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RESEARCH ARTICLE OPEN ACCESS

Isolation and Characterization of Dominant Fungi from Rhizospheric Soil of *Saussurea obvallata* (DC.) Edgew. (Brahma Kamal) of the Indian Himalayan Region

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Abstract

The Himalayan region is conferred by diverse wealth in term of flora and fauna. Among Himalayan plant biodiversity, *Saussurea obvallata* (common name *Brahma Kamal*) has an immense spiritual and medicinal significance. Owing to high demand, this herb is on the verge of extinction. Numerous studies have been carried out for preservation and *in vitro* regeneration of *Saussurea obvallata*. In view of above, this is the first study which aims to isolate and identify rhizospheric fungi associated with this plant. Soil samples were collected from three locations in Kedarnath valley and total 34 fungal isolates were isolated. Out of 34, three fungal isolates i.e. MaHaD1, MaHaD2 and MaHaD3 were selected for morphological and molecular characterization. The 16S rDNA sequencing identified MaHaD1, MaHaD2 and MaHaD3 as *Phanerochaete chrysosporium, Trichoderma longibrachiatum* and *Aspergillus fumigatus* respectively. The presence of these three dominant fungi in rhizospheric region seems to be results of climatic conditions and plant physiology. Results indicate that the presence of fungal microflora may play a vital role in survival and proliferation of *Saussurea obvallata* plant by providing tolerance and resistance to abiotic stress and fungal/nematode pathogens respectively.

Keywords: *Saussurea obvallata*, *Aspergillus fumigatus, Phanerochaete chrysosporium, Trichoderma longibrachiatum,* Rhizospheric soil, rDNA-ITS Sequencing.

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INTRODUCTION

Saussurea obvallata (DC.) Edgew. (Brahma Kamal) is one of the valuable traditional herb of the Himalayan region belonging to the family Asteraceae[1]. Generally, it is found in different states of the Indian Himalayan region (Kashmir, Himachal Pradesh, Uttarakhand, Sikkim and Arunanchal Pradesh) and other countries (Nepal, Bhutan, Myanmar, China and Pakistan)[2] with an altitudinal range from 3,000 - 4,800 m above mean sea level[3,4,5]. The whole plant of *Saussurea obvallata* is used by the local people of the Himalaya, for traditional, cultural, and religious purposes[6,7]. The Conservation Assessment and Management Plan (CAMP) had categorized *Saussurea obvallata* as an endangered species. Several investigations have been carried out to study reproductive biology, genetic diversity, cultivation and propagation of *S. obvallata* in view of conservation and management of the endangered medicinal herb[3,6] (Fig. 1). To the best of our knowledge, no study has been done to study rhizospheric microbes of *Saussurea obvallata* till now. As rhizospheric microflora plays an important role in germination, growth and survival of plant in respective niche. In order to know the role of rhizospheric microflora in growth of *Saussurea obvallata,* this study was conducted to isolate the fungal community of *Saussurea obvallata* rhizospheric region. In the present study, we revealed that the potential fungal community present in the rhizospheric soil of *Saussurea obvallata*.

Fig. 1. Soil sample collection sites (Photo: P. Semwal)

Table 1. Growth conditions and collection site details of selected isolates

Isolate Name	Growth Conditions		Soil Sample
	Temperature	Medium	
MaHaD1	25±3.0°C	Fungal Broth and PDA	MG
MaHaD2	25±3.0°C	Fungal Broth and PDA	MP
MaHaD3	25±3.0°C	Fungal Broth and PDA	HP

MATERIALS AND METHODS

Isolation of Fungus

The rhizospheric soil samples of *Saussurea obvallata* were collected from Madhu Ganga (MG), HathiParwat (HP) and MahaPanth (MP) in Kedarnath Valley (Fig. 1), Uttarakhand, India[8]. The samples were packed and sealed in plastic bags and immediately brought to the laboratory and stored in refrigerator till further use. The three soil samples were serially diluted and 100µl of dilution sample (10^{0-5}) was plated onto potato dextrose agar (PDA) (CDH, JO-0013, India) supplemented with antibiotic (50 mg L^{-1} of chloramphenicol) for the isolation of fungi and incubated for seven days at 20°C.

