Seed characterization and standardization of *in vitro* seed germination protocol in *Rhynchostylis retusa* (L.) Blume.

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KERALA, INDIA

2023

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by

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THESIS

Submitted in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BREEDING AND GENETICS
COLLEGE OF AGRICULTURE, VELLAYANI
THIRUVANANTHAPURAM-695522
KERALA, INDIA

2023

DECLARATION

I, hereby declare that this thesis entitled **"Seed characterization and standardization of *in vitro* seed germination protocol in *Rhynchostylis retusa* (L.) Blume."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani

Date: 16-03-2023

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CERTIFICATE

Certified that this thesis, entitled **"Seed characterization and standardization of *in vitro* seed germination protocol in *Rhynchostylis retusa* (L.) Blume."** is a record of research work done independently by **Ms. Akhila Rajan** (2020-11-098) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Date: 16.3.2023

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We, the undersigned members of the advisory committee of **Ms. Akhila Rajan** (2020-11-098), a candidate for the degree of **Master of Science in Agriculture**, with major in Plant Breeding and Genetics, agree that the thesis entitled **"Seed characterization and standardization of *in vitro* seed germination protocol in *Rhynchostylis retusa* (L.) Blume."** may be submitted by **Ms. Akhila Rajan** (2020-11-098), in partial fulfilment of the requirement for the degree.

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ACKNOWLEDGEMENT

First of all I thank Almighty God for all the blessings he showered upon me throughout my life. I feel immensely grateful for this experience and feel indebted to all the members of my advisory committee.

To start with, I am extremely thankful to my Major Advisor, ***Dr. Seeja G.,*** *Professor, Department of Plant Breeding and Genetics, College of Agriculture, Vellayani, Thiruvanathapuram. Her guidance throughout the journey was the most important factor which led to successful completion of this project. I am most thankful to her for the exposure and opportunities received during this period. Her sincerity and dedication to work, shall remain an inspiration for the rest of my life.*

I extend my gratitude to ***Dr. Arya K.,*** *Professor and Head of the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani for her valuable criticism and suggestions and keen interest in the work and for helping in completion of the work in time.*

I would like to mention ***Dr. Joy M.,*** *Professor, Plant Pathology who gave great support and encouragement in pursuing this project and lending wise counsel when needed.*

I would like to thank ***Dr. Biju C.K.,*** *Principal Scientist, JNTBGRI, Puthenthope for his patience in instruction and invaluable direction throughout my PG programme. I also acknowledge the technical guidance he had given in handling of samples, microscope and associated software packages. I express my sincere appreciation for being there for me whenever I needed help and carefully monitoring the progress in work.*

I express my heartfelt gratitude to ***Dr. Sreekumar S.,*** *Principal Scientist, JNTBGRI, Puthenthope, Thiruvananthapuam for teaching me the science and the skill of tissue culture technology. I acknowledge the pain he had taken to demonstrate the techniques and to take care of the infrastructure and facilities required for the research. Thank you for your teachings of subject and ethics of research, for the principles imparted and for helping me develop a proper mindset for the same.*

I sincerely express my obligation and respect to teachers of Department of Plant Breeding and Genetics, ***Smt. Adheena Ram A., Dr. Beena Thomas, Dr. G. Gayathri, Dr. Lovely B.*** *and* ***Dr. Ninitha Nath C.*** *for their constant encouragement and support.*

I also express my gratitude to ***Dr. Jayalekshmy V.G.****, Professor (RC), Department of Seed Science and technology for her co-operation throughout my course work.*

Words cannot express my feelings of indebtedness to my dear friend Athira who was there to assure me through the moments of fear and self-doubt. I benefitted greatly from her understanding and comfort when it was overwhelming for me and when I felt lost. Thank you for bearing with me and lending a helping hand uncountable number of times even before I had the idea of asking. Without you this endeavour would have been far behind successful.

The staff at Saraswathy Thangavelu Extension Centre-JNTBGRI, Puthenthope had been a source of great energy and enthusiasm. To begin, I would like to say how much indebted

*I am to **Remya chechi** and **Manjima** who spend their valuable time in providing technical assistance. Very special thanks to **Akshara, Shefin itha, Keerthi chechi, Leksmi chechi, Aswathy** and **Amina** who were always there for discussions on anything that I was unsure of.*

*Let me take a moment to thank my friends for keeping me sane and making the last two years fly by. A big appreciation to my batchmates **Ayana, Parvathy, Varadha, Aakash, Deepika, Jyothi, Manasa, Aravind** for their helping hands. I also thank my seniors **Anand chettan** and **Bhaskar chettan** for showing direction. Many thanks to **Sandra, AnuKrishna, Navyashikha, Farha, Gayathri GR, Keerthana and Kalyani**. Thank you all for the people you are.*

*I gratefully acknowledge JNTBGRI, Puthenthope for providing all the facilities and materials. I thank Kerala Agricultural University for the opportunity in carrying out my study and research work. I would like to express my sincere thanks to **Librarian**, library staffs and all other staffs of the department at COA, Vellayani for their support during my study period.*

*Further, yet importantly, sense of respect goes to my parents **Mr. Rajan P.P.** and **Mrs. Shiby Rajan** for their strong support during this journey, and my beloved sister **Dr. Alkha Rajan** who is an endless source of energy and encouragement. Thank you for the for choosing to listen without judgment. Thank you for believing in me. I understand that the prayers of my family are there with me at all times.*

A lot of people were involved in this endeavour constructively whose names are not mentioned but their contribution will be remembered.

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Introduction

1. INTRODUCTION

Orchids are among the most highly prized of ornamental plants. In addition to their geographical and taxonomic diversity, orchids are harvested and traded for a variety of purposes, including as ornamental plants, medicinal products and food. There are many features unique to the Orchidaceae family which led to their dominance in the globe in terms of number of species. Orchids represent the epitomy of plant ecological interaction for reproductive success. They have complex pollinator interaction. They have literally dust like seeds which are produced in millions in a fruit and the seed requires a symbiotic relationship with fungus for germination and establishment. Because of their complex biology, orchids are excellent indicators of overall forest diversity in an area (Christenson, 2003).

Rhynchostylis is an Indo-Southeast Asian epiphytic genus characterized by short stems, thick and fleshy leaves and produce lateral racemose inflorescence of closely arranged flowers annually. In India the genus is represented by a single species *Rhynchotylis retusa* (L.) Bl**.** This was first described and illustrated by Van Rheede from Kerala (Rasmussen *et al.,* 2022). *R. retusa,* is an endangered epiphytic Vanda alliance orchid species with floriculture and therapeutic importance (Zhang *et al.*, 2013). It is popularly called foxtail orchid because its pendulous inflorescence bearing almost a hundred flowers in dense cluster looks like a furry fox's tail (Naing *et al.,* 2010). Some vernacular names in Malayalam are 'Dhroupati mala', 'Annaan thali'and 'Annaan vaalan'. Due to its special flower shape, multitude of flower colour, long lasting flowers, fragrance and large cluster of flowers in an inflorescence, it has become popular. Additionally, reliable manipulation of flowering time and ability to produce high quality spikes are important commercial considerations.

Rhynchostylis plant - based preparation have been used in traditional medicine to cure a number of diseases. The species is of cultural importance for the people of Assam and other north eastern states. Due to the medicinal properties as well as ornamental value, the plant has been over exploited from their natural habitats throughout centuries till become endangered. To prevent over exploitation from natural habitat, it is listed under Appendix II of CITES (Convention on International Trade in Endangered Species).

Orchids have non-endospermic seeds and thus needs an appropriate fungal stimulus (mycotrophy) for germination. The seed germination percentage of the species is very low (less

than 1%). Furthermore, this species requires specific natural habitat like open forests or forest margins at elevations of 300– 1500 m and open sunlight with intermittent raining for flowering (Adhikari and Fischer, 2011). Considering its exceptionally low seed germination and survival percentage in wild habitat, conservation importance and its increasing demand in the commercial market, development of efficient seed germination protocol by the application of biotechnological intervention have become urgent requirement.

Seed germination and early-stage seedling development of orchids are mycobiont mediated (Rasmussen *et al.*, 2015). This limitation can be overcome by *in vitro* culture in which exogenous supply of carbon and other minerals as furnished by the fungal partner in natural conditions. Pierik (1987) reported that an orchid plant produces 1300 to 4 million seeds per capsule and only 0.2-0.3 per cent germinate in nature (Singh, 1992) due to the lack of endosperm or very small endosperm which is inefficient for the seed germination. Its roots and leaves are taken as explant for multiplication, but asymbiotic germination of *R. retusa* seeds is very popular to propagate the plant quickly (Kumar *et al.*, 2002).

Many orchid species produce hybrids (inter-specific to inter-generic) with ease and this feature has been exploited for the development of novel inter-specific and plurigeneric hybrids. The success of hybridisation depends largely upon asymbiotic seed germination, which is an assured means of establishment of the hybrid seedlings. The hybrids can combine good characters from both the parents and may help to survive better in challenging environments.

Out of 2-3 per cent seed germination, only very few survive and become mature plant since it needs microclimate of favourable temperature, humus, humidity, etc. in nature. Being an epiphyte in trees of the rainforests *R. retusa* grows under canopy filtered sunlight and high humid microclimate with peculiar nutrition requirement not accessible from soil.

Generally, orchids are propagated vegetatively but due to its slow growth and limited production of suckers, its propagation rate is very low. Propagation of these orchids has been hampered by the naturally slow growth rate of the plant, which renders it very difficult to be propagated through conventional methods. *In vitro* culture techniques have provided a useful alternative technology for propagating this recalcitrant species. Moreover, to promote genetic divergence and evolutionary adaptations, seed propagation is to be inevitable. This necessitates the application of *in vitro* seed propagation technique for large scale production of orchids with

conservation and commercial importance. Seed propagation helps in producing variability of significance in population which can be identified and maintained permanently.

Although great advances are being made, orchid seed physiology, ecology, and whole plant ecology still needs to be understood. Natural pollination in Orchidaceae has coevolved with the pollinator and this coevolution sometime leads to the decline of orchid species as their pollinator is declining regularly. Standardization of *in vitro* culture techniques also helps in valuable germplasm conservation and makes it possible to conserve the most threatened species (Magrini *et al.*, 2019).

Knowledge about seed characteristic features is inevitable for the successful development of germination technology. Besides, it also contributes taxonomy and phylogenetic understanding. Hence, documentation of seed morphology, morphometric and viability data are inevitable for enriching the basic knowledge as well as developing successful germination protocol. Additionally, pollen and seed characters are direct indicators of fitness of a species. Documentation of plant characters enable understanding of regional variability and inheritance studies. In the light of these, the present investigation is aimed to document seed morphological traits and to standardize protocol for *in vitro* seed germination in *R. retusa*.

Review of Literature

2. REVIEW OF LITERATURE

Taxonomic position (Saxena, 2020)

Kingdom: Plantae

Clade: Tracheophytes

Clade: Angiosperms

Clade: Monocots

Order: Asparagales

Family: Orchidaceae

Subfamily: Epidendroideae

Genus: *Rhynchostylis*

Species: *retusa*

2.1. EVALUATION OF FLORAL CHARACTERS

2.1.1. *Floral characters (qualitative)*

2.1.1.1. Flower colour

The floral characteristics of this genus consist of light coloured flowers with fragrant, plump inflorescences. The colour of *R. retusa* shows polymorphism of several shades of red and pink (Arditti, 1967). Flowers with purple blotches on sepals and petals and dark purple labellum is the species character of *R. retusa* (Buragohain *et al.,* 2015). Higgins (2012) observed pendant inflorescence having white flowers with pink, red or violet markings. The plant bear one or more pendent inflorescence(s) with closely arranged white flowers with pink spots (Bhatti *et al.*, 2016). Flowers are densely arranged in a raceme and are pink, white and deep magenta in colour. Pedicel is purplish; sepals and petals are white with a faint pinkish tinge. Column is short and slender dotted with magenta (Sibin and Gangaprasad, 2012).

2.1.1.2. Nature of flowering – free or seasonal

Xi *et al.* (2021) in his study based on the orchids in China, made an interesting note that the flower or inflorescence was given as a gift during the Spring Festival there because it was flowering around at that time. In Assamese culture, the long inflorescence is worn by the brides as ‘gajra’ or hair ornament and girls wear them in a hair-bun during folkdance on their states’ harvest festival, ‘Rongali Bihu’. It is a symbol of religious purity there (Saxena, 2020).

2.1.1.3. Season of flowering

Flowering occurs from summer to autumn, especially in May - June (Sinha and Jahan, 2012). Flowering season is reported as June – July by Thakur and Dongarwar (2019) and around June by Tongbram *et al.* (2012). Both conducted their study in India. The endangered orchid species grows in moist areas and blossoms during monsoon (Bhattacharjee and Islam 2015). *Rhynchostylis retusa* anthesis is in February; the rainy season being October to April in Java Island of Indonesia (Higgins, 2012).

2.1.1.4. Time of anthesis

Anthesis in some orchids is a genetically controlled trait (Sanford, 1971). The mechanism of continuous flowering in orchids is tightly linked to dormancy and bud release. A number of key floral regulators MADS box genes, such as *FT*, *SOC1*, *ELF*, *COL5*, *COL9*, key hormonal regulators of ABA and GA, and the autonomous pathway regulators (*FY*, *FCA* and *FPA*) regulate flowering behaviour. These, along with circadian clock agents (*GI* and *CCA1*) make multiple regulatory conduits that may drive continuous flowering in bamboo orchid, *A. graminifolia*. (Ahmed *et al.*, 2022). The 'pollinator tracking phenomenon' suggests that flowering patterns are determined by trends in pollinator availability (Zimmerman *et al.*, 1989).

2.1.1.5. Time of stigma receptivity

Pollinating young, fully open flowers is recommended in case of artificial pollination in orchids in general since pollen and stigma is most receptive 1– 8 days after flowers are open (Proctor, 1998; Shiau *et al.*, 2002). Young flowers (1-8 days) of *R. retusa* show maximum receptivity of stigma and pollen. Young pollinia of upto 7 days old after anthesis and stigma between 2-8 day of anthesis were in combination (like 1/1, 1/2, 1/3…. pollinia age/stigma age etc.) shows maximum receptivity and high pollination percentage. Morning time from 6 to 10 am was the best time for pollination success from the rest of day (Thakur and Dongarwar, 2019).

2.1.2. *Pollen characters*

2.1.2.1. Pollen morphology

Palynological observation of *Rhynchostylis* orchid was made by Mulgaonkar and Dabhade (2004). They reported that the species has circular pollinium. Pollen is also circular and very small in size when seen under light microscope. Detailed observation under Scanning Electron Microscope revealed the ultrastructure of pollinia and pollen. Pollinium is yellow coloured and circular long with granulate surface. Pollen is monoporate circular or slightly ovate in shape.

In most orchids, the pollen grains are either remain in tetrads or are united into larger masses. When the pollen grains are clustered into tetrads or even larger units, the grains usually have thick exine on the outer surface of the pollinium or aggregation, though there may be little or no exine developed on the surfaces between the grains within the aggregation (Dressler, 1993).

Some Epidendroideae with soft pollinia have more or less heavily sculptured pollen grains, but the more advanced members of the Epidendroideae have thick but relatively smooth exine on the outer walls of the pollinia (Dressler, 1993).

2.1.2.2. Pollen production per pollinium

Number of pollen grains to ovule ratios is several orders of magnitude lower in orchids than in plants with powdery or granular pollen. This may due to the lack of wastage during transport to the stigma (Lukasiewicz, 1999). The number of grains per pollen dispersal unit (PDU) and the number of ovules per ovary have genetic and ecological consequences: the more grains/PDU, the greater the probability that seeds in a fruit will have the same male parent; the greater the amount of pollen that shall adhere to the stigma, the greater the male competitiveness; and the greater the number of ovules per ovary, the greater the female competition, especially if only a few pollen grains glue to the stigma (Pacini and Franchi, 1999). Pollen load per pollinium varied from variety to variety within an orchid species (Nair *et al.,* 1964).

2.1.2.3. Pollen fertility percentage

The common and most reliable methods of determination of pollen viability are *in vitro* and *in vivo* germination tests. Viability is also indicated by fruit set after pollination. Both tests need not give identical results (Visser, 1955; Meeyot, 1968). Pollinium of one to four days old is more viable for effecting pollination in *R. retusa* (Nitin and Uma, 2014). Environmental conditions have influence on pollen longevity (Pacini *et al.,* 1997).

2.1.2.4. Pollen germination percentage

Pollen grains of orchids with pollinia germinate at least twenty four hours after pollination because the pollen grains per tetrad must swell and make space for the pollen tubes to grow (Pacini and Hesse, 2002).

2.1.3. *Capsule characters*

2.1.3.1. Days taken for pod setting after anthesis

Pollinia and stigma combination of less than one week old from flower opening shows maximum receptivity and high pollination percentage (Thakur and Dongarwar, 2019).

2.1.3.2. Days to mature capsule harvest

The appropriate age for the collection of pods from plant for *in vitro* embryo culture has been identified to be 180 days or six months after pollination. Beyond this period the fruit will dry and dehisce (Fosenka, 2020). Sibin and Gangaprasad (2012) reported that seeds of eight months old capsules got maximum seed germination for *R. retusa*.

2.1.3.3. Length of capsule (cm)

Length of fruit is about 3.8 cm upon reaching maturity (Fosenka, 2020). At six months capsule is 2.86 cm long (Sibin and Gangaprasad, 2012). Average pod length was maximum after 11 weeks (Fosenka, 2020).

2.1.3.4. Width of capsule (cm)

Mature pods are dark green with prominent ridges and both length and girth are about 3.8 cm. During first three months the length and the girth increase. Average pod length was maximum after 11 weeks (Fosenka, 2020). Six-month old green capsule is on an average 22.06 cm wide (Sibin and Gangaprasad, 2012).

2.1.3.5. Percentage of filled seeds

There is no effect of pollen age on percentage of non-embryonated seeds (Proctor, 1998).

2.2. MORPHOLOGICAL AND MORPHOMETRIC CHARACTERIZATION OF SEEDS

2.2.1. *Morphological characters*

2.2.1.1. Seed colour

Six months after effecting pollination the seeds within the pod resembled white powder with intermingling white hairs within the capsule (Fosenka, 2020).

