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Isolation and Identification of Rhizospheric and Endophytic Fungi Associated with *Habenaria brachyphylla* (Lindl.) Aitch.: An Endemic and Rare Orchid of the Western Ghats of Maharashtra, India

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ABSTRACT: Orchidaceae is one of the largest families among the flowering plants extensively distributed in the Eastern Himalaya, Eastern part of Western Himalaya, and Western Ghats. A Terrestrial orchid is an enormous group among the orchidaceous family which needs to be conserved as they are on the verge of being threatened. The fungal partner associated with the roots of terrestrial orchids plays an important role in the germination of seeds and in providing nutrients to orchids. Therefore, this study aims to isolate and identify the fungi associated with *Habenaria brachyphylla* (Lindl.) Aitch from the Western Ghats of Maharashtra can lead to the development of efficient conservation strategies for this endemic and rare orchid.

In our present study, the fungal isolates of endophytic and rhizospheric regions were isolated and identified. All the isolates found belonged to the phylum Ascomycota. Amongst which *Aspergillus sydowii* was found in the root peloton however other species such as *Acremonium* spp., *Clonostachys* spp., *Fusarium* spp., *Penicillium* spp., *Talaromyces* spp., showed the association with the rhizospheric region.

Keywords: Terrestrial orchid, *Habenaria*, Soil fungi, Peloton, Molecular identification.

INTRODUCTION

Orchidaceae is one of the largest and species-rich families among the flowering plants distributed in almost every habitat with specificity (Fay and Chase, 2009). The maximum diversity of orchids in India has been observed in the Eastern Himalayas, Eastern part of the Western Himalaya and Western Ghats (Arisdason and Lakshminarasimhan 2020). In India, 186 genera with 1331 orchid species have been reported among which 130 species with 38 genera endemic to peninsular India out of which 34 species were found to be threatened (Jalal, 2012; De and Medhi 2014). Terrestrial orchids are an enormously threatened species due to their specialized habitat, therefore, need to be conserved otherwise ultimately results in the complete loss of their knowledge or hamper the development of new insights (IUCN, 2001; Wonkka *et al.*, 2012).

The conservation of orchids in recent years was extensively done through the reintroduction of *in-vitro* propagated plantlets to their natural habitat by asymbiotic and symbiotic seed germination methods (Aggarwal and Zettler 2010; Abraham *et al.*, 2012; Chen *et al.*, 2015). For symbiotic seed germination, it is therefore essential to identify the fungal partners which are associated with the orchid species (Khamchatra *et al.*, 2016; Zhao *et al.*, 2021). So far, a large number of mycobiont have been isolated from orchids but still about 7% out of 1.5 million fungal taxa are known (Xi *et al.*, 2020). The various endophytes of an orchid were isolated and identified for their symbiotic seed germination. In recent research, it has been found that, along with the endophytic mycorrhizal fungi, the rhizospheric non-mycorrhizal fungi of orchid's also play an important ecological role (Chand *et al.*, 2020). Rhizosphere is a portion of a soil around the roots of plants in which there are presence of many soil microorganisms including fungi. Rhizosphere fungi play a significant role in plant growth through different activities like absorption of nutrients, growth hormone production and control of pathogenic attack (Larekeng *et al.*, 2019; Sun *et al.* 2022).

In India, among the terrestrial orchid species, about 45 species of *Habenaria* have been reported in the Western Ghats of India among which 21 are endemic (Jalal and Jayanthi 2012). As orchid seeds, lack of endosperm, they rely on fungal partners to supply the carbon source as well as other nutrients in nature for their germination (Rasmussen, 1995). Due to many intrinsic and extrinsic

factors, *H. brachyphylla* coupled with low frequency of seed formation and ultimately limited evidence of seed germination (Avhad *et al.*, 2021). Hence the species being rare and threatened in the wild are included under CITES provisions.

Hence, this study aims to isolate and identify the fungal partners associated with *Habenaria brachyphylla* (Lindl.) Aitch from the Western Ghats of Maharashtra which can lead to the development of efficient conservation strategies of this endemic and rare orchid.

