Full Length Research Article

CARAS
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Research Journal of Agricultural Sciences
Volume 17; Issue 01 (Jan–Feb 2026); pp 73–79

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

Morphological and Molecular Identification of Fungi Isolated from the Cotton Rhizosphere Soil in the Bhokardan Region of Maharashtra

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Received: 11 Dec 2025; Revised accepted: 22 Jan 2026

Abstract

The present study deals with the isolation and identification of seven fungal isolates (AF1, AF2, AF3, AF4, AF5, AF6 and AF7) from the rhizosphere soil of cotton collected from the Bhokardan region, Jalna district. The fungal isolates were identified morphologically and were identified to be *Aspergillus flavus*, *Penicillium chrysogenum*, *Trichoderma harzianum*, *T. asperellum*, *T. viride*, *Trichoderma* sp. isolate yi1007, and *Trichoderma* sp. BAB-45021. The molecular characterization of the fungal isolates was performed using ITS sequencing, followed by BLAST analysis. The isolates presented highest similarity to rhizosphere fungi, *Aspergillus flavus*, *Penicillium chrysogenum, Trichoderma harzianum, T. asperellum*, *T. viride*, and two *Trichoderma* spp. The sequences submitted to the NCBI and accession numbers of isolated fungi were AF1 PX802230 AF2 PX811100, AF3 PX802359, AF4 PX802417, AF5 PX802429, AF6 PX802433, AF7 PX802453. Morphological and molecular identification could be a beneficial tool for identifying fungi isolated from the rhizosphere soil of cotton.

Key words: Cotton Rhizosphere Soil, Isolation, Fungal, Bhokardan, *Trichoderma*, Molecular, BLAST analysis

Soil is a highly complex and dynamic ecosystem that harbors an enormous diversity of microorganisms, among which fungi play a pivotal role in maintaining soil health, nutrient cycling, and plant productivity. The rhizosphere the narrow zone of soil directly influenced by plant roots is particularly rich in microbial diversity due to the continuous release of root exudates, mucilage, and sloughed cells that provide nutrients and signaling molecules for microbial colonization. Fungi inhabiting the rhizosphere are integral to plant soil interactions, contributing to organic matter decomposition, mineral solubilization, nutrient uptake, and suppression of soil-borne pathogens. Consequently, understanding the diversity and identity of rhizospheric fungi is essential for sustainable crop production and soil ecosystem management.

Cotton (*Gossypium* spp.) is one of the most important commercial fiber crops in India and plays a crucial role in the agrarian economy, particularly in semi-arid regions. Maharashtra is a leading cotton-producing state, where crop productivity is strongly influenced by soil fertility, microbial activity, and the prevalence of soil-borne diseases. The cotton rhizosphere supports a diverse assemblage of fungal communities that can exert both beneficial and detrimental effects on plant growth. Beneficial fungi such as *Trichoderma*, *Penicillium*, and mycorrhizal species enhance nutrient availability, stimulate plant growth, and provide biological control against pathogens, whereas pathogenic fungi including *Fusarium*, *Rhizoctonia*, and *Aspergillus* species may cause severe yield losses. Therefore, accurate identification of rhizospheric fungi associated with cotton is vital for developing effective disease management strategies and exploiting beneficial microbial resources.

Traditional identification of fungi has largely relied on morphological characteristics such as colony appearance, growth pattern, pigmentation, spore morphology, and reproductive structures. While morphological methods remain fundamental in mycological studies, they often present limitations due to phenotypic plasticity, overlapping characters among taxa, and the inability to reliably identify cryptic or closely related species. These challenges necessitate the integration of molecular approaches to achieve precise and reproducible identification.

Molecular identification techniques, particularly those based on DNA sequencing of conserved genomic regions such as the internal transcribed spacer (ITS) region of ribosomal DNA, have revolutionized fungal taxonomy and systematics. ITS-based analysis is widely accepted as a universal DNA barcode for fungi and allows accurate discrimination at the species level. The combined use of morphological and molecular tools provides a robust framework for the comprehensive characterization of fungal diversity, enabling reliable identification and improving our understanding of fungal ecology in the rhizosphere.