According to Thomas and Michael (2007), immature seeds obtained from 2 months old pods were light green in colour and can be differentiate from mature seeds easily.
Each seed of *R. retusa* has ~~a~~ pale-yellow colour when viewed through light microscopic lens (Tongbram *et al.,* 2012).

2.2.1.2. Seed shape

Rhynchostylis has fusiform shaped seeds (Verma *et al.,* 2014). Seed shape has an evolutionary significance, with fusiform seeds found in more primitive orchids (Koene *et al.,* 2020).

2.2.1.3. Seed testa ornamentation

Testa walls of *R. retusa* is straight or sinuous (Verma *et al.,* 2014). The seed coat usually consists of uniform cells. Shape of individual testa cells in orchids is most commonly tetragonal, hexagonal or polygonal; sometimes the cell shape is irregular. Three kinds of testa cell shape are distinguished: (1) all cells are more or less iso-diametric; (2) the cells are elongate in the longitudinal axis of the seed (prosenchymatic) and rectangular; and (3) the cells are elongate but rounded at the ends. Within a single seed coat, all cells are either equal in size (regardless of their shape) or the medial cells are highly elongate in comparison to the cells at the poles. Both these patterns result from cell division in the outer integument (Barthlott *et al.,* 2014). The cell wall may have longitudinal thickenings, transverse thickenings, or netlike thickenings, or it may be covered with wax deposits in many Epidendreae (Dressler, 1993). Testa cells of 'Vanda type' orchids are always so strongly elongate that the longitudinal anticlinal walls are in contact with the surface of the seed (Tongbram *et al.,* 2012).

2.2.2. *Morphometric characters*

2.2.2.1. Seed length (*μ*m)

Seeds of orchids range from 100 µm to 6000 µm in length. Medium-size (500-900 µm) represents the average (Dressler, 1993). In *Rhynchostylis retusa,* seed length is 275 μm (Tongbram *et al.,* 2012). Mean length of the species seeds was 282.4 µm were reported by Sibin and Gangaprasad (2012).

2.2.2.2. Seed width (μm)

Rhynchostylis retusa seed width was 81.25 μm (Tongbram *et al.,* 2012). The average width of the seeds was 138.95 µm (Sibin and Gangaprasad, 2012).

2.2.2.3. Seed length/width ratio

In *R. retusa* ratio of seed length/ seed width is 5.583+/-0.377%. Seeds with length/width (L/W) ratio of <6.0 were designated as truncated and those with L/W of >6.0 as elongated (Verma *et al.,* 2014).

Elongated seeds have SL/SW ratio >5 while, truncated seeds have this ratio <5 (Barthlott *et al.,* 2014; Diantina *et al.,* 2020).

2.2.2.4. Seed volume (μm^3)

Seed volume of *R. retusa*- 479.4×10^3 μm^3 (Tongbram *et al.,* 2012). Seed volume of *R. retusa* 0.105 ± 0.020 $mm^3 \times 10^{-3}$ (Verma *et al.,* 2014).

2.2.2.5. Embryo volume (μm^3)

Embryos of *R. retusa* are large occupying majority of seed space (Bhatti *et al.,* 2016). Embryo ratio of *R. retusa* ($mm^3 \times 10^{-3}$) is 0.094+/-0.021 (Verma *et al.,* 2014). Embryo volume (*Rhynchostylis retusa)*- 447.4×10^3 μm^3 (Tongbram *et al.,* 2012). Embryo had an average length and width of 85.86 µm and 44.63 µm in *R. retusa* (Sibin and Gangaprasad, 2012).

In Japanese orchid *Liparis* (Epidendroideae), the seed volumes and air spaces were found to be significantly different between epiphytes and terrestrial species. Differences were found in embryo volumes, seed volumes, and air spaces, as well as in the length and width of the embryos. Therefore, it can be concluded that embryo size is correlated with the evolution of epiphytism and that larger embryos may be an advantage for the epiphytic life form (Tsutsumi *et al.,* 2007).

2.2.2.6. Percentage of air space between seed testa and embryo

Orchid seeds resemble balloons, they can float in air for long periods, and that floatation time bears a relationship to percentage air space (Gandawijaja and Arditti, 1983). A lower s/e volume ratio (1.182 ± 0.265) is observed in *R. retusa* which is a member of subfamily Epidendroideae, seed air space (%) is 11.122+/-8.742 (Verma *et al.,* 2014). 6.4% is the air space in *R. retusa* reported in a study based on plant material from north east India (Tongbram *et al.,* 2012).

2.2.2.7. Seed viability percentage

Lal *et al.* (2020) based on micropropagation study of *R. retusa*, made an interesting estimation that the viability of seeds was found to be 100 per cent when tested and observed under light microscope. The capsules possess 89% viable seeds (Sibin and Gangaprasad, 2012**)**. The tetrazolium seed viability test on the species revealed that 97.15±0.33 of the seeds were viable (Bhatti *et al.,* 2016).

Tetrazolium salt- based test is the most commonly used test but Evan's Blue test is better reliable for viability check in orchids (Pradhan *et al.,* 2022).

2.3. STANDARDISATION OF *IN VITRO* SEED GERMINATION

2.3.1. Stages of germination

Seed germination and subsequent development was categorized on a scale of 0–4 following Aggarwal *et al.* (2012) with slight modification. In the first stage, there is swelling of embryo but no rupture of seed coat; further swelling of embryo and rupture of seed coat is the next stage; following this there is development of polarity and formation of protocorm; the next stage is the emergence of first leaf; and lastly differentiation of first root. Seeds with enlarged embryo and ruptured seed coat were considered germinated irrespective of further changes occurring in them. Comparatively lesser seed germination was observed for fully mature seeds than immature green seeds (Tongbram *et al.,* 2012).

Aseptic culture of seeds and its development process is crudely defined as: Firstly, the seed expands with water, and the chalazal end of the embryo distends the seed coat longitudinally to form the protocorm, which then forms a protocorm with terminal or lateral meristem. The rhizoid first appears from the tip of the protocorm and then overgrows the entire

surface. Next, a cotyledon forms on one side of the apical meristem. Afterward, true leaves begin to appear, protocorm elongate into rhizomes, and eventually grow into seedlings (Xi *et al.,* 2021).

2.3.2. Growth of culture

Percentage of seed germination was highest in MS (72.60%) followed by ½MS (60.60%). Germination was marked by swelling and emergence of the embryo from the testa. Seed germination was first evident by swelling and within 7-8 weeks the undifferentiated embryos formed an irregular shaped cell mass as spherules. After 1-2 weeks, these spherules turned green and formed round structures as protocorms. Protocorms became visible after 11 weeks of culture initiation and showed at the vegetative apex. This was followed by the development of 2-3 leaf primordia when MS basal medium or half strength of MS medium or B5 media were used. Out of four media MS showed most effective on the development of leaf primordia than others. Protocorms cultured in PM medium did not develop beyond the vegetative apex stage, while half-strength of MS and B5 supported only poor growth of protocorms in comparison to MS medium. Protocorm like bodies development initiated after 30 days of culture. The maximum number of PLBs (16.00± 0.41) per protocorm was observed after 60 days of culture in MS medium that contained BAP (1.0 mgL^{-1}) and NAA (1.0 mgL^{-1}), followed by BAP (0.5 mgL^{-1}) and NAA (0.5 mgL^{-1}). Almost all the PLBs were converted into plantlets in the following 20-30 days, giving rise to multiple numbers of shoots on the same media (Bhattacharjee and Islam, 2015).

BAP at lower concentrations was found to be beneficial for multiplication of PLBs; however, a decrease in BAP concentration in the medium resulted in higher conversion of PLBs into plantlets (Kumar *et al.,* 2002).

2.3.3. Callus formation and protocorm multiplication and secondary protocorm formation

To assess the effectiveness of auxin and cytokinin for protocorm multiplication, 60 days old primary protocorms were sub-cultured on fresh MS medium supplemented with different concentrations of BAP, Kinetin, NAA, Picloram, IBA and IAA alone and in combinations. The primary protocorms responded significantly higher in combination with 1.0 mgL^{-1} BAP and 1.0 mgL^{-1} NAA. This resulted in the generation of secondary protocorms (PLBs) instead of shoot formation directly (Bhattacharjee and Islam, 2015).

Leaf and root explants of *R. retusa* induced callus in NAA and BAP containing medium while root explants form callus in medium with NAA and BAP. Calli are fragile and white in colour in the initial stage of induction. Later, after 2-3 subcultures, calli turn creamish and PLBs are induced from the superficial portion of the cream-coloured calli (Sunitibala Devi, and Neelashree, 2018).

In the medium containing BA, IAA and banana puree, callus grew profoundly after transfer to appropriate media for 20 days, and embryogenic calli were noticed. The protocorm developed into buds and calli further proliferated after 30 days. With the occurrence of protocorm, cluster buds were generated on each callus block and obvious rhizoids were seen on the surface of callus after 50 days. Then, lots of cluster buds produced from the protocorm increased, callus and protocorm proliferated continuously after 70 days. After 90 days, with the callus proliferation, the produced cluster buds from the protocorm grew rapidly. In the process of callus proliferation and bud induction, callus of *R. retusa* had a strong embryogenesis and a lot of protocorms occurred on each callus, accompanied by the production of rhizoids. The protocorm first inducted buds and then rooted to grow into seedlings. Meanwhile, callus also continuously proliferated, produced more protocorms and appeared similar to the phenomenon of adventitious cluster buds. When the material was transferred into the 1/2 MS medium with 100 g L^{-1} banana puree, 1.0 mgL^{-1} NAA, 2.0 mgL^{-1} 6-BA, and 1.0 g L^{-1} activated charcoal around 20 days, the callus at the base began to proliferate, the roots of the buds grew rapidly and the leaves began to extend. After that, the leaves of the buds were further extended, and the callus at the base was also significantly increased about 40 days. After 50 days, the buds had 4–6 new leaves, and callus at the base turned green due to the large number of protocorms proliferated. Then a lot of protocorms occurred on callus after 70 days. After 90 days small plants grew from the young buds and more young buds came from bottom again (Xi *et al.,* 2021).

Materials and Methods

3. MATERIALS AND METHODS

The present investigation was carried out at College of Agriculture, Vellayani and at Saraswathy Thangavelu Extension Centre of JNTBGRI, Puthenthope, Thiruvananthapuram during 2021-2022.

Rhynchostylis retusa floral characters were observed and its capsules were collected for seed characterization and *in vitro* seed germination study from the *g*ermplasm repository maintained at Saraswathy Thangavelu Extension Centre of JNTBGRI, Puthenthope, Thiruvananthapuram.

3.1. EXPERIMENT I- EVALUATION OF FLORAL CHARACTERS

3.1.1. *Floral characters (qualitative)*

3.1.1.1. Flower colour

Flower colour was visually observed and compared with the report in the reference book entitled “Introduction to Orchids” (Abraham and Vatsala,1981) and described in subjective terms.

3.1.1.2. Nature of flowering – free or seasonal

Emergence of inflorescence and its blooming period during a year was observed and recorded.

3.1.1.3. Season of flowering

Season of inflorescence emergence and blooming was observed and recorded.

3.1.1.4. Time of anthesis

Time of opening of the flowers in the inflorescence was noted.

3.1.1.5. Time of stigma receptivity

Total number of days of the stigma receptivity for effective pollination in a flower was taken.

3.1.2. *Pollen characters*

3.1.2.1. Pollen morphology

Pollinia were collected from freshly opened flowers and crushed and pollen grains were stained in acetocarmine which was mounted on a slide and observed under microscope for shape, ornamentation and nature of pollen grains and these were recorded.

3.1.2.2. Pollen production per pollinium

The pollen production per pollinium was estimated using haemocytometer. Pollinium was collected in an Eppendorf tube and crushed well. The prepared pollen suspension was gently filled into the two chambers underneath the coverslip of the haemocytometer and the pollen grains were observed under microscope and counted in four sets of sixteen corner squares and centre square of 25 sub squares calculated the average number of pollen grains present in pollinium.

3.1.2.3. Pollen fertility percentage

Pollen fertility was estimated using acetocarmine staining technique. Pollinia were crushed with needle and pollen grains liberated were transferred into acetocarmine stain for one minute. These were mounted on a slide and the pollen grains were observed under the microscope. Stained pollen grains were counted as viable and unstained ones as non-viable. The viable pollen grains were counted from five different microscopic fields at random and mean was expressed in percentage.

Pollen fertility percentage = (Number of stained pollen grains / Total number of pollen grains) ×100

3.1.2.4. Pollen germination percentage

Pollinia was crushed in 10 per cent sucrose solution and kept it for one day. After that slide was prepared and observed under the microscope. Pollen germination and pollen tube formation is noted.

Pollen germination percentage= (Number of germinated pollen / Total number of pollen) × 100

3.1.3. *Capsule characters*

3.1.3.1. Days taken for pod setting after anthesis

Number of days taken from the day of pollination to greening of ovary and swelling of column or gynostegium (mainly ovary) was recorded.

3.1.3.2. Days to mature capsule harvest

Number of days from pollination to harvesting of green undehisced mature capsule was recorded.

3.1.3.3. Length of capsule (cm)

Distance from the base of pedicel to the base of distal end of the capsule from ten plants was measured. Observation taken at longest axis with a ruler and mean was calculated.

3.1.3.4. Width of capsule (cm)

Diameter of capsule was measured at the broadest axis. Measurements were taken from ten plants using a ruler and mean was worked out.

3.1.3.5. Percentage of filled seeds

Matured capsules were taken and seeds were scooped out. It was dipped in water and five samples were taken and it was mounted on a slide. From each sample five microscopic fields were observed and counted total number of seeds and number of seeds with well filled embryo inside seed coat and estimated percentage of filled seeds.

$$\text{Percentage of filled seeds } = \frac{Total\ number\ of\ filled\ seeds}{Total\ number\ of\ seeds} \times 100$$

Statistical analysis was done using the software application GRAPES of KAU.

3.2. EXPERIMENT II- MORPHOLOGICAL AND MORPHOMETRIC CHARACTERIZATION OF SEEDS

3.2.1. *Morphological characters*

3.2.1.1. Seed colour

The colour of seed mass inside the capsule was observed. After opening the capsule it was visually assessed and compared based on the general orchid seed colour proposed by Barthlott *et al.* (2014). The colour of seed and embryo was also observed under compound light microscope.

3.2.1.2. Seed shape

Seed shape was visually assessed under a light compound microscope and recorded. Then it was compared based on the general orchid seed shape proposed by Barthlott *et al.* (2014).

3.2.1.3. Seed testa ornamentation

Seed testa was visually assessed under a light compound microscope and recorded.

3.2.2. *Morphometric characters*

3.2.2.1. Seed length (μm)

Seed length was measured under light microscope and using Leica LAZ 500 microscope software from five plants and mean was calculated. Length was measured at the longest axis of the seed.

3.2.2.2. Seed width (μm)

Seed width was measured under light microscope using Leica LAZ 500 microscope software from five plants and mean was calculated. Width was measured at the widest axis of the seed.

3.2.2.3. Seed length/width ratio

From the estimated seed length and seed width measurements, the ratio was calculated.

3.2.2.4. Seed volume (μm^3)

The approximate seed, embryo and air-space volume were calculated following Prasongsom *et al*. (2017) as below:

Based on seed length and width measurements, seed volume was calculated for fusiform seeds as below:

$SV= 2[(SL/2)(SW/2)^2(1.047)]$ where, SV is seed volume, SL is seed length, SW is seed width.

3.2.2.5. Embryo volume (μm^3)

Based on seed length and width measurements seed volume was calculated for prolate spheroid embryos as:

$EV= 4.19\ (EL/2)\ (EW/2)^2$ where EV is embryo volume, EL is embryo length, EW is embryo width.

3.2.2.6. Percentage of air space between seed testa and embryo

From seed volume and embryo volume percentage of air space is calculated as:

$$\text{Percentage of air space} = \frac{SV-EV}{SV} \times 100$$

where, SV is seed volume, EV is embryo volume.

3.2.2.7. Seed viability percentage

Method for testing viability of seed was done as per reported by Pradhan *et al.* (2022).

3.2.2.7.1. Using Tetrazolium salt

Seeds were extracted from mature capsules and were dipped in 1 per cent tetrazolium solution for 24 h. Seeds were mounted on slides and observed through microscope. Count was taken for total number of seeds, total number of seeds stained and total number of seeds unstained present in each microscopic field and it average was worked out. From this estimated seed viability percentage by the following formula.

Seed viability (%) = Total number of stained seeds (embryo) / Total number of seeds x 100

3.2.2.7.2. Using Evan's Blue stain

The seeds were soaked for 16 h in distilled water, following which the water was discarded and the seeds were suspended for one hour in 1% (w/v) Evan's blue solution. The syringe was shaken frequently to facilitate proper contact between the seeds and the Evan's blue solution. Finally, seeds were rinsed thoroughly several times with distilled water to remove excess stain and the seeds were placed on a microscopic glass slide, and viewed under the compound light microscope. The seeds with unstained embryos were considered as viable and those with embryos stained blue as non-viable.

Seed viability (%) = Total number of unstained seeds (embryo)/ Total number of seeds x 100

$$\text{Seed viability (\%)} = \frac{Total\ number\ of\ unstained\ seeds\ (embryo)}{Total\ number\ of\ seeds} \times 100$$

Hand pollination procedure

Selected freshly opened flower and the pollinium from the flower was removed and collected on a clean paper from the tip of anther cap and placed in the flower's own stigma with the help of a needle. Different ages of flowers were selfed to observe maximum receptivity at different interval of time from 6 am to 6 pm. Artificially pollinated flowers were monitored

for flower senescence, capsule development, and capsule dehiscence. Capsules were harvested at different maturity for asymbiotic seed germination.

3.3. EXPERIMENT III- PREPARATION OF *IN VITRO* SEED GERMINATION MEDIA

The immature capsule of *R. retusa* used as source of seed for seed (embryo) culture and these were collected from JNTBGRI, Puthenthope.

In the present investigation four different media both solid and liquid were examined for *in vitro* seed germination. Full-strength Murashige and Skoog (MS) media (Murashige and Skoog, 1962), half-strength Murashige and Skoog (1/2 MS) media, Knudson C media (Knudson, 1946) and Woody Plant Media or WPM (Lloyd and McCown, 1980)

The pH of the medium was adjusted to 5.7 with either 1.0 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl) before being solidified with 8 gL^{-1} agar in case of solid media. Thereafter, approximately 10/50 ml of the medium was dispensed into culture tubes/bottles and sterilized using pressure cooker.