MATERIALS AND METHODS

A. Selection of soil sample and roots

The study sites were located approximately at 18º 10′59.76″ N and 73º 48′58.89″ E at the Western Ghats of Maharashtra in India. The wild, bloomed and healthy *H. brachyphylla* (Lindl.) Aitch. orchid root and rhizosphere soil samples were collected between August-October 2021 during the rainy season and kept in sterile plastic bags and used within 24 hrs for isolation of fungal partners (Fig. 1). Roots were carefully removed from the plant and then the plant was again replanted. The roots and the soil around the roots were taken into two different 100 ml beakers. The roots were washed in running tap water for 10 min. to remove soil and surface debris then used as explants (Zhu *et al.*, 2008).

Fig. 1. Bloomed orchid habit of *H. brachyphylla.*

B. Anatomy of roots for fungal colonization

Thin and uniform transverse sections of the roots were taken and stained with trypan blue (0.1% Lactophenol) and observed under Leica microscope (LAS Ver 4.4) for the presence of pelotons (Fig. 2) in cortical cells of the roots of *H. brachyphylla* (Lindl.) Aitch. Microphotography was done at 10X, 40X and 100X (Bertolini *et al.*, 2014; Pereira *et al.*, 2018).

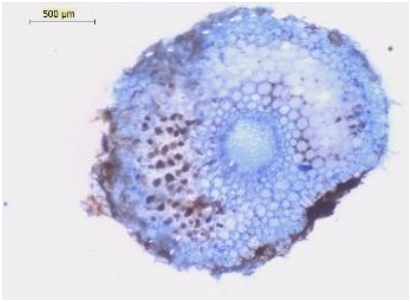

Fig. 2. T.S. of the root of *H. brachyphylla.*

C. *Isolation of endophytic fungi*

The samples showing the presence of pelotons were chosen and washed with distilled water and surface sterilized with 0.5% sodium hypochlorite solution for 3 min. and then rinsed with sterile distilled water 3-4 times. Final disinfection was done by treating it with 70% alcohol for 30 sec. and rinsed with distilled water 3-4 times. The cross sections of sterilized roots were taken about 2 mm thickness by a sterile surgical blade and plated on to 9 cm petri plate containing fungal isolating potato dextrose agar medium. Around five explants were inoculated per petri plate with six replicates. The Petri plates were then incubated in the dark at 25±2ºC for 7 days. The fungal hyphal tips that arise from root cells containing peloton were transferred to fresh PDA to maintain the pure fungal culture (Ezeonuegbu *et al.*, 2022). The fungi grown from the inner portion of the root section were considered probable endophytic fungi.

D. Preparation of media

Potato Dextrose Agar (PDA) medium was prepared by using potato infusion (200 g sliced potatoes) in 1000 ml DW for 30 min. After boiling the mixture was squeezed through a muslin cloth to get the pulp of it. Dextrose (20 g) was added to it until the completely dissolved. Then pH was adjusted to 5.6 and agar (15 g) was added for solidification. The prepared media was digested and sterilized at 15lbs pressure for 15 min. in an Autoclave. After autoclaving 20 ml media was poured into each petri dish and allowed to solidify. Before pouring the media antibiotic streptomycin 5mg/L was added to it to inhibit the growth of bacteria.

E. Isolation of rhizospheric fungi

The soil dilution plate method (Waksman, 1922) was used to prepare the soil dilutions for the isolation of rhizospheric fungi of *H. brachyphylla.*

The suspension of 1g of rhizospheric soil of *H. brachyphylla* in 10 ml of sterile distilled water was done. Soil dilutions from 10^1 to 10^5 were made from which 1ml of each concentration was added to freshly prepare sterile PDA petri dishes and swabbed to ensure the uniform distribution of inoculum. A total of six replicates of each dilution were prepared and labelled properly. The petri dishes were then incubated at 25±2ºC for 4-7 days in the dark. The pure fungal colony isolation was carried out by taking out a single colony on a single PDA petri dish and slant with six replicates. Then the isolates were maintained and stored in the refrigerator for further identification in the form of PDA slants.

