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Asymbiotic Seed Germination, Mass propagation and Conservation of Fox-tail orchid, *Rhynchostylis retusa* L. Blume: An endangered orchid

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ABSTRACT

Experiments were carried out to identify the correct maturity stage for *in-vitro* seed germination and to develop an *in-vitro* protocol for mass propagation of *Rhynchostylis retusa*. After hand pollination of the flowers the length, girth and color changes of the pod were observed in weekly intervals in order to determine the morphological indicators for maturity. *In-vitro* seed germination was done 3, 4, 5, 6 and 7 months after pollination on nine media viz 1. Murashige and Skoog medium (MS) 2. MS with PVP (2g/L) 3. MS with charcoal (2g/L) 4. Knudson C medium (KNC) 5.KNC with PVP (2g/L) 6. KNC with charcoal (2g/L) 7. KNC with banana extracts (75g/L) 8.Vacin and Went medium (V&W) 9. V&W with banana extract (75g/L) and coconut water (150ml/L). Results revealed that six months after pollination was the best stage for *in-vitro* seed germination of *R. retusa.* Significant germination was observed only on MS with PVP, MS with charcoal, KNC with banana extracts and V&W with banana extracts and coconut water ($p \leq 0.05$). KNC with banana extracts and V&W with banana extracts and coconut water gave 100% seed germination. Six- month-old pods took 20 days to germinate. In vitro derived plantlets were acclimatized in coconut husk chips with 75% survival.

Key words: biodiversity, conservation, embryo culture, endangered orchids, mass propagation, tissue culture

INTRODUCTION

In Sri Lanka, Orchids (belongs to family Orchidaceae) are among prominent flora, which is adapted to a wide range of eco-climatological zones. However, their existence has been endangered due to various pressures on the environment imposed by man. Orchids with showy flowers and with medicinal properties encounter an added disadvantage due to over-collection from the wild. Although, legislative measures play an important role, the most effective measure to conserve orchids is to make available the species in the required amounts and reintroduce them to their natural habitats.

Rhynchostylis retusa (L.) Blume, which has been identified as an endangered species is an epiphytic plant with a robust stem of about 25 cm long. Pendulous inflorescences of the plant are densely flowered with attractive flowers and about 60 cm long (Sinha and Jahan, 2012). The plant is reported to grow in Sri Lanka, India and Bangladesh up to Philippines. *Rhynchostylis retusa* has been used to treat conditions like paralysis, rheumatism, allergies, abnormal menstruation (Akhter *et al*., 2017) and malaria fever (Tiwari *et al*., 2012) in traditional medicine. 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. The suggested approach to preserve this orchid requires high capacity multiplication techniques to generate the required plants. Reports on mass production of *R. retusa* through *in vitro* propagation are limited. Hence, the present study investigated an *in-vitro* method using embryo culture aimed at generating the required plants for the proposed conservation plan.

MATERIALS AND METHODS

Mother plants were collected from their natural habitats (Bibila, Nilgala area in Sri Lanka) after getting permission from Department of Wildlife Conservation and Forest Department, Sri Lanka. Natural habitats were found from revised handbook to the "Flora of Ceylon" and referring IUCN, the world conservation union maps.

(A) Morphological indicators of maturity

Flowers of *R. retusa*, which are maintained under greenhouse conditions, were hand pollinated. Pod length, girth and visual observations such as color, appearance and texture were recorded at weekly intervals.

(B) Effect of pod age on germination

Developed pods were removed from the mother plant at different intervals (3, 4, 5, 6 and 7 months after pollination). Harvested pods were kept under running tap water for half an hour. The water was removed and flask was taken to laminar flow cabinet. Then the flask was filled with 10% Clorox solution and shaken for 20 minutes. After, the Clorox solution was removed and pods were washed three times with sterilized distilled water. Then the pod was dipped in absolute ethanol and then the alcohol is burnt off. This was to burn off any bacteria or fungi on its surface. Pod was then placed on a sterilized surface and cut lengthwise. The seeds within the pod resembled white color. Then the seeds were scraped onto the medium.

(C) Effect of media on germination

The Murashige & Skoog (MS), Knudson C (KNC) and

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Vacin & Went (V & W) basal media and their modifications were tested for seed germination as listed below (Table 1).