Sterile glasswares, chemicals, additives, plant growth regulators, a precision weighing balance, precision pH meter, distilled water, spatula, tissue paper, microwave oven were the materials and equipments required for media preparation. Inoculation is carried out in Laminar Air Flow Chamber.

3.4. EXPERIMENT IV- STANDARDISATION OF *IN VITRO* SEED GERMINATION

The prepared media was transferred to sterile glasswares in an immaculate environment and autoclaved before taking to the Laminar Air Flow Cabinet. pH of the medium was adjusted to 5.8 before autoclaving. The mature capsules before dehiscence were collected in paper bags. The capsules were first washed in running tap water followed by cleaning in solution of liquid detergent for 3-5 minutes. The seeds were surface disinfected with 0.1 per cent mercuric chloride for 3 minutes with occasional agitation, and rinsed with distilled water three to four times followed by dipped in 70% ethanol for 10-30 sec and flaming under spirit lamp with the help of a sterilized spatula and burned off alcohol. Flaming was repeated two to three times. This was to burn off any bacteria or fungi on its surface. The capsules were kept in a sterile petri plate near to the spirit lamp. The sterilized capsules were cut longitudinally with a sterile

surgical blade in the laminar air flow cabinet. Feathery seeds were collected with the help of a sterilized spatula and small mass of the aggregated seeds were inoculated on different culture media for *in vitro* asymbiotic seed germination. Observations were made by using five replicates for each medium.

Cultures were maintained at 25 ± 5°C. Germination percentage was calculated on the basis of seed germinated out of total seeds inoculated (Approximately). Growth characteristics of the plantlets in every media were observed to check that which medium is the best for germination and plantlets development. The data of different stages of germination were recorded on a particular week like week 6 and week 14 etc.

The following observations were recorded in frequent intervals.

3.4.1. Number of days taken for seed with chlorophyll embryo in different media after inoculation

Number of days taken for greening from the day of seed inoculation was observed and average was worked out in days.

3.4.2. Number of days taken for protocorm development

Number of days taken for protocorm development from the day of seed inoculation was observed and average was worked out in days.

3.4.3. Number of days taken for first leaf

Number of days taken for forming first leaf from the day of seed inoculation was observed and average was worked out in days.

3.4.4. Number of days taken for root initiation

Number of days taken for root initiation from the day of seed inoculation was observed and average was worked out in days.

3.4.5. Number of seeds with chlorophyll embryo

Number of seeds with chlorophyll embryo per culture bottle was approximately counted and average was worked out.

3.4.6. Number of protocorm with shoot apex

Number of protocorm with shoot apex per culture bottle was counted and average was worked out.

3.4.7. Number of protocorm with 1-2 leaves

Number of protocorm with 1-2 leaves was counted and average was worked out.

3.4.8. Number of protocorm with two or more leaves and root (plantlet)

Number of protocorm with leaves and roots was counted and average was worked out. Statistical analysis was done using GRAPES 1.0.0 (General R based Analysis Platform Empowered by Statistics) software developed by Kerala Agricultural University.

Results

4. RESULTS

4.1. EVALUATION OF FLORAL CHARACTERS

4.1.1. *Floral characters (qualitative)*

Rhynchostylis retusa inflorescence is pendant, racemose. The anthesis is not in strict acropetal or basipetal succession.

4.1.1.1. Flower colour

Flowers were white in colour with purple blush or purple spots on dorsal and lateral sepals and lateral petals. Labellum was deep purple colour with white colour at tip on the frontside and purple color with central median band of white colour on the backside. Labellum was spurred towards the base; spur was white in colour (Plate 1).

4.1.1.2. Nature of flowering – free or seasonal

R. retusa was noted as a seasonal and annual flowering orchid.

4.1.1.3. Season of flowering

In the present investigation, the species came to bloom during June to August when the southwest showers were plentiful.

4.1.1.4. Time of anthesis

Flower opening was between 5 pm and 8 am, i.e., flower opens at dark hours of the day, during early morning or late evening.

4.1.1.5. Time of stigma receptivity

Stigma remains receptive upto five days after anthesis. It was found to be highly receptive during morning hours when the pollinia sticks easily on the stigmatic surface. After 10.30 to 11.00 am during daytime receptivity declined considerably and gradually drying up of stigmatic surface occurred after five days.

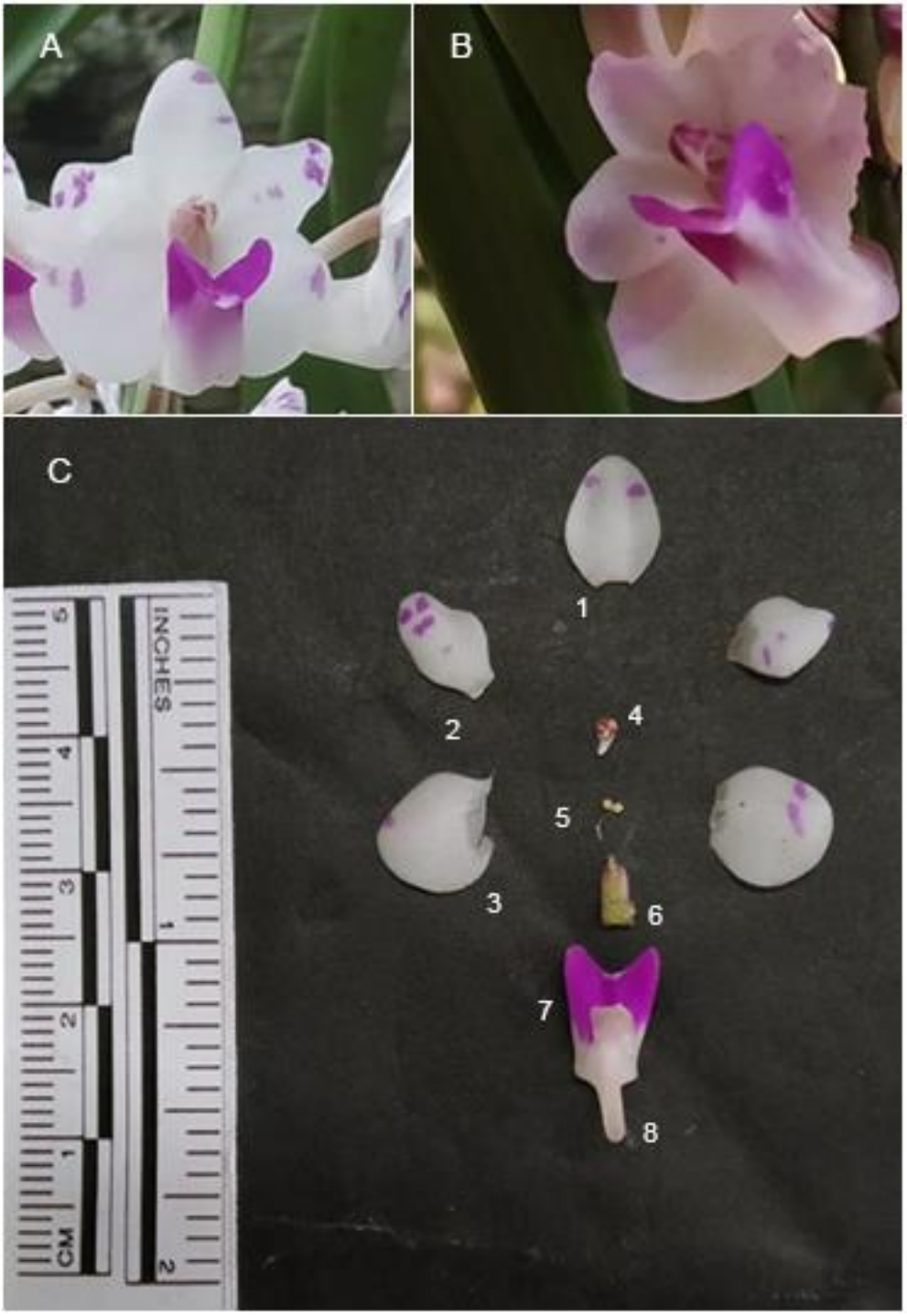


Plate 1: Flower morphology (A) Flower with purple spots; (B) Flower with purple blush; (C) Dissection of a flower, 1. Dorsal sepal, 2. Laterala petal, 3. Lateral sepal, 4. Anther cap, 5. pollinia, 6. Stigma, 7. Labellum, 8. spur.

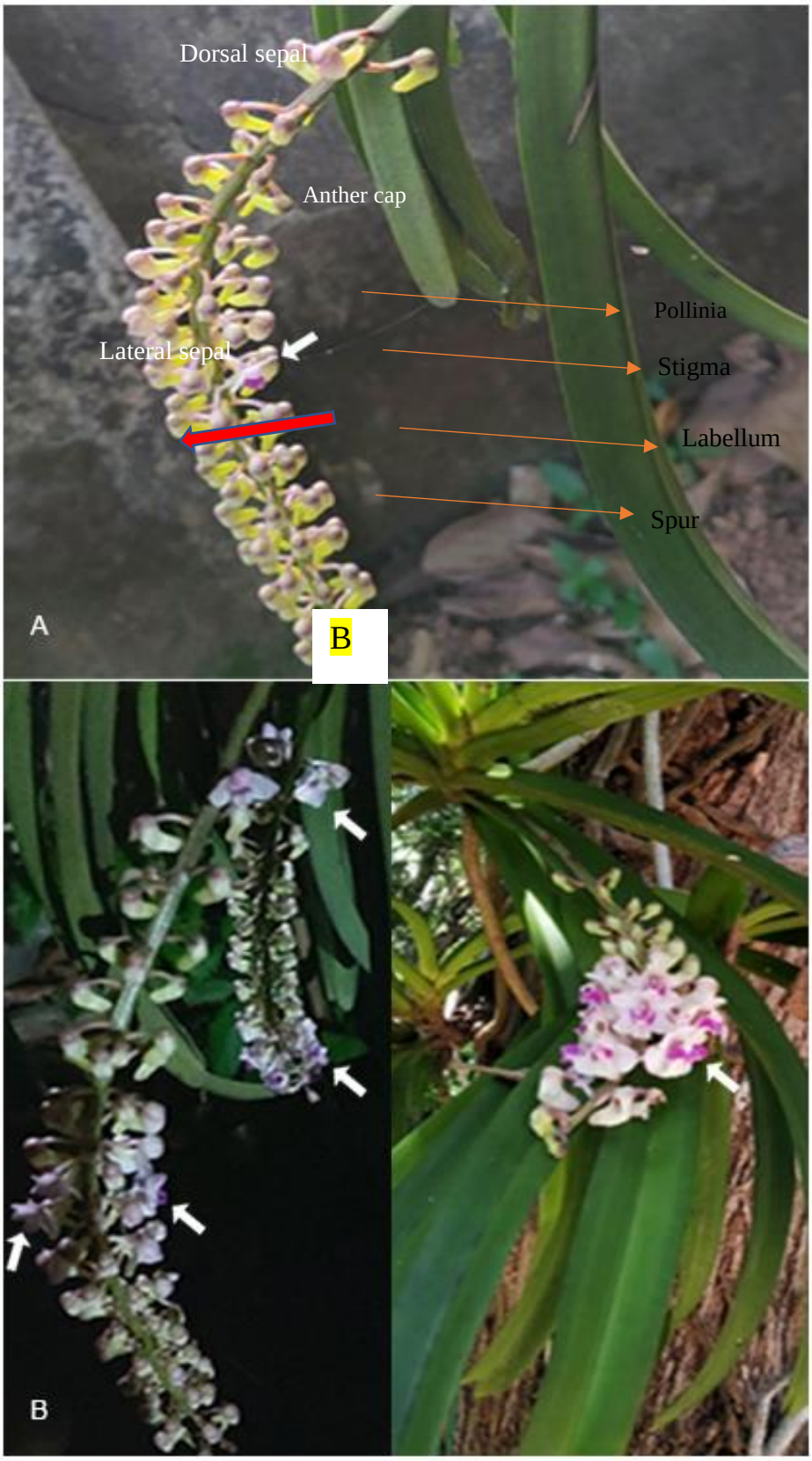


Plate 2: Pattern of anthesis (A) First flower opening (B) Variable pattern of blooming in an inflorescence

Plate 3: Post pollination changes in flower (A) after one day, (B) after one week

Post pollination changes in a flower:

Pollination if effected can be noticed within a day. Visible signs observed were stigmatic closure, flower begins to fade and wilt, sepals and petals wither and dry, stigmatic end of column bulges, labellum retracts closer to column, proximal end of pedicel *i.e.*, ovary turns light greenish colour (Plate 3).

4.1.2. *Pollen characters*

4.1.2.1. Pollen morphology

Pollen grains were obovoid to triangular in shape. Exine or outer layer was smooth and thin and intine was thickened. Mostly pollen grain was present as tetrad. Triad and dyad stage were also present (Plate 4).

4.1.2.2. Pollen production per pollinium

The pollen load was estimated to be 2,06,000 pollen per pollinia (Plate 4).

4.1.2.3. Pollen fertility percentage

Most of the pollen (99.6%) was found to be fertile (Plate 4).

4.1.2.4. Pollen germination percentage

73.64 percent of pollen in a pollinia germinated under suitable conditions (Plate 4).

4.1.3. *Capsule characters*

4.1.3.1. Days taken for pod setting after anthesis

Under natural conditions pod setting was observed within seven days of anthesis.

4.1.3.2. Days taken to mature capsule harvest

The days to harvest of yellowish green mature pods varied from 135 to 180 days i.e., between four and half to six months duration.

4.1.3.3. Length of capsule (cm)

Length of the capsule measured was 2.18 cm on an average (Plate 5).

4.1.3.4. Width of capsule (cm)

Average capsule width was measured to be 0.86 cm (Plate 5).

4.1.3.5. Percentage of filled seeds

The percentage of embryonated seeds was 41.85. Rest of the seeds were without embryo and or non-viable one (Plate 7).

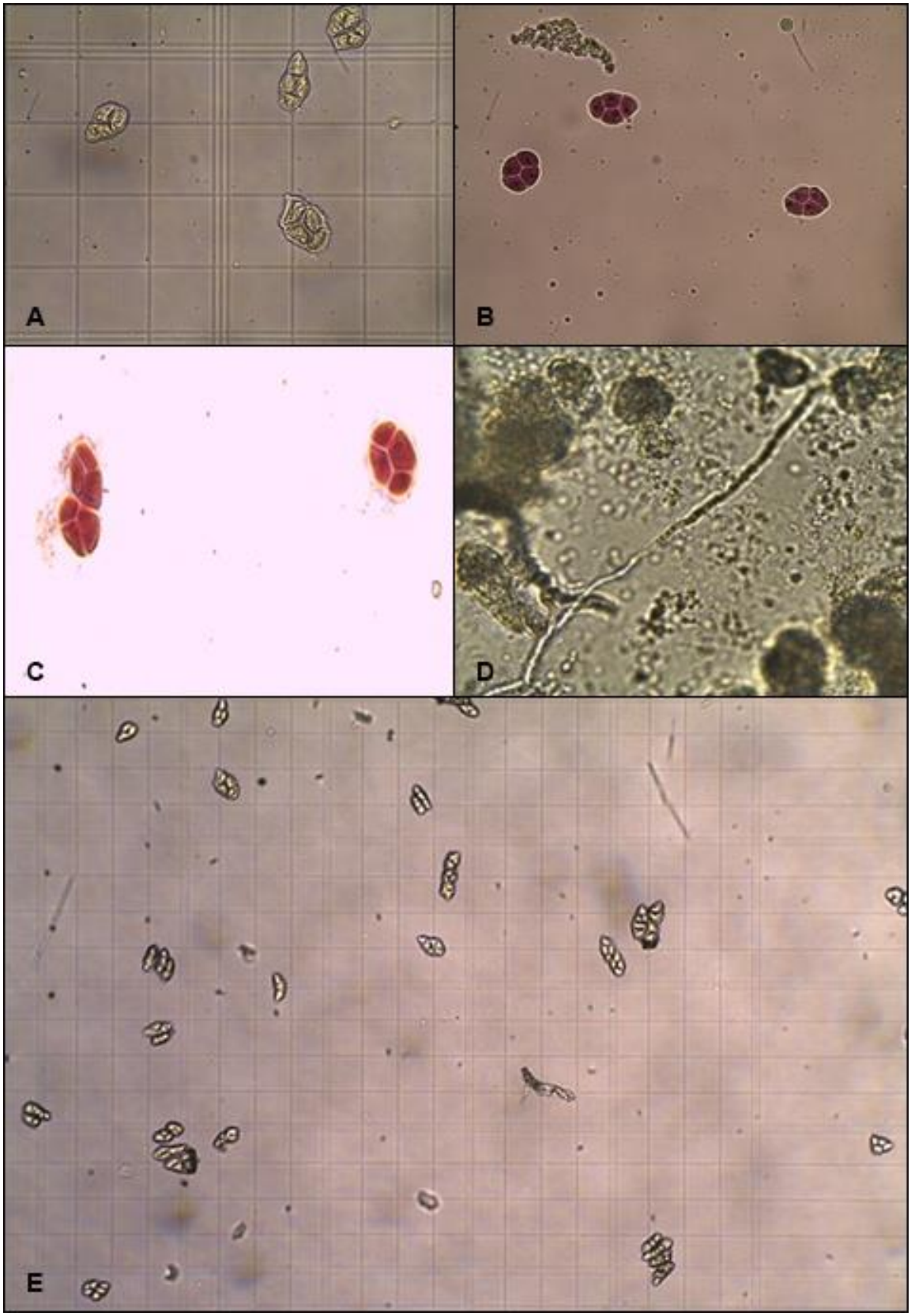


Plate 4: Pollen characters (A, B) Pollen morphology (C) Viable pollen (D) Growing pollen tube (E) Microscopic field view of 25 central squares in haemocytometer

Table 1. Correlation analysis of capsule and pollen characters

	Capsule length (cm)	Capsule width (cm)	Percentage of filled seeds	Pollen fertility percentage	Pollen germination percentage
Capsule length (cm)	1				
Capsule width (cm)	0.177	1			
Percentage of filled seeds	-0.61	-0.134	1		
Pollen fertility percentage	0.452	-0.077	0.129	1	
Pollen germination percentage	0.541	0.076	-0.268	0.888*	1

* Correlation is significant at 0.05 level (two tailed)- significant

Correlation analysis has shown that pollen germination is significantly positive correlated with pollen fertility (Table 1).

