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SPECIES SPECIFIC POLLINATION AND SEED GERMINATION IN *RHYNCHOSTYLIS RETUSA* BLUME (ORCHIDACEAE)

Thakur U N[1*], Dongarwar N M[2]

1. Department of Botany, Institute of Science, Nagpur, Maharashtra, India.
2. Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, University Campus, Amravati Road, Nagpur, Maharashtra, India.

ABSTRACT: Natural pollination in Orchidaceae is coevolved with the pollinator and this coevolution sometime leads to the decline of orchid species as their pollinator is declining regularly. In present investigation pollination biology of *Rhynchostylis retusa* was studies. Different ages of pollinia and stigma were used for artificial pollination. Capsule developed by artificial pollination was subjected to asymbiotic seed germination prior to capsule dehiscence. Different parameters were studied as per the nutritional need of the taxa like five asymbiotic media (Vacin and Went, Modified Knudson C, ½ MS, Lindeman and BM1), three photoperiods (0/24, 16/8, 24/0 h L/D) for asymbiotic germination and three photoperiods (8/16, 12/12, 16/8 h L/D) were examined for seedling development. Seed germination percentage was highest on BM1 and ½ MS (BM 1, 86.66±1.20%) (½ MS, 79.01±6.08%).

KEYWORDS: Artificial, Pollination, Asymbiotic, Germination, Seed.

Corresponding Author: Dr. Thakur U N*

Department of Botany, Institute of Science, Nagpur, Maharashtra, India.

Email Address: umanthakur@gmail.com

1. INTRODUCTION

The Orchidaceae is one of the largest, most diverse categories of botanically and commercially significant flowering plants with 20,000–30,000 species [1], [2], [3], [4], [5], [6], [7], [8], [9]. *Rhynchostylis* have very beautiful and attractive pink colored flowers which increases the value of that plant in the ornamentals. It is an epiphytic orchid which grow on the trees. In wild state, the plants fix themselves to the trunk and branches of living trees. In present investigation explorations

were made in the month of June- July for the collection of plants. Plant specimens had been collected from Semadoh (Chikaldara, Amravati District). Orchids have very specialized and diversified pollination system which is species specific [10], [11]. Infact, it is believed that pollinator and Pollination mechanism adopted by the species is coevolved due to which particular species facing problem in pollination if pollinator is absent [12], [13], [14], [15], [16], [17]. During exploration it was observed that the pollination rate was very high in nature, but when those plants were collected and artificially pollination was tried the pollination rate was very low. For knowing that region different pollination experiments were tried in the species.

2. MATERIALS AND METHODS

Procured plants were acclimatized in the poly house of Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University. Extra care was taken to protect the plant from external stress in order to increase survival percentage of the species. Artificial pollination was tried to develop the capsules and to study the breeding system of the species. The *R. retusa* was also observed for autopollination (flowers were bagged before anthesis using paper bags until senescence, excluding pollinators), apomixes (before anthesis, the entire pollinium was removed with forceps, and the flowers were then bagged until senescence), self- compatibility(the pollinium was removed and flowers were bagged until stigma receptivity, flowers were then pollinated with the whole pollinium from another flower on the same inflorescence), cross pollination (flowers were pollinated with pollen from another individual of the same species) and natural pollination.

Hand pollination procedure

Before pollination male and female part of the flower were identified. The Pollinium from male parent was removed and collected on a clean paper from the tip of anther cap in the same way pollinum was also taken out from the female parent. Now pollinium of the male parent was transferred over stigmatic surface of the female parent with the help of a needle. Different ages of pollinia and stigma were used to observe maximum receptivity at different interval of time form 6a.m. to 6p.m. Artificially pollinated flowers were monitored for flower senescence, capsule development, and capsule dehiscence. Capsules were harvested before dehiscence for asymbiotic seed germination.

Asymbiotic media screen

In the present investigation five different media like (Vacin and Went, Modified Kundson C, ½ MS , Lindemann and BM1) were examined for asymbiotic germination (Table 1). Best selected medium was observed for seed germination in three light treatments (0/24, 16/8, 24/0 h L/D) and further three.

