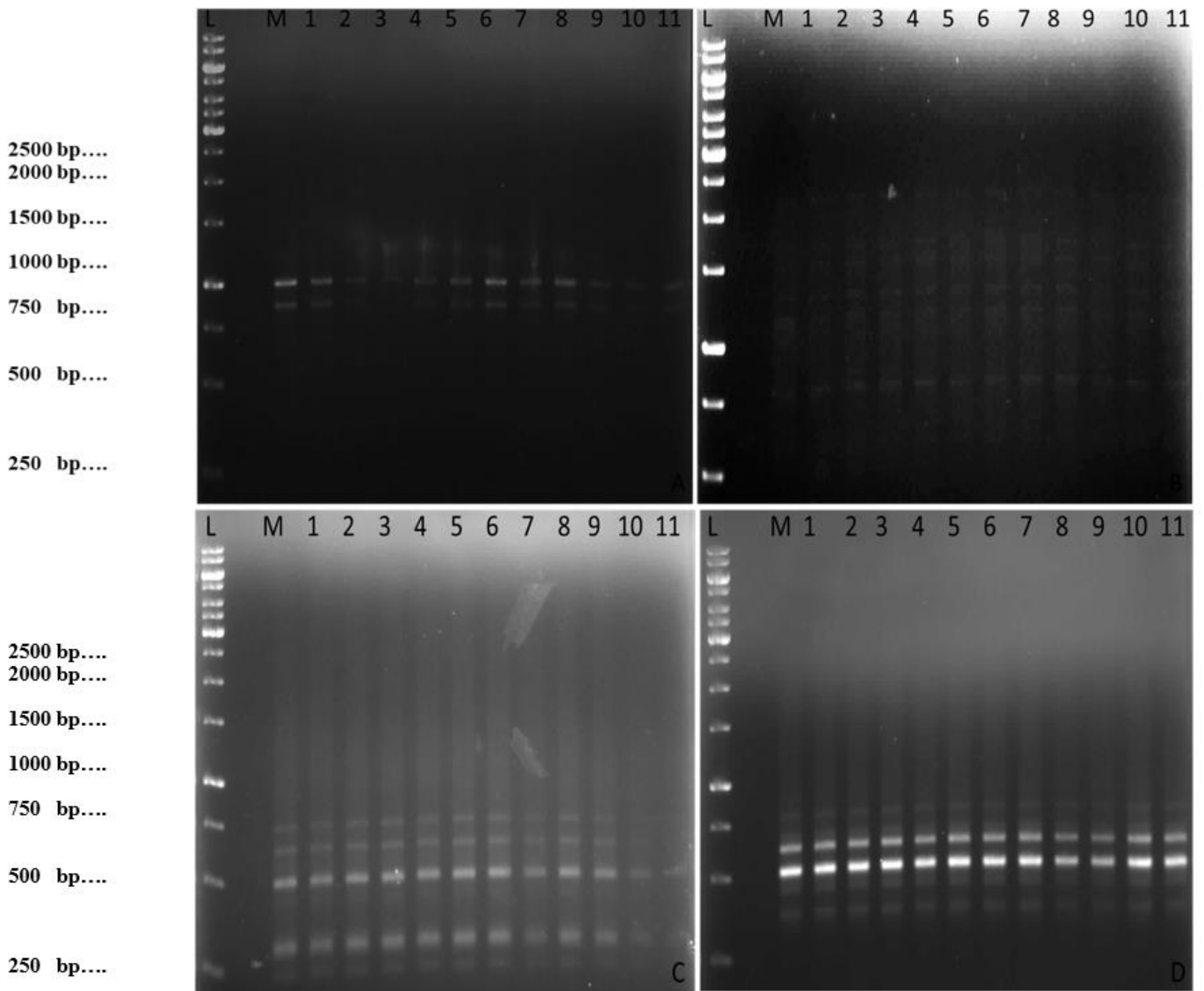

Figure 4– Agarose gel electrophoresis of RAPD and ISSR products of regenerated leaves of *Rhynchostylis retusa*, **A-D** Genetic profiles of regenerated leaves and mother plant using **A and B** – RAPD, **C and D** – ISSR, **L** – Ladder, **M** – Mother plant, **1-11** – Regenerated leaves.