4.2. MORPHOLOGICAL AND MORPHOMETRIC CHARACTERIZATION OF SEEDS

4.2.1. *Morphological characters*

4.2.1.1. Seed colour

When the capsules were cut open the mass of millions of seeds present inside appeared as white to visible eye. A seed when observed under microscope had yellowish green colour which was the seed coat colour. The embryo inside had deep purple to violet colour. Embryo of seeds of immature capsules had yellowish colour when viewed through microscopic lens (Plate 6).

4.2.1.2. Seed shape

R. retusa was observed to have fusiform shaped seeds tapered at one end and blunt at the other end. The embryo is spheroid shaped i.e., prolate spheroid (Plate 6).

Plate 5: Capsule formation and its characteristics (A, B) Size of mature capsule (C) Capsules formed after selfing (D, E, F) Stages of growth of inflorescence: (D)elongation of axis, (E) flower bud differentiation, (F) after bloom.

4.2.1.3. Seed testa ornamentation

Testa or seed coat appeared reticulated or net like, testa cells longitudinally oriented with transverse testa cells connecting them at short distance looking like irregular rectangles (Plate 6).

4.2.2. *Morphometric characters*

4.2.2.1. Seed length (μm)

Seeds were 242.42 μm long.

4.2.2.2. Seed width (μm)

Width of seed was 46.88 μm.

4.2.2.3. Seed length/width ratio

From the dimensions of seed, its length and width ratio were worked out to be 5.29, i.e., *R. retusa* has truncated to slightly elongated seed shape.

4.2.2.4. Seed volume (μm^3)

The amount of room inside a seed was 202600.65 μm^3.

4.2.2.5. Embryo volume (μm^3)

39963.94 μm^3 was the estimated embryo volume in seed of the species.

4.2.2.6. Percentage of air space between seed testa and embryo

Air space inside a seed was averaged to 78.74 per cent. Almost three fourth of the seed volume was space of air and embryo occupied only a small space in most of the seeds.

4.2.2.7. Seed viability percentage

78.87 per cent of seeds in a capsule were viable (Plate 7).

Plate 6: Seed morphology (A) Opened capsule (B) Mature seed (C) Immature seed (D) Seed testa ornamentation

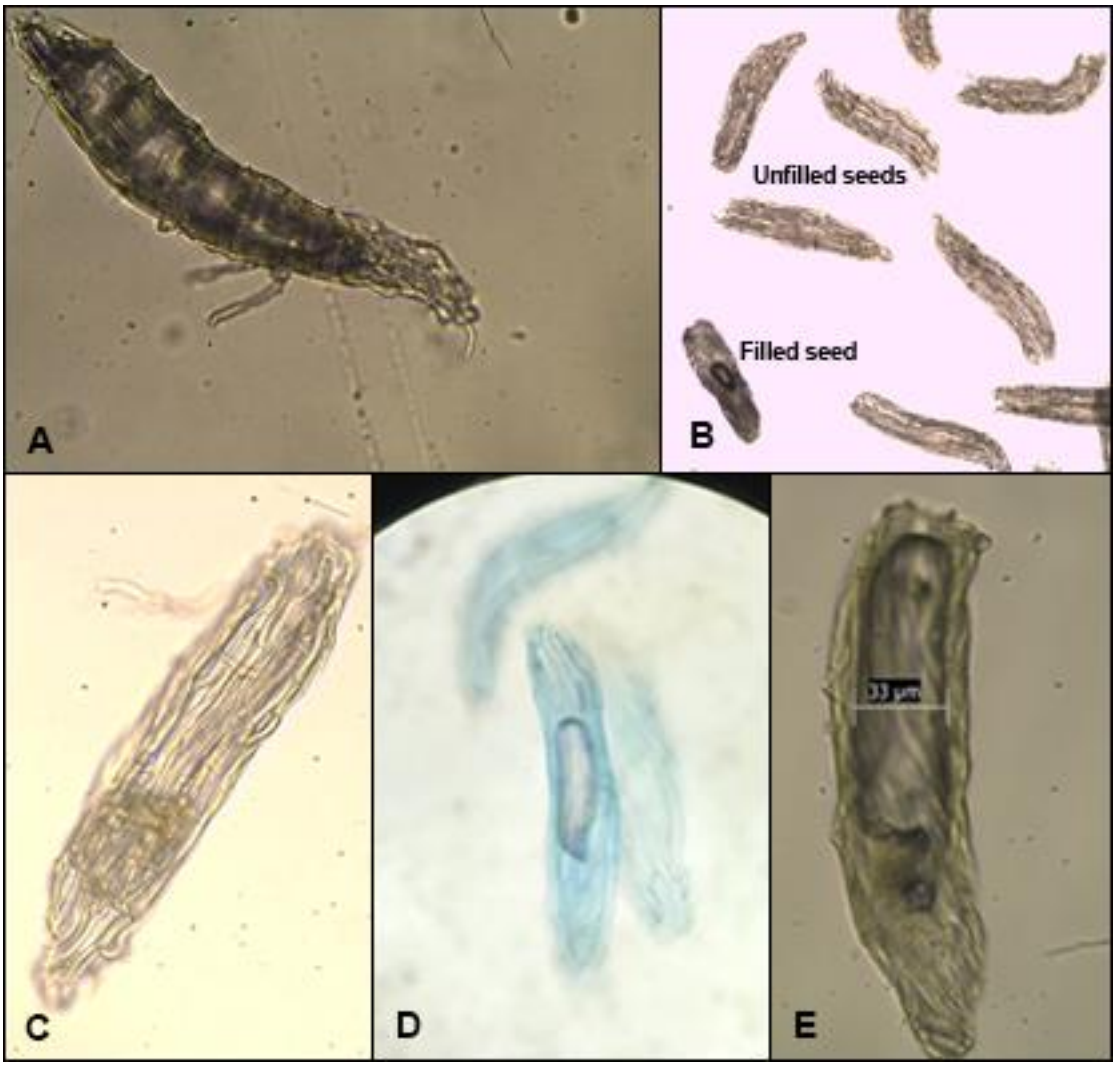


Plate 7: Seed morphometry (A) A well embryonated seed (B) Filled and unfilled seeds (C) Viable seed of tetrazolium test (D) Viable seed of Evans blue test (E) Size of seed

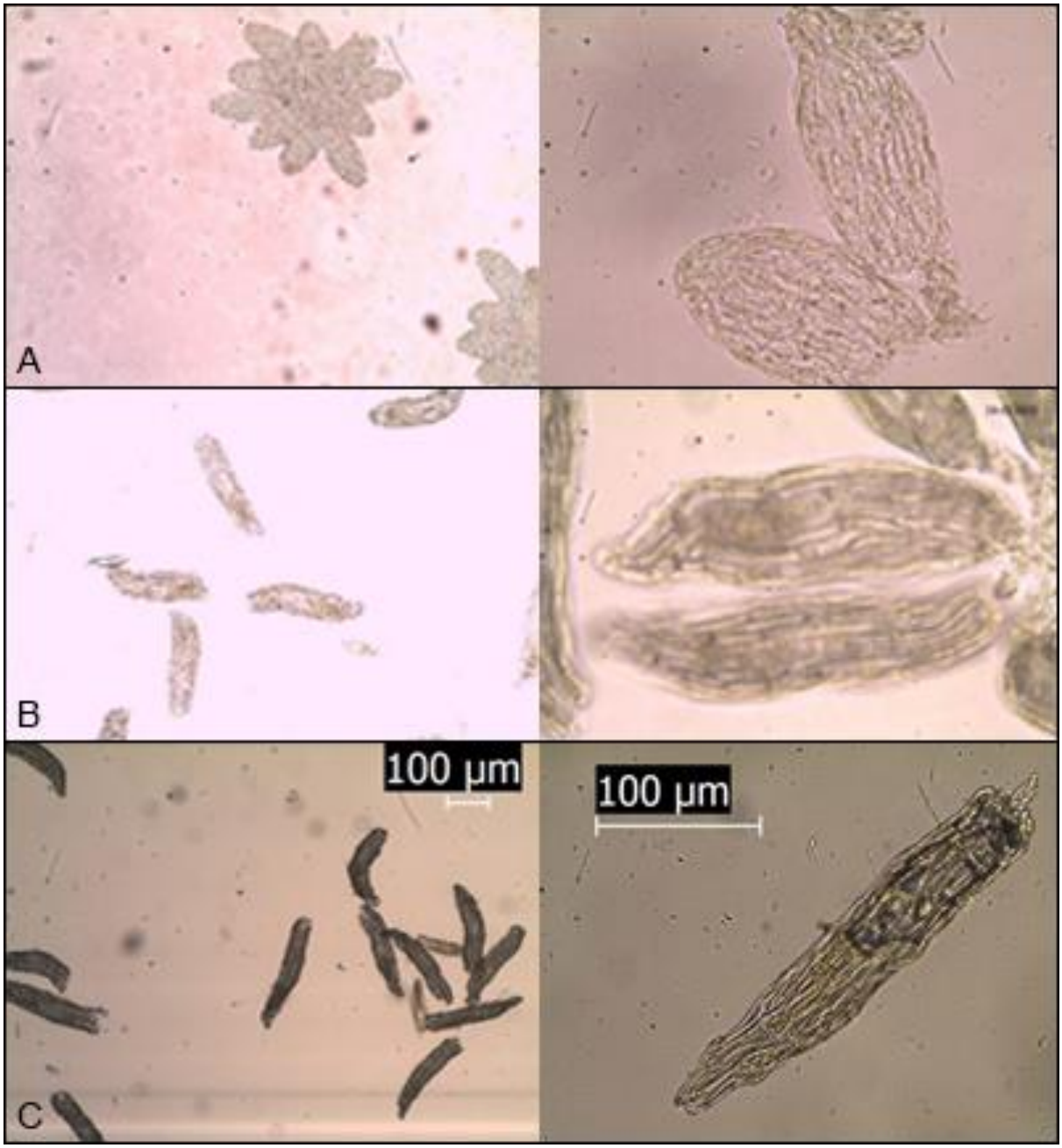


Plate 8: Seed differentiation in capsules at different ages (A) Immature seeds cluster from 3.5 months old capsule, (B) Seeds from 4.5 months old capsule with undifferentiated embryonic region, (C) Mature seed with developed embryo and maximum air space.

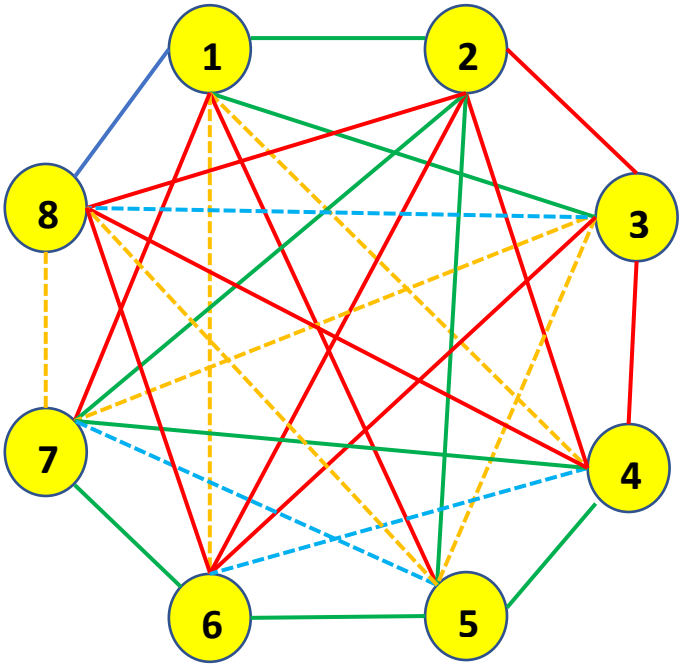
Table 2. Correlation analysis of seed morphometric characters

	Seed length (μm)	Seed width (μm)	SL/SW	Embryo length (μm)	Embryo width (μm)	Seed volume (μm^3)	Embryo volume (μm^3)	Air space
Seed length (μm)	1							
Seed width (μm)	0.521	1						
SL/SW	0.642	-0.321	1					
Embryo length (μm)	-0.982 **	-0.351	-0.774	1				
Embryo width (μm)	-0.445	0.533	-0.972 **	0.605	1			
Seed volume (μm^3)	-0.994 ***	-0.428	-0.719	0.997 ***	0.536	1		
Embryo volume (μm^3)	-0.682	0.269	-0.999 ***	0.807	0.958*	0.755	1	
Air space	0.61	-0.359	0.999 ***	-0.748	-0.981 **	-0.69	-0.996 ***	1

*** Correlation is significant at 0.001 level (two tailed)- very highly significant
** Correlation is significant at 0.01 level (two tailed)- highly significant
* Correlation is significant at 0.05 level (two tailed)- significant

Correlation among seed morphometric characters is depicted in Figure 1. Length of the embryo was negative and highly significant and seed volume was negative and very highly significantly correlated with the length of the seed. Embryo width was negative and highly significantly

correlated with the seed length/ width ratio. Embryo volume was negative and highly significant and air space was positive and very highly significantly correlated with the ratio of seed length and width. Seed volume was positive and very highly correlated with embryo length. Embryo volume was positively significant to embryo width and air space was negatively and highly significant to embryo width. Embryo volume and air space was very highly and negatively significantly correlated (Table 2).



1. Seed length
2. Seed width
3. SL/SW
4. Embryo length
5. Embryo width
6. Seed volume
7. Embryo volume
8. Air space

Negative

Negative Significant

Positive

Positive significant

Figure 1: Diagrammatic representation showing correlation of seed characters

4.3 STANDARDISATION OF *IN VITRO* SEED GERMINATION

4.3.1. Number of days taken for seed with chlorophyll embryo in different media after inoculation

Seeds were scooped out from capsule and inoculated to liquid media. In MS and half MS media seed greening was observed after 60 days. It took about 75 days in Knudson C medium for the same change.

4.3.2. Number of days taken for protocorm development

Protocorm like bodies developed in 60 days for MS and half MS media. In Knudson C medium it took 90 days for formation of protocorm.

4.3.3. Number of days taken for first leaf

Root and leaf primordia emerged from protocorm and leaf was developed in 160 days for MS medium. While it took only 120 days for culture in half MS medium to show the same growth.

4.3.4. Number of days taken for root initiation

Root development was noted in 240 days and 210 days for half MS and MS media respectively.

4.3.5. Number of seeds with chlorophyll embryo

In half MS media liquid- 91, Knudson C liquid- 20.5, MS media liquid -55.67 were the number of green embryos.

Analysis of variance revealed treatment means were significantly different. Half MS (liquid) was the best media for formation of seeds with chlorophyll embryo. Next best result was for MS media followed by Knudson C media for chlorophyllous embryo formation (Plate 9).

Table 3. Analysis of variance for number of seeds with chlorophyll embryo

Media	Treatment means
HMS (l)	91.000[a]
KC (l)	20.500[b]

MS (l)	55.667[c]

Table 4. LSD test Matrix of CD values for number of seeds with chlorophyll embryo

	HMS	KC	MS
HMS	0	24.993	22.354
KC	0	0.000	24.993
MS	0	0.000	0.000

4.3.6. Number of protocorm with shoot apex

Number of protocorm with shoot apex in different media was observed as follows: MS medium liquid with 0.5 mgL^{-1} BAP- 54.33, half MS liquid with 0.5 BAP- 88 and Knudson C liquid media- 3.5.

Analysis of variance revealed that the best media for protocorm formation and development were half strength MS media with 0.5 mgL^{-1} BAP (liquid) followed by liquid MS media with 0.5 mgL^{-1} BAP (Plate 9).

Table 5. Analysis of variance for number of protocorm with shoot apex

Media	Treatment means
HMS + 0.5 BAP (l)	88.000[a]
KC (l)	3.500[b]
MS + 0.5 BAP (l)	54.333[c]

Table 6. LSD test Matrix of CD values for number of protocorm with shoot apex

	HMS+0.5BAP	KC	MS+0.5 BAP
HMS + 0.5BAP	0	27.51	24.605
KC	0	0.00	27.510
MS + 0.5 BAP	0	0.00	0.000

4.3.7. Number of protocorm with 1-2 leaves

Number of protocorms in half MS media solid media with 0.5 mgL^{-1} BAP and 1 mgL^{-1} NAA was 84.333, while in MS solid medium with 0.5 mgL^{-1} BAP and 1 mgL^{-1} NAA – 53.

Analysis of variance revealed that the treatment means of culture media for protocorm formation with 1-2 leaves was significantly different and half MS media supplemented with 0.5 mgL^{-1} BAP and 1 mgL^{-1} NAA was found to be the best (Plate 10).

Table 7. Analysis of variance for number of protocorm with 1-2 leaves

Media	Treatment means
HMS + 0.5 BAP+1NAA (s)	84.333[a]
MS+0.5 BAP+1 NAA (s)	53.000[b]

Table 8. LSD test Matrix of CD values for number of protocorm with 1-2 leaves

	HMS+0.5 BAP+1NAA	MS+0.5 BAP+1 NAA
HMS + 0.5 BAP + 1NAA	0	26.209
MS + 0.5 BAP + 1 NAA	0	0.000

Seeds from three months maturity to dehisced capsules of present and previous years pods were used for inoculating culture medium.

Within a few weeks (2-3 weeks) of inoculation for *in vitro* culture of seeds in yellowish green capsules, the embryo inside the seed began to swell and as size increased seed coat ruptured and released the embryo. There was chlorophyll synthesis and seeds began to be visible as very minute green spherical structures (spherules) to the visible eye. This stage was seed greening which was observed in between 1-2 months. Protocorm like bodies (PLBs) were formed green in colour after two months. Greening and protocorm development coincided for some culture bottles. As they developed and became grain like protocorm colour was cream to greenish yellow. If the protocorm like bodies were not subcultured at proper time or if they were left for more than forty to sixty days without subculture progressive degeneration of these structures were the result as depicted in Plate 9.

