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ISOLATION, IDENTIFICATION, AND FUNCTIONAL CHARACTERIZATION OF MYCORRHIZAL *TULASNELLA* SP. FROM A TERRESTRIAL ORCHID, *OREORCHIS INDICA* (LINDL.) HOOK.F. THROUGH MICROMOLECULAR TECHNIQUES: A PIONEER CASE STUDY IN INDIA

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Abstract

Climate change, unregulated commercial collections, and poor natural regeneration have adversely affected habitats and natural populations of charismatic orchids which have complex life strategies. Mycorrhizal fungi are obligatory for the growth and development of orchids as these are associated with stimulating seed germination, protocorm development, and seedling growth *in vivo*. In the present study, fungal isolate was recovered from root sample of a terrestrial orchid, *Oreorchis indica* (Lindl.) Hook.f. and was identified based on micro-morphological features and molecular characteristics [sequencing the internal transcribed spacer (ITS) region of ribosomal DNA]. The slow growing isolated fungus showed barrel shaped moniloid cells and ITS sequence of isolate (accession number OP467009) possesed 92.24% similarity with *Tulasnella* spp. (Basidiomycetes) in NCBI mega BLAST search. These mycorrhizal interactions have a referential value to be utilized in symbiotic seed germination experiments and thus protecting rare and endemic orchid plants. This is the first report of fungal endophyte (*Tulasnella* spp.) isolation and identification through morpho-molecular techniques in *Oreorchis indica* from India. Mature micro-seeds procured from the dehisced capsules (12 WAP) were also evaluated for *in vitro* germination potential under both symbiotic and asymbiotic environment. The germinating entities failed to exhibit any response, either symbiotically or asymbiotically, despite repeated subculturing on fresh media even after four months of inoculation, indicating thereby that either the isolated fungus was incompatible or the nutrient medium and culture conditions were not suitable. Therefore, our findings suggest that orchids may require different fungal partners during symbiotic germination of seeds and seedling development.

Introduction

THE FAMILY Orchidaceae is a diverse and widespread family of flowering plants and most advanced amongst the monocots (Willis, 2017). This family comprises of about 29,481 species distributed in 693 genera (POWO, 2025; WFO, 2023). Orchids are easily distinguished from other plants, as they have characteristic features namely, bilateral symmetry of the flower, highly modified petal (labellum), fused stamens, highly complex pollination mechanism, and extremely small non-endospermic seeds (Prakash and Pathak, 2020a,b, 2022). However, their mode of life in association with mycorrhizal fungus is one of the most significant ecological interactions facilitating seed germination and further seedling development. The complexity of floral structures, great diversity of plant pollinator interactions, and orchid habitats are best understood in the light of mycotrophy (Lal *et al.,* 2021; Prakash and Pathak, 2019). Orchid mycorrhizas are phylogenetically distinctive from other major type of mycorrhiza as they consistently use an alternative nutritional system in which fungus supplies the plant with energy (Hadley, 1982; Rasmussen, 1995). Though, the minute non-endospermic seeds of orchids must colonize compatible mycorrhizal fungi to provide the embryo with carbon and other nutrients to initiate the germination stage and stimulate the critical symbiosis throughout their lives, yet less than 1% orchid seeds germinate in nature (Pathak *et al.,* 2022). The parasitism on fungus can lead to permanent loss of photosynthetic capacity as observed in most plants, but this is not the case with all orchids. Most of these species have retained photosynthetic capacity along with the requirement of mycorrhizal fungus for seed germination and subsequent seedling development (Rasmussen, 1995). Fully mycoheterotrophic taxa which have entirely lost their photosynthetic ability are also more common in the Orchidaceae than in other families (Bidartondo, 2005). During early life stages, there is complete reliance on mycotrophy for nutrition and this might have led to the evolution of life-long mycoheterotrophy in orchids, while during subsequent developmental stages, they utilizes both mycotrophy and photosynthesis for nutrition, either alternatively or one supplementing the other (Leake, 1994; Rasmussen, 1995). The production of fungicidal phytoalexins in reaction to symbiotic infection is widespread in orchids which restrict the growth of mycorrhizal fungi to specific cells. These also provide the plant with broad-spectrum protection against parasites and pathogens (Chen *et al*., 2003; Hadley, 1982; Suz *et al*., 2018). Once the

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symbiotic relationship is established, orchids obtain nutrient by hydrolytic enzyme mediated digestion of fungal hyphal coils known as pelotons (Hadley, 1982; Merckx, 2013; Sakamoto *et al*., 2016).

As the nature of mycorrhizal associations has not been fully investigated in many orchid species and merely assumptions may be made on their fungal assistance for nutrition. Isolation and identification of mycorrhizal endophyte is the pioneer step of its significance for commercial orchid propagation and conservation (Zhang and Zhou, 2004). Traditionally, the research into orchid mycorrhizal identity involved morphological comparison of isolated cultures from single peloton or infected tissue of mature plant. These methods, however, used in combination with molecular data, permit accurate implication of nature of orchid mycorhizal association (Shefferson *et al*., 2005).