Table 1: Comparative mineral salt content of five asymbiotic Orchid Seed germination media

	VW	KC	BM1	LM	½ MS
Macronutrients (mM)					
Ammonium	3.78	10.02		15.14	10.31
Calcium	0.4	2.12		2.12	1.5
Chlorine		3.35	0.0021	14.08	3.1
Magnesium	1.01	1.01	0.83	0.49	0.75
Nitrate	5.19	8.37		2.12	19.7
Potassium	7.02	5.19	2.2	15.07	10.89
Phosphate	2.47	1.84	2.2	0.99	0.63
Sulfate	5.3	4.92	1.1	8.1	0.86
Sodium	0.1		0.2		0.1
Micronutrients (µM)					
Boron			161.7	16.4	50
Cobalt			0.11		0.053
Copper			0.1	0.1	0.5
Iron	183	90	100.2	17.96	50
Iodine				0.6	2.5
Manganese	33	37	147.9	37.63	50
Molybedenum			1.03		0.52
Zinc			34.8	3.5	14.95
Nickel				0.24	
Organics (mg/l)					
D-Biotin			0.05		
Casein hydrolysate			500		
Folic acid			0.5		
L-Glutamine			100		
Glycine			2	2	2
myo-Inositol			100	100	100
Nicotinic acid			5	1	0.5
Pyridoxine. HCl			0.5	1	0.5
Thaimine. HCl			0.5	10	0.1
Total mineral salt concentration (mM)	25.27	36.82	6.53	58.11	47.84
Total N	8.97	18.39	0	17.26	30.01
NH4:NO3	0.73	1.12	0	7.14	0.52

light treatment (8/16, 12/12, 16/8 h L/D) for seedling development. pH was adjusted to 5.8 before autoclaving. The mature capsules before dehiscence were collected in paper bags and seeds were used for germination. The seeds were surface disinfected with 0.1% Mercuric Chloride for 3minutes, followed by 70% ethanol for 30 sec and rinsed with distilled water. Seeds were collected with the help of a sterilized spatula and small mass of the aggregated seeds was sown on different culture media for *in vitro* asymbiotic seed germination. Observations were made by using five replicates for each medium. Germination and development stages were observed as per mentioned in table 2. Cultures were maintained at 25 ± 5 C°. Germination percentages were calculated on the basis of seed germinated by total seeds inoculated (Approximately). Growth characteristic of the seedling in every media were observed to check that which medium is best for germination and seedling

development. The data of different stages of germination was recorded on a particular week like week 6 and week 14. All the data were subjected to one-way analysis of variance (ANOVA), and the means were compared by Duncan's multiple comparison test ($\alpha = 0.05$) using DSAASTAT version 1.1. Means are presented with standard error (±SE).

Table 2: Seed germination and protocorm development in *R. retusa*

Stage	Description
0	Hyaline embryo, testa intact
1	Embryo swollen, rhizoids present (=germination)
2	Continued embryo enlargement, testa ruptured
3	Emergence of first leaf
4	Elongation of first leaf and further development
5	Complete developed plant

3. RESULTS AND DISCUSSION

The fragrant orchid, *R. retusa* blooms in the month of June- July. Bright pink colored flowers, with amazing fragrance may be pollinated by honey bees or wasp in nature. The pollination percentage was quite high in nature (Table 3). But when plants were procured and acclimatized in our locality the pollination was negligible, may be because of absence of pollinator. Self and cross pollination tried in the plant for developing seed sets. During present investigation, it was observed that *R. retusa* was self incompatible, non autopollination and non apomictic plant. The durability of unpollinated flowers remains for many days, but as pollination accomplished, flowers start wilting and senescence.

Table 3: Pollination biology of *R. retusa*

No. of flowers	Treatments	No. of capsule developed	Pollination %
200	Natural pollination in nature	165	82.2
45	Self pollination	0	0
30	Autogamy	0	0
43	Apomixis	0	0
250	Cross pollination (2009)	140	56
186	Cross pollination (2010)	109	58.60
167	Cross pollination (2011)	120	71.85
105	Cross pollination (2012)	86	81.90

The artificial pollination percentage was quite small in poly house (Table 3), so different ages of stigma and pollinium was tried to develop capsule at different time intervals from 6a.m. to 6p.m. In first year the pollination success rate was very low. Different ages of pollinia and stigma was tried for the pollination and come to conclusion that young pollinia (1-7) and stigma (2-8 day) 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 6a.m. to 10a.m. was the best time for pollination success from the rest of day. After finding the maximum receptivity of the stigma and pollinia in the next year the pollination rate was increased quite high (Table 3).