Protocorms when subcultured to solid media grew and root and shoot primordia was developed. Shooting occurred from calloid like structure also after four months of subculturing. Multiple shoots arose from each of these. Each shoot was transferred to new medium to develop to individual plantlets after rooting after 7-8 months from inoculation. Flow chart showing different stages of *in vitro* seed germination and plantlet development is depicted in Figure 2.

Basal liquid media without additives was effective for seed germination upto protocorm stage. For further development and support solid (agar- 8 g L^{-1}) or semisolid (agar- 5 g L^{-1}) media with hormones such are BAP and NAA gave good results.

In half MS media fast and better results were obtained for asymbiotic embryo culture. In Knudson C media though germination occurred, developing to plantlets were noted in MS and half MS media only. WPM media did not give satisfactory results in *in vitro* culture.

Best media for seed greening and protocorm formation was found to be liquid half strength MS media. Best media for callusing and shoot proliferation was found as solid half MS media with 0.5 mgL^{-1} BAP. Best media for multiple shoot and root formation was found as half MS media with 0.5 mgL^{-1} BAP and 1 mgL^{-1} NAA.

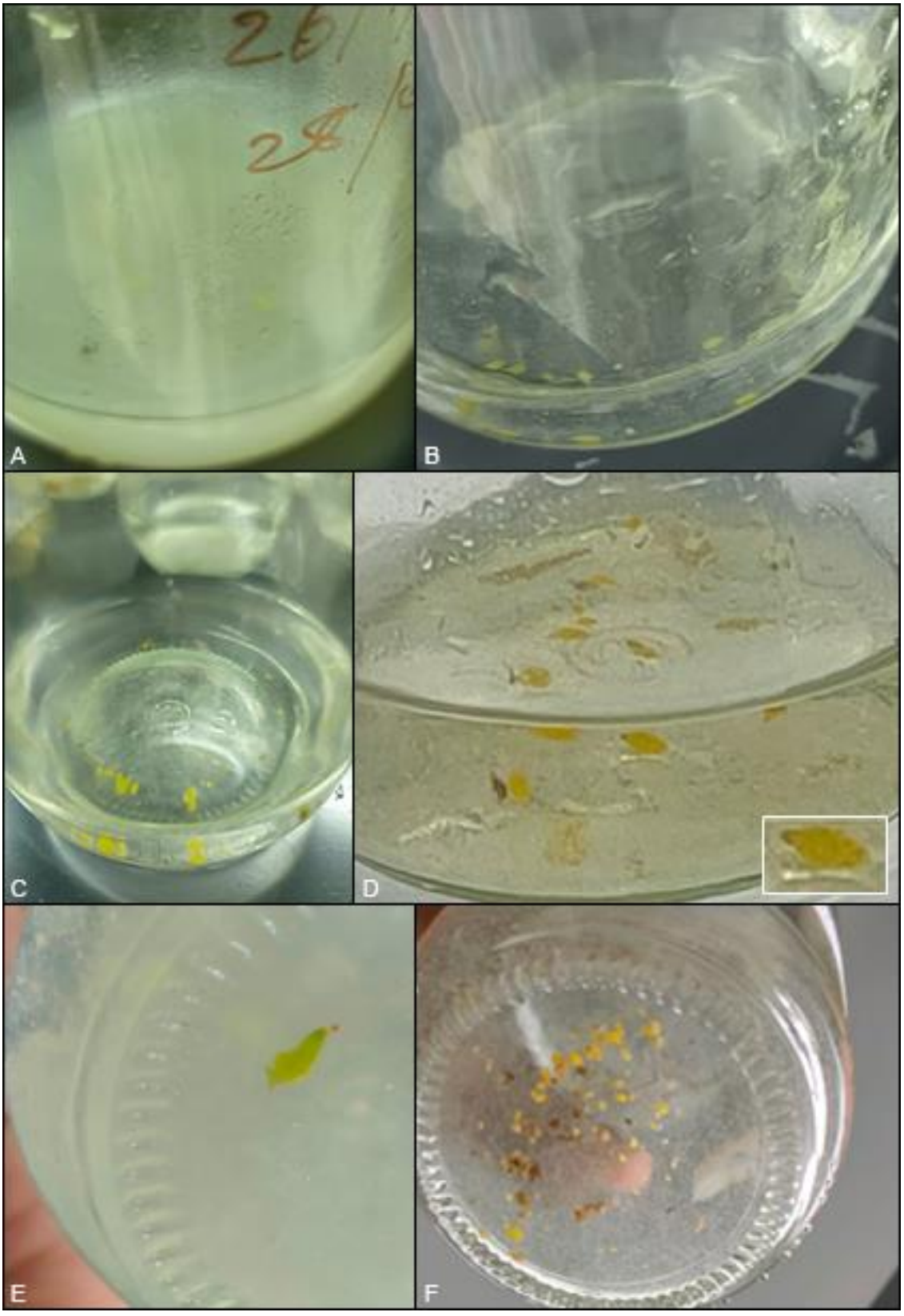


Plate 9: Stages of *in vitro* seed germination (A) Greening (B) Chlorophyll embryo (C) Protocorm like bodies (D) Typical grain like protocorm, insight enlarged protocorm (E) Initiation of primordium in protocorms (F) Degeneration of protocorms

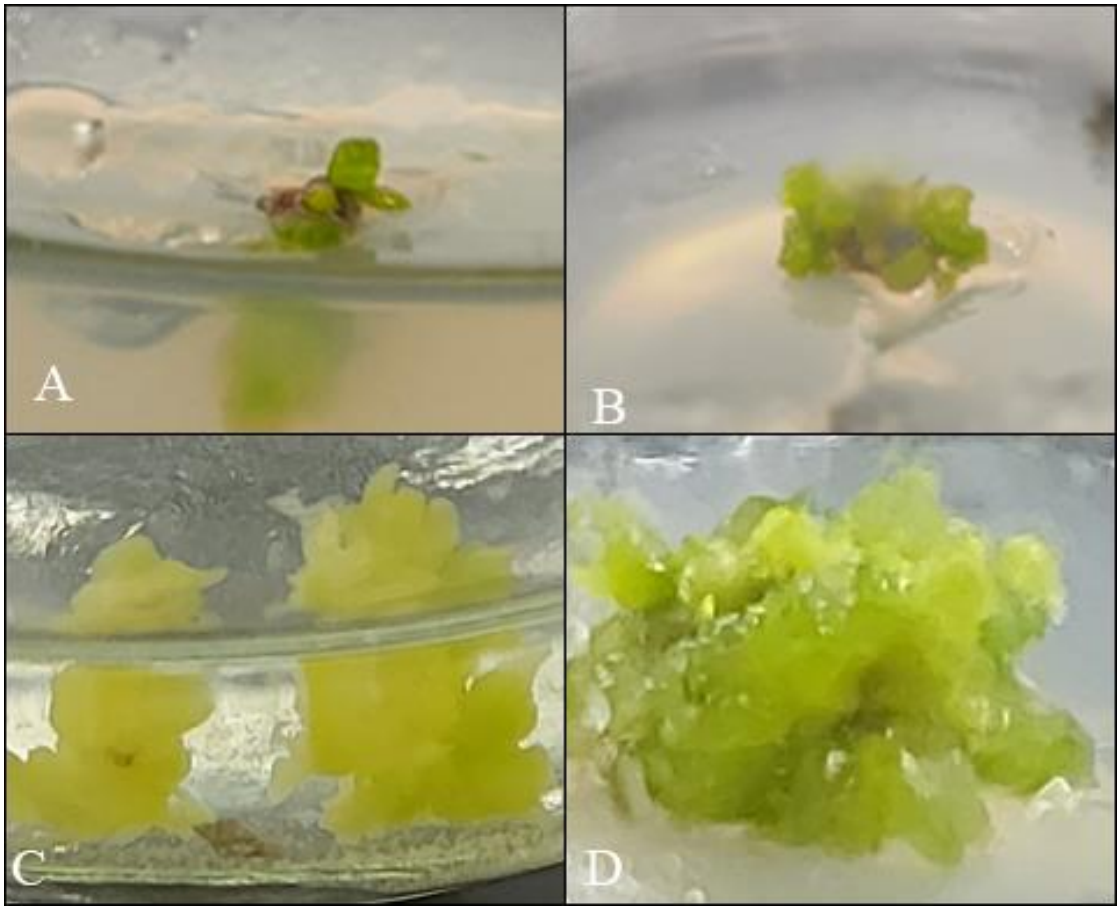


Plate 10: Development of plantlets (A) Individual plantlets with root and leaf (B) Callusing and shooting (C) Multiple shoot formation in liquid media (D) Multiple shoot formation in solid media

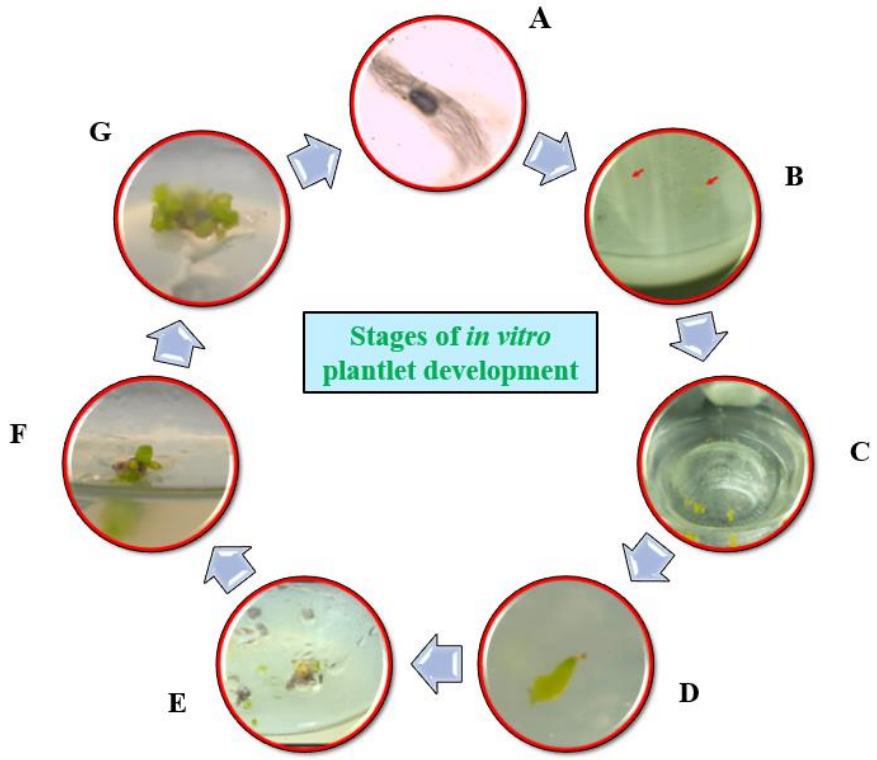


Figure 2: Flow chart showing stages of *in vitro* seed germination and plantlet development (A) Seed (B) Seed greening (C) Protocorm formation (D) Leaf and root primordia (E) Callusing and shooting (F) Multiple shoot formation (G) Plantlet

Discussion

5. DISCUSSION

The Orchidaceae, one of the largest families of angiosperms, has great value in the market. Orchids are a fascinating group of flowering plants with an astonishing number of species of more than 22000 and remarkable ability to thrive and adapt. They are evolved to have many unique characteristics and adaptations which help them in the evolutionary race. Orchids represent to be the epitome of angiosperm evo[l]ution. The reproductive biology of the Orchidaceae family of plant which aid in perfect ecological interaction with pollinators and elements of nature has left botanists and breeders in amazement for a long time.

Under the tribe Vandeae, sub tribe Aeridinae, and family Orchidaceae (Dressler, 1993), the *Rhynchostylis* is a small genus consisting of only four species. The genus is closely allied to the genus *Vanda*. Three species, *R. gigantea, R. retusa and R. coelestis* are reported as endemic in southeast Asian countries. *R. retusa* species was originally found in Indonesia. The most common name is fox-tail orchid due to the appearance of the inflorescence. *R. retusa* is an epiphytic orchid with a monopodial growth type that has exotic and potential interest for commercial production.

The present investigation focused on the reproductive aspects of the plant, morphological characters of flowers, and characterization of seeds qualitatively and quantitatively. A protocol for *in vitro* germination of seeds and its development was also standardized.

The results pertaining to the investigation done are discussed herewith.

5.1. EVALUATION OF FLORAL CHARACTERS

5.1.1. *Floral characters (qualitative)*

5.1.1.1. Flower colour

The flower colour of *R. retusa* was observed to be white with purple blushing or spotting on sepals and lateral petals, labellum being deeper purple and spur was white. This result is in close correspondence with other authors. Polymorphism in terms of flower colour has also been reported.

The purple flowers of *R. retusa* consist of dark purple labellum, and purple blotches on the lateral petals (Buragohain *et al.,* 2015). The colour of *R. retusa* exhibiting polymorphism,

including red, pink, white, blue, orange and other colours (Arditti, 1967). Inflorescence is arching or pendent, flowers white marked with pink, red or violet, spur is not bent (Higgins, 2012).

Polymorphism in terms of flower colour is a cue to attract different pollinators each preferring a different colour or pattern of floral parts in order to visit them. It could be seen as a sign of widening ecological adaptation of a plant species (Ortiz *et al.,* 2015).

5.1.1.2. Nature of flowering – free or seasonal

The species was observed to be flowering once in a year during particular season only. Anthesis was between rainy and spring months. Similar was the observation of other authors.

In China, owing to the unique flower shape, long flowering period and the opening around the Spring Festival can be used as a spring festival gift, deeply loved by people (Xi, *et al.,* 2021). However, flowering was also reported in summer to autumn seasons i.e., May - June months according to Sinha and Jahan (2012).

5.1.1.3. Season of flowering

R. retusa flowering was during South West monsoon rains or shortly after it i.e., during the spring. The months of blooming were from June to August.

Authors like Thakur and Dongarvar (2019) and Tongbram *et al.* (2012) agreed that June and July months, the flower opens in Indian conditions. Higgins (2012) also reported that in Java blooming is in February where the rainy season is from October to April. However, according to Buragohain *et al.* (2015), the anthesis in *R. retusa* initiates from the last week of March and persists up to early May in Assam state, India.

5.1.1.4. Time of anthesis

Flower opening was between 5 pm to 8 am, i.e., flower opens at dark hours of the day, during early morning or late evening.

Sanford (1971) concluded that anthesis in at least some orchids is a genetically controlled trait. White colour of flower should be related to flower opening at dark.

Night and dusk have abiotic features particularly weather parameters that differ from the day. Light intensity, wind speeds, turbulence, and temperatures are lower while humidity

may be higher at night. Nocturnal pollination occurred in thirty percent of angiosperm families across 68% of orders. Out of these 97% of families are equipped with C_3 photosynthetic pathway, two-thirds of families with crassulacean acid metabolism (CAM), and 71% among dicot families with C_4 photosynthesis. Nocturnal pollination occurs in more angiosperms with xerophytic adaptations than halophytes or mesophytes, suggesting that nocturnal flowering is primarily an adaptation to water stress because flowering is one of the water-intensive processes (Borges *et al.,* 2016).

5.1.1.5. Time of stigma receptivity

Stigma remained receptive upto five days after anthesis. It was found to be highly receptive during morning hours when the pollinia sticks easily to the stigmatic surface. Present study also made a notice that after 10.30-11.00 am during daytime receptivity declines considerably and gradually drying up of stigmatic surface occurs after five days.

In *R. retusa* young pollinia and stigma shows maximum receptivity and high effective pollination percentage. Morning time from 6 am to 10 am was the best time for pollination success from the rest of day (Thakur and Dongarwar, 2019). Knowledge of this fact could lead to higher success in hand pollination according to them.

5.1.2. *Pollen characters*

5.1.2.1. Pollen morphology

Pollen grains were observed to be obovoid to triangular in shape. Exine or outer layer was smooth and thin and intine was thickened. Mostly pollen was present as tetrad. Triad and dyad stage were also present.

The pollen grains as monads, or single grains, seen in Apostasioideae, Cypripedioideae, the Vanilleae, some Diurideae, and a few Neottieae. In all other orchids, the pollen grains either remain in tetrads or are united into larger masses. Some Epidendroideae with soft pollinia also have more or less heavily sculptured pollen grains, but the more advanced members of the Epidendroideae have thick but relatively smooth exine on the outer walls of the pollinia (Dressler,1993).

When the pollen grains are clustered into tetrads or even larger units, the grains usually have thick exine on the outer surface of the pollinium or aggregation, though there may be little or no exine developed on the surfaces between the grains within the aggregation (Dressler, 1993).

5.1.2.2. Pollen production per pollinium

The pollen number per pollinium in the species was calculated to be 2,06,000 in this investigation.

Given the fact that there are millions of ovules in orchid flower, to obtain a substantially good number of fertile seeds sufficient pollen load has to be ensured. In orchids pollen to ovule number ratios are several orders of magnitude lower than in other angiosperms with powdery pollen due to the lack of wastage during transport to the stigmatic surface (Lukasiewicz, 1999).

The number of pollen grains per pollen dispersal unit has both genetic and ecological consequences. The most important being the more grains per pollinia, the greater probability that seeds in a pod will have the same male parent; the greater the amount of pollen adhering to the stigma and fertilizing it, the greater the male competition inspiring greater female competition leading to reproductive fitness of the species in its ecology (Pacini and Franchi, 1999).

5.1.2.3. Pollen fertility percentage

Pollinium of one to four days old was most viable for effecting pollination in *R. retusa* (Nitin and Uma, 2014).

Pollen grains maintain a prolonged capacity for *in vitro* germination, even 100 per cent fertilization and embryonate seed development five days after their dislodgement from the original flowers (Bellusci *et al.,* 2010). Present research is in accordance to this understanding where 99.6 per cent pollen was viable.

5.1.2.4. Pollen germination percentage

73.64 percent of pollen in a pollinia can germinate under suitable conditions. This is in correspondence to the pollen germination in the species as reported by Seeja (2018).

5.1.3. *Capsule characters*

5.1.3.1. Days taken for pod setting after anthesis

Young flowers of upto eight days shows maximum receptivity and high pollination percentage under natural conditions (Shiau *et al.*, 2002) and (Proctor, 1998). This justifies that the symptoms of pod setting if any could be noticed within a week or ten days of anthesis of each flower in an inflorescence.