The genus *Oreorchis* is partially mycoheterotrophic (Suetsugu *et al*., 2021) with broad distribution from the Himalayas across China to Taiwan, Korea, Japan, and India (Himachal Pradesh, Sikkim, Uttarakhand) (Pearce and Cribb, 1997; Singh *et al*., 2019). Partially mycoheterotrophic as it is closely related to leafless *Corallorhiza* and possesses coralloid rhizomes often found in fully mycoheterotrophic orchids (Suetsugu *et al*., 2021). Owing to typically small and isolated population of *Oreorchis indica* (Lindl.) Hook.f., the species has been classified as critically endangered in Japan (Ministry of Environment of Japan, 2015). An attempt was presently made to isolate, identify, and functionally characterize the mycorrhizal associate of terrestrial orchid, *O. indica* following morpho-molecular techniques.

Material and Methods

Sample Collection and Fungal Isolation

Whole plants of *Oreorchis indica* were collected from Kufri hills (31.09833°N, 77.26295°E; 2400-2650 m amsl) of Shimla (Himachal Pradesh, India) during its active vegetative growth phase (Fig. 1). Live specimens were maintained at the Orchid Greenhouse, Department of Botany, Panjab University, Chandigarh, India and the fungus was isolated from the roots of actively growing plants. Hand cut transverse sections of washed roots were stained with lactophenol cotton blue (0.5%); only those with fungal colonization were selected for isolation of endophytes. The selected roots were washed with running tap water for 15-20 min to remove surface debris. Under sterile conditions in a laminar air flow cabinet, the root pieces were surface sterilized with 70% ethanol and then treated with 0.1% $HgCl_2$ solution for 2-3 min followed by rinsing with sterilized distilled water for three times. Surface sterilized roots were then sliced into small segments (8 mm long) and were cultured on H_1-oat medium with added streptomycin to reduce bacterial growth. The emerged hyphal tips from cut ends were carefully transferred to Potato Dextrose Agar (PDA) medium and serially transferred for 3-4 times until pure cultures were obtained.

Morphological Characterization of Fungal Isolate

For morphological characterization, isolate was cultured on PDA at 25±2°C and observed for colour and growth of colony, and presence or absence of moniloid cells. The mycelium of the isolated fungi was taken on glass slides for microscopic observation. Lactophenol cotton blue was used as a staining agent for observing hyphal characteristics and Nikon Eclipse E200 microscope for photography.

Molecular Identification of Fungal Isolate

For genomic DNA extraction, freshly grown pure culture growth was transferred to mortar and pestle followed by treatment with liquid nitrogen. Crushed mycelia were suspended in 500 µl of lysis buffer followed by incubation at 56°C for 3 min. DNA was then extracted by the Phenol-Chloroform-Isoamyl alcohol (25:24:1) (PCI) method (Shivaprakash *et al*., 2011). Briefly, the PCI mixture (Himedia, Mumbai, India) was added and mixed well with the lysed specimen followed by centrifugation at 13,000 rpm for 15 min. Then, the aqueous phase was transferred to a sterile microcentrifuge tube and DNA was precipitated with absolute ethanol at -20°C overnight. DNA was then centrifuged followed by 70% ethanol wash and air-dried. It was suspended by thoroughly mixing in 50 µl of sterile, deionized water (Milli Q, Millipore systems). The yield and quality of the DNA were quantified by spectrophotometric measurement at a wavelength of 260 nm using a NanoDrop™ 2000 spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, USA).

The PCR reaction mixture consisted of 1 µl of total DNA, 15 µl of PCR buffer, 1 µl each of dNTPs, Taq polymerase (Ampliqon, Herlev, Denmark) and ITS primers; ITS4 (52 -TCCTCCGCTTATTGATATGC-32) and ITS5 (52 -GGAAGTAAAAGTCGTAAC-32). The PCR cycling condition comprised of an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 2 min followed by final incubation period of 10 min at 72°C. The amplified products were visualised by resolving in 1.2% agarose gel containing 2 µl ethidiumbromide. Molecular identification was done by sequencing the Internal Transcribed Spacer (ITS) region of ribosomal DNA as described previously (White *et al*., 1990).