Morphology of Isolates

The fungi were identified based on the macroscopic (colony morphology, texture) and microscopic (fruiting bodies *i.e.* conidia and hyphae) culture characters. For microscopic identification fungal isolates were stained with lactophenol cotton blue stain and were observed at 40x and 100x. The classifications of the fungi were carried out following standard procedures. All the isolated fungi were named and stored in fungal broth containing 15% (v/v) glycerol at -20°C for further use.

DNA extraction, gene amplification and sequencing

Genomic DNA was isolated from the isolates *viz.* MaHaD1, MaHaD2 and MaHaD3 using the ultrapure DNA isolation kit.The identification of isolates carried out by internal spacer transcribed (ITS) region amplification and sequencing. The ITS region of rDNA was amplified by PCR using fungal universal primer pairs ITS4 and ITS5[9]. The sequencing PCR was set up with ABI BigDye®Terminatorv3.1 Sequence kit. The raw sequences were obtained from ABI 3100 automated DNA sequencer and aligned with publicly available sequences and analyzed for identification.

Sequence analysis and Multiple sequence alignment

The sequence data were aligned using BLASTN 2.8.1 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and sequences were deposited in NCBI (https://submit.ncbi.nlm.nih.gov/subs/genbank/) for accession number. The phylogenetic analysis was done *via.* MEGA6-ClustalW sequence program followed by evolutionary tree constructionusing software MEGA6based on the Tamura-Neimodel[10]. Multiple sequence alignment (MSA) of fungal isolates *i.e.* MaHaD1, MaHaD2 and MaHaD3 were carried out using T-Coffee (Version_11.00.d625267)(http://tcoffee.crg.cat/), a consistency-based MSA program[11].

Deposition of fungus isolates in culture collection center

MaHaD1, MaHaD2 and MaHaD3 fungal isolates were deposited in the National Fungal Culture Collection of India (NFCCI) (http://nfcci.aripune.org/) for the accession no. and future research works.

RESULTS AND DISCUSSION

Fungal Isolates

Total 34, 29 and 31 fungal isolates were isolated from rhizospheric soil samples of *Saussurea obvallata* collected from MG, HP and MP sites respectively. For further study one fungal isolate (MaHaD1, MaHaD2 and MaHaD3) from each collection site i.e. MG, MP and HP were selected on the basis of the best growth (Table 1).

Morphological identification

Morphological identification of MaHaD1, MaHaD2 and MaHaD3 isolates were carried out using of lactophenol cotton blue staining method (Fig. 2). The colony appearance and microscopic morphological details revealed by staining are summarized in Table 2.

Molecular identification

The ITS sequence of *Phanerochaete chrysosporium* MaHaD1 showed 99% maximum similarity with accession no. HM171940.1, MH854905.1, MG646957.1, KT188591.1 and KP135093.1 sequences while *Trichoderma longibrachiatum* MaHaD2 showed 97% similarity with accession no. MG836641.1, MG836590.1, MK015045.1, MF632112.1 and KY495196.1. Third isolate i.e. *Aspergillus fumigatus* MaHaD3 showed 98% similarity with accession no. MH185963.1,