5.1.3.2. Days to mature capsule harvest

The days to harvest of yellowish green mature pods varied from 135 to 180 days i.e., between four and half to six months duration.

To be successful through *in vitro* mass propagation of *R. retusa*, the appropriate age for collecting fruits in days was identified as 180 by Fosenka (2020). Afterwards the pod dehisces as per the study. Eight months old capsules when harvested and cultured immediately got maximum seed germination in *R. retusa* (Sibin and Gangaprasad, 2012).

5.1.3.3. Length of capsule (cm)

Length of the capsule measured was 2.18 cm on an average.

Six-month old mature green capsule has an average length of 2.86 cm long (Sibin and Gangaprasad, 2012). Both length and girth of pods were averaged to about 3.8 cm at maturity (Fosenka, 2020).

5.1.3.4. Width of capsule (cm)

Average capsule width was measured to be 0.86 cm.

Capsule width reported by Sibin and Gangaprasad (2012) and Fosenka (2020) were almost equal to length of the pod which were 2.06 and 3.8 respectively. Average pod length was maximum after 11 weeks (Fosenka, 2020).

However, capsules taken for the present research had much lower width. This might be because pods of different maturity duration were harvested for standardization of asymbiotic embryo culture leading to bias in analysis and at the time maximum length was already attained but not maximum width. When yellow or brown coloured capsule was harvested because of start of drying and pod lose dry weight along with moisture and shrink.

5.1.3.5. Percentage of filled seeds

The proportion of embryonated seeds out of the total seed in a capsule was found as 41.85%.

Female reproductive success in orchids can be highlighted by the proportion of seeds with embryos (Proctor and Harder, 1994; Lock and Profita, 1975; Nilsson, 1980; Patt, *et al.*, 1989).

5.2. MORPHOLOGICAL AND MORPHOMETRIC CHARACTERIZATION OF SEEDS

5.2.1. *Morphological characters*

5.2.1.1. Seed colour

When the mature capsule was cut open the mass of millions of seeds present inside appears white to visible eye. This is in agreement with the findings of Fosenka (2020). According to Thomas and Michael (2007), immature seeds obtained from 50 days old pods were light green in colour and can be differentiated from mature seeds easily.

A seed when observed under microscope had yellowish green colour which was the colour of the seed coat. The embryo inside had deep purple to violet colour. Embryo of seeds of immature capsules was yellowish colour when viewed through microscopic lens. Individual seeds were pale yellow in *Rhynchostylis retusa* when observed under light microscope (Tongbram *et al.*, 2012).

During maturation, in the seeds of the capsules, there is an increase in percent air space. Increase in the air space is due to an increase in the cell length of the testa and not because of an increase in the number of cells in the seed coat (Swamy *et al.*, 2004). This variation in seed space with age might be the reason why in immature seeds the region of embryo cannot be seen with a definite boundary in light microscope and region of undifferentiated embryo appears yellow as a reflection of colour from intact testa cells.

5.2.1.2. Seed shape

R. retusa was viewed to have fusiform shaped seeds tapered at one end and blunt at the other end. Same was the finding of Verma *et al.* (2014) who reported *Rhynchostylis* to be fusiform shaped with embryo spheroid in shape i.e., prolate spheroid.

The shape of the seeds is also thought to have phylogenetic significance. Fusiform seeds that are found in all of the orchid subfamilies (Arditti *et al.*, 1979; Arditti, 1980; Healey *et al.*,

1980; Rasmussen, 1995; Verma *et al.,* 2014) appear to be basic form in orchids, from which all other seed shapes might have evolved.

5.2.1.3. Seed testa ornamentation

Testa or seed coat was reticulated or net like, testa cells running longitudinally with transverse testa cells connecting them at short distance. Similar was the observation of Tongbram (2012) that testa cells of 'Vanda type' orchids are always so strongly elongate that the longitudinal anticlinal walls are in contact with the surface of the seed.

Testa walls of *Rhynchostylis retusa* is straight or sinuous. Some orchids possess uneven deposition of thickening materials on their periclinal or anticlinal walls, and such ornamentation(s) is of taxonomic importance (Verma *et al.,* 2014).

5.2.2. Morphometric characters

5.2.2.1. Seed length (μm)

Seeds were 242.42 μm long was the result of the present investigation. *Rhynchostylis retusa* has a seed length of 275 μm (Tongbram *et al.,* 2012). Even greater average length (282.4 μm) of the seeds was reported by Sibin and Gangaprasad, (2012).

On the basis of classification of seed size based on seed length by Dressler (1993), *R. retusa* falls in the range of small (200-500 μm) sized seed.

5.2.2.2. Seed width (μm)

Width of seed that used for *in vitro* culture was 46.88 μm. However, seed width of 81.25 μm and 138.95 μm was presented by Tongbram *et al.* (2012) and Sibin and Gangaprasad (2012) respectively.

5.2.2.3. Seed length/width ratio

From the dimensions of seed, its length: width ratio was calculated as 5.29 indicating that Rhynchostylis has truncated to slightly elongated seed shape.

L/W ratio of seed comes to 5.583 +/- 0.377% for *R.retusa* (Verma *et al.,* 2014).

Seeds with length/width (L/W) ratio of >6.0 were designated as elongated by Verma *et al.* (2014). While if the value was > 5 the seed was classified elongated according to (Barthlott *et al.* (2014) and Diantina *et al.* (2020).

5.2.2.4. Seed volume (μm^3)

The seed volume of *Rhynchostylis* was estimated to be $202.6 \times 10^3\ \mu m^3$ in present inquiry and a seed volume of $479.4 \times 10^3\ \mu m^3$ was reported by Tongbram *et al.* (2012).

5.2.2.5. Embryo volume (μm^3)

Estimated embryo volume of the species was $39.96 \times 10^3\ \mu m^3$. A much bigger embryo of $447.4 \times 10^3\ \mu m^3$ volume was found by Tongbram *et al.* (2012).

In *R. retusa,* EL/EW is 3.705+/-0.851 and embryo volume ($mm^3 \times 10^{-3}$)= 0.094+/-0.021 (Verma *et al.*, 2014).

EL/EW ratios above 1 show that the orchid species have prolate spheroid shaped embryos Diantina *et al.* (2020) where EL is embryo length and EW is embryo width.

5.2.2.6. Percentage of air space between seed testa and embryo

Air space in a seed was averaged to 78.74 per cent of the seed volume. Contrary to this, 6.4 per cent is the air space inside the seed coat of the species according to Tongbram *et al.*, (2012). Also, Verma *et al.* (2014) studied the seed morphometry of some threatened Himalayan orchid species and found that *R. retusa* has lower air space volume (11.122%) than many other members of the subfamily Epidendroideae. Seed volume/ embryo volume for *R. retusa* was 1.182 in their estimation. The larger space for air in this research study might be because of inappropriate storage of capsules.

Seeds with higher ratio of seed volume/embryo volume are expected to more buoyant than those with a lower ratio of seed volume/embryo volume. There is a direct correlation apparent between the seed buoyancy and distribution pattern (Swamy *et al.*, 2004). Morphometric characterization of seeds point that the seeds used for the present enquiry was generally smaller in size (length, width and volume) than expected based on previous reports.

5.2.2.7. Seed viability percentage

78.87 per cent of seeds used for asymbiotic seed germination was viable according to tetrazolium test in the present research. Similar tetrazolium test by Bhatti *et al.* (2016) revealed that 97.15±0.33 of the seeds of the species was viable. The viability of seeds of *R. retusa* was found to be 100 per cent as observed under light microscope after staining with tetrazolium (Lal *et al.*, 2020). Each capsule possesses 89% viable seeds (Sibin and Gangaprasad, 2012).

Considerable reduction in viability in present investigation would be due to prolonged storage especially of dried pods, inappropriate storage or refrigeration and inadequate pre-treatment before viability testing or bias in sampling or estimation.

Correlation analysis was performed for eight characters- seed length, seed width, seed length/ seed width ratio, embryo length, embryo width, seed volume, embryo volume and air space. Seed length showed highly significant and very highly significant negative correlation with embryo length and seed volume respectively. Seed length by seed width ratio had negative highly significant correlation with embryo width. Ratio of seed length and width is positively correlated with air space volume and negatively correlated with embryo volume, both characters very highly significant with the ratio. Embryo length is positive and very highly significantly correlated with seed volume. Embryo width is significantly positive correlated with embryo volume and air space is negative highly significant with embryo width. Air space is negative very highly significant to embryo volume. Monroy *et al.* (2017) reported a significant positive correlation between the seed size and embryo size and suggested that larger seeds with larger embryos might contribute to higher success in germination.

5.3. STANDARDISATION OF *IN VITRO* SEED GERMINATION

5.3.1. Effect of pod age

Capsules of different age were harvested for standardizing *in vitro* seed (embryo) culture. Fully matured yellowish green capsules showed results in asymbiotic culture.

Comparatively lesser seed germination was observed for fully mature dry seeds than mature green seeds. This can be attributed to the high maturity level of inoculated seeds; the immature seeds have been reported to exhibit better germination because of their distended testa cells, metabolically awakened embryos and absence of dormancy factors (Linden, 1980; Yam and Weatherhead, 1988; Arditti *et al.*, 1981).

The inner integument of the ovule usually fails to develop further after fertilization, the cells then collapse around the embryo proper forming a hard 'shell', termed as 'carapace' and it wraps the embryo (Veyret, 1969; Yam and Weatherhead, 1988). The carapace can offer protection to the embryo. Additional deposition of phenolic compounds and wax like materials further reduces the permeability of the carapace. The low percentage of mature seed germination observed in *C. falcata* is attributed to the accumulation of substances such as lignin in the inner integument, a possible cause of germination inhibition (Yamazaki and Miyoshi, 2006). In easy-to-germinate species such as *Orchis morio* and *Serapias lingua*, a carapace is incompletely formed around the embryo (Veyret, 1969). In the native environment, carapace closely surrounding embryo prolong seed survival, enhancing their chance of finding a suitable mycorrhizal symbiont. However, it can be one of the major causes in inhibiting germination of mature seeds *in vitro* (Yeung, 2017).

5.3.2. Protocorm development

Greening of culture bottles and prominent green embryo stage of irregular sized spherical structures were seen in one to two months in the present investigation.

Germination was marked by swelling and emergence of the embryo from the testa. Seed germination was first evident by swelling and within 7-8 weeks the undifferentiated embryos formed an irregular shaped cell mass as spherules (Bhattacharjee and Islam, 2015).

Spherules and protocorm formation were around the same time in many culture bottles. This might be because some seeds are more responsive and show greater vigour than others.

After 1-2 weeks these spherules turn green and formed round structures as protocorms. Protocorms became visible after 11 weeks of culture initiation and showed at the vegetative apex (Bhattacharjee and Islam, 2015).

Development of protocorms was observed after 12.50 ± 0.50 weeks after inoculating (Bhatti *et al.*, 2016).

5.3.3. Liquid consistency of medium

Germination of seeds in *in vitro* culture was allowed in liquid media upto protocorm stage and there was good result. However, earlier works in the species for micropropagation concentrated on solidified media for embryo culture. It can be concluded that liquid media can

facilitate better hydration of embryos and nutrient availability. Periodic light agitation of culture bottles helps to maintain homogeneity of ions in solution.

Sibin and Gangaprasad (2012) did their investigation in liquid Knudson C medium in the same field and reported good result for seed germination but not for protocorm formation and further growth. It is in agreement with present results.

5.3.4. Protocorm death

Protocorm like bodies (PLBs) in some culture bottles were green initially but turned yellow and later degraded and turned brown (Plate 10). This process once initiated led to progressive of PLBs one after the other. This might be because of phenolic oxidation which release polyphenol oxidase another toxic compounds for the plants to the medium. Another possible reason might be nutrient competition or exhaustion or failure of completion of maturity program in seeds used for culture.

Segmentation made in leaf segments of *Phalaenopsis* to induce PLBs results in a process called phenolic oxidation, which is the release of polyphenol oxidase (PPO) and other compounds toxic to plant tissue, which may cause its death, consequently reducing the induction of PLBs (Cardoso *et al.*, 2020; Chuanjun *et al.*, 2015).

The advantage of activated charcoal can be considered by at least two aspects, reduction of light at the base of explants, which provides an environment conductive to the accumulation of auxin or cofactors (Druart and Wulf, 1993) and or absorption of inhibitory substances such as polyphenols which can be harmful to plant growth and proliferation (Fridborg and Eriksson, 1975).

Browning followed by death of the protocorm were a common occurrence after seed germination in many terrestrial orchids (Stoutamire, 1974; Harvais, 1982). Protocorm death may be due to imbalance among nutrient sources or insufficiency of growth stimulating compounds in the culture medium (Stoutamire 1974). The degenerated protocorm usually lacks a well-differentiated meristematic region (Perner *et al.*, 2022).

5.3.5. Effect of nutrient composition

Percentage of germination varied with different media tested. The present study found that the half MS medium promoted better seed germination and PLB's formation was higher.

The concentrations of ions in basal medium can influence the growth and development of explants, using a suitable basal medium can be a key factor for successful *in vitro* cultures (Ichihahsi, 1992). Nutrient requirements for orchid seed germination are thought to be species specific (Kauth *et al*., 2008).

Nitrogen is a very critical component for growth of plants and its source has been shown to have an effect on the germination of different orchid species. The MS medium contains nitrate nitrogen and ammonium stimulated seed germination (Popova *et al*., 2003). It is reported that seed germination of orchids could be improved by the addition of amino acids to the media and the reduction of inorganic nitrogen sources (Malmgren, 1992; Anderson, 1996). These researchers have suggested that organic sources of nitrogen (i.e., amino acids) may be more readily available to a seed than an alternative inorganic nitrogen source. In *R. retusa* both organic and inorganic form of nitrogen is helping in seed germination and seedling development ½ MS initiated seed germination and supported advanced protocorm development (Thakur and Dongarwar, 2019).

5.3.6. Growth and development

First leaf developed after 18.00±0.71 weeks, and first root after 26.80±0.57 weeks. After development of one or two green leaves, and emergence of first root, the developing entities were subcultured. Seedlings with 2–3 leaves and 1–2 roots were obtained after 31.50±1.12 weeks (Bhatti *et al.*, 2016).

Summary

6. SUMMARY

Orchids are known for their floral exquisiteness, a very important component of the total floricultural trade at national and international level. The unique assembly of floral organs and superior shelf life makes them best commodity for the market as cut flowers and potted plants. However, their reproduction by sexual means is difficult because of the seed's dependency on a fungal symbiont for germination.

Rhynchostylis retusa is a traditionally important orchid species in our country. Its significance is deep rooted in our culture especially of people in north east India where the plant is a symbol of sanctity and religious purity. Though it has medicinal importance, but the ornamental usage outweighs its medicinal ones. Its fragrant plump inflorescence with a large number of closely spaced flowers is a thing of absolute beauty. However, in depth and comprehensive understanding of the species in all dimensions is not yet attempted. Difficulties in conventional propagation methods and unrestrained commercial exploitation have led to a rapid deterioration of the orchid in its forest home. Unstable weather parameters and fast penetrating climate change effects might be other reasons which threaten the species. Being an epiphytic orchid, it is more exposed to the risk of extinction due to forest fires, clearing, etc. Actions for conserving this endangered species is an urgent call.

The present experiment was aimed at documentation of morphological and morphometrical seed characters and standardization of *in vitro* seed germination protocol in *Rhynchostylis retusa* (L.) Blume. It was conducted at College of Agriculture, Vellayani and Saraswathy Thangavelu Extension Centre of Jawaharlal Nehru Tropical Botanic Garden and Research Institute during 2020-2022 period.

The study included four experiments viz., (i) evaluation of floral characters; (ii) morphological and morphometric characterization of seeds; (iii) preparation of *in vitro* seed germination media and (iv) *in vitro* seed germination in culture media and evaluation

Qualitative characters of inflorescence, pollinia and capsules were subjectively described. Seed and pollen characters were studied using a compound light microscope and software application Leica LAZ 500. All experiments were carried out in five replications including 10 plants in total. *R. retusa* was found to be seasonal in flowering behaviour, the season of blooming being June to August. Based on flower colour patterns there are two types

of plants, one with purple spots or tessellation and other with blush of purple on white background for both types. The anthesis of the species is not at daytime but between late evening and early morning when it was dark. The stigma is receptive in young flowers upto 5 days after anthesis after which there is considerable decline in effecting pollination. Moreover, morning hours until 10.30 am showed maximum receptivity compared to other times of the day. This is in line with the finding that in natural conditions pod setting is noticed within seven days of flower open.

Morphology of pollinia was observed with visible eye and they were crushed and stained using acetocarmine to understand the individual pollen structure, number, arrangement, viability etc. Pollen grain is obovoid to triangular shaped, mostly present as tetrad with smooth exine and thick intine. The pollen number per pollinium was estimated using a haemocytometer and the value averaged to 2,06,000. Furthermore, almost all the pollen was found to be viable and 73.64 per cent of them germinated in sucrose solution of 10 per cent strength when kept in it for a day. The pollen characters study is important because it has a profound influence on character of the pod and seed formed after selfing. The fruit or capsule is 2.18 cm long and 0.86 cm wide after ripening. Hand pollination was practiced to collect capsules for embryo culture. Undehisced mature capsules of 145-180 days of pollination was found to be best for *in vitro* culture.

Seeds of *R. retusa* were characterized morphologically under compound light microscope and morphometric data analysed using microscopic software package Leica LAZ 500. The seeds are fusiform shaped, long, with prolate spheroid embryo of violet colour without staining. The testa cells are yellowish white and reticulated. At maturity 78.74 percent of the seed volume is air and only one-fourth of the volume is occupied by embryo which may or may not be present at the center. The viability of seeds used for embryo culture was worked out to be 78.87 percentage. Out of the millions of seeds in a capsule 41.87 percentage of seeds were well embryonated with embryo of regular shape.

For the *in vitro* seed germination studies four basal solid and liquid media viz., MS, half-strength MS, Knudson C orchid medium and WPM medium were made with different combinations of plant growth hormones such as BAP, NAA, IBA, etc. The minor nutrients and vitamins were added from stock solutions. At protocorm stage they were transferred to fresh solid media supplemented with additives to promote further growth and development. The seeds from mature capsules were disinfected and inoculated under sterile conditions in 250 mL

culture bottles each containing about 50 mL of the prepared media. Five replicates of each media were inoculated. The capsules at different stages of maturity were inoculated. Dry and dehisced capsules were tied with a thread to cover the grooves before disinfection and culturing. Surface sterilization was done by laboratory detergent (5min), 0.1% mercuric chloride (2-3 min), distilled water to wash off these agents, followed by dipping in 70% alcohol and flaming for 2-3 times.