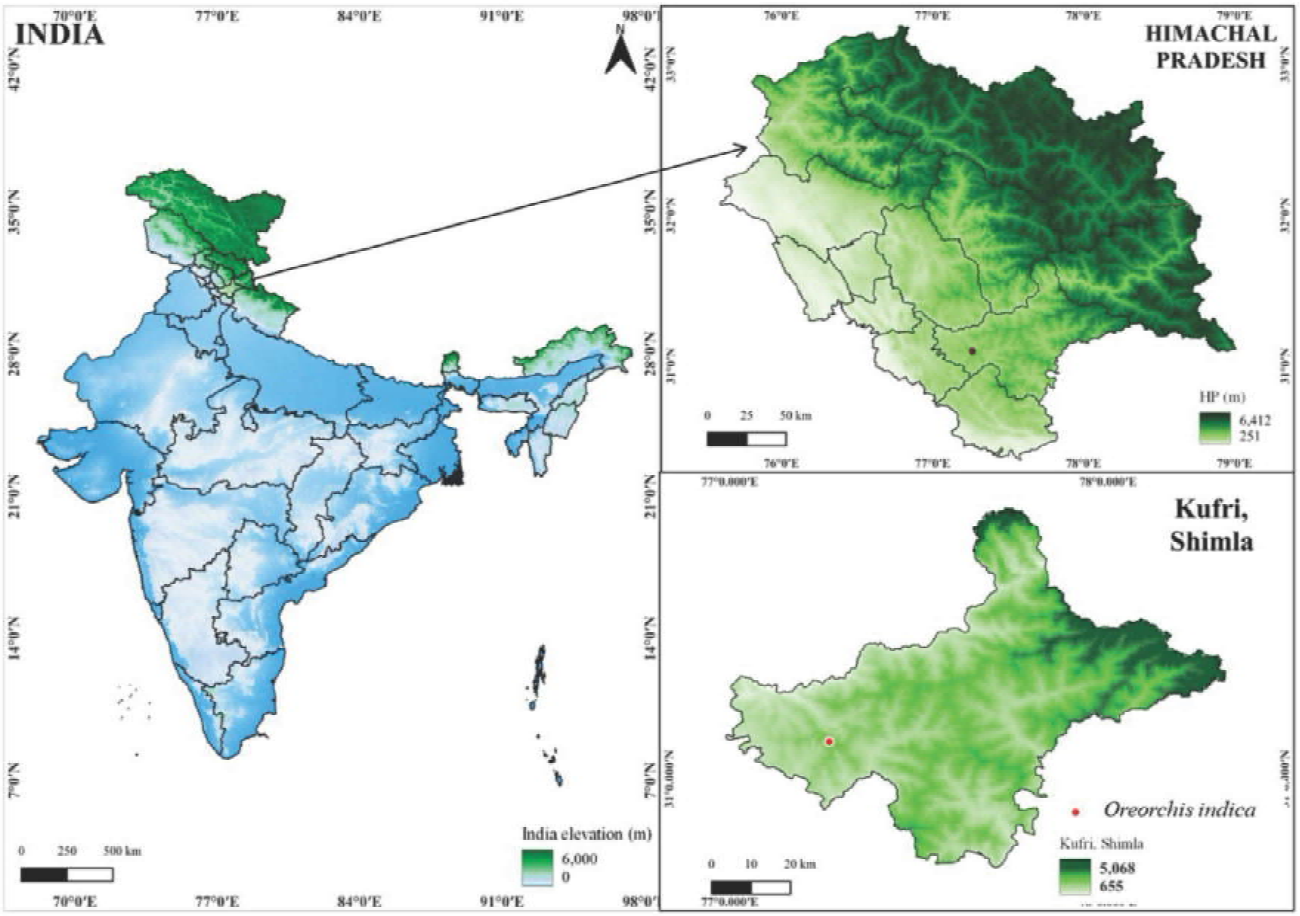

Fig. 1. Map of study area, Kufri in Himachal Pradesh, India. The red circle marks the location where the sample was collected.

After sequencing, the consensus nucleotide sequence was analysed and placed into web based Basic Local Alignment Search Tool (BLAST) of NCBI GenBank (https://blast.ncbi.nlm.nih.gov/) to find out similar species/isolates (Altschul *et al.*, 1990) and phylogenetic analysis were conducted with MEGA version 7 (Saitou and Nei, 1987).

Symbiotic and Asymbiotic Seed Germination

Mature micro-seeds procured from the dehisced capsules [12 wks after pollination (WAP)] were evaluated for germination potential under both under *in vitro* symbiotic and asymbiotic environment. Seeds were taken out of cold storage and examined for their viability using 2,3,5- triphenyltetrazolium chloride (TTC; 1%). The micro-seeds were placed on a glass slide, stained with TTC solution and incubated in darkness for 24 hr. The seeds were then observed under a light microscope and considered viable if the embryo turned red while unstained embryos were scored non-viable (Van Waes and Debergh, 1986). The seeds were sterilized under aseptic conditions in a laminar air flow hood by immersing and swirling them in 5% NaOCl solution for 20 min (Sweet and Bolton, 1979). The seeds were then transferred onto a Whatman No. 1 filter paper and rinsed three times with sterilized distilled water.

