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EMBRYO CULTURE OF *RHYNCHOSTYLIS RETUSA*, AN EXQUISITE ORCHID OF WESTERN GHATS

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ABSTRACT

Six months old seeds released from the green pod of an exquisite epiphytic orchid, *Rhynchostylis retusa* were cultured in Mitra *et al* and Knudson C liquid nutrient medium supplemented with casein acid hydrolysate (CH), yeast extract (YE), peptone and coconut water (CW). Mitra *et al* liquid medium supplemented with 20% (v/v) to achieve 78% seed germination and maximum protocorms growth in 90 days. YE and CH also supported proliferation and growth of the protocorm. Developed protocorms after 90 days in culture were transferred to solid Mitra *et al* medium containing the same additive. After the 2nd subculture, seedlings transferred to banana pulp containing medium, healthy shoots with roots were obtained. Seedlings were established at 93% rate in the nursery without hardening. Six month old established seedlings in community pots were introduced into different trees available in the Department Garden, at Kariyavattom and 83% rates of establishment after 12 months. The results indicate the conservation of this exquisite orchid through *in vitro* multiplication and introduction into other habitats.

Keywords: Green pod, Protocorms, Epiphytic Orchid

INTRODUCTION

Biodiversity, which encompasses all life forms on earth, can be auto- sustainable and self regenerating if there are no natural or manmade perturbations. India is one of the 12 mega biodiversity countries of the world. Indiscriminate exploitation and various

anthropogenic pressures lead to depletion of this valuable gift of nature. Western Ghats, one of the richest floristic regions in the country is having about 4000 flowering plant species which is about 30% of the flowering plants in the country **[1]**.

According to the IUCN Action Plan, orchids are among the world's most diverse and widely distributed plants. Orchids are beautiful, fascinating flowers that have long held a grip on the human imagination, perhaps due to their sexual appearance. The bloom of an orchid plant is so gorgeous and distinctive that one botanist was led to describe orchids as 'living jewels'. These perennial plants have adapted to almost every environment on earth, and this has led to a great diversity in orchids. There are between 25,000 to 30,000 different kinds through the world. Additionally, there are also approximately 60,000 known types of orchid hybrids that have been created by orchid growers. They have emerged as leaders in floriculture and account for multimillion dollar cut flower industry in several countries **[2]**. The majority of orchids in cultivation are native of tropical belt and occur in their profusion in humid tropical forests of central and South America, India, Sri Lanka, Burma, south China, Australia, Thailand, Malaysia, Philippines, New Guinea etc.

The widely diverse climatic regions of India are reflected in the wide diversity of its orchid flora. India with 1129 species in 184 genera **[3]** is one of the major orchid habitats of the world. The Indian Himalayan Region alone harbours about 876 species in 151 genera **[4]**. The Indian orchids grow at an altitude up to 5,000 m, and in areas having an annual rainfall of as low as 600 mm to as high as 1,100 mm. The epiphytic orchids are abundant up to 1,800 m and their frequency progressively decreases with further increase in altitude. Majority of the terrestrial orchids, on the other hand, are confined to temperate regions.

The Western Ghats region in the peninsular India is a known mega diversity centre and is one of the richest orchid habitats in the world. Altogether, the Western Ghats harbour 288 species of orchids in 76 genera of which 79 species belonging to 45 genera were reported from the Agasthyamala region alone, a hot spot in the Western Ghats **[5]**. It has been reported that many of our native orchids are an untapped resource. They are showy and promising and hence have great potential in breeding programme to raise novel hybrids of immense beauty.

Conservation of the shrinking plant gene pool especially of the rare and endangered taxa of known potential economic source is a well-debated issue all over the world. Plant tissue culture is an effective tool to

conserve plant genes and guarantee the survival of endangered and overexploited genotype is derived from the fact that it makes sure of small units (cell and tissues) without losing the mother plant, take the pressure of waning wild populations and makes available large number of faithful copies of plants for reintroduction and wide distribution. Particularly for orchids, seed (embryo) cultures offer opportunities of easy and rapid multiplication, maintenance of diversity within species and facilitate reintroduction and restoration of taxa into the native habitat. The Ministry of Environment and Forest, Govt. of India and IUCN have recommended applications of seed and tissue culture to save and multiply endangered taxa and their conservation through reintroduction.