F. Slide culture technique of fungi for morphological identification (Harris, 1986).

(i) Slide culture. All the glass wares like petri dishes, slides and coverslips as well as forceps, blade, blade holder, scalpel, spatula were sterilized in an autoclave for 30 minutes at 121°C temperature and a pressure of 15 psi. The bent glass rod was placed in petri dish on the disc of sterile filter paper, and a sterile glass slide was put on the glass rod. With the help of sterile blade 1 × 1 cm block of potato dextrose agar (PDA) cut and

then was transferred to the centre of glass slide with the help of sterile scalpel.

Then, using nichrome wire loop, the fungal mycelium of pure culture was inoculated from culture plate to the all sides of PDA agar block. Sterile cover slip was placed on the agar block with slight pressure. About 4 mL of sterile water was poured on the filter paper disc at the bottom of petri dish along with the 20% glycerine to prevent fogging and then it was closed with plate cover. The petri dishes then incubated at room temperature 25±2ºC for around 7days covered with aluminium foil. The entire procedure was then executed for each of culture.

(ii) Staining. After the incubation period, a drop of 0.1% lactophenol cotton blue was placed on the microscope slide. The cover slip containing hyphae and spores was removed from the agar block carefully and placed on a slide. The excess medium was removed from the slide and then sealed with the help of DPX (Diesteryenedibutylpthalate xylol) or wax. The fungal characteristics were observed for the morphological identification of the species under the Euromex microscope.

G. DNA isolation and sequencing

The fungal isolates were inoculated on sterilized potato dextrose agar (PDA) medium and incubated in an incubator for two weeks. DNA was extracted from 15 days old cultures. The genomic DNA was isolated by the standard phenol/chloroform extraction method (Sambrook *et al.,* 1989), followed by PCR amplification of the ITS regions using universal primers ITS1 [5'-TCC GTA GGT GAA CCT GCG G -3'] and ITS4 [5'-TCC TCC GCT TAT TGA TAT GC -3']. The amplified ITS PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequence (Applied Biosystems, Inc., Foster City, CA). Essentially, sequencing was carried out from both ends so that each position was read at least twice. Assembly was carried out using Lasergene package followed by NCBI BLAST against sequences from type material for the identification (Boratyn *et al.,* 2013).

RESULT AND DISCUSSION

A total of seven fungal isolates were isolated from a rhizospheric and endophytic region of the root among which six were rhizospheric and one was endophytic. Morphological and molecular identification showed that all of the isolates belonged to the phylum Ascomycota.

A. Colony morphology and microscopic characterization of fungal isolates

The fungi were identified using the standard literature like Handbook of Soil Fungi (Nagamani *et al.,* 2006), Pictorial Atlas of Soil and Seed Fungi Morphologies of Cultured Fungi and Key to Species (Tsuneo Watanabe, 2000).

For the morphological identification, macromorphological characters of the fungal colony like colour on upper and reverse, topography, pigmentation, exudence, texture, diameter etc. were considered comprising micromorphological characters like septate or aseptate hyphae, branching, pigmentation, size and shape of the spores etc.

Key for morphological identification

AAHbEn

***Aspergillus* spp.**

The key is based upon Samson (1994) and the description on Raper and Fennell (1965), Domsch *et al*., Vol 1 (1980)

Phialides strictly uniseriate ……...1

1b. Conidial heads radiate to columnar, variable in colour; vesicles globose to subclavate orturbinate ……....2

2a. Conidia heads radiate to loosely definitely columnar; osmophilic; cleistothecia present……...…….....Subgenus ***Aspergillus***

Colonies grown well, velvety, dark green to greyish; the reverse of the colony honey brown; conidial heads globose; vesicles globose to hemi-spherical bearing two series of hyaline phialides; conidia globose to subglobose.

AAHbFs

***Fusarium* spp.**

Colony fast growing, pink coloured; aerial mycelium felty; conodiophores solitary and conidiogenous cells phialidic; phialides slender, tapering distally, macroconidia large, septate, hyaline, fusiform, cylindric, curved, frequently naviculate (boat shaped), microconidia smaller, non-septate or one septate, in chains……..*Fusarium* **spp**.