For symbiotic germination, the seeds were scooped out and evenly distributed on the surface of sterile filter paper strips (1×3 cm, Whatman No. 1) placed atop the Oat Meal Agar medium (OMA) in test tubes, with a 10 mm^3 PDA block containing isolated pure fungus placed at the lower edge of the sterile strips (Hollick, 2004). The innoculated test tubes were then sealed with cotton plugs and incubated at 25±2°C temperature under a 12 hr light cycle using day-light fluorescent tubes (40W; Philips India Ltd., Mumbai, India). The cultures were regularly examined and various morphogenetic responses (swelling of embryos, seed coat rupturing, sperule and protocorms development) were documented based on visual and microscopic observations. Seed morphogenetic development was assessed utilizing a 0-5 scale, where Stage 0 represents no germination, Stage 1 indicates seed coat rupture due to swelling of embryo, Stage 2 signifies the presence of rhizoids, Stage 3 denotes the emergence of the leaf primordium (shoot), Stage 4 indicates the

appearance of the first leaf, and Stage 5 marks leaf elongation and root differentiation (Zettler and McInnis, 1994). Symbiotically inoculated swollen micro-seeds with ruptured seed coats (Stage 1) were classified as initially germinated. However, this rupture can occur passively, independent of mycorrhizal activity; Stage 3 marked by the emergence of the leaf primordium was considered the earliest growth stage attributed to mycotrophy.

For asymbiotic germination, sterilized seeds were inoculated on the surface of four different chemically defined nutrient media namely KC (Knudson C modified, 1946), M (Mitra *et al*., 1976), MS (Murashige and Skoog, 1962), and VW (Vacin and Went, 1949) singly or in combination with plant growth regulators (auxins, cytokinins; 1mgL^{-1} each), growth additives (CH, P, YE; 1gL^{-1} each), and activated charcoal (AC; 2gL^{-1}). After inoculations, the culture vessels were securely sealed with cotton buns and incubated under light condition in the culture room. Temporary mounts in 10% glycerine were prepared to observe microscopic stages of the morphogenetic development during seed germination. Photomicrographs were taken using a Nikon camera (Nikon Eclipse E 200, DS-Fi1) under eyepiece (10x) and objectives (4x, 10x, 40x, 100x) lenses magnification.

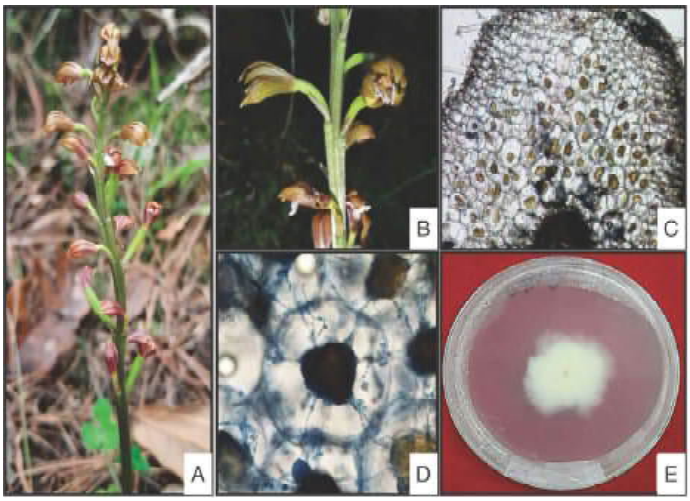

Fig. 2. A-E. Morphology and fungal infection in *Oreorchis indica*: A-B, A Plant in full bloom; C, Transverse section of root showing extent of fungal penetration; D, Interconnected fungal clumps of cortical cells; E, Seven day old culture on PDA.

Results

Identification of Fungal Isolate

The fungal endophytes showed intra- and intercellular colonization of the cortical cells with peloton formation within the mature roots of *Oreorchis indica* collected from their natural habitat (Fig. 2A-D). Root hair were found protruding out of the epiblema layer and some of them were found containing fungal hyphae. Therefore, fungal hyphae were believed to have entered inside the root tissue directly through root hair. Root colonization extent was higher in June-July than in November-December. Enlargement of infected cortical cells was also observed. The vascular bundles were fungus free. In the isolation medium, fungal hyphae emerged out from the cut ends of the inoculated segments within a week and then spread slowly in the form of a mat on the medium. Pure cultures were then obtained by transferring hyphae onto PDA medium. Mycelia procured from pure cultures were kept in 10% glycerol at -20°C for long term preservation.

The isolated fungus was subcultured on PDA medium and its growth behaviour was studied. The colonies appeared white to cream coloured with undulate and submerged edge (Fig. 2E). The hyphal growth rate was slow and it was measured at <0.1mm/hr at 25°C; hyphae from cultures was hyaline and regularly septate with branching at right angles (Fig. 3A). The moniloid cells were hyaline, barrel shaped, often in short branched chains, and interwined into loose aggregate of sclerotia (Fig. 3B).