The Bhokardan region of Maharashtra represents an important cotton-growing area characterized by distinct agro-climatic and soil conditions. Despite the agricultural significance of this region, systematic studies on the diversity and identification of fungi inhabiting the cotton rhizosphere soil are limited. Exploring the fungal communities in this region is essential not only for documenting native fungal diversity but

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Citation: Gavande DL, Pochhi VU, Kolte MD. 2026. Morphological and molecular identification of fungi isolated from the cotton rhizosphere soil in the Bhokardan region of Maharashtra. *Res. Jr. Agril. Sci.* 17(1): 73-79.

also for identifying potential plant growth–promoting fungi and soil-borne pathogens that influence cotton health and productivity.

In this context, the present study aims to isolate fungi from the cotton rhizosphere soil of the Bhokardan region and to identify them using a combination of morphological and molecular approaches. By integrating classical mycological techniques with molecular characterization, this research seeks to generate reliable baseline data on rhizospheric fungal diversity associated with cotton, which may contribute to sustainable crop management, biological control strategies, and future microbiome-based interventions in cotton-growing agro-ecosystems.

There are seven layers in the Earth's crust, of which the topmost layer is soil that functions as a reservoir for the storage of essential nutrients and water. Soil diversifies in its habitat as it endures the genetic diversity of the earth. It provides a complex environment for the microorganisms for their growth [1]. The rhizosphere soil is that part of the soil that directly adheres to the plants; thus, this area sustains the majority of the biodiversity of soil microbes. The plant rhizosphere and soil influence physical, chemical and biological processes and enhance the nutrient cycle [2].

Fungi encompass a major share of soil micro-biota; they are ubiquitous in nature and regulate the activity of the eco-system including decomposition of organic matter, plant growth, bio-geochemical cycles and disease control. The rhizosphere fungi are closely associated with plant growth and health of the plant due to their role as antagonizing pathogens, plant residue decomposers and providing nutrients to the plants [3]. The present study deals with the isolation of fungi from the Bhokardan region, Jalna district of Maharashtra. The fungal isolates were identified using morphological and molecular methods.

MATERIALS AND METHODS

Isolation of fungi from cotton rhizosphere soil

Materials: Potato Dextrose Agar (PDA), Streptomycin (100 µg/mL), Sterile distilled water, Sterile Petri dishes, test tubes, pipettes, autoclave, laminar airflow cabinet, incubator (25–28°C).

Samples of cotton rhizosphere soil were collected from different locations of Bhokardan Tehsil (collected from 5–15 cm depth, closely adhering to the roots). Sterile diluent and PDA medium with antibiotics were prepared. Initial suspension using 1 g of soil in 10 mL of sterile water (10^{-1} dilution) was made and serially diluted up to 10^{-6}, and 0.1 mL from selected dilutions was plated onto PDA plates using the spread plate method. The plates were incubated at 25–28°C for 3–7 days. The colonies were purified by repeated sub-culturing until pure isolates were obtained.

Isolation and identification of fungi from the rhizosphere soil of cotton

Isolation and Identification of fungi from the rhizosphere soil of Cotton in the Bhokardan region was performed by studying the macroscopic and microscopic characteristics of the isolates. The isolated sample IDs are AF1, AF2, AF3, AF4, AF5, AF6, and AF7.

Identification of fungi from the rhizosphere soil of cotton based on the ITS region

Extraction of genomic DNA

In the present study, the fungal Culture samples were identified based on molecular identifications. The DNA was extracted by ProGenome Life Science DNA Extraction Kit and quality checked on 1% agarose gel electrophoresis. The gel was visualized using Gel Documentation System - UV Transilluminator (Himedia). A fragment of ITS gene was amplified by ITS 1 and ITS 4 primers. A single discrete PCR amplicon band was observed when resolved on 1.3 % Agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The ITS gene sequence was used to perform a BLAST search against the NCBI GenBank database. Based on the maximum identity score and alignments using multiple alignment software program Clustal W. Distance matrix was generated. Finally, a phylogenetic tree was constructed using MEGA 11 software [4].

Sequencing

PCR products were processed for cleanup to remove unincorporated nucleotide and residual primers using Exonuclease-I and Shrimp Alkaline phosphatase enzyme (1unit/µL) followed by cycle sequencing reaction using BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.). For ITS amplicon sequencing same PCR primers were used. The thermal cycler conditions were an initial denaturation of 2 min at 96^0C and 35 cycles of 30 sec at 96^0C, 15 sec at 55^0C, and 4 min at 60^0C. The Cycle sequencing is followed by sequencing cleanup by ethanol precipitation, followed by dissolving the template in HiDi formamide and bidirectionally sequenced in ABI Genetic analyzer.