Asymbiotic seed germination

Seeds began swelling within two weeks after inoculation and germination commenced within three weeks after inoculation on BM1 and ½ MS. Germination was observed in all the media but the time of germination varies with the media. Out of all media tested, BM1 commenced highest germination percentages (86.66 ± 1.20%) and earliest protocorm development. On MS medium, the seed germination was (79.01 ± 6.08%) and seedling development was in similar way as on BM1. Germination percentage on KC, LM, and VW are 30.1 ± 1.47%, 39.8 ± 2.55%, 33 ± 1.45% respectively but further development was quite slow. To describe the response of different media on germination, we had discussed the week 6 and week 14 and all the media were screened for different developmental stages (0, 1, 2….5). On 6th week, ½ MS and BM1 showing that 58.2 ± 3.85 and 25.68 ± 2.62 (respectively) number of seeds were in stage 2, it conclude that the germination rate is high in ½ MS and BM1. On the same day it was observed that in other media growth of the seeds was supported upto stage 1.On the basis of 6th week it was cleared that germination started early on ½ MS but the development of seedling was early on BM1 (week 14) that is why Stage 5 was more in BM1 (19.46 ± 0.04) than ½ MS (11.28 ± 0.19). Seeds on other media were responding slowly. In all the media seedlings development were observed but the time of development was very slow on VW, KC and LM. The best media for asymbiotic germination of *R. retusa* were BM1 and ½ MS. Once the medium was decided for the seed germination and development further photoperiod effect was tested on both germination and seedling development.

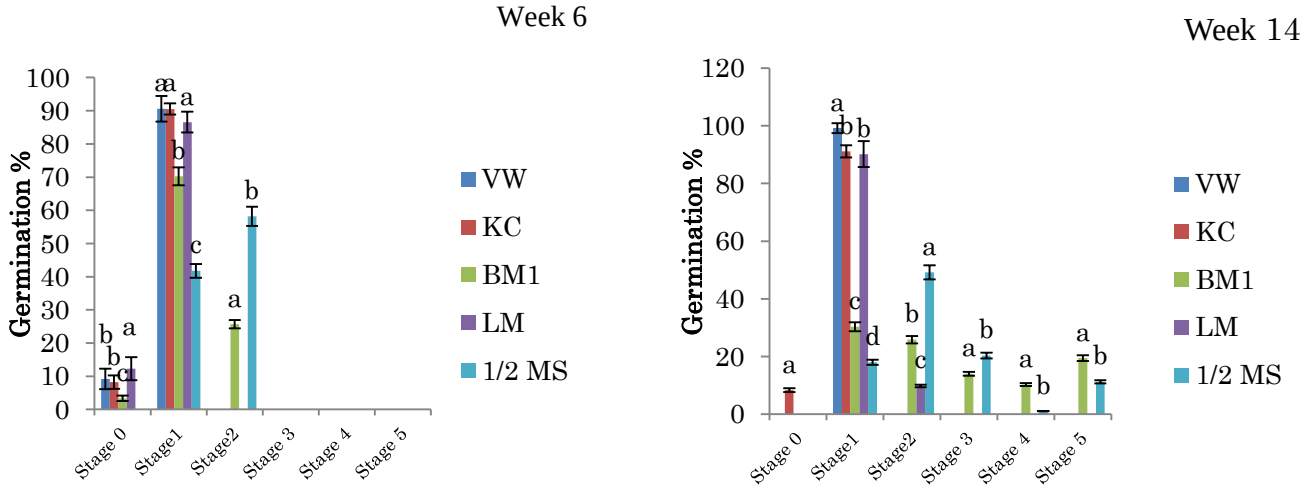

Fig 1: Effects of culture media on percent seed germination and protocorm development of *R. retusa* after 6 & 14 weeks. Histobars with the same letter are not significantly different within stages (α= 0.05).