Table 1. Media tested for *in-vitro* germination

Basal media	Modification of the media
MS	1.MS
	2.MS+PVP (2g /L)
	3. MS + Charcoal (2g /L)
KNC	4. KNC
	5. KNC+ PVP (2g /L)
	6. KNC + Charcoal (2g /L)
	7. KNC + Banana extracts (75g/L)
V& W	8. V& W
	9. V& W + coconut water (150ml/L) + Banana extract (75g/L)

(D) Hardening of the plantlets

Developed plantlets were taken out from the flasks and washed thoroughly with tap water. Dead leaves and roots were removed and plantlets were again washed with lukewarm water to remove agar completely. Then plantlets were dipped in mild fungicide solution for 5–10 minutes. Plantlets were established in charcoal medium and kept in a propagator. All experiments were arranged as Complete Randomized Design and treatments were replicated in four times. Data were analyzed using SAS 9.3 software package.

RESULTS AND DISCUSSION

(A) Morphological indicators of maturity

With maturity, pods, which were pale green initially, turned into dark green. During first three months the length and the girth increased (Table 2) and after that pod filling was observed. Average pod length was maximum after 11 weeks (Figure 1). When the pods were fully mature the ridges became prominent and remained light green and appeared as a ribbon (Plate 1-c).

Though records on *in vitro* propagation of *R. retusa* are limited, according to Bhatti *et al*. (2016) there are some records on mass propagation through symbiotic seed and organ culture. *In vitro* propagation through a symbiotic seed germination can be identified as an alternative method of mass propagation for *R. retusa* (Thomas and Michael, 2007).

Development of an *in vitro* propagation protocol through seeds for any orchid can be specific to the species and depend on factors like pod maturity (Ávila-Díaz *et al*., 2009). According to Kumar *et al*., 2002. *R. retusa* has been *in vitro* propagated through green pods. But, the morphological factors to identify the correct maturity stage of pods was not indicated. According to the current study mature pods are dark green in color with prominent light green ridges and both length and girth are about 3.8 cm.

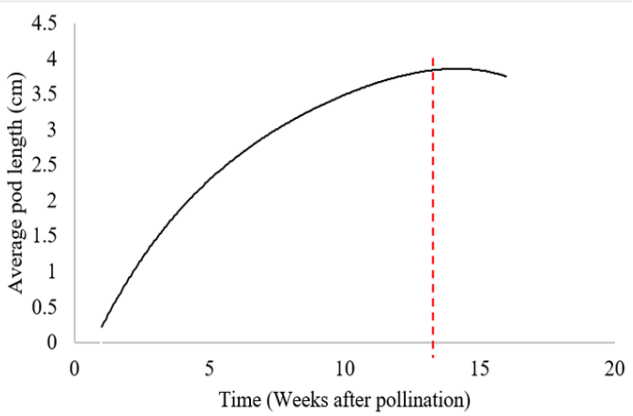


Figure 1. Change in average pod length of *Rhynchostylis retusa*

(B) Effect of pod age on germination

Green pods were harvested at 3, 4, 5, 6 and 7 months after pollination. 6 months after pollination the seeds within the pod resembled white powder with white hairs. Seeds collected from 6- month- old – pods took 20 days

Plate 1. Developmental stages of the pods of *Rhynchostylis retusa* (a = flowers; b = developing pods; c = fully mature pods)

Table 2. Pod length and girth of *Rhynchostylis retusa*

months	1			2				3				4			
weeks	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Avg. pod length (cm)	1.2	1.6	1.8	2.0	2.5	3.1	3.1	3.4	3.5	3.6	3.8	3.8	3.8	3.8	3.8
Avg. girth (cm)	1.0	1.5	1.8	2.0	2.4	2.6	2.7	2.9	3.0	3.2	3.4	3.5	3.8	3.8	3.8

to germinate and 100% germination was observed (Table 3). Immature seeds (3, 4, 5 months old) turned into black and did not germinate. Addition of charcoal and PVP to the medium did not show any improvement. After six months pods did not show any sign of germination. Seeds cannot be collected after seven months due to dehiscent of pods.