Observations were recorded on the number of days for showing growth at initial stage of seed greening and chlorophyll embryo, number of days for protocorm development, number of protocorms per culture bottle and number of protocorms with shoot apex and multiple shoots, number of days for root and leaf initiation was registered.

The above study revealed that liquid half MS medium is the best among the media used for asymbiotic seed germination of *R. retusa.* Initiation of germination became evident within two months of culture inoculation as seed greening and spherule formation. At around the same time within 70 days PLBs (Protocorm-like bodies) developed. The grain shaped protocorm needed to be subcultured at proper time and growth regulators were needed for further growth. Shoot and root primordia was developed from the protocorm, leading to development of shoots and roots. Calloid like growth of protocorm was also seen. Analysis of variance have shown that there is significant difference between treatment means for seed germination and further development of protocorm like bodies (PLBs). The best media for seed germination is liquid basal half MS media. The best culture media for calloid formation and shooting from protocorm was solid half MS with 0.5 mgL^{-1} BAP. Callus with multiple shoots was divided to separate units and each unit sub cultured to grow as separate plantlets. For multiple shooting and establishment half MS media (solid) with 0.5 mgL^{-1} BAP and 1 mgL^{-1} NAA gave good results.

Standardizing of the *in vitro* seed germination protocol helps in the restoration of the species in its habitat. Additionally, hybrids without sterility could be developed by embryo rescue and mass multiplication of plant propagule for commercial purposes could be effected. Selfing of this naturally cross-pollinated species gave hundred percent results with fruits of good viability seeds which revealed that this species is self-compatible and there is no presence of male- sterility. Characterization of seeds is the initial step towards maintaining variability. It helps in understanding distribution patterns, adaptive features and taxonomic position.

References

7. REFERENCES

Abraham, A. and Vatsala, P. 1981. *Introduction to Orchids with Illustrations and Descriptions of 150 South Indian Orchids.* Tropical Botanic Garden and Research Institute, Thiruvananthapuram, India, Kerala, 533p.

Adhikari, Y.P. and Fischer, A. 2011. Distribution pattern of the epiphytic orchid *Rhynchostylis retusa* under strong human influence in Kathmandu valley, Nepal. *Botanica Orientalis: J. Plant Sci.* 8: 90-99.

Aggarwal, S., Nirmala, C., Beri, S., Rastogi, S., and Adholeya, A. 2012. *In vitro* symbiotic seed germination and molecular characterization of associated endophytic fungi in a commercially important and endangered Indian orchid *Vanda coerulea* Griff. Ex Lindl. *Eur. J. Environ. Sci.* 2(1).

Ahmad, S., Gao, J., Wei, Y., Lu, C., Zhu, G., and Yang, F., 2022. The transcriptome profiling of flavonoids and bibenzyls reveals medicinal importance of rare orchid *Arundina graminifolia. Frontiers Plant Sci.* 13, p.1934.

Anderson, A.B. 1996. The reintroduction of *Platanthera ciliaris* in Canada. In: Allen, C. (ed.), *North American native terrestrial orchids propagation and production.* North American Native Terrestrial Orchid Conference, Germantown, Maryland, pp. 73–76.

Arditti, J. 1967. Factors affecting the germination of orchid seeds. *Bot. Rev.* 33(1): 1–97.

Arditti, J. 1980. Aspects of the physiology of orchids. In: *Advances in botanical research,* Academic Press, 7: 421-655

Arditti, J., Michaud, J.D., and Healey, P.L. 1979. Morphometry of orchid seeds. I. Paphiopedilum and native California and related species of Cypripedium. *Am. J. Bot.* 66(10): 1128-1137.

Arditti, J., Michaud, J.D., and Oliva, A.P. 1981. Seed germination of North American orchids. I. native California and related species of Calypso, Epipactis, Goodyera, Piperia, and Platanthera. *Bot. Gaz.* 142(4): 442-453.

Barthlott, W., Grobe-Veldmann, B., and Korotkova, N. 2014. Orchid seed diversity: a scanning electron microscopy survey. *Englera,* 32: 3-245.

Bellusci, F., Musacchio, A., Stabile, R., and Pellegrino, G. 2010. Differences in pollen viability in relation to different deceptive pollination strategies in Mediterranean orchids. *Ann. Bot.* 106(5): 769-774.

Bhattacharjee, B. and Islam, S.M. 2015. Plant regeneration through somatic embryogenesis from leaf and root explants of *Rhynchostylis retusa* (L.) Blume. *Appl. Biol. Res.* 17(2): 158-165.

Bhatti, S.K., Verma, J., Sembi, J.K., and Kumar, A. 2016. Mycobiont mediated *in vitro* seed germination of an endangered 'fox-tail'orchid, *Rhynchostylis retusa* (L.) Blume. *J. Pure and Appl. Microbiol.* 10(1): 663-670.

Borges, R.M., Somanathan, H., and Kelber, A. 2016. Patterns and processes in nocturnal and crepuscular pollination services. *The Quart. Rev. Biol.* 91(4): 389-418.

Buragohain, B., Chaturvedi, S.K. and Puro, N., 2015. Biotic Pollination in *Rhynchostylis retusa* (L.) Bl. (Orchidaceae). *Int. J. Plant Reproductive Biol.* 7(1): 78-83.

Cardoso, J.C., Zanello, C.A., and Chen, J.T. 2020. An overview of orchid protocorm-like bodies: Mass propagation, biotechnology, molecular aspects, and breeding. *Int. J. Mol. Sci.* 21(3): 985.

Christenson, E. 2003. Mschu Picchu: A Handbook to the Orchids of the Machu Picchu National Sanctuary. The Peruvian Trust Fund for National Parks and Protected Areas (PROFONAPE), Lima, Peru.

Chuanjun, X.U., Zhiwei, R.U., Ling, L.I., Biyu, Z., Junmei, H., Wen, H., and Ou, H. 2015. The effects of polyphenol oxidase and cycloheximide on the early stage of browning in Phalaenopsis explants. *Hortic. Plant J.* 1(3):172-180.

Diantina, S., McGill, C., Millner, J., Nadarajan, J.W., Pritchard, H., and Clavijo McCormick, A. 2020. Comparative seed morphology of tropical and temperate orchid species with different growth habits. *Plants,* 9(2): 161.

Dressler, R.L. 1993. *Phylogeny and Classification of the Orchid Family*. Cambridge University Press, pp. 1-314.

Druart, P. and De Wulf, O. 1993. Activated charcoal catalyses sucrose hydrolysis during autoclaving. *Plant cell, tissue and organ cult.* 32: 97-99.

Fonseka, K. 2020. Asymbiotic seed germination, mass propagation and conservation of fox-tail orchid, *Rhynchostylis retusa* L. Blume: An endangered orchid. *Asian J. Conserv. Biol.* 9(2): 144.

Fridborg, G. and Eriksson, T. 1975. Effects of activated charcoal on growth and morphogenesis in cell cultures. *Physiologia Plantarum*, 34(4): 306-308.

Gandawijaja, D. and Arditti, J. 1983. The orchids of Krakatau: evidence for a mode of transport, *Ann. of Bot.* 52(2): 127–130.

Harvais, G., 1982. An improved culture medium for growing the orchid *Cypripedium reginae* axenically. *Can. J. Bot.* 60(12): 2547-2555.

Healey, P.L., Michaud, J.D. and Arditti, J. 1980. Morphometry of orchid seeds. III. Native California and related species of Goodyera, Piperia, Platanthera and Spiranthes. *Am. J. Bot. 67*(4): 508-518.

Higgins, W.E. 2012. *A New Name for a Distinctive Rhynchostylis (Orchidaceae) and The Misapplication of Rhynchostylis praemorsa. Selbyana*, 31(1): 40-43.

Ichihashi, S. 1992. Micropropagation of Phalaenopsis through the culture of lateral buds from young flower stalks. *Lindleyana*, 7: 208-215.

Kauth, P.J., Dutra, D., Johnson, T.R., Stewart, S.L., Kane, M.E., and Vendrame, W. 2008. Techniques and applications of *in vitro* orchid seed germination. In: Ongena, M.A.R.C. and Thonart, P. (eds), *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues, 5*: 375-391.

Knudson, C. 1946. A new nutrient solution for germination of orchid seed. *Am. Orchid Soc. Bull.* 15: 214-217.

Koene, F.M., Amano, E., Smidt, E.D.C., and Ribas, L.L.F. 2020. Asymbiotic germination and morphological studies of seeds of Atlantic Rainforest micro-orchids (Pleurothallidinae). *Plos one*, 15(12): e0243297.

Kumar, A., Nandi, S.K., Bag, N., and Palni, L.M.S. 2002. Tissue culture studies in two important orchid taxa: *Rhynchostylis retusa* (L.) Bl. and *Cymbidium elegans* Lindl. *Gyanodaya Prakashan, Nainital, India*, pp.113-124.

Lal, A., Pant, M., Palni, L.M.S., and Kumar, A. 2020. Development of rapid micropropagation protocol for germplasm conservation of two orchid species-*Aerides multiflora* Roxb. and *Rhynchostylis retusa* (L.) Blume. *Asian J. Conserv. Biol.* 9(2): 341-347.

Lindén, B. 1980. Aseptic germination of seeds of northern terrestrial orchids. In: *Ann. Bot. Fennici*. Finnish Botanical Publishing Board, pp. 174-182.

Lloyd, G. and McCown, B. 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Proceedings of the International Plant Propagator's Society, 30: 421–427.

Lock, J.M. and Profita, J.C. 1975. Pollination of *Eulophia cristata* (Sw.) Steud. (Orchidaceae) in Southern Ghana. *Acta Botanica Neerlandica*, 24(2): 135-138.

Lukasiewicz, M.J. 1999. *Maternal Investment, Pollination Efficiency and Pollen: Ovule Ratios in Alberta orchids*. University of Calgary, pp. 1-123.

Magrini, S., De Vitis, M., Torelli, D., Santi, L., and Zucconi, L. 2019. Seed banking of terrestrial orchids: evaluation of seed quality in Anacamptis following 4-year dry storage. *Plant Biol.* 21(3): 544-550.

Malmgren, S. 1992. Large-scale asymbiotic propagation of *Cypripedium calceolus*-plant physiology from a surgeon's point of view. Bot. Gard. Micropropagation News 1:59–63.

Meeyot, W. 1968. *Studies on storage of orchid pollen* (Doctoral dissertation, University of Hawaii).

Monroy, F., Calevo, J., Giovannini, A., Cornara, L., and Peccenini, S. 2017. *Orchis patens*: seed morphology of an endangered Mediterranean orchid. *Plant Biosystems,* 151(5): 770-774.

Mulgaonkar, M.S. and Dabhade, G.T. 2004. Palynological observations of some corticolous orchids from Maharashtra under SEM. *Phytotaxonomy*, 4: 131 – 134.

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum,* 15(3): 473-497.

Naing, A.H., Park, I.S., Hwang, Y.J., Chung, J.D. and Lim, K.B. 2010. *In Vitro* Micropropagation and Conservation of *Rhynchostylis retusa* BL. *Hortic. Environ. and Biotechnol.* 51(5): 440-444.

Nair, P.C.S., Balasubramanyam, V.R., and Khan, H.H. 1964. Palynological investigations of some guava varieties. *Indian J. Hortic.* 21: 79-84.

Nilsson, L.A. 1980. The pollination ecology of *Dactylorhiza sambucina* (Orchidaceae). *Botaniska Notiser,* 133: 367-385.

Nitin, D. and Uma, T. 2014. Artificial pollination and its role in orchid conservation, In: *A Glimpse of current vistas in Plant Science Research.* Nagpur, India: Hislop Coll. Publ. Cell, pp.123-140.

Ortiz, P.L., Berjano, R., Talavera, M., Rodríguez-Zayas, L., and Arista, M. 2015. Flower colour polymorphism in Lysimachia arvensis: how is the red morph maintained in Mediterranean environments? *Perspectives Plant Ecol., Evol. Syst. 17*(2): 142-150.

Pacini, E. and Hesse, M. 2002. Types of pollen dispersal units in orchids, and their consequences for germination and fertilization. *Ann. Bot.* 89(6): 653-664.

Pacini, E. and Franchi G. G. 1999. Types of pollen dispersal units and pollen competition. In: Clément, C., Pacini, E., and Audran J.C. (eds), *Anther and pollen: from biology to biotechnology* Berlin: Springer-Verlag, Berlin, pp. 1–11.

Pacini, E., Franchi, G.G., Lisci, M. and Nepi, M. 1997. Pollen viability related to type of pollination in six angiosperm species. *Ann. Bot.* 80(1): 83-87.

Patt, J. M., Merchant, M. W., Williams, E. R., and Meeuse, B.J. 1989. Pollination biology of *Platanthera stricta* (Orchidaceae) in Olympic National Park, Washington. *Am. J. Bot.* 76(8): 1097-1106.

Perner, H., Zhou, R., Perner, W., Jiang, H. and Lee, Y.I., 2022. Cypripedium subtropicum embryo development and cytokinin requirements for asymbiotic germination. *Bot. Stud.* 63(1): 28.

Pierik, R.L.M. 1987. *In vitro Culture of Higher Plants*. Martinus Higoff Publ., Netherlands, p344.

Popova, E.V., Nikishina, T.V., Kolomeitseva, G.L., and Popov, A.S. 2003. The effect of seed cryopreservation on the development of protocorms by the hybrid orchid Bratonia. *Russian J. Plant Physiol.* 50: 672-677.

Pradhan, N., Fan, X., Martini, F., Chen, H., Liu, H., Gao, J., and Goodale, U.M. 2022. Seed viability testing for research and conservation of epiphytic and terrestrial orchids. *Bot. Stud.* 63(1): 1-14.

Prasongsom, S., Thammasiri, K., and Pritchard, H.W. 2017. Seed micromorphology and *ex vitro* germination of Dendrobium orchids. *Acta Hortic.* 1167: 339-344.

Proctor HC. 1998. Effect of pollen age on fruit set, fruit weight, and seed set in three orchid species. *Can. J. Bot.* 76: 420–427.

Proctor, H.C. and Harder, L.D. 1994. Pollen load, capsule weight, and seed production in three orchid species. *Can. J. Bot.* 72(2): 249-255.

Rasmussen, H.N. 1995. *Terrestrial Orchids: from Seed to Mycotrophic Plant*. Cambridge University Press, pp 7-358.

Rasmussen, H.N., Dixon, K.W., Jersáková, J., and Teoh, E.S. 2022. Rhynchostylis Bl. In: *Orchid Species from Himalaya and Southeast Asia Vol. 3 (R-Z)*, Cham: Springer International Publishing, pp. 25-34.

Rasmussen, H.N., Dixon, K.W., Jersáková, J. and Těšitelová, T., 2015. Germination and seedling establishment in orchids: a complex of requirements. *Ann. Bot.* 116(3): 391-402.

Sanford, W.W. 1971. The flowering time of West African orchids. *Bot. J. Linn. Soc.* 64: 163-181.

Saxena, S., 2020. The current research status of endangered *Rhynchostylis retusa* (L.) Blume: a review. *Asian J. Res. Bot.* 4(2): 16-25.

Seeja, G. 2018. Distant hybridization and compatibility studies in wild orchids. Ph.D. thesis, Kerala Agricultural University, Thrissur-Kerala, India, pp. 6-93.

Shiau, Y.J., Sagare, A.P., Chen, U.C., Yang, S.R., and Tsay, H.S. 2002. Conservation of *Anoectochilus formosanus* Hayata by artificial cross-pollination and *in vitro* culture of seeds. *Bot. Bull. Acad. Sin*. 43: 123–130.

Sibin, N.T. and Gangaprasad, A. 2012. Embryo culture of *Rhynchostylis retusa*, an exquisite orchid of Western Ghats. *Int. J. Biol. Pharm. Allied Sci.* 1(7): 973-981.

Singh, F. 1992. *In vitro* propagation of orchids 'state of art'. *J. Orchid Soc. India,* 6: 11-14.

Sinha, P. and Jahan, M.A.A. 2012. Clonal propagation of *Rhynchostylis retusa* (Lin.) Blume through *in vitro* culture and their establishment in the nursery. *Plant Tissue Culture and Biotechnol. 22*(1): 1-11.

Stoutamire, W.P. 1974. Terrestrial orchid seedlings In: Withner, C.L. (eds), The Orchids Scientific Studies John Wiley and Sons, New York, pp. 101–128

Sunitibala Devi, Y. and Neelashree, N. 2018. Micropropagation of the monopodial orchid, *Rhynchostylis retusa* (L.). *Int. J. Life Sci.* 6(1): 181-186.

Swamy, K.K., Krishnakumar, H.N., Ramakrishna, T.M., and Ramaswamy, S.N. 2004. Studies on distribution and phenology of orchids in Karnataka. *J. Orchid Soc. India,* 18(1-2): 81-96.

Thakur, U.N. and Dongarwar, N.M. 2019. Species specific pollination and seed germination in *Rhynchostylis retusa* Blume (Orchidaceae). *Res. J. Life Sci. Bioinform. Pharm. Chem. Sci.* 5(2): 387-395.

Thomas, T.D. and Michael, A. 2007. High-frequency plantlet regeneration and multiple shoot induction from cultured immature seeds of *Rhynchostylis retusa* Blume., an exquisite orchid. *Plant Biotechnol Rep.* 1: 243-249.

Tongbram, J., Rao, A.N., and Vij, S.P. 2012. Seed morphometric studies in some orchids from Manipur. *J. Orch. Soc. India*, 26(1-2): 25-29.

Tsutsumi, C., Yukawa, T., Lee, N.S., Lee, C.S., and Kato, M. 2007. Phylogeny and comparative seed morphology of epiphytic and terrestrial species of Liparis (Orchidaceae) in Japan. *J. Plant Res.* 120(3): 405-412.

Verma, J., Sharma, K., Thakur, K., Sembi, J.K., and Vij, S.P. 2014. Study on seed morphometry of some threatened Western Himalayan orchids. *Turkish J. Bot.* 38(2): 234-251.