Sequence alignment and assembly

PCR products were then processed for direct bi-directional sequencing using ABI PRISM 3730× 1 Genetic Analyzer (Applied Biosystems, USA). The resulting DNA sequences were aligned using CLUSTALW embedded in MEGA 11 [4-6], manually trimmed and edited to obtain complete sequences. The confirmation of species depends on the sequence similarity score. Homology searches were carried out using the BLASTn program against the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast. cgi). NJ tree was constructed using MEGA 11, with all positions containing gaps and missing data were included for analysis [6-7]. Clade supports were calculated based on 1,000 bootstrap re-samplings [8].

RESULTS AND DISCUSSION

Morphological identification

The micro-ecological zone that surrounds the plant roots is called the rhizosphere and it affects the physical, chemical and biological properties of soil [9]. The present study deals with the isolation and identification of fungal isolates from the cotton rhizosphere soil of the Bhokardan region. The seven isolates obtained during the study were investigated for their macroscopic and microscopic characteristics, as mentioned in (Table 1). All the possible morphological characters of the seven isolates were taken into account and represented in (Fig 1). Azmi *et al*. [10] studied the rhizosphere soil of Glasshouse and Nursery Complex (GNC), International Islamic University Malaysia, Kuantan, Pahang, with the help of serial dilution and plating techniques. The results of the study showed the isolation of 10 isolates of soil fungi and were identified based on cultural and morphological characteristics. The fungi were identified to be Cladorrhinum, Penicillium, Paecilomyces and Aspergillus. Makut *et al*. [11] isolated fungi from the plant rhizosphere

region in Keffi, Nigeria. The fungal isolates observed during the study were *Aspergillus flavus*, *Verticillium dahlia*, *Trichoderma harzarium*, *Rhizopus sp*., and *Mucor sp*., The observations were similar to those of the present study.

Table 1 Macroscopic and microscopic characteristics of isolated fungi

Isolate	Growth	Colour	Texture	Special feature	Hyphae	Conidiophores	Phialades	Conidia	Overall Structure
AF1 *Aspergillus flavus*	Rapid	Back: White, developed into a yellow-green due to conidial masses with white borders. Reverse: white to brown	Powdery	Produces white-to-brown sclerotia	Thread-like, branching	Colorless, thick-walled, rough (pitted) stipes (stalks)	Uniseriate and bi-seriate	Globose	Vesicle: Globose (round) to sub-globose, radiating conidial head (apical sac). Seriation: Biseriate
AF2 *Penicillium chrysogenum*	Often forms large colonies with concentric rings on some media	Blue-green to green	Velvety	Margin Distinct white	Colorless (hyaline), slender, branched, septate (divided by walls) filaments	A specialized stalk arising from hyphae, often thick, with branches (metulae)	Flask shaped cells borne on metulae, producing spores	Small, round to oval, smooth spores in long, dry chains (basipetal succession)	The conidiophores and phialides create a characteristic brush-like (penicillus) appearance
AF3 *Trichoderma harzaneum*	Rapid, often forming dense mats that cover the plate	Starts white/dull whitish, quickly developing green pigmentation (dark green to light green) as conidia form.	Mycelium is thick and fluffy	Frequently grows in distinct concentric rings	Septate (divided by walls), white	Erect, frequently branched, often forming whorls (verticillate).	Short, flask-shaped (ampulliform or lageniform), arranged in whorls (2-6) on the conidiophores	Globose to subglobose (roundish), pale green, produced in slimy clusters	Chlamydospores: Round to subglobose, found singly or in pairs along the hyphae
AF4 *Trichoderma asperellum*	Rapid, covering the plate quickly with thick, dense mycelium	Varies, often starting whitish and turning dark green, light green, or yellowish-green with age; some show yellow rings or pigmentation on the reverse	Cottony or woolly surface	Shape: Round, often filling the medium	Creamy white, septate (divided by cross-walls)	Upright, profusely or sparsely branched, with branches often paired	Short, thick, cylindrical, and often clustered at the tips of branches	Green, small, globose to oval, borne in slimy masses (conidial pustules) on phialides, with uneven distributio	Chlamydospores: Round or sub-globose, thick-walled, often found at hyphal tips or middle of hyphae, indicating stress tolerance
AF5 *Trichoderma viride*	Extremely rapid, often overgrowing plates.	White to yellowish, turning green (often dark green) with age due to spore formation, sometimes with faint yellow reverse.	Floccose (woolly/cottony), arachnoid (spiderweb-like), with dense, powdery, granular areas (pustules) where spores form	Pattern: May show concentric rings (zonation). Odor: Often described as distinctive, sometimes like coconut	Hyaline (clear), septate (divided by walls).	Erect, branched, arising from aerial mycelium, branching towards the tip.	Short, flask-shaped (ampulliform), arranged in tight clusters (penicillus) at the ends of branches, often 3-6 in a group, with a sterile tip	Small, spherical to elliptical, green, smooth or slightly rough, formed in slimy balls at the tips of phialides	Chlamydospores: May be present, thick-walled, round, often formed in the middle of hyphae
AF6 *Trichoderma* sp. Isolate yi1007	Rapid, often forming dense mats that cover the plate.	Rapid, often forming dense mats that cover the plate.	Starts white, slowly developing green pigmentation. Colony underside (reverse) is often dull whitish-colored	Mycelium is thick and fluffy		Septate, white	Short, flask-shaped (ampulliform or lageniform), arranged in whorls (2-6) on the conidiophores	Globose, pale green, produced in slimy clusters	
AF7 *Trichoderma* sp. BAB-4501	Rapid, covering the plate quickly with thick, dense mycelium	Varies, often starting whitish and turning dark green	Cottony surface	Shape: Round, often filling the medium	Creamy white, septate (divided by cross-walls).	Upright, profusely or sparsely branched, with branches often paired.	Short, thick, cylindrical, and often clustered at the tips of branches	Green, small, globose to oval	

