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Mycobiont Mediated *In vitro* Seed Germination of an Endangered 'Fox-tail' Orchid, *Rhynchostylis retusa* (L.) Blume

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***Rhynchostylis retusa* is an endangered species of epiphytic orchids. It bears beautiful pendent inflorescences, and is of high floriculture and therapeutic importance. Natural populations of this species are gradually declining because of unscrupulous collection pressures. The species has earlier been propagated through asymbiotic seed germination and organ culture but there is no report of raising it symbiotically. Therefore present study was undertaken to isolate its mycobionts and to investigate their role in inducing seed germination *in vitro*. For this, the surface sterilized root segments (10-15 mm long) were cultured on Oat Meal Agar medium and three fungal endophytes (RR201 - RR203) were discerned based upon colony and micromorphological characteristics. Mature seeds were co-cultured separately with all of these fungal isolates. The embryos showed initial swelling after 2.10±0.02 weeks of culturing. Seed coat ruptured after 4.62±0.18 weeks in more than seventy five percent seeds irrespective of the fungal isolate used. However, the further morphogenetic changes occurred only with isolate RR202. It was therefore characterized by sequencing the internal transcribed spacer (ITS) regions of ribosomal RNA gene. The sequence showed 97% similarity with a Basidiomycetes taxon, *Ceratobasidum* sp. Fungal hyphae entered into seed from general seed surface and colonized the embryonic cells. The protocorms developed after 12.50±0.50 weeks. First leaf and root emerged after 18.00±0.71 and 26.80±0.57 weeks respectively. Seedlings transferred to greenhouse showed 98.34±0.28 percent survival. *Ceratobasidium* sp. can be utilized for efficient mass propagation of *R. retusa* symbiotically.**

Keywords: Mycobiont, symbiotic seed germination, orchid, protocorm.

Orchids enjoy a place of pride in the floriculture industry because of their beautiful foliage and attractive flowers of varying shapes and sizes. With about 25000 species, they represent one of the largest plant families, the Orchidaceae[1]. Orchid seeds are the smallest in the plant kingdom. They are produced in very large numbers but their embryos lack access to nutrient reserves. Their natural germination is, therefore, exceptionally low (less than 1%) and they totally depend upon appropriate fungal stimulus (mycotrophy) for this. Fungal partner helps the orchid host in a number of ways. It augments carbohydrate nutrition by breaking down the complex organic compounds in the soil and facilitating their subsequent release in the plants[2]. One of the important roles of endophytic fungi is to initiate the biological degradiation of dead and dying host plant, which is necessary for nutrient recycling[3]. Endophytic fungi generally live peacefully within their hosts,

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but may behave as facultative pathogens under certain circumstances [4]. Earlier, *Rhizoctonia* was thought to be the only mycobiont of orchids, but later on, a number of fungi were identified which resemble *Rhizoctonia* in many aspects; they were therefore termed *Rhizoctonia*–like fungi. Till now, many fungal symbionts have been isolated from orchids. They belong mainly to Basidiomycetes (*Ceratobasidium, Ceratorhiza, Epulorhiza, Mycena, Sebacina, Thanatephorus, Tulasnella,* etc.) and Ascomycetes (*Alternaria, Bionectria, Cladosporium, Cochliobolus, Fusarium, Trichoderma, Xylaria,* etc.) genera [5,6,7,8,9,10]. Symbiotic seed germination signifies an efficient technique to investigate the orchid–fungus association and specificity[11].