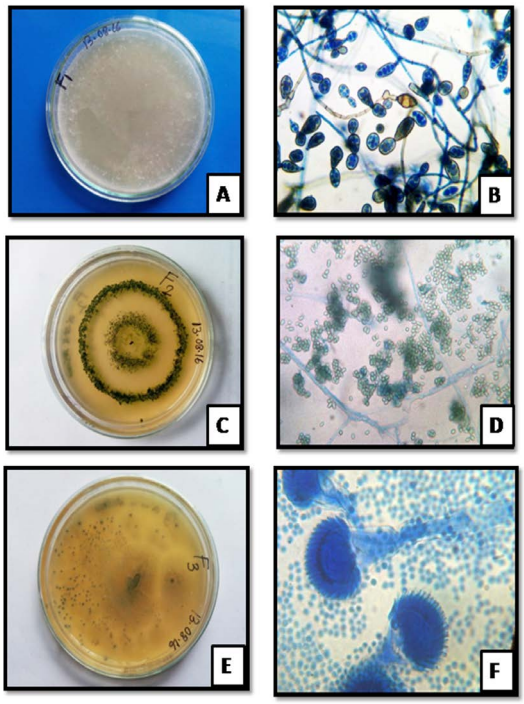

Fig. 2. Colony morphology of MaHaD1 (A) MaHaD2 (C) and MaHaD3 (E) isolates on PDA plate (after 48hrs at 28°C) respectively; Microscopic structure of MaHaD1 (B) MaHaD2 (D) and MaHaD3 (F) isolates at 100x respectively.

Table 2. Colony morphology and microscopic details of selected isoates

Isolates Name	Morphological details
MaHaD1	White cottony colonies,septate hyphae, club-shaped basidia, cylindrical to ellipsoidal shaped spores
MaHaD2	Colony greenish-yellowish coloured, conidia shape is globose/ ellipsoidal/ obovoidal, or short-cylindrical
MaHaD3	Green colored colony, echinulate conidia borne directly on broad clavate vesicles

MG972741.1, MH305231.1, MH305230.1 and MH305226.1 from nBLAST. Isolates molecular identification details from NCBI database are shown in Table 3.

Phylogenetic Analysis and MSA

Strain name and accession no. of the related fungus was retrieved from NCBI-BLAST and MSA was performed using ClustalW. Phylogenetic tree for the same data was obtained by neighbor joining method with Bootstrap values 1000 alignment with HM171940.1, MH854905.1, MG646957.1, KT188591.1, KP135093.1, MG836641.1, MG836590.1, MK015045.1, MF632112.1, KY495196.1, MH185963.1, MG972741.1, MH305231.1, MH305230.1, MH305226.1, MH911420.1, MH911400.1 and evolutionary tree shown in Fig. 3. MSA of *Phanerochaete chrysosporium* MaHaD1 (MK172050), *Trichoderma longibrachiatum* MaHaD2 (MK172051) and *Aspergillus fumigatus* (MK172052) in T-Coffee analysis (Fig. 4).

Isolates deposite report

All three fungal isolates (MaHaD1, MaHaD2 and MaHaD3) were submitted to National Fungal Culture Collection of India (NFCCI) and accesion no given to *Phanerochaete chrysosporium* MaHaD1 (MK172050), *Trichoderma longibrachiatum* MaHaD2 (MK172051) and *Aspergillus fumigatus* (MK172052) are NFCCI-4192, NFCCI-4193 and NFCCI-4194 respectively.