Veyret, Y. 1969. The structure of Orehidaeae seeds and their ability to germinate *in vitro* in pure cultures (trans. French), *Mus. Hist. Nat. Paris, Trav. Lab. The Jaysinia*, 3: 89-98.

Visser, T. 1955. *Germination and storage of pollen* (Doctoral dissertation, Veenman).

Xi, Y., Zeng, B. and Huang, H. 2021. Rapid propagation of *Rhynchostylis retusa in vitro*. *Phyton*, 90(3): 987.

Yam, T.W. and Weatherhead, M.A. 1988. Germination and seedling development of some Hong Kong orchids. Lindleyana, 3: 156–160.

Yamazaki, J. and Miyoshi, K. 2006. *In vitro* asymbiotic germination of immature seed and formation of protocorm by *Cephalanthera falcata* (Orchidaceae). *Ann. Bot.* 98(6): 1197-1206.

Yeung, E.C. 2017. A perspective on orchid seed and protocorm development. *Bot. Stud.* 58(1): 1-14.

Zhang, G.Q., Liu, K.W., Chen, L.J., Xiao, X.J., Zhai, J.W., Li, L.Q., Cai, J., Hsiao, Y.Y., Rao, W.H., Huang, J., and Ma, X.Y. 2013. A new molecular phylogeny and a new genus, Pendulorchis, of the Aerides–Vanda alliance (Orchidaceae: Epidendroideae). *PLoS One*, 8(4): 60-97.

Zimmerman, J.K., Roubik, D.W. and Ackerman, J.D. 1989. Asynchronous phenologies of a neotropical orchid and its euglossine bee pollinator. *Ecology*, 70(4): 1192-1195.

Seed characterization and standardization of *in vitro* seed germination protocol in *Rhynchostylis retusa* (L.) Blume.

by

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(2020-11-098)

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BREEDING AND GENETICS

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KERALA, INDIA

2023

ABSTRACT

The present study entitled "Seed characterization and standardization of *in vitro* seed germination protocol in *Rhynchostylis retusa* (L.) Blume." was carried out in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during the year 2020-22. The experiment was aimed at seed characterization and standardization of asymbiotic seed germination protocol in *Rhynchostylis retusa* (L.) Blume.

Germplasm repository maintained at Saraswathy Thangavelu Extension Centre of JNTBGRI, Puthenthope, Thiruvananthapuram was utilized to observe the flower characters and the capsules for the characterization and *in vitro* seed germination study.

The research work was divided into four experiments. Experiment I - evaluation of floral characters, experiment II -morphological and morphometric characterization of seeds, experiment III- preparation of *in vitro* seed germination media, and experiment IV -*in vitro* seed germination in culture media and evaluation. All the experiments were carried out in five replicates and mean was calculated.

In the first experiment, various floral, pollen and capsule characters were noted and qualitative characters were expressed descriptively after observing the plants behaviour closely and quantitative characters were assessed and mean was worked out. *Rhynchostylis retusa* was seasonal (June-August) in flowering behaviour. Two types of plants were observed, one with white flowers having purple spots or tessellation and other with blush of purple on white background. Anthesis was between late evening and early morning. The stigma was receptive in young flowers until five days after anthesis and after which the stigma become dried out. Morning hours until 10.30 am showed maximum receptivity than other times of the day. Under natural conditions capsule setting was noticed within seven days of flowering.

Morphology of pollinia was observed visually. They were crushed and stained using acetocarmine to understand the individual pollen structure, number, arrangement, viability etc. The pollen load per pollinium estimated by haemocytometer averaged to 2,06,000. Furthermore, almost all the pollen was found to be viable and 73.64% of them germinated in sucrose solution of 10 per cent strength. The fruit or capsule at maturity was 2.18 cm long and 0.86 cm wide. Undehisced mature capsules of 145-180 days of pollination was found to be best for *in vitro* culture.

In experiment II, seeds of *R. retusa* was characterized morphologically under compound light microscope and morphometric data analysed using microscopic software package Leica LAZ 500. The seeds were fusiform shaped, elongated, with prolate spheroid embryo of violet colour. The testa cells were yellowish white and reticulated. At maturity 78.74 percent of the seed volume was filled with air space. The viability of seeds used for seed culture was worked out to be 78.87 percentage. Out of the millions of seeds in a capsule 41.87 percentage of seeds were found to be well filled with large embryo of regular shape.

In experiment III- for the *in vitro* seed germination studies four different media viz., MS, half-strength MS, Knudson C orchid media and WPM including liquid and solid with and without growth hormones were prepared and used.

The seeds from mature disinfected capsules were scooped out and inoculated under sterile conditions. Five replicates of each media was inoculated with seeds. Observations were recorded on the number of days for showing seed greening and chlorophyll embryo, for protocorm development, for number of protocorms produced and number of protocorms with shoot apex and multiple shoots, number of days for root and leaf initiation. Half MS medium (liquid) was observed to be the best among the different media used for asymbiotic seed germination of *Rhynchostylis retusa.* Half MS was best media for greening and protocorm development. If we compare the overall response for different stages of development half MS with 0.5 mgL^{-1} BAP (solid) was found best for callusing and shoot proliferation. Half MS with 0.5 mgL^{-1} BAP and 1 mgL^{-1} NAA (solid) for multiple shoots, small leaves and root development. Initiation of germination was seen within two months of culture inoculation. The grain shaped protocorm needed to be subcultured at proper time and calloid like structures with multiple shoots could be divided to separate units and each unit developed to separate plantlets.

Seed characterization provides an insight into understanding biology, ecology and evolutionary background. Mass multiplication by asymbiotic seed culture can be a viable option for restoring this threatened species and can be utilized for further genetic improvement programme.

സംഗ്രഹം

"അണ്ണാൻ താളി" (വന്യ ഓർക്കിഡ്) സസ്യവിത്ത് സ്വഭാവപഠനവും അതിനോടൊപ്പം അവയുടെ ഇൻ-വിട്രോ വിത്ത് മുളപ്പിക്കൽ പ്രോട്ടോക്കോൾ തയ്യാറാക്കൽ" എന്ന വിഷയം സംബന്ധിച്ച ഗവേഷണ പഠനം വെള്ളായണി കാർഷിക കോളേജിലെ സസ്യ പ്രജനന ജനിതക ശാസ്ത്ര വിഭാഗത്തിൽ 2020-22 കാലയാളവിൽ നടത്തുകയുണ്ടായി. അണ്ണാൻ താളി വിത്തിന്റെ സ്വഭാവം പഠിക്കുക എന്നതും ഇൻ-വിട്രോ വഴി വിത്ത് മുളപ്പിക്കലിന്റെ പ്രോട്ടോക്കോൾ വികസിപ്പിക്കുക എന്നതുമായിരുന്നു പരീക്ഷണത്തിന്റെ ലക്ഷ്യം.

ജവഹർലാൽ നെഹ്റു ട്രോപ്പിക്കൽ ബൊട്ടാണിക്കൽ ഗാർഡൻ ആൻഡ് റീസർച്ച് ഇൻസ്റ്റിറ്റ്യൂട്ട് പുത്തൻതോപ്പ് -ൽ (JNTBGRI, Puthenthope) പരിപാലിച്ചുവരുന്ന സസ്യവൈവിധ്യ ശേഖരത്തിൽ നിന്നു പരീക്ഷണ വസ്തുക്കൾ ശേഖരിച്ചു.

ഗവേഷണ പ്രവർത്തനങ്ങൾ നാല് പരീക്ഷണങ്ങളായി തിരിച്ചിരിക്കുന്നു. പരീക്ഷണം I - പുഷ്പത്തിന്റെ സ്വഭാവവിലയിരുത്തൽ, പരീക്ഷണം II- വിത്തുകളുടെ രൂപഘടനയും മോർഫോമെട്രിക് സ്വഭാവവും വിലയിരുത്തൽ, പരീക്ഷണം III- ഇൻ വിട്രോ വിത്ത് മുളയ്ക്കൽ മാധ്യമം തയ്യാറാക്കൽ, ഒപ്പം പരീക്ഷണം IV- ഇൻ വിട്രോ വിത്ത് മുളപ്പിക്കലിന്റെ വിവിധ പരീക്ഷണങ്ങളും മൂല്യനിർണ്ണയവും. എല്ലാ പരീക്ഷണങ്ങളും അഞ്ച് പകർപ്പുകളിലായി നടത്തുകയും ശരാശരി കണക്കാക്കുകയും ചെയ്തു.

ആദ്യ പരീക്ഷണത്തിൽ വിവിധ പുഷ്പങ്ങൾ, പൂമ്പൊടി, കായ്കൾ എന്നിവ യുടെ പ്രതീകങ്ങൾ രേഖപ്പെടുത്തുകയും സസ്യങ്ങളുടെ സ്വഭാവം സൂക്ഷ്മമായി നിരീക്ഷിച്ച് ഗുണപരമായ പ്രതീകങ്ങൾ വിവരിക്കുകയും അളവിലുള്ള പ്രതീകങ്ങൾ വിലയിരുത്തുകയും ശരാശരി രൂപപ്പെടുത്തുകയും ചെയ്തു. പൂവിടുന്ന സ്വഭാവത്തിൽ റിങ്കോസ്റ്റെലിസ് റെറ്റൂസ സീസണൽ (ജൂൺ-ഓഗസ്റ്റ്) ആയിരുന്നു. രണ്ട് തരം സസ്യങ്ങൾ നിരീക്ഷിച്ചു, ഒന്ന് വെളുത്ത പൂക്കളിൽ ധൂമ്രനൂൽ പാടുകളോ ടെസ്സലേഷനോ ഉള്ളതും മറ്റൊന്ന് വെളുത്ത പശ്ചാത്തലത്തിൽ ധൂമ്രനൂൽ നിറമുള്ളതും.

വൈകുന്നേരത്തിനും അതിരാവിലെയ്ക്കും ഇടയിലായിരുന്നു പൂവിടരുന്നത്. പൂവിടരുന്ന സമയം മുതൽ അഞ്ച് ദിവസം വരെ ഇളം പൂക്കളിൽ ജനിതലം പൂമ്പൊടിയോടു സ്വീകാര്യമായിരുന്നു, അതിനുശേഷം സ്റ്റിഗ്മാലായിനി ഉണങ്ങുന്നു. രാവിലെ 10.30 വരെയുള്ള സമയങ്ങളിൽ ദിവസത്തിലെ മറ്റ് സമയങ്ങളെ അപേക്ഷിച്ച് പരമാവധി സ്വീകാര്യത കാണിച്ചു. സ്വാഭാവിക സാഹചര്യങ്ങളിൽ കായ്കൾ രൂപപ്പെടുന്നത് ഏഴു ദിവസത്തിനുള്ളിൽ ശ്രദ്ധയിൽപ്പെട്ടു.

പോളിനിയ (പരാഗരേണുകളുടെ കൂട്ടം) യുടെ രൂപഘടന ദൃശ്യപരമായി നിരീക്ഷിച്ചു. വ്യക്തിഗത പൂമ്പൊടിയുടെ ഘടന, സംഖ്യ, ക്രമീകരണം, പ്രവർത്തനക്ഷമത മുതലായവ മനസ്സിലാക്കാൻ അവയെ ചതച്ചശേശം അസറ്റോകാർമൈൻ സ്റ്റെൻ ഉപയോഗിച്ച് നിറം നൽകി. ഹീമോസൈറ്റോമീറ്റർ കണക്കാക്കിയ പോളിനിയത്തിലെ പൂമ്പൊടിയുടെ അളവ് ശരാശരി 2,06,000 ആണ്. കൂടാതെ, മിക്കവാറും എല്ലാ കൂമ്പോളയും പ്രവർത്തനക്ഷമമാണെന്ന് കണ്ടെത്തി, അവയിൽ 73.64 ശതമാനവും 10 ശതമാനം വീര്യമുള്ള പഞ്ചസാര ലായനിയിൽ മുളച്ചു. കായ്കൾ അല്ലെങ്കിൽ കാപ്സ്യൂൾ പാകമാകുമ്പോൾ 2.18cm നീളവും 0.86cm വീതിയും ഉണ്ടായിരുന്നു. പരാഗണത്തിനു ശേഷം145-180 ദിവസം പകമായ കായകൾ ഇൻ വിട്രോ കൾച്ചറിന് മികച്ചതാണെന്ന് കണ്ടെത്തി.

പരീക്ഷണം II-ൽ, റിങ്കോസ്റ്റെലിസ് റെറ്റൂസയുടെ വിത്തുകൾ സൂക്ഷ്മദർശിനി- കോമ്പൗണ്ട് ലൈറ്റ് മൈക്രോസ്കോപ്പിന് കീഴിൽ രൂപഘടനാപരമായി ചിത്രീകരിക്കുകയും മോർഫോമെട്രിക് വിവരശേഖരണത്തിനു മൈക്രോസ്കോപ്പിക് സോഫ്റ്റ്വെയർ പാക്കേജ് ലെയ്ക ലാസ് 500 ഉപയോഗിച്ച് വിശകലനം ചെയ്യുകയും ചെയ്തു. വിത്തുകൾ ഫ്യൂസിഫോം ആകൃതിയിലുള്ളതും നീളമേറിയതും വയലറ്റ് നിറത്തിലുള്ള പ്രോലേറ്റ് സ്ഫെറോയിഡ് ഭ്രൂണത്തോടുകൂടിയതുമാണ്. ടെസ്റ്റ കോശങ്ങൾ മഞ്ഞകലർന്ന വെള്ളയും ജാലിതങ്ങളുമായിരുന്നു. പാകമാകുമ്പോൾ വിത്തിന്റെ 78.74 ശതമാനവും വായുവിൽ നിറഞ്ഞു. വിത്ത് സംസ്ക്കരണത്തിന് ഉപയോഗിച്ച വിത്തുകളുടെ പ്രവർത്തനക്ഷമത 78.87 ശതമാനമായിരുന്നു. ഒരു കായയിലെ ദശലക്ഷക്കണക്കിന്

വിത്തുകളിൽ 41.87 ശതമാനം വിത്തുകളും സാധാരണ ആകൃതിയിലുള്ള വലിയ ഭ്രൂണത്താൽ നന്നായി നിറഞ്ഞിരിക്കുന്നതായി കണ്ടെത്തി.

പരീക്ഷണം III- ഇൽ വിട്രോ വിത്ത് മുളയ്ക്കൽ പഠനത്തിനായി നാല് വ്യത്യസ്ത മാധ്യമങ്ങൾ, അതായത് എംഎസ് (MS), ഹാഫ് എംഎസ് (half MS), നഡ്സൺ സി (Knudson C) ഓർക്കിഡ് മീഡിയ, ഡബ്ലിയു. പി.എം (WPM) എന്നിവ വളർച്ചാ ഹോർമോണുകളുള്ളതും അല്ലാത്തതുമായ ദ്രാവകവും ഖരവും ഉൾപ്പെടെയുള്ള മാധ്യമങ്ങൾ തയ്യാറാക്കി ഉപയോഗിച്ചു.

പരീക്ഷണം IV- ഇൽ പ്രായപൂർത്തിയായ അണുവിമുക്തമാക്കിയ കാപ്സ്യൂളുകളിൽ നിന്നുള്ള വിത്തുകൾ അണുവിമുക്തമായ അവസ്ഥയിൽ പുറത്തെടുക്കുകയും മാധ്യമത്തിൽ വയ്ക്കുകയും ചെയ്തു. ഓരോ മാധ്യമത്തിന്റെയും അഞ്ച് പകർപ്പുകൾ വിത്തുകൾ ഉപയോഗിച്ച് നിറയ്ക്കപ്പെട്ടു. വിത്ത് ഹരിതവൽക്കരണവും ക്ലോറോഫിൽ ഭ്രൂണവും കാണിക്കുന്നതിനുള്ള ദിവസങ്ങളുടെ എണ്ണം, പ്രോട്ടോകോം വികസനം, ഉൽപ്പാദിപ്പിക്കുന്ന പ്രോട്ടോകോമുകളുടെ എണ്ണം, കാണ്ഡഉത്പാദനവും ഒന്നിലധികം ചിനപ്പുപൊട്ടലുകളുമുള്ള പ്രോട്ടോകോർമുകളുടെ എണ്ണം, വേരുകളും ഇലകളും മുളക്കുന്നതിനുള്ള ദിവസങ്ങളുടെ എണ്ണം എന്നിവയിൽ നിരീക്ഷണങ്ങൾ രേഖപ്പെടുത്തി. റിങ്കോസ്റ്റെലിസ് റെറ്റൂസയുടെ അസിംബയോട്ടിക് വിത്ത് മുളയ്ക്കുന്നതിന് ഉപയോഗിക്കുന്ന വ്യത്യസ്ത മാധ്യമങ്ങളിൽ ഹാഫ് എംഎസ് മീഡിയം (ദ്രാവകം) ഏറ്റവും മികച്ചതായി നിരീക്ഷിച്ചു. ഹരിതവൽക്കരണത്തിനും പ്രോട്ടോകോം വികസനത്തിനും ഹാഫ് എംഎസ് മികച്ച മാധ്യമം ആയിരുന്നു. വികസനത്തിന്റെ വിവിധ ഘട്ടങ്ങളിലെ മൊത്തത്തിലുള്ള പ്രതികരണം താരതമ്യം ചെയ്താൽ പകുതി എംഎസ് 0.5 mg/L BAP (സോളിഡ്) ഉള്ളത് ക്യാലസിംഗിനും തണ്ട് വളർച്ചയ്ക്കും മികച്ചതായി കണ്ടെത്തി.

ജീവശാസ്ത്രം, പരിസ്ഥിതിശാസ്ത്രം, പരിണാമ പശ്ചാത്തലം എന്നിവ മനസ്സിലാക്കുന്നതിനുള്ള ഉൾക്കാഴ്ച വിത്ത് സ്വഭാവം നൽകുന്നു. വംശനാശഭീഷണി നേരിടുന്ന ഈ ജീവിവർഗത്തെ പുനഃസ്ഥാപിക്കുന്നതിനുള്ള

ഒരു പ്രായോഗികമായ ഉപാധിയാണ് അസിംബയോട്ടിക് സീഡ് കൾച്ചർ വഴിയുള്ള വൻതോതിലുള്ള വിഭജനം.