Rhynchostylis Blume is a small orchid genus of 3-4 species. It is represented by a single species, *R. retusa* (L.) Blume in India[12]. The plants bear one or more beautiful pendent inflorescence(s) with densely arranged white flowers spotted with pink. It is therefore popularly known as 'fox-tail' orchid. The species is also of high therapeutic value. The whole plant preparations are used to treat rheumatic disease, tuberculosis, epilepsy, blood dysentery, menstrual disorders, gout, asthma, skin diseases and external inflammations[13]. Leaf juice is used against ear pain, and roots for curing malarial fever[14,15]. The plant also shows antibacterial activity against *Bacillus subtilis* and *Escherichia coli*[16]. There are many reports of its mass propagation through asymbiotic seed and organ culture[17,18,19,20,21,22]. But no information is available on its mycobiont mediated (symbiotic) seed germination. Therefore, an attempt was made to isolate mycorrhizal associates of *R. retusa* and to investigate their role in the symbiotic seed germination and seedling development *in vitro*. The study will facilitate achieving efficient mass propagation of *R. retusa* by raising seedlings manifested with symbiotic mycorrhizal partner.

MATERIALS AND METHODS

Collection of plant material, and isolation and characterization of the fungal endophyte

Rhynchostylis retusa is an epiphytic orchid (Fig. 1a). Roots of a vigorously growing plant were collected from Tihra town (31º46´N, 76º40´E, 1057 m) of Mandi district in Himachal Pradesh. Collection was made during active vegetative growth phase of the plants (mid March, 2013). They were rinsed with tap water and scrubbed with a soft brush to remove surface debris. Hand-cut transverse sections of roots were stained with lactophenol triglycero cotton blue and observed under light microscope for the presence/ absence of fungal colonization. Only those roots were selected for further experimentation which showed the presence of fungal pelotons. Isolation of the mycorrhizal endophyte was carried out following Currah *et al.*[23] with slight modification. For this, roots were cut in to approximately 15 mm long segments and washed with detergent (Teepol) under running tap water. Further surface sterilization was carried out under aseptic conditions in a laminar air flow cabinet. They were initially dipped in 70% alcohol for 5 sec. and then treated with 0.1% solution of $HgCl_2$ for 3-4 min. They were thoroughly rinsed twice in sterile distilled water and their cut ends discarded. The segments were then inoculated on Oat Meal Agar (OMA) medium in 6×1 inch test tubes. The cultures were incubated in the dark at 25±2°C until hyphae emerged out from the inoculated segments and spread on the medium. Pure cultures were obtained by carefully transferring hyphal tips on to the Potato Dextrose Agar (PDA) medium dispensed in 90 mm glass Petri dishes. The mycobionts were initially delimited using morphological features (colony color, hyphal characteristics, presence/ absence of conidia, moniliod cells, etc.). Molecular characterization was done only in case of that isolate(s), which established successful symbiotic seedgermination. For this, amplification and sequencing of the internal transcribed spacer (ITS) regions of ribosomal RNA gene using specific primers ITS1 and ITS4 was done[24] (Chromous Biotech, Bangalore, India). The sequence similarity with the database available through National Centre for Biotechnology Information (NCBI) platform was investigated using Basic Local Alignment Search Tool (BLAST) algorithm. A dendrogram was constructed using MEGA4 software employing neighbor–joining method[25] with the bootstrap value of 1000 replicates.

Symbiotic seed germination and morphogenetic changes

For symbiotic germination, seeds were procured from a mature undehisced green fruit of

the same plant that previously yielded roots. To clean outer surface, it was scrubbed with detergent (Teepol) using a soft brush and washed under running tap water for 10 min. Further sterilization was done in laminar air flow cabinet by treatment with 0.1% $HgCl_2$ for 5 min. with occasional hand-stirring. The fruit was washed with sterile distilled water thrice, dipped in absolute alcohol for 2 sec. and flamed. It was then placed on a sterilized filter paper in a 90 mm glass Petri dish and dissected longitudinally with the help of sterile surgical blade. Seeds were scooped out and distributed over the surface of a sterile filter paper (Whatman No. 1) strip (10×30 mm) resting on the surface of OMA medium dispensed in 6×1 inch test tubes. For symbiotic germination, a PDA block (10 mm^3) with isolated fungus was placed at the lower edge of this strip. All fungal isolates, delimited initially on the basis of morphological characteristics (RR201–RR203), were tested for symbiotic seed germination *in vitro*. The test tubes were incubated at 25±2°C under continuous 12 hr/12hr light/dark photoperiod. Some seeds were taken for testing their viability by performing a tetrazolium test[26].