Histological studies

Histological analysis showed that shoot bud is initiating from the callus induced on the leaf sample. The cells of the callus showed intense cellular divisions and get organised to meristematic pockets (Figure 5A). These pockets led to the development of shoot buds having vascular connection with the underlying leaf tissue (Figure 5B).

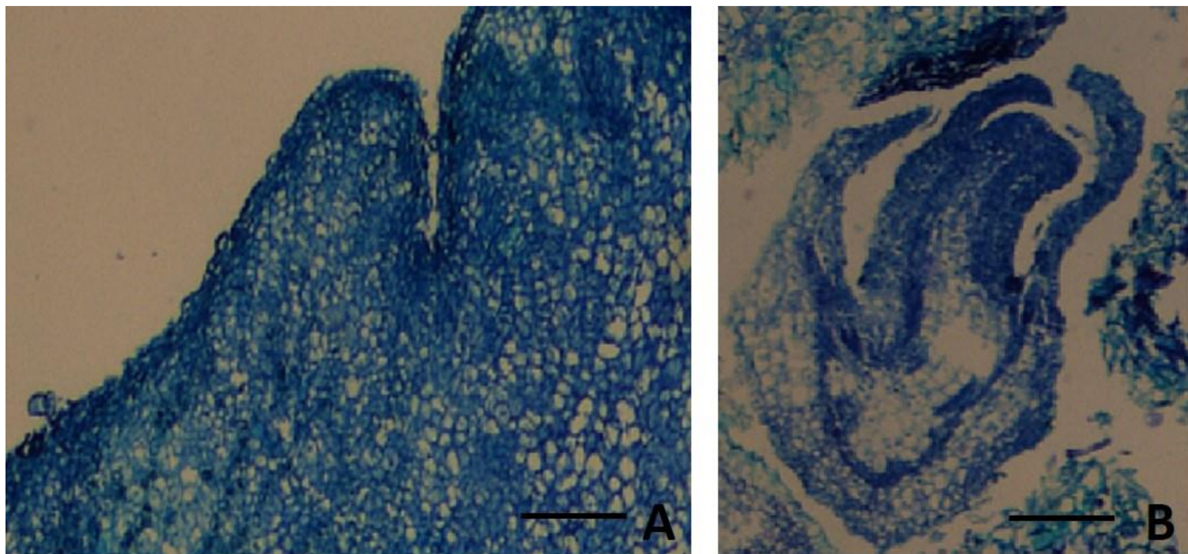

Figure 5 - Histological study of leaf explants showing shoot bud differentiation on MS medium supplemented with BA (2.5 µM). **A** meristematic pocket developed by cellular divisions from callus **B** Shoot bud having vascular connection with underlying tissue

The effect of storage days and temperature on germination of artificial seeds

To assess post storage longevity of encapsulated PLBs, beads were stored at different temperature (4 °C and 25 °C) for different storage period (0-60 days). The present study revealed that encapsulated PLBs stored at 4 °C were having higher multiplication efficiency in comparison with storage at 25 °C. The germination of encapsulated PLBs was initiated after 10-12 days of culture on MS medium. It was observed that at both storage temperatures, multiplication frequency of beads decreased with increase in storage duration. Encapsulated PLBs stored at 4 °C were found to retain viability for longer period in comparison with those stored at 25 °C (Figure 6 and 7). Encapsulated PLBs stored at 4 °C were observed to have higher multiplication potential (26.66%) even after storage of 60 days and contrastingly, at 25 °C storage temperature, encapsulated PLBs were observed to be shrunken, necrotic and brown in nature.

Figure 6 - Source of explants and multiplication of encapsulated PLBs, **A**- 21 days old culture of *Rhynchostylis retusa* used as a source of PLBs, **B**-Encapsulated PLBs; **C**-Regeneration and growth of encapsulated PLBs after different period of culture.

Table 9 – Influence of storage days and storage temperature on percentage multiplication frequency of encapsulated protocorm like bodies.

Storage duration (in Days)	Multiplication frequency of encapsulated PLBs stored at 4°C storage	Multiplication frequency of encapsulated PLBs stored at 25°C storage
0	46.6b	46.6a
15	66.6ab	53.3a
30	53.3ab	33.3b
45	33.3ab	13.3c
60	26.6b	6.6cd

Data were recorded after 70 days of culture and analysed using ANOVA. Means were compared using Newman Keul's test at $P<0.05$. The values followed by same lowercase letters are not significantly different at $P<0.05$.