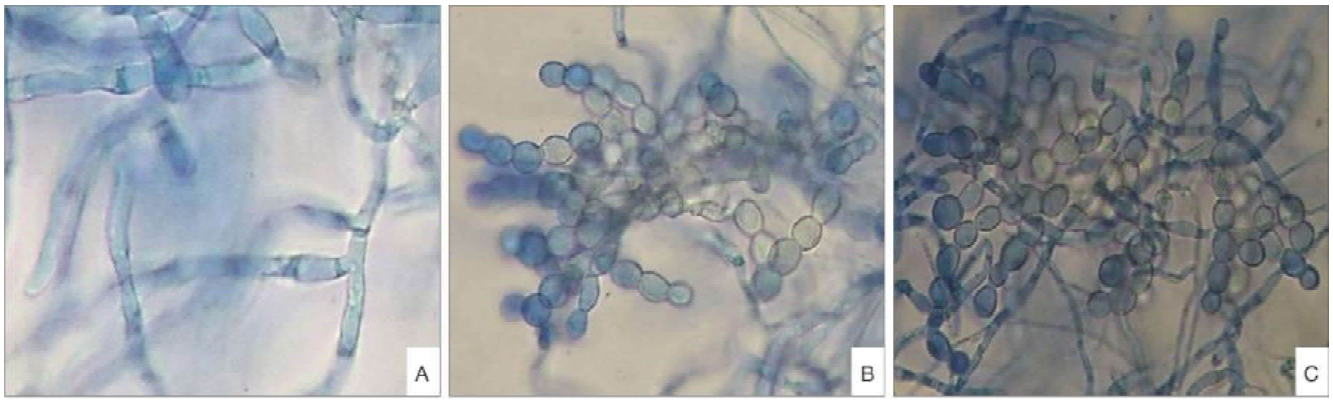

Fig. 3. A-C. Morphological feature of endophytic fungus: A, Hyphae with branching at right angles; B-C, Moniloids in dense aggregates forming sclerotia.

Hyphae typically lack clamp connection and no sexual reproductive structures were observed. The isolated fungus was also characterized by PCR amplification of the ITS region using the primer pair ITS4 and ITS5 and sequencing the amplified product. This was followed by identification through comparing the sequence obtained with already available sequence in NCBI Genbank database using BLAST search tool. The isolated fungus (Accession number OP467009) showed 92.24% similarity with *Tulasnella* spp. (Accession number JQ247565) of NCBI data bank. A phylogenetic tree using MEGA 7 software program was constructed to reveal the phylogenetic relationship between the fungal isolate obtained from roots of *O. indica* and related isolates from other orchids, registered in the NCBI (Fig. 4).

Symbiotic and Asymbiotic Seed Germination

Mature micro-seeds (12 WAP) procured from the dehisced capsules were tested for their *in vitro* germination potential under both symbiotic and asymbiotic conditions. All seeds appeared monoembryonic, consisting of an undifferentiated globular mass of cells enclosed in reticulately thickened seed coats, with a viability rate of 85.60% confirmed through TTC testing (Fig. 5A). When the seeds were co-cultured with the isolated fungal endophyte (*Tulasnella* sp.) on OMA medium; 14% germinated, showing embryo swelling within two weeks of inoculation (Fig. 5F). The fungal mycelium completely enveloped the seeds and was observed to enter through the general surface (Fig. 5C); moniloid cells formed on surface of some seeds (Fig. 5D) as well as inside the seed coats (Fig. 5E). Despite repeated subculturing on fresh OMA medium, the germinating entities showed no further development even after 4 months of inoculation. In contrast, no germination response was reported in seeds cultured on OMA medium without fungal inoculum. In asymbiotic conditions, mature seeds were inoculated on various nutrient media namely Knudson C, 1946 (KC), Mitra *et al*., 1976 (M), Murashige and Skoog, 1962 (MS), and Vacin and Went, 1949 (VW) singly or in combination with plant growth regulators (auxins, cytokinins; 1mgL^{-1} each), growth additives (CH, P, YE; 1gL^{-1} each), and activated charcoal (AC; 2gL^{-1}). The monoembryonic seeds invariably failed to exhibit any germination response on all nutrient media used during the investigation despite repeated subculturing.