Data recording and analysis

Observations were made at three days intervals to follow various morphogenetic changes. Seed germination and subsequent development was scored on a scale of 0–4 following Aggarwal *et al.*[27] with slight modification. Stage 0: Swelling of embryo but no rupture of seed coat; Stage1: Further swelling of embryo and rupture of seed coat; Stage 2: Development of polarity and formation of protocorm; Stage 3: Emergence of first leaf; and Stage 4: Differentiation of first root. Seeds that entered Stage 1 were considered germinated irrespective of further changes occurring in them. The data for each fungal isolate were collected in five replicates and the values were expressed as their means (Table 1). Results were analyzed using a completely random design and subjected to one–way analysis of variance (ANOVA), and post hoc tests were employed to detect the significant differences ($p \leq 0.05$) among different fungal isolates, using SPSS 17.0 (SPSS Inc., USA).

RESULTS AND DISCUSSION

Mycorrhizal characterization

Transverse sections of *Rhynchostylis retusa* roots revealed that their cortical cells were colonized by mycorrhizal fungi (Fig. 1b). This is in accord with the generalization that under natural conditions, orchids are associated with some fungal partner at least at some period of their life cycle[28]. The extent of fungal manifestation has been, however, reported to differ with respect to the habit, habitat and life cycle stages of various orchid taxa[5,7]. Exact place of fungal entry was not noticed during present study. Though the roots possessed a number of root hairs, but no fungal mycelium was observed to enter through them. Probably, the fungus might have entered inside the tissue well before root collection. Literature studies revealed that fungus usually penetrate inside root either through root hairs or directly through epiblema cells[29,30,31,32].

Earlier, the colony characteristics and micromorphological features were used for identification of orchid mycobionts[10], and the same were employed for initial recognition of fungal endophytes isolated presently. A total of three fungal endophytes were isolated which fall under two groups based upon colony characteristics and

Table 1. *In vitro* symbiotic germination and successive morphogenetic stages of *Rhynchostylis retusa* seeds co-cultured with three mycobionts on Oat Meal Agar medium

Mycobiont seed	Time taken in weeks for onset of					Percent
	Stage0	Stage1	Stage2	Stage3	Stage4	germination
RR201	2.10±0.02[a]	4.69±0.13[a,b]	-	-	-	77.68±2.60[a]
RR202	2.10±0.03[a]	4.65±0.28[a]	12.50±0.50[a]	18.00±0.71[a]	26.80±0.57[a]	86.40±1.52[b]
RR203	2.14±0.02[a]	4.62±0.18[a]	-	-	-	83.06±1.86[b]
-	2.12±0.04[a]	4.72±0.22[b]	-	-	-	75.40±1.14[a]

Data are shown as mean ± standard deviation. Values in a column with the same superscripts are not significantly different at $p \leq 0.05$.

micromorphological studies. In Group I (isolates RR201 and RR203) the colonies were cottony white; hyphae hyaline and septate, branched at nearly right angles; conidiophores branched and conidia green or yellow; and monilioid cells absent. Under Group II (isolate RR202) the colonies were white; hypae septate, binucleate or uninucleate, branches arising at nearly right angles; conidia absent; monilioid cells spherical. As seedling development was observed only with isolate RR202 (Table 1), it was characterized using ITS sequencing method. This molecular method is very precise and has been effectively used by researchers for identifying mycorrhizal fungi in recent times[8,9,10,27, 33,34]. The sequence of presently studied fungus (RR202) was submitted to NCBI GenBank (Accession No. KR149122) and it showed 97% similarity with *Ceratobasidum* sp. (Accession No. DQ102434) of NCBI Data Bank (Fig. 2). This Basidiomycete genus and its allied genera (*Rhizoctonia, Ceratorhiza, Epulorhiza,* etc.) are frequent associates of orchids[8],[35,36]. *Ceratobasidum* sp. has earlier been isolated from *Rhynchostylis retusa*[35]. Aggarwal and Zettler[36] recorded 100% seed germination of *Dactylorhiza hatagirea* seeds with *Ceratobasidium* sp. isolated from its own roots.