Rhynchotylis an Indo-Southeast Asian epiphytic genus characterized by short stems, thick and fleshy leaves and lateral inflorescence of closely arranged flowers. In India the genus is represented by a single species *Rhynchotylis retusa* (L.) Bl **(Figure 1).** This was first described and illustrated by Van Rheede from Kerala. Stem woody, covered with old leaf base. Leaves thick, leathery, 15-20 cm long, 2 cm wide. Leaf tip blunt unequally lobed. Inflorescence axillary, 1-7 per plant and drooping. Raceme 20-30 cm long. Flowers at the tip opening first densely arranged, pink, white and deep magenta in colour. Pedicel purplish, Flowers 1.8 cm across. Sepals and petals white with a faint pinkish tinge. Lip spurred. Column short, slender dotted with magenta.

The Objective of the study was to develop appropriate micro propagation methods for multiplication of this rare orchid and strengthen the resource base through restoration.

MATERIALS AND METHODS

Embryo culture

Six-month old green capsule with an average length of 2.86 cm long and 2.06 cm wide were collected from Bonnacard forest area of Western Ghats **(Figure 2)**. The average length and width of the seeds were 282.4 µm and 138.95 µm respectively and embryo with an average length and width of 85.86 µm and 44.63 µm respectively were used for embryo culture **(Figure 3)**.

Media

Liquid media were used for seed germination and protocorm growth and solid medium was employed for protocorm proliferation and seedling development. Knudson C medium **[6]** and Mitra *et al* medium **[7]** with different organic additives like casein acid hydrolysate (0.05%), peptone (0.05%), yeast extract (0.05%), coconut water (20 %), were tried. The salt of respective media were weighed accurately and individually added to a small

volume of distilled water. Vitamins were added from stock solutions. The carbon source and other additives were added and made up to the required volume. Required pH of the medium viz. Knudson C (5.2) and Mitra et al (5.2) were adjusted using 1N HCl or 1N Na OH using a digital pH meter (Systronic India Ltd, Mumbai). Agar (0.8%) for solid medium was added to the media and melted in a water bath and dispensed into conical flasks or culture tubes and autoclaved (National Steel Equipment Pvt Ltd, Mumbai) for 18 min and 1210C and 111.1kg/cm^2. For subculture of protocorm Mitra *et al* solid medium supplemented with 0.05% CH, P, YE and 2.5- 10 % banana pulp were tried.

Surface Sterilization and Inoculation

The capsule were collected from the Bonnacord forest area during the month of December and brought to the laboratory. The green pods were washed in running tap water using 10 % detergent labolene (Qualigen India Ltd, Mumbai) solution and washed in tap water. The washed pods were rinsed in distilled water and taken inside a laminar air flow hood where the green capsules were immersed in 0.1% Hg Cl_2 solution for 12 min. The capsules were then washed thrice in sterile distilled water. The surface decontaminated capsules were taken in a sterile Petri plate, split open vertically and the seeds were scooped into 20 ml of sterile distilled water. Uniform seed suspension was inoculated into liquid Knudson C and Mitra *et al* medium containing various organic additives. An aliquot of seed suspension was tested for its seed viability.

Culture Conditions

Cultures were raised in a gyratory shaker at 80 rpm and incubated at 25 ± 2^0C under 12hr photoperiod with an illumination of 1500 lux provided by Philips day light fluorescent tubes. Observations were made at weekly intervals. After 90 days representative samples were taken from each flask and fresh weights as well as diameter measurements were recorded.

Subculture

Protocorms 90 day old raised in Mitra *et al* medium basal and containing organic additives were subculture in solid Mitra medium containing same organic additives like CH, YE, peptone and CW and banana pulp. Observations were made after 3rd subculture in terms of number of roots, number of leaves, length of roots and length of shoots.