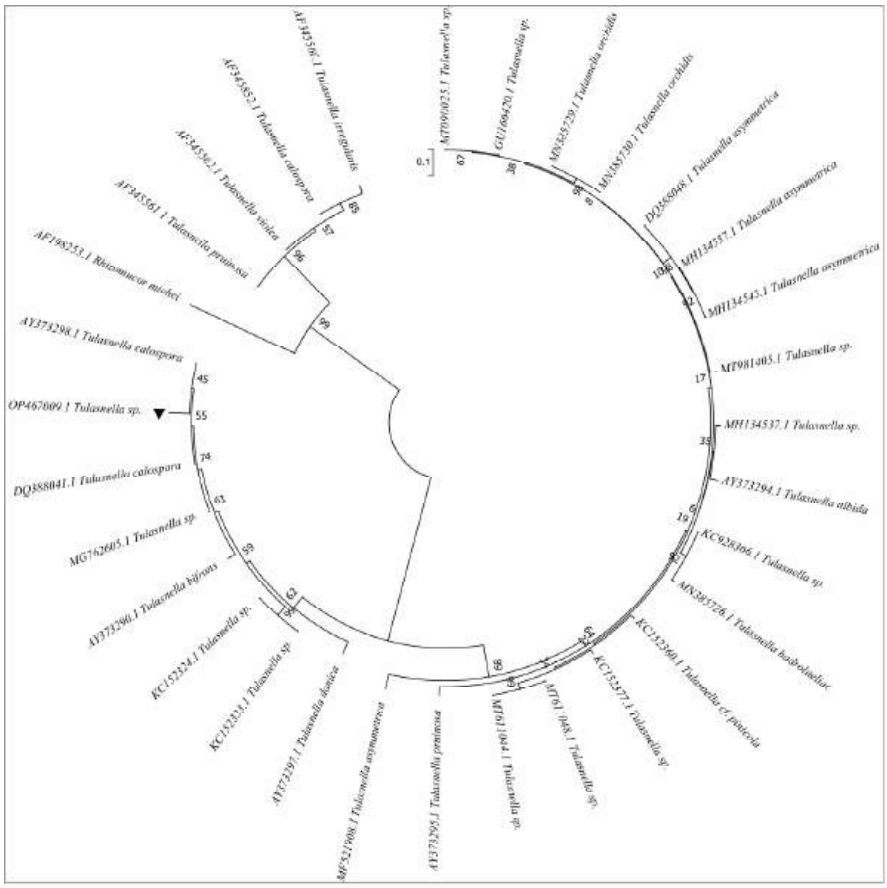

Fig. 4. Neighbour joining tree obtained from alignment of ITS sequence of fungus isolated from *Oreorchis indica* with sequences of related fungi obtained from Genbank. The numbers in the branches are percentage bootstrap value (out of 1000 trials) as indicated.

Discussion

Orchids have been described as mycotrophs (fungus feeders) as these are associated with mycorrhizal fungi at one or other stage of their life cycle under natural conditions (Zettler, 1997). During the present study, a systematic investigation of mycorrhizal fungi was performed in the roots of *Oreorchis indica,* a terrestrial and partially mycoheterotrophic orchid species. Fungal hyphae were believed to enter into root tissue through root hair or directly through epiblema cells or through both of these structures (Burgeff, 1936; Nurfadilah *et al*., 2013; Sathiyadash *et al*., 2012; Vij and Sharma, 1983). Tight interwoven coils (pelotons) formation in cortex reflects the establishment of stable symbiosis and is considered to be the most distinctive character of an orchid mycorrhiza as also earlier been indicated by Zettler (1997). Presently, the fungal colonization was observed mainly in middle layer of the cortex and some root hair were found protruding out of the epiblema layer containing