Reports of orchid–fungus specificity/ non–specificity are well documented in literature. Salifah *et al*.[37] isolated six species of *Fusarium* from roots of *Grammatophyllum speciosum* but not all of them were able to induce germination in its seeds. Zhang *et al*.[9] isolated 65 endophytic fungi from *Dendrobium officinale* roots but only one of them stimulated growth and development of its protocorms. Sathiyadash *et al*.[8] successfully germinated the seeds of *Coelogyne nervosa* by co–culturing with *Epulorhiza* sp. isolated for the roots of *Eulophia epidendrea*. Therefore, it has been suggested that there might be a narrow checkpoint for mycorrhizal range during seedling growth relative to the more promiscuous germination and mature stages of these plants' life cycle[38]. An orchid may be associated with a

Fig. 1. Stages of Mycobiont mediated Seed Germination in *Rhynchostylis retusa* (L.) Blume

1a. Plant growing in natural habitat. 1b. Cortical cells colonized by mycorrhizal fungi. 1c. A mature seed showing embryo and seed coat. 1d. Fungal hyphae surrounding a seed. 1e. Swollen embryo with intact seed coat. 1f. Broken seed coat and released embryo. 1g. A protocorm with mycorrhizal pelotons. 1h. Developing seedlings subcultured on OMA medium without any fungal associate. *Abbrivations:* em, embryo; fh, fungal hyphae; hc, hyphal connection between two root cells; sc, seed coat; se, swollen embryo. *Scale bars:* a, 1 cm; b – g, 100 µm; h, 1 mm.

number of fungal endophytes during its life cycle and it appears true in case of *Rhynchostlis retusa*. Where RR202 is found to be actively involved in its germination and protocorm formation, rest of the mycobionts (RR201, RR203) might have some different functional consequences during some other life stage(s). The endophytic fungi in orchid plants are very abundant but *Rhizoctonia*–like fungi often form mycorrhizal associations with them[39]. Investigations dealing with orchids and their mycorrhizal associates are of tremendous importance in orchid commercialization and conservation[40].

Morphogenetic changes occurring during symbiotic seed germination

The tetrazolium test revealed that 97.15±0.33 of the presently inoculated seeds was viable. Embryos are large occupying majority of seed space (Fig. 1c). *Ceratobasidium* sp. induced symbiotic seed germination in nearly eighty six percent of the seeds. Earlier workers got 43 – 94 percent seed germination of immature seeds of this species on artificial nutrient medium (asymbiotic germination) and reported 55–75% seed germination in majority of cases[17,18,19,20,21,22]. Comparatively lesser seed germination can be attributed to the high maturity level of presently 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[41,42,43].

The morphogenetic changes occurring in seeds inoculated with fungal isolates are summarized in Table 1 and presented below in detail. Initial sign of germination (swelling of embryo; stage 0) was detected within 2.10-2.14 weeks of inoculation. Time taken to reach stage 0 varied slightly in different cultures; it was however, not significantly different in any case including control. Hyphae in case of each isolate surrounded the seeds initially, but only those of RR202 were able to enter inside them. As many as 86.40±1.52 percent seeds inoculated with this isolate entered stage 1 (seed coat rupturing) within 4.65±0.28 weeks. The percentage of seeds reaching this stage was significantly lesser (75.40±1.14–77.68±2.60) in all other combinations except those inoculated with RR203. The germinating entities in isolates RR201 and RR203, and control ceased to develop beyond stage 1, and further morphogenetic changes were observed only in seeds co–cultured with isolate RR202 (*Ceratobasidum* sp.). Earlier investigations also reveal that seed germination percentage may be higher even under non–symbiotic *in vivo/ in vitro* conditions, but the germinating entities fail to develop further[5,8].