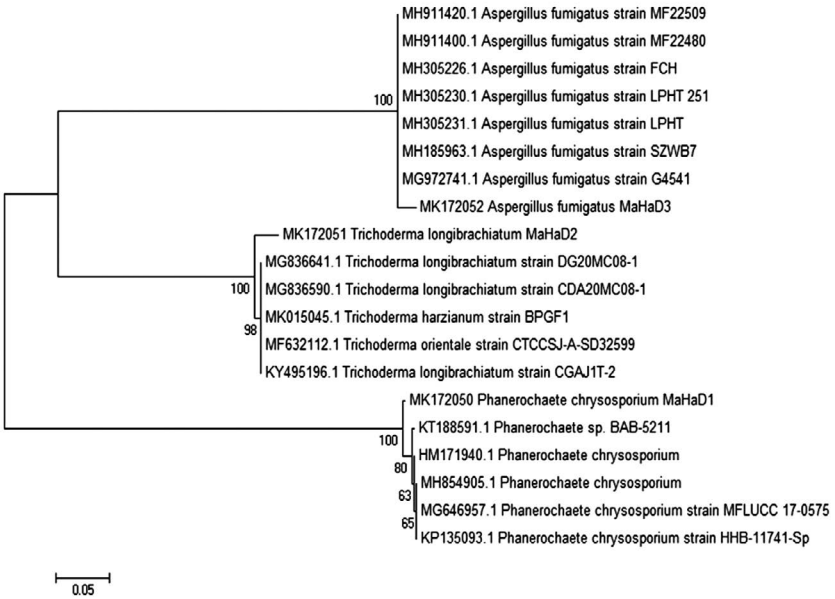

Fig. 3. Neighbors joining molecular phylogenetic tree showing relationship between *P. chrysosporium* MaHaD1, *T. longibrachiatum* MaHaD2 and *A. fumigatus* with other isolates, based on their ITS sequence

Table 3. Molecular identification details of isolates

Isolates	Isolates identification	Sequence length	Accession no.	Ident	Ident	Accession no.
MaHaD1	*P. chrysosporium*	584bp	MK172050	99%	*P. chrysosporium* isolate liu	HM171940.1
MaHaD2	*T. longibrachiatum*	547bp	MK172051	97%	*T. longibrachiatum* strain DG20MC08-1	MG836641.1
MaHaD3	*A. fumigatus*	519bp	MK172052	98%	*A. fumigatus* isolate DFS 2	MK116584.1

Fig. 4. MSA (T-Coffee) results of *P. chrysosporium* MaHaD1 (MK172050), *T. longibrachiatum* MaHaD2 (MK172051) and *A. fumigatus* (MK172052)

DISCUSSION

In our study, isolation, morphological and molecular characterization of fungal isolates from rhizospheric region of *Saussurea obvallata* suggested occourence of *Phanerochaete chrysosporium*, *Trichoderma longibrachiatum* and *A. fumigatus* as dominant genera. These fungal genera have effective role in organic decomposition, bioremediation and sustainable development[12,13]. *Aspergillus fumigatus* is saprophytic, airborne opportunistic pathogen. The ubiquitous prevalence of *Aspergillus fumigatus* in comparison to other sp. of *Aspergillus* is due to tolerance to various stresses (freezing, low pH, dehydration) and growth on decaying vegetation and debris. Although *Aspergillus fumigatus* does not have any direct role in plant growth promotion, some studies reported vital role of *Aspergillus fumigatus* in C and N recycling[14,15].

The *Phanerochaete chrysosporium* commonly known as white rot fungi[16] is well known for digestion of fragrant poisons, lignin and lipids and remediation of various pollutants such as PAH, heavy metals and dyes[17]. *Phanerochaete chrysosporium* is considered an uncommon organism for its capacity to depolymerize and change lignin, cellulose and hemicellulose, in comparison with different basidiomycetes[18].

Trichoderma longibrachiatum uses cellulases to digest cellulose from decaying plant biomass, and chitinases to digest the chitinous walls of other fungi and nematode which indicate role of *Trichoderma* as a biocontrol agent[19,20,21,22]. Moscatiello *et al.*[23] investigated that a mechanism of plant perception of HYTLO1, a hydrophobin abundantly secreted by *T.longibrachiatum*, which may assume an imperative role in the beginning times of the plant-parasite association. Some *Trichoderma* sp. are also known to provide tolerance/resistance to abiotic stresses, disease in addition to better plant growth[24,22]. The results suggest roles played by these fungal isolates in survival of *S. obvallata* plants. The probable mechanism of which may be by protecting plants from pests, inducing systemic resistance to plants for abiotic stress and improve soil nutrition acquisition. Further study is required to confirm the mechanism and role demonstrated by rhizospheric microorganisms in survival and growth of *S. obvallata*.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

LMSP and AR conceived the idea, designed the study, analyzed and interpreted data. DM, NU, KS and AC generated data. PS and PA provide the soil sample and screening. AR and DM drafted the paper and critically revised it for important intellectual content. All authors gave final approval of the version to be published.

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DATA AVAILABILITY

All data is freely available from the corresponding author on request.

ETHICS STATEMENT

Not applicable

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