Table 3. Pod age and time taken for germination

Age at maturity (days)	Germination %	Observation time taken for Germination (days)
90	0	30
120	0	30
150	0	30
180	100	20
210	0	30

To get success through *in vitro* mass propagation of *R. retusa,* the appropriate pod age (Days after pollination) for seed collection for mature seed culture has been identified as 180 days. Forty to eighty days old pods were used for immature seed culture of *R. retusa* (Parab and Krishnan, 2012) in early studies. According to Thomas and Michael (2007), immature seeds obtained from 50 days old pods were light green in color and can be differentiate from mature seeds easily. For the orchid *Dendrobium nobile* Lindl., mature seeds harvested in158 days have given a higher frequency of seed germination than immature seeds harvested in 96 and 116 days (Vasudevan and Staden, 2010). Therefore, seed maturity can be identified as a crucial factor for *in vitro* seed germination of orchids.

(C) Effect of media on germination

Germination was observed in all media (plate 2). But significant germination was observed only on MS with PVP, MS with charcoal, KNC with banana extracts and V&W with banana extracts and coconut water ($p \leq 0.05$).KNC with banana extracts and V&W with banana extracts and coconut water gave 100% seed germination (Plate 3). Percentage of germination varied with different media tested. Results of germination percentages are given in Figure 2.