AAHbGr

***Penicillium* spp.**

Slow growth of colony, green coloured, reverse brown coloured, conidiophores conspicuous, more or less erect, sometimes aggregated into synnemata, septate, branching at the apex, branches divergent or adpressed to main conidiophore axis, giving brush-like appearance; conidia borne in long chains, globose to ovoid...……..…***Penicillium* spp.**

AAHbGs

***Acremonium* spp.**

Colony hyaline, green coloured, reverse pale coloured, mycelium fine, simpleaw-shaped, simple to compound conidiophores, confined to lower part; conidia one celled, hyaline or pigmented, in droplets……...……***Acremonium* spp.**

AAHbWr

***Clonostachys* spp.**

White-coloured colony, reverse pale coloured, hyphae hyaline, branched, septate, conidiophores erect or suberect, penicillate fashion with metulae and phialides; phialides sometimes divergent; conidia hyaline, ovoid or flattened on one side……...…***Clonostachys* spp.**

AAHbWs

***Fusarium* spp.**

White coloured colony, reverse pale coloured, conodiophores solitary; phialides slender, macroconidia large, septate, hyaline, curved, frequently naviculate (boat shaped), microconidia smaller, non-septate or one septate, in chains...…….....***Fusarium* spp.**

AAHbYs

***Talaromyces* spp.**

Colonies slow growing, yellowish green white colony, reverse pale to brown coloured, loosely interwoven hyphae, asci spheroidal to ellipsoidal, borne in short chains; ascospores ellipsoidal……..……..***Talaromyces* spp.**

The cultural pictures of surface characteristics of **Fig. 3a** and **3b** show the reverse characteristics and microscopic features of fungal isolates of AAHbFs, AAHbGr, AAHbWr, AAHbEn, AAHbYs, AAHbWs and AAHbGs respectively.

The observation of microscopic structures by the optical microscopy showed that the filamentous fungi AAHbFs and AAHbWs were *Fusarium* spp., however AAHbEn, AAHbWr, AAHbGs, and AAHbGr were *Aspergillus* sp., *Clonostachys* sp., *Acremonium* sp., *and Penicillium* sp. respectively. The analysis of AAHbGr and AAHbYs showed similarities in the structures such as conidiophore, the branching pattern of conidiophores, and cleistothecium. All these isolates were then confirmed using internal transcribed spacer (ITS) ribosomal DNA sequencing (Tsuneo Watanabe, 2000; Nagamani *et al.*, 2006).

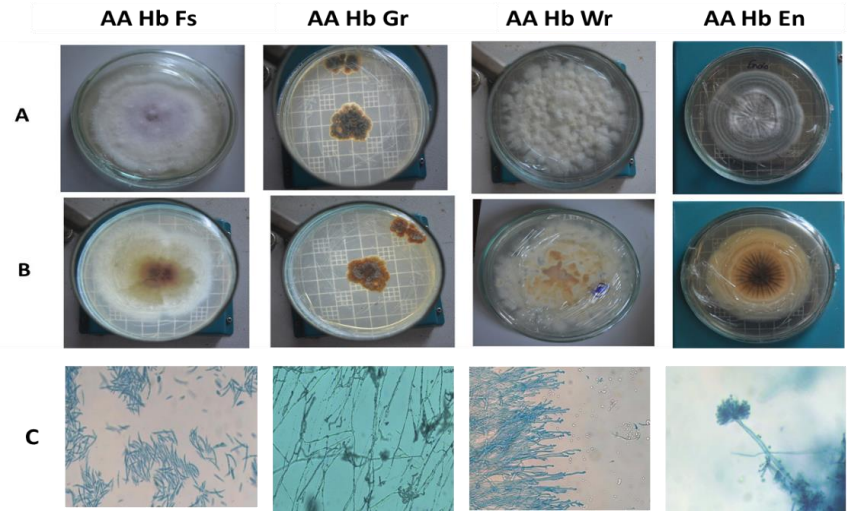

Fig. 3a. Cultural pictures of fungal isolates (A) Surface characteristics; (B) Reverse characteristics (C) Microscopic features.