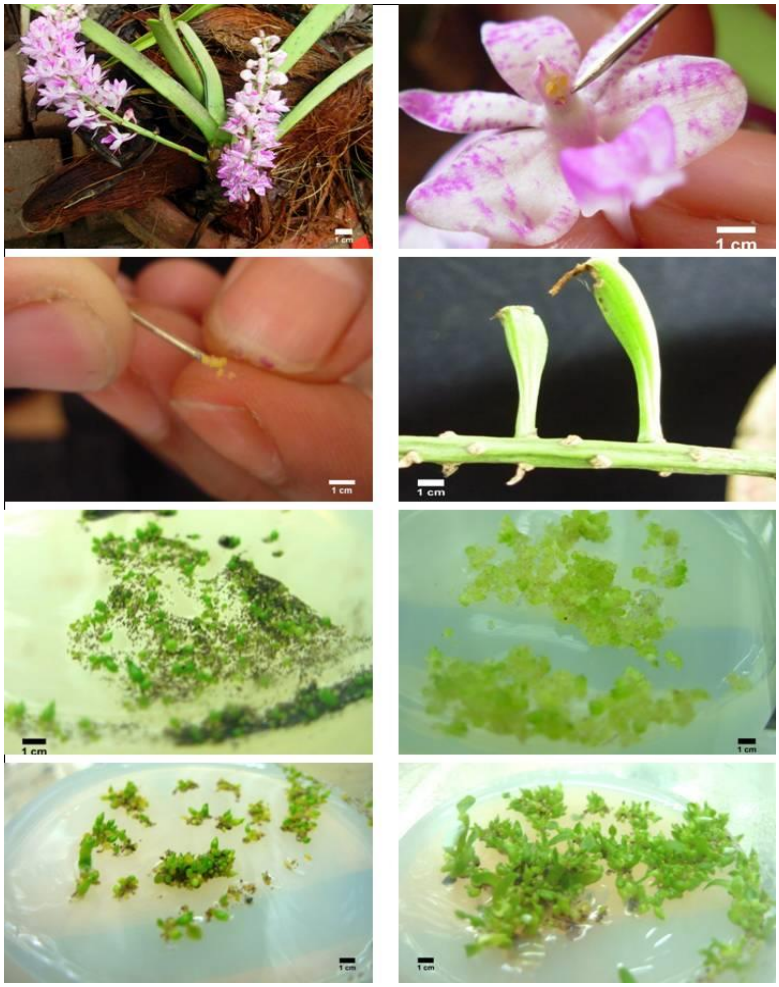

Fig 2: *R. retusa* a. *R. retusa* inflorescence in green house b. Removal of pollinia from flower c, Pollinia d. Capsule formation after pollination e. f. g. h. Various stages of asymbiotic seed germination.

Photoperiod is an important factor in germination of seeds and development of seedlings. Germination percentage was significantly different in three photoperiods 0/24, 16/8, 24/0 are 51.86 ± 1.15%, 80.16 ± 1.96% and 65.33 ± 4.21% respectively on BM1. Highest germination was observed when seeds were kept in 16/8 L/D cycle. Three photoperiods were analyzed for further development of the seedlings 8/16, 12/12, 16/8 h L/D but no significant difference was observed. All developmental stages (Stage 0, 1, 2, 3, 4, and 5) were observed in all three photoperiods irrespective of the time. Statistically Analysis was done by One Way ANOVA and Duncan's Multiple Range Test at 0.05. The data presented here is highly significant. Statistical analysis of particular stage in week 6 and week 14 was calculated by number of seeds in that particular stage divided by total number of seeds germinated.

DISCUSSION

Pollinating young, fully open flowers is recommended since pollen and stigma is most receptive 1–8 days after flowers are open [18] [19]. In present investigation, Young flowers (1-8 day) show maximum receptivity of sigma and pollen because of knowing this fact pollination percentage was increased in consecutive years (Table 3). Self-incompatible plant of *R. retusa* when cross pollinated,

capsules were developed. Artificial pollination in *R. retusa was* also studied earlier, but they have focused their research on biochemical changes in the senescing flower not on capsule development [20]. Similar results like senescence of the flowers were observed in present investigation after artificial pollination.

Asymbiotic germination

Many Scientists have worked on different aspects of *R. retusa* like clonal propagation, embryo culture and seed germination [21], [22], [23], [24] [25]. They have collected the immature capsule and germinated on Mitra et al., and Knudson C liquid nutrient medium. On Mitra et al., they achieved 78% germination and protocorm in 90 days. Another scientist also used the immature capsule of *R. retusa* and cultured on VW media supplemented with BA, NAA and CW. They observed callus and later PLB and complete plant development. They also tried the MS and Mitra et al., media [26]. In one of the research ½ MS along with different concentrations of CW (coconut water) for seed germination of *R. retusa* was used [27]. Seedlings were developed in 90 days then seedlings were transferred to different concentrations of BA and NAA for multiple shooting. IBA was used for rooting of the multiple shoots. In present investigation effect of different media were observed for asymbiotic seed germination and to find out the nutritional need of the plant. Every media used in this research has nitrogen source can be in organic or inorganic form which helps in early grow of the seedling (Table 1). Knudson C and VW media contains inorganic source, BM1 contains only amino acids as a nitrogen source and rest of the media contain both organic and inorganic together. All media initiated seed germination and supported protocorm development to all stages of development, suggesting that nitrogen in any form and concentration will support asymbiotic seed germination in *R. retusa.* Interestingly, BM1 and ½ MS initiated seed germination and supported advanced protocorm development (Stage 5) early as compared to other media. But early seedling development was noticed in BM1. 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 [28], [29], [30], [31]. These researchers have suggested that organic sources of nitrogen (i.e., amino acids) may be more readily available to a seed or plant than an alternative inorganic nitrogen source. BM1 contain amino acids (glutamine Glycine) as a nitrogen source. In *R. retusa* both organic and inorganic form of nitrogen is helping in seed germination and seedling development as results observed in both BM1 and ½ MS media.

4. CONCLUSION

The current study demonstrate pollination mechanism in green house and their comparison with that of natural pollination. Natural pollination always yields more seeds as compare to artificial pollination but artificial pollination is great where natural pollination is missing. In present investigation asymbiotic seed germination was also studied to check the nutritional need of the plant in different developmental stages.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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