The experiments were properly planned to facilitate statistical analysis. Randamised Block Design (RBD) was performed. All the experiments were repeated thrice and identical physical conditions were maintained for the entire period of investigation and for every replicate.

RESULTS

The capsules possess 89 % viable seeds. After twelve days of inoculations, the seeds started to germinate followed by yellow pigmentations within 20 days. After 20 days, greening of protocorms occurred in Mitra medium. But in Knudson C medium all the protocorms appears to be creamy white or yellow in colour.

Mitra *et al* medium containing 20 % CW was selected as the best for protocorms development **(Figure 4)** followed by peptone, YE and CH. Knudson C basal medium and supplanted medium also supported seed germination but the growth of the protocorm was inferior to Mitra *el al* medium **(Table 1)**.

Morphogenesis of the protocorm was observed after 10 days of subculture in Mitra *et al* medium containing additives. Irrespective of the medium in which the protocorms were raised, CW supported high rates of shoot proliferation and vigorous growth of the seedlings **(Figure 5)**. YE and CH also supported proliferation and growth of the protocorm to some extent resulting reduced vigour compared to CW and peptone. After the 2nd subculture, seedlings were transferred to Banana pulp containing medium, healthy shoots with roots were obtained. After the 3rd subculture lasting 45 days were deflasked and transferred to community pots. Maximum healthy seedlings were obtained in medium containing 7.5% banana pulp **(Table 2)**.

Seedlings were established at 93% rate in the nursery without hardening. Six moth old established seedlings in community pots were introduced into different trees available in the Department Garden, at Kariyavattom and 83% rates of establishment after 12 months. The established protocol is efficient to propagate this rare and exquisite orchid which is necessary to keep pace with the need and keep of the species from extinction.

Table 1: Green Pod Culture of *Rhyncostylis retusa* in Different Nutrient Medium

Media	Additives	Germination (%)	Pigmentation	Protocorm Fresh wt Mean ±SD n=3	Diameter of the protocorm (mm)
Mitra	Nil	43	Creamy white	3.73±1.65	1.27±0.69
	0.05%CH	73	Green	13.48±0.98	2.58±0.56
	0.05%YE	50	Pale green	7.32±1.98	2.4±0.54
	0.05%P	71	Pale green	10.83±1.25	2.48±0.39
	20%CW	78	Yellowish green	15.95±1.78	3.12±0.74
Knudson C	Nil	46	Creamy white	1.73±130	1.37±0.73
	0.05%CH	68	Green	3.84±1.87	1.57±0.67
	0.05%YE	53	Pale green	3.43±2.12	1.29±0.67
	0.05%P	71	Pale green	4.1±1.94	1.54±0.63
	20%CW	73	Yellow	5.4±1.82	1.73±0.68

Observation were made after 90 days of culture

Table 2: Relative Growth of Seedlings of *R. retusa* in Different Concentrations of BP

S. No	Medium	BP (%)	Response
1	Mitra	2.5	3 leaves 3 roots
2	Mitra	5.0	3leaves 4 roots
3	Mitra	7.0	5 leaves 4 roots
4	Mitra	10.0	3 leaves 2 roots