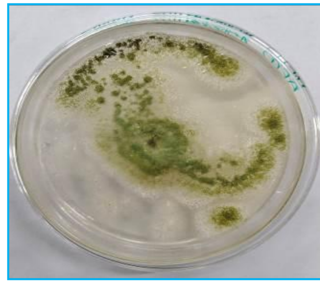
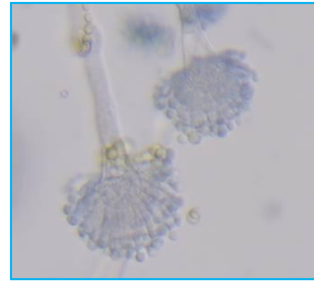
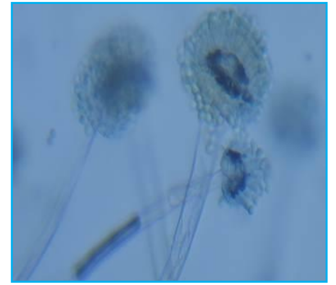
Table 2 Sample IDs showing similarity searches in sequence alignment

Sample ID	Accession No.	Description	Max score	Total core	Query cover	E-value	Per Ident
AF1	PX802230	Aspergillus flavus clone EF_356 small subunit ribosomal RNA gene, partial sequence; interna	1002	1002	100%	0.0	99.82%
AF2	PX811100	Penicillium chrysogenum strain P13 small subunit ribosomal RNA gene, partial seq	220	220	94%	5e-53	89.33%
AF3	PX802359	Trichoderma harzianum strain SC24g small subunit ribosomal RNA gene, partial seq	1003	1003	100%	0.0	99.82%
AF4	PX802417	Trichoderma asperellum strain IIPRPPF1-2 small subunit ribosomal RNA gene, partial sequence	985	985	100%	0.0	99.09%
AF5	PX802429	Trichoderma viride strain SBTTv-001 18S ribosomal RNA gene, partial sequence	998	998	99%	0.0	99.82%
AF6	PX802433	Trichoderma sp. Isolate yi1007_1 internal transcribed spacer 1, partial sequence	1000	1000	99%	0.0	99.82%
AF7	PX802453	Trichoderma sp. BAB-4501 18S ribosomal RNA gene, partial sequence; internal transcribed	1038	1038	98%	0.0	99.82%