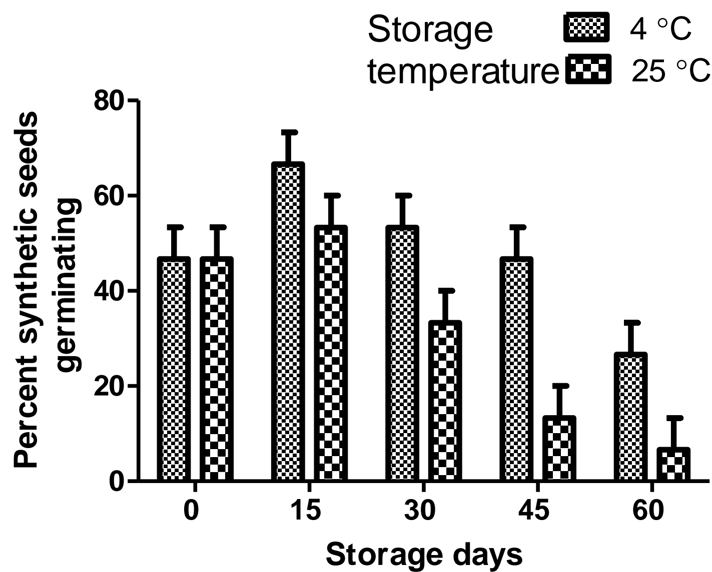

Figure 7 – Effect of storage duration (0-60 days) and temperature (4 °C and 25 °C) on germination (%) of encapsulated PLBs.

DISCUSSION

Rhynchostylis retusa, an important ornamental plant is enlisted as endangered species in Convention on International trade in endangered species (CITES) due to inefficient seed germination and lower efficiency of conventional measures of propagation. Furthermore, the continuous efforts to conserve the plant and increase its ornamental value through genetic manipulations makes in vitro studies of the plant, an important aspect of research. To undertake plant improvement programmes, the development of reliable and reproducible shoot organogenesis protocol is a prerequisite. Although shoot organogenesis is reported in *R. retusa,* but these studies are very limited (Bhattacharjee and Islam 2015; Vij *et al*., 1984). Thus, in the present study, efficient shoot organogenesis protocol was developed using leaf explants of *R. retusa*. The study was further elaborated to assessment of clonal fidelity of regenerated explants and production of synthetic seeds.

To begin with the studies, aseptic cultures were established by inoculating seeds of *R. retusa* on MS medium. The culture initiation leads to the formation of PLBs which were maintained and multiplied on basal MS medium for further experimentation. In *R. retusa* the culture initiation has already been reported using different explants such as apical nodal (Sinha and Jahan, 2012) segments, leaves (Islam *et al*., 2015) and green pods (Kumar *et al*., 2015). Further, the use of basal MS medium for culture establishment has also been reported in *R. retusa* (Bhattacharjee and Islam 2015).

PLB cultures of *R. retusa* were found to be devoid of any leaves. Therefore, for preparation of the explants and to attain the desired size of leaves, PLB clumps were cultured on MS medium supplemented with different concentrations and combinations of plant growth

regulators (NAA, BA and GA_3). Among the different combinations tested, maximum number of leaves per PLB clump were observed on MS medium supplemented with 2.5 µM NAA, 1 µM BA and 1 µM GA_3. As per our knowledge, no similar study has been reported in *R. retusa*. However, similar kind of study has been taken out in *Dendrobium* hybrids (Martin and Madassery, 2006).

R. retusa is an orchid of economic importance and various efforts have been made to improve the colour and quality of its flowers (Arditti *et al*., 1993; Thammasiri *et al* 2015). To undertake plant improvement programmes, the establishment of efficient and reliable regeneration protocol is a prerequisite. Shoot regeneration system has been well reported in *Rhynchostylis* species using various explants such as leaf and immature seeds (Vij *et al*., 1984; Sinha and Jahan, 2012; Niknejad et al., 2011). In these studies, the use of different plant growth regulators and other nutrients such as coconut water has also been mentioned to attain shoot organogenesis in *R. retusa* (Thomas and Michael, 2007; Parab and Krishnan, 2012). However, in the present study, BA was used as sole source of PGRs and maximum regeneration was achieved on MS medium supplemented with 2.5 µM BA. The maximum number of shoots regenerated per explant was also observed on the same medium combination. It was important to note that among various auxins (NAA, IAA and 2,4-D) tested, regeneration was only observed on MS medium containing 2.5 µM and 5 µM NAA and no regeneration was observed on other auxins. Previously, the requirement of cytokinin for shoot organogenesis has been reported in many plants (Aggarwal et al. 2010; Kaur et al. 2017; Vij *et al*., 1984). It has also been reported that the cytokinins are required for differentiation of cells, an important phenomenon for shoot organogenesis (Prakash and Gurumurthi, 2005).