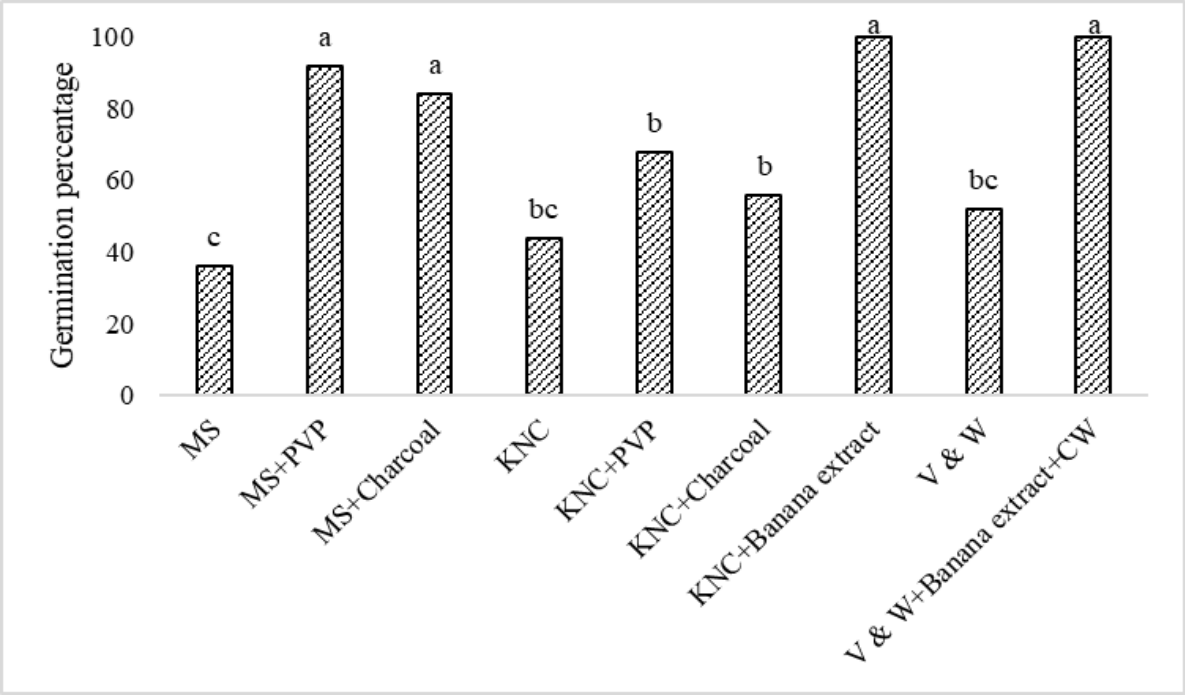


Figure 2. Germination percentages of *Rhynchostylis retusa*

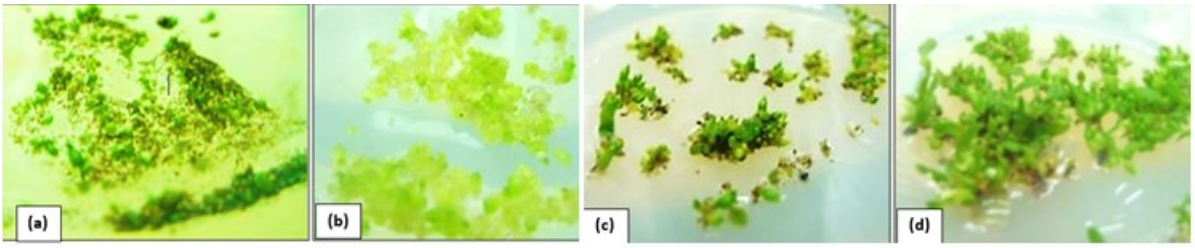
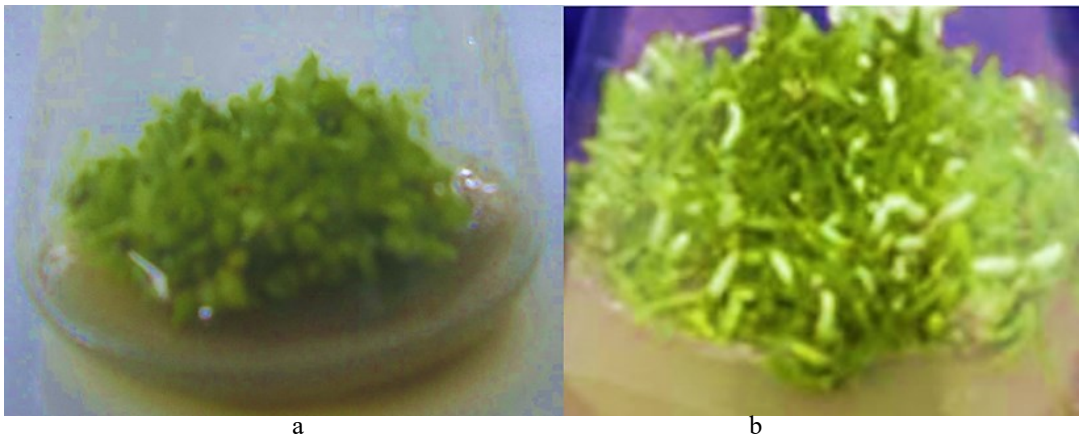


Plate 2. Stages of seed germination of *Rhynchostylis retusa*



a b

Plate 3. Germinating seeds of *Rhynchostylis retusa* (a = KNC + Banana extract, b = V & W + Banana extract + Coconut water

Hence the concentrations of irons 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). Records on suitable media for *R. retusa* seed culture is limited as H_3P_4 medium with 3 mg/L Hyponex and 4g/L Peptone (Naing *et al*., 2010) for mature seeds and Murashige and Skoog (MS) medium with 1 mg/L BA, lmg/L NAA and 15% coconut water (v/v) (Parab and Krishnan, 2012). Seedling growth of *R. retusa* was recorded to been maximum in MS medium supplemented with 6 µM BA, 0.2 µM NAA and 1g/L activated charcoal (Thomas and Michael, 2007) and MS medium supplemented with 1 mg/LNAA and 1.1 mg/L TDZ (Naing *et al*., 2010). Other than for seeds and seedlings, media for explants as shoot tips, leaves, root tips and nodal segments obtained from *R. retusa* have been optimized in previous studies. According to Sunithibala and Neelashree (2018), Green protocorm – like bodies and calli which later differentiate into plantlets were obtained from shoot tip, leaf and root tip explants of using Vacin and Went medium supplemented with 0.1 mg/L NAA along with either 1.0 mg/L Kinetin or 1,0 mg/L BAP. Half strength MS medium with 2% sucrose, 1.5 mg/L BA, 0.5 mg/L NAA 2 g/L peptone, 10% (v/v) coconut water and 0.5 gL-1 activated charcoal reported to produce higher amount of micro shoots from nodal segments of *R. retusa.*

The technique standardized here may be useful in the re-establishment of *R. retusa* in its natural habitat and for large scale multiplication for other commercial uses. The technique is efficient, quick and highly reproducible and can be used in advance biotechnological research not only for this specie but also for the micropropagation of other orchids.

CONCLUSION

Embryo culture can be successfully applied for mass propagation of *Rhynchostylis retusa.* Correct maturity stage has to be identified for *in-vitro* seed germination. Six months after pollination was the correct maturity stage for *R. retusa* and 6 months old pods took 20 days for *In-vitro* seed germination.

Addition of charcoal (2 g/L) and PVP (2g/L) to the MS medium and V & W with banana extracts (75g/L) and coconut water (100ml/L), KNC with banana extracts (75g/L) increased the germination of *R. retusa* significantly.

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