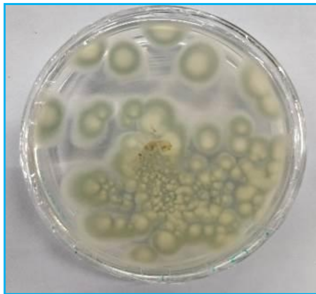
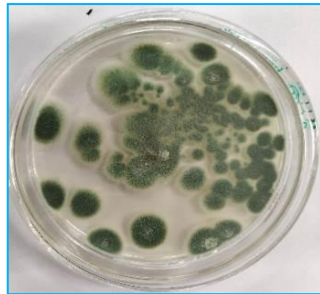
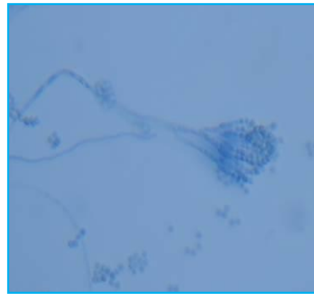
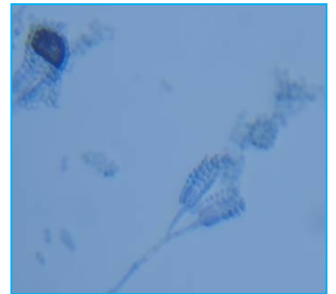
Molecular identification and phylogenetic analysis

The six main regions were identified as possible Deoxyribonucleic acid (DNA) barcodes for fungi, the second largest kingdom of eukaryotic life, by a multinational, multi-laboratory consortium [4]. The genomic Deoxyribonucleic acid (DNA) of 7 fungi from the Rhizosphere Soil was extracted using a commercially available kit, and the isolation procedure was performed according to the manufacturer's guide. The purity and Deoxyribonucleic acid (DNA) concentration were determined. The Deoxyribonucleic acid (DNA) so obtained was pure and of a high concentration. These DNA samples isolated from fungi in the Rhizosphere Soil were subjected to further

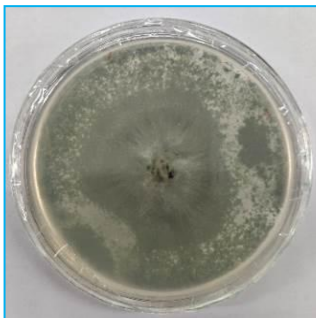
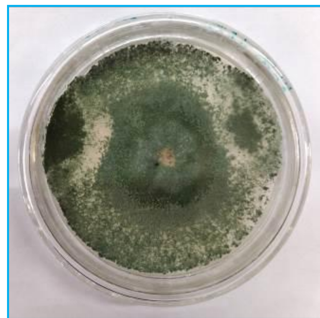
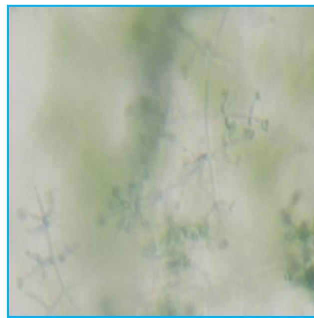
PCR amplification (Table 2). Total DNA of 7 isolates was extracted and ITS regions were amplified using universal ITS1 and ITS4 primers, ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').

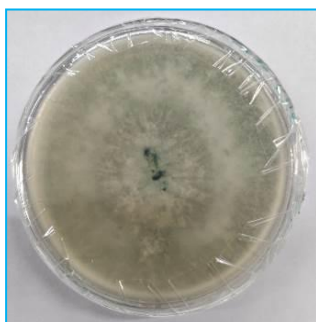
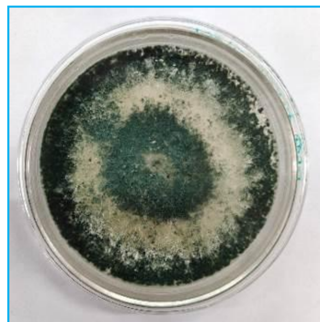
Aspergillus flavus