Since in the present study, the shoot organogenesis was attained through intermediate callus phase, thus the chances of somaclonal variations cannot be neglected. Therefore, the clonal fidelity of regenerated plants was studied using PCR based molecular markers (RAPD and

ISSR). The use of molecular markers such as RAPD, ISSR, SSR, SNPs etc. for testing the clonal fidelity of regenerated shoots is widely reported earlier in many plants including orchids (Kaur *et al*., 2017; Aggarwal *et al*., 2010; Bansal *et al*., 2014). In the present study, 23 markers of RAPD and 12 markers of ISSR were scored and all the scored markers were found to be monomorphic. This indicates the clonal uniformity of regenerated plantlets. Previously it has been reported that the length of culture and the presence of a disorganized growth phase in tissue culture are major factors causing somaclonal variations (Rani and Raina, 2000). Thus, the clonal uniformity in the present study could be due to short exposures time in the culture medium.

To figure out the pathway of shoot organogenesis, histological studies using regenerated tissues was carried out. The histological analysis revealed that shoot buds were initiated from surface layer of the explant. Some surface cells show meristematic activity, which further organized to form shoot buds. Similar observations in shoot differentiation has been reported in Gladiolus (Kumar *et al*., 1999).

The orchid protocorms are very fragile and need proper and steady nutrient supply for complete differentiation into plantlets, their transportation is usually difficult and expensive. Synthetic seeds are produced by encapsulation of protocorm like bodies in an alginate matrix. This system serves as a low cost, high volume propagation system. Encapsulation of various explants and their subsequent conversion into plantlets had been earlier reported in many plant species (Ghosh and Sen 1994; Ganapathi *et al*.1992).

The parameters like the storage temperature and duration of storage are known to affect the viability of the encapsulated PLBs (Ikhlaq *et al*., 2010) in various plants including *R. retusa* but the work is limited. Thus, in the present study the effect of two temperatures i.e. 4 °C and 25 °C on synthetic seed viability were evaluated. It was observed that storage at lower

temperatures (4 °C) was able to retain viability of encapsulated PLBs for longer period of time. Previously, storage at 4 °C has been demonstrated as an optimum storage condition for synthetic seeds of *Cymbidium elegans* and Coerulea orchid (Gantait and Sinnah, 2013).

To conclude, the present study reports an efficient and highly reproducible micropropagules, shoot organogenesis system in *R. retusa*. The regenerated plants were found to be true to type. The study was extended to find the effect of different temperatures on synthetic seed viability and 4 °C was found to be helpful in retaining the seed germination potential even after 60 days of storage period. Overall, the outcomes of the present study can be used as a platform for plant improvement and its conservation.

Conclusions

Rhynchostylis retusa is a monopodial epiphytic plant with a long foxtail tapering tail. It is an important therapeutic orchid having multiple medicinal properties and ornamental values.

1. PLB cultures of *R. retusa* were established by culturing disinfected seeds on MS medium.
2. Leaves growth and expansion in the established PLB cultures was attained by inoculating PLB clumps on MS medium supplemented with BA (1 µM), NAA (2.5 µM) and GA_3 (1 µM).
3. Maximum shoot organogenesis from leaf explants of *R. retusa* was observed on MS medium fortified with BA (2.5 µM).
4. Shoot organogenesis was not observed on medium devoid of cytokinin and containing auxins alone.
5. Monomorphic bands were observed for all the regenerated plantlets indicating their clonal uniformity with the mother plant.
6. Histological sections of leaf sample revealed the shoot bud was initiated from the callus and cells showed intense cellular division which were organised to form meristematic pockets
7. Encapsulation of PLBs followed by storage at 4°C were observed to have higher germination potential (26.66 %) even after period of 60 days in comparison with storage at 25 °C where encapsulated PLBs were found to be shrunken, necrotic and brown only after 40 days of storage.

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