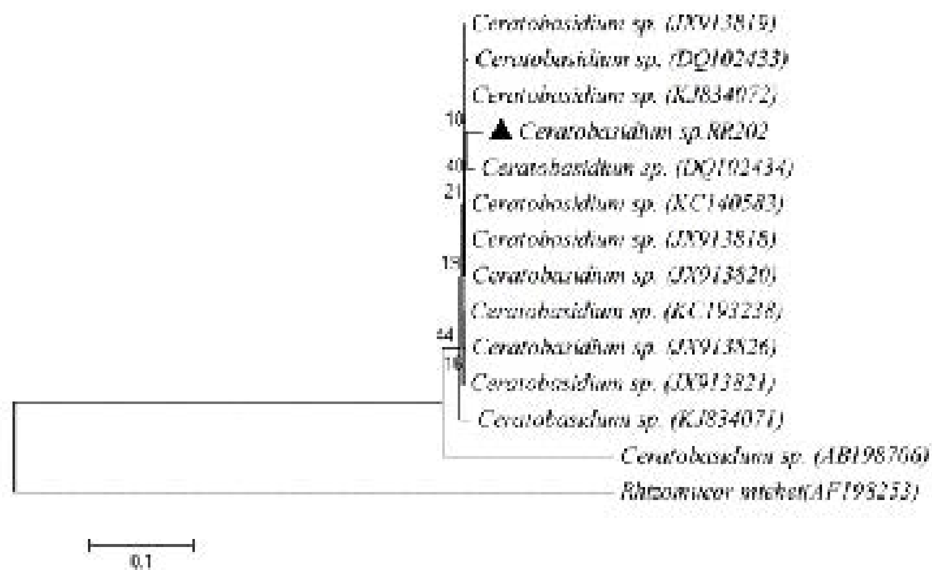

Fig. 2. A dendrogram constructed employing neighbor–joining method by alignment of ITS 1 and ITS4 regions of rRNA gene with *Rhizomucor miehei* out–group. The numbers in the branches are percentage bootstrap value (out of 1000 trials) as indicated. Accession numbers followed by names represent sequences from Gene Bank. Name preceded by a triangle represents sequence obtained presently

The fungal hyphae surrounded the seeds and penetrated inside from their general surface (Fig. 1d). According to earlier reports, hyphal entry occurs either from general seed surface, through embryonic rhizoids or from micropylar end[5,8,10,34,36,37,44,45]. After entry, fungus started colonizing the embryonic cells forming loose networks i.e. pelotons. Subsequently, the embryo size increases (Fig. 1e) and testa broke to release it (swollen embryo) on to the culture medium (Fig. 1f). According to Clements[46], presence of pelotons is an important indicator of the successful symbiotic relationship which is essential for getting protocorms transformed into seedlings. The pelotons act as an interface for nutritional exchange between fungus and the host[5]. Development of protocorms (Fig.1g) was observed after 12.50±0.50 weeks. 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 leaf/ leaves, and emergence of first root, the developing entities were subcultured on OMA medium without any fungal associate (Fig. 1h). Seedlings with 2–3 leaves and 1–2 roots were obtained after 31.50±1.12 weeks; the transverse sections of their roots showed presence of fungal pelotons. They were transferred to greenhouse conditions where 98.34±0.28 percent of them survived after six weeks of shifting. Such a high survival rate is in line with earlier investigations that symbiotically raised seedlings exhibit higher survival rate during lab to land transfer[47,48,36]. *In vitro* symbiotic seed germination is becoming a useful and favoured practice for orchid mass propagation and restoration now.

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