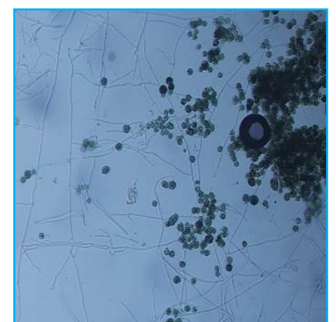





Penicillium chrysogenum

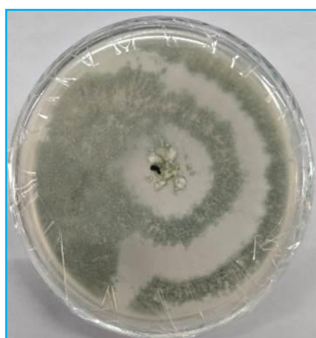




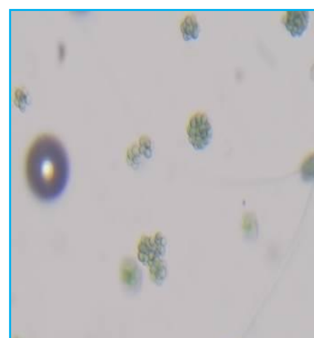
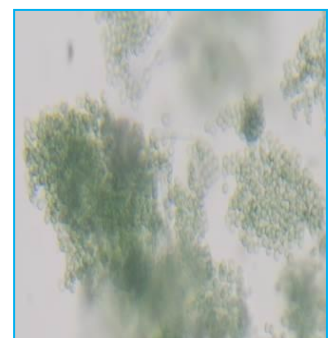
Trichoderma harzanium

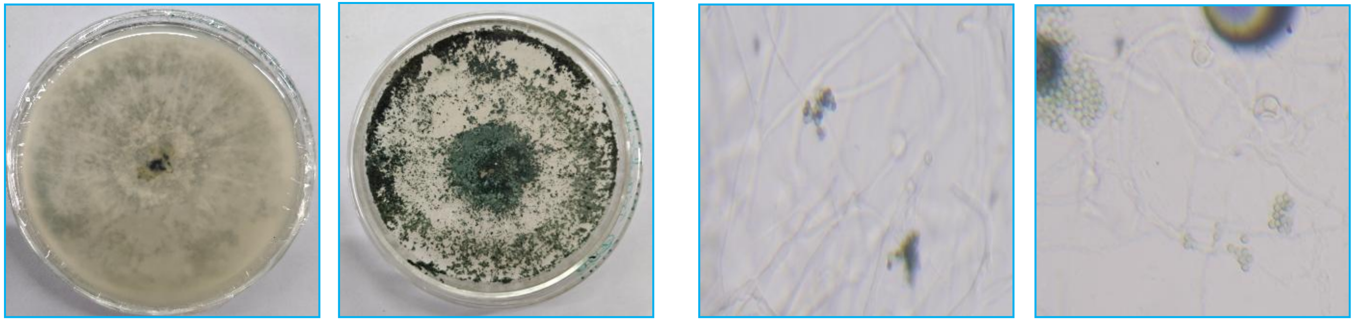


Trichoderma asperellum



Trichoderma viride



Trichoderma sp. BAB-4501

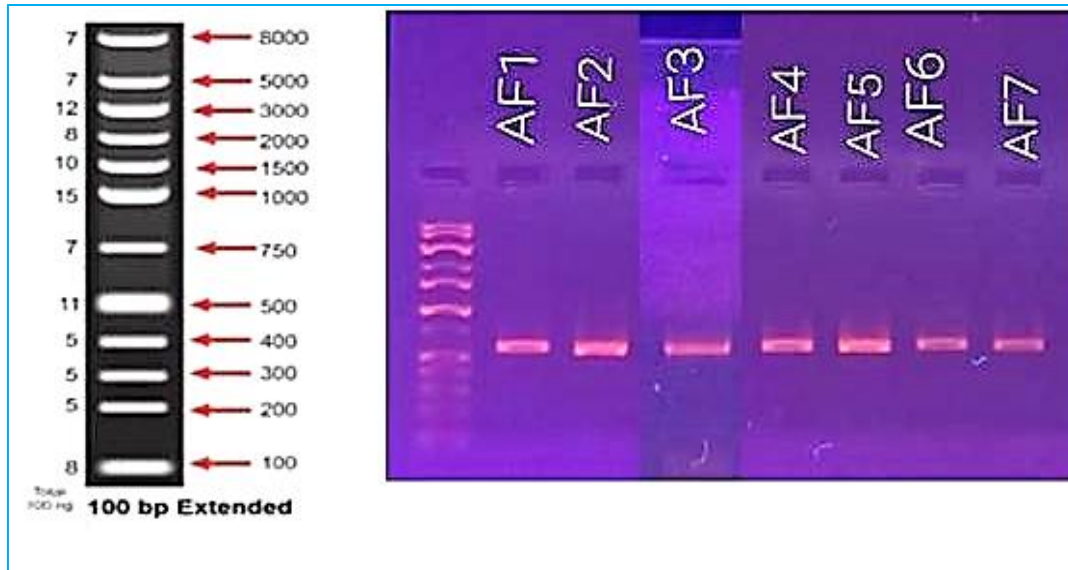


Fig 2 Molecular identification of fungi

The expected size of the PCR product in ITS region was observed to be approximately 300 to 500 bp on 1.5% Agarose gel after electrophoresis (Fig 2). PCR products were processed for cleanup to remove unincorporated nucleotide and residual primers using Exonuclease-I and Shrimp Alkaline phosphatase enzyme (1unit/µL) followed by cycle sequencing reaction using BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.).