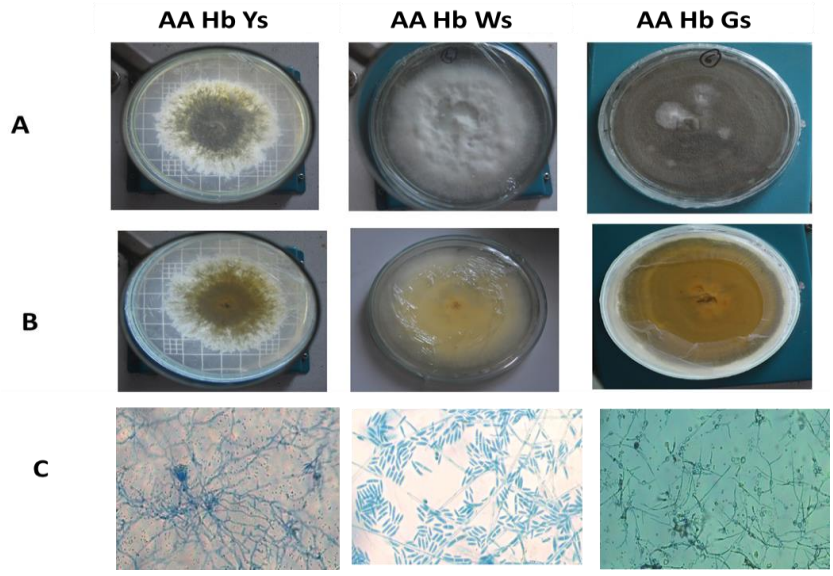

Fig. 3b. Cultural pictures of fungal isolates (A) Surface characteristics; (B) Reverse characteristics (C) Microscopic features.

B. Molecular identification of fungal isolates

The molecular identification of isolates was carried out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. At the facility centre a total of seven fungal isolates were identified.

Most of the fungal genera show macro and micro morphological similar structures hence along with morphological identification molecular identification is much essential. In the current work, we found the *Penicillium* and *Talaromyces* sp. which showed similarities in the microscopic structures. Therefore, in the present study in addition to morphological identification, molecular identification has been done (Sun *et al.*, 2022).

Table 1: Cultural characteristics and molecular identification of fungal isolates.

Fungal Strain	Colour of fungal isolate	Taxonomic Designation	Phylum	Accession no.	% similarity
AA Hb En	Dark green to greyish	*Aspergillus sydowii* CBS 593.65	Ascomycota	NR_131259.1	100
AA Hb Fs	Pink	*Fusarium foetens* CBS 110286	Ascomycota	NR_159865.1	99.12
AA Hb Ws	White	*Fusarium gamtoosensis* strain CBS 146502	Ascomycota	MW173063.1	100
AA Hb Wr	White	*Clonostachys rosea* f. catenulata CBS 154.27	Ascomycota	AF358231.1	99.38
AA Hb Gs	Green	*Acremonium cellulolyticus* Y-94	Ascomycota	AB474749.2	100
AA Hb Gr	Green	*Penicillium guanacastense* DAOM 239912	Ascomycota	NR_111673.1	99.81
AA Hb Ys	Yellowish green white	*Talaromyces australis* IBT 14256	Ascomycota	NR_147431.1	97.64
		*Talaromyces yunnanensis*KUMCC 18-0208		MT152339.1	97.56
		Talaromyces pratensis NRRL 62170		NR_165529.1	97.41

The cultural characters and molecular identification of fungal isolates with the GenBank Accession numbers and percentage of similarities are showed in Table 1. Endophytic fungal isolate of AAHbEn was identified as *Aspergillus sydowii* CBS 593.65 (GeneBank accession no. NR_131259.1) however the rhizospheric fungi AA Hb Fs, AA Hb Ws, AA Hb Wr, AA Hb Gs, AA Hb Gr and AA Hb Ys were identified as *Fusarium foetens* CBS 110286 (NR_159865.1), *Fusarium gamtoosensis* strain CBS 146502 (MW173063.1), *Clonostachys rosea* f. catenulata CBS 154.27 (AF358231.1), *Acremonium cellulolyticus* Y-94 (AB474749.2), *Penicillium guanacastense* DAOM 239912 (NR_111673.1), *Talaromyce saustralis* IBT 14256 (NR_147431.1) respectively.

C. Phylogenetic analysis

A phylogenetic tree of the fungal samples has been generated to reveal the isolates in grouping patterns of a close resemblance. All fungal species had cluster identities of above 97% with those from GenBank.