Observation were made after 45 days of culture

Figure 1: *Rhynchotylis retusa* (L.) Bl

Figure 2: Six-month Old Green Capsule

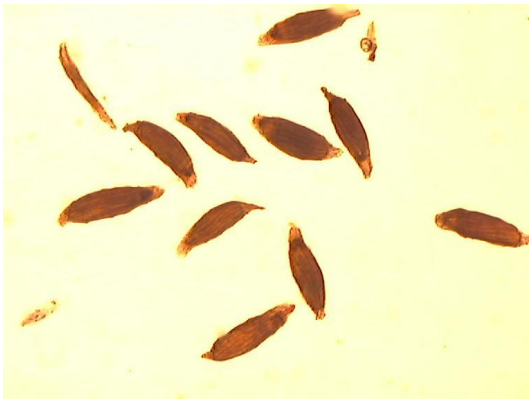

Figure 3: Seeds Used for Embryo Culture

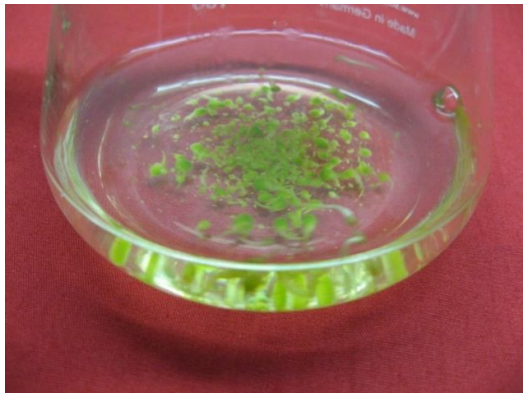

Figure 4: Protocorms Development

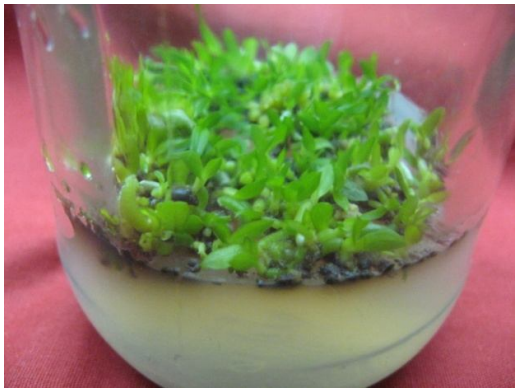

Figure 5: Shoot Proliferation and Vigorous Growth of the Seedlings

DISCUSSION

Seed cultures of a number of species including tropical epiphytes, tropical lithophytes, tropical terrestrials and temperate terrestrials developed with varied levels of success **[8]**. In orchid seed germination, the protocorms represents the intermediate stage between germination and organogensis **[9]**. In the present study on *R. retusa*, asymbiotic seed germination, protocom formation and development of seedlings largely depended on the culture media, additives used and age of the capsule. Mitra medium was superior to Knudson C medium. The lesser amount of ammonium salt, nitrates of calcium and potassium and phosphate ions and various minor salts and vitamins in Mitra *et al* medium are related to its enhanced influence on seed germination **[10]**. Eight month old capsules got maximum seed germination in *R. retusa*. The relative time taken after pollination wherever embryos / ovules could be successfully germinated seems to be varying with species **[11]**. Immature seeds dissected out of green pods as followed in the present study are more preferred not only for the ease of surface sterilisation but also for obtaining highest germination percentages **[12]**.

A large number and bewildering number of organic additives, undefined and unwanted or untried in other plants are routinely used for orchid seed, protocorm and seedling culture. Of the different growth additives used, 20% coconut water was found be the best additives. The promotary effect of coconut water (20%), the most frequently used complex additive may be related to its highly active natural cytokinins and other minerals present in coconut water and CW is the liquid endosperm of coconut frequently used as the complex additives in tissue culture media **[13]**.

The growth stimulating effects of banana pulp on seedlings are well documented **[14]** though the reasons are not clear **[15]**. The conditions of weaning away the seedlings, potting and post transplantation care in a nursery were sufficient to ensure reasonably high percentage (93%) survival without hardening. The established protocol is efficient to propagate this beautiful rare orchid. Six moth old established seedlings in community pots were introduced into different trees available in the Department Garden, at Kariyavattom and 83% rates of establishment after 12 months. The results indicate the conservation of this exquisite orchid through *in vitro* multiplication and introduction into other habitats.

CONCLUSION

Seedlings were raised from green pod of *R. retusa* in Mitra et al medium fortified with 20% CW. Seedling thus raised were established at 93% success rate in

community pots without hardening. Six month old community potted plants were successfully introduced in to native tree species available in the Department garden. The successful establishment revealed the amenability of the embryo culture derived plants for restoring forest region of Western Ghats with orchids thereby conserving rare and exquisite species.

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