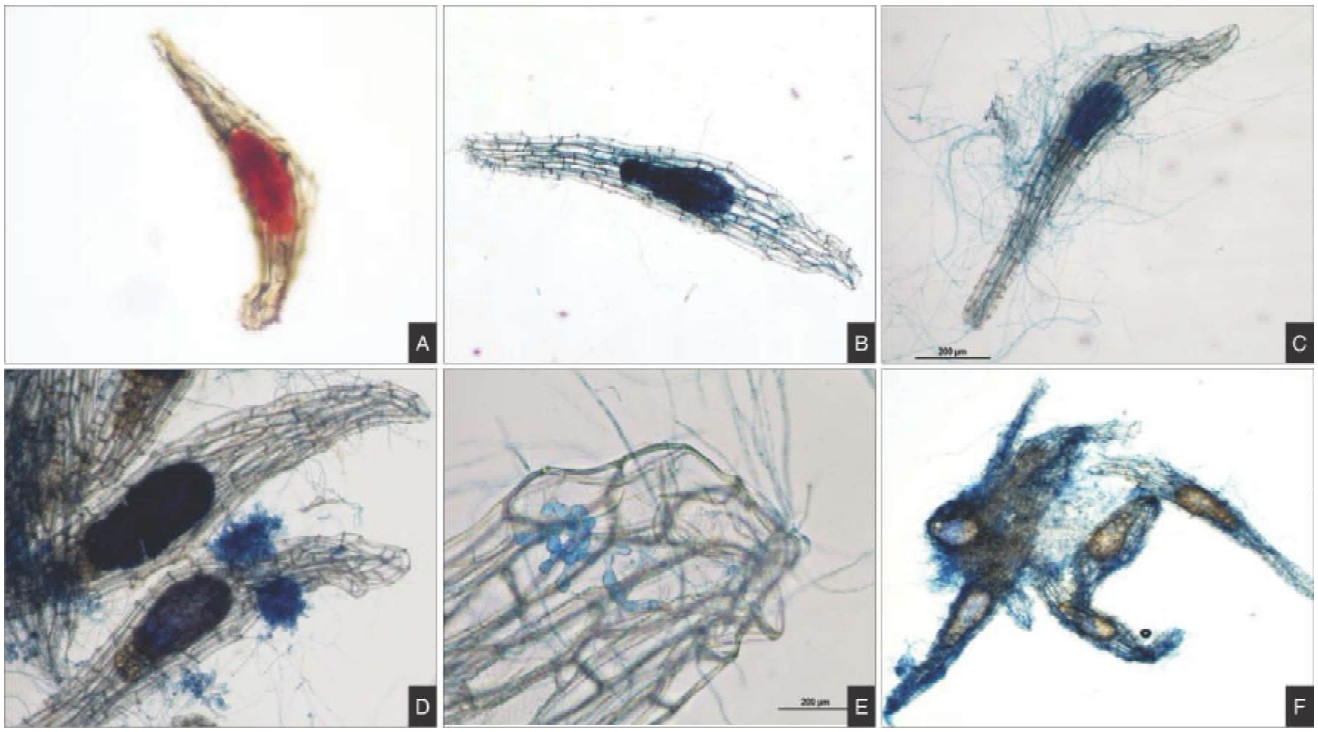

Fig. 5. A-F. *In vitro* symbiotic seed germination in *Oreorchis indica*: A, Viable seed after TTC testing (×10); B, Seed at the time of inoculation (×10); C, Entry of fungus into the seed (×10); D-E, Formation of moniloid cells on the seed surface and inside seed coat, respectively (×10, ×40); F, Swelling of embryos (×4).

fungal hyphae. During active growth season (June-July), the extent of pelotonization was more as compared to winter season. Seasonal variations of fungal pelotonization in orchid roots were also reported by Hossain *et al.* (2013). Higher pelotonization during the summer and rainy season may probably be due to active vegetative growth of plant and flowering as compared to the sluggish growth of winter season. The present observations also substantiated the earlier reports of higher colonization by compatible fungi to meet up the extreme nutrient demand for active vegetative growth and phenology of orchids (Hossain *et al*., 2013; Kaur and Deb, 2025; Lekshmi and Decruse, 2023; Masuhara and Kutsuya 1992; Raj *et al*., 2023; Shagufta *et al*., 1993).

Earlier, the mycorrhizal associates were identified using micro-morphological characteristics and *Rhizoctonia* spp. were considered to be the only root harbouring fungal endophyte. Later on, other fungi isolated and identified from orchids showed similitude in many aspects to *Rhizoctonia* henceforth are collectively called *Rhizoctonia*-like fungi (Shan *et al.* 2002; Sharon *et al.,* 2008). Earlier, Dearnaley *et al*. (2012) indicated that most green orchids form associations with rhizoctonias, a polyphyletic group of species from the families *Tulasnellaceae*, *Ceratobasidiaceae*, and *Serendipitaceae*. The conventional methods to identify orchid mycorrhizal endophytes have limitations as most of the fungi are *mycelia sterilia*. Consequently, the broad vegetative measures for identification have ensued in paraphyletic taxonomy, with several unrelated fungi being grouped together. These limitations entailed the application of molecular techniques for accurate identification (Dearnaley, 2007; Shan *et al*., 2002). The ITS region has several features that mark its strong candidature to be used as a universal fungal identification barcode as it is easy to amplify due to high copy number, relatively few primer sets are needed as a result of the highly conserved SSU (small sub-unit), LSU (large sub-unit) flanking regions, and varies relatively little within species but profoundly between species, and far better represented in GenBank than other loci in fungi (Taylor and McCormick, 2008; Zettler and Corey, 2018). Nowadays, molecular techniques along with conventional methods are being employed for correct identification.

In the present investigation, *Tulasnella* spp., a universal orchid symbiont, was found as mycorrhizal endophyte in the roots. *Tulasnella* (Tulasnellaceae) genus was described by Schroter in 1888, with *Tulasnella lilacina* J. Schröt. as the type species, and nowadays 73 accepted species are in *Index Fungorum* (Freitas *et al*., 2020). Species of this genus has worldwide distribution and known for associating with a wide range of endangered orchids. It has been isolated from orchids in Asia (Ding *et al*., 2014), Australia (De *et al*., 2013),