All the isolated samples from fungi were sent for ITS region DNA sequencing. After comprehensive authentication, all the above 7 fungi sequences were submitted to the National Center for Biotechnology Information (NCBI) database through the online submission policy. The ITS sequences obtained for all the fungi species were added to the Basic Local Alignment Search Tool (BLAST) network service, which is available online free of cost. Subsequently, the nucleotide sequences of isolated fungi spp. were compared with all the sequences available in nucleotide databases (e.g. NCBI). As per the availability of similarity tools in the software, the homology search was performed within the non-redundant databases of GeneBank using the BLAST analysis found at http://www.ncbi.nlm.nih.gov/BLAST/of NCB. The isolated fungi showed the highest similarity: *AF1- Aspergillus flavus, AF 2- Penicillium chrysogenum, AF-3 Trichoderma harzianum, AF- 4 Trichoderma asperellum, AF-5 Trichoderma viride, AF-6 Trichoderma sp.* Isolate and *AF- 7 Trichoderma* sp respectively. The sample IDs show similarity searches in sequence alignment (Table 3). The sequences submitted to the NCBI and accession numbers of isolated fungi were AF PX802230 AF2 PX811100, AF3 PX802359, AF4 PX802417, AF5 PX802429, AF6 PX802433, AF7 PX802453. In line with our study results, similar results have been reported in previous studies where ITS-based molecular identification successfully distinguished *Aspergillus*, *Penicillium*, and *Trichoderma* species from rhizosphere soils [6], [13-15].

Table 3 Details of ITS polymerase chain reaction composition

Component	Component volume
REDy MasterMix	12.5 µL
ITS1 primer	1.5 µL
ITS4 Primer	1.5 µL
Template DNA	3.0 µL
Nuclease-Free Water	6.5 µL
Total reaction volume	25 µL

CONCLUSION

The current study isolated and identified seven fungal species from the rhizosphere soil of cotton collected from the Bhokardan region. Moreover, molecular characterization using ITS sequencing, followed by BLAST analysis, has been demonstrated to be an efficient and consistent approach for accurate species identification. The isolates presented high similarity to well-known rhizosphere fungi, including *Aspergillus flavus*, *Penicillium chrysogenum*, *Trichoderma harzianum*, *T. asperellum*, *T. viride*, and two *Trichoderma* spp. The occurrence of multiple *Trichoderma* species highlights the ecological significance of beneficial fungi in cotton rhizospheres, particularly their potential roles in plant growth promotion and biological control. This study concluded valuable baseline information on fungal diversity in cotton-

growing soils of the Bhokardan region and provided a foundation for future studies discovering their functional roles and likely applications in sustainable agriculture. In the future, we will study the antagonistic activity of isolated fungal species from the rhizosphere soil of cotton collected from the Bhokardan region.

Sequences:

>AF1
TGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTT
CCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC
CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCC
GGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGA
CACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTT
GATTGTATCGCAATCAGTTAAAACTTTCAACAATGGA
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ATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAAT
CATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT
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CGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAA
ATCAATCTTTTACCAGGATGACCTCGGATCAGGT

>AF2
TGCCTGTCCTAGCGTCATTGCTGCCATCAAGCAAGGA
TTGTGTGCTGGGCCCCGTCCTCCGATCCCGGGGGACG
GGCCCGAAAGGCAGCCGCGACCCCGCGTCCGGTCCT
CGAGCGTATGGGCATTCGAAAACCGCTGTGTAGGCC
CTGCCGGCGCATCCCGTATGCAATTCCAAATTAATTT
AACCTAG

>AF3
TTCCGTAGGTGAACCTGCGGAGGGATCATTACCGAG
TTTACAACTCCCAAACCCAATGTGAACGTTACCAAAC
TGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGC
AGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCA
AAACTCTTTTTGTATACCCCCTCGCGGGTTTTTTATA
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CGGGAGCGCGGCGCGTCCACAGCCGTTAAACA

>AF4
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