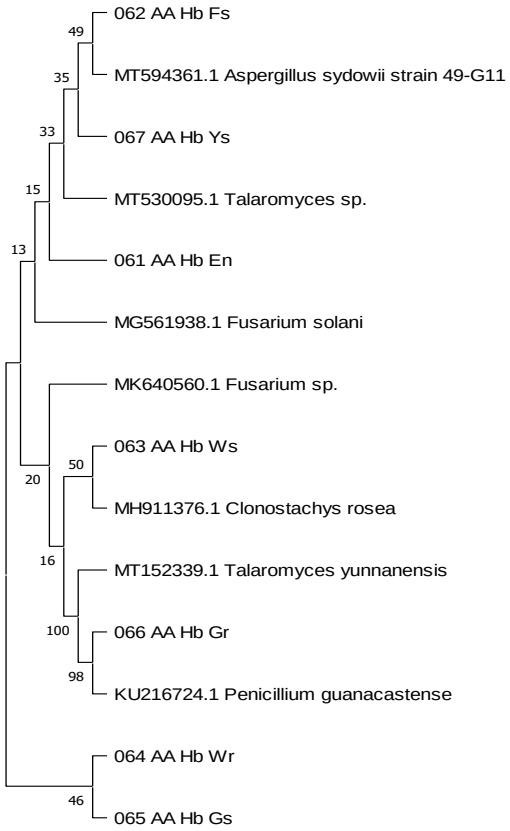

Fig. 4. Phylogenetic tree based on ITS-rDNA sequence of endophytic and rhizospheric fungi isolated from *Habenaria brachyphylla* (Lindl.) *Aitch.*

Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura *et al.*, 2004). This analysis involved 14

nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 603 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.,* 2021).

A phylogenetic tree was created with ITS nucleotide sequence to check homologues from different fungi (Fig. 4). According to morphological and molecular characteristics, the strain was identified as *Aspergillus sydowii*. The ITS nucleotide sequence completely showed homology to *Aspergillus sydowii* (accession number NR_131259.1). Likewise, *Fusarium gamtoosensis* strain CBS 146502 and *Acremonium cellulolyticus* Y-94 also showed 100% homology according to the GeneBank database. However, *Penicillium guanacastense* DAOM 239912, *Clonostachys rosea* f. catenulata CBS 154.27, *Fusarium foetens* CBS 110286 and *Talaromyces australis* IBT 14256 showed 99.81%, 99.38%, 99.12% and 97.64% homology respectively.

DISCUSSION

The aim of the study was to determine the endophytic and rhizospheric fungi associated with the *Habenaria brachyphylla* (Lindl.) Aitch. This is the first report of isolation and identification of fungal isolates from *Habenaria brachyphylla* (Lindl.) Aitch. The great diversity of association of fungi and their metabolites showed the pharmacological properties found in orchids, which would represent a benefit to the plant (Ma *et al.,* 2015).

In the present study, it was found that the fungi associated with the orchid *H. brachyphyalla* belong to the phylum Ascomycota although pezizalean Ascomycota species have been reported as mycorrhizal fungi from *Epipactis* spp. (Tesitelova *et al.,* 2012). However, *Dactylorhiza sambucina* possesses several asco- and basidiomycetes in their roots (Pellegrino and Bellusci 2009). Nonetheless, basidiomycetes are mostly reported as orchid mycorrhiza than ascomycetes (Rasmussen, 2002). It is known that carbon is the source of nutrition required at the time of the protocorm stage, but at a later stage, other nutrients also play an important role in the growth and development of the plant (Stockel *et al*., 2014). However, in the present study *A. sydowii* was identified in the root peloton which has the ability to produce the enzymes, cellulase, and ligninase, in addition to being responsible to facilitate the uptake of phosphate by plants (Baron *et al.,* 2018). *Fusarium foetens* have been found to be associated with the *H. brachyphylla* however; *F. oxysporum* non-pathogenic mycorrhizal fungal isolate was identified from *Bletilla striata* and *Pecteilis susannae* (L.) Rafin based on ITS sequencing (Chutima *et al*., 2011; Jiang *et al.,* 2019). Several *Fusarium* spp. isolates have been disclosed in the seed germination of *Cypripedium reginae* (Vujanovic *et al.,* 2000) and to form endomycorrhiza in *Eulophia alta* (Johnson *et al.,* 2007). Along with the plant and human pathogens, plant endophytes and saprophytes, the *F. oxysporum* species was reported as a root rotting agent in *Cymbidium* orchids (Benyon *et al.,* 1996). *Fusarium gamtoosensis* i.e. *Neocosmospora* and other species such as *Aspergillus, Penicillium, Gymnopilus, Pyrenochaeta, Trichoderma* has been showed the mycorrhizal association with the orchid *Bletilla striata* (Zeng *et al.,* 2021).