Europe (Warcup and Talbot, 1967), and South America (Nogueira *et al*., 2014). Several isolates have also been reported to increase seed germination and subsequent seedling development (Khamchatra *et al*., 2016; McCormick *et al*., 2004; Meng *et al*., 2019; Zi *et al*., 2014). Earlier, Rasmussen (1995) utilized *Ceratobasidium* and *Tulasnella* to successfully germinate seeds of *Dactylorhiza majalis,* but only the *Tulasnella* strain prompted further seedling development. Yamamoto *et al.* (2017) studied the successful symbiotic germination of *Bletilla striata* with *Tulasnella* spp. while Liu *et al.* (2022) reported that *Tulasnella* sp. enhanced seed germination and seedling growth by producing plant hormones. Basidiomycetous fungi (*Tomentella*) and Helotiales were earlier recovered from roots of *Oreorchis indica* (Suetsugu *et al*., 2021). Orchid taxa vary in their fungal specificity and the embryos of photosynthetic orchids appear to form mycorrhizal associations with a fewer fungal species than adults. It is relatively common to find many endophytic fungal species in adult orchids. Many orchid genera have been investigated for their endophytic fungal diversity and the endophytes mainly belong to Basidiomycetes (*Ceratobasidium, Ceratorhiza, Epulorhiza, Mycena, Rhizoctonia, Sebacina, Thanetophorus, Tulasnella, Tomentella etc.*) and Ascomycetes (*Alternaria, Fusarium, Trichoderma, Xylaria etc.*) genera (Alexander and Hadley, 1983; Bhatti *et al*., 2016; Chen *et al*., 2011; Rasmussen, 2002; Sathiyadash *et al*., 2014; Suetsugu *et al*., 2021; Yamamoto *et al.,* 2017). Endophytic fungi are recognized as mutualists that enhance plant resistance to diseases, salinity, water scarcities and heat stress, while also promoting plant growth. In the present study, the isolated *Tulasnella* fungi from roots of selected orchid, were unable to allow seed germination. Although 14% of seeds germinated symbiotically, displaying embryo swelling within two weeks of inoculation, this stage does not necessarily indicate true germination, as even dead embryos can absorb water and swell (Meng *et al*., 2019). This result is similar to earlier reports where orchid germination fails to progress beyond early stages (swelling of embryo) when associated with *Fusarium* (Vujanovic *et al*., 2000) or *Mycena* (Guo *et al*., 1997) species. However, the latent roles of mycorrhizal fungus in adult plants require further investigation, especially since they are isolated from the roots of mature plants. The germinating entities failed to exhibit any response, either symbiotically or asymbiotically, despite repeated subculturing on fresh media even after four months of inoculation, indicating that either the isolated fungus was incompatible or the culture conditions were unsuitable (Meng *et al.,* 2019). Chen *et al*. (2011, 2013) also reported that *Xylaria* species were the dominant fungi in several *Dendrobium* species across tropical regions of China, though their specific roles remain unclear. Most significantly, we recorded that in *O. indica*, the *Tulasnella* mycorrhizal fungi isolated from adult plants are ineffective at supporting germination. The capability to switch fungal partners may help orchid plants adapt to environmental fluctuations and disturbances, particularly as their metabolism changes in adulthood (McCormick *et al*., 2004; Tesitelova *et al*., 2012). Furthermore, a shift in mycobiont reduces competition with adult plants by utilizing different fungal partners. While lifelong association may provide a positive maternal effect on advanced seedlings (Jacquemyn *et al*., 2011; Phillips *et al*., 2011), avoiding fungal partner sharing with adults seems to offer an ecological advantage.

The morpho-molecular approaches established for identification of mycorrhizal fungi from *Oreorchis indica* may facilitate to expound the diversity and variability of the mycorrhizal species. These mycorrhizal interactions have a referential value to be utilized in symbiotic seed germination experiments and thus protecting RET (rare, endangered, and threatened) and endemic orchids. Further interpretation of the ecological functions of each fungal taxon and a clear classification and phylogenetic analysis to completely clarify the roles and relationship between fungi, soil, and plant ecosphere is still needed (Standing *et al*., 2007). On the other hand, *in vitro* asymbiotic seed germination in a few commercially important and endangered orchid species has earlier been successfully carried out by some workers (Anuprabha and Pathak, 2019; Bhowmik and Rahman, 2020, 2022,2023; Dhillon and Pathak, 2023; Gangaprasad *et al*., 2024; Jaryal *et al*., 2025; Lalduhsanga *et al.,* 2021; Mutum *et al.,* 2022; Pathak *et al*., 2016, 2017, 2023; Sunita *et al.,* 2021; Thakur and Pathak, 2020, 2021; Tripura *et al.,* 2022; Vasundhra *et al.,* 2021) so as to conserve these valuable orchids. The efforts are still not commensurate with the large size of the orchid family.

Conclusion

The eco-friendly mycorrhizal fungi have the potential to enhance nutrient uptake and stress resilience in plants, thus, improving the sustainability of commercial orchid cultivation by reducing the requirement for costly and environment damaging chemical inputs such as fertilizers and pesticides. The geophytic orchid seeds obligatorily require establishing an association with fungus in early stages of growth *in vivo*. Moreover these species have a reputation of being very difficult to grow asymbiotically *in vitro*. Therefore, the symbiotic seed germination signifies

an efficient technique to look into the orchid-fungus relationship, specificity in a precise manner, and facilitating their restoration into native habitats. An additional advantage of symbiotic seed germination is that the resulting seedlings can be utilized as both plant material and inoculum for conservation efforts. The isolation of a suitable mycobiont for *Oreorchis indica* is a promising step forward in on-going efforts to develop reintroduction and conservation protocols for this species, as well as other RET orchid species. Further researches should emphasis on *in vitro* symbiotic and asymbiotic seed germination, acclimatization, and *in situ* establishment methodologies to advance progress in conservation of *O. indica*.

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