The genus *Clonostachys* has been found to stimulate the protocorm development in *Pogoniopsis schenckii* Cogn. an aclorophyllated and mycoheterotrophic (MH) orchid (Sisti*et al* 2019). The inoculation of *Clonostachys rosea* f. *catenulate* showed the plant growth-promoting effect and bio-control of the pathogenic fungus *Pythium ultimum* in *Euphorbia pulcherima* (Camps and Sigg 2015). *C. rosea was* reported as an aggressive mycoparasite as it produces chitinases and glucanases which degrades the cell walls of diverse plant pathogens from the oomycete genus *Pythium* and *Fusarium* (Inglis and Kawchuk 2002; Jensen *et al.,* 2021). The *C. rosea* is important in sustainable plant protection and conservation strategy however there were few reports of root rot on orchids (Lee *et al.,* 2020).

Cellulase specific activity and glucose yield from lignocellulosic materials were observed with the *A. cellulolyticus* inoculant. (Fujii *et al.,* 2009). The rhizospheric non-mycorrhizal fungi such as *Fusarium, Acremonium, Cylindrocarpon* and *Phlebiopsis flavidoalba* has been found in *Arundinagramini folia*s pecies which were behaved as symptomless endophytes (Meng *et al.,* 2019). However, *A. cellulolyticus* has been isolated from rhizosphere and endosphere of endangered plant *Cypripedium japonicum.* (Geun-Hye *et al*., 2017), while in the present study *A. cellulolyticus* was isolated from rhizospheric region of *H. brachyphylla.* The antimicrobial potential was reported from endophytic fungi of *A. cellulolyticus* isolated from *Melastoma malabathricum* L. against the *P. aeruginosa, E. coli, S. aureus, F. oxysporum, F. Graminearum* and *F. Culmorum,* which might show potential inhibitors of these microbial strains in the *H. brachyphylla.* (Mishra*et al*., 2016).

*Penicillium guanacastense*was isolated from the *H. brachyphylla* however *Penicilium* sp. along with the other fungal isolates such as *Trichoderma, Aspergillus, Rhizoctonia*-like fungi, were identified in the root peloton of *Caladeniaformosa* (Huynh *et al.,* 2009). Themembers of the genera *Asper gillus* spp., *Penicillium* spp. and *Talaromyces* has been found to synthesise the L-asparaginase which is essential to convert the asparagine into aspartate and ammonia, and promotes the production of other compounds (Herrera-Rus *et al.,* 2020).

Inclusive of *Penicillium* sp. other species of *Aspergillus* were isolated form *Crepidium acuminatum* (D. Don) Szlach, *Bulbophyllum neilgherrense* and *Vanda testacea* (Sudheep and Sridhar 2012). The fungal species *of Xylaria, Trichoderma,* and *Fusarium* are non-mycorrhizalendophytes mostly found in association with orchids (Ma *et al.,* 2015).

CONCLUSIONS

In this study, the isolation and molecular identification of rhizospheric and endophytic fungal isolates of *Habenaria brachyphylla (*Lindl.) *Aitch.* from the Western Ghats of Maharashtra, India was studied. The results showed that the rhizospheric and endophytic fungi of *H. brachyphylla* were non-mycorrhizal and all belonged to the phylum Ascomycota.

FUTURE SCOPE

The fungal isolates of rhizosphere and root endophyte can be employed single and/ or in a consortium for the symbiotic seed germination of *H. brachyphylla* to conserve this rare and endemic orchid species as a future prospect.

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Conflict